ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF
ENVIRONMENTAL HEALTH SCIENCES

FISCAL YEAR 1971

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH

ANNUAL REPORT

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U.S. NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES

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July 1, 1970 through June 30, 1971

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Summary Statement

The close of the fiscal year brought a change in the leadership of the Institute with the resignation of Dr. Paul Kotin as Director and the appointment of Dr. David P. Rall as his successor. Dr. Kotin, the Director of the Institute from its creation as the Division of Environmental Health Sciences in November 1966, played the major role in formulating the mission, structure, policy and philosophy of the organization. Success in the recruitment of professional staff as well as the nurturing of cooperative relationships with the Research Triangle Foundation, local universities and other scientific organizations in the area was largely a result of the personal efforts and skill of Dr. Kotin.

In March 1971, a ribbon-cutting ceremony attended by distinguished local, state and national leaders marked the first major expansion of the interim facilities at the National Environmental Health Sciences Center in Research Triangle Park, North Carolina. The completion of the new facilities doubles the laboratory space for the Institute and makes possible the continued growth of the intramural research program.

The newly-appointed director now has available facilities that will be a valuable asset in the continuing recruitment of scientific staff and the broadening of the laboratory research program.

The completion, by a joint venture of two architectural and engineering firms, of the site planning of the 509 acre tract on which will be located the Institute's permanent facilities sets the stage for further development of long-range plans and programs.

During this year the continuation of a successful scientific liaison program with other Federal agencies having activities related to environmental health has been complicated by the contemplated governmental reorganization and the subsequent creation of the Environmental Protection Agency (EPA) from segments of several different departments, including major programs within HEW. The wisdom of developing liaison activities and information exchange on a scientist-to-scientist basis rather than on organization structure has been proven. Even in the reorganizational period and in the formative stages of EPA it has been possible to continue scientific exchange on specific subjects and problems of mutual interest.

The Institute has continued its participation in the planning of the 1972 UN Conference on the Human Environment and is developing background information, including four book-length documents on topics relevant to the proposed conference, in cooperation with the Fogarty International Center.

At the request of Dr. Colin MacLeod, Chairman of the U.S. delegation for the U.S.-Japan Cooperative Medical Science Program, the Director attended a meeting of the sub-committee on Program review in Honolulu on February 4 and 5, 1971, to consider the introduction of environmental studies into the program. As the areas of possible cooperation are defined, the Institute is formulating its role in the program if the new panel is created.

The Institute also participated in the Second U.S.-Japan Conference on Environmental Pollution in Washington, D.C. The Director was the senior HEW representative at the technical sessions on May 27 and 28 and, with the Surgeon General, attended the ministerial level Conference on June 1 and 2, 1971.

The Staff of the Office of the Director has participated in various ways in the work of the Office of Science and Technology. For example, a Task Force with members from several agencies in HEW, the Atomic Energy Commission, the National Science Foundation, and the Council on Environmental Quality prepared a report of Research Needs for Environmental Health Research; a working group prepared recommendations for the creation of an activity in technological forecasting for environmental health following the general recommendations in the report "Man's Health and the Environment -- Some Research Needs"; and a committee studied the need for epidemiology in environmental health and explored means and mechanisms for increasing the scope of epidemiological studies.

The Director of the Institute served as the Chairman of the Environment Committee of the Health Options Group in the preparation of recommendations for meeting the needs of the nation more effectively.

As a member of a Task Force to study research areas of interest to both HEW and the Environmental Protection Agency, the Director participated in the preparation of a report on Health Effects Research and the Environment.

The Director and other scientists from NIEHS continued to furnish technical information to the Secretary of HEW and congressional committees on a variety of environmental problems including components of detergents, pesticides, metals and hazards associated with uranium mining.

Requests for the report "Man's Health and the Environment -- Some Research Needs" necessitated a second printing. It was used as the basis for the work of the OST Task Force on Research Needs for Environmental Health Research as well as the OST Committee on Technological Forecasting. The Health Effects Task Force, chaired by Dr. Theodore Cooper, found the report a valuable summary of needs for research in specific environmental problem areas, for research on methods and specific disease conditions, and for needs related to the social and behaviorial sciences, technological trends, training and organizational needs. Steps are being taken to establish a mechanism to periodically up-date the report.

In planning and developing a program in epidemiology, it has become apparent that one of the more important and immediate considerations has to do with the need for improved biomedical data resources. In addition to the fact that environmental health problems are of progressive complexity and

require improved data resources for evaluation and resolution, it is essential that such improved resources be developed to facilitate problem recognition. This Institute has coordinated efforts to document the spectrum in nature of such needs and hopes to provide leadership in this area in the future.

In summary, fiscal year 1971 has been a year of continuing program development and growing acceptance of the Institute's role in Federal science activities. The coming year should bring continuing program maturation and, with the availability of new laboratory resources, the development of full-scale research programs in areas where only modest efforts were heretofore possible.

OFFICE OF THE SCIENTIFIC DIRECTOR

OFFICE OF THE SCIENTIFIC DIRECTOR
Summmary Statement

As in previous years the Director of the Institute served also in the capacity of the Scientific Director, and most of the activities of the Director are reported only once in the Summary Statement of the Office of the Director.

During this fiscal year some of the long-hoped for developments of this Institute have come to fruition. Of greatest importance is the addition of specialized laboratory space in a complex of three modern buildings which will allow this Institute to carry out experiments essential to its mission that could not be undertaken in the past.

The new animal facility allows scientists in the intramural program to attack problems of long-range exposure of animals to toxicants under conditions where the health of the animals can be monitored adequately, and all precautions can be taken to avoid unplanned intercurrent infections or problems of temperature or humidity control. Even though modern design features and equipment have been incorporated into this animal facility, intensive training of supportive personnel is necessary in procedures that will minimize the possibilities of cross-contamination between animals and assure the integrity of each experiment.

The aerotoxicology facility will meet one of the urgent needs of this Institute: the capability of exposing animals to environmental toxicants in a form to which man may actually be exposed, although the anatomical and physiological differences between rodent species and man must be recognized. Nonetheless, comparative studies on inhalation toxicology are imperative for this intramural program if the Institute is to provide answers that are comparable to human exposures to aerosols, gases and particulate matter which can be inhaled. Recruiting for this facility during this fiscal year has been slow because great care was exercised to attract personnel and scientists with capabilities in this specialized area, and experts in this field are not abundant. Dr. Drew has joined the Institute's Pharmacology and Toxicology Branch and has brought his knowledge and enthusiasm in aerotoxicology research for planning a very active participation of the aerotoxicology group in the Institute's programs.

The third facility of the new complex, the laboratory building, will provide an opportunity for scientists of the several branches to develop closer associations when their interests are in similar directions and to share common facilities and instrumentation. Thus, the tissue culture and mycotoxicology facilities of the Cell Biology Branch will bring together personnel working in these areas.

Although hope that an Associate Director for Epidemiology and Biometry might be found during this fiscal year was optimistically expressed in last year's report from this office, unfortunately the Institute did not find such a person; however, this effort will be continued and it is hoped success may be forthcoming in the near future. The search for a Chief of the Pathologic Physiology Branch also is continuing under the new Director. In the meantime, pathologists from local universities consult with Institute scientists when there is a need for their knowledge and expertise. These consultants have special knowledge and a specific interest in animal research and the program of the Institute. Their past efforts have focused on specific target organs, such as the kidney, the lung and the brain. To further meet the needs for scientific input by pathologists to the overall program of the Institute, Dr. Robert Goyer of the University of North Carolina School of Medicine will spend his sabbatical at the Institute carrying out research of high relevance to the mission of the NIEHS and, at the same time, contributing his special knowledge on the toxicology of heavy metals.

The Chief of the Pharmacology and Toxicology Branch, Dr. James Fouts, has come on board and has initiated a program in enzyme induction which was one of the unmet requirements of this Institute. It has been realized that most of the environmental toxicants like pesticides or certain air pollutants, but also food additives and food components, have the ability to induce enzymes at levels of intake which may be 100 times less than those at which toxicity becomes apparent. It has never been determined whether this capability of enzyme induction is beneficial or detrimental to the organisms, but it is certain that both answers will be correct under specific circumstances. The need to explore enzyme induction in tissues other than the liver is another important area of concern, and the Pharmacology and Toxicology Branch plans to exert considerable efforts in this direction. The teratology program of the Branch will be enlarged next fiscal year to cope with urgent needs of the Institute as they may arise.

With the new facility in operation and a small increase in personnel, some increase in activities for the proposed Biophysics and Biomedical Instrumentation Branch is anticipated in that the microwave program will be able to answer important questions on possible non-thermal health effects of microwaves and a modest program in noise will be started. This Branch still has not been activated, but considerable contributions to the overall program of the Institute with relation to physical factors affecting the health of man are being made by scientists located at present in the Research Services Branch.

During this fiscal year the many calls made upon this Institute for information on problems of environmental health and the requests by Government officials inside and outside the Department of Health, Education and Welfare for information on specific problems, as well as the desire on the part of other agencies to have this Institute carry out research on various problems, all recognize that the Institute's mission is of great importance and its function of supplying, advising or giving information and consultation is highly essential to other organizations, national and international. The summary statements of the individual Branch Chiefs and Associate Directors give many examples of these functions fulfilled during this fiscal year.

CONTRACT NARRATIVES

BATTELLE NORTHWEST-PACIFIC NORTHWEST LABORATORY, RICHLAND, WASHINGTON
(Interagency Agreement Between NIEHS and AEC)

TITLE: Research on the "Combined Effects of Inhalation of Uranium Ore Dust and Radon Daughters with Cigarette Smoke or Diesel Exhaust Fumes on the Lungs of Dogs and Hamsters"

CONTRACTOR'S PROJECT DIRECTORS: J. F. Park, Ph.D. and B. O. Stuart, Ph.D.

PROJECT OFFICER (NIEHS): Hans L. Falk, Ph.D.

ASSOCIATE PROJECT OFFICER: Phillip J. Walsh, Ph.D.

DATE CONTRACT INITIATED: June 1, 1968

CURRENT ANNUAL LEVEL: $331,795

PROJECT DESCRIPTION

OBJECTIVES: As a major part of an integrated research program on the effects of exposure to radon and daughters on the development of lung cancer in uranium miners, the following animal studies were initiated:

1. Groups of hamsters are to be exposed to the following combinations:

   a. radon daughters on room air nuclei,
   b. radon daughters and carnotite ore dust,
   c. diesel engine exhaust alone,
   d. diesel exhaust fumes, radon daughters and ore dust,
   e. carnotite ore dust.

Analysis and control of concentrations of radon and daughters, composition of ore dust, and concentrations of other toxic agents in the diesel oil exhaust fumes will be made by the most advanced techniques available. After being exposed to alpha particles irradiation to predetermined cumulative dosage levels comparable to the WLM exposures of high-level exposed miners, the animals will be maintained for life for the observation of pathological changes, particularly lung cancer.

2. To test specifically the possible synergistic effects of radon and daughters and cigarette smoking, studies were initiated on three groups of 18 dogs as described below:

   a. dogs exposed to radon and daughters on carnotite ore--exposure for four hours per day, 7 days per week at concentrations of 15 nanocuries per liter; with sham smoking;

   b. dogs exposed to radon plus daughters on ore as in (a), plus two working shifts of smoking (total of 10 cigarettes per day with resting on alternate hours);

c. two shifts of smoking only, 10 cigarettes per day as in (b).

Regular clinical, radiological and radiographic examinations will be made during and after the smoking and radon exposures. The dogs, except for one in each group which will be sacrificed for early histopathological studies, will be held for their remaining life span with observations of pulmonary changes and lung cancer as the end point.

MAJOR FINDINGS: The hamster group exposed to 30 WL radon daughter concentration and the groups exposed to 600 WL and 600 WL plus ore dust have lived out their life spans. Exposures of the groups to diesel engine exhaust and to diesel plus 600 WL radon daughters plus ore dust were initiated in 1970. There have been no statistically significant changes in mortality patterns, weight changes or hematology. In the groups exposed to 600 WL and 600 WL plus ore dust, hyperplasia and metaplasia were evident in the medium sized bronchioles. Neoplasia has not been observed. Only one case of hyperplasia has been observed in the 30 WL group at 19 months into the experiment. The analysis of the results is continuing and a group at 1200 WL exposure has been added.

All the dog groups are currently being exposed and tests are being conducted as outlined under proposed course. No significant changes are apparent at this time.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These experiments are designed to determine, in both a short-lived animal, the hamster (life-span 2-3 years) and a long-lived species, the dog (life-span 12-13 years), the possible synergistic effects of chemical agents--dust, cigarette smoking, and diesel exhaust fumes operating singly and in concert with exposure to radon and daughters upon the production of lung cancer in animals. To the extent possible in the laboratory, the exposure conditions are designed to simulate those to which uranium miners have been exposed, and in whom an increased incidence of lung cancer is now appearing.

PROPOSED COURSE: The hamsters exposed to 30 WL radon daughters and to 600 WL radon daughters and 600 WL radon daughters plus uranium ore dust have reached their life spans and results are being analyzed. The exposures to diesel exhaust and radon daughters will continue.

The dog exposures to cigarette smoke, ore dust and radon daughters will also continue.

COLORADO STATE UNIVERSITY, FORT COLLINS, COLORADO (PH-43-68-1326)

TITLE: "Radon Progeny Inhalation Exposures to Uranium Miners"

CONTRACTOR'S PROJECT DIRECTOR: Keith Schiager, Ph.D.

PROJECT OFFICER (NIEHS): William W. Payne, Sc.D.

ALTERNATE PROJECT OFFICER (NIEHS): Phillip J. Walsh, Ph.D.

DATE CONTRACT INITIATED: June 23, 1968

CURRENT ANNUAL LEVEL: $30,010

PROJECT DESCRIPTION

OBJECTIVES: This research was initiated as part of an integrated program to determine the effects of inhalation of radon and its daughters upon the induction of bronchogenic carcinoma in uranium miners. The objective was to develop a reliable personal dosimeter and a reliable bioassay procedure that would indicate cumulative exposures over definite time periods.

METHODS EMPLOYED: Forty actively working uranium miners were selected from two mines on the Colorado plateau along with ten control subjects residing in the area who had no known occupational exposure to radon progeny or mine environments. The miners were equipped with personal dosimeters consisting of a battery-operated air sampling pump and a thermoluminescent dosimeter (TLD) worn on the miner's hat and designed to sample and measure the radioactivity in the air which the miner actually breathed. Blood and whisker samples were collected on a regular basis for measurement of polonium-210 and lead-210 to determine the correlation between the measured Working Level Month (WLM) exposures to radon progeny and the subsequent deposition of long-lived radioactive decay products in the whiskers.

MAJOR FINDINGS: Thermoluminescent dosimetry, as applied in this contract, does not appear to be a likely candidate for personal miner dosimetry. Improper handling of the dosimeters by the miners and problems with buildup of particulates on the integral surfaces of the dosimeters introduced large uncertainty into the exposure measurements.

The value of the whisker content of ^{210}Pb or ^{210}Po as a biological dosimeter cannot be properly assessed because of lack of reliable individual exposure estimates for the miners. The correlation coefficients between net ^{210}Pb and ^{210}Po levels and available exposure estimates are high enough to be encouraging but additional studies would be necessary to confirm the results.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: For several years the PHS has borne a large share of the responsibility for the epidemiological studies of the increased incidence of lung cancer in uranium miners. One of the major problems revealed by the epidemiological work was the difficulty of establishing a clear dose-effect relationship between low-

level exposures in past years and the statistically significant increase in lung cancer now appearing in miners in the lower exposure categories. Part of the problem was due to a lack of knowledge of the metabolic fate of inhaled radon progeny in the working miner and part to the lack of dosimeters of sufficient sensitivity to measure the radioactivity actually inhaled by the individual miner. This research was aimed at investigating possible solutions to these problems.

PROPOSED COURSE: Support for the work on this contract ended in June 1970.

Duke University Medical Center, Durham, N. C.
(PH-43-69-73)

PROJECT TITLE: "The Effects of Toxic Chemicals on Cell Membranes"

CONTRACTOR'S PROJECT DIRECTOR: D. C. Tosteson, M.D.

PROJECT OFFICER (NIEHS): Paul Kotin, M.D.

ALTERNATE PROJECT OFFICER (NIEHS): R. G. Owens, Ph.D.

DATE CONTRACT INITIATED: January 16, 1968

CURRENT ANNUAL LEVEL: $108,620

PROJECT DESCRIPTION

OBJECTIVES: To characterize the interactions of various environmental toxicants with cell membranes and to determine the kinds of interactions most important to human health.

METHODS EMPLOYED: A number of scientists were involved, each with different test systems. Methodology utilized for the various study areas are indicated in the following section.

MAJOR FINDINGS: Studies on the mode of action of insecticides on nerve and muscle membranes were conducted on sensory cells of the cockroach and on the giant axon of the crayfish. It was found that p,p'-DDD did not increase very effectively the negative after-potential. Repetitive after-discharges were elicited in some cases by a single electrical stimulus. However, a more remarkable change was observed in the spike phase of the action potential, e.g., partial suppression of the amplitude. The resting potential was unchanged. This suggests that the action of p,p'-DDD was on sodium conduction, which is in contrast to the lack of effect of p,p'-DDT on this process.

The effects of DDT on the cell membrane of the marine alga Valonia were also determined. DDT was applied to both the outer and inner (vacuolar) membranes. No effect could be detected on membrane voltage, membrane resistance, or short ciruit current (which reflects the inward active transport of ions) at concentrations of DDT up to 10^{-4} M.

Studies on the effects of cobalt on the transient and steady state currents in uterine smooth muscle show that both the transient current mechanism and steady state current were depressed. This was previously attributed to competition with Ca^{++}. However, the effects of Co^{++} closely resemble those of La^{+++}, suggesting that suppression of current may be due to nonspecific interaction with anionic sites and not specifically those concerned with calcium-induced current.

Effects of Co^{++} and a number of other heavy metal ions were also conducted on the neurons of the central nervous system of the cat in vivo. Five-barreled micropipettes were used to administer the ions to the neurons in the cerebral cortex and in the brainstem. All ions tested caused depression of the discharge rate of nerve cells firing spontaneously and of those excited by application of glutamate. Cd, Co and Ni were found to be more potent depressors than Mn, Zn and Fe. The potency of Pb and Be could not be determined accurately because both cations formed insoluble salts in the micropipettes and failed to deliver current. Currents of 5nA or less carried by the depressant ion were sufficient to cause marked depression of the discharge rate.

Studies were continued on the hemolysis of red blood cells by the Prymnesium parvum toxin, prymnesin. Hemolytic rate studies show that the rate of membrane lysis is a function of both concentration of prymnesin and temperature. Lysis is inhibited by cholesterol and cephalin at ca. 10^{-5} M, which apparently form complexes with the toxin.

Binding of prymnesin to erythrocyte membranes was determined by use of tritium labeled toxin. It was found that intact cells will bind about 10 per cent of the available toxin while RBC ghosts will bind about 45 per cent. This probably indicates that the toxin does not penetrate intact cells but induces lysis by attack on the outer membrane surface.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Membranes are vital structures in all living cells and there are many lines of evidence which converge to implicate membrane interactions with various kinds of compounds in the bioactivity of drugs and toxicants. Toxicant-membrane interactions may be particularly important in nerve and muscle cells where electrical impulses associated with membrane potentials serve to control movement, both voluntary and involuntary, and sensory phenomena. Thus, an understanding of membrane properties and associated phenomena and the effects of various toxicants on these properties is fundamental to appreciation, evaluation and, eventually, control of potentially hazardous compounds capable of interacting with membrane components.

PROPOSED COURSE: This project has been terminated.

PUBLICATIONS

Padilla, G.M.: J. Protozool. 17:456, 1970.

Rauckman, B. and Padilla, G.M.: Abstract, 14th Annual Meeting Biophys. Soc., Baltimore, Md., 1970.

Rozear, M., DeGroof, R. and Somjen, G.: Effects of micro-ionophoretic administration of divalent metal ions on neurons of the central nervous system of cats. (In Press)

MASSACHUSETTS GENERAL HOSPITAL, BOSTON, MASSACHUSETTS (NIH-69-77)

TITLE: "Beryllium Case Registry"

CONTRACTOR'S PROJECT DIRECTOR: H. Kazemi, M.D., Chief, Pulmonary Unit

PROJECT OFFICER (NIEHS): Douglas H. K. Lee, M.D., Ph.D.

DATE CONTRACT INITIATED: December 19, 1968

CURRENT ANNUAL LEVEL: $35,000

PROJECT DESCRIPTION

OBJECTIVES: The Beryllium Case Registry was set up by Dr. Harriet Hardy in 1952 to provide for: (a) periodic reporting by physicians of suspected cases of beryllium poisoning; (b) examination of reports by an expert panel to decide admission to the Registry; (c) acquisition of additional evidence of diagnostic significance; (d) diagnostic assistance to the reporting physician; (e) periodic follow-up on progress of cases; and (f) critical study of disease processes.

METHODS EMPLOYED: The Registry is in full operation at the Massachusetts General Hospital. It consists of 806 cases, of which 275 are dead. Contact has been re-established with one quarter of the 531 presumed living cases. Attempts are being made to extend the contacts by direct communication with the patients. Cooperation has been established with the Pennsylvania State Department of Health and 60 living cases have been added to the Registry from this source. Another 60 dead cases not previously in the Registry are being added from this source. 6 new cases were added to the Registry in 1970, and 7 "doubtfuls" will probably be added. The radiographs are being systematically reviewed.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The Registry is being maintained as a source of reference material because: (a) knowledge of late manifestations of Be poisoning is poor; (b) the patterns of exposure are changing with widespread and new uses of Be , alloys, and compounds; and (c) numerous uncertainties exist about the basic patho-physiological processes involved.

The material is available for study purposes, but the identity of the patients is kept confidential.

PROPOSED COURSE: Continue in its present form, with critical review of contents against new standards. Examination of salient features of new cases or advent of new disease manifestations as need arises. Conference with participating physicians to improve follow-up procedures.

North Carolina State University, Raleigh, N. C.
(PH-43-68-1375)

TITLE: "Role of Fungi in the Production of Toxins in Cigarette Smoke."

CONTRACTOR'S PROJECT DIRECTOR: G. B. Lucas, Ph.D.

PROJECT OFFICER (NIEHS): Robert G. Owens, Ph.D.

ALTERNATE PROJECT OFFICER (NIEHS): Ronald W. Pero, Ph.D.

COLLABORATORS (NIEHS): R. G. Owens, Ph.D. and Ronald W. Pero, Ph.D.

DATE CONTRACT INITIATED: July 1, 1968

CURRENT ANNUAL LEVEL: $70,000

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to determine the kinds and amounts of toxins contributed by molds to the solids of cigarette smoke. This involves acquisition of reference standards for the major toxins produced by molds associated with tobacco. In most cases it is necessary to isolate these from the molds of interest since few of the toxins are available from commercial or other sources. It also involves development of methods for isolation and quantitation of the toxins. As these methods are developed the toxin contents of moldy tobacco and smoke condensates are determined. The influence of environmental factors and strain characteristics on toxin production by the various organisms will also be determined.

MAJOR FINDINGS: One of the organisms of interest associated with tobacco is an Alternaria sp. that causes the brown spot disease of tobacco. These studies have prompted closer scrutiny of the taxonomy of the species and development of evidence that the correct name is Alternaria alternata (Fries) Keissler instead of the current name Alternaria tenuis.

A number of strains of A. alternata have been examined in order tc obtain maximal production of toxins. A high-yielding strain was found and large scale cultures were grown for toxin extraction. Crude extracts (acetone: water, 7:3 v/v) were obtained from some 2000 cultures and delivered to NIEHS for separation and purification of the toxins alternariol, alternariol monomethyl ether, and altenuene for toxicological tests.

In addition to screening of strains of A. alternata for high toxin yields, studies were conducted to determine the cultural parameters most favorable for toxin production. It was found that high carbon, low nitrogen ratios are conducive to toxin accumulation and that sucrose and asparagine are the best carbon and nitrogen sources tested.

Temperature also had a marked effect on both growth and toxin production. The optimum for toxin production was 25 degrees centigrade with little or no production at temperature extremes. However, when growth was initiated at the temperature optimum and the fungus held subsequently at the temperature extremes, toxin production was about equivalent to that in cultures held constantly at 25 degrees. This is relevant to the storage and processing of tobacco at different temperatures and final return of the tobacco to ambient room temperature in finished cigarettes.

A number of samples of tobacco taken at different stages in the curing and storage processing and with differing mold counts and brown spot lesions were analyzed by gas chromatographic methods developed at NIEHS for the three major toxins. None contained detectable amounts of any of the toxins. This is in accord with previous experiments with green tobacco in which alternariol was injected into leaf tissue. The compound rapidly disappeared after injection, suggesting rapid alteration by living tobacco tissues.

Subsequently, the fungus was cultured on shredded tobacco wet with Czapek's broth. Under these conditions considerable quantities of alternariol and alternariol monomethyl ether were produced. However, the studies on natural, heavily diseased tobacco appear to preclude important accumulations of these toxins in marketed tobacco.

A number of other toxigenic species associated with tobacco were also assayed for known toxins: Aspergillus ochraceus, A. clavatus, A. versicolor, Penicillium cyclopium and Epicoccum sp. These were found to produce substances toxic to Bacillus megaterium and B. mycoides in the disc assay procedure when cultured on rice or other culture media. Penicillic acid and patulin, both potent carcinogens, were isolated from Aspergillus ochraceus and Penicillium cyclopium, respectively. However, neither appears to be produced by the organisms cultured on shredded tobacco. Thus, tobacco appears to be a poor substrate for production of these toxins as well as for aflatoxins, kojic acid and other toxins, as reported previously. Moreover, cigarettes spiked with 10 mg. of penicillic acid each yielded no detectable toxin in smoke condensates. We conclude that neither penicillic acid nor patulin constitute any health hazard in marketed flue-cured tobacco or in cigarette smoke condensates.

Further observations have been made on actinomycetes isolated from tobacco. Two strains of Streptomyces albus have a wide spectrum of antagonism against the fungi of tobacco, including species of Aspergillus, Penicillium, Alternaria, Cladosporium and Epicoccum.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Some forty million Americans are smokers and, therefore, may inhale products of toxigenic fungi associated with tobacco along with the pyrolysis products of tobacco per se. Some fungi associated with stored tobacco have been shown to produce known carcinogenic compounds in peanuts, grains, and other agricultural products, but little work has been done previously on the

capibilities of the fungi to produce volatile toxins in tobacco. This study was initiated to determine if fungi do constitute a hazard to smokers' health beyond that already recognized to be due to tobacco products themselves, and, if so, to determine the magnitude of the problem.

PROPOSED COURSE: The studies thus far have given no indication of an appreciable contribution of toxins by fungi to the solids of cigarette smoke or of marketed tobacco. Therefore, this project will be terminated at the end of the contract period (about December 1, 1971).

PUBLICATIONS

Lucas, G.B.: Alternaria alternata (Fries) Keissler, the correct name for A. tenuis and A. longipes. Tob. Sci. 15: 40-44, 1971.

Lukic, A.M., Lucas, G.B., and Welty, R.E.: The main characteristics of Actinomycetes from tobacco. Beograd. Mikrobiologija. (In Press)

Lucas, G.B., Pero, R.W., Snow, J.P., and Harvan, D.: Analysis of tobacco for the presence of the Alternaria toxins alternariol and alternariol monomethyl ether. Jour. Food & Agric. Chem. (In Press)

UNIVERSITY OF NORTH CAROLINA, CHAPEL HILL, N. C. (PH-43-68-74)

TITLE: "The Subcellular Pathology of Heavy Metal Intoxication"

CONTRACTOR'S PROJECT DIRECTOR: Robert A. Goyer, M.D.

PROJECT OFFICERS (NIEHS): Hans L. Falk, Ph.D. and Paul Kotin, M.D.

DATE CONTRACT INITIATED: January 16, 1968

CURRENT ANNUAL LEVEL: $115,000

PROJECT DESCRIPTION

OBJECTIVES: To investigate pathologic changes in cellular metabolism due to metallic environmental contaminants, specifically lead, in order to relate structural and biochemical alterations in subcellular organelles with functional ability of the cell.

MAJOR FINDINGS: Ultrastructural changes in proximal tubular lining cells of rat kidney could be correlated with renal tubular dysfunction in animals ingesting lead acetate with the drinking water. Aminoaciduria due to lead poisoning is secondary to renal tubular dysfunction and differs from drug-induced aminoaciduria. Cytochrome content of renal mitochondria is reduced in lead poisoning and oxidative and phosphorylative ability is reduced but not completely impaired.

Intranuclear inclusion bodies are found in lead poisoning and represent a sensitive index of lead intoxication. These lead-protein complexes also contain calcium, as shown by electron probe studies and seem to represent a detoxification mechanism, a theory substantiated by the observation that lead toxicity is enhanced on a low calcium diet, which may represent a very important observation on its own.

Both acute and chronic effects of lead intoxication in the rat are similar to those observed clinically in man. Renal effects of lead poisoning are progressive.

Additional observations have been made on the localization of lead in the central nervous system of the puppy and on lead-induced changes in myelination of the CNS in the rat. The effects of lead on reproduction have been investigated in rats, mice and chicken, and the effects on development of the rat's cerebral cortex under different nutritional situations have been studied.

Additional investigations have been started involving other metals, i.e. a comparison was made of bismuth-induced inclusion bodies in the rat kidney with lead-induced inclusion bodies, and the hepatic and renal toxicology of cadmium has been explored in the rabbit.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Heavy metals have been known for their poisonous properties for a long time. In recent times the increasing pollution of our environment has become a tough problem and, consequently, toxic metals for which degradation and elimination from the environment does not offer an easy way out must be studied in depth to appreciate their toxic action.

PROPOSED COURSE: The contract has been discontinued, but the project director has applied for a grant and is receiving NIH support, so that this important effort is not stopped or delayed.

PUBLICATIONS

Goyer, R.A., May, P., Cates, M., and Krigman, M.R.: Lead and protein content of isolated intranuclear inclusion bodies from kidneys of lead-poisoned rats. Lab. Invest. 22: 245-251, 1970.

Goyer, R.A., Leonard, D.L., Moore, J.R., Rhyne, B. and Krigman, M.R.: Lead dosage and the role of the intranuclear inclusion body. Arch. Environ. Health. 20: 705-711, 1970.

Carroll, K.G., Spinelli, F.R. and Goyer, R.A.: Electron probe microanalyzer localization of lead in kidney tissue of lead-poisoned rats. Nature 227: 1056, 1970.

Goyer, R.A.: Lead Toxicity: A problem in environmental pathology. Am. J. Path. (In press).

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Six, K.M. and Goyer, R.A.: Experimental enhancement of lead toxicity by low dietary calcium. Lab. Clin. Med. 76: 933-945, 1970.

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Rhyne, B.C. and Goyer, R.A.: Cytochrome content of kidney mitochondria in experimental lead poisoning. Exp. Molec. Path. (In press).

Six, K.M. and Goyer, R.A.: Lead toxicity: Synergism and antagonism. Chapter in National Research Council Monograph on Health Effects of Low Levels of Lead. (In press).

Goyer, R.A. and Chisolm, J.J., Jr.: In D.H.K. Lee and P.G. Condliff (Eds.) Chapter in Metallic Contaminants and Human Health. (In press).

UNIVERSITY OF PITTSBURGH, PITTSBURGH, PENNSYLVANIA (PH-43-62-147)

TITLE: "Carcinogenic Hazards in Industrial Populations (Steel)"

CONTRACTOR'S PROJECT DIRECTOR: Antonio Ciocco, Sc.D.

PROJECT OFFICERS (NIEHS): J. William Lloyd, Sc.D. and Frank E. Lundin, Jr., M.D.

DATE CONTRACT INITIATED: December 1961

CURRENT ANNUAL LEVEL: $80,000 (obligated in FY 1970)

PROJECT DESCRIPTION

OBJECTIVES: Detection of carcinogenic exposures in industry for the protection of workers and fuller understanding of the susceptibility of human subjects to chemical carcinogens are objectives of the project. Other objectives include the determination of non-carcinogenic effects of occupational exposures and the development of methods to discriminate between causal and non-etiologic associations.

METHODS EMPLOYED: A cohort of approximately 59,000 steelworkers who were employed in the Pittsburgh are in 1953 has been followed for mortality analysis from 1953 through 1961. Five additional years of mortality experience has been assembled. Employees in or around 1953 of ten coke-oven plants were compared with matched control groups of steelworkers in widely scattered geographic areas in the United States and Canada with regard to mortality by cause. Records of the cohorts who left the industry prior to January 1, 1967, were checked with the Post Office Department, the Bureau of Old Age and Survivors Insurance, and other governmental agencies to determine the status of these employees as of December 31, 1966. Death certificates for employees who expired in the study period were obtained from state vital statistics divisions and were classified as to cause of death.

MAJOR FINDINGS: Men employed on the topside of coke ovens have a six-fold excess of lung cancer.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The uncovering of industrial hazards will lead to safer work practices within industry and to information concerning the etiology of and susceptibility to cancer. These studies are of great value in developing methodologies for field studies of chronic illness consequent to occupational exposures.

PROPOSED COURSE: A paper on mortality by work area has been published. It is noted in this report that men employed in the coke plant experienced three times the expected level of respiratory cancer mortality. A more detailed analysis of mortality by work area within the coke plant has been published. This project has been transferred to the Bureau of Occupational Safety and Health.

UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE
(Interagency Agreement Between NIEHS and AEC)

TITLE: "Uranium Mining - Health Hazards Associated with Radon Daughters"

CONTRACTOR'S PROJECT DIRECTOR: William F. Bale, M.D.

PROJECT OFFICER (NIEHS): William W. Payne, Sc.D.

ASSOCIATE PROJECT OFFICER (NIEHS): Phillip J. Walsh, Ph.D.

DATE CONTRACT INITIATED: June 30, 1968

CURRENT ANNUAL LEVEL: $119,000

PROJECT DESCRIPTION

OBJECTIVES: In five separate but closely interrelated projects, senior scientists are conducting the following studies:

Project A - Dr. Donald Morken has exposed dogs to radon and radon daughters on room air nuclei. The magnitude and intensity of exposure versus early histopathological effects are important initially.

Project B-1 - Dr. John Hursh is studying the bone and tissue distribution of ^{210}Pb as well as excretion of ^{210}Pb and ^{210}Po using dogs exposed in Project A.

Project B-2 - Dr. Hursh has measured the transport time of ^{212}Pb from human lungs.

Project C - Dr. Thomas Mercer has characterized the radon daughter carrying aerosols used in the studies by Morken and Hursh.

Project D - Dr. R. W. Helmkamp and Dr. W. F. Bale developed new techniques for the measurement of ^{210}Po and ^{210}Pb in urine.

MAJOR FINDINGS: Project A - Effects on Dogs of Radon Decay Products - A total of 40 dogs have been exposed to radon daughter levels from 250 - 10,000 WLM. Six dogs exposed to 1,000 WLM in 1968 were sacrificed at six months and one year post-exposure and showed no significant early pathological changes. Six of the 30 dogs exposed in 1969 were sacrificed at one year post-exposure. Three dogs with 250 WLM exposures and three dogs with 3,750 WLM exposures showed only subtle early pathological changes with the three at the higher exposure level showing more frequent changes. The findings for ten additional dogs will be available by June 1971 at various exposure levels and post-exposure times.

Project B-1 - Measure of ^{210}Pb Effective Half-Live in Dogs - The measurement of the exretion of ^{210}Pb from dogs indicate that the excretion curve is similar

to radium. The retention at 400 days appears to be about 10 percent. Whole body counting overestimated the excretion as compared to excretion measurement in urine and feces because of burial of ^{210}Pb in bone and other tissues.

Project B-2 - The Transport of ^{212}Pb from the Lung in Man - The effective half life for the loss rate of ^{212}Pb from the human lung was measured to be 10 - 12 hours.

Project C - Research on Attachment of Radon Decay Products to Airborne Particles - The atmospheres used by Morken for the dog exposures and by Hursh for the ^{212}Pb loss rate study were characterized according to activity distribution on the carrier aerosol and the percent of unattached activity. Basic information on the properties of atmospheres containing radon daughters was accumulated. New samplers which segregate the radio-activity by particle size have been developed and have been made available for use in uranium mines.

Project D - Methods for Improved Bioassay of ^{210}Pb Body Burdens by Determination of ^{210}Pb and ^{210}Po in Urine and Feces - Dr. Helmkamp and Dr. Bale have developed a method which appears to quantitatively recover ^{210}Po from two liter collections of urine. A specially designed ionization chamber counter is used to improve counting efficiency; in effect eliminating many of the preparation steps previously necessary.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project is an integral part of research directed toward the resolution of the uranium miner-lung cancer problem. This project should yield useful information on induction of lung cancer and on heavy metal metabolism.

PROPOSED COURSE: Projects B-2, and C have been completed. The techniques developed in Project D will be combined with the work in Project B-1 to correlate ^{210}Pb-^{210}Po excretion in the Project A dogs with their cumulative exposures. Plans for Project A dogs will be developed according to the results of recent sacrifices.

PUBLICATIONS

Hursh, J. B. and Mercer, T. T.: Measurement of ^{212}Pb loss rate from human lungs. Journal of Applied Physiology. (In press).

Mercer, T. T.: The deposition of Unattached Decay Product Atoms in an Impaction Stage. Health Physics 17:259 (1969).

Blair, H. A.: Dose-time relations for induction of lung cancer in uranium miners. Reprint from symposium on "Radiation-Induced Cancer." International Atomic Energy Agency, Vienna (1969).

UNIVERSITY OF TEXAS; AUSTIN, TEXAS (NIH-70-2288)

TITLE: "Detection of Human Somatic Mutations"

CONTRACTORS PROJECT DIRECTOR: H. Eldon Sutton, Ph.D.

PROJECT OFFICER (NIEHS): W. G. Flamm, Ph.D.

DATE CONTRACT INITIATED: June 22, 1970

CURRENT ANNUAL LEVEL: $25,000

PROJECT DESCRIPTION

OBJECTIVES: This investigation is designed to develop methods for measuring the frequency of somatic mutations at several genetic loci in individual persons. Blood cells are the tissue studied, since the production of these from a stemline may lead to the accumulation of many more mutations than occurs in tissues with slower turnover of cells. The system currently under investigation is the X-linked enzyme glucose-6-phosphate dehydrogenase in white cells. An effort will be made to detect mutant cells in peripheral blood and to develop methods of screening for such mutant cells which can be applied to large-scale monitoring.

METHODS EMPLOYED: Histochemical techniques are used to quantitatively assess the presence of rare mutant variants occurring at the glucose-6-phosphate dehydrogenase (G6PD) locus on the X-chromosome. This involves the employment of a substrate (2-deoxyglucose-6-phosphate) and co-factor (DPN) which cannot be metabolized or utilized by the wild-type form of G6PD. The mutant cell, with its altered G6PD enzyme, will oxidize 2-deoxyglucose (to 2-deoxygluconic acid) and reduce DPN to DPNH. By adding phenozinemethosulfate and a tetrazolium salt, DPNH reacts to form a dark blue, insoluble formazan which is microscopically visible. In the absence of added substrate, but in the presence of DPN no detectable production of formazan occurs.

MAJOR FINDINGS: Many of the laboratory variables influencing the ability of white blood cells to utilize 2-deoxyglucose phosphate as a dehydrogenase substrate have been identified and controlled. It is now possible to detect variant cells which utilize this abnormal substrate with reliability. The frequency of such cells is dependent on methods of preparation, however, and we are concerned that there may be differential survival of cell type during processing. It is also possible that some of the positive variant cells arise as a result of treatment, even though there is reason to believe this is not true for the majority of variants. Cultured lymphocyte cells have been examined and show the same variant behavior with low frequency; and paired daughter cells appear always to be identical, either both normal or both variant.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The development of systems which can be used to monitor the exposure of individuals to mutagenic events is extremely important. Such exposures undoubtedly occur as a consequence of certain occupations and as a result of generally available mutagenic agents in the environment.

PROPOSED COURSE: Attempts will be made to clone lymphocytes on filters where larger numbers of cells of common origin can be studied. Also, establishing the relationship between an individual's age and the incidence of variant cells will reflect on the same question. In this way, it is hoped that the mutational origin of these cells can be demonstrated. This continues to be the primary objective.

WAYNE STATE UNIVERSITY (PH-43-66-53)

TITLE: "Epidemiologic Studies of Occupational Exposure to Cutting Oil Mists"

CONTRACTOR'S PROJECT DIRECTOR: Thieu Lenh Nghiem, M.D., M.Sc., D.P.H.

PROJECT OFFICERS (NIEHS): Frank E. Lundin, Jr., M.D. and Leonard G. Salvin, A.B.

DATE CONTRACT INITIATED: August 10, 1965

CURRENT ANNUAL LEVEL: $105,000 (obligated in FY 1970)

PROJECT DESCRIPTION

OBJECTIVES: To determine whether occupational exposure to cutting oil mists produces significant mortality from diseases of the respiratory tract.

METHODS EMPLOYED: A mortality study is made of approximately 24,000 subjects who were employed for one or more years between 1937 and 1967. Approximately 10,000 to 12,000 of these subjects have been exposed to cutting oil mists. Length, time, and intensity of exposure to cutting oil mists is determined from employment records. Death certificates are located through retirement and insurance records and the Social Security Administration. Using a life-table technique, person-years-at-risk by age, race and year are determined. The cause-specific mortality among the study group, classified according to the duration of exposure to cutting oil mists, is compared with that for the general population of the area.

Approximately 1300 employees currently exposed to cutting oil mists and an equal number of control subjects completed questionnaires which include smoking histories. This information will be used to estimate the effects on lung cancer mortality of differences in smoking habits among subgroups of the study population classified by degree of exposure to cutting oil mists.

MAJOR FINDINGS: There have been no major findings at this time.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Carcinogenic hazards for man can be identified by epidemiologic studies of the effects of selected occupational exposures. In this way, preventive measures may be undertaken.

PROPOSED COURSE: This project is being transferred to the Bureau of Occupational Safety and Health.

RESEARCH SERVICES BRANCH

RESEARCH SERVICES BRANCH
Summary Statement

The Research Services Branch is responsible for planning, coordinating, and providing support services for the National Institute of Environmental Health Sciences including: 1) research facilities planning, design and construction; 2) plant engineering, alteration, operation and maintenance; 3) physical factors research and instrumentation development and maintenance; 4) environmental sanitation and engineering services; 5) sterile glassware and media preparation; 6) security; 7) safety; and 8) landscaping and grounds maintenance.

The Institute's research program on the effects of physical agents has been initiated in this branch because of its close relationship to instrumentation development and maintenance. While administrative direction is provided by the Research Services Branch, the research activities are discussed with and reviewed by the Associate Director for Laboratory Research so that this program will operate as an integral part of the intramural research program. When additional resources become available, it is expected that the Biophysics and Biomedical Instrumentation Branch will be activated and that research on physical factors will be included in the new branch.

A microwave generating system has been installed and is operating. The system operates at 2450 MHz with a power range of 0.01 to 200 mW/cm^2. The system has been calibrated three dimensionally and is now functional for developing dosimetry techniques and exposing biological systems to defined microwave fields. Some studies have already commenced collaboratively with staff of the Cell Biology Branch to determine thermal and possible non-thermal effects of microwave exposure to various biological test systems. A second microwave system is under design and will be purchased this fiscal year which will permit defined exposures to frequencies from 1,000 to 10,000 MHz at power levels of 0.01 to 10 mW/cm^2.

Studies involving the impedance of the middle ear are being initiated with the return to the Institute of Reginald O. Cook from graduate studies at North Carolina State University. The major initial objective will be to design and develop improved instrumentation for measuring impedance of the middle ear.

The engineering design and construction group supervised the completion of the Phase II facilities and a new office building (Bldg. #17). Phase II will house approximately 165 additional staff and Building #17 approximately 30.

Serial No.: NIEHS-RSB-001
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Respiratory Tract Dose Model for Uranium Miners"

PREVIOUS SERIAL NUMBER: NIEHS-RSB-01

PRINCIPAL INVESTIGATORS: P. J. Walsh, Ph.D.

OTHER INVESTIGATORS: D. I. McRee, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	1.2
Professional:	1.1
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to develop a respiratory tract dose model that will provide reliable estimates of the dose of radiation to the basal cells of the bronchial epithelium of the respiratory tract in the case of inhalation of alpha emitters.

METHODS EMPLOYED: Calculation of the dose to the basal cells in each anatomical region of the respiratory tract requires a detailed description of the exposure atmosphere, a detailed respiratory tract model, accurate techniques of deposition calculation and an accurate analysis of the effects of clearance.

MAJOR FINDINGS: The dose to the basal cells in each subdivision of the tracheobronchial tree cannot be reliably calculated at present. The reasons are:

1. The uranium mine atmosphere has not been characterized to the degree required.
2. Respiratory tract models are not detailed enough.
3. Equations to be used for deposition calculations are not strictly applicable.
4. The locations of the basal cells below the mucous blanket are highly variable and not yet fully known.

5. The geometrical and physiological properties of the mucous blanket and bronchial epithelium vary between people and are altered by various deposited materials.

6. Calculations based upon uncertain theory show that regional depositions are strongly influenced by the characteristics of the atmosphere, flow rates, breathing patterns and the respiratory tract model assumed.

The dose averaged over the bronchial epithelium of the entire tracheobronchial tree can be estimated much more reliably than the regional doses and is preferred for interim dose calculations. The relation between the exposure to radon daughters in units of working level months and dose in rads has been quantified. The average dose to the basal cells in the tracheobronchial tree per working-level-month (WLM) exposure has been calculated taking into account the contribution of the fraction of uncombined radon daughters. A confident estimate of the cancer related dose cannot be made at present. A conservative estimate of the dose/exposure conversion factor, based upon present information, is 6 rems/WLM. The differences in depth-dose curves for alpha particles from area sources and point sources have been described. The possible differences in biological response to hot spot versus uniform exposure have been discussed and experiments which should yield insight into the response of whole organs have been suggested.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The development of an ideal respiratory tract dose model would require improved input information. This information would be crucial to the assessment of the risk associated with the possible inhalation of any toxic material. Such a model would have immediate applicability to the studies of the health hazards of uranium mining.

PROPOSED COURSE: Experimental measurement of the deposition of ions and particulates in models of the tracheobronchial tree is one example of information needed to develop a comprehensive deposition theory (see NIEHS-RSB-007). The effects of the wall characteristics and eddy currents at bifurcations will be important considerations. Additional physiological and biological information is also needed. The respiratory tract dose model will be elaborated in accord with availability of basic input information.

PUBLICATIONS

Walsh, P. J.: Radiation dose to the respiratory tract of uranium miners - A review of the literature. Environmental Research 3:14-36, 1970.

Walsh, P. J. and McRee, D. I.: Depth-dose curves for alpha particles from area sources and point sources. Health Physics 20:352, 1971.

Serial No.: NIEHS-RSB-002
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Energy Loss Characteristics of Radiation in Matter"

PREVIOUS SERIAL NUMBER: NIEHS-RSB-02

PRINCIPAL INVESTIGATOR: P. J. Walsh, Ph.D.

OTHER INVESTIGATORS: D. I. McRee, Ph.D.; Frances Pendergrass, Technician

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	0.3
Other:	0.7

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to obtain more information on the main interactions of ionizing radiation with matter, such as ionization and excitation of atoms and molecules, and the more subtle interactions of radiation with molecules, such as the effect of the penetrating radiation upon molecular bonds. Such information will be basic to further analysis of the connections between physical energy loss and absorption processes and biological effects.

METHODS EMPLOYED: The measurement of the energy loss of radiation is accomplished by using a solid state radiation spectrometer. The complete spectrometer consists of a measurement chamber containing an alpha, beta or gamma radiation source, multiple samples of the material to be studied and a solid state radiation detector. The doses and dose rates of alpha, beta and gamma radiation can be varied at will by adjusting the source-absorber geometry; and the solid state spectrometer allows an accurate determination of the energy loss in the absorber at the same time. This provides an experimental check on theoretical dose estimation. Biological absorbers can be studied with this system.

MAJOR FINDINGS: Polyethylene, polypropylene, polystyrene and tissue equivalent plastic absorbers have been used to study the energy loss characteristics of alpha particles. Comparison of this data to alpha particle data for ethylene and propane gas indicate that hydrocarbon compounds with single carbon to

carbon bonds have a higher stopping power than compounds with double carbon to carbon bonds and that double carbon to carbon bonds have a higher stopping power than triple carbon to carbon bonds. Also the stopping power of the plastics is apparently greater than equivalent amounts of the gas. From earlier work and from data available in the literature, the energy loss and range of alpha particles in tissue equivalent gas has been calculated. These calculations neglected chemical binding effects. Experimental data for tissue equivalent plastic shows that the stopping power of the plastic is higher than that calculated for the gas. The range of alpha particles in tissue would then be less than was calculated from the gas data.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Further information on the nature of the interactions of ionizing radiation with matter is necessary for more accurate dose calculation and to improve our understanding of the connections between damaging insults to atoms and molecules and subsequent biological effects.

PROPOSED COURSE: This project may be expanded to:

1. Study the energy loss characteristics of radiation in cell suspensions and cell cultures and develop quantitative dosimetric concepts which describe the results;

2. Deliver known doses of radiation to cells at various dose rates to determine dose-response relationships as a function of dose rate and turnover time of the cells;

3. Study the relative effectiveness of alpha, beta, and gamma radiation for a given end point;

4. Use chemical or biological toxicants or protectors in conjunction with the radiation. Such studies could identify the most important molecular components in terms of biological response.

PUBLICATIONS

Walsh, P. J.: On the application of the uncertainty principle to stopping power theories. Health Physics 19:574, 1970.

Walsh, P. J.: Stopping power and range of alpha particles. Health Physics 19:312-316, 1970.

Walsh, P. J.: Possible effects of dose rate and turnover time of cells on dose response relationships: implications for radiation protection guidance. International Journal of Environmental Studies. (In press).

Serial No.: NIEHS-RSB-003
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Microwave Absorption by Biological Material"

PREVIOUS SERIAL NUMBER: NIEHS-RSB-03

PRINCIPAL INVESTIGATORS: D. I. McRee, Ph.D. and P. J. Walsh, Ph.D.

OTHER INVESTIGATORS: G. W. Flamm, Ph.D. and Donald Clive, Ph.D.

COOPERATING UNIT: Cell Biology Branch

MAN YEARS:

Total:	.5
Professional:	.5
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to determine the absorption of microwave radiation by biological material, to investigate the contribution of cell structure to the absorption, and to attempt to explain the mechanism of interaction between microwaves and the biological material.

METHODS EMPLOYED: DNA, RNA, protein, cell cultures and biologically simulated material (alcohol-water solutions) will be exposed to 2450 MHz microwave radiation. The amount of energy absorption will be measured, and the changes in the media will be evaluated with the assistance of the Cell Biology Branch.

MAJOR FINDINGS: Studies have been made which tend to suggest that non-thermal microwave fields at various frequencies interact with intracellular moieties to alter metabolic and/or genetic processes. Interpretation of these effects is difficult since mechanisms are not specified. The absorption of microwave energy by RNA, DNA, protein, and complete cells appear to be very frequency dependent. It is not known whether the absorption of 2450 MHz microwave radiation is different in the above media.

Some preliminary exposure of DNA and mouse lymphoma cells have been made. Drs. Flamm and Clive have been involved in these studies. The breaking of bonds in the DNA molecules occur at a very definite temperature. The specific temperature at which this occurs depends upon the percentage DNA and saline in the mixture. Samples of DNA-saline were exposed to microwave radiation

with the temperature rise less than that needed to break the bonds. The samples were then observed using a spectrophotometer. There was some change in absorbance of the sample. More experiments under better controlled conditions are necessary before definite conclusions can be drawn as to the effects of the microwave exposure.

Mouse lymphoma cells were exposed to 2450 MHz radiation. The cells were exposed at a power level of 90 mW/cm^2. From model experiments, it was determined that an equilibrium temperature of 37°C could be maintained at this power level. The cells were exposed for 1 hour, 2 hour, and 4 hour durations with specimens taken at the end of each exposure period. The effects of the microwaves on the cells are presently being evaluated by Dr. Clive of the Cell Biology Branch.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The mechanisms of interaction of microwave radiation and biological material need to be specified before an accurate evaluation of effects can be obtained. Many investigators believe that all effects, so far observed, are thermal effects. However, some studies have shown effects which were not due to rise in temperature. More basic information about the interaction mechanisms will assist in the interpretation of these non-thermal effects.

PROPOSED COURSE: Studies in the absorption of microwave radiation by simulated biological material will be performed in order to obtain a measure of the electrical properties of biological specimens. Cell components such as RNA, DNA, and protein as well as the cells themselves will be exposed, and the energy absorption measured. By studying the absorption of particular molecules which make up the cell, an insight into the mechanism of interaction will be obtained. Dr. Gary Flamm and Dr. Don Clive of the Cell Biology Branch will assist in the evaluation of the effects of the exposure on the specimens.

Serial No.: NIEHS-RSB-004
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Interagency Review of the Health Hazards of Uranium Mining - Comparison of Experimental to Empirical Results"

PREVIOUS SERIAL NUMBER: NIEHS-RSB-04

PRINCIPAL INVESTIGATOR: P. J. Walsh, Ph.D. for NIEHS

OTHER INVESTIGATORS: Subgroup I-B of the Interagency Review Group composed of scientists from NIH, EPA, BOHS, AEC

COOPERATING UNITS: Radiation Control Office, EPA, Bureau of Occupational Safety and Health, DHEW and the Atomic Energy Commission

MAN YEARS:

Total:	.5
Professional:	.5
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: This project is part of a Federal Interagency Review of the health hazards of uranium mining. Scientists from all the federal agencies are participating in providing information for the review and in reviewing the total body of information available on radiogenic lung cancer. The subgroup dealing with radiogenic lung cancer has identified responsible agencies and due dates for the various parts of the review. The NIEHS is the responsible agency for a comparison of empirical and experimental information on radiogenic lung cancer.

METHODS EMPLOYED: The epidemiological studies and toxicological studies were reviewed by other agencies. With their reviews as back-up information, the NIEHS reviewed the theoretical dose calculations and the relationship between the dose calculations and the epidemiological and toxicological results.

MAJOR FINDINGS: Summary and Conclusions of the Subgroup
Based on a comprehensive review of a large body of experimental and epidemiological information on the etiologic role of ionizing radiation in the induction of lung cancer, the following general conclusions are reached:

1. There is some consistence between the epidemiologic and the experimental

data regarding the sensitivity of the lung to radiation induced cancer. Lung cancer has been induced in both man and animal under a variety of exposure conditions. The epidemiologic data and data from two of the animal studies corroborate the provisional classification by ICRP Committee I that considers the lung to have a radiosensitivity with respect to radiation-induced malignancy approaching that of hematopoietic tissue.

2. The experimental animal data suggest that for the radiation induction of lung cancer, alpha emitters are more effective than X-rays. On the other hand, in the case of human exposure to alpha emitters, the epidemiologic data suggest that X or gamma rays be as effective and/or lower dose rates may be less effective than acute dosages in the radiation induction of respiratory cancer for a given total dose.

   However, the animal data are considered to provide a better basis for the derivation of relative effectiveness because the human studies are especially complicated by factors such as the amount of "wasted" dose, age distributions of the exposed populations, unexpressed latency and simultaneous exposure to other agents which may have potentiated the effects.

3. Neither the animal nor the human data can confirm or reject the existence of a threshold dose below which respiratory cancer would not be induced by radiation. In one study, respiratory lung cancers were associated in the dog with doses as low as 100 rad from acute external radiation exposures, and in another study involving rats with protracted doses as low as 70 rad following the inhalation of polonium 210. Human respiratory cancers were associated with dosages as low as 100 rad under conditions of acute exposure and respiratory cancers were associated with cumulative exposures greater than 120 WLM (dosages in excess of 120 rad) several years (usually more than 10) after the start of underground uranium mining inhalation exposure to radon and radon daughters.

   Based upon the above data, if a threshold does exist in the case of occupational exposure to radiation in underground uranium mines, it probably is below 120 WLM.

4. There is evidence that other factors may also be operative in the induction of lung cancer associated with radiation exposures. Particularly important in this regard is cigarette smoking. Other factors or agents associated with radiation in some of the reported studies which might interact with radiation as protectors, promoters, co-factors or synergists include respiratory infections, silica, ferric oxide, nickel, arsenic, diesel fumes and uranium ore dust.

5. Based on the information reviewed in this report, radiation emerges as a major etiologic factor in the induction of lung cancer even though other factors may be acting as promoters, co-factors or synergists in some of the studies. The importance of these other factors relative to the radiosensitivity of the lung per se cannot be evaluated at this time. Attempts should be made to reduce the risk from these other factors as they may become better identified. In the meantime standards for radon daughters should be developed which apply to the environment in which the miners

continue to work and live.

6. The above conclusions can be interpreted in the context of setting radiation standards for underground uranium miners as follows:

   a. The theoretical approach is ordinarily used in the setting of standards for occupational radiation exposures and generally employs an annual permissible dose of 15 rem to the lung. Using this approach, it can be conservatively estimated that a concentration of 1 WL (12 working level months per year) would result in an average dose to the bronchial epithelium of 12-24 rad per year. The highest regional doses at 1 WL would amount to roughly 120 rad per year. A confident estimate of the cancer related dose cannot be made at present. A conservative estimate of the dose/exposure conversion factor, based upon present information, is 6 rems per working level month.

   b. When considering the empirical approach, it should be recognized that cumulative exposures to greater than 120 WLM are associated with a statistically significant risk of excess respiratory lung cancer. Based on the dose effect model described in the report of IUMRRG Subgroup I.A., the excess cancer risk at 120 WLM is between 2 and 4 times the expected incidence, and the risk of excess lung cancer at cumulative exposure levels below 120 WLM would decrease at a rate which is somewhat less than proportional to the decrease in dose.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Information can be obtained from further advances in the studies of the health hazards of uranium mining which would be basic to inhalation toxicology and cancer research in general.

PROPOSED COURSE: The present review was completed in January 1971.

Serial No.: NIEHS-RSB-005
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Microwave Exposure System and Microwave Dosimetry"

PREVIOUS SERIAL NUMBER: NIEHS-RSB-05

PRINCIPAL INVESTIGATOR: D. I. McRee, Ph.D.

OTHER INVESTIGATORS: P. J. Walsh, Ph.D.

COOPERATING UNITS: Georgia Institute of Technology, Engineering Experimental Station, Electronics Division

MAN YEARS:

Total:	1.0
Professional:	0.9
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to develop a microwave exposure system for biological material which has a well-defined, uniform field characteristic; to study the mechanisms of interaction of microwave radiation with matter; and to develop dosimetric relationships for energy absorption.

METHODS EMPLOYED: A thorough study of the research requirements on the biological effects of microwaves was completed. Based on these studies, a set of specifications was written for a microwave exposure system which would generate a uniform, low-level microwave field in a small laboratory space. At present, it is difficult to accurately measure low level microwave fields and to determine the mechanisms by which the fields interact with matter. It is necessary, therefore, to conduct dosimetric studies so as to develop analytical interaction models. Microcalorimetry, thermister and thermocouple detectors, chemical reactions, liquid crystals, and photographic film are some of the technics which will be investigated.

MAJOR FINDINGS: A microwave exposure system with a frequency of 2450 MHz has been designed, built and the field calibrated. The microwave field at the specimen location was determined to be uniform within the design specifications with variation in power density as follows:

6 inch diameter area - ±5%
9 inch diameter area - ±10%
12 inch diameter area - ±15%

The system provides a power density range of 0.01 mW/cm^2 to 200 mW/cm^2. A paper has been written on the system and upon approval will be submitted for publication to "The Review of Scientific Instruments."

Thermisters and thermocouples have been used to measure the absorption of microwave energy in various specimens. The thermister appears to be an excellent device for measuring energy absorption by a biological specimen. Further studies will be performed to investigate if there is any interaction between the thermister and microwave field and if the presence of the thermister in the medium changes the absorbance of microwave energy. Thermoluminescent chips supplied by the University of North Carolina were exposed to 2450 MHz microwave field. These samples were checked with control samples. The microwave exposed chips were not different from the controls. We do not plan any further studies on thermoluminescence.

An analytical model for the power-time thresholds for lenticular damage has been developed. The absorption of energy in the eye results in a rise in temperature of the fluid in the lens. Determining the absorption and dissipation rate constants of the lens from existing data in the literature, an analytical model was developed for power-time thresholds for cataractogenesis.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The well defined microwave field will allow the Institute to perform accurate quantitative studies of the effects of microwave radiation on biological systems at a frequency of 2450 MHz. Using the thermister to measure the temperature profile of a specimen during irradiation enables us to duplicate this temperature in the controls using ordinary heating techniques. This procedure will enable us to separate thermal and non-thermal effects. The analytical model for threshold levels for the development of eye cataracts enables interested scientists to determine safe exposure levels and provides a technique for extention to all frequencies as more experimental data become available.

PROPOSED COURSE: Investigations to evaluate the thermister and other techniques in measuring the absorption and interaction of microwave radiation with matter will be continued. Determination of the electrical properties of biological material, and the physical and chemical mechanisms of interaction between microwave radiation and biological material will be studied.

PUBLICATIONS

McRee, D. I.: Thresholds for lenticular damage in the rabbit eye due to single exposure to CW microwave radiation: an analysis of the experimental information at a frequency of 2.45 GHz. Health Physics Journal. (In press).

Serial No.: NIEHS-RSB-006
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Ultraviolet Dosimetry"

PREVIOUS SERIAL NUMBER: NIEHS-RSB-06

PRINCIPAL INVESTIGATOR: D. I. McRee, Ph.D.

OTHER INVESTIGATORS: P. J. Walsh, Ph.D. and G. Flamm, Ph.D.

COOPERATING UNIT: Cell Biology Branch

MAN YEARS:

Total:	.2
Professional:	.2
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: The objectives of this project are to measure the actual dose to the cells when exposed to ultraviolet radiation and to develop analytical dose-response relationships. A comparison of the relative biological effectiveness of the ultraviolet radiation with ionizing radiation will be studied.

METHODS EMPLOYED: An ultraviolet exposure unit using 2537 Angstrom wavelength source is being designed. The unit will be used to produce damage in cell cultures. A study of the DNA repair mechanisms will be performed by Dr. Gary Flamm of the Cell Biology Branch. A UV photomultiplier tube will be used to determine the amount of energy absorbed by the cells.

MAJOR FINDINGS: An ultraviolet source has been obtained and calibrated. An exposure chamber which allows a variation in dose rate has been built. Seventy-seven percent of the energy output of the source is at 2537 Angstrom wavelength. The power density of the source ranges from 2 ergs/sec-mm^2 at 40 inches to 90 ergs/sec-mm^2 at 6 inches. In order to use the pulse height analyzer to measure the energy absorbed by the cells, the quantum density of the source must be drastically reduced. Spherical integrators made of silver lined spheres have been obtained. These spherical integrators will reduce the quantum density and maintain the spectral integrity of the source.

The ultraviolet exposure system is presently being used by Dr. Brandt to produce damage in cells. He is studying the damage and repair of mouse lymphoma

and Hela cells and is trying to develop an UV resistant or super repair cell.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Information on the amount of ultraviolet energy absorbed by a cell to a given amount of damage is important in studying the repair mechanisms and in comparing this damage to that caused by other types of radiation or agents. Knowledge of the actual dose to the cells can lead to analytical description of the interaction which may be applied to other types of cell damage.

PROPOSED COURSE: The spherical integrators will be incorporated into the UV readout system so that not only the exposure to the cells but also the dose to or absorption of the cells can be measured using the photomultiplier-pulse height analyzer system.

The dose of ultraviolet radiation to cell suspensions and cultures will be measured and theoretical dosimetric concepts which describe the phenomena will be made. A comparison of the relative biological effectiveness of the ultraviolet radiation to ionizing radiation will be performed in an attempt to develop broad dose-response relationships.

Serial No.: NIEHS-RSB-007
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Deposition of Aerosols In Tracheobronchial Tree Models"

PREVIOUS SERIAL NUMBER: NIEHS-RSB-07

PRINCIPAL INVESTIGATOR: Phillip J. Walsh, Ph.D.

OTHER INVESTIGATORS: D. I. McRee, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	.15
Professional:	.15
Other:	.00

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to study the deposition of aerosols in respiratory tract models. Accurate measurements of the deposition will be made and an improved theory formulated for calculating the magnitude of deposition along the walls and at bifurcations.

METHODS EMPLOYED: Aerosols with radioactive tracers will be passed through tracheobronchial tree models at flows common to man's breathing rates. Small solid state detectors will be used to survey the deposition throughout the models. An analytical description using fluid mechanical principles will be attempted in order to predict the amount of deposition and the mechanisms by which the deposition occurs.

MAJOR FINDINGS: A major problem in all inhalation studies is the determination of the deposition pattern in the respiratory tract. Present theory, used to calculate the deposition, has proven inadequate. The dose to given tissue cannot be determined until the deposition throughout the respiratory tract is better determined. Techniques of model preparation and deposition measurement are available. A contract has been initiated to begin work in this area.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The ability to predict the deposition of toxic materials in the respiratory tract will be very important in all inhalation studies. It will be possible to more accurately determine the dose to the tissue at different locations in the

respiratory tract as a result of this investigation.

PROPOSED COURSE: Experiments to measure the deposition of aerosols with radioactive tracers in tracheobronchial tree models will be performed. The flow rate and particle size will be varied so that the dependence of the deposition upon these variables can be determined. Theoretical studies will be made using fluid mechanical principles to describe the experimental results.

Serial No.: NIEHS-RSB-008
1. Research Services Branch
2. Biophysics and Instrumentation Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Variable Frequency Microwave Exposure System"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: D. I. McRee, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	.20
Professional:	.20
Other:	.00

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to design, build, and calibrate a variable frequency exposure system for biological material.

METHODS EMPLOYED: Specifications were written for the desired system. A field uniformity at the specimen of 90 percent over a 4 inch diameter circle, a continuous frequency variation in the range of 1 to 10 GHz, and a power level range at the specimen of 0.010 to 10 mW/cm^2 were specified. A complete search of the available microwave components was performed and from these components a system designed. Components for the system are now being ordered.

MAJOR FINDINGS: Many of the biological effects of microwave radiation is frequency dependent. In terms of whole body irradiation, a frequency of 150-1,200 MHz affects the internal organs, whereas, a frequency of 10,000 MHz and above affects primarily the skin. Components of cells such as RNA, DNA, and protein were found to have maximum absorption at different frequencies. Therefore, it is necessary to have the capability of varying frequency when investigating microwave effects on biological systems. The frequency range of 1 to 10 GHz was chosen because it is within this range of frequencies to which the general population is exposed.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Many biological effects of microwave radiation appear to be frequency specific. The variable frequency exposure system will provide the researchers of the

Institute the capability of studying the effects of different frequencies on biological systems.

PROPOSED COURSE: All the components will be ordered and assembled. The microwave field will be calibrated at the specimen location for power levels of 0.010 to 10 mW/cm^2 and throughout the entire frequency range. Since there is no accurate detector available for some of the frequency range, investigations will be made into power measurements at these frequencies.

Serial No.: NIEHS-RSB-009
1. Research Services Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Development of Device for Measuring Impedance of the Middle Ear"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: R. O. Cook

OTHER INVESTIGATORS: L. Royster, Ph.D., North Carolina State University,
G. Thomas, Ph.D., University of North Carolina (advisors)

COOPERATING UNITS: None

MAN YEARS:

Total:	1.1
Professional:	1.0
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: Better methods are needed for measurement of middle ear impedances. The subject project will involve design, modeling and prototype development of a device capable of generating, sensing and transmitting the necessary parameters.

The investigator has returned from outside-the-service training in acoustics.

METHODS EMPLOYED: As in any development project, efforts will be divided between design and prototype building. The size, sensitivity and reliability of the transducers, since they must fit down in the ear canal, will present formidable obstacles.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Present impedance measuring devices are limited to very low frequencies, are unwieldy, require very precise measurements of ear canal volume and near immobilization of the subject, and are limited as to range of atmospheric pressures which can be tolerated. For instance, a number of subjects in the Duke University hyperbaric chamber studies have experienced temporary threshold shifts, and some have experienced permanent threshold shift. It is thought that a major contributor may be impedance change attributable to the high pressure helium environment.

PROPOSED COURSE: Minaturized, adequate pressure and velocity transducers appear to be available. They will be acquired and tested. Design of the energizing transducer and its testing will occupy a major part of the effort.

Serial No.: NIEHS-RSB-010
1. Research Services Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Adaptation of Zwicker Loudness Calculation Scheme for Annoyance Evaluation"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: R. O. Cook

OTHER INVESTIGATORS: F. D. Hart, Ph.D., Director, Center for Acoustic Studies, North Carolina State University; S. E. Dunn, Ph.D., North Carolina State University

COOPERATING UNITS: None

MAN YEARS

Total:	.1
Professional:	.1
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: The objective is to redesign the Zwicker Nomographs for calculations which measure the annoyance rather than the loudness of sounds.

METHODS EMPLOYED: Mathematical and graphical techniques, along with the curves for equal annoyance developed by K. D. Kryter, will be employed. Results will be compared with results of judgment tests previously published in the literature.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The method developed by Zwicker mimics the sound energy summation properties of the human auditory system by correctly accounting for spread of masking and critical band properties. However, it was developed for subjective loudness calculations, which seem to be the preferred subjective parameter in Europe, whereas the annoyance parameter has been more widely used in this country. The methods developed by Kryter for annoyance estimation give good results as long as the spectrum is a relatively broad band, but are subject to error where energy peaks are widely separated in the spectrum.

PROPOSED COURSE: To redesign the Zwicker Nomographs for calculations which measure the annoyance of sounds.

Serial No.: NIEHS-RSB-011
1. Research Services Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Determination of Impedance of Middle Ear at two to three atmospheres pressure (in air)"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: J. Farmer, M.D., Duke University; G. Thomas, Ph.D., University of North Carolina; Reginald O. Cook, NIEHS

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	.2
Professional:	.2
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: Objectives are to gain information relative to the change in impedance with increased pressure.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: At present, no data exists on the sound transmissibility of the ear under several atmospheres pressure. Knowledge of transmissibility characteristics will make possible a better understanding of the permanent and temporary threshold shifts noted in personnel using the Duke University hyper-baric chamber.

OFFICE OF THE ASSOCIATE DIRECTOR FOR LABORATORY RESEARCH

OFFICE OF THE ASSOCIATE DIRECTOR FOR LABORATORY RESEARCH
Summary Statement

During this fiscal year the intramural laboratory research program has grown in width as well as in depth in response to the need for additional research to understand the new hazards in our environment, to forestall disasters, and to make scientifically sound recommendations for revisions in the use of questionable agents.

Because questions have been raised repeatedly by interested parties in and outside of Government regarding the activities of the National Institute of Environmental Health Sciences and how it may differ in mission from similar newly created institutes and facilities, it may be worth-while restating in practical terms the approach which has been taken by the NIEHS in contributing to the solution of environmental health problems.

The intramural program has aimed at developing a high degree of competency in fundamental research so that chemicals which may cause environmental health problems can be studied adequately with regard to their degradation, before and after use, and for their purity as they reach the market. Such information is frequently not available when a problem arises, but inhouse research can immediately go to work. When such information is available, it should be corroborated and extended.

Competency has been developed at the Center in determining the absorption, retention, metabolism and elimination of chemicals. Thus, comparative data can be obtained for those species in which the tests for potential toxicity are to be carried out. Adequate steps have not as yet been completed to obtain the most important species for this comparison, namely man.

For evaluation of physical factors which may present human health hazards, efforts are under way for adequate quantitation of the dosage of exposure to the potential hazards before major studies on interaction of such factors with the experimental animal will be initiated.

Evaluation of toxicologic properties of environmental agents relies on the usual toxicologic techniques for preliminary studies, and much attention is directed towards determining early noticeable effects which may under many circumstances not be harmful but even beneficial. Enzyme induction in the hepatic microsomal fraction by many environmental chemicals needs careful evaluation of its existence in those tissues foremost in contact with environmental chemicals: the respiratory and gastro-intestinal tissues. These tissues have the capability of responding by enzyme induction. Of great importance, however, is the interference with enzyme induction by certain common air pollutants, such as carbon monoxide or lead. It is necessary to investigate this process as it is not certain whether we are dealing with a protective process which when fully understood can be

utilized to advantage, or with a response which on continuous stimulation may actually produce untoward effects. An important point may be that for most of the typical enzyme inducers which also show toxicologic expressions for specific organs, there is a difference in dosage for the two parameters which allow for a considerable degree of safety before the toxic level is reached, but leaves little time before the dose for induction of enzymes is encountered by man in his daily life. The toxicologic studies will duplicate man's route of exposure whenever possible; thus, his exposure to air pollutants will be studied by inhalation. The usual toxicologic studies on oral intake of the chemical will also be followed. Chronic studies can now be carried out with the assured hope that undesired and unplanned, intercurrent stresses to the animals can be avoided by the use of new adequate animal housing facilities. Emphasis on interference with growth and development by environmental chemicals will be continued and expanded. These studies will, hopefully, take into consideration the types of human exposure and will control other factors which may alter the host's responses.

The importance of adequate surveillance of environmental hazards which may express themselves by increasing the mutation rate of the population has long been recognized by this Institute. The knowledge in that field, including the recommendations by the NIEHS Task Force in 1969 and the publication of a textbook on chemical mutagens by scientists at the Institute, does not allow us to relax about the state of the art. On the contrary, new and adequate test systems are still not available and much effort at this Institute goes into the development of sensitive test systems for rapid use.

The most frequent complaint deals with problems of extrapolation of laboratory data to the actual exposure of man as it happens today or may become in the future. It is important that the experiments have the foundation for careful extrapolation to man, so that the results may serve as solid ground for action to be recommended by agencies responsible for such decisions. Scientists usually rely on young and healthy rodents which are deliberately kept from all other stresses and exposures, so as to avoid complicated results. This, of course, is far from the experience of the average person, who throughout life is exposed to a myriad of stresses which may not wait to come in succession but may coincide. These multiple stresses of a physical, chemical, biological and psychological nature must be considered and evaluated to come to a realistic appraisal of the true hazard for man.

The Institute has adopted this panorama of interacting influences on the health of man as its mission and even though some of the experiments cannot be carried out in all the ramifications suggested above, the interpretation of all data must consider these various synergistic or antagonistic environmental insults as well as the unavoidable debilitation of host defenses which must occur with the passing of time. Built-in obsolescence applies to all organs of the human body and must be given serious attention.

To evaluate specific situations observed in animal models as they may apply to man, scientists at the Institute develop new models by the choice of different but unique species, like the opossum or the galago, or the choice of organ systems, tissues, or subcellular organelles, to obtain meaningful answers. With such systems fully developed, these scientists are then in a position to find answers for specific questions about environmental pollutants as these may arise.

Because of the multiplicity of problems under investigation, a detailed overall summary of laboratory research activity would be too large. This summary statement will therefore restrict itself to the highlights of activity of this office as it relates to laboratory research activity of the Institute, referring the reader to the Branch Chiefs' summary statements for more information.

If a specific chemical should be singled out that brought attention this fiscal year to one research activity at the Center, it is the polyphosphate replacement in household detergents, nitrilotriacetate or NTA.

After a quiet beginning in April of 1970 when the Federal Water Quality Administration requested the cooperation of this Institute and others of HEW to help advise them on the safety of NTA, preliminary results of these studies have become known and have produced a great deal of concern.

On September 8, Canadian and British visitors representing their industry's point of view came to discuss the problem of polyphosphate replacement by NTA. It was the feeling of the British that their island did not have a detergent problem and of the Canadians that there were other solutions which would not require replacing polyphosphates in detergents.

On December 16, a visit to the Center was made by a Monsanto Chemical Company scientist to discuss NTA. This meeting was held shortly before the Surgeon General's release of information on the teratogenicity of NTA metal complexes and was timed poorly in that NIEHS' information could not be made available, while Dr. Hill informed us in detail about the company's research effort to establish whether nitrosamines could be formed during degradation of NTA and whether this compound would be stable. Valuable information was also presented on degradation of NTA under realistic conditions, as well as on degradation of specific metal chelates which showed that at higher levels (5 ppm), several of the metal chelates were no longer degradable. This suggested that organisms or specific enzymes can be inactivated by contact with these heavy metals.

On December 18, the Surgeon General presented the NIEHS data on NTA to industry. As a result of that meeting and based on a feeling of uncertainty whether NTA would be a safe product under all circumstances, the companies decided to hold off large-scale production of NTA-containing detergents until they could come up with scientific data to supplement those obtained by NIEHS. This data should clarify beyond a doubt whether a potential hazard due to NTA metal chelates exists.

On December 21 a number of scientists from the Canadian Department of Health and Welfare visited the Institute and our scientists, Dr. Courtney and Dr. Chernoff, who had been responsible for the teratogenicity studies of NTA. Because of Canada's involvement in a ban on polyphosphates, these scientists needed to know in all detail what the findings on NTA had been and what these findings might imply.

The next day this office received an inquiry from Mr. Nader's office on potential toxicity of new non-polyphosphate, non-NTA detergent formulations.

On December 23, Proctor and Gamble requested a meeting between industry's scientists and those at the Center involved in the testing of NTA. This meeting was held at the Center, and protocol as well as results of the teratogenic studies were reviewed in depth.

On January 20, a group of Swedish scientists came to the Center to talk to our scientists and the Associate Director about the NTA data, their interpretation, and future research developments in this area. They presented an industrial point of view, similar to that of Proctor and Gamble and Monsanto, and similar arguments were presented by them as had been heard before.

The Associate Director appeared with the Special Assistant to the Surgeon General before staff members of the Committee on Public Works, Jennings Randolph, Chairman, on January 21, 1971. They answered questions regarding the toxicologic testing of NTA, to serve the committee as illustration for future decisions on legislative needs to control the "safety testing" of chemicals before their release into the environment. The case of NTA was used as example.

This office responded to a letter from Congressman Cleveland addressed to the Surgeon General regarding NTA's earlier "adequate evaluation," specifically answering questions on nitrosamine formation and metal chelate toxicity tests.

On March 13, the Associate Director was asked to attend a meeting of the Panel on Chemicals and Health of the President's Science Advisory Committee, Dr. John W. Tuckey, Chairman, to report on the research carried out at this Institute with NTA. The opportunity was also given at that time to discuss data regarding the carcinogenicity of optical brighteners in the presence of ultraviolet light. This committee concerned itself very seriously with the problem of responsibility for investigation of environmental hazards not covered by the Food & Drug Act. The case of NTA toxicity was considered a good example of the need to strive for a clear, authoritative Governmental responsibility in this respect.

Next, the detergent industry requested a meeting in the Surgeon General's office on April 12 to bring his office up-to-date on their own results with NTA. At this meeting attended by NIEHS, no data were presented that could be reviewed and analyzed in depth, but the general conclusion was presented that there were no hazardous results from exposing animals to

NTA and heavy metals beyond the expected toxicity due to heavy metals. Because experiments had not been done to confirm or contradict the original findings obtained by NIEHS, the Surgeon General's office recommended that industry attempt to repeat as best as possible these experiments. To follow that recommendation, a meeting of industry and NIEHS scientists was held at the Center on April 20 to devise the best protocol which would duplicate and improve the experiments done previously. Such a protocol, with approval of all scientists concerned and the blessings of the Director of NIEHS, the industry representatives hoped will become the definitive experiment on which final decision about the safety of NTA could be determined. A follow-up telephone call on April 26 regarding this protocol was received by this office and another meeting at HEW was held with industry representatives on April 29.

Meanwhile, scientists from American companies visited the Center to discuss new NTA replacements, and a visitor from the German industry discussed NTA metal chelates and the absence of their degradation under certain conditions. Also, Swedish scientists entered the discussions.

As a spinoff of this story on metal chelation, interest was stimulated regarding actual health hazards of heavy metals reaching the environment.

A science writer of a Washington paper was invited to the Center in order to learn about heavy metals and their chelation as a potential hazard. Some of the information has been published in the newspaper following that interview.

Science writers from Fortune Magazine visited the Center to discuss health problems relating to trace elements. They spent several hours discussing all aspects of environmental contamination and the magnitude of the problem in the light of what appeared to them as conflicting statements made by Dr. Schroeder and the Surgeon General. Although the two points of view seemed mutually exclusive, an explanation of the apparent contradiction could be offered.

On March 2, The Associate Director was visited by Dr. Agthe of the Unit of Chemical Carcinogenesis, International Agency for Research on Cancer, World Health Organization, Geneva, Switzerland. Dr. Agthe discussed the function of his agency and the special problem for which he visited the Center; namely, to invite the Associate Director to participate in the writing and reviewing of documents on evidence of carcinogenicity of the same metal compounds for man and animals. These monographs, also to be compiled on other carcinogenic chemicals, will be published in order to serve as a baseline for future international research in carcinogenesis and extrapolation of these results.

Of all the heavy metals, much of the adverse publicity in recent months had focused on alkylmercury compounds which were shown to be exceedingly toxic. They were shown to reach man through certain food staples which had not been expected: Metallic mercury was transformed into alkylmercury by biologic systems and in this form found its way into the food chain.

To bring all interested scientists of the Center up-to-date on the mercury problem, a joint meeting was held with the FDA in Washington. Subsequently, an inhouse meeting at the Center alerted all scientists to the problem. Research activities were thus initiated in every Branch of the Institute. Half-a-year later a second alkylmercury conference was held at the Center to discuss the results and conclusions of this research activity. The complete discussion was recorded and a document was prepared on NIEHS findings on methylmercury toxicity.

A telephone request from the Council of Environmental Quality asked for recent findings on methylmercury toxicity which were made available by telephone and letter.

Information on the safe handling of methylmercury was sent by our scientists as a letter to the editor of Science and was accepted.

Steps were taken to protect all personnel from any accidental exposure, to monitor blood concentrations, and to review future studies involving methylmercury.

In this connection the disposal of mercury and other toxic metals came under scrutiny. Recommendations were made by the Associate Director regarding the disposal of mercury, silver, osmium, and a number of other compounds which were discussed by the Director at a meeting of the Scientific Directorate of NIH.

Other metals were not forgotten while much of the emphasis focused on mercury. The Associate Director responded to a call from the Surgeon General's office to review the carcinogenicity and mutagenicity of lead compounds. The Associate Director also responded to requests for review for NAPCA of the first draft of the Air Quality Criteria document on lead.

The Associate Director reviewed other Air Quality Criteria documents for NAPCA, specifically one on fluoride and on oxides of nitrogen. The comments on these documents as presented to NAPCA were also requested by the Surgeon General's office.

A major effort was the review of the document entitled "Particulate Polycyclic Organic Matter" which the National Research Council was preparing on request for NAPCA. The Associate Director has already committed himself to review another document proposed by the National Research Council on other carcinogenic air pollutants.

On January 22, members of the Federal Trade Commission visited the Associate Director to discuss the scientific background regarding air pollution by automobile emission and fraudulent claims made by an oil company in their advertising regarding reduction of air pollution. This in-depth review of all facets of air pollution, visible and invisible, was quite involved because all the FTC visitors had legal backgrounds rather than scientific. Reprints on the topic under discussion were supplied to enable them to solidify their scientific background in this field.

Because of the importance of the potential hazard of nitrates and nitrites in food, the Associate Director evaluated the existing literature and prepared background documents for discussion and presentation, particularly on the topic of nitrosamine formation. A monograph was prepared by the Institute.

The problem of cigarette smoking and health was not ignored. Several activities of this office were focused on reviewing documents and holding discussions on progress in research on smoking and health. On invitation by the U.S.D.A. Tobacco Research Laboratory at Oxford, the Associate Director spoke to scientists of Federal and state laboratories on problems of smoking and health and how changes in tobacco growing, culturing and processing may affect the properties of the smoke with regard to health.

On invitation by the University of Kentucky, Tobacco and Health Research, the Associate Director attended the Tobacco Health Research Conference on March 17-19 as a panel member and discussed bioassay procedures for evaluating cigarette smoke and health effects, as well as synergistic effects between viral infection and air pollutants. These discussions will be published in the proceedings of that conference. The conference allowed scientists from Government and industry to exchange ideas and to plan further meetings.

The Associate Director was asked to review for the National Clearing House for Smoking and Health two documents: a chapter on "Cancer and Smoking" for their annual report to Congress and a document on "The Effect of Secondary Smoking." The evaluation of the latter document was given directly to Dr. Horn on the telephone, and a second draft will be submitted later.

As a member of the Tobacco Working Group of the Lung Cancer Task Force, the Associate Director reviewed the NCI's suggestions for new contract activity and made numerous recommendations regarding priorities. On November 19, the Associate Director was visited by representatives of Pittsburgh Carbon to discuss the problems of effective charcoal filters for cigarettes in relation to recent FTC rulings on advertising of the tar and nicotine content of cigarettes.

As much of the activity of this Institute has always been focused on the toxicologic evaluation of pesticides, it is obvious that this office saw a good deal of activity in this regard. Of particular importance were reviews on the hazards of pesticide synergists.

On March 31, a meeting was held at the Center with scientists from Burroughs Wellcome & Company; Food, Machinery, Chemicals Corp.; and Cooper Technical Bureau from England on the toxicity of piperonyl butoxide and other pesticide synergists. The members of industry have been very much concerned with health hazards and wanted to be brought up-to-date on recent findings. Advice on pesticide synergists was also given to the National Communicable Disease Center scientists regarding their utilization in sprays in airplane cabins, and a summary report was invited by the German journal UMSCHAU on research on the toxicity of pesticide synergists carried out earlier by the Associate Director.

On April 8, Dr. Wasserman, Head, Department of Occupational Health, The Hebrew University-Hadassah Medical School, University of Jerusalem, visited the Center to talk to interested scientists about DDT residues in different populations by country, age, sex, ethnic origin and occupation. People who have high levels of DDT and derivatives in their tissue and blood will be followed clinically for pathologic and biochemical changes.

On invitation the Associate Director will attend a seminar on "Environmental Ecology and Pesticides" sponsored by the U.S. Navy Disease Vector Ecology and Control Center, Naval Air Station, Alameda, California, to discuss in detail pesticides and their carcinogenic hazards.

The toxicity of mirex was used by the Environmental Defense Fund in going to court against USDA to stop the spraying of mirex for controlling the imported fire ant. The Office of the Surgeon General was called upon to evaluate the health hazards resulting from the use of the chemical on a large scale. This Institute analyzed, in collaboration with other Federal laboratories, shellfish, shrimp and catfish. Marginal amounts of mirex were present in shellfish and shrimp, but several hundred ppb of mirex were present in catfish which had lived in waters where mirex pollution could be anticipated. On the basis of carcinogenicity data obtained by Bionetics Laboratories, findings of cataract formation in offspring whose mothers had been given mirex, as well as the apparent inability of species so far studied to metabolize this chemical, this office wrote, on request, several negative recommendations regarding the large scale use of this insecticide to the Surgeon General's office.

A meeting was held with scientists from Monsanto on chlorobiphenyls regarding their world-wide distribution and the means of controlling further wide-spread dissemination of these compounds.

A different and unique technique of pest control was the proposed utilization of the nuclear polyhedrosis virus as a harmless replacement for chemical insecticides which needed to be evaluated for the FDA regarding any future hazards. Several critical evaluations were made by this office for use by scientists at the NCI.

Following the publication of a paper on the combined activity of ultraviolet light and optical brighteners in producing cancer on the skin of mice, this office met with representatives of industry to discuss protocol for additional studies to clarify the problem and to determine whether enough evidence exists to suggest that brighteners be removed by industry from contact with man. Such information was also communicated on their request to scientists in Sweden and Great Britain.

On request by the Division of Biological Standards, the Associate Director discussed on separate occasions the protocol for carcinogenicity and cocarcinogenicity testing of adjuvants which had proved carcinogenic in a previous study with scientists of the Division and of Midwest Research Institute. Earlier this office and scientists of the Institute had served as a review board for the Division.

On invitation by the Heart and Lung Institute, the Associate Director presented data before the American Heart Association meeting in Atlantic City on November 11-12 on experimental evidence of carcinogenicity resulting from high intake of dietary fats in rodents. This paper entitled "Lipids and Experimental Carcinogenesis" was presented as a desired background document regarding a presentation by Drs. Pearce and Dayton of UCLA who documented that the polyunsaturated diet given to veterans after they had suffered a heart attack led to a low incidence of cardiovascular accidents but an increased incidence of cancer in this group in an eight-year follow-up study.

On January 15, this office advised a scientist from Columbia University on steps to be taken to further substantiate his findings on a long-term feeding study that specific unsaturated oils produced cardiac fibrosis in rats. Such observations were not made in rats on animal fat diets, and may present a new risk in shifting from animal fats to plant oils in the human diet. Contact was established with the Director of the National Heart and Lung Institute on this matter.

Although the herbicide 2,4,5-T filled much space in last year's summary report of the Office of the Associate Director, this year the topic is in comparatively low key. The activity existed in reviewing the Office of Science and Technology's reports on 2,4,5-T and 2,4-D for the Director of NIH. In addition, the teratogenicity of 2,4,5-T in rodents was discussed with a scientist of the National Institute of Dental Research and Drs. Courtney, Moore, and Chernoff. The data obtained in the two Institutes were compared. The additional research done by scientists at this Center confirmed that esters of 2,4,5-T possessed similar teratogenic activity as the free acid, while silvex, a closely related herbicide, was without teratogenic effect in mice. On request by the EPA, Dr. Courtney from this Institute presented once more all data to a review committee set up by EPA to evaluate the 2,4,5-T teratogenicity results and to explain the reasons why negative results were obtained by industry in a follow-up study.

Another meeting was held at the Center, bringing together scientists of FDA and NIEHS to discuss progress relating to research on "dioxins," the highly toxic impurity found in the earlier sample of 2,4,5-T. This liaison between scientists of two institutions served to produce a good scientist-to-scientist relationship. The discussion also covered research on mycotoxins carried out at the two facilities. Several visitors from Sweden representing the National Environmental Protection Board and the Department of Food Hygiene came to discuss a number of major problems in public health. The Swedish points of view were compared to our own on "dioxins," the chick edema factor and 2,4,5-T, among others.

As a member of the program committee for the Environmental Mutagen Information Center representing the NIEHS, the Associate Director attended a meeting on December 18 on future activity of this essential program supported by several agencies and carried out at Oak Ridge National Laboratory. There was general agreement on the need and usefulness of

this effort to compile and critically review information on mutagenic chemicals, but problems of financial support for this effort remained unsolved.

On invitation and request, the Associate Director attended a Senate hearing before the Subcommittee on Reorganization and Government Research, Senator Ribicoff, Chairman, to discuss the health hazard of mutagenic environmental chemicals. Dr. Flamm and Dr. Fishbein read their prepared statements and answered questions.

Invited lectures were given by the Associate Director on "Chemical Carcinogenesis" at Duke University in the Clinical Cancer Training Program, and at North Carolina State University on "Micro Contamination and Potential Carcinogenic Hazards." Additional services were rendered to scientists of the NCI in making a contract site visit, advising on problems in carcinogenesis, etc. On invitation by Drs. Homburger and Van Duuren, a chapter for Progress in Experimental Cancer Research, Vol. 14 was written on "Anti-Carcinogenesis - An Alternative."

On request by the Surgeon General, the Associate Director took part in project OPERATION CHASE to help in the solution of a public health hazard in a disposal problem originating with the Army.

Honors and Awards - The Associate Director was invited to be included in Who's Who in Government and to be listed again in American Men of Sciences, 12th Edition.

ANALYTICAL AND SYNTHETIC CHEMISTRY BRANCH

ANALYTICAL AND SYNTHETIC CHEMISTRY BRANCH
Summary Statement

The programs of the Chemistry Branch are designed to conduct and augment research related to the possibly harmful effects in a wide area of environmental exposures (e.g., pesticides, food additives, trace metals, fungal toxins, and air and water pollutants—including their metabolic and degradation products). They include the chemical analysis of multiple components in the elaboration of synergistic and potentiating effects of these agents.

The Branch is engaged in the development, improvement and standardization of new and improved analytical techniques for the determination of a spectrum of specific metabolic and degradation products arising from inter- and intra-Branch investigations as well as studies of the Branch involving other governmental laboratories. It utilizes diverse analytical procedures consisting of spectroscopy (visible, ultra-violet, infra-red, emission, fluorescence, mass and nuclear magnetic resonance) and chromatography (paper, thin-layer, column, radioautography and gas-liquid), using in the latter instance a variety of detectors such as flame, thermal conductivity and electron-capture for greatest efficiency.

Synthetic procedures are employed for the preparation of required intermediates for the confirmation of identity of metabolites and degradation products as well as for other Branch investigations; e.g., pharmacological, teratological, mutagenic and DNA-binding studies.

An important class of environmental agents is represented by the methylenedioxy moiety which is found widely distributed in essential oils, alkaloids and other physiologically active compounds of natural origin. Compounds containing this moiety are widely used in insecticides (primarily as synergists), food additives, perfumes and medicinals.

Studies are in progress to evaluate whether a potential hazard exists on exposure to these agents and to understand the mechanisms controlling enzymic detoxification of this important class of environmental agents. These studies include: the unequivocal identification of the nature of the metabolites of methylenedioxyphenyl food additives and synergists, their tissue distribution and rates of elimination following several routes of administration in several species and their effect on microsomal drug metabolizing enzymes.

Safrole (4-allyl-1,2-methylenedioxybenzene) is a major constituent of essential oils (sassafras, camphor and anise) and a minor constituent of oils of nutmeg, mace, cinnamon leaf, wild ginger and California bay laurel. It has been widely used in the past as a flavoring agent in root beer, chewing gum, toothpastes and certain pharmaceutical preparations. It is also used to scent

soaps and cosmetics and with isosafrole is employed in the production of piperonal and as an intermediate in the preparation of the insecticidal synergist piperonyl butoxide. Following oral or intraperitoneal administration of safrole to male rats and guinea pigs three tertiary aminomethylenedioxy propiophenones are excreted in the urine, e.g., 3-N,N-dimethylamino-1-, 3-piperidyl-1-, and 3-pyrrolidinyl-1-(3',4'-methylenedioxyphenyl)-1-propanones. All three amino derivatives decompose to form 1-(3',4'-methylenedioxyphenyl)-3-propen-1-one.

Myristicin (1-allyl-3-methoxy-4,5-methylenedioxybenzene) is a constituent of widely used food additives, e.g., nutmeg, mace and the oils of nutmeg, parsley and celery seed and is also found in parsnips, carrots and bananas as well as in flavoring agents used in tobacco. It possesses insecticidal, (pesticidal), synergistic and possibly psychotropic properties. Following oral or intraperitoneal administration of myristicin in the rat, the major urinary metabolite was found to be 3-piperidyl-1-(3' methylenedioxyphenyl)-1-propanone. In addition, the rat and guinea pig excrete trace quantities of the pyrrolidinyl ketone. In contrast to the urinary basic metabolites of safrole, no detectable quantity of N,N-dimethylamino ketone was present in either rat or guinea pig urine after administration of myristicin.

The finding of nitrogen-containing metabolites following administration of both safrole and myristicin may contribute to the better understanding of the physiological as well as the pathological action of the allylic and propenylic benzene derivatives in natural food products and essential oils.

The study of the retention, localization and rates of elimination of the labeled pesticidal synergist (e.g., ^{14}C-methylenedioxy- and alpha-methylene-^{14}C-piperonyl butoxide) in the rat following i.v., oral and intratracheal administration has revealed many similarities in a number of biliary, urinary and fecal metabolites as evidenced by identical R_F values in several solvent systems following co-chromatography and radioautography. Their identity as well as the further separation of metabolites following acid, beta-glucuronidase and sulfatase hydrolysis is currently under investigation. The elaboration of structure of two metabolites found in lung following i.v., oral and intratracheal administration of piperonyl butoxide tentatively suggests metabolites formed by successive removal of the $-OC_4H_9$ terminal group and ethylene glycol, respectively, with the methylenedioxy ring and alpha-propyl sidechain remaining intact. Additional parameters of purification of commercial sources of piperonyl butoxide have been elucidated involving column and TLC techniques permitting the recovery of chromatographically pure synergists.

The synergistic action of methylenedioxyphenyl derivatives with various pesticides is attributed to inhibition of enzyme systems or prevention of enzyme induction both concerned with the metabolism of the pesticides. Pesticide synergists are used in aerosols of insecticides which are widely employed for home use. Studies are in progress to elaborate the mixed-function oxidase enzymes of mammalian lung and the effects of inhaled xenobiotics such as pesticidal synergists and carbon monoxide on these enzymes. If the mixed-function oxidase enzymes of the lungs serve to protect the lungs and consequently the individual from the effects of inhaled xenobiotics, inhibition of these enzymes by the contents of household aerosols and urban air may play a

significant role in lowering the resistance of the exposed individual to the actions of any inhaled xenobiotics.

Initial in vitro studies with rabbit lung have indicated that mixed-function oxidase systems are inhibited by insecticidal synergists (I_{50} values are in the range of 10^{-5}M), and also inhibited 15-25% when incubated under an atmosphere containing 0.1% carbon monoxide.

Despite the increasing curtailment of use of DDT, its ubiquitous ecological distribution and extremely long biological half-life warrant further study into its retention in mammalian tissue, the nature of its products and possible enzyme interactions. In the latter regard, the mode of action of DDT and its metabolites as well as other chlorinated hydrocarbons is being elaborated using calf brain Na-ATPase as a test system.

Brain Na-ATPase and Na-independent ATPase represent enzymes associated with the neuronal membrane. The Na-ATPase is generally assumed to play a major role in neuronal depolarization-repolarization phenomena in addition to or in conjunction with its role in regulating alkali metal ion transport and cellular electrolyte balance. Initial studies indicate that reversal of (Na^++K^+)ATPase inhibition produced by DDT and other chlorinated hydrocarbon pesticides could be effected by crude commercial phospholipid preparation, and the component responsible was identified as phosphatidyl serine. Commercial, chromatographically homogeneous preparations of phosphatidyl ethanolamine and phosphatidyl inositol inhibited the enzyme and offered no protection. The dose-response relationships to the chlorinated hydrocarbon pesticide inhibitors were found to be quantitatively similar to those involving oleic acid (tris salt) and since inhibition by both pesticide and fatty acid may be prevented or reversed by phosphatidyl serine, it is tentatively suggested that these two classes of inhibitors probably effect inhibition by emulsifying or otherwise interacting with phospholipids which are required for normal enzyme activation.

The behavior of DDT in solution with a variety of model compounds has been investigated with the aid of proton magnetic resonance spectroscopy and indicates that the phosphate group may bind DDT via an interaction with benzhydryl hydrogen much more strongly than carbonyl amide nitrogen or aromatic functions. This observation appears consistent with the suggestion that DDT inhibition results from interference with phospholipid activation of the (Na^++K^+)ATPase.

Relative toxicities of DDT and its metabolites and analogs might also be correlated with relative affinities for target proteins or specific transport systems in the blood. Pesticide binding studies are in progress utilizing DDT and its metabolites as well as other chlorinated hydrocarbons and cyclodienes to determine (a) the types of binding interactions (co-valent, hydrophobic, charge-transfer, π-interactions, etc.) involved in the biologically significant associations of pesticides with proteins; and (b) whether a correlation can be found between binding forces and biological activity and (c) to develop methods for the fractionation of serum and tissue proteins that will allow isolation of purified proteins without displacement of bound pesticides. Initial findings suggest that besides serum albumin, the only serum protein fraction that appears to have a high affinity for DDT is that which contains the low-density lipoproteins, and that this lipoprotein will not displace DDD already bound to

albumin. The binding of DDT to serum proteins appears to be relatively weak in that the DDT is preferentially absorbed on cellulose or cellulose acetate.

Dieldrin is representative of a class of pesticides of major importance, the cyclodienes. A study of the metabolism, storage and rates of excretion of dieldrin in the rat following administration of doses possibly analogous to that resulting from environmental exposure (e.g., a single 10 ppm oral administration) has indicated that dieldrin is metabolized and excreted much more rapidly by male rats than by females. The major metabolite excreted in the urine by the male rat has been identified previously and designated as "Klein's" metabolite. Female rats apparently do not excrete this metabolite. Both sexes excrete an unknown water soluble metabolite in the urine and a small amount of aldrin-transdiol in both the urine and feces. Male rats excrete very little (approx. 2%) unmetabolized dieldrin compared to approx. 25% of unmetabolized dieldrin excreted by females. In the female tissues and several tissues in the male dieldrin was stored as almost 100% unmetabolized insecticide. In other tissues of the male animals, particularly the kidneys and lungs, a large portion of the stored insecticide existed as "Klein's" metabolite.

Proton magnetic spectroscopy has been employed to clarify the seemingly anomalous results surrounding the structural and stereochemical identification of the major metabolites of dieldrin and aldrin. Re-investigations of the spectral and chemical properties of the major fecal metabolite (F-1) of dieldrin in the rat have led to the postulation of a new structure for this metabolite. It has also been demonstrated that the F-1 metabolite can be converted to the major urinary metabolite of dieldrin (U-1 or U-1 "type" bridged isomers of increased polarity) compounds by both oxidative and photolytic pathways which may also be of biological significance.

Mirex is an insecticide used for the irradication of the fire ant in the Southeastern United States. However, residues of Mirex have already been found in both shellfish and catfish in fresh water and ocean sources. If Mirex is not biodegradable, its further use could create a major pesticide residue problem. Studies are in progress to determine if Mirex undergoes biodegradation and if so to elaborate the rate and nature of the products via both *in vitro* and *in vivo* investigations.

A number of cyclic and linear metallodithiocarbamates have wide utility as fungicides and rubber accelerators and hence are of environmental significance. Their rates of decomposition as well as the nature of their decomposition products are being elaborated by variable temperature nuclear magnetic resonance spectroscopy.

The increasingly ubiquitous nature of mercury in the environment as well as its conversion to methyl mercury and the latter's apparent toxicological significance is a matter of increasing concern. Studies are in progress to elaborate the mammalian effects of repeated storage to sub-lethal amounts of mercury in terms of tissue storage, identification of the form of mercury stored, rate of storage and/or conversion, and concomitant effects on microsomal activity. In initial studies involving the oral administration of a total of 6 and 12 mg of methyl mercuric hydroxide/kg body weight over a two-week interval, no large changes of rat liver microsomal enzyme activity were

found, e.g., p-nitroanisole demethylation levels were equal to that of controls; P-450 levels were not increased significantly and a small increase in aniline hydroxylation was observed. Greatest total mercury storage was found in the kidney with the next highest concentrations in the spleen. Marked dose-related mercury accumulations were found in the lung and liver while lower mercury levels were found in brain and muscle. Additional studies are in progress involving the administration of methyl mercuric hydroxide over longer time periods with measurement of tissue residues and enzyme assays at intermittent periods.

The banning of the food additive cyclamate due to its role in bladder tumor induction as well as metabolic, mutagenic and teratogenic aspects of its metabolites coupled with the more recent finding of the induction of bladder tumors in the rat following implantation of saccharin pellets have focused on the need to study the above compounds and related derivatives for such potential hazards. Accordingly, the preparation of potential metabolites of saccharin and cyclamate, e.g., N-hydroxy, N-acetoxy, methoxy, nitroso and nitrile, dihydrosaccharin, etc., has been in progress since they are vital for the metabolic, mutagenic, teratogenic, carcinogenic and DNA-binding studies of the above food additives and their degradation products. The *in vitro* and *in vivo* metabolism of ^{14}C-saccharin is in progress. Initial studies with a rat liver enzyme preparation indicate the formation of 4 metabolites.

Another aspect of the activities of the Chemistry Branch relates to the elaboration of potential environmental hazards from natural products. Studies to determine whether exogenous prostaglandins should be considered environmental health factors, either relative to ingestion or topical exposure as well as the determination as to whether exogenous prostaglandins play a significant role in the response of animals to drugs, pesticides, antigens, etc., are in progress. Edible portions of plant sources such as wheat, wheat bran, corn, soybean meal and oats have not been found to contain prostaglandins in detectable amounts. Wheat and wheat bran, however, did contain polyhydroxy acids which are of interest because of their structural analogy to di-nor-prostaglandin F. Preliminary findings also suggest that prostaglandins may be synthesized by mast cells from post-partum female rats; however, no synthesis from arachidonic acid by mast cells of male rats has been found thus far. Techniques for the recovery and identification of sub-microgram quantities of newly-synthesized prostaglandins as well as the isolation of clean, metabolically active mast cells have been developed or refined.

The development of new and the improvement of established techniques is an integral requirement for the solution of diverse problems confronting scientists at the Institute. The development of analytical methodology includes: (a) TLC and GLC determination of N-hydroxy and related derivatives and metabolites of cyclamate and saccharin; (b) TLC and GLC separation, detection and estimation of inorganic and organic forms of mercury for *in vivo* studies; (c) TLC and GLC determinations of Mirex from tissue residues; (d) TLC and GLC separation and determination of diverse coumarin derivatives of biological interest; (e) TLC and GLC determinations of urinary, fecal and biliary metabolites of methylenedioxyphenyl synergists; (f) spectrochemical and GLC determination of nitrogen bases, e.g., ammonia, hydroxylamine, nitrosamines or conjugates and metabolites in baby foods and food additives;

(g) the GLC determination of tryptophan and L-dopa metabolites in urine and (h) GLC and TLC determination of chlorinated dibenzodioxins and chlorinated dibenzofurans in polychlorphenols, chlorophenoxy acetic acids, pentachloronitrobenzene and polychlorinated biphenyls.

The widespread use of sodium nitrite as a preservative in meat, fish and cheese, the possible conversion of nitrate to nitrite in foods and water and the role of nitrite in the formation of (a) nitrosamines and (b) methemoglobin and the latter's possible role in methemoglobinemia in infants all focus on the need to evaluate the potential environmental hazard of this food additive. Studies have been carried out to determine conditions which could enhance the formation of nitrite as well as other reduction products, e.g., hydroxylamine, ammonia and their derivatives. The levels of nitrite and nitrate in a spectrum of baby foods were initially established under parameters of ambient and elevated temperatures. The effects of microorganisms, e.g., B. mycoides, M. lysodeiktus, E. coli and Sarcina lutea, on baby foods at room temperature and 35°C for periods of up to two weeks were elaborated. In all of the vegetables tested, e.g., beets, squash, carrots, spinach and green beans, the presence of B. mycoides and M. lysodeiktus within 5 days (particularly at 35°C) resulted in statistically significant decreases in the nitrate levels with a concomitant significant increase in nitrites for beets, a small increase in nitrites for spinach and no increase in nitrites for squash, carrots or green beans. In addition, traces of hydroxylamine (0.04 p.p.m.) were found in two samples of beets treated with M. lysodeiktus at 35°C for 4 days. This was the only instance of hydroxylamine found in any of the microbial treatments at ambient and elevated temperatures. Exposure of baby foods with E. coli at room temperature for periods of up to 2 weeks resulted in elevated levels of nitrite (approx. 40 p.p.m. after 2 weeks) with decreased levels of nitrate only in the case of beets. Analogous studies with Sarcina lutea revealed elevated levels of nitrite for both beets and spinach (approx. 4.0 p.p.m. after 2 weeks) and decreased levels of nitrate. In no cases have any traces of nitrosamines been detected in baby foods even in cases under which nitrite was present (beets and spinach). The levels of pesticides (particularly chlorinated hydrocarbons, DDT and metabolites) in baby foods is being currently evaluated to ascertain whether a potential correlation exists with their presence and elevated levels of nitrate in vegetables (e.g., beets and spinach).

PERSONNEL

Dr. Fishbein is authoring a three volume series for Elsevier Press, "Chromatography of Environmental Hazards", and has completed the first volume, Carcinogens, Teratogens, Mutagens and Chromosome Breaking Agents. Volumes II and III in preparation are Air, Water, Industrial Pollutants and Pesticide Residues and Drugs; and Narcotics, Psychotropic Agents and Related Compounds, Hallucinogens and Miscellaneous Agents, respectively. He has participated at the Fogarty International Workshop on Mutagenic Effects on Environmental Contaminations for the United Nations Conference on the Human Environment in Stockholm in 1972; and his chapter on "Pesticidal, Industrial, Food Additives and Drug Mutagens" will be incorporated in the bound proceedings on Mutagenic Effects on Environmental Contaminations. He has also participated in presenting

invited papers at the 2nd International Congress of Pesticide Chemistry in Tel-Aviv, Israel and the 2nd Environmental Mutagen Society Meeting in Washington at which latter meeting he also chaired a session on Environmental Chemical Mutagens.

Dr. Fishbein has also presented invited testimony at Senator Ribicoff's Subcommittee on Executive Reorganization and Environment Research hearings in Washington on mutagenic hazards due to environmental chemicals.

PUBLICATIONS

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Fishbein, L.: Pesticidal, industrial, food additive and drug mutagens, Fogarty International Center Workshop on Mutagenic Effects on Environmental Contaminations, Washington, D. C., March 29-31, 1971, (in press).

Fishbein, L. and Flamm, W. G.: Potential environmental chemical hazards. I. Drugs, II. Food additives and pesticides and III. Industrial and miscellaneous. Science of Total Environment, (in press).

Serial No.: NIEHS-ASC-001
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Metabolism of Pesticidal Synergists"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-001

PRINCIPAL INVESTIGATOR: L. Fishbein, Ph.D.

OTHER INVESTIGATORS: J. Fawkes and S. Jordan

COOPERATING UNITS: None

MAN YEARS:

Total:	1.6
Professional:	0.4
Other:	1.2

PROJECT DESCRIPTION

OBJECTIVES: To study the retention, localization and rates of elimination of ^{14}C-methylenedioxy-, and alpha-methylene ^{14}C-piperonyl butoxide in the rat following I.V., oral, intratracheal and aerosol administration.

METHODS EMPLOYED: Paper, thin-layer and gas-liquid chromatography, radioautography and liquid scintillation techniques.

MAJOR FINDINGS: (1) Many similarities were found in a number of biliary, urinary and fecal metabolites (as evidenced by identical C_f values in several solvent systems following co-chromatography and radioautography) following the I.V., oral and intratracheal administration of both α-methylene ^{14}C- and methylenedioxy ^{14}C-piperonyl butoxide. Their identity as well as the further separation of metabolites following acid, beta-glucuronidase and sulfatase hydrolysis is currently under investigation. (2) The elaboration of structure of two metabolites found in lung following I.V., oral and intratracheal administration of ^{14}C-piperonyl butoxide tentatively suggests metabolites formed by successive removal of the OC_4H_9 terminal group and ethylene glycol respectively with the methylenedioxy group and α-propyl side chains remaining intact. (3) Additional parameters of purification of piperonyl butoxide have been elucidated (involving column and TLC techniques) permitting the recovery of chromatographically pure synergist.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Unequivocal identification of the nature of the metabolites of pesticidal

synergists, their rates of elimination and tissue distribution following various routes of administration would be of importance in evaluating whether or not a potential hazard to man exists by exposure to these agents, as well as understanding the mechanism controlling enzymic detoxification of these important agents.

PROPOSED COURSE: (1) Further separation and elaboration of metabolites following I.V., oral, intratracheal administration of labeled synergists will be done. (2) Studies to determine whether synergists, such as piperonyl butoxide, possess enzyme inhibitory activity after absorption from the lung, skin or G.I. tract.

PUBLICATIONS

Fishbein, L., Falk, H. L., Fawkes, J., and Jordan, S.: The metabolism of ^{14}C-piperonyl butoxide in the rat. 2nd International Congress of Pesticide Chemistry, IUPAC, Tel-Aviv, Israel, Feb. 21-26, 1971 (in press).

Serial No.: NIEHS-ASC-003
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Metabolism of Myristicin and Congeners"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-003

PRINCIPAL INVESTIGATOR: Edward Oswald, Ph.D.

OTHER INVESTIGATORS: B. Corbett, M. P. Walker, L. Fishbein, Ph.D.

COOPERATING UNITS: Pathology

MAN YEARS:

Total:	2.5
Professional:	1.0
Other:	1.5

PROJECT DESCRIPTION

OBJECTIVES: Substituted allyl and propenyl benzene derivatives are widely distributed in nature, e.g., in nutmeg, parsley, parsnip, carrots, and bananas as well as in processed tobacco and flavoring agents. In order to determine whether these environmental agents constitute a potential hazard, investigations are in progress to isolate and identify the various urinary metabolites of myristicin, safrole, isosafrole, asarone, beta asarone and dihydrosafrole following administration by various routes to the rat.

METHODS EMPLOYED: Chromatography (TLC, Column and GLC), NMR, I.R. and mass spectroscopy.

MAJOR FINDINGS: (1) Following oral or intraperitoneal administration of safrole to male rats and guinea pigs, three tertiary aminomethylenedioxypropiophenones are excreted in the urine, e.g., 3-N,N-dimethylamino-1-, 3-piperidyl-1-, and 3-pyrrolidinyl-1-(3',4'-methylenedioxyphenyl)-1-propanones. All three amino derivatives decompose to form 1-(3',4'-methylenedioxyphenyl)-3-propen-1-one. (2) The major urinary metabolite of myristicin in the rat is 3-piperidyl-1-(3'-methoxy-4',5'-methylenedioxyphenyl)-1-propanone. In addition, the rat and the guinea pig excrete trace quantities of the pyrrolidinyl and piperidyl ketone, respectively. In contrast to the urinary basic metabolites of safrole, no detectable quantity of N,N-dimethylamino ketone was present in either rat or guinea pig urine after administration of myristicin.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Un-

equivocal identification of the metabolites and knowledge of the rates of elimination of these environmental agents are essential in determining whether the exposure or ingestion of these agents and related compounds constitutes a potential hazard. The finding of nitrogen-containing metabolites following administration of both safrole and myristicin may contribute to the better understanding of the physiological as well as the pathological action of the allylic and propenylic benzene derivatives in natural food products and essential oils.

PROPOSED COURSE: (a) Complete identification and characterization of both the basic ninhydrin positive and non-basic urinary metabolites of safrole, myristicin, elemicin, eugenol and the asarones, (b) preparation of C^{14}-labeled myristicin, safrole, elemicin and eugenol, and (c) the elaboration of these drugs following introduction into whole animals and finally cell-free systems will be undertaken.

PUBLICATIONS

Oswald, E. O., Fishbein, L. and Corbett, J.: Metabolism of naturally occurring propenyl benzene derivatives. J. Chromatog. 45: 437-445, 1969.

Oswald, E. O., Fishbein, L., Corbett, B. J. and Walker, M. P.: Identification of tertiary aminoethylenedioxy propiophenones as urinary metabolites of safrole in the rat and the guinea pig. Biochem. et Biophys. Acta 230: 237-247, 1971.

Oswald, E. O., Fishbein, L., Corbett, B. J. and Walker, M. P.: Urinary excretion of methoxy-methylenedioxy propiophenones as metabolites of myristicin in the rat and guinea pig. Biochem. et Biophys. Acta, (in press).

Serial No.: NIEHS-ASC-006
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Development of Analytical Methodology"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-006

PRINCIPAL INVESTIGATOR: Lawrence Fishbein, Ph.D.

OTHER INVESTIGATORS: P. Albro, Ph.D., N. K. Wilson, Ph.D., J. McKinney, Ph.D., A. Latimer

COOPERATING UNITS: None

MAN YEARS:

Total:	1.2
Professional:	0.7
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: The development of new as well as the improvement of established analytical techniques is an integral requirement for the solution of diverse problems confronting the Chemistry Branch as well as other groups within the Institute. The development of analytical methodology programs include: (a) TLC and GLC determination of N-hydroxy and related derivatives and metabolism of food additives (saccharin and cyclamate). (b) TLC and GLC separation, detection and estimation of inorganic and organic forms of mercury for in vivo studies. (c) The spectrochemical and GLC chromatographic determination of nitrogen bases, e.g., ammonia, hydroxylamine, nitrosamines or conjugates and metabolites in baby food and food additives. (d) TLC and GLC separation and determination of diverse coumarin derivatives of biological interest. (e) GLC determination of Tryptophan metabolites in human urine following administration of L-Dopa. (f) TLC and GLC determination of Mirex from tissue residues of shellfish and catfish.

METHODS EMPLOYED: TLC and radioautographic techniques, gas-liquid chromatography (flame ionization, electron-capture and thermal conductivity), NMR and mass spectroscopy, uv and visible spectroscopy.

MAJOR FINDINGS: (1) TLC techniques have been developed for the separation and detection of potential metabolites of saccharin and diverse coumarin derivatives of biological interest. (2) GLC procedures have been elaborated for the separation and estimation of the metabolites of dopa and tryptophan. (3) Column and

GLC methodology has been developed for the separation and determination of Mirex from fish and shellfish.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Successful development of the analytical methodology in the areas delineated above will accelerate the successful elaboration of a number of metabolic and degradation studies in progress in the Chemistry Branch as well as be of utility for other studies within the Center.

PROPOSED COURSE: Efforts will be continued for the analytical separation and quantification of metabolites and degradation products of environmental agents, e.g., pesticides, food additives, toxins, utilizing primarily TLC, GLC, NMR and IR and mass spectroscopic techniques.

Serial No.: NIEHS-ASC-008
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Relationship Between Responsiveness of Calf Brain (Na^++K^+)-ATPase and Structural Characteristics of Inhibitory Pesticides, Fungicides and Drugs"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-008

PRINCIPAL INVESTIGATOR: W. E. Wilson, Ph.D.

OTHER INVESTIGATORS: S. T. Clements, C. Roy, A. Latimer, R. Ferguson and N. K. Wilson

COOPERATING UNITS: None

MAN YEARS:

Total:	3.0
Professional:	1.0
Other:	2.0

PROJECT DESCRIPTION

OBJECTIVES: The major objective is to ascertain the most probable mode of action of the chlorinated hydrocarbon pesticides as inhibitors of brain membranal (Na^++K^+)ATPase and of Na^+-independent ATPase activities. An understanding of the mode of action of these pesticides as inhibitors of the membranal (Na^++K^+)ATPase system is of interest as this enzyme system is believed to be of fundamental importance in (a) cellular electrolyte balance, (b) neuronal depolarization-repolarization phenomena and (c) heart muscle contraction. Thus, such an understanding could provide additional insight into a variety of poorly understood biological control mechanisms which are related to the energy transduction phenomena carried on by the (Na^++K^+)ATPase system.

METHODS EMPLOYED: Autoanalyzer analysis, various chromatographic techniques, electron microscopy, ultracentrifugation, and various spectroscopic techniques.

MAJOR FINDINGS: (1) In attempts to determine a satisfactory method for reversing the (Na^++K^+)ATPase inhibition produced by DDT and other chlorinated hydrocarbon pesticides (CHP's), it was found that reversal could be effected by exposing the enzyme to a crude commercial Folch Fraction III phospholipid preparation; the component responsible in the above fraction for reversal of enzyme inhibition was identified as phosphatidyl serine. (2) An examination

of the four main classes of phospholipids for their ability to protect the enzyme from inhibition by the CHP's revealed the following: (a) a commercial chromatographed preparation of phosphatidyl choline did not activate or inhibit the enzyme but did protect it from DDT inhibition; (b) commercial, chromatographically homogeneous preparations of phosphatidyl ethanolamine and phosphatidyl inositol inhibited the enzyme and offered no protection. (3) The recent finding that a beef brain phosphatidyl inositol-like material activated the enzyme about as well as phosphatidyl serine suggests that the latter is not exclusively responsible for enzyme protection. (4) The dose-response relationships to the CHP inhibitors are quantitatively similar to those involving oleic acid (tris salt) and since inhibition by both pesticide and fatty acid may be prevented or reversed by phosphatidyl serine, it is suggested that these two classes of inhibitors probably effect inhibition by emulsifying or otherwise interacting with phospholipids which are required for normal enzyme activation. (5) The solution behavior of DDT with a variety of model compounds has been investigated with the aid of NMR spectroscopy. The results of these studies indicate that the phosphate group may bind DDT (interact with the benzhydryl hydrogen) much more strongly than carbonyl, amide N, or aromatic functionalities. This observation is consistent with the suggestion that DDT inhibition results from interference with phospholipid activation of the $(Na^+ + K^+)$ATPase.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The only known deleterious effect of the chlorinated hydrocarbon pesticides in vivo in mammals is their interference with central nervous system function (neurotoxicity). The neurotoxicity is reported to result from interference of the passage of Na^+ ion-dependent current across the membrane. Such interference may have resulted from inhibition of membranal $(Na^+ + K^+)$ATPase activity. The observation that this membranal enzyme system can be protected by phospholipids that are also capable of reconstituting the $(Na^+ + K^+)$ATPase suggests that the mode of neurotoxicity of these pesticides involves an interference with phospholipid activation of this enzyme system. Follow-up of these observations could lead to a better understanding of the unique molecular organization in membranal enzyme systems as well as other, non-enzymatic membranal phenomena. It is quite possible that a similar pesticide interference with Ca^{+2} ion transport and/or carbonic anhydrase activity in egg-shell forming glands could account for the formation of very thin egg shells in certain avian species. Additionally, interference with biological membrane structural organization could conceivably account for induction of de novo protein synthesis in response to the chlorinated hydrocarbon pesticides.

PROPOSED COURSE: Further clarification of the mode of action of the chlorinated hydrocarbon pesticides as inhibitors of the membranal $(Na^+ + K^+)$ATPase system depends upon the following: (a) the aquisition of a supply of a variety of chromatographically homogeneous beef brain phospholipids as well as several synthetic phospholipids—(dibutyl, dioleoyl, and distearoyl-phosphatidyl serines would represent a minimum variety of synthetic phospholipids); (b) the $(Na^+ + K^+)$ATPase must be more extensively purified and this can be accomplished using known techniques; (c) a much more extensive kinetic characterization is required in regard to the interactions of enzyme protein with both phospholipid and pesticides; and (d) initiation of in vivo studies in rats to ascertain the protective effects of the phospholipids to the treatment of observable symptoms of DDT administration via an examination of

$(Na^{+}+K^{+})$ATPase levels in brain and other tissues before and after administration of varying doses of DDT is needed.

PUBLICATIONS

Wilson, W. E., and Hanna, L. T.: Automation of calf brain NaATPase and Na-independent ATPase analyses. In Advan. Automated Anal., 1969 Technicon Int. Congr., 1970, vol. 1, p. 133.

Wilson, W. E., Sivitz, W. I., and Hanna, L. T.: Inhibition of calf brain membranal sodium- and potassium-dependent adenosine triphosphatase by cardioactive sterols. A Binding Site Model. Mol. Pharmacol. 6: 449, 1970.

Wilson, W. E., Fishbein, L., and Clements, S. T.: DDT participation in ultraviolet-detectable charge-transfer complexation. Science 171: 180, 1971.

Serial No.: NIEHS-ASC-009
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Metabolism and Physiological Aspects of Ingested Hydrocarbons"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-009

PRINCIPAL INVESTIGATOR: P. W. Albro, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.75
Professional:	0.75
Other:	0.00

PROJECT DESCRIPTION

OBJECTIVES: (1) To investigate the relationship(s) of accumulation of dietary paraffins in animal tissues to such suspected pathologic effects as lipidoses, co-carcinogenesis, and interference with reticular endothelial functions. (2) To determine the specificities of various animal tissues for the accumulation of individual hydrocarbons from defined diets. (3) To elucidate the pathway for catabolism of ingested hydrocarbons, particularly with respect to interference by drugs, pesticides, and synergists.

METHODS EMPLOYED: The basic test organism is the rat, to which hydrocarbons are administered orally in corn oil, mixed with rat chow, injected I.P., I.V. or I.M. In some cases radioactive tracers (1-^{14}C-hexadecane, 1-^{14}C-octadecane, U-^{14}C-phytane and 2,3-^{3}H-phytane, the latter two synthesized here) are used in metabolic studies. Standard chromatographic, immunologic, radioassay and other biochemical techniques are used.

MAJOR FINDINGS: (1) Quantitative aspects on the intestinal absorption of dietary paraffins in rats have been elaborated. A possibly significant finding was the depression of hydrocarbon absorption in rats treated with a variety of antibiotics. The opposite effects, e.g., increased retention of hydrocarbons in rat tissues caused by piperonyl butoxide, was also elucidated. (2) It was also observed that the presence of pristane (tetramethylpentadecane) in the diet of rats for a few weeks prior to immunization with egg albumin results in anaphylaxis and death upon re-exposure to egg albumin (no adverse effects are seen in the absence of pristane). The inclusion or exclusion of Freund's Complete

Adjuvant during immunization does not modify the response. Phytane (tetramethylhexadecane) is absorbed by rats and accumulates in various tissues; however, it is rapidly eliminated from all body stores when it is removed from the diet. The main route of elimination is catabolism followed by excretion of polar metabolites in the urine, with little if any conversion to CO_2. The catabolic process is being investigated in an attempt to identify the metabolites found in the urine.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The ingestion of paraffins from a variety of substances as well as its accumulation (as well as other hydrocarbons) in human tissues in cases of lipoid pneumonia, follicular lipidosis of the spleen and meningioma all suggest that tissue accumulation of hydrocarbons is undesirable. Present work has clarified the parameters involved in access of hydrocarbons to the tissues, and has demonstrated one type of interference of hydrocarbons with reticularendothelial function (immunogenesis).

PROPOSED COURSE: It is presently clear that the metabolism of branched hydrocarbons differs radically from that of n-paraffins. This novel catabolic pathway will be investigated through the use of radiotracer compounds. As it has not been possible to detect antibodies against ovalbumin with the relatively insensitive ager diffusion technique, further examination of the effects of branched hydrocarbons on immunogenesis using fluorescent (fluorescein-conjugated) egg albumin will be initiated. These studies are preliminary to returning to work on the effects of pesticides, etc., on hydrocarbon utilization.

PUBLICATIONS

Albro, P. W. and Fishbein, L.: Absorption of aliphatic hydrocarbon by rats. Biochim. Biophys. Acta 219: 437-446, 1970.

Albro, P. W. and Fishbein, L.: Short-term effects of piperonyl butoxide on the deposition of dietary hydrocarbon in rat tissues. Life Sciences 9: 729-739, 1970.

Serial No.: NIEHS-ASC-013
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Chemistry of the Chlorinated Polycyclodiene Pesticides and Their Metabolites:

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-013

PRINCIPAL INVESTIGATOR: J. D. McKinney, Ph.D.

OTHER INVESTIGATORS: H. B. Matthews, Ph.D., L. Barling, and L. H. Keith, Ph.D.

COOPERATING UNITS: Southwest Water Laboratory, WQO, Environmental Protection Agency

MAN YEARS:

Total:	0.5
Professional:	0.3
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: (1) To clarify the seemingly anomalous results surrounding the structure identification of the major metabolites of dieldrin and aldrin. (2) To establish any stereo-selectivities associated with dieldrin and aldrin metabolism. (3) To investigate other degradative routes, i.e., photolysis, etc., for the so-called "terminal" residues in the metabolism of the chlorinated polycyclodiene pesticides.

METHODS EMPLOYED: A variety of synthetic, chromatographic and spectroscopic techniques, particularly high resolution proton magnetic resonance (PMR) spectroscopy.

MAJOR FINDINGS: PMR spectroscopy has been employed for the rapid and accurate determination of the relative stereochemistry of some postulated chlorinated polycyclodiene pesticide metabolites without excessive dependence on chemical methods and should prove valuable in elucidating the overall structures of unknown metabolites of these systems. Reinvestigation of the spectral and chemical properties of the major fecal metabolite (F-1) of C^{14}-dieldrin in the rat have led to the postulation of a new structure for this metabolite which seems to clarify many anomalous results. It has also been demonstrated that F-1 can be converted to the major urinary metabolite (U-1) or U-1 "type" (bridged isomers of increased polarity) compounds by both oxidative and photolytic pathways. These results may be of biological consequence.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The chlorinated polycyclodiene pesticides are among the most stable and persistent insecticides that can contaminate our environment. Continued use of these pesticides necessitates the elucidation of their metabolic pathways in mammalian systems and other degradative pathways of so-called metabolic "terminal" residues. Such elucidation and elimination of metabolic and degradative possibilities will illuminate the possible differences in toxicity due to chemical (or stereochemical) factors, i.e., F-1 → more toxic U-1.

PROPOSED COURSE: (1) To investigate further the C^{14}-metabolites of dieldrin in light of our understanding of the stereochemical properties of these systems. Further studies should reveal other stereoselectivities associated with dieldrin metabolism, i.e., the intermediacy of a cis-aldrin diol in the metabolism of both dieldrin and aldrin. (2) To study other degradative routes, i.e., photolysis, microorganisms, etc., for chlorinated polycyclodiene pesticide systems.

PUBLICATIONS

Keith, L. H., Alford, A. L., and McKinney, J. D.: Long range coupling in the chlorinated polycyclodiene pesticides. Tetrahedron Letters 28: 2489, 1970.

McKinney, J. D., Keith, L. H., and Alford, A. L.: PMR spectra of some chlorinated polycyclodiene pesticide metabolites. Rapid assessment of stereochemistry. Can. J. Chem. (in press) 1971.

Serial No.: NIEHS-ASC-014
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "NMR Investigations of the Stability of Metallic Linear and Cyclic Dithiocarbamates and Related Derivatives"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-014

PRINCIPAL INVESTIGATOR: N. K. Wilson, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.5
Professional:	0.5
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To determine the specific rate constant at various temperatures for the decomposition of a variety of fungicidal and related cyclic and linear metallodithiocarbamates, to calculate the activation energies and thermodynamic parameters for the process, and to develop an experimental procedure to be adapted to similar studies on related compounds. (2) Determination of the degree of partial double-bond character of the carbon-nitrogen bond (the barrier to rotation about this bond) in tetraalkyl thiuram sulfides.

METHODS EMPLOYED: Variable temperature proton magnetic resonance spectroscopy was employed.

MAJOR FINDINGS: The rotational barrier was found to be about 15 Kcal/mol., and higher in monosulfides than in disulfides. This was attributed to greater double-bond character and greater steric hindrance to rotation in the monosulfides.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The cyclic and linear metallodithiocarbamates have wide utility as fungicides and rubber accelerators and hence are of environmental significance. Ethylselenac has also been shown to be an effective enzyme inhibitor. The exact species responsible and the mode of inhibition are not known. Knowledge of the rate of decomposition and the nature of the degradation products of this fungicide as well as related compounds should help determine the possible

extent of its in vivo activity.

PROPOSED COURSE: Continue the NMR spectroscopic studies of ethylselenac and related derivatives.

PUBLICATIONS

Wilson, N. K.: Hindered rotation about the S_2C-NR_2 bond in N,N,N',N'-tetra-alkyl thiuram disulfides and monosulfides. J. Phys. Chem. 75: 1067-1072, 1971.

Serial No.: NIEHS-ASC-016
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Synthesis of N-Hydroxy- and Related Possible Metabolites of Environmental Agents"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-016

PRINCIPAL INVESTIGATOR: L. Levy, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	1.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The preparation of potential metabolites of saccharin and cyclamate, e.g., N-hydroxy, N-acetoxy, methoxy, nitroso and nitrite, dihydrosaccharin, etc., are vital for the metabolic, mutagenic, teratogenic, carcinogenic and DNA-binding elaboration of the above food additives and their degradation products.

METHODS EMPLOYED: Some thirteen or more different synthetic sequences and appropriate spectroscopic techniques have been utilized in attempts to prepare and identify N-hydroxysaccharin and related derivatives.

MAJOR FINDINGS: Although it has not been possible to prepare this compound, numerous saccharin derivatives which are potential metabolites have been synthesized. Of special note has been the synthesis of N-methoxydihydrosaccharin (and by extension of this idea, although not yet in fact, N-hydroxydihydrosaccharin), the first N-oxygenated saccharin derivative of any sort. These compounds have been tested against some of Dr. R. Pero's fungi as possible fungistatic agents. The results were not encouraging. Along the way several interesting bits of chemistry tangentially connected with the problem of saccharin metabolites have been discovered. These will be further investigated as time permits. The synthesis of C-14 labeled saccharin has been performed so as to be able to study more easily the metabolic fate of saccharin.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This work has been carried out because of the scarcity of knowledge about the metabolism of saccharin, a food additive with an extremely widespread usage in our daily existence. Furthermore, the concept of N-hydroxylation of ingested substances leading to potentially hazardous metabolites is not limited to saccharin but extends to a variety of drugs as well.

PROPOSED COURSE: The techniques developed in this study will be equally applicable to other nitrogen-containing environmental agents, e.g., dilatin. Work is continuing on those pathways which are most promising with regard to the synthesis of N-hydroxysaccharin. At the same time continuous work is being undertaken to prepare other possible metabolites of saccharin.

Serial No.: NIEHS-ASC-017
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Investigation of Nitrite-Nitrate and Related Nitrogen Bases in Baby Foods"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-017

PRINCIPAL INVESTIGATOR: L. Fishbein, Ph.D.

OTHER INVESTIGATORS: C. Roy

COOPERATING UNITS: None

MAN YEARS:

Total:	0.5
Professional:	0.2
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: To determine the levels of nitrite and nitrate as well as possible additional nitrogen bases, e.g., ammonia and/or hydroxylamine, when a spectra of baby foods are exposed to a variety of microorganisms under parameters of ambient and elevated temperatures.

METHODS EMPLOYED: U.V. and visible spectrophotometry, column and thin-layer chromatography.

MAJOR FINDINGS: (1) In all of the vegetables tested (e.g., beets, squash, carrots, spinach, green beans), the presence of B. mycoides and M. lysodeiktus within five days (particularly at 35°C) resulted in statistically significant decreases in the nitrate levels with a concomitantly significant increase in the nitrite content of beets and a small increase in nitrites for spinach and no increase in the nitrite content for squash, carrots or green beans. (2) Trace quantities of hydroxylamine (0.04 ppm) have been found in two samples of beets treated with M. lysodeiktus at 35°C for four days. This is the only instance of hydroxylamine found in any of the microbial treatments of baby food at room or elevated temperatures. (3) Exposure of baby foods with E. coli at room temperature for periods of up to two weeks resulted in elevated levels of nitrite (approx. 40 ppm after 2 weeks) with decreased levels of nitrate only in the case of beets. (4) Analogous studies with Sarcina lutea revealed elevated levels of nitrite for both beets and spinach (approx. 4.0 ppm after 2 weeks) and decreased levels of nitrite. (5) The treatment of the baby foods with the microorganisms screened resulted in the formation of

ammonia at different rates over the two-week experimental period. However, this formation cannot be attributed solely to nitrate precursors as other endogenous nitrogen sources are available in the foods, e.g., amino acids and possible ammonium salts. (6) In no cases have any traces of nitrosamines been detected in the baby foods even in cases where nitrite was present (beets and spinach) (3 and 4 above).

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Since excessive nitrite content in baby foods may contribute to the onset of methemoglobinemia, it is of vital importance to ascertain the conditions which may prevail that can possibly enhance the formation of nitrite as well as other toxic nitrogen derivatives, e.g., hydroxylamine, nitrosamines, ammonia and/or their derivatives.

PROPOSED COURSE: (1) Determine the effect of pesticide levels (particularly chlorinated hydrocarbons) on nitrite oxidation with a view toward elaborating the nature of any resultant transformation products.

Serial No.: NIEHS-ASC-020
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Pesticide Binding Studies"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-020

PRINCIPAL INVESTIGATOR: P. W. Albro, Ph.D.

OTHER INVESTIGATORS: R. Thomas

COOPERATING UNITS: None

MAN YEARS:

Total:	0.2
Professional:	0.1
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: (1) To develop methods for the fractionation of serum and tissue proteins that will allow isolation of purified proteins without displacement of bound pesticides. (2) To determine the types of binding interactions (covalent, hydrophobic, charge-transfer, π-interactions, etc.) involved in the biologically significant association of pesticides with proteins. (3) To determine whether a correlation can be made between binding forces and biological activity.

METHODS EMPLOYED: Ion-exchange chromatography, electrophoresis, differential centrifugation and discontinuous density gradient centrifugation and selective denaturation, TLC and GLC, have been employed.

MAJOR FINDINGS: Other than serum albumin, the only serum protein fraction that appears to have a high affinity for DDT is that which contains the low-density lipoproteins. However, preliminary experiments indicate that this lipoprotein will not displace DDT already bound to albumin. The binding of DDT to serum proteins appears to be relatively weak, in that the DDT is preferentially absorbed on cellulose or cellulose acetate.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The relative toxicity of DDT and its analogs and metabolites might be correlated with relative binding affinities of target protein or possibly with relative capacities of specific transport systems in the blood; hence, definite information regarding the nature of the binding of this class of environmental agent

is of importance.

PROPOSED COURSE: Purified proteins will be compared in their ability to bind DDT. Distribution of DDT among blood constituents will be examined in detail.

Serial No.: NIEHS-ASC-021
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Prostaglandins as Environmental Factors"

PREVIOUS SERIAL NUMBER: NIEHS-ASC-01-021

PRINCIPAL INVESTIGATOR: P. W. Albro, Ph.D.

OTHER INVESTIGATORS: R. O. Thomas

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	0.5
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: (1) The development of a highly sensitive, specific analytical procedure for qualitative and quantitative determination of the prostaglandins. (2) Evaluation as to whether exogenous prostaglandins should be considered environmental health factors, either relative to ingestion or topical exposure. (3) Determination as to whether endogenous prostaglandins play a significant role in the response of animals to drugs, pesticides, antigens and the like.

METHODS EMPLOYED: (1) Thin-layer and gas-liquid chromatography; (2) the use of arachidonic acid-1-^{14}C as a prostaglandin precursor to detect the synthesis of PGE_2 and $PGF_{2\alpha}$ in various systems as well as ^{3}H-PGF, α as a marker for the determination of uptake and release of prostaglandins by various cell types.

MAJOR FINDINGS: (1) Edible portions of plant sources such as wheat, wheat bran, corn, soybean meal and oats have not been found to contain prostaglandins in detectable amounts. Wheat and wheat bran did contain polyhydroxy acids which are of interest because of their structural analogy to di- nor- prostaglandin F. However, these acids did not exhibit prostaglandin-like activity in muscle-contraction assays. Preliminary findings suggest that prostaglandins may be synthesized by mast cells from post-partum female rats; no synthesis from arachidonic acid by mast cells from male rats has been found. (2) Techniques for the recovery and identification of submicrogram quantities of newly synthesized prostaglandins as well as the isolation of clear, metabolically active mast cells have been developed or refined.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: (1) Prostaglandins are released in the lungs during allergic reactions; we are attempting to determine whether these prostaglandins are derived from the mast cells, and if so, whether they are primarily protective in activity. (2) It has been reported that abortions can be induced in cats by oral administration of prostaglandins. This observation increases the need for examination of foodstuffs for the presence of prostaglandins.

PROPOSED COURSE: The screening procedures that have previously been applied to plant materials will be used to examine milk, eggs and the like. The ability of cooking to destroy the more stable prostaglandins (PGA, PGF) will be examined. Synthesis, uptake, and release of prostaglandins by mast cells will be studied.

Serial No.: NIEHS-ASC-022
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Chronic Exposure in the Rat to Organo-Metallics"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: M. Folsom, Ph.D.

OTHER INVESTIGATORS: L. Fishbein, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	1.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To determine the effects of long-term exposure in the rat to small amounts of organo-metallics such as methylmercury derivatives via an examination of tissue residues of total mercury and inorganic and organic mercury in brain, muscle, liver, kidneys, lung and spleen as well as concomittant elaboration of microsomal enzyme activity.

METHODS EMPLOYED: Neutron activation, thin-layer and gas-liquid chromatography, p-nitroanisole demethylation, P-450 and aniline hydroxylation spectrophotometric assays and histological studies of tissues have been utilized.

MAJOR FINDINGS: (1) Following oral administration of a total of 6 and 12 mg/ methyl mercuric hydroxide/Kg body weight over a two-week interval no large changes of liver microsomal enzyme activity were found (p-nitroanisole demethylation was equal to that of controls; P-450 levels were not increased significantly and a small increase in aniline hydroxylation was observed). (2) Slight histological changes were observed particularly in the rats in the higher treatment group. An increase in the size of liver cord cells as well as mild degenerative changes in kidney tubules and glomeruli were observed. However, pronounced weight decrease, symptomatic of mercury poisoning, was not observed. (3) The greatest total mercury storage was found in the kidney. This amounted to 46 µg of mercury/gm. of wet tissue in animals receiving 12 mg of mercury and 20 µg mercury/gm tissue in animals receiving 6 mg mercury. The second highest concentrations of mercury were found in the spleen. Marked dose-related mercury accumulations were found in the lung and liver while lower mercury levels were found in brain and muscle.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The increasingly ubiquitous nature of mercury in the environment as well as its conversion to methylmercury and the latter's apparent toxicological significance is a matter of increasing concern. The knowledge of the mammalian effect of repeated exposure to sub-lethal amounts of mercury stored, rate of storage and/or conversion, and committant effects on microsomal activity would be of benefit in elaborating the possible dimensions and nature of this hazard.

PROPOSED COURSE: Studies involving the administration of methylmercuric hydroxide over longer time periods with measurements of tissue residues and enzyme assays at intermittent experimental points are in progress. The development of analytical methodology utilizing primarily thin-layer, gas-liquid and column chromatography and atomic absorption for the separation, detection and measurement of total inorganic and organic mercury is in progress. Other enzyme systems will be studied as well as with other mercurial derivatives.

Serial No.: NIEHS-ASC-023
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Lung Mixed Function Oxidase Systems"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: H. B. Matthews, Ph.D.

OTHER INVESTIGATORS: M. Fields

COOPERATING UNITS: None

MAN YEARS:

Total:	1.5
Professional:	0.6
Other:	0.9

PROJECT DESCRIPTION

OBJECTIVES: To study the mixed-function oxidase enzymes of mammalian lung and the effects of inhaled xenobiotics on these enzymes.

METHODS EMPLOYED: Ultra-violet spectrophotometric determinations of the mixed-function oxidase enzyme activity in the subcellular fractions of rabbit lung were made.

MAJOR FINDINGS: The mixed-function oxidase enzymes of lungs are similar in many ways to the mixed-function oxidase enzymes of liver which have been studied extensively, the most notable exceptions being the techniques necessary for the isolation of these enzymes, the variability in activity from animal to animal and the lower yield of enzyme per gram of lung tissue. *In vitro* the lung mixed-function oxidase enzymes are inhibited by insecticide synergists (I_{50} values are in the range of 10^{-5}M), and piperonyl butoxide, one of the insecticide synergists tested, has been shown to be metabolized by these enzymes. Also, *in vitro* these enzymes have been shown to be inhibited 15-25% when incubated under an atmosphere containing 0.1% carbon monoxide.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Pesticide synergists are used in aerosols of insecticides which are used in the home and in many other areas frequented by people. The atmospheres of several large urban areas have daily peak concentrations of near 0.01% carbon monoxide and higher in restricted areas. If, as is probably the case, the mixed-function oxidase enzymes of the lungs serve to protect the lungs and consequently the individual from the effects of inhaled xenobiotics, inhibition

of these enzymes by the contents of household aerosols and urban air may play a significant role in lowering the resistance of the exposed individual to the actions of any inhaled toxicant.

PROPOSED COURSE: (1) Continued elaboration of the effects of other pesticidal synergists and other common gaseous air pollutants for their *in vitro* effect on the mixed-function oxidase enzymes of rabbit lung. (2) Initiation of *in vivo* studies involving the effect of gaseous pollutants, synergists and combination of compounds on mixed-function oxidase systems.

Serial No.: NIEHS-ASC-024
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Metabolism of Dieldrin"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: H. B. Matthews, Ph.D.

OTHER INVESTIGATORS: J. D. McKinney, Ph.D.; G. W. Lucier, Ph.D.

COOPERATING UNITS: Cell Biology Branch

MAN YEARS:

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To study the metabolism, storage and the rate of excretion of dieldrin in the rat following administration of doses possibly analagous to that resulting from environmental exposure (e.g., a single 10 ppm oral admin.).

METHODS EMPLOYED: Thin-layer chromatography, liquid scintillation and NMR spectroscopy have been employed.

MAJOR FINDINGS: Dieldrin is metabolized and excreted much more rapidly by male rats than by females. The major metabolite excreted by each sex is excreted in the feces. The major metabolite excreted in the urine by the males has been previously identified and designated "Kline's" metabolite. Female rats apparently do not excrete this dieldrin metabolite. Both sexes excrete an unknown water soluble metabolite in the urine and a small amount of aldrin-transdiol in both the urine and feces. Male rats excrete very little unmetabolized dieldrin (approx. 2%) whereas the females excrete approximately 25% of the dildrin unmetabolized.

As expected, the primary site of dieldrin storage in each sex was the adipose tissue. In the tissues of the female and several tissues of the males, dieldrin was stored as almost 100% unmetabolized insecticide. In other tissues of the males, particularly the kidneys and lungs, a large portion of the stored insecticide existed as "Kline's" metabolite.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Dieldrin

is a "hard" pesticide and even though its use is declining, its existence in the environment will continue for some time; therefore, definitive knowledge of its metabolism is essential.

PROPOSED COURSE: The elaboration of the toxicity of the dieldrin metabolites as well as the effects of multiple administration of small doses of dieldrin in the rat will be undertaken.

PUBLICATIONS

Matthews, H. B., McKinney, J. D. and Lucier, G.: Dieldrin metabolism, excretion and storage in male and female rats. J. Ag. Food Chem. (in press).

Serial No.: NIEHS-ASC-025
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Metabolism of Mirex"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: H. B. Matthews, Ph.D.

OTHER INVESTIGATORS: M. Fields

MAN YEARS:

Total:	0.10
Professional:	0.05
Other:	0.05

PROJECT DESCRIPTION

OBJECTIVES: To determine if Mirex undergoes biodegradation and, if so, to elaborate the rate and nature of the products via both *in vitro* and *in vivo* studies.

METHODS EMPLOYED: To date, only *in vitro* enzyme studies have been employed.

MAJOR FINDINGS: As yet, no loss of parent compound or formation of a metabolite has been observed.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It has been proposed that Mirex be used in a major program to irradicate the fire ant from the Southeastern United States. Residues of Mirex have already been found in both shellfish and catfish in fresh water and ocean sources. If Mirex is not biodegradable, its further use could create a major pesticide resude problem. Identification of the nature of the Mirex residues would help delineate the scope of the possible residue problem.

PROPOSED COURSE: Additional *in vitro* as well as feeding studies in the rat will be undertaken to study the excretion and storage of this insecticide. The bacterial degradation of Mirex will also be undertaken.

Serial No.: NIEHS-ASC-026
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Chemistry of Substituted Allyl and Propenyl Benzenes and Their Proposed Metabolites"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: J. D. McKinney, Ph.D.

OTHER INVESTIGATORS: E. O. Oswald, Ph.D., L. Fishbein, Ph.D., and M. Walker

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	0.4
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: (1) To investigate the utility of β-nitrostyrenes as chemical intermediates for positioning of labels in the substituted allyl and propenyl benzenes and for studying conjugative effects on a methylenedioxyphenyl ring induced by substituents in the side chain. (2) To study reaction types of the allyl and propenyl benzenes that could lead to Mannich bases, proposed metabolites of safrole.

METHODS EMPLOYED: Various organic techniques, thin layer and gas-liquid chromatography and NMR spectroscopy have been utilized.

MAJOR FINDINGS: Attempts at the reductive hydrolysis of 3,4-methylenedioxy-β-nitrostyrene to the corresponding acetaldehyde derivative have indicated that a number of products are formed. However, reductive hydrolysis of 3,4-diacetoxy-β-nitrostyrene appears to be a convenient route for synthesizing the corresponding diacetoxy acetaldehyde derivative and suggests that the methylenedioxyphenyl ring definitely plays a part in reactions of the nitroolefin side chain. The synthesis of safrole from this diacetoxyphenyl-acetaldehyde requires the formation of the allyl side chain via a Wittig reaction and the methylenedioxyphenyl ring via hydrolysis of the diacetate and coupling of the dihydroxy compound with methylene dibromide. This synthetic scheme permits maximum variability in positioning of radioactive labels and should afford the greatest number of compounds that may be of biological interest, i.e., the allyl and propenyl benzenes themselves, aminoalcohols, aminoketones, propional-

dehydes, acetaldehydes, the corresponding unsaturated aldehydes, vinyl ketones, etc.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The substituted allyl and propenyl benzenes are widely distributed in nature and some are known to be pesticidal synergists. Therefore, it is necessary to isolate, quantitate, and identify their metabolites and other conversion products to ascertain whether or not exposure or ingestion of these agents represent a potential environmental hazard. A theoretical understanding of the organic chemistry of these agents should facilitate their localization and characterization.

PROPOSED COURSE: (1) To synthesize safrole both with the methylenedioxy ring intact and in the absence of this group. (2) To investigate the chemistry of the synthetical compounds with three basic reaction types in mind: (a) Oxidation of side chain, allylic, etc.; (b) Oxidation - addition [Michael additions to α,β-unsaturated ketones by nucleophiles]; (c) Isomerization - oxidation and addition. This chemistry should provide a basis for delineating the metabolic pathways of safrole leading to Mannich bases as metabolites.

Serial No.: NIEHS-ASC-027
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "NMR Spectroscopic Examination of Complex Equilibria Involving DDT and Related Chlorinated Hydrocarbons"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: N. K. Wilson, Ph.D.

OTHER INVESTIGATORS: R. Ferguson

COOPERATING UNITS: None

MAN YEARS:

Total:	0.8
Professional:	0.5
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: To determine the thermodynamic parameters, e.g., energies of interaction, for the formation of complexes between the pesticide p,p'-DDT and model compounds of biological interest bearing ester, amide and peptide configurations.

METHODS EMPLOYED: Variable temperature proton magnetic resonance was employed.

MAJOR FINDINGS: Two types of complexes have been observed, one where the primary site of interaction is the benzhydryl proton, and the other involving donation of electrons into the DDT aromatic π-electron system. Thermodynamic parameters have been elaborated for several systems.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The ubiquitous distribution and retention of DDT in mammals require a more precise characterization of the possible molecular association phenomena involved.

PROPOSED COURSE: An extension of the study of the thermodynamic parameters involving DDT with additional models of biological interest as well as an elaboration of other chlorinated hydrocarbons and cyclodienes such as methoxychlor, dieldrin and endrin will be undertaken to help clarify their mode of biological activity.

Serial No.: NIEHS-ASC-028
1. Analytical & Synthetic Chemistry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Metabolism of Saccharin"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: H. B. Matthews, Ph.D.

OTHER INVESTIGATORS: L. Fishbein, Ph.D.; A. Latimer

COOPERATING UNITS: None

MAN YEARS:

Total:	0.3
Professional:	0.2
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To elaborate the *in vitro* and *in vivo* metabolism of saccharin and derivatives.

METHODS EMPLOYED: (1) Incubation of ^{14}C-saccharin with various enzyme preparations and TLC analysis of saccharin and possible metabolites were undertaken.

MAJOR FINDINGS: Saccharin is metabolized by an enzyme preparation from rat liver to 4 metabolites, the nature of which are being elaborated.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The metabolism of this important food additive has not been fully elucidated despite its extensive utilization for over many decades. The possibility of potential harmful metabolites of saccharin, e.g., N-hydroxy and related derivatives, cannot be ruled out.

PROPOSED COURSE: Further *in vitro* studies with enzyme preparations from other organs, as well as identification of the metabolites and correlation of these results with those obtained from *in vivo* studies of saccharin metabolism will be undertaken.

ANIMAL SCIENCE AND TECHNOLOGY BRANCH

ANIMAL SCIENCE AND TECHNOLOGY BRANCH
Summary Statement

The Animal Science and Technology Branch has, as a primary mission, the responsibility for procurement, housing, maintenance and health status of research animals and breeding colonies used at the Institute. To accomplish this goal, in addition to the development of excellence in animal care, support laboratories proficient in clinical medicine, clinical pathology, comparative biology, diagnostic pathology, and microbiology must be maintained. Secondarily, these laboratories are expected to conduct relevant research within their spheres of competence.

During the current year efforts were expended toward further development or strengthening of basic service activities. Special emphasis was given to those functions vital to the smooth and effective occupancy of additional animal facilities. Completion of this structure late in the fiscal year should markedly alleviate the problem of inadequate quality and quantity of animal housing.

The greatest emphasis has been in the proper orientation and training of existing animal care personnel. As a group they were marginally trained, devoid of career ambition, and had not developed a sense of job purpose--a reason for being at NIEHS--a feeling for their role in the proper function of the Institute. The challenge was three-fold: to instill in them a cognizant goal, a perceptible means for attaining this goal, and a desire to strive for that goal. The animal facilities offered a tangible point of focus. Regularly scheduled training sessions, at least weekly in frequency, were initiated. Concepts that they could grasp and apply were stressed. As training progressed, their function in the total NIEHS effort became apparent and the desire to learn in order to "do their job right" subtly took form. The most important step, to which any measure of training program success can be attributed, was the identification and subsequent appointment of competent, concerned, first line supervisors. Their enthusiasm and quiet persistence with the program is most gratifying.

A second training program was also initiated for training new animal care personnel. This endeavor involving 6 trainees is a joint effort between this Branch and the "New Careers Program" of Operation Breakthrough, Inc., an agency funded by the U. S. Department of Labor. The objective is to initially select individuals from a financially and culturally deprived background and through orientation, remedial education, plus on-the-job training, qualify them for anticipated vacancies in which they should adequately perform. Additional groups may be started dependant upon program success in meeting objectives.

Advances were not made in the further development of laboratories supporting the service mission of the Branch. Factors which contributed to the static quality of this program were: failure to effect redirection of professional activities in the virology-serology laboratory; loss of virology technicians through transfer to another Institute; delay in identifying a veterinary pathologist to direct our Diagnostic Pathology Laboratory; inordinate demands on professional staff time in the planning, guidance, and execution of the in-service training program; and, the extensive effort required in the design, planning, and purchase of equipment for new facilities. Marked improvement in the quality of Branch laboratory efforts is expected. Dr. Loren Koller, a veterinary pathologist, is to assume the direction of our Diagnostic Pathology Laboratory at the end of this fiscal year. Additionally, the successful fruition of current recruiting efforts should then provide an excellent base from which to advance with our laboratory support and research efforts.

The Disease Surveillance Program generated results that provided a rational basis for the selection of commercial animal sources. It is observed that marked fluctuation occurs in animals from the most reputable sources underlining the necessity for surveillance procedures to be of a continuing nature.

Modest success was attained with the opossum program. Disease morbidity and mortality levels have undergone a sharp decrease from previous years. Rigid control of animal sources, combined with better husbandry and sanitation, probably account for this finding. A group of captive-born opossums were reared and maintained free from parasites. Their subsequent mating has resulted in the birth of an F-2 generation in 42% of the females, a percentage without previous parallel. A further reduction in the incidence of endocarditis coupled with continued advances in female fertility should result in the formation and sustenance of a closed, captive-born colony in which a majority of extraneous variables have been controlled.

A small colony of greater bushbabies, Galago crassicaudatus, has been assembled through the cooperative efforts of Dr. Robert Cooper of the San Diego Zoological Society and NCI. Initial efforts have been in familiarizing NIEHS personnel with the husbandry, biology, and reproduction of this prosimian primate. This colony is to serve as a resource from which Branch scientists are to characterize and evaluate the species' potential as a research animal.

Continued research dealing with the chlorinated phenoxyacid herbicide proved most interesting. The ability of 2,4,5-trichlorophenoxyacetic acid to produce teratologic effects (cleft palate) in mice was confirmed. It was further found that the oral, as well as the subcutaneous route, produced similar effects. Preliminary data indicates that exposure to 2,4,5-T at time of palate closure, rather than chronic exposure throughout gestation, produces the observed teratogenic effect. The butyl, iso octyl, and propylene glycol butyl ether esters of 2,4,5-T, forms in which this herbicide is often used, were also found to be teratogenic.

Silvex (2,4,5-trichlorophenoxyproprionic acid) did not produce a teratological response in mice. Comparative studies on the absorption, metabolism, and excretion of 2,4,5-T and Silvex have begun. Initial results show that serum levels of Silvex are lower and disappear earlier than do those of 2,4,5-T. It is hoped that observed variations in the metabolism and excretion of these

compounds will allow for development of a hypothesis which accounts for their opposite teratological properties.

The Branch Chief appeared before the 2,4,5-T Advisory Committee of the Environmental Protection Agency and subsequently forwarded research data it requested. Another activity of note is the Branch Chief's current membership on the Committee of Laboratory Animal Ecology, Institute of Laboratory Animal Resources, National Research Council.

Research, on a markedly reduced level, continued on the interaction of chemicals and viral infections. Much of this work involved the organic arsenical 4,3,BAA (4 hydroxy, 3 nitro benzenearsonic acid). Variable results were observed when this compound was interacted with different viruses.

Serial No.: NIEHS-AST-001-2
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Interactions Between Environmental Chemicals and Viral Infections in Mice and Tissue Cultures"

PREVIOUS SERIAL NUMBER: NIEHS-AST-01.001

PRINCIPAL INVESTIGATOR: Joseph H. Gainer, D.V.M.

OTHER INVESTIGATORS: Worth I. Capps
Paul R. Hill

COOPERATING UNITS: None

MAN YEARS:

Total:	1.6
Professional:	1.6
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To test for interactions between sublethal doses of various chemicals, principally salts of heavy metals and viral infections in mice--to look for probable mechanisms of action.

METHODS EMPLOYED: Mice are injected with or are fed the chemical in their drinking water for various periods before or after the administration of an LD_{10-20} of a necrotizing virus. Mice are weighed periodically to determine whether the chemical alone is reducing the growth of the animals.

When enhancements have occurred between certain viruses and certain chemicals, interferon levels in plasma and tissues have been determined to attempt to explain mechanisms. Interferon is produced by poly I/poly C in mice or in tissue culture. It is determined in primary mouse embryo cultures by the plaque assay procedure.

MAJOR FINDINGS: CD-1 male mice, 4 weeks of age, administered $NiSO_4$, .001 or .005 M solution in their drinking water for two weeks experienced a 2-fold increase in mortality [$p<.05$] over controls when inoculated with the encephalomyocarditis (EMC) virus; other mice administered the same concentrations of $CuSO_4$ or $FeCl_3$ had no such increase in mortality to the EMC virus. This increase in susceptibility to EMC from Ni toxicity follows the increased susceptibility to EMC as caused by $CoSO_4$ reported a year ago. [Ni accounted for only a slight

depression in growth of mice over controls prior to virus challenge.] $HgCl_2$ at levels which accounted for depressed growth, .0003 M and .0001 M did not add to EMC or to pseudorabies virus (PRV) mortality.

Studies have continued with the arsenicals, including the organic arsenical 4,3 BAA (4-hydroxy, 3-nitro benzenearsonic acid). When administered in the drinking water to 4 to 8 week old CD-1 male mice, 4,3 BAA is of the same order of toxicity as is $NaAsO_2$ or Na_2HAsO_4, that is, either of these 3 compounds becomes lethal when administered at concentrations greater than .002 M; and a concentration of .002 M severely retards growth of immature mice. The finding that the organic arsenicals are as toxic as the inorganic salts is very important for up to now it has been held that the organic arsenicals are much less toxic than are their inorganic counterparts.

4,3 BAA at .002 M to CD-1 male mice, 4 weeks of age, for 1 week synergized with Saint Louis Encephalitis virus (SLE), enhancing its mortality two-fold over controls ($p<.05$), whereas WE (western encephalitis) virus mortality was not so affected. When mice were given 4,3 BAA, 1 day after virus, both SLE and WE mortality were now synergized. These data suggest that interferon (IF) may play a strong role in the prevention of SLE infection, the synthesis of the IF being inhibited by 4,3 BAA. In the case of WE, IF may not play such a role in the primary prevention of infection, but it may have value later on in the course of the infection.

4,3 BAA also synergized with EMC and PRV mortalities in mice and it accounted for within cage transmission of EMC but not of PRV. A low percent of 4,3 BAA control mice exhibit encephalitis or paralysis.

Interactions of arsenicals with interferon have been studied. Sodium arsenite (NAS) partially reversed the protective effect of poly I/poly C for EMC mortality. NAS enhanced EMC virus titers in the spleens of the mice and reduced IF levels therein. NAS inhibited the production of IF in primary rabbit kidney cell cultures as stimulated by poly I/poly C, and as assayed for with pseudorabies virus. NAS reversed the action of mouse IF in mouse embryo cultures through cell interaction, not through direct binding of the As to the IF nor through binding to the cell through pre-treatment of the cells with the NAS. There was an excellent dose-response relationship between concentration of NAS and inhibition of IF action, the NAS being active through concentrations from $10^{-4.5}$ M to $10^{-6.5}$ M. When low concentrations of NAS were tested with low concentrations of IF, a slight stimulatory effect of NAS on IF action followed.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The finding that arsenicals, both inorganic and organic, at the same concentrations, enhance the susceptibility to certain viral infections, presents several significant considerations as follows: 1) What are safe levels of arsenicals for man? 2) New considerations about the addition of organic arsenicals to animal feeds. 3) May arsenicals be cocarcinogenic with oncogenic viruses in man? That arsenicals enhance certain viral diseases through the inhibition of interferon formation and action is of basic scientific importance relative to the understanding of the biochemistry of interferon. Conversely, the studies demonstrating that arsenicals depress the susceptibility to other viral diseases, probably through the inhibition of the uncoating of these viruses and

consequently the prevention of infection by them, illustrates another significant finding in the diversity of the biochemical aspects of viral infections.

PROPOSED COURSE: To interact other viruses, such as the adenoviruses, with arsenicals in tissue cultures; for this purpose, human embryonic cells will be used. To assay $NiSO_4$ effect on interferon formation and action; $AsCo^{2+}$ enhances VS in tissue culture and inhibits IF action, and it should be tested for its effect on IF formation.

There is another phase of this work which should be examined; that is, does a pesticide, such as lead arsenate, synergize with an arbovirus in the anthropod, particularly if the anthropod is resistant to the pesticide? It is hoped that a proposal of this nature may be implemented either with the Entomology Department at North Carolina State University or through the National Communicable Disease Center in Atlanta.

Other compounds for considered study on the "Enhancement of Viral Disease by Environmental Chemicals" are (1) the anti-cholinesterases and complement activity (one component of complement is an esterase which is degradable by those compounds); (2) lead, which we have examined to some extent but without obtaining consistently reproducible results in terms of enhancement of viral disease; and (3) dieldrin, with which we have done some work but which needs additional study. We have shown that dieldrin will enhance the EMC virus and the pseudorabies virus.

PUBLICATIONS

Gainer, J.H., Long, J., Jr., Capps, W.I., and Hill, P.R.: Inactivation of the pseudorabies virus by dithiothreitol. Virology. (In Press)

Serial No.: NIEHS-AST-004-2
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Interactions Between Environmental Chemicals and Viral-Induced Disease in Domestic Chickens"

PREVIOUS SERIAL NUMBER: NIEHS-AST-01.004

PRINCIPAL INVESTIGATOR: Joseph H. Gainer, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: Dr. Ray Harris
Dr. Pat Hamilton
North Carolina State University

MAN YEARS:

Total:	0.1
Professional:	0.1
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To study interactions between environmental chemicals and viral-induced disease in domestic chickens.

METHODS EMPLOYED: Slides prepared from turkeys fed varying concentrations of aflatoxin in their feeds have been examined.

MAJOR FINDINGS: Aflatoxin at 5 ppm, the lowest level fed, induced mild changes in the livers of the turkeys, in contrast to no disease in control birds. Higher levels induced progressively more severe hepatic changes including hemorrhage, fatty degeneration, but with some degree of healing.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These findings illustrate that turkeys are susceptible to aflatoxin B-1 poisoning and that they are substantially more susceptible than are domestic chickens to poisoning by aflatoxin B-1.

PROPOSED COURSE: Slides from chickens fed varying levels of aflatoxin are still at hand for study. Providing time permits, it is proposed that new experiments be conducted utilizing higher levels of the organic arsenical 4-hydroxy 3-nitro-benzenearsonic acid (4,3 BAA) first to establish its toxicity for chickens and secondly to interact it with Marek's Disease virus and/or Newcastle Disease virus.

These considerations stem from our observations in mice that 4,3 BAA is just as toxic as are the inorganic arsenic salts for young mice (AST-001-2 current report). These studies will be pursued at North Carolina State University.

Serial No.: NIEHS-AST-005-2
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Spontaneous Diseases of the Wild-Captive Virginia Opossum (Didelphis virginiana)"

PREVIOUS SERIAL NUMBER: NIEHS-AST-01.005

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.4
Professional:	0.2
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES:

1. To continue monitoring of spontaneous disease of captive wild opossums.

2. To evaluate the effectiveness of eradicating Salmonella spp. from the opossum by use of antibiotics.

METHODS EMPLOYED: Necropsies were performed on dead or debilitated opossums. Specimens were collected and submitted for microbiological and histopathological examination when indicated. Salmonella reactors received therapeutic doses (50 mg/lb) of tetracycline in drinking water daily for 30 days. Feces was subsequently cultured for Salmonella every 2 weeks for 6 weeks. Clinically ill opossums were treated with therapeutic doses of repository penicillin or chloramphenicol.

MAJOR FINDINGS: Overall mortality decreased from the previous year. The incidence of vegetative endocarditis decreased but was still observed in a majority of animals. Many of the opossums were septicemic; evidence of bite wounds were often observed.

Sick opossums treated with antibiotics occasionally recovered but most ultimately died.

Tetracycline, as administered, was ineffective in eliminating Salmonella from reactive opossums.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: A decrease in the incidence of spontaneous disease is probably attributable to better husbandry practices and solitary housing of animals. The opossum seems to be an excellent natural model for the study of vegetative endocarditis. The pathogenesis of the disease has not been determined but the evidence suggests that the opossum is prone to septicemias, some of which may be due to fight wounds. Many septicemias terminate in an acute death; opossums which survive develop vegetative lesions on the heart and thromboembolic lesions in other organs. The significance of this pathologic syndrome as it relates to experimental variability must be recognized by the investigator.

PROPOSED COURSE: Continued monitoring of the colony disease is planned.

Serial No.: NIEHS-AST-006-2
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Helminth Parasites of the Virginia Opossum (Didelphis virginiana)"

PREVIOUS SERIAL NUMBER: NIEHS-AST-01.006

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.6
Professional:	0.3
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: To identify helminth parasites infesting wild-captive opossums and to determine what lesions (if any) are inflicted upon the host. To compare the parasites infesting offspring born in captivity with the helminths infesting their mother.

METHODS EMPLOYED: Standard techniques were employed for collection, fixation and preservation of specimens. Identification of parasites was through original published descriptions and from comparison of specimens at the U. S. National Museum Helminth Collection, U. S. Department of Agriculture, Beltsville, Maryland.

MAJOR FINDINGS: A total of 17 helminth species were identified from 211 necropsies; 14 of these species inhabited the gastrointestinal tract. No deaths could be attributed to parasitism. Four helminth species caused obvious destruction of host tissue. Two of these species, Acanthocephala sp. and Paragonimus kellicotti were rarely found and are not considered to be significant. Ninety-two percent of all opossums examined had pulmonary lesions associated with infestation by the lungworm, Capillaria aerophila. The pathogenic response was judged never to be severe enough to significantly impair lung function.

The most significantly destructive parasite was the stomach worm, Physaloptera turgida, which caused focal necrosis of the gastric mucosa. Perforation of the stomach wall was not observed in this study, but is known to occur. Massive Physaloptera infestation conceivably could occlude the pylorus.

Opossums born in captivity and weaned at 3 months of age were free of all parasites, except for transient unidentified larvae which were no longer recovered in animals older than 5 months of age.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study is a descriptive and visual guide to identification of opossum parasites and is intended to aid investigators utilizing this animal. Although most of the parasites are innocuous, elimination or at least drastic reduction of the "parasite load" will concomitantly reduce extraneous variables in experimental data. Production and maintenance of young in vermin free premises interrupts the life cycle of the parasites thus a parasite free colony is easily attained.

PROPOSED COURSE: It is planned to publish a guide to identification of opossum helminths and to evaluate the efficiency of various anthelmintic regimens (see NIEHS-AST-013).

Serial No.: NIEHS-AST-007-2
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin"

PREVIOUS SERIAL NUMBER: NIEHS-AST-01.007

PRINCIPAL INVESTIGATOR: John A. Moore, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.2
Professional:	0.1
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: Identify the sequent of toxicologic processes which develop following exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

METHODS EMPLOYED: Initial work with the rat consisted of a screen, incorporating many clinical procedures, to ascertain which organs were affected and the earliest time interval at which abnormalities were detected.

Later studies will focus on characterizing the sequence and magnitude of those metabolic dyscrasias that are involved in the toxic process. Selected hematologic, clinical chemistry, enzyme chemistry and histologic procedures would be employed.

MAJOR FINDINGS: In the initial study, rats that received a 10 µg/kg dose of TCDD for 10 days exhibited a cessation of weight gain and subsequent weight loss. Hematologic tests indicated a reduction in circulating thrombocyte values and increased serum transaminase levels. Histologic examination of selected tissues showed an acute, diffuse, toxic hepatitis. Electron photomicrographs of liver revealed a marked reduction in the amount of rough endoplasmic reticulum present in each cell; other organelles were usually not affected.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Data pertaining to TCDD's toxicologic properties and the mechanism by which it produces its toxic effect are requisite for rational evaluation of its role as a health hazard.

PROPOSED COURSE: Work on this project has been currently suspended. It is anticipated that it will be reactivated once collaborative efforts with a pathologist and clinical chemist are initiated.

Serial No.: NIEHS-AST-008
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Teratogenic evaluation of selected esters of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T)"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: John A. Moore, D.V.M.

OTHER INVESTIGATORS: K. Diane Courtney, Ph.D., Pharmacology and Toxicology Branch

COOPERATING UNITS: None

MAN YEARS:

Total:	0.6
Professional:	0.1
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES:

1. Evaluate the teratogenic potential of three commonly used esters of 2,4,5-T.

2. Ascertain if there is variability in teratogenic effects of the esters as compared to the free acid.

METHODS EMPLOYED: The butyl-isobutyl, iso octyl, propylene glycol butyl ether esters were studied. Date bred pregnant mice received doses equivalent to 100 mg/kg free acid on days 6 thru 15 of gestation of one of three esters. Mice were killed on day 17 of gestation; fetuses were harvested, weighed, and preserved for subsequent examination by gross necropsy. Fetal mortality, maternal weight gain and maternal liver to body weight ratio were also recorded and evaluated.

MAJOR FINDINGS: All three esters of 2,4,5-T were teratogenic as evidenced by the production of cleft palates. A significant reduction in fetal weight was also observed.

The ranking of esters as to teratogenic potential was not possible since the incidence of cleft palate, although always observed, showed marked fluctuation. The fluctuation in cleft palate incidence may be inherent in the original experimental design. Several factors to be considered are genetic heterogeneity

in the mouse stock tested, calculation of dose on an average group weight, and the fact that doses employed may be at the threshold dose, above and below which there is essentially an "all or nothing" response.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Esterification of the free acid of 2,4,5-T to either the butyl-isobutyl, iso octyl, or propylene glycol butyl ether ester did not reduce the herbicide's teratogenic effects. Whether certain esters are teratogenic at dose levels below those of the free acid should be ascertained since the commercial production of the herbicide is often in the ester form.

PROPOSED COURSE: Replicate studies in an inbred strain of mouse are underway. Blood levels of each compound will be determined. Based on these results, subsequent doses may be altered to see if there is variability among esters in the minimal dose required to cause cleft palate. Should variability between esters be found, investigations would commence to identify the factors contributing to this variability.

Serial No.: NIEHS-AST-009
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "2,4,5-T: Interrelationship of dose, dose frequency and fetal age on the incidence of cleft palate in mice"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: John A. Moore, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.6
Professional:	0.1
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES:

1. Determine the minimum time frame during which exposure to 2,4,5-T will result in cleft palate.

2. Determine if an interrelationship exists between dose, route, and dose frequency on cleft palate incidence.

METHODS EMPLOYED: Mice, all of which have been bred within a known 8-hour time period, receive an oral dose of 2,4,5-T, 100 mg/kg or 125 mg/kg, on various days immediately preceding palate closure. Mice are killed on gestation day 17; the fetuses are harvested, weighed and checked for presence of cleft palate. Variations in fetal weight, fetal mortality, maternal weight gain, etc. are also recorded and evaluated.

MAJOR FINDINGS: Findings are still incomplete. However, cleft palates are observed in litters from females who received 125 mg/kg of 2,4,5-T for either four, three or two consecutive days. The litter incidence of cleft palate appears to correlate positively with dose frequency. Similar results are obtained when the herbicide is administered by the oral or subcutaneous route.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Previous work (NIEHS-PT-005) showed that 2,4,5-T produced cleft palate in litters from female mice which received a daily, parenteral dose of 100 mg/kg on days 6 thru 15 of gestation. There is a preliminary indication that three or even two doses of 2,4,5-T administered at the time of palate closure will result in cleft palate. This suggests that the herbicide produced cleft palate in mice through maternal exposure at a critical time of palate development and, repeated daily maternal exposure throughout fetal organogenesis and maturation is not required.

PROPOSED COURSE: This project will be continued.

Serial No.: NIEHS-AST-010
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Teratogenic studies on 2,4,5-trichlorophenoxyproprionic acid (Silvex)"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: John A. Moore, D.V.M.

OTHER INVESTIGATORS: K. Diane Courtney, Ph.D., Pharmacology and Toxicology Branch

COOPERATING UNITS: None

MAN YEARS:

Total:	0.5
Professional:	0.1
Other:	0.4

PROJECT DESCRIPTION

OBJECTIVES: To make a comparative teratogenic evaluation of the α proprionic acid and the acetic acid form of the 2,4,5-trichlorophenoxyacid moiety.

METHODS EMPLOYED: Date bred pregnant mice and rats received varied subcutaneous doses of Silvex on days 6 thru 15 of gestation. Fetuses were harvested prior to parturition, weighed and preserved for subsequent gross necropsy. Fetal mortality, maternal weight gain and maternal liver to body weight ratio were also recorded and evaluated.

MAJOR FINDINGS: Silvex did not evoke any observed teratogenic effects in mice that received doses of 106, 159, or 265 mg/kg. There was a reduction in fetal weight observed at a dose level of 265 mg/kg.

Silvex was not teratogenic in the rat although there was a significant reduction in maternal weight gain at the 53 and 106 mg/kg doses. Marked toxicity and maternal death occurred at the 265 mg/kg dose level. A significant increase in fetal mortality, i.e., early resorption, was found at the 106 mg/kg dose.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The 2,4,5-trichloro configuration of the phenoxyacid is assumed to be essential for teratogenicity to be observed. Substitution of acetic acid by proprioin acid (bonded to the phenoxy radical at the α position) abrogates the teratologic response. The proprionic acids' lack of teratogenicity may be associated with

different distribution, metabolism or excretion, which may spare the fetus' exposure to the compound. Should credence be given to the extrapolation of results observed in mice to man, practical application could be substitution of the proprionic acid form for 2,4,5-T in field application.

PROPOSED COURSE: The objective of the projects were obtained. The results prompted further study which is reported in Project No. NIEHS-AST-011.

Serial No.: NIEHS-AST-011
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Comparative Studies on the Absorption, Metabolism and Excretion of 2,4,5-Trichlorophenoxyacetic Acid and 2,4,5-Trichlorophenoxyproprionic Acid in Mice and Rats"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: John A. Moore, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.8
Professional:	0.2
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: To compare the metabolism of 2,4,5-T and Silvex in mice and rats in an attempt to formulate a rational hypotheses for the difference in teratogenic effects of the two compounds.

METHODS EMPLOYED: 2,4,5-T and Silvex are assayed using a spectrophotometric method. The levels of these compounds and their distribution in serum and urine over time are determined for several dose levels.

MAJOR FINDINGS: A graph plotting mouse serum levels against time shows that curves for 2,4,5-T and Silvex are parallel with Silvex values 50 - 70 µg/ml lower than those obtained for 2,4,5-T.

Serum levels in rats are similar for the first 24 hours but whereas 2,4,5-T levels at 48 hours approach zero, those for Silvex persist through 72 - 96 hours.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Understanding the factors producing differences in blood levels, distribution, or excretion of 2,4,5-T and Silvex may shed insight on the mechanisms by which a methyl group addition to 2,4,5-T abrogates the teratogenicity of the compound.

PROPOSED COURSE: The project will continue with emphasis on rats and form in which the compounds appear in the urine. Collation of results with comparative fetal studies will occur.

Serial No.: NIEHS-AST-012
1. Animal Science and Technology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Sedation and Anesthesia of the Virginia Opossum (Didelphis virginiana)"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.2
Professional:	0.1
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To evaluate the effectiveness of a series of sedatives and anesthetics in immobilizing the opossum for pre-determined periods of time. Drug efficacy was based upon diminution of aggressive behavior, loss of retention or consciousness, analgesic effect, and degree of muscular relaxation.

METHODS EMPLOYED: Four drugs selected for rapid intramuscular injection included: promazine hydrochloride, phencyclidine hydrochloride, ketamine hydrochloride, and, fentanyl-droperidol. Methoxyflurane was evaluated as an inhalant anesthetic for prolonged surgical anesthesia.

MAJOR FINDINGS: Ketamine hydrochloride (20-25 mg/kg) and fentanyl-droperidol (0.75-1.0 cc/kg) were equally effective in immobilizing the opossum for approximately 1 hour. Fentanyl-droperidol demonstrated a superior analgesic effect.

Phencyclidine hydrochloride (5-6 mg/kg) caused lateral recumbency but muscular tension was exaggerated.

Promazine hydrochloride failed to immobilize the opossum at dosages 5 to 10 times greater than recommended for other small animals (1-2 mg/kg).

A methoxyflurane atmosphere induced loss of consciousness in 5 to 8 minutes with sufficient relaxation to permit endotracheal intubation. The animal was then attached to an anesthetic machine containing methoxyflurane. Surgical

plane of anesthesis was subsequently reached and maintained for 2 hours. Recovery was uneventful in 12 minutes.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The ability to rapidly immobilize an opossum by intramuscular injection of a suitable drug decreases the danger of handlers being bitten or clawed during experimental procedures.

PROPOSED COURSE: This project has been completed.

PUBLICATIONS

Feldman, D.B. and Self, J.L.: Immobilization and anesthesia of the virginia opossum Didelphis virginiana. Laboratory Animal Science. (In Press)

Serial No.: NIEHS-AST-013
1. Animal Science and Technology Brar
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Anthelmintic Treatment of Opossums in a Breeding Colony"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.4
Professional:	0.1
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: To evaluate a series of anthelmintics, singly and in combination against the common opossum helminths in the attempt to eliminate as many para sites as possible without subjecting the animal to repeated treatments.

METHODS EMPLOYED: Six anthelmintics were evaluated: carbon disulfide-pipera zine complex; dichlorvos; drocarbil; l-tetramisole; thiabendazole; and thia- bendazole-piperazine complex. Combinations of the above were subsequently ad ministered.

Experimental animals were necropsied 7-14 days post-treatment. The number of parasites remaining in the gastrointestinal tract and lungs was tabulated.

MAJOR FINDINGS: No anthelmintic employed affected the lungworm, Capillaria aerophila. The CS_2-piperazine complex was effective in eliminating the stoma worm, Physaloptera turgida, and significantly reducing the oxyurid population Trematodes numbers were reduced with dichlorvos but the trichostronglyle popu lation was unaffected. In contrast, l-tetramisole produced an opposite effe

The combination of l-tetramisole and piperazine-CS_2 complex was judged most superior in that Physaloptera turgida was eliminated and population of the most common nematode species were markedly reduced.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study demonstrated the difficulty in eliminating all internal parasites from wild-captive opossums by employing anthelmintics. These drugs are too parasite specific to affect the entire diverse population infesting the opossum. It is apparent that a colony of parasite free opossums will be realized only through the establishment and management of a captive-born colony.

PROPOSED COURSE: Termination of the above study and subsequent publication of results is planned.

Serial No.: NIEHS-AST-014
1. Animal Science and Technology Bran
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Evaluation of a Captive-Born and Reared Colony of Opossums"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Donald B. Feldman, D.V.M.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.2
Professional:	0.1
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: To evaluate the physiologic, pathologic, and reproductive parameters of a "closed" colony of captive-born opossums.

METHODS EMPLOYED: Sixty-four captive-born opossums of both sexes, weaned at approximately 3 months of age, were housed in a separate quarantine building. Animals were reared individually in a screened cage provided with a nesting box. The diet consisted of 3 parts dry cat chow moistened with 1 part ground bovine liver. No dietary supplements were added. Water was provided ad libitum.

Animals were periodically examined for internal parasites and Salmonellae through routine fecal examination. All moribund animals were euthanatized and necropsied. With the onset of the breeding season, partitions separating a female and male opossum were removed and the animals permitted to breed at will. Records of births were maintained.

MAJOR FINDINGS: Thirty-four of the original 64 opossums still survive. Losses are equally attributable to these factors: animals weaned too young, endocarditis, and deaths of male due to fighting of breeding pair.

Salmonellae of antigenic groups identical to the mother were inconsistently transmitted to some, but not all, young in a litter. All animals remain free of parasites.

Of 22 females mated in mid January, 8 litters (36%) averaging 10 young per litter have been born. Neonatal mortality has been essentially non-existent.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study has indicated the feasibility of maintaining a "closed" or quarantined nucleus of captive-born opossums for breeding purposes. The high mortality encountered can be markedly reduced through changes in management practices; i.e., wean young at a later age, pair male opossums with females only at time of estrus. The captive-born opossums were susceptible to endocarditis but the incidence was considerably lower than among wild-trapped animals.

Evidence has been presented that these animals are more uniform than wild animals in that the burden of parasitism has been eliminated; the incidence of endocarditis reduced; thus, these animals are superior candidates for bio-medical research by presenting few extraneous variables.

PROPOSED COURSE: Maintenance, enlargement, and continued monitoring of a captive-born closed colony is planned.

CELL BIOLOGY BRANCH

CELL BIOLOGY BRANCH
Summary Statement

The purpose of the Cell Biology Branch is to evaluate the influence of environmental contaminants on the fundamental determinants of heredity and cellular function. Areas of responsibility are wide-ranging by definition and in concept. However, present research deals principally with bioenergetics, the genome and mutagenesis, teratogenesis, protein synthesis, biotransformation of endogenous and xenobiotic compounds, chemical resistance mechanisms, chemical transport, biochemical mechanisms of intoxication, and mycotoxicology.

Bioenergetics

Only those aspects of bioenergetics related directly to mitochondrial activities are under study at the present time. Efforts are being made to identify heretofore undetermined components of the electron transport system, to determine the relationships between molecular architecture and function of the components, and to clarify chemical events on both sides of the oxidation-phosphorylation couple upon which energy conservation is dependent. Equally essential studies are in progress concurrently to identify environmental agents capable of interfering with mitochondrial activities, to localize effects with respect to specific molecular targets, and to relate in vivo findings to cytopathology in target tissues of mammals at realistic environmental toxicant levels.

Current studies have added substantially to understanding the roles of coenzyme Q (CoQ) and Cytochrome b (Cyt b) in electron transport and energy conservation. By use of the antibiotic Antimycin (AA), which inhibits electron transfer from cytochrome b to cytochrome c, it can be demonstrated that Cyt b exists in two allosteric forms with different redox potentials. It is during the transition in states that the potential is raised to the level necessary to supply energy for ATP formation from ADP and P_i. CoQ, which is positioned between succinic dehydrogenase and Cyt b in the electron transport chain, acts not only as a redox participant but also as an allosteric effector with respect to the two conformational forms of cyt b and with respect to succinic dehydrogenase. This is indicated by the fact that the thermodynamic equilibrium in the respiratory chain strongly favors the flow of electrons toward oxygen when CoQ is present. However, if CoQ is removed (by extraction) from the particles and the iron chelating agent 2-thenoyltrifluoroacetone is added, the flow of electrons under pressure of excess ATP is reversed and Cyt b is reoxidized even though communication with succinic dehydrogenase is completely blocked. These studies now make it feasible to localize activity of toxicants in the first part of the electron

transport chain and further to determine the nature of interactions at the molecular level.

Representatives of a number of classes of important pesticides have been evaluated for effects on mitochondrial functions and membranes. These include trichloromethylthioimides, dithiocarbamates, cyclodienes, DDT and its analogs and metabolites, methylenedioxylphenyl derivatives, and phenylmercuric acetate.

As expected from the considerable work by others, perchloromethylthioimides inhibit enzymes known to have functional sulfhdryl groups. However, at high concentrations the compounds also affect membrane integrity, as indicated by alterations in H^+ permeability. Thus, they are capable of multiple effects, but at minimum-effect levels, sulfhydryl enzymes appear to be the primary sites of detectable damage. This is relevant also to the mode of action of certain other pesticides discussed below.

Several methylenedioxyphenyl derivatives, including the insecticide synergist piperonyl butoxide, were shown previously to affect mitochondrial function in vitro, while others had no effect. The more active compounds are piperonyl butoxide, tropital, and myristicin, which inhibit state 3 (ADP-controlled) respiration and uncouple phosphorylation from oxidation. A number of other derivatives with the methylenedioxyphenyl moiety but without the lipophilic side chain were relatively inactive as phosphorylation uncouplers. These compounds are also relatively ineffective synergists.

Attempts to establish a relationship between in vitro findings and synergistic effects in rats in vivo were negative. No effect of piperonyl butoxide could be demonstrated on mitochondrial systems in rats administered up to 880 mg/kg. This is in contrast to reported inhibition of microsomal mixed function oxidases in vivo by 100 to 200 mg piperonyl butoxide/kg. These experiments appear to preclude mitochondria in vivo as a contributing factor in synergistic action, despite the sensitivity of phosphorylation in isolated mitochondria.

Experiments reported last year indicated that chlordane, heptachlor, heptachlor epoxide, aldrin, and dieldrin are potent and perhaps primarily, inhibitors of oxidative phosphorylation. The pattern of inhibition is similar for all of the compounds and shows three distinguishable concentration-dependent phases. At low concentrations (5 to 20 mμmoles/mg protein) state 3 respiration (phosphorylation)is inhibited. At 20 to 40 mμmoles, phosphorylation is uncoupled and substrate respiration is stimulated. Finally, at 50 mμmoles, the mitochondrial membrane is completely destroyed. This illustrates again that phosphorylation may be inhibited by certain pesticides without visible damage to membranes. This is relevant also to mode of action studies on phenylmercuric acetate discussed below.

More detailed studies on binding of chlordane in mitochondria show that only about 10% of the available chlordane is bound when ambient concentrations are sufficient to inhibit phosphorylation. Half of this is bound to the outer membrane. The other half is bound by the inner membrane where the

enzymes for oxidative phosphorylation are located. Assuming even intracellular distribution of chlordane in liver cells *in vivo* among nuclei, mitochondria, microsomes, and cell sap, which is an adversely biased assumption (since many pesticides accumulate preferentially in mitochondria), the pesticide content of the mitochondria can be shown to be in the range of overt chlordane toxicity symptoms in the liver of mammals.

Further evidence of localization of primary effects of chlordane in mitochondria was obtained with yeast cells. The active cyclodienes mentioned above inhibit yeast cell growth in the presence of nonfermentable carbon sources, such as ethanol, lactate, and glycerol, but have no effect on cells supplied with fermentable substrates (glucose, fructose). This shows that the cyclodienes are inhibitory only when there is obligative dependence of the cells on mitochondrial function for energy, and not when energy can be supplied through fermentation in facultative anaerobic organisms. It indicates further that endergonic reactions, such as protein synthesis are not inhibited by direct interaction with the pesticides.

All of these findings are consistent with the premise that oxidative phosphorylation is the primary target in the toxicity of cyclodienes. Moreover, they provide rather compelling arguments against the view that these compounds act directly on nerve impulse conductance mechanisms *per se*, since the amount necessary to block nerve impulses *in vitro* is 2 to 3 orders of magnitude higher than that required to inhibit phosphorylation. However, target cells *in vivo* have not yet been identified.

Studies similar to those on the cyclodienes were made on phenylmercuric acetate (PMA). Kidney, brain, and liver mitochondria were examined since the kidney is involved in acute toxicity of some mercurials and the brain is the critical site of damage in chronic, low-level exposure, especially in the case of alkyl mercurials.

Phosphorylation in mitochondria from all sources was highly sensitive to PMA, with ID_{100} values in the range of 15 to 20 mμmoles/mg protein and threshhold effects at less than 1 mμmole. This is equivalent to less than 1/20 the amount of mercury found in the brains of rats administered as little as 1 mg Hg/kg in a single dose, which is well below the range of concentrations inducing visible brain damage. Substrate-supported (state 4) respiration, with glutamate, succinate, or β-hydroxybutyrate as the substrate, was unaffected or stimulated at these levels. ADP:O ratios and respiratory control data show that phosphorylation was not uncoupled from oxidation in the manner analogous to classical uncouplers such as nitrophenols. It is concluded, therefore, that PMA has a direct effect on the phosphorylation side of the oxidation-phosphorylation couple. This system is the most sensitive of the mitochondrial activities tested and was completely inhibited without visible damage to mitochondrial membranes as viewed by electron microscopy.

Tests on yeast cell growth, as indicated above for the cyclodienes, reveal that PMA inhibits growth in the presence of fermentable or nonfermentable substrates about equally, but at concentrations somewhat above those necessary to inhibit phosphorylation in mitochondria *in vitro*. Thus, this test did not rule out phosphorylation as the primary target and basis of cell damage, but

it also did not preclude the possibility that protein synthesis or other synthetic reactions may be primary in cell damage. This point is of some importance since inhibition of protein synthesis has been postulated to be the primary biochemical effect of methyl mercury in brain cells. However, both phosphorylation and protein synthesis are sufficiently sensitive to account for cell damage, and further studies are required to determine which is indeed primary.

Genetics and Mutagenesis

Perhaps one of the greatest concerns arising from chemical contamination of our ecosystem is the risk of induced genomal changes which will affect the course of human evolution, and, more immediately, the quality of metabolism and various aspects of cerebral function. Thus, efforts are being made to develop techniques for detecting mutations in the mammalian body, to evaluate environmental agents with respect to mutagenic properties, and to provide insight into the relationships between chemical properties and specific effects at genomal targets. Toward the latter goal, normal patterns of DNA replication, interaction of DNA with selected chemical agents, and the repair of DNA are being investigated.

Current studies on mouse DNA show that under normal circumstances nucleotide sequences synthesized at different periods during S-phase of the cell cycle differ somewhat in base composition. At the beginning of S-phase, the sequences contain relatively more guanine and cytosine than sequences replicated later. The first and last sequences differ by about 10% with respect to these two bases. This mode of replication applies to genetically inert DNA in the centromeric and constitutive heterochromatic regions of mouse chromosomes as well as to the genetically active areas. Although the basic mechanisms and ramifications of time-related pattern changes are not yet understood, compositional differences may be of value as markers of specific genes whose expression, repression, or change might be detectable at the metabolic level.

Some time has been devoted to clarification of the chemistry of interaction between selected compounds, especially alkylating agents, and DNA. Bifunctional alkylating agents generally are more toxic than monofunctional analogs. Current studies indicate that this may be due to the ability of bifunctional agents to form intrastrand and interstrand crosslinks, both of which are lethal.

It was shown previously that replication in the heterochromatic regions of metaphase chromosomes is selectively inhibited by mustards. Since these regions are replicated only near the end of S-phase, these findings suggest possible differential sensitivity in DNA synthesis at different times, possibly related to differential rates of guanine and cytosine incorporation. Further experiments, however, indicate no effect of mustards on the rate of replication within S-phase. On the other hand, 2 to 3 times more crosslinks are formed in the light strand of heterochromatic DNA, which might account for the differential sensitivity of replication in this area.

Some members of classes of chemicals other than recognized alkylating agents also react with DNA. The chemistry and sites of interaction of many of these compounds have not yet been elucidated. Thus, during the year, a two dimensional thin-layer chromatographic procedure was developed which should be generally useful for locating specific bases and chemical groups that react with potential mutagens either in vivo or in vitro. After incubation of the cells with radiolabeled bases, the DNA is extracted, hydrolyzed, and the bases and/or base-toxicant derivatives separated by thin-layer chromatography. Where labeled test compounds are available, the cells may be incubated with the labeled compound instead of with labeled bases. Reaction products of certain N-hydroxy compounds have already been identified and the target bases thus located. This procedure should facilitate studies of the interrelationships between dosimetry and toxicity and mutagenesis, and it should be especially useful in identification of the specific base alterations that give rise to identifiable mutagenic lesions.

While DNA may react with numerous potentially mutagenic compounds, modified DNA fragments do not necessarily persist. This is due to the presence in most normal cells of enzymes which cleave the DNA molecule in the vicinity of the modified base and excise the altered fragment. The excised bases are then replaced by a normal fragment and rejoined. This repair process is believed to maintain hereditary fidelity, except in a few cases where repair mistakes occur or where repair cannot keep pace with DNA damage. This system, therefore, constitutes an important defense against genome changes induced by environmental agents. Thus, efforts are being made by use of mouse lymphoma cells to elucidate the components of the systems, the chemical mechanisms of the repair process, and to evaluate environmental agents with respect to effects on the various steps in the process.

Lymphoma cells are able to effect excission of alkylated nucleotides but they do not effectively carry out the repair process. Moreover, sulfur and nitrogen mustards have been found unsuitable as experimental tools because of their general toxicity. However, some repair has been detected by use of ultraviolet radiation to induce DNA damage. Efforts are now being made to select UV-resistant mutants in which the repair system may operate efficiently (although it is recognized also that resistance may be conferred by mechanisms unrelated to repair). One resistant clone has been obtained which exhibits a 10-fold increase in survival rate over the wild type. Cells from this clone will be used for future studies on the repair system per se and in assessing repair inhibitors, if feasible.

Studies were initiated this year toward development of mutagenicity assay techniques and tools. This is concerned primarily with selection of cultured mammalian cells grown in suspension with well-defined characteristics and development of culture conditions and techniques conducive to rapid and accurate determination of both the frequency and type of mutation. Notable advancements were made in several areas.

Soft-agar cloning medium used for selection of mutants was found to cause considerable variation in cloning efficiency. Thus, a study was made

of the variable components of the medium in an effort to eliminate the variability. It was found that the cells have an absolute requirement for pyruvate and that growth is further enhanced when linoleic acid, lipoic acid, and putrescine are supplied along with pyruvate. With these supplements, the problem of variability has been largely eliminated and it is now possible to clone effectively from a single cell.

A number of mutant L 5178 Y lymphoma cells have been isolated by plating in soft agar medium supplemented with Methotrexate to inhibit folic acid-dependent pathways. These clones have been characterized biologically and biochemically at the HGPRT and TK loci (see NIEHS-CB - 012). Cells with forward mutations to $HGPRT^-$ and TK^- have also been isolated.

It is anticipated that mutants induced in cultures will be used in a mouse-mouse host-mediated assay as genetic indicators for both experimental and environmental chemical mutagens. Initial trials indicated that recovery of cells injected directly into the peritoneal cavity of mice is not practical; thus, experiments are now being carried out to test the feasibility of confinement of the cells in dialysis bags, which permit diffusion of the mutagen and its metabolites, implanted in the peritoneal cavity. If this test system is satisfactory, it will be used to obtain data on mutation rates relative to specific mutagens and on the mutability of specific gene loci. Use of known mutants with previously characterized mutable loci may be helpful in characterizing mutagens with respect to the types of mutations they induce and their genome targets. It is recognized, however, that the relevance of this kind of assay to the ultimate effects of mutagens *in vivo* on specific stem cells *in situ* and in different animals remains to be established.

Teratogenesis

It was previously shown that chlorcyclizine and a number of other benzhydrylpiperazines produce fetal abnormalities, including cleft palate, thorax enlargement (edema), receded mandible, small mouth, some limb shortening, and hypocalcification of the spine. Cleft palate was correlated with thorax enlargement and remained correlated despite three tests designed to dissociate the two phenomena. Further experiments show that the diuretic triamterene reduces the incidence of cleft palate and the degree of thorax enlargement when co-administered with chlorcylizine. Whether these effects were due directly to the diuretic action of triamterene has not been established, but the experiments indicate persistent correlation between chlorcyclizine-induced cleft palate and thorax enlargement and edema.

The effects of chlorcyclizine were compared with those induced by four additional agents known to produce cleft palate; e.g. β-aminopropionitrile, triamcinolone acetonide, cortisone acetate, and N-nitroso-N-methylurea. The compounds were administered at doses that produce up to 100% cleft palate. The glucocorticoids, triamcinolone and cortisone, induce horizontal-type cleft palates but neither induce thorax enlargement in the mouse. N-nitroso-N-methylurea causes vertical-type palates with initial reduction in thorax size but return of the thorax to normal relative to fetal size on day 21.

Since there was no correlation between thorax enlargement with the latter compounds and cleft palate, it is clear that cleft palate may be induced via mechanisms not related to thorax pressure against the mandible. On the other hand, this does not preclude thorax pressure as one possible explanation for cleft palate in specific cases.

Other experiments concerning fetal development dealt with passage of pesticides across the placenta and pre- and postnatal effects. Studies on the herbicides diquat and paraquat show that diquat passes the placenta more effectively than does paraquat but that the latter is more persistent in the maternal blood stream. Moreover, the two compounds were distributed somewhat differently in the fetus. Diquat was found primarily in the fetal liver, kidney and amniotic fluid whereas paraquat accumulated also in the lung.

Experiments on placental passage of dieldrin show that passage increases with gestational age. Passage is reduced by phenobarbital but the mechanism of phenobarbital action has not been determined. Experiments now in progress show that most of the dieldrin in the maternal blood stream is associated with albumin and beta-globulins and that the amount associated with the latter increases with gestational age. The rate of dieldrin passage appears to be highly dependent upon the quantity of plasma protein circulating on the fetal side. No dieldrin was detected in fetuses perfused with saline but increasing amounts were found when the protein concentration of the fetal perfusate was increased.

Some testing of mycotoxins for fetal effects was done in support of the mycotoxicology program. Toxic metabolites from the fungus Alternaria tenuis (alternariol and alternariol monomethyl ether) were found to induce some malformations or increase in in utero death at the 50 mg/kg level.

Protein Synthesis

All metabolic processes and cell growth depend ultimately upon the flow of genetic information through transcription and, subsequently, translation into both structural and enzyme proteins. Thus, protein synthesis is relevant to environmental health in its own right, and is also a fundamental consideration in the responses of cells to nutrition and xenobiotic compounds relative to enzyme induction.

Current studies are concerned with effects of mercurials, certain pesticides, pesticide synergists, and the chelating agent nitrilotriacetic acid on protein synthesis and enzyme induction. It was found that methyl mercury induces disturbances in both liver microsomal enzyme activity and in protein synthesis in brain, liver, and kidney of rats. In animals treated for 7 days, there is a decrease of protein in the liver and kidney. Brain protein levels remain unchanged. Although RNA levels are unaffected, there are significant alterations in the levels of various ribonucleoprotein particles, particularly ribosomal subunits, monoribosomes, and polysomes. Polysomes decreased as much as 50%, with a proportional increase in subunit and monoribosome levels. This

suggests that methyl mercury either dissociates polysomes or prevents aggregation of the ribosomes and subunits.

In contrast to the situation in brain and kidney tissues, the methyl mercury induced a generalized increase in all ribosomal components in liver, suggestive of the response of the liver to other hepatotoxins. This is seen also in electron micrographs of the endoplasmic reticulum of liver cells, although there appears to be little or no change in physical structure (sedimentation characteristics) of the ribosomes. Changes in ribosomes and subunits indicate effects of the mercurial at the translation (protein synthesis) level. The consequence of this is expected to be early termination of polypeptide synthesis, which is in accord with the findings of Yoshina in his studies of the Minamata disease.

Studies on the effects of chlordane, piperonyl butoxide, and MGK-264 indicate that all of these compounds induce an increase in total RNA, ribosomal subunits and polysomes, but a small decrease in monoribosomes in the liver. These changes are concomitant with a 2-fold increase in both mixed function oxidases and UDP-glucuronyltransferases. Electron micrographs also indicate proliferation of rough and smooth endoplasmic reticulum. This appears to be the first work to relate induction of oxidases and transferases (detoxification enzymes) to fundamental indices of protein synthesis.

Some work was done on the influence of nitrilotriacetic acid (NTA) on cell growth and protein synthesis in response to proposed large-scale use of NTA as a substitute for phosphates in detergents. It was found that large amounts of NTA are necessary to inhibit cell growth or protein synthesis. In general, the effects in both cases were attributable to chelation of essential cations (Mg^{++} in the case of protein synthesis).

Toxicant Biotransformations

Mixed function oxidases and conjugative enzymes represent a first line of defense of the mammalian body against overload and intoxication by endogenous and xenobiotic compounds. Thus, a new program was initiated this year to determine the effects of various toxicants singly and in various combinations on these systems, to identify more clearly the nature of the enzymes responsible for detoxication of xenobiotics, and to elucidate the factors and fundamental processes associated with induction of the various enzymes.

As a first step toward facilitating studies on microsomal UDP-glucuronyltransferase, a simple quantitative radioassay technique was developed for the enzyme. Radiolabeled enzyme substrates are used and the reaction is carried out in scintillation counting vials. At the end of the reaction, scintillation fluid is added and the vial is shaken and then placed in the counting chamber. Unreacted substrate partitions into the scintillation fluid while the conjugate remains in the aqueous phase and the conjugated substrate is calculated by difference. This simple technique

greatly facilitates assay of large numbers of samples and is considerably more sophisticated than conventional colorimetric assay procedures.

Initial experiments, designed to determine optimal assay conditions, showed that microsomal UDP-glucuronyltransferase activity with some substrates may be stimulated as much as 10-fold by use of the surfactant Triton X-100. However, testosterone glucuronidation was inhibited. Mg^{++}, Mn^{++}, and Co^{++} were also stimulatory while Zn^{++} was inhibitory. Triton X-100 and Mg^{++} in combination effected a 30-fold stimulation. It appears, therefore, that this enzyme may be present in inactive or bound form under some conditions. This may be relevant to the mode of activation or "induction" of the enzyme by lipophilic or surface active compounds. Differential activity on different substrates suggests, further, that there may be two or more transferases in some cells.

A number of pesticides and drugs have been tested for inhibition and induction of transferases, and, in some cases, microsomal oxidases. The insecticide synergists piperonyl butoxide and MGK-264 were strongly inhibitory, with ID_{50} values comparable to those of microsomal oxidases. This suggests that if inhibition of pesticide metabolism is the basis of synergistic action, inhibition of conjugating enzymes may also be a factor in potentiation by certain compounds, especially phenolics and other mono- and polyhydric ring compounds.

Methyl mercury at high doses was also found to inhibit transferases in rats, but it had little effect at low doses. On the other hand, microsomal cytochromes P-450 (Cyt P-450) and b_5, were markedly reduced at methyl mercury doses as low as 2.5 mg/kg/day for 2 days. Aminopyrine demethylation and aniline hydroxylation were inhibited in proportion to the decrease in Cyt P-450. It appears, therefore, that the effects of the mercurial on mixed function oxidases is attributable to reduction of Cyt P-450 levels.

It was shown further that the depression of Cyt P-450 and associated enzymes was due to a reduction in smooth endoplasmic reticulum. Proliferation of rough reticulum was increased under the same conditions without effect on oxidase activity. It appears, therefore, that the smooth reticulum is the critical site relative to the effects of methyl mercury on mixed function oxidases.

A number of compounds were found to induce UDP-glucuronyltransferases when given singly or in combination to rats. Both piperonyl butoxide and MGK-264 pretreatment of male or female rats induced approximately 2-fold increases in the males and slightly more in the females. Induction occurred in both the smooth and rough ER with these compounds. This was in contrast to induction only in the smooth ER by chlordane.

Further experiments show that chlordane acts also as an inducer of microsomal hydroxylation of the carbamate insecticides carbaryl and carbofuran. N-hydroxymethyl carbaryl formation was enhanced 6-fold and ring hydroxylation at the 4 and 5 positions approximately 3-fold. Both chlordane and dieldrin also induce UDP-glucuronyltransferases, so that the rate of

conjugation and subsequent excretion of carbamates can be expected to be considerably enhanced by pretreatment with at least some of the chlorinated hydrocarbons.

Studies on conjugative enzymes in vitro were also carried out in connection with DDT resistance of cultured mouse lymphoma cells and in relation to fetal and neonatal development. Transferase activity in DDT resistant cells was 15 times that in control or chlordane resistant cells. However, other studies indicate that conjugation cannot account for DDT or for chlordane resistance. In the developmental studies, it was found that the lung has little UDP-glucuronyltransferase activity up to 3 days after birth whereas there is a marked rise in the enzyme level in liver just prior to birth. β-Glucuronidase activity appears prior to birth in both the lung and the liver. Further studies are planned on the age-related status of these enzymes in developing mammals in relation to susceptibility and body burdens of environmental agents.

Resistance to Pesticides

It was shown last year that L 5178 Y mouse lymphoma cell lines could be selected that show considerable resistance to DDT. DDT-resistant cells continue to show a high degree of tolerance to DDT and DDT analogs and metabolites. They are also resistant to chlordane, lindane, dieldrin, and heptachlor, but not to piperonyl butoxide. However, piperonyl butoxide does not synergize DDT toxicity in resistant or nonresistant cells.

Another resistant cell line has been selected through exposure to chlordane. This line exhibits equal resistance to chlordane and DDT, and is as resistant to DDT as DDT-selected cells. However, there are some metabolic differences in the two lines.

Normally, drug and pesticide resistance depends at least in part upon the action of microsomal enzymes found in the liver and elsewhere in the body. In the case of DDT, resistance may also be due to the DDT dehydrochlorinase. The resistance exhibited by DDT- and chlordane-resistant lines is due to a different, but as yet undefined, mechanism. No evidence for mixed function oxidases in these lines has been found and they contain no detectable cytochrome P-450 or b_5. On the other hand, high levels of UDP-glucuronyltransferases are present in the DDT-Resistant line. Levels are 3- and 15-fold greater than in control cells with testerone and 1-naphthol, respectively, as substrates. Levels of this enzyme are the same in control and in chlordane-resistant lines. Moreover, resistant cells are equally as sensitive as control cells to 1-naphthol despite the higher activity of transferases in the resistant line. Thus, it is doubtful that resistance is due to conjugation, especially in view of the absence of hydroxylation which must precede conjugation.

This type of resistance would appear to represent a heretofore unrecognized mechanism of defense of cells against foreign compounds, and further work on its nature and mechanisms in continuing.

Mycotoxicology

This program is wide-ranging, as is the conventional field of toxicology. One of its aims is to identify potentially hazardous fungi and the environmental parameters associated with their evolution and, more immediately, their toxin-producing capabilities. The principal effort, however, is directed toward isolation and identification of mycotoxins, description of mammalian pathologies associated with consumption or other modes of contact with the fungi, and development of appropriate methodology for assay of the toxins in consumable products and in the tissues of man and meat animals.

Toxigenic fungi are ubiquitous and toxin-producing species or strains are known in many genera. Current work, however, has been confined by necessity to three of the more common genera which have historically been associated in mycotoxicoses, e.g., Penicillium, Alternaria, and Aspergillus. The Alternaria have been studied most extensively and a number of metabolites from one species, A. tenuis, have been identified. This species is the causal agent of the brown spot disease of tobacco, but also occurs widely as a facultative parasite or saprophyte in other agricultural products.

One of the metabolites was previously unidentified. Its structure has now been determined and the compound was given the trivial name altenuene. It is a dibenzolactone derivative very closely related to two other known toxins, alternariol and alternariol monomethyl ether. A number of nontoxic metabolites, which are generally present in fungi and plant residues, have also been identified. Although they are of no importance as fungal hazards, identification of these was necessasy because they are normally encountered during gas chromatographic analysis of fungal extracts and, in some cases, confound analysis of toxins.

In addition to the tobacco isolate of Alternaria, dozens of other toxigenic species or strains in this genus have been acquired. Ten known toxic isolates were provided by scientists at the University of Minnesota. Most of these are more toxic to mice than the tobacco strain. In addition, some 140 isolates were obtained from Dr. E. G. Simmons, at the U. S. Army Natick Laboratories. Of these, 39 are toxic to bacteria in the agar plate test and 22 exhibit toxicity to mice, with LD_{50} values ranging from 200 to 1200 mg/kg for chloroform extractible solids.

A number of Aspergillus spp. from tobacco have been tested for toxin production, both in conventional media and in shredded tobacco. Among these are A. flavus, A. ochraceus, A. tamarii, and A. clavatus. A. flavus produces large amounts of aflatoxins B_1 and G_1 and kojic acid when cultured on rice. A. ochraceus produces several ochratoxins and patulin, A. tamarii produces both kojic acid and terrein, and A. clavatus produces substantial amounts of patulin in appropriate culture. However, none of these yield detectable amounts of the respective toxins when grown on shredded tobacco fortified with Czapek's-Dox broth. Thus, despite the fact that they are among the more abundant fungi in stored tobacco and produce virulent toxins under some conditions, they apparently do not create any particular hazard with respect to known toxins in tobacco.

One Penicillium species, P. cyclopium, which is a major tobacco storage fungus, has been studied and found to produce substantial amounts of penicillic acid in culture. This compound is carcinogenic at very low doses and was found to be produced in significant quantities by the fungus growing on tobacco. Consequently, tobacco containing the fungus was made into cigarettes, and the cigarettes were smoked by machine. Some cigarettes were also spiked with known amounts of the purified compound. No penicillic acid was detectable in smoke condensates even though the compound can be steam distilled and is volatile at elevated temperatures.

All aspects of methodology development have progressed quite rapidly. Satisfactory column procedures either exist or have been worked out for separation of all of the major toxins in crude extracts of all genera. These are now being used routinely to isolate sufficient quantities of the various toxins for toxicological tests in vitro and in small animals. Gas chromatographic and thin-layer chromatographic procedures have been developed for quantitation and confirmation of all of the toxins for which pure reference standards are available. In addition, current work with mixtures of toxins and nontoxic fungal metabolites indicates that it is quite feasible to separate and quantitate at least 12 components of interest simultaneously by gas chromatography. This is particularly important in analysis of moldy farm commodities or animal tissues where mixed fungal populations grow or where ingestion of food containing mixed populations occurs.

Several refinements have been made in primary biological assays for mycotoxins also. Bacterial assays of crude extracts and of column fractions during purification of compounds from crude extracts have been standardized. They are now based upon responses of universally available stocks of bacteria from the American Type Culture. A fungus assay, modified for extremely small amounts of test compound, has also been included in the screening procedure to broaden the spectrum of test organisms. Currently, efforts are being made to scale down the assay procedure for testing against mammalian cells in culture. This will substantially reduce the quantities of scarce compounds necessary for the test and permit a larger number of tests to be made with the small amounts of some toxins obtainable.

Toxicity of compounds on hand has been determined for bacteria, fungi, cultured lymphoma cells, and mice. Alternariol (AOH), alternariol monomethyl ether (AME), and altenuene are more toxic to lymphoma cells than to bacterial or fungal cells, with the ID_{50} at about 6 and 8 ppm, respectively, for AOH and AME. AOH effects two phases of the cell cycle. Initiation of G-2 phase is especially sensitive, but the effect is reversible. This appears to be associated with inhibition of RNA synthesis. AOH also inhibits cytokinesis in cells synchronized at metaphase and subsequently released. This also appears to be due to inhibition of RNA synthesis. The mechanism of these effects is as yet undetermined.

Among the more interesting effects of AOH and AME were those produced in the bacterial assay. It was found that neither compound when tested separately inhibited bacteria at less than 500 µg per disc in the agar plate

test. However, when AOH was tested in combination with either AME or altenuene, or both, only 0.25 µg of each compound was necessary to produce easily detectable inhibition zones. This represents a 2000-fold potentiation of AOH toxicity in this system. It is perhaps also significant from a mechanistic standpoint that no synergism was exhibited when the compounds were first dissolved and then added to either cultured lymphoma cells or bacteria in broth. However, if the bacterial culture tubes were coated with mixtures of the compounds and dried, the synergistic effect was apparent in the broth cultures. This strongly suggests that synergism in this case is dependent upon some kind of physical association between AOH and AME which occurs upon coprecipitation or drying. Studies designed to elucidate the mechanism of synergism exhibited by these two compounds in combination have been initiated. While it is recognized that synergism may result from interactions by more than one mechanism, we are confident that the principle involved in this system will provide understanding of this important phenomenon in other systems.

Tests for fetal effects of the mycotoxins in mice and rats were negative. The LD_{50} for mice is about 100 mg/kg. However, chronic treatment produces marked internal effects at less than 10 mg/kg, especially spleen and liver enlargement and either enlargement or necrosis of the thymus, depending upon dosage and length of exposure. Histopathological examination of the spleen and thymus revealed extensive stem cell necrosis. This suggests special susceptibility of rapidly dividing cells to the toxins and is reminiscent of the inhibition of cytokinesis in cultured lymphoma cells noted previously.

Some work was also initiated this year on the mutability of fungi and effects of mutations on toxin production, and on the control of biosynthesis of toxins. Five variants of Alternaria mali were selected from cultures grown in the presence of the fungicide maneb, 4 mutants from cultures treated with zineb, and 3 variants from those treated with ferbam. In general, the variants tended to be less toxigenic than the original isolate.

Variants of Helminthosporium maydis, the causal organism of southern corn blight, induced by UV radiation were also screened. This strain of H. maydis is not toxigenic, but three of mutants obtained were slightly toxigenic to mice.

Studies on the control of biosynthesis of Alternaria toxins have just been initiated. Preliminary tests show that glutamate at levels higher than 0.06 M in the growth medium completely suppresses synthesis of AOH, AME, and altenuene. No effects were seen on fatty acid synthesis. Aspartate, on the other hand, stimulated toxin production. These findings indicate rather specific controls related to metabolism of specific amino acids and not to nitrogen metabolism in general. Efforts are now being made to identify the metabolic pathways in toxin biogenesis and those steps that appear to be affected by glutamate and/or its metabolites.

Serial No.: NIEHS-CB-001
1. Cell Biology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Alkylating Agents in Target Tissues."

PREVIOUS SERIAL NO.: NIEHS-CB-01-1

PRINCIPAL INVESTIGATOR: Robert G. Owens, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: Physiological Pathology Branch

MAN YEARS:

Total:	0.6
Professional:	0.1
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: Alkylating agents are widely distributed in the environment, so that exposure to compounds with alkylating properties in common can come from many sources simultaneously, including pesticides, drugs, industrial intermediates and byproducts, and from toxigenic organisms. These agents are often general poisons that react with electron rich groups characteristic of all proteins, nucleic acids, and many other body constituents. Despite ubiquitous distribution of $-NH_2$, -OH, and -SH groups in tissuesand cells, these toxicants are often remarkably specific with respect to organs, cells, and even molecular components. The purpose of this project is to determine the molecular basis of preferential accumulation and action in these target areas under conditions of chronic exposure.

METHODS EMPLOYED: Charles River rats were used for all tests. The test compounds under study were phenylmercuric acetate (PMA), chloroethylmethane sulfonate (CEMS), and triethylohosphoramide (TEPA). The compounds are administered orally, singly and in combination, three times a week.

Histopathology is done by standard procedures and includes heart, lung, liver, pancreas, spleen, kidney, muscle, brain, and genitals.

MAJOR FINDINGS: It was shown previously that the three compounds administered in combination at 3.125 mg/kg produced essentially the same mortality as PMA administered alone, which indicated that PMA was the primary toxicant. Studies this year were concerned with minimal-effect time-dosages of PMA and

location of the primary target organ at these doses. Previous tests showed that 3.125 mg PMA/kg produced 100% mortality in less than a year. Follow-up experiments were carried out with 0.5 and 1.0 mg/kg in order to approach the threshhold lethal chronic dose. At 1 mg/kg, the LD_{50} was obtained in 7 months. At 0.5 mg/kg, the LD_{10} was obtained in the same period. At that time the experiment was terminated because protozoan parasites were found in the brains of some of the animals.

At these low PMA levels, histopathological examination of moribund animals revealed no recognizable abnormalities in any organs except the brain. Brain damage consisted of hemorrhagic lesions similar to those described for alkyl mercury damage in the Minamata disease and in experimental animals.

These and previous findings are consistent with the idea that phenylmercury compounds may produce two kinds of symptoms, depending upon dose and length of exposure period. At high levels, symptoms of toxicity resemble those reported for inorganic mercuric ions and death may occur before brain damage is obvious. At very low levels, where inorganic mercury symptoms are minimal or nonexistent, brain damage eventually occurs after long and persistent exposure. A recent quote from Norseth and Clarkson (Arch. Environ. Health 21: 717, 1970) is, therefore, worthy of note: "Organomercurials such as aryl mercury compounds, known to rapidly release inorganic mercury in vivo, have not been reported to produce central nervous system damage even if unchanged organomercurial must be expected to be able to penetrate the brain." Thus, our experiments appear to be the first to show that aryl mercurials do, in fact, cause central nervous system damage under chronic exposure conditions and do so at levels not greatly exceeding damaging levels of alkyl mercurials.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The hazard of alkyl mercury in the environment and in food products has been well publicized. Relatively much less significance has been attributed to aryl mercurials because they are more rapidly hydrolyzed and no dramatic episodes of human poisoning have been recorded. This study suggests that aryl mercury compounds cannot be safely discounted as environmental or occupational hazards, particularly in view of the fact that PMA has been one of the more important mercurials used in agriculture, in medicinals, and as a fungicide and algicide in paints, swimming pools, and in the paper industry.

PROPOSED COURSE: Long-term (life-time) studies are planned on other important alkylating agents, particularly some halogenated compounds, cyanates, isocyanates (and thio analogues), epoxides, lactones, and lactams of industrial and natural origins when facilities for long-term studies are operational. No additional long-term studies on PMA are anticipated, but studies of PMA effects on mitochondria, lysosomes, protein synthesis and other subcellular targets, described in Project No. NIEHS-CB-002, will be continued to clarify the modes of action of various forms of mercury.

Serial No.: NIEHS-CB-002
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Biochemical Basis of Phenylmercuric Acetate Toxicity"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Robert G. Owens, Ph.D.

OTHER INVESTIGATORS: B. Deal Nelson, Ph.D. and Paul E. Brubaker, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	0.6
Professional:	0.1
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: Experiments reported in Project No. CB-001 have shown that phenylmercuric acetate (PMA) at low-level, chronic doses produces brain lesions in rats. Previous metabolic studies in our laboratory indicate little effect of PMA at low levels on respiration or other processes examined. This suggests that brain lesions, which may occur several months after initiation of treatments, must result from subtle and cumulative effects of PMA. The purpose of this study is to elucidate the more sensitive biochemical processes and organelles to PMA and to relate effects at these loci to tissue degeneration. The particular processes of interest, based on our work and studies by others, are protein synthesis, energy metabolism, and tissue destruction resulting from release of lysosomal enzymes.

MAJOR FINDINGS: Electron Microscopy of Mitochondria. Reports of damage in cells and tissues by mercurials based on electron microscopical studies have not been consistent with respect to mitochondrial effects. In some cases, mitochondrial damage has been reported to be among the first visible signs of intracellular change. Other studies suggest little or no damage to mitochondria until extensive intracellular disorganization of endoplasmic reticulum, golgi apparatus, and other structures has occurred.

In our studies, isolated mitochondria were examined for structural changes upon aging for 2 hours, at which time all capacity for phosphorylation was lost spontaneously, and after treatment with PMA, which is a potent inhibitor of phosphorylation. No changes in appearance of the mitochondria were

detectable. This indicates that appearance of the mitochondria is useless in assessing damage to mitochondrial function, particularly phosphorylation, and it is counter to implications in the literature that lack of visible structural damage eliminates mitochondria as a primary target in mercurial toxicity. It is well established that phosphorylation and transport of certain ions in mitochondria are affected by some inhibitors at less than 1/10 the concentration necessary to affect mitochondrial structure.

Metabolic Studies: Dosage-response of phosphorylation and substrate-supported oxidations to PMA was determined with mitochondria from rat liver, kidney, and brain. Mitochondria from all sources were highly sensitive to PMA. ID_{100} values of 15-20 muM were found for ADP-stimulated respiration (phosphorylation). Perhaps more pertinent to long term in vivo effects is the fact that threshhold inhibition with brain mitochondria was obtained at less than 1 muM of PMA. This is equivalent to less than 1/20 the amount of mercury found in the brain of rats administered as little as 1 mg. Hg/Kg in a single dose (Norseth and Clarkson, Arch. Environ. Health 21: 717, 1970). Thus, sensitivity of phosphorylation in mitochondria is well within the range of damaging accumulations of mercury in the brain and in mitochondria per se in vivo, even at sublethal doses.

Substrate-supported respiration with glutamate, succinate, or β-hydroxybutyrate was not inhibited at these levels, and, in fact, was sometimes stimulated. Despite the stimulation of oxygen uptake, ADP:O ratios and respiratory control data did not indicate uncoupling of phosphorylation from oxidation in a manner analogous to classical uncouplers such as nitrophenols.

Yoshina et al. found that protein synthesis was affected in brain tissue slices by alkylmercury at concentrations that had no effect on respiration of glucose. They did not study effects on phosphorylation, however. Our studies indicate that phosphorylation may be affected without change in the rate of oxidation. Studies are now underway to determine the relative sensitivity of protein synthesis and phosphorylation in order to clarify cause and effect relationships in the initial or primary target processes.

Studies with yeast cells. It has been shown that compounds which act directly and specifically on mitochondria will inhibit the growth of yeast cells supplied with fermentable carbon sources such as glucose or fructose. This has been used to distinguish the action of antibiotics and of chlordane directly on mitochondria as opposed to the glycolytic system. This test was applied in the case of PMA to determine if mitochondrial activities are more sensitive than glycolysis. Some slight difference in sensitivity of the yeast cells supplied with ethanol or glucose was found. However, the difference was so slight as to suggest that energy yield from fermentation and mitochondrial mediated respiration are about equally sensitive to PMA, or that protein synthesis was blocked which would negate the importance of energy supply, regardless of its source. However, since the sensitivity of the yeast cells was exhibited at concentrations of PMA somewhat above that of phosphorylation in isolated mitochondria, this system ruled out neither phosphorylation nor protein synthesis as primary targets.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In chronic, low-level exposure to mercury and some of its compounds, the brain is the critical site of damage. This is somewhat enigmatic in view of the fact that considerably higher levels of mercury are accummulated in other tissues; e.g. kidneys, liver, and blood. It is important, therefore, to understand the basis of unusual susceptibility of specific tissues to mercurials and other toxicants, since clinical evaluation of toxicant levels often cannot be made directly on tissues involved. Basic information, however, about specific modes of action can provide a degree of predictability of clinical effects when threshhold tolerances, tissue repair rates, and degree of absolute dependence of the organ upon target processes are known. Thus, we can reasonably expect that knowledge of the principle target and molecular basis of mercury damage will contribute not only to understanding of mercury toxicity but also help to clarify the mode of action and localization of effects of other heavy metals and other types of toxic environmental agents with similar chemical properties.

PROPOSED COURSE: Protein synthesis and other endergonic processes obviously cannot be maintained without ATP synthesis. On the other hand, polymerases and other enzymes directly responsible for protein synthesis may be more sensitive than phosphorylation to mercurials. Since both phosphorylation and protein synthesis are now known to exhibit an order of sensitivity that might account for the initial or primary effects of mercury in the brain, we plan to determine which of these processes is primary in brain damage. Thus, simultaneous determinations of ATP synthesis (or maintenance) and protein synthesis will be made in brain tissue slices *in vitro* and in rats *in vivo* by use of appropriately labeled phosphate and amino acids. Attempts will be made also (in collaboration with Dr. Brubaker) to pinpoint primary effects in polysomal systems capable of protein synthesis, using ATP and mitochondria + ADP as alternative energy sources. This should indicate unequivocably whether the protein synthesizing system *per se* or generation of chemical energy is more sensitive to PMA. Similar tests with other mercurials are also planned and, depending upon feasibility, this system may be standardized as a basic test system for evaluating environmental agents with regard to effects on protein synthesis and bioenergetics.

Serial No.: NIEHS-CB-003
1. Cell Biology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Environmental Agents on the Biochemistry and Biogenesis of Mitochondria"

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-10

PRINCIPAL INVESTIGATOR: B. Dean Nelson, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	1.0
Professional:	0.5
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: (1) To study the interaction of toxic agents with mitochondrial membranes through analysis of oxidative phosphorylation and related enzyme reactions. (2) To elucidate the action of environmental agents on membrane formation during biogenesis of mitochondria.

MAJOR FINDINGS: Cyclodiene Pesticides. A. Studies with yeast systems. We have previously reported (see last year's annual report) the use of a yeast system to screen for toxic agents which act specifically on oxidative phosphorylation in intact cells. Our recent results show that chlordane, heptachlor, heptachlor epoxide, aldrin and dieldrin inhibit cell growth when the energy source is a nonfermentable substrate, and have no effect on growth when the energy source is fermentable. Endrin and Mirex did not effect growth on either energy source. These findings are in agreement with studies on isolated rat liver mitochondria in which these pesticides were shown to inhibit oxidative phosphorylation. These results indicate that in yeast, and quite probably in mammals, the action of cyclodiene pesticides is to disrupt oxidative phosphorylation. In yeast this is further substantiated by results showing that the concentration of chlordane needed to inhibit oxidative phosphorylation (10^{-6} to 10^{-7} M) was 2 to 3 orders of magnitude lower than that necessary to inhibit nerve impulses in mammalian tissue in vitro. The present studies with yeast also indicate that inhibition of oxidative phosphorylation is probably due to interference with

energy conservation rather than electron transport since the pesticides did not inhibit the reduction of tetrazolium dyes (a measure of electron transport function) under conditions in which cell growth on oxidiable substrate was inhibited. In agreement with these findings, our studies on isolated rat liver mitochondria also indicate that the action of the pesticide is on the energy transfer system. Whether the effect is due to a direct interaction of pesticide with some component(s) of the energy transfer system, or to interference with the genetic apparatus resulting in altered synthesis of some component(s) in this system, will remain the subject of future experimentation.

B. Rat liver mitochondria. In vitro studies on liver mitochondria show that chlordane, heptachlor, heptachlor epoxide, aldrin and dieldrin all inhibit oxidative phosphorylation, while isodrin and Mirex were ineffective. The pattern of inhibition was similar for all pesticides and they occurred in 3 distinct phases. At low concentrations (5 to 20 mμmoles/mg of protein) phosphorylation is limited as shown by inhibition of State 3 respiration (ADP controlled), valinomycin-induced mitochondrial swelling with either succinate, ATP or ascorbate + TMPD as electron donors, and by lack of inhibition of the ADP:O ratio. At intermediate concentrations (20 to 40 mμmoles/mg protein), oxidative phosphorylation is uncoupled as shown by activation of State 4 respiration (substrate), by the decreased ADP:O ratios, and by inhibition of reactions which require energy such as Ca^{++} uptake, H^+ transfer, and energy dependent mitochondrial swelling. Also, at these concentrations arsenate-activated respiration (which is inhibited at low concentrations of pesticide) is reactivated, indicating that low concentrations of pesticide inhibit by stabilizing some intermediate form in the phosphorylating sequence, probably a phosphorylated form. Finally, at high concentrations (50 mμmoles/mg), the mitochondrial membrane is completely destroyed as shown by H^+ ejection, inhibition of electron transport, and loss of membrane integrity as seen in electron micrographs. These reactions are strictly protein concentration dependent and in all cases pesticide titration curves show the 3 phases to be easily distinguishable and highly reproducable. Furthermore, these studies confirm our observations on intact yeast cells; e.g., that cyclodiene pesticides inhibit oxidative phosphorylation by interfering with phosphorylation rather than electron transport.

To determine how much pesticide is located at the inhibitable site when phosphorylation is disrupted, methods have been developed for determining the binding of chlordane and its distribution within the mitochondria. Approximately 10% of the chlordane added is actually bound to the mitochondria, i.e., when phosphorylation is inhibited (at 20 mμmoles chlordane/mg protein) only 2 mμmoles/mg is bound. Fractionation studies show that the bound material is distributed evenly between the inner membranes (where the enzymes for oxidative phosphorylation are located) and the outer membrane. However, the specific activity of the outer membrane is 2-3 fold higher than the inner membrane, probably because of its high lipid content. Based on these findings, and with the assumption that in liver tissue chlordane distributes itself evenly among the four compartments (nuclear, mitochondrial, microsomal, and soluble), we have calculated the total liver concentration of pesticide which would be expected when mitochondrial levels are sufficient to inhibit

phosphorylation. This value is 150 to 250 ppm, which is very close to that reported for liver when chlordane produces first toxic symptoms in intact organisms.

Methylenedioxyphenyl Synergists. Piperonyl butoxide (PB) is an insecticide synergist. Its synergistic action is thought to be due to competitive inhibition of microsomal enzymes which metabolize pesticides, thereby prolonging their biological halflives and increasing their apparent toxicities. The present study was concerned with whether part, or all of the synergistic effect could be due to uncoupling of oxidative phosphorylation.

A. In vitro studies. Experiments with rat liver mitochondria show that PB at concentrations which inhibit microsomal enzymes also inhibits oxidative phosphorylation. The most sensitive sites involve energy transfer, as shown by inhibition of ADP-controlled respiration and by the lowering of phosphorylating efficiency (reduced ADP:O and P:O ratios). The I_{50} values for PB (mμmoles PB/mg protein to give 50% inhibition) on State 3 respiration was 100 with succinate and 50 with NAD-linked substrate, glutamate, and β-hydroxybutyrate. In contrast, the I_{50} value for succinoxidase and NADH oxidase in nonphosphorylating particles was 15-25 times higher, i.e., 2,500 and 1500 mμmoles/mg protein respectively. These findings indicate that PB acts primarily on events leading to phosphorylation rather than on electron transport directly. However, the direct action of PB on the respiratory chain is somewhat specific for the NADH dehydrogenase as shown by the 5-fold difference in the I_{50} for succinoxidase and NADH oxidase. Structure-activity relationships were investigated using only compounds in which the side chain rather than the methylenedioxyphenyl moiety was altered. Results show that inhibition of oxidative phosphorylation is due only to the length of the side chain, i.e., the longer and more lipid soluble the side chain, the more effective the compound as an uncoupler. In contrast, the sites active in inhibition of microsomal enzymes are located on the methylene dioxybenzene ring.

B. In vivo studies. Since PB inhibited mitochondrial function in vitro at concentrations similar to those needed for inhibition of microsomal enzymes in vitro, further tests were carried out to determine whether the in vivo synergistic properties of PB could be, in part, due to its action on the mitochondria. Several attempts were made to determine the effect of PB on microsomal and on mitochondrial function by injecting rats with PB, removing the microsomes and mitochondria, and assaying the various functions in vitro. Experiments were conducted on normal rats and on rats in which the hydroxylating system was induced to high levels with phenobarbital. No effect of PB was observed on either the mitochondrial or microsomal systems. The explanation for the lack of an effect of PB on microsomal enzymes is that PB acts as a competitive inhibitor of the substrates to be hydroxylated. As a result, the small amount of PB bound to enzyme after isolation will be rapidly removed by substrate under assay conditions where substrate concentration is very high. The lack of an effect on mitochondria can not be explained this way, however, since inhibition of mitochondria in vitro can not be reversed by washing in BSA containing solutions. Concentrations up to 880 mg PB/kg body weight had no effect on

mitochondrial function. The above finding, along with literature reports that 100 to 200 mg PB/kg body weight is sufficient to inhibit hydroxylating reactions in vivo, are interpreted to mean that PB has little, if any, effect on mitochondria in vivo. It is concluded that the synergistic effect of PB is due primarily to inhibition of drug metabolism and prolongation of the biological life of the toxicant.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The effect of environmental agents on mitochondrial biochemistry and cellular energy metabolism is an important consideration in environmental health today. The present studies indicate for the first time the possibility that certain chlorinated pesticides, whose toxic action heretofore were thought to be non-specific, act rather specifically by interfering with energy transfer reactions in mitochondria. These findings point to the need for substantial work on alterations in cellular metabolism which result from inhibitory, yet nontoxic, concentrations of pesticides at the site of cellular control center, i.e., the mitochondria.

PROPOSED COURSE: Studies will continue on the binding of toxic agents to mitochondrial membranes and the resulting alterations in mitochondrial function and morphology. The action of toxic agents on membrane formation will be investigated in vitro with submitochondrial particles. The work with micro-organisms will be continued since these organisms afford a test system in which mitochondrial genesis can be controlled through manipulation of the growth media.

PUBLICATIONS

Nelson, B.D.: The action of the fungicides captan and folpet on rat liver mitochondria. Biochem. Pharmacol. 19: 1971 (In Press).

Nelson, B.D. and Williams, C.: Action of cyclodiene pesticides on oxidative metabolism in the yeast Saccharomyces cerevisiae. J. Agricul. & Food Chem. (In Press)

Nelson, B.D.: Induction of mitochondrial swelling by the fungicide captan. Biochem. Pharmacol. 19: 1971 (In Press).

Nelson, B.D., Drake, R., and McDaniel. O.S.: In vitro and in vivo effects of methylenedioxyphenyl compounds on oxidative phosphorylation in rat liver mitochondria. Biochem. Pharmacol. 19: 1971 (In Press).

Serial No.: NIEHS-CB-004
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Role of Coenzyme Q in Electron Transport"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: B. Dean Nelson, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.25
Professional:	0.25
Other:	0.00

PROJECT DESCRIPTION

OBJECTIVES: (1) To determine the role of Coenzyme Q (CoQ) in the electron transport chain, and (2) to determine the nature of the sites responsible for binding succinic dehydrogenase (SDH) to cytochrome b and the transfer of electrons between the two.

METHODS EMPLOYED: These studies involved primarily spectrophotometric analysis of cytochrome b under conditions described in the following section.

MAJOR FINDINGS: TTFA (2-thenoyltrifluoroacetone) is an iron chelating agent which inhibits succinic dehydrogenase, supposedly by chelating the non-heme iron moiety in the enzyme. We have found that the nature of the interaction of TTFA is quite different in the presence and absence of CoQ. When CoQ is extracted from submitochondrial particles, SDH no longer communicates with cytochrome b as shown by the fact that cyto b is not reduced with succinate. However, if the antibiotic Antimycin A (AA) is added all of the cytochrome b is reduced by succinate. Thus, AA, which binds to cytochrome b, produces a conformational change which positions the cyto b to SDH. If under these conditions (Q is extracted and cyto b is reduced with succinate and AA) TTFA is added, a rapid and complete reoxidation of cyto b occurs. The reoxidation is due to backward flow of electrons in the electron transport chain and is observed only in the absence of CoQ, since reincorporation of CoQ prevents the effect of TTFA. The significance of this finding is that in the presence of CoQ, or in particles capable of participating in phosphorylation, the

thermodynamic equilibrium of the respiratory chain strongly favors reaction going towards oxygen. Only in phosphorylating (coupled) particles in the presence of a high energy source such as ATP can electron transport be reversed and cyto b oxidized by reversal of the respiratory chain. One of the current theories of energy conservation for oxidative phosphorylation is that cytochrome b exists in two conformational states having different redox potentials. It is during the transition in states that the redox potential is raised to that necessary to supply the energy to drive the formation of a phosphate ester in ATP. The high potential form of cyto b is in equilibrium with the previous step in the respiratory chain and thus, under suitable conditions, can pass electrons to that member (reverse electron transport). We are probing the possibility that CoQ and non-heme iron play a role in this potential change, and are trying to determine if TTFA reversal of electron transport can be used as a simple model to study the basis for energy conservation at this site in particles which do not make ATP.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE MISSION OF THE INSTITUTE: Previous studies have shown that many environmental agents are potent inhibitors of electron transport and/or oxidative phosphorylation. Only in a few cases, where unusual specifity obtains, is the specific site of interference known. An impediment to full understanding of the mode of operation of environmental agents in these processes is a basic lack of information about details of the electron transport system itself. These studies are designed to clarify the components and mechanisms of electron transport and, subsequently, to facilitate understanding of interference mechanisms of specific environmental agents.

PROPOSED COURSE: The tenure in Dr. Ernster's Laboratory will terminate in June. However, work is expected to continue on this project at NIEHS toward the same goals.

Serial No.: NIEHS-CB-005
1. Cell Biology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Role of Cytochrome b in Electron Transport"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: B. Dean Nelson, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.25
Professional:	0.25
Other:	0.00

PROJECT DESCRIPTION

OBJECTIVES: To determine the cofactor binding sites and conformation of cytochrome b in relation to electron transport and energy conservation.

METHODS: These studies involved primarily spectrophotometric analysis of cytochrome b under conditions and treatmemts described in the following sections.

MAJOR FINDINGS: The antibiotic Antimycin A (AA) inhibits electron transfer from cytochrome b to cytochrome in mammalian respiratory chains. It is one of the most specific and potent inhibitors of electron transport known. Binding of AA occurs at, or near, cytochrome b as indicated by the spectral shift in the cytochrome to a longer wavelength after addition of AA. The binding of AA to this site is so significant because one of the energy conservation sites in the respiratory chain is located between cytochrome b and c_1. In a current theoretical scheme describing the chemical events in energy conservation at this site it is postulated that AA binds to only the oxidized, high energy form of cytochrome b. Little is known, however, about the nature of the binding of AA to cyto b, especially in regard to the chemical nature of the binding or the role of other components of the respiratory chain, such as non-heme iron or CoQ which are positioned very near cyto b. We have undertaken a series of experiments on the binding of AA to cytochrome b, with particular emphasis on the role of CoQ on this phenomenom. AA exhibits a high fluorescence when bound to BSA. In our experiments we have constructed AA binding curves by adding increasing

concentrations of AA to BSA solutions containing submitochondrial particles, removing the particles by centrifugation and measuring the fluorescence remaining in the supernate. From this a Scatchard plot is constructed. This is a plot of the ration of bound/free AA vs. bound AA. When only one binding site is present this relationship is negative and linear, but when two or more binding sites are present, the relation is negative and nonlinear. Where the experimentally determined lines cross the X axis the value of N (total number of binding sites) can be calculated, and where they cross the Y axis, K (the dissociation constant) can be derived. From K one can easily calculate the binding constant. In our experiments the AA binding curves did not fit a classical Scatchard plot. At low concentrations of AA a plateau was obtained. The plateau extended to a point where 40 to 50% of the cytochrome b was titrated as indicated by AA titration curves for cyto b reduction and inhibition of succinoxidase. These findings are interpreted as follows: Cytochrome b exists in two conformations and AA acts as an allosteric effector converting one form of cyto b to the other. The plateau region is the region in which the allosteric effect occurs. When a molecule of AA binds, it induces a conformational change exposing additional binding sites, thus the value of N increases. When the allosteric sites are saturated a more linear, and negative slope appears. The allosteric sites are saturated at approximately 0.08 - 0.10 ug AA/mg protein and the total number of binding sites are 0.16 to 0.20/ mg protein.

When CoQ depleted particles were examined in this manner, the general shape of the plot, the total number of binding sites, and the number of allosteric binding sites were not altered, but the binding constant was increased. Upon reincorporation of CoQ into the particles the value of K was restored to normal. These data suggest that CoQ regulates in some manner the binding of AA to cytochrome b. To study this further, we developed a method for determining the affinity of the AA binding site for AA by measuring the rate of transfer of AA from BSA to cytochrome b. This is done by adding AA to particles suspended in BSA and measuring the rate at which cyto b is reduced. BSA retards AA binding to the extent that the transfer of AA is several orders of magnitude slower than the rate limiting component of the respiratory chain. Thus, cyto b reduction measures the actual binding of AA. Titration curves for Q-containing and Q-extracted particles show that at low concentrations of AA, binding is retarded in CoQ-containing particles and accelerated in CoQ-deficient particles. Once the initial binding sites (probably the allosteric sites) are saturated the rate of binding in both types of particles becomes identical. These findings are in agreement with fluorescence measurements and they indicate that the affinity for AA is higher in the absence of CoQ. These experiments, however, add another dimension. They indicate that the affinity of only those binding sites involved in the allosteric effect of AA are influenced by CoQ. If one assumes that allosteric (conformational) changes in cyto b, similar to those induced by AA, occur during electron transport or energy conservation (as suggested by several investigators), then the results suggest that CoQ may have a role in controlling these conformational transitions. Because CoQ is the preceding member of the electron transport chain, it is possible that changes in the redox state of Q could favor one cyto b conformation over the other. Further experiments will be conducted on the role of CoQ in cyto b

conformational transitions and the relationship of the transitions to electron transfer and energy conservation.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE MISSION OF THE INSTITUTE: Previous studies have shown that many environmental agents are potent inhibitors of electron transport and/or oxidative phosphorylation. Only in a few cases, where unusual specifity obtains, is the specific site of interference known. An impediment to full understanding of the mode of operation of environmental agents in these processes is a basic lack of information about details of the electron transport system itself. These studies are designed to clarify the components and mechanisms of electron transport and, subsequently, to facilitate understanding of interference mechanisms of specific environmental agents.

PROPOSED COURSE: The tenure in Dr. Ernster's Laboratory will terminate in June. However, work is expected to continue on this probject at NIEHS toward the same goals.

Serial No.: NIEHS-CB-006
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Repair Replication of DNA and the Resealing of Single-Stranded DNA Breaks in Mammalian Cells"

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-12

PRINCIPAL INVESTIGATORS: W. G. Flamm, Ph.D., W. Brandt, M.D.

OTHER INVESTIGATOR: N. J. Bernheim

COOPERATING UNITS: None

MAN YEARS:

Total:	0.4
Professional:	0.4
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The process that mediates gene repair must necessarily involve a set sequence of metabolic events. The first step involves the induction of single-stranded breaks in DNA which facilitates the removal of the genetic lesion, e.g., the removal of an alkylated guanine. Secondly, the excised nucleotides are replaced by a new set of bases (but, presumably, in proper Watson-Crick register) through the function of a special repair enzyme or by the normal DNA polymerase. The last step in the process includes a resealing (esterification) of 5' and 3' ends. It is our purpose to quantitate each of the above steps so that they may be related to data on dealkylation and induced mutation frequencies. We want to know how many single-stranded breaks are produced as each lesion is removed and the number of new nucleotides inserted in response to the event. We also want to assess the fidelity of repair and to learn whether the process increases or decreases mutation frequencies.

METHODS EMPLOYED: The presence and extent of repair replication could be determined by the incorporation of DNA-precursors into DNA were it not for the fact that two different processes are responsible for the incorporation; i.e., one involving gene-duplication and the other, repair replication. These processes, however, can be resolved since gene-duplication is semi-conservative while repair replication is not. In practical terms, resolution depends upon separating, on density gradients of CsCl, the molecules undergoing semi-conservative replication from those engaged in repair. This can be done

by incorporating into DNA a radioactive precursor that also contains a density label, i.e., incorporation of tritiated BUdR. As a practical matter we use a combination of BUdR and ^{3}H-labeled thymidine. The quantitation of single-stranded breaks can be performed by determining the changes, as they occur, in the molecular size of single-stranded DNA following the exposure of cell cultures to chemical mutagens. For this assay to be meaningful, it is obligatory that the DNA be of great size (greater than 100 million daltons). This is possible only when the cells are lysed, the DNA is extracted under special conditions in which great care is taken to avoid shear, and the DNA is centrifuged directly into alkaline sucrose density-gradients.

MAJOR FINDINGS: Mouse lymphoma cells appear to be very ineffective in terms of repairing lesions induced by poly-functional alkylating agents. While the initial events of excision-repair (those involving dealkylation via the excision of alkylated nucleotides) proceed adequately, a fault seems to lie within that step of the process concerned with repair-replication. Since the enzyme responsible for repair-replication functions only after the lesion has been removed, the chemical cause and nature of the lesion should not directly influence the extent or quality of repair-replication. The agent used to produce the lesion is of importance, however, in the sense that it should be well tolerated by the cell and the lesion produced should be readily removable by enzymic excision so as to expose a large number of single-stranded gaps on chromosomal DNA. On this basis, ultraviolet light is the agent of choice and has the third advantage of having been extensively employed in a great variety of other biological systems. Our findings show that mouse lymphoma cultures, irradiated at high dosage levels with ultraviolet light, engage in a small but detectable level of repair-replication. Efforts are now being made to isolate ultraviolet-resistant mutants in which detection of repair-replication should be easier. One such clonal line has been obtained and preliminary experiments suggest a 10-fold difference in cell-survival between UV-resistant and wild-type cultures. Since we have shown that photoreactivation does not occur in these cells it is expected that increased UV-resistance should correlate with increased repair-replication. If it does not, then presumably other forms of repair exist which are not detected by current procedures.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Repair of genetic damage before the damage causes an irreparable genetic change appears to constitute the primary means by which genes are properly maintained and protected from harmful environmental influences. Employment of the above system in which each step of repair is individually assessed and related to the overall process would reveal which chemical and physical agents interrupt and uncouple the sequence of repair. Such interruptions could conceivably cause more damage to the cell's genetic material than the initial lesion. For instance, repair enzymes might produce multiple breaks in DNA and yet be prevented from resealing them by exogenous, environmental influences.

PROPOSED COURSE: By comparing the mutability of UV-resistant to UV-sensitive cells, we hope to discover whether repair-replication contributes to the processes of mutagenesis. In other words, by determining the relative

mutabilities of repair plus and repair minus strains, we should be able to learn about the fidelity of repair-replication and thereby be better able to assess and predict the biological consequences presented by inhibitors of repair.

PUBLICATIONS

Fishbein, L., Flamm, W.G., and Falk, L.: Chemical Mutagens: Environmental Effects on Biological Systems, N.Y., London, Academic Press, 1970, 360 pp.

Serial Number: NIEHS-CB-007
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Investigation of Normal Semiconservative Replication Patterns of DNA"

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-15

PRINCIPAL INVESTIGATOR: W. G. Flamm, Ph.D., D. Clive, Ph.D.

OTHER INVESTIGATORS: N. J. Bernheim, P. E. Brubaker, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	0.3
Professional:	0.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: A large number and variety of chemical compounds inhibit DNA synthesis and prevent the attainment of an appropriate G2 or 4n amount of DNA. In contrast, certain other substances, including some alkylating agents, fail to inhibit DNA synthesis but induce the accumulation of a greater than normal amount of DNA in G2 cells. Doubtlessly, other compounds effect the process of replication in even more subtle ways and yet these are likely to lead to lethal or mutagenic events. Efforts to more fully characterize normal replication of the mammalian genome is predicated on the belief that it will help us to understand better the possible mutagenic effects of such environmental agents as certain alkylating agents, caffeine, DDT, and certain antibiotics.

METHODS EMPLOYED: Mouse lymphoma cells (L-5178-Y) are blocked in metaphase with colcemid and in some experiments held at the end of G1 with fresh medium containing 5-flurodeoxyuridine. Following reversal and the beginning of S-phase, DNA replication is assessed by incubating the cultures in medium containing either tritium labeled thymidine or unlabeled 5-bromodeoxyuridine. DNA is extracted and analyzed by isopycnic centrifugation in CsCl density gradients.

MAJOR FINDINGS: The mouse genome replicates in such a fashion that the nucleotide sequences (genes) undergoing synthesis in early S-phase are of a different chemical composition from those genes or nucleotide sequences

which replicate in middle or late S-phase. At the beginning of S-phase, only those nucleotide sequences which have a high guanine plus cytosine content undergo replication and, as replication proceeds, the guanine plus cytosine content of replicating sequences gradually decrease. From the beginning to the end of S-phase a change of 10% in guanine plus cytosine content is observed. It now appears that this is a general phenomenon applicable to, perhaps, all mammalian cells (including those of human origin) and while the underlying mechanism is not understood, these observations show that specific nucleotide sequences replicate at fixed times within S-phase. This is also true of the genetically inert sequences which comprise the centromeric and constituative heterochromatic regions of mouse chromosomes. These sequences, which can be isolated from cesium salt gradients, normally replicate only in the third quarter of S-phase. Their replication, however, can be selectively inhibited by sulfur mustard and, as shown by others, induced by polyoma virus to replicate out of phase.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The above study has provided the basis for testing and evaluating the potential of envrionmental agents in altering normal patterns of DNA replication and may help explain the spectrum of biological and genetic effects produced by these agents.

PROPOSED COURSE: The above study indicates that specific genes are replicated at fixed times within S-phase which should enable us to isolate preparations of DNA which are greatly enriched in certain genes for transformation studies. We are presently attempting to learn when ribosomal and transfer RNA genes are replicated within S-phase.

PUBLICATIONS

Flamm, W.G., Bernheim, N.J., and Brubaker, P.E.: Density gradient analysis of newly replicated DNA from synchronized mouse lymphoma cells, Expt. Cell Research 64: 97-104, 1971

Flamm, W.G., Birnstiel, M.L., and Walker, P.M.B.: Isopycnic centrifugation of DNA: methods and application. In Bernie, G.D. (Ed): Subcellular Components: Isolation and Fractionation. 2 Ad. edition. London, Butterworth's, 1971 (in press)

Flamm, W.G.: Highly repetitive sequences of DNA in Chromosomes. In J.F. Danielli (Ed.): International Review of Cytology. New York, Academic Press, 1971 (in press)

Serial No.: NIEHS-CB-008
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Inhibition and Alteration of the Semi-Conservative Replication of DNA"

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-16

PRINCIPAL INVESTIGATOR: W. G. Flamm, Ph.D., D. Clive, Ph.D.

OTHER INVESTIGATOR: N. J. Bernheim, P. E. Brubaker, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

TOTAL:	0.3
PROFESSIONAL:	0.3
OTHER:	0.0

PROJECT DESCRIPTION

OBJECTIVES: In virtually all cases, those chemical or physical agents which alter normal semiconservative replication of DNA exhibit either lethal or mutagenic properties. This is so whether the inhibition is mediated through an interaction of the agent with DNA or due to a suppression of one or more of the metabolic precursors of DNA. In either event and because there are several known routes by which chemical agents can interrupt normal replication, a wide variety of chemically diverse compounds come under consideration. It is our objective to test various classes of environmentally important compounds for their ability to interfer with or otherwise effect semi-conservative replication and to determine the consequences in terms of mutagenic and lethal effects.

METHODS EMPLOYED: Synchronized and asynchronous cultures of mammalian cells have served as the biological test system. Replication of DNA is assessed either by the incorporation of radioactive precursors into DNA, in which case either chemical or cytological procedures can be used, or by the density marker method utilizing 5-bromodeoxyuridine. In the later approach, analytical ultracentrifugation is employed to separate and quantitate the fraction of newly synthesized DNA.

MAJOR FINDINGS: We have shown that bifunctional alkylating agents such as sulfur and nitrogen mustard cause a selective inhibition of the synthesis of heterochromatic regions of metaphase chromosomes. Because these regions are replicated only near the end of S-phase, we considered the possibility that

the above results were a consequence of cells in late S-phase being more sensitive to the inhibition induced by sulfur mustard than those of early or middle S-phase. To examine this question we determined the nucleotide composition of just those sequences which underwent replication after the exposure of cell-cultures to mustard gas. These experiments indicated that DNA sequences from both early, middle and late S-phase were probably undergoing replication at comparable rates (see NIEHS-CB- 007) and hence, a differential sensitivity among such cells could not account for the effect. The principal cause seemingly relates to the fact that the light strand of heterochromatic DNA acquires, upon exposure to mustard, 2 to 3 times as many intrastrand crosslinks as do the sequences of the main band. This is particularly significant since the monofunctional half-mustard fails to produce the effect and because intrastrand crosslinks are known to inactivate bacteriophage viruses and prevent DNA replication. A limited number of other compounds are also being tested (e.g.; chlordane, NTA) but preliminary evidence has yet to reveal obvious changes.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Because of the empirical correlation between chemical mutagens and inhibitors or modifiers of DNA synthesis and because of compelling mechanistic reasons for believing that a correlation should exist, we believe that monitoring compounds for their ability to alter normal replication patterns constitutes a relatively rapid and simple means of establishing mutagenic potential for certain environmental agents.

PROPOSED COURSE: Using synchronized cell-cultures, we plan to determine whether the staging of DNA replication (as outlined in NIEHS-CB- 007) is disturbed or permuted by alkylating agents and other compounds of environmental concern such as, metabolites of artificial sweeteners, caffeine, chlorinated hydrocarbons and carbamate insecticides.

PUBLICATIONS

Flamm, W. G., Bernheim, N. J., and Spalding, J. W.: Selective inhibition of the semiconservative replication of mouse satellite DNA. Biochem. Biophys. Acta. 195: 272-275, 1969.

Brubaker, P. E., Flamm, W. G., and Bernheim, N. J.: Effect of gamma-chlordane on synchronized lymphoma cells and inhibition of cell division. Nature 226: 548-549, 1970.

Fishbein, L., and Flamm, W. G.: Potential environmental chemical hazards. The Science of the Total Environment, 1971 (in press).

Serial No.: NIEHS-CB-009
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Nature of cross-links in alkylated DNA"

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-13

PRINCIPAL INVESTIGATOR: W. G. Flamm, Ph.D.

OTHER INVESTIGATORS: L. Fishbein, Ph.D. and N. J. Bernheim

COOPERATING UNITS: Analytical and Synthetic Chemistry Branch

MAN YEARS:

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: It is well known that bifunctional alkylating agents such as nitrogen and sulfur mustard are more toxic to eucaryotic and procaryotic cells than their monofunctional analogs. The basis of this enchanced toxicity was thought to be the result of bifunctional agents crosslinking the opposite strands of a DNA duplex via the alkylation of two guanines positioned nearly opposite each other in the duplex. It was, in fact, thought for some time that essentially all diguaninyl alkylation products were derived from interstrand crosslinks. It has been the purpose of this project to learn whether intrastrand crosslinks are formed, how readily they form, and the extent to which they occur based on the total amount of alkylation of DNA.

METHODS EMPLOYED: To provide evidence of a direct nature for the existence of intrastrand crosslinks we have selected some unusual DNA fractions for alkylation with S^{35} labeled sulfur mustard. These DNA's are from mammalian sources and represent the so-called mouse and guinea pig satellite. The special advantage afforded by these duplexes relates to their interstrand compositional bias and to the fact their individual complementary strands can be isolated. Standard chromatographic methods were employed for separation and analysis of alkylation products.

MAJOR FINDINGS: We have found that isolated single strands, which according to our earlier studies remain single-stranded and do not associate, contain both mono- and diguaninyl products following alkylation. The relatively high proportion of diguaninyl derivative associated with alkylated single

strands (19, 29, & 30% for mouse satellite H and L strands and guinea pig H α strand respectively) argues in favor of intrastrand crosslinks since hydrolysis of the unreacted arm of the mustard would preclude most intermolecular reactions. Furthermore, no change in the molecular weight of DNA is observed as would be the case were intermolecular reactions responsible for the diguaninyl derivative. It should be recognized, however, that on account of structural differences, duplexed DNA would not necessarily behave in a manner similar to individual single strands (i.e., develop intrastrand crosslinks). For this reason, the satellite duplex of the guinea pig was examined, following alkylation, since of its two strands, the $H\alpha$ contains 36% guanine while its complement the $L\alpha$ contains only 2% guanine. Thus, only a very small proportion of the alkylated guanines on the H strand would have an opportunity of undergoing a second reaction with a guanine on the opposite strand. The fact that diguaninyl derivatives were found to constitute 26% of the total alkylation products from the satellite duplex indicates that the majority of these derivatives must be of intrastrand origin and establishes that intrastrand crosslinking of adjacent guanines does indeed occur.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: To understand the types of biologic or genetic effects that various classes of compounds are likely to exert (e.g., carcinogenic, mutagenic, lethal) it is imperative we develop a detailed understanding of how these substances interact with the genetic material of a living cell and the kinds of alterations they produce.

PROPOSED COURSE: Project is complete insofar as sulfur mustard is concerned and any continuation would involve other types of bifunctional or polyfunctional agents.

PUBLICATIONS

Flamm, W.G., Bernheim, N.J., and Fishbein, L.: On the existence of intrastrand crosslinks in DNA alkylated with sulfur mustard. Biochim et Biophys. Acta 224: 657-659, 1970.

Flamm, W.G., Walker, P.M.B., & McCallum, M.: Renaturation and isolation of single strands from the nuclear DNA of the guinea pig. J. Mol. Bio. 42:441-455, 1969.

Serial No.: NIEHS-CB-010
1.
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Interaction of Environmental Agents with DNA"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: W.G. Flamm, Ph.D.

OTHER INVESTIGATOR: L. J. Swaim, Jr., N. J. Bernheim, and L. Fishbein, Ph.D.

COOPERATING UNITS: Analytical and Synthetic Chemistry Branch

MAN YEARS:

Total:	0.6
Professional:	0.3
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: Certain classes of environmental agents are well known for their ability to interact either physically or chemically with DNA. Such classes include alkylating agents, certain N-hydroxy derivatives, nitrous acid and nitrites, bisulfites, certain purines and pyrimidines, a variety of antibiotics and mycotoxins, heavy metal ions and various polycyclic aromatic compounds containing either oxygen or nitrogen in the ring system. In addition, other compounds such as polycyclic hydrocarbons (dimethylbenzanthracene) heterocyclics (mitomycin C) and nitrosamines (dimethylnitrosamine) are metabolically converted to substances which attack nucleotide bases in DNA. It has been our purpose to devise a series of procedures, primarily chromatographic ones, by which the interaction of various environmental agents with DNA could be detected and the nature of the reaction products characterized.

METHODS EMPLOYED: In order to learn whether a compound chemically reacts with DNA and to characterize the reaction products we have prepared a series of isotopically labeled DNA's in which the following moieties were specifically labeled: (a) the phosphate of nucleotides with ^{32}P (b) the guanine base with tritium (c) the adenine base with tritium and (d) the cytosine base with ^{14}C. In addition to interacting unlabeled compounds with labeled DNA, we have and plan to do the opposite, incubating such compounds as ^{35}S-mustards or ^{14}C-metabolites with unlabeled DNA. Following the presumed reaction, DNA is hydrolyzed, either chemically with acid or enzymically with a combination of deoxyribonuclease and snake-venom phosphodiesterase, to its constituent

nucleotides or free bases which are then chromatographically separated by thin-layer and paper chromatography. Autoradiographic or isotope-scanning techniques are then applied for the ultrasensitive detection of even minor reaction products.

MAJOR FINDINGS: We have developed a new two-dimensional thin-layer chromatographic procedure whereby the nucleotide composition of ^{32}P- labeled DNA can be determined by a combination of autoradiography and scintillation counting of autoradiographic spots. Using this procedure we have successfully identified reaction products of certain N-hydroxy derivatives with DNA, have determined the conditions under which the reation occurs and have identified the specific bases involved in the reaction. Preliminary evidence indicates that the mycotoxin, alternariol, reacts specifically with deoxycytidine moeities in DNA though, the conditions of this reaction have yet to be worked out. In addition, we are studying the reaction products of certain alkylating agents such as ethyl- and methyl- methane sulfonate with the hope of establishing their dosimetry relative to cytotoxic and mutagenic effects and the possibility of identifying the primary mutagenic lesion.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: All known mutagens appear to function by interacting with DNA or with its replication and insofar as mammalian systems are concerned this has invariably involved a chemical reaction in which new covalent bonds are formed. By examining whether certain untested environmental compounds and their metabolites interact with DNA and the conditions under which the reaction takes place we derive information relative to their potential mutagenicity.

PROPOSED COURSE: It will be necessary to confirm some of the observations reported here and it is hoped that they can be extended to include other environmental agents; e.g., N-hydroxy metabolites of saccharine and certain drugs. Similar studies might also be carried out with nitrosamines (e.g. dimethylnitrosamine) though it is recognized that, in these cases, the addition of a liver homogenate to the incubation mixture will be necessary.

Serial No.: NIEHS-CB -011
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Nutritional Studies on Fischer Lymphoma Cells (L 5178 Y)"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: D. Clive, Ph.D.

OTHER INVESTIGATORS: W. G. Flamm, Ph.D. and M. R. Machesko

COOPERATING UNITS: None

MAN YEARS:

Total:	0.2
Professional:	0.1
Others:	0.1

PROJECT DESCRIPTION

OBJECTIVE: During our early use of the soft agar cloning technique, much variation in cloning efficiency was noted. Two critical components of this cloning medium (insofar as contribution to nutritional variation is concerned) seemed to be horse serum and "conditioned" medium (filter-sterilized supernatant of a cell culture that had been grown for 15 hours). This project was initiated to determine the critical and presumably varying nutrients of these components, and to the extent feasible, the composition of our suspension and cloning media.

METHODS EMPLOYED: Fischer lymphoma cells (L 5178 Y) were grown in suspension culture using inoculum sizes, media and supplements as described under Major Findings. Cloning medium originally consisted of 0.33% agar, 20% horse serum and 18% "conditioned" medium in Fischer's medium. Cells were added to this, mixed, aliquoted into Falcon flasks, chilled in a freezer to gel the agar and incubated at 37°C. After 7-10 days, at which time each plated cell had given rise to a colony of 1 mm diameter or larger, colony counts were made to evaluate the adequacy of each medium.

MAJOR FINDIIGS: Cell growth was evaluated in a variety of media. It was found that cells failed to grow when (1) Fischer's medium with less than 2% horse serum was employed; (2) Bovine serum albumin (BSA) was used as a replacement for horse serum in Fischer's medium; and (3) Ham's F-12 medium (more complex than Fischer's) at any BSA concentration was used.

The cells would grow, however, in a 1:1 mixture of Fischer's and Ham's media containing 0.4 - 0.6% BSA. By adding the ingredients unique to Ham's medium singly and in combination to Fischer's containing 0.6% BSA, an absolute requirement for pyruvate (100 µg/ml) was discovered. Moreover, further growth enhancement was obtained when linoleic acid, lipoic acid and putrescine were supplied along with pyruvate. With the addition of pyruvate to Fischer's medium containing horse serum we can grow 50 ml suspension cultures from a single cell inoculum. The generation time was found to be constant (10.8 ± 0.1 hours) over the entire range of cell concentrations from 10^0 - 10^5 cells/ml. These facts suggest that pyruvate might satisfy fully the role of "conditioned" medium in our cloning technique and this has indeed been borne out by experiment. Cloning medium is now prepared using pyruvate in place of "conditioned" medium. Phosphoenolpyruvate (PEP) and lactate were compared with pyruvate for their ability to support suspension growth of small inocula. PEP and pyruvate appeared comparable while lactate was less effective in this regard.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The validity of our in vitro and host-mediated mutagenesis projects (Project Numbers NIEHS-CB-012 and NIEHS-CB-013) depend on the ability to clone mutant and wild-type cells both accurately and precisely. The initial inadequacies of the soft-agar cloning technique have been overcome as mentioned, yielding greater reliability for our subsequent studies on mutagenesis.

PROPOSED COURSE: As new mutant stocks are accumulated, each with their unique metabolic idiosyncracies, adjustments to growth media will be made as required.

Serial No.: NIEHS-CB-012
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "In Vitro Studies of Chemical Mutagenesis in Mammalian Cells"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: D. Clive, Ph.D. and W. G. Flamm, Ph.D.

OTHER INVESTIGATORS: M. R. Machesko, N. J. Bernheim

COOPERATING UNITS: None

MAN YEARS:

TOTAL:	1.0
PROFESSIONAL:	0.5
OTHERS:	0.5

PROJECT DESCRIPTION

OBJECTIVE: Because of their large numbers, relatively short generation times, biological relevance to mammalian genetics, and ease of handling, mammalian cells grown in suspension culture offer a highly sensitive system for assaying the mutagenicity of environmental agents. Elucidation of the mechanism of mutagenicity of such agents requires the establishment of a number of well-defined mutant stocks produced by mutagens with a known mechanism of action. Back-mutation induced by an undefined agent of, say, a transition-type mutant but not of an addition-deletion type mutant, would characterize the unknown mutagenic agent as causing transition-type base substitutions. It is our objective to develop the mutant stocks and the proper selective media required for assaying and characterizing the mutagenicity of environmental agents. Inherent in this is the evaluation of the critical parameters (plated cell concentrations, concentration of selective agents, recovery of mutants) affecting the accurate determinations of mutation rate.

METHODS EMPLOYED: Fischer lymphoma cells (L 5178 Y) plated in a soft-agar medium supplemented with Methotrexate (M) to inhibit folic acid-dependent pathways, thymidine (T), hypoxanthine (H), and glycine (G) (THMG medium), give rise to visible $THMG^r$ clones in a week to 10 days. A number of these have been isolated, grown in suspension culture, and characterized biologically and biochemically at the HGPRT and TK loci. The former was accomplished by use of toxic analogs: i.e., $HGPRT^+$ cells ribosephosphorylate 8-azaguanine (azg) or 6-mercaptopurine (6 MP), each of which is subsequently incorporated into DNA with lethal results. TK^+ cells phosphorylate 5-bromodeoxyuridine (BUdR), also with lethal results. Radioactively-labeled precursors ([^{3}H]-hypoxanthine

and [^{3}H]-thymidine or [^{3}H]-BUdR) were used to assess the allelic form of TK and HGPRT.

Forward mutations to HGPRT$^-$ and TK$^-$ are being isolated and studied by taking advantage of the presumed relationships: (1) (THMG)^r = TK$^+$ & HGPRT$^+$ = BUdR^s & azg^s & 6MP^s; (2) HGPRT$^-$ =azg^r (but see Major Findings) = 6MP^r; (3) TK$^-$ = BUdR^r. These mutants are products of forward mutations and are unambiguously characterized as follows: (1) ability to incorporate [^{3}H]-thymidine or [^{3}H]-hypoxanthine into DNA and RNA; and (2) for their cell-free extracts to phosphorylate or ribosephosphorylate thymidine and hypoxanthine, respectively. The enzymes are being assayed by a combination of autoradiographic and chromatographic techniques. Mutants with amorphic alleles have been used in reconstruction experiments for determining the critical parameters of a successful in vitro mutation assay system for each of the two loci discussed.

MAJOR FINDINGS: As expected, THMG^r cells are azg^s at 20 µg/ml and 6 MP^s at 10 µg/ml and are able to incorporate into their DNA high levels of [^{3}H]-hypoxanthine (7700 dpm/µg DNA), [^{3}H]-thymidine (50,000 dpm/µg DNA), and [^{3}H]-BUdR (60,000 dpm/µg DNA).

Early attempts to isolate HGPRT$^-$ mutants using a selective medium containing 8-azaguanine proved the infeasibility of an azg^s azg^r mutation assay for this locus. First, azg^r mutants proved to have significantly varying rates of [^{3}H]-hypoxanthine incorporation into their DNA's ranging from 25 to 500 dpm/µg DNA. Second, one of these mutants (azg^r-4 which incorporated 500 dpm of [^{3}H]-hypoxanthine/µg DNA), was not selected against by THMG medium (all other azg^r mutants were killed in this medium) and yet was still resistant to 50 µg of 8-azaguanine/ml. Thus we were warned of the possibility that the accumulation of such leaky mutants in THMG cultures would yield falsely high mutation rates. Third, at cell concentrations above 10^3 HGPRT$^+$ cells/ml, cloning medium containing 8-azaguanine produced surviving clones (which later tested as azg^s) at a frequency which increased with the square of the number of cells plated, and, in fact, followed a "coincidence" curve. This problem was circumvented by the use of 6 MP as selective agent which (1) killed azg^r-4 while not affecting the other (THMG)^s-azg^r mutants; (2) gave no "coincidence" effects at high cell plating concentrations; (3) gave no cross-feeding effects in reconstruction experiments at 10^3 HGPRT$^+$ cells/ml; and (4) yielded a mutation rate for HGPRT$^+ \rightarrow$ HGPRT$^-$ (6 MP$^s \rightarrow$ 6 MP^r) of approximately 10^{-7} mutations/locus/generation.

One presumptive TK$^-$ mutant has been isolated ([^{3}H]-thymidine incorporation = 8 dpm/µg DNA; resistant to 50 µg BUdR/ml; killed in THMG medium) and has been used in reconstruction experiments (no cross-feeding at 10^5 (THMG)^r cells/ml in 50 µg BUdR/ml) and for a preliminary calculation of the spontaneous mutation rate the TK locus (approximately 5×10^{-11} mutations per locus per generation). This last finding makes it likely that the TK locus is present in diploid condition and hence, probably autosomal.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project is integrated with the development of a mouse-mouse host-mediated assay for chemical mutagens (Project No. NIEHS-CB-013) and shares its significance. Further, this in vitro system permits both quantitative and qualitative (i.e., mechanistic) evaluation of a chemical's mutagenic effects on the mammalian

genome. By studying forward- and back-mutation frequencies at either the HGPRT or the TK locus, one can characterize a compound as being, for example, a mildly potent, addition- or deletion-type mutagen. In conjunction with the host-mediated assay, it should be possible to distinguish between mutagenicity of the parent compound and of its metabolite(s).

PROPOSED COURSE: It would appear (see Major Findings) that the TK locus is present in a diploid condition. We would like to produce a chromosomal or large gene deletion at one of these loci to obtain a cell line which is in essence haploid for the TK locus. Forward point-mutants (TK^-) of known type (e.g. addition-deletion, transition, transversion) can be constructed at this haploid locus using well-defined chemical mutagens known to produce only additions or deletions, or only transitions, etc. An uncharacterized environmental mutagen can then be defined by determining which type of mutant it is capable of back-mutating.

Serial No.: NIEHS-CB-013
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "A Mouse-Mouse Host-Mediated Assay for Chemical Mutagens"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: D. Clive, Ph.D. and W. G. Flamm, Ph.D.

OTHER INVESTIGATORS: M. R. Machesko

COOPERATING UNITS: None

MAN YEARS:

TOTAL:	0.4
PROFESSIONAL:	0.2
OTHERS:	0.2

PROJECT DESCRIPTION

OBJECTIVE: Some chemicals have been shown to exhibit species-specificity in their mutagenic activity. Such variation has been attributed to differences in host metabolism of the compound (converting non-mutagens to mutagens or vice versa), or to differences in chromosomal architecture between, say, bacteria and mammals. The ideal mutagen assay system should combine the operational ease and high sensitivity of bacterial test systems with the biological relevance of expensive, time-consuming whole mammal test systems. This system should, in effect, expose a large number of mammalian genomes to the test compound and its products of mammalian metabolism, in a mammalian milieu. We intend to use mouse lymphoma cells (the mammalian genetic indicator) implanted within a mouse peritoneum (the mammalian metabolism and milieu) to realize these ideals.

METHODS EMPLOYED: Fischer lymphoma cells (L 5178 Y), of known genetic composition, are put in a dialysis bag and implanted intraperitoneally in DBA/2J mice, to which is then administered (by various routes) a known or suspected mutagen. After 1-4 days, the cells are removed, a small aliquot is cloned for determination of survival, and the bulk of the cells are cloned in a selective medium to determine mutation frequency. (The soft agar cloning technique used for determining viability and the rationale for the various selective media are described in detail under Project No. NIEHS-CB-012).

MAJOR FINDINGS: To date only the preliminary details of mutant isolation, the biological and biochemical characterizations of these mutants, the characteristics and limitations of three in vitro selective media for two genetic

loci, and initial studies on forward mutation rates at each of these loci have been evaluated. (These are described under Project No. NIEHS-CB-012). In addition, our investigation of techniques for in vivo incubation of lymphoma cells has shown the impracticality of recovering cells injected directly into the mouse peritoneum, and have demonstrated the desirability of containing them in a manner which permits free diffusion of the mutagen and its metabolites.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The past half century has seen the creation and dispersal of an unknown number of actual and potential mutagens, physical, chemical and biological. If we are to avoid the generally agreed-upon long-term disastrous effects of rapidly accumulated mutations within any biological population we urgently need methods which aid in the detection and control of mutagens. Test systems for the detection of mutagens exist; each of them has limitations in terms of sensitivity, ease of operation, and biological relevance to man and other economically important species. The mouse-mouse host-mediated assay for chemical mutagens which has been described above, combines operational convenience and high sensitivity with biological relevance to mammals.

PROPOSED COURSE: We plan to make this assay operational using as a criterion the stimulation of mutation frequency by known mutagens administered intraperitoneally. Later, environmentally significant chemicals (e.g. DDT, food additives such as nitrites) will be tested for their mutagenicity by administering them via non-intraperitoneal routes.

Serial No.: NIEHS-CB-014
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Effects of NTA on Cultured Mammalian Cells"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: D. Clive, Ph.D. and W. G. Flamm, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	0.4
Professional:	0.2
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: To establish a dose-response relationship for NTA on Fischer lymphoma cells (L 5178 Y) cloned in a soft-agar medium, and to elucidate the mechanism of toxicity of NTA.

METHODS EMPLOYED: A known number of Fischer lymphoma cells (L 5178 Y) were cloned in our standard soft-agar cloning medium supplemented with various concentrations of NTA, EDTA, $CaCl_2$, and $MgCl_2$ as required. After 7-10 days incubation, visible colonies were counted as an index to the biological response of the additive(s).

MAJOR FINDINGS: (1) NTA, even at concentrations as high as 10 mM, appears to have no effect on the initial rate of DNA synthesis (done in suspension culture); (2) NTA had no effect on the cloning efficiency of these cells below 0.8 mM; (3) ID_{95} for NTA was 3.0 mM; (4) Cloning efficiency was a linear function of NTA concentration between 0.8 and 3.0 mM; (5) EDTA gave results similar to those of NTA, the relative effects of the two compounds being quantitatively related to their dissociation constants with Mg^{++} and Ca^{++}; (6) Exogenous Ca^{++} (but not Mg^{++}, within the range of concentrations practical) when added to the cloning medium together with an ID_{95} concentration of NTA or EDTA, caused a complete reversal of the toxic effects of each chelating compound; (7) The Ca^{++} concentration required to accomplish this reversal was somewhat higher than that calculated to restore normal free Ca^{++} levels in the medium; (8) Hence, the toxicity of NTA appears to be a consequence of its ability to chelate cations, though other factors may be involved; (9) NTA is

able to reduce slightly the toxicity of $HgCl_2$ on mouse lymphoma cells; and (10) Methyl mercuric chloride is 2-3 times as potent as $HgCl_2$, at the ID_{50} dose.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project was initiated in order to learn whether NTA exerts cytotoxic effects which become evident in tissue culture systems. This seemed important because of the recently proposed use of NTA as a detergent additive.

PROPOSED COURSE: These studies might be extended to include tests for mutagenicity at either the TK or HGPRT locus (See NIEHS-CB-012).

Serial No.: NIEHS-CB-015
1. Cell Biology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Subcellular Effects of Methyl Mercury in adult male rats: Effects observed at the site of gene translation in brain, kidneys and liver."

PREVIOUS SERIAL NO.: None

PRINCIPAL INVESTIGATOR: Paul E. Brubaker, Ph.D.

OTHER INVESTIGATORS: Sheldon Herman, M.D., Ronald Kline, M.D., George Lucier, Ph.D., Lovest T. Alexander and Merritt Long

COOPERATING UNITS: Pathologic Physiology Branch

MAN YEARS:

Total:	1.0
Professional:	0.5
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVE: Organomercurial compounds are a hazard to the health of both man and animals through direct and indirect exposure to increasing levels in the environment. Acute renal failure is observed in animals treated with inorganic mercuric chloride. Aryl and alkyl derivatives affect other organs. Of these the brain is particularly important since it has been shown to be the primary site of damage and responsible for both death and debility due to alkyl mercury exposure. Among these compounds methyl mercury hydroxide is the alkyl compound commonly found in the environment. Recent investigations have indicated inhibitory effects on protein synthesis and decreased microsomal enzyme activity in animals treated with this compound. The objective of this project is to investigate alterations in protein synthesis in three target organs of treated animals, the brain, liver and kidney.

The molecular model for this approach deals with subcellar constituents and organelles involved with protein synthesis. The nucleolus give rise to ribosomal subunits, which upon entrance to the cytoplasm, complex with messenger RNA templates to form polyribosomal aggregates, the cellular site of gene translation (protein synthesis). The relevant amount of individual monoribosomes, polyribosomes and ribosomal subunits, along with

levels of total ribonucleic acid and protein, provides an index of disturbances in flow and expression of genetic information. These effects will be correlated with levels of mercury found in each individual organ.

METHODS EMPLOYED: Sprague-Dawley male rats are used; tissue homogenization and subfractionation by ultracentrifugation; biochemical determinations of total RNA and protein and resolution of various ribonucleoprotein particles by sucrose density gradient ultracentrifugation are employed.

MAJOR FINDINGS: "Minamata Disease" has been produced in adult male rats that provides an experimental model for investigating the mode of action of methyl mercury hydroxide in three target organs; e.g., brain, liver, and kidney. Animal treated for 1, 2,5 and 7 days with methyl mercury hydroxide display subcellular disturbances in both liver microsomal enzyme activity and components involved in protein synthesis in all three organs. Analysis of organ body weight ratio indicate little or no change in organ weight in animals treated for 1, 3, 5 and 7 days. Total protein levels in the brain remain unchanged while a 15-20% reduction occurs in both liver and kidney levels in animals treated for seven days. Although total ribonucleic acid levels are unaffected in organs of all treated animals, there are significant alterations in levels of various ribonucleoprotein particles isolated from these organs in treated animals.

A maximal 42% increase in total liver ribosomes is observed in animals receiving treatments for two days. This is followed by a reduction to levels 7% above controls following treatment for seven days. While a 3-6% increase is observed in kidneys of animals treated for 5 days, there is a maximal 21% increase in seven-day treated animals. There were no changes in levels of ribosomes observed in the brain.

In addition to changes in levels of ribonucleoprotein particles, there are differences observed in the relative proportion of ribosomal subunits, monoribosomes and polyribosomal aggregates. A 50% reduction of polyribosomes occurs in both brain and kidney of 7-day-treated animals. Concomitantly, there is a 250% and a 60% increase in subunit and monoribosome levels, respectively. A generalized increase in levels of all the ribosomal components is observed in liver with variations in levels of the individual components in 1, 2, 5 and 7-day-treated animals. This is an opposite response to that observed in the kidney and brain. A 30% increase in liver polyribosomes occurs in animals treated for only one day. A 45% increase is observed in livers of animals treated for two days. From this point, the polyribosomes appear to undergo dissociation to levels 8% above controls in animals treated for 7 days. These polyribosome changes are accompanied by up to a 300% increase in levels of subunits and a 40% increase in monoribosomes.

While no pathological disturbances are observed at the light microscopy levels, changes on nucleolar structure are observed in electron micrographs of the liver. Alterations in nucleolar structure are classically used as an index to inhibitory effects on ribonucleic acid synthesis and suggests interaction of this compound at the level of the gene (nucleolar ribosomal

DNA templates).

There appears to be little or no change in physical structure of ribosomes extracted from the three organs of treated animals since their sedimentation in sucrose gradients remains identical to those of controls.

Preliminary evidence indicates a graduated increase in mercury levels among the three organs (kidney > liver > brain) excised from animals treated with 1.0, 2.5, 5.0 and 10 mg/kg of methyl mercury hydroxide. The alterations in polyribosomal profiles and changes in levels of ribosomes and their constituent subunits indicate inhibitory effects at the level of gene translation, i.e. protein synthesis. The dissociative effects on the polyribosomes observed in the kidney and brain suggests an early release of ribosomes from translational activity. The consequence of this effect is early termination of the polypeptide chain synthesis with production of incomplete and possibly inactive enzymes. The increase in polyribosomes of larger size also suggest inhibitory effects by the opposite mechanism in which ribosomes are not released at the proper time. In this situation it is conceivable that either more of one particular polypeptide is synthesized which in turn could inhibit enzyme activity th ough distortions in stochiometry. Decreased activity of microsomal enzymes in the liver has been demonstrated.

The generalized increase in all the varieties of ribonucleoprotein particles along with decreased protein levels and no change in total RNA suggests a redistribution of ribosomal material. These alterations could occur as an artifact of tissue homogenization or pronounced alterations in the endoplasmic reticulum (ER). A large portion of the ER is present in the post-mitochondrial fraction. Evidence supporting conclusions suggesting ER alterations is found in the observed "stacking phenomenon" of numerous sections of the liver excised from all treated animals. Furthermore, these observations point to a smooth to rough conversion of the ER and is substantiated by the large increase in ribosomes found in microsomal preparations. Redistribution of ribosomes suggests failure of ribosomes to be released from the ER that ordinarily may occur in the absence of mercury. Therefore, this evidence points to the inhibition of enzymes involved in ribosome degradation and turnover.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE MISSION OF THE INSTITUTE:
Contamination of the environment with heavy metals has recently been recognized to be a major problem. Mercury is a particularly hazardous element, not only because of its high, broad-spectrum toxicity, but because elemental mercury and inorganic mercurials are converted into alkyl mercury by microorganism and can cycle indefinitely in the food chain.

It is important to fully understand the behavior of mercurials in the body and the primary biochemical mechanisms by which it acts in order to recognize its danger signals in clincial diagnosis and in symptomatology. Such understanding should also be useful in development of rationale and methodology for countering acute and chronic effects of mercurials.

PROPOSED COURSE: (1) Studies are underway to determine effects on synthesis of both ribosomes and proteins through use of appropriate radioactive metabolic precursors.

(2) Experiments are now being initiated to evaluate the levels of methyl mercury hydroxide, and other organomercurials, required to interfere with protein synthesis. An *in vitro* protein synthesizing system will be used for these studies. This test system also can be used to screen the functional integrity of other treated organelles (mitochondria).

(3) Since the surface of the ribosome has been shown to have free sulfhydryl groups present that are essential to their activity, investigations will be conducted to determine the degree to which mercurial compounds are bound to these particles.

(4) Magnesium is essential to the integrity of polyribosomes and to the action of enzymes involved in RNA and protein synthesis. Magnesium - mercury ratio's will be determined in target organs of treated animals to determine if polyribosome dissociation is due to magnesium displacement or activation of a ribonucleace. These studies have particular reference to the kidney and the brain.

(5) Information derived from these experiments could lead to studies dealing with a means of reversing the effects of various organic mercurial compounds.

Serial No.: NIEHS-CB-016
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Induction of Oxidative and Conjugative Enzymes in Rat Liver by Several Pesticides and Synergistic Agents: Their Influence at the Level of Gene Translation"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Paul E. Brubaker, Ph.D.

OTHER INVESTIGATORS: G. W. Lucier, Ph.D., L. Alexander, and M. Long

COOPERATING UNITS: None

MAN YEARS

Total:	0.3
Professional:	0.1
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: The biological integrity of the entire ecosystem has been threatened by the ubiquity and increasing residue levels of various pesticidal chlorinated hydrocarbons. The toxicity of many of the compounds has been shown to be enhanced by including other aromatic hydrocarbons in pesticide formulations. Among the subcellular effects observed in mammals exposed to sublethal levels of these pesticides are decreased mitochondrial respiration, delay of cell division and induction of hepatic drug metabolizing enzymes. All of these processes depend upon flow and expression of genetic information required to maintain normal cell metabolism. The ribosomes have been described as a programmed enzyme that establishes peptide bonds between genetically selected amino acids, and polyribosomes represent the cellular site of protein synthesis. The purpose of this project is to investigate the effects of various pesticides and selected synergistic agents on protein synthesis in rat liver. This information will be correlated with assays of several microsomal enzymes to provide clues to the nature of induced enzyme activity.

MAJOR FINDINGS: Preliminary experiments indicate an increase in liver size over a 3 day period in adult male rats treated with gamma chlordane (30 mgm/kg/day). This is accompanied by an initial increase in total RNA followed by increasing levels of total protein each reaching a maximal 90% and 25% respectively over this time period. There is a 27-35% increase in levels of polyribosomes, a 130% increase in ribosomal subunits and a 10% decrease in

levels of monoribosomes isolated from the post-mitochondrial supernatant of treated rat liver. The same general pattern is also observed with synergistic compounds; piperonyl butoxide and MGK-264, both at 150 mgm/kg/day over a 3 day period. Electron micrographs of excised livers from treated animals indicate disturbances in the endoplasmic reticulum that suggest proliferation of rough and smooth endoplasmic reticulum. This observation substantiates the increased levels of total ribonucleoprotein particles isolated from treated animals.

The activity of microsomal mixed function oxidases (aminopyrine demethylase), cytochrome P-450 content and the UDP glucuronyltransferases are increased by piperonyl butoxide, chlordane and MGK-264. Induction is approximately two-fold for both mixed function oxidases and UDP glucuronyltransferases and occurs in both smooth and rough endoplasmic reticulum.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Oxidative and conjugative enzymes are functional in normal steroid metabolism and detoxification of environmental chemicals. Various xenobiotics enhance the activity of these enzymes. This could be attributed to direct interaction of these agents with the enzymes, their cofactors, substrates or metabolism of the enzyme itself. This project hopes to provide clues to the basic underlying mechanism of the inductive process.

PROPOSED COURSE: A study correlating protein synthesis kinetics and enzyme activity will be undertaken to determine relative rates of induction. The influence of ribosome-membrane association on enzyme activity will also be assessed. The interaction of various xenobiotics at the level of gene transcription could be evaluated by investigating effects of nucleolar ribosome properties and processings. This information will be correlated with alteration in RNA polymerase activity.

Serial No.: NIEHS-CB-017
1. Cell Biology Branch
2.
3. Research Triangle Park, NC

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Influence of Nitrilotriacetic Acid on Growth and Macromolecular Metabolism of Cultured Mammalian Cells"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Paul E. Brubaker, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	0.50
Professional:	0.25
Other:	0.25

PROJECT DESCRIPTION

OBJECTIVES: The growth of mammalian cells in culture offers a means of evaluating the influence of various environmental agents on (a) the capacity of a cell to undergo division, (b) the flow and expression of genetic information within a cell, and (c) the molecular interactions regulating various metabolic processes. The information obtained provides clues to the molecular mode of biological interaction of specific compounds as well as an aide to assessing the toxicology observed in vivo.

In attempts to relieve eutrophication of lakes and streams, Nitrilotriacetic Acid (NTA) has been considered as either a complete or partial substitute for polyphosphates in many detergent formulations. NTA forms stable chelates with divalent metal cations similar to those of ethylenediametetracetic acid (EDTA) in the physiological pH range of 7.2-7.4. While a great deal is known about the biological interaction of EDTA there is little information relative to NTA. The objective of this project is to contrast the effects of NTA on cell growth, division and aspects of macromolecular metabolism in mammalian cells with those of EDTA.

METHODS EMPLOYED: Tissue culture, electronic cell enumeration, radioactive isotope techniques and density gradient ultracentrifugation were employed.

MAJOR FINDINGS: About twice as much NTA (2.1×10^3 ppm; 0.0076M) as EDTA (1.1×10^3 ppm; 0.0028M) is required to inhibit growth of mouse lymphoma

cells 25% within one generation time (12 hours). Attempts to reverse this degree of inhibition by removing treated cells from chelate-containing medium and placing them in conditioned growth medium (medium from cultures undergoing exponential growth) failed. The G_1 and early S-phase stages of the cell cycle appear to be the more sensitive periods of interphase. There is little or no effect on the capacity to complete karyo- and cytokinesis once cells have entered mitosis.

Total cellular protein is reduced by 25-30% in cells treated for 12 hours with ID_{25} levels of NTA and EDTA. Ribonucleic acid levels are also reduced. A 40% reduction in RNA levels is observed in treated cells at 6 hours following initial exposure. Since 80% of the total cellular RNA is ribosomal these findings suggest alterations in levels of cytoplasmic ribonucleoprotein particles and incorporation of uridine and amino acids in treated cells.

There is 5-10% dissociation of polyribosomes into free ribosomal subunits in ID_{25} treated cells over a 6 hour exposure period. In vitro studies were undertaken to determine the levels of chelating agent required to induce polyribosome dissociation and to determine differences in mode of dissociation induced by NTA and EDTA. It requires about 11 times the amount of NTA (16.59×10^3 ppm; 0.06 M) over that of EDTA (1.54×10^3 ppm; 0.004 M) to induce complete dissociation of isolated polyribosomes. These levels are 7.9 and 1.6 times the ID_{25} for NTA and EDTA, respectively. There are subtle differences in mode of dissociation of polyribosomes induced by these two chelators. EDTA-induced dissociation leads to release of ribosomal subunits and an increase in levels of monoribosomes. There are also changes in sedimentation characteristics of the released particles that become more pronounced with increasing concentration of EDTA. The large 60 S ribosomal subunit and 80 S monoribosome become progressively slower in sedimenting through sucrose density gradients. There are no changes in levels of monoribosomes observed with increasing concentrations of NTA. Of the released ribosomal subunits there is a relatively slight modification of the sedimentation characteristics of the large ribosomal subunits. Dissociative effects similar to those observed with EDTA occur when polyribosomes are placed in magnesium deficient isolation medium.

At various intervals over a 6 hour exposure of cells to ID_{25} levels of NTA and EDTA indicate no changes in levels of cytoplasmic ribonucleoprotein levels relative to controls. While the distribution of incorporated ^{3}H-5-uridine is essentially identical to untreated cells the overall specific activity becomes progressively reduced in chelate-treated cells. The specific activity of total cellular RNA is inhibited by 56% following exposure to ID_{25} levels of EDTA. Cells treated with ID_{25} levels of NTA exhibit a 17% reduction in specific activity of cellular RNA over the same time period. Determinations of the amount of exogenous radioactive uridine remaining in the growth medium following treatment indicates failure of treated cells to incorporate the labeled RNA precursor.

The incorporation of C^{14}-labeled leucine presents a different pattern from that of uridine labeling in treated cells. Both NTA and EDTA stimulate the

incorporation of this labeled amino acid within a 60 minute exposure period. Subsequently, the specific activity of cellular protein is reduced by 15% at 12 hours following exposure to ID_{25} levels of both NTA and EDTA. However, the distribution of C^{14}-leucine among polyribosomes is similar to that of untreated control cells.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The levels of NTA required to induce biochemical and growth alterations in rapidly dividing cells are approximately 1000 times the calculated 2 ppm to be found in the environment upon complete substitution of polyphosphates in detergent formulations. The effects on nucleic acid (RNA) metabolism and protein synthesis appears to be the consequence of divalent ion deficiency due to their sequesteration in growth medium. The evidence presented points to disturbances in cellular mechanisms concerned with transport and uptake of metabolic precursors.

PROPOSED COURSE: No further work is anticipated on this project.

Serial No.: NIEHS-CB-018
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of organic mercurials on hepatic microsomal enzymes "

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: G. W. Lucier, Ph.D.

OTHER INVESTIGATORS: O. S. McDaniel, R. Klein, M.D., S. Herman, M.D., P. E. Brubaker, Ph.D.

COOPERATING UNITS: Pathological Physiology Branch

MAN YEARS:

TOTAL:	0.8
PROFESSIONAL:	0.8
OTHER:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The health hazards of organic mercury in our environment are becoming increasingly evident. However, most attention has been focused on neurological effects, with essentially no studies conducted on *in vivo* effects of the hepatic drug metabolizing enzymes. Our investigations were initiated to; (1) determine the effects of methyl mercury hydroxide and other organic mercurials on microsomal enzyme systems (UDP glucuronyltransferases and mixed function oxidases); (2) to evaluate effects of organic mercurials on the metabolism of the carbamate pesticides, which require rapid hydroxylation and conjugation to be detoxified and excreted; (3) to determine the mechanism by which organic mercurials reduce cytochrome P-450 and cytochrome b_5 content; and (4) to determine effects resulting from the interaction of inducing agents (chlordane, dieldrin, phenobarbital, and piperonyl butoxide) and organic mercurials on the drug-metabolizing enzymes.

METHODS EMPLOYED: Livers from male rats, Sprague-Dawley derived, were used for preparation of microsomes and all other studies, Other relevant methods are described in the Major Findings section.

MAJOR FINDINGS: Mercury accumulation in liver. Following sub-cutaneous administration of methyl mercury hydroxide (MMH) to male rats for 2 days at 10 mg/kg/day, the livers contained approximately 60 µg mercury/gram liver, which corresponds to approximately 10% of the administered dose. Mercury bound to the microsomal fraction represented 0.25% of the administered dose and corresponded to 0.1 µg mercury/mg microsomal protein. Mercury levels detected in

the whole liver and the microsomal fractions were not directly proportional to the amount of administered MMH.

Effects on microsomal enzymes. The microsomal cytochromes, P-450 and b_5, were markedly reduced following MMH administration at levels as low as 2.5 mg/kg/day for 2 days. The reduction was approximately 2-fold, whether animals received either 2.5 mg/kg/day or 10 mg/kg/day for 2 days. Doses of less than 2 mg/kg resulted in only a slight decrease of the microsomal cytochromes. The observed effects of MMH on the rate of aminopyrine demethylation and aniline hydroxylation were identical to those observed for cytochrome P-450. Decreased levels of mixed function oxidase activity can therefore be explained by a decrease in hepatic cytochrome P-450 levels. The UDP glucuronyltransferase conjugating 1-naphthol and p-nitrophenol was unaffected by MMH treatment, until the dosage rates were increased to 10 mg/kg/day for five days, when a marked reduction in enzyme activity was observed. At all dosage levels used the animals exhibited no neurological symptoms, no weight loss and no liver histopathology as determined by light microscopy. Reduction of aminopyrine demethylase was maximal 24 hours after a single treatment of MMH. The effect gradually diminished, so that by 1 week after treatment there was no effect on microsomal enzyme activity.

Effects on submicrosomal distribution of hepatic microsomal enzymes. The depression of cytochrome P-450 and associated enzymes was accounted for by a reduction of the smooth endoplasmic reticulum fraction (SER). The rough endoplasmic reticulum (RER) was markedly proliferated by MMH treatment but this was not associated with an increase in mixed function oxidase activity. The observed biochemical effects on the submicrosomal fractions were verified by electron microscopy, which also demonstrated proliferation of RER at the expense of the SER. The large increase in RER was accompanied by a correspondingly large increase in polyribosomes and monoribosomes while total RNA levels were unchanged by MMH pretreatment.

Redistribution of mercury and I_{50} values. To determine if the observed effects of MMH were related to redistribution of mercury from the red blood cells during homogenization, livers were perfused prior to microsome preparation. The reduction of P-450 content and activities of related enzymes were the same using either perfused or non-perfused livers from treated rats. Also, the levels of mercury in the microsomal fraction of treated rats were too low to inhibit the microsomal enzymes *in vitro*. The I_{50} values for MMH for either 1-naphthol glucuronyltransferase or aminopyrine demethylase were approximately 10^{-5} M.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: MMH has been shown to interfere with oxidative enzymes which function in the degradation of xenobiotics as well as the normal metabolism of many endogenous compounds. Thus, organic mercurials are likely an environmental hazard, since the normal regulation of steroid hormones may be upset by exposures to methyl mercury compounds. The carbamate pesticides and many other environmental agents and drugs are considered relatively safe because they are rapidly detoxified by hydroxylation reactions but if the normal rapid rate of hydroxylation is inhibited, these compounds may become considerably more toxic to animals.

PROPOSED COURSE: The effects of MMH on cytochrome P-450 content might be related to a decreased rate of synthesis of microsomal cytochromes or an increased rate of degradation. To resolve this question, the rate of incorporation and subsequent degradation of labeled S-aminolevulinic acid (ALA) into the microsomal CO-binding particles will be studied in treated and control rats. The possible effects on ALA synthetase will also be investigated. Studies will continue on the effects of organic mercurials on the ability of whole animals and microsomes to detoxify and eliminate environmental agents such as pesticides, food additives and mycotoxins. The joint action of organic mercurials and inducing agents on UDP glucuronyltransferase and the mixed function oxidases will be investigated in relation to environmental health hazards. Studies are being initiated to screen chelating agents which are rapidly eliminated *in vivo* in order to obtain a compound which can rapidly flush mercury from the body.

Serial No.: NIEHS-CB-019
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Chlorinated Hydrocarbons on the Metabolism of Carbamate Insecticides by the Mixed Function Oxidases, UDP Glucuronyltransferases and Sulfokinases"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: G. W. Lucier, Ph.D.

OTHER INVESTIGATOR: O. S. McDaniel

COOPERATIVE UNITS: None

MAN YEARS:

Total:	0.3
Professional:	0.3
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Chlorinated hydrocarbons have been shown to induce mixed function oxidase activity, and they may also induce the UDP glucuronyltransferases and sulfokinases. Thus, the chlorinated hydrocarbons might increase the rate of detoxication and excretion of carbamate insecticides by effecting both "primary" and "secondary" detoxication. The objectives of this study are to evaluate the effects of dieldrin, chlordane, and DDT on the *in vitro* and *in vivo* metabolism of carbaryl and carbofuran.

METHODS EMPLOYED: Male and female rats and guinea pigs are used in the metabolism studies. The chlorinated hydrocarbons are administered orally and radiolabeled carbaryl and carbofuran are used to evaluate effects on metabolism. Metabolites are identified by co-chromatography and isotope dilution techniques.

MAJOR FINDINGS: Following chlordane administration (20 mg/kg/day for 3 days) to either male or female rats, a marked increase in the rate of microsomal hydroxylations of carbaryl and carbofuran was observed. N-Hydroxymethyl carbaryl formation was enhanced 6-fold and ring hydroxylations at the 4 and 5 positions were enhanced approximately 3-fold. Ring hydroxylation of carbofuran at the 3 position was increased 3-fold, while N-hydroxymethyl formation increased 5-fold. Chlordane and dieldrin pretreatment of rats were shown to induce UDP glucuronyltransferase and are therefore expected to increase the rate of conjugation and subsequent excretion of hydroxylated carbamates.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: With the increasing use of the less persistent carbamate and organophosphate pesticides it is necessary to have information concerning their rate of enzymatic degradation as modified by other environmental agents for evaluation of potential long-range hazards. Inducers of oxidative and conjugative enzymes will generally protect animals against pesticide poisoning, whereas inhibitory agents should cause the animal to be more susceptible.

PROPOSED COURSE: Studies are continuing to determine qualitative and quantitative effects of chlorinated hydrocarbons on the *in vivo* metabolism of the carbamate insecticides, mediated by the mixed function oxidases, UDP glucuronyltransferases, and sulfokinases.

Serial No.: NIEHS-CB-020
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Insecticide Synergists on Microsomal UDP Glucuronyltransferase"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: G. W. Lucier, Ph.D.

OTHER INVESTIGATORS: O. S. McDaniel, H. B. Matthews, Ph.D., and P. E. Brubaker, Ph.D.

COOPERATING UNITS: Analytical and Synthetic Chemistry Branch

MAN YEARS:

TOTAL:	0.6
PROFESSIONAL:	0.6
OTHER:	0.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To characterize optimal *in vitro* reaction conditions and to develop rapid and sensitive assay techniques for measuring activity of conjugative enzyme systems. (2) To evaluate *in vitro* inhibition of conjugative enzymes by insecticide synergists. (3) To evaluate *in vivo* effects of insecticide synergists on conjugative enzymes with special reference to enzyme induction and protein synthesis.

METHODS EMPLOYED: Livers from male and female rats (CD strain, 125-150 g), were used for preparation of microsomes and all other studies. Other relevant methods are described with the Major Findings.

MAJOR FINDINGS: Radioassay for UDP glucuronyltransferase. Radiolabeled substrates (1-naphthol-^{14}C or testosterone-^{14}C) were added to a scintillation vial. Amounts of the respective unlabeled substrate were added to achieve desired substrate concentrations. After addition of the necessary co-factors and divalent cations the vial contents were incubated under nitrogen at 37°C and the reaction stopped by the addition of a non-aqueous scintillation fluid. Unmetabolized substrate partioned into the non-aqueous phase, whereas all glucuronide formed remained in the aqueous phase. Therefore disappearance of substrate was measured by scintillation counting against blanks incubated without UDPGA. In parallel experiments, results obtained using the rapid method were similar to those obtained from the more laborious conventional extraction procedures. Oxidative reactions were shown not to be a factor in the assay procedure. The new and rapid radioassay has several advantages

over the standard colorimetric assays; greater sensitivity, less variability, ease of obtaining kinetic data, shorter incubation periods, and limited transfer of liquids.

Activation of Enzyme. Triton X-100 activates microsomal UDP glucuronyltransferase approximately 10-fold in vitro using 1-naphthol or p-nitrophenol as substrates while testosterone glucuronidation was inhibited by detergent. Activation was not accompanied by a change in Km for 1-naphthol or UDPGA, which indicates that activation is not related to an increase in enzyme affinity for substrate or co-factor. Magnesium, manganese and cobalt ions were shown to stimulate enzyme activity, while Zn^{++} was strongly inhibitory. A total activation factor of 30 was observed when both Triton X-100 and Mg^{++} were added to the incubation medium. Stimulation of glucuronyltransferases by divalent cations is a general phenomenon since effects were similar with enzyme from unactivated, detergent-activated, and aged microsomes as well as enzyme derived from the smooth or rough-surfaced endoplasmic reticulum. The mechanism of enzyme activation was investigated but no conclusion has been reached, as yet.

In vitro inhibition by insecticide synergists. The UDP glucuronyltransferases conjugating 1-naphthol, testosterone, and a hydroxylated dieldrin metabolite were inhibited by a variety of insecticide synergists (methylenedioxyphenyl and N-alkyl compounds). Piperonyl butoxide had respective I_{50} values of 7.3 x 10^{-6} M and 9.0 x 10^{-5} M for testerone and 1-naphthol glucuronyltransferase. Inhibition of enzyme from detergent-activated microsomes was considerably less than that from unactivated or aged microsomes. Methylenedioxybenzene and piperonal were not inhibitory suggesting that the methylenedioxyphenyl group is not the sole inhibitory moiety.

Induction Studies. Piperonyl butoxide or MGK-264 pretreatment of male or female rats resulted in an induction of the UDP glucuronyltransferases conjugating p-nitrophenol and 1-naphthol. Induction was approximately 2-fold and was slightly higher in females than in males. The 2-fold induction was obtained in detergent-activated, aged and unactivated microsomes from piperonyl butoxide-treated rats and the inductive effect was not altered when livers were homogenized with buffer containing $MgCl_2$. Induction of UDP glucuronyltransferase was not accompanied by a change in Km for 1-naphthol or UDPGA. Only V_{max} of the enzyme reaction was affected. Piperonyl butoxide-pretreatment induced enzyme in both the smooth and rough surfaced endoplasmic reticulum fractions whereas other inducing agents, such as chlordane, were shown to increase enzyme activity solely in the smooth endoplasmic reticulum. Piperonyl butoxide and MGK-264 also caused a 2-fold induction of mixed function oxidase activity accompanied by an increase in CO binding particles. Time-course effects on the conjugative and oxidative enzyme systems were extensively studied following a single dose of piperonyl butoxide or MGK-264. These results showed an initial inhibition of enzyme activity followed by induction.

Effects on Polyribosomes and RNA Levels. Following pretreatment of male or female rats with piperonyl butoxide or MGK-264, the polyribosome content of the hepatic post-mitochondrial fraction was increased approximately 30%. This increase in polyribosomes was accompanied by an increase in RNA levels and a decrease in monoribosomes. Thus, enzyme induction, in this case, appears to be related to an increased level of protein synthesis.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: UDP glucuronyltransferases play an important role in the metabolism and excretion of numerous zenobiotics and endogenous compounds. Any disruption in the detoxication rate of foreign compounds or rate of metabolism and excretion of endogenous compounds might result in a serious health hazard. Insecticide synergists which are commonly used in household sprays and in conjunction with many other insecticide applications were believed to exert their primary action by serving as alternative substrates for the mixed function oxidase enzymes. However, evidence presented here indicates that the UDP glucuronyltransferases are involved as well. The radiolabeled assay procedure described here has applications for many other enzyme assays, such as the β-glucuronidases, sulfatases, esterases, and sulfokinases.

PROPOSED COURSE: Studies are continuing on the mode of inhibition and subsequent induction of UDP glucuronyltransferase by insecticide synergists. The effects on *in vivo* conjugation of several other environmental agents such as the carbamate insecticides will be investigated. Attempts will be made to purify UDP glucuronyltransferase.

Serial No.: NIEHS-CB- 021
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "UDP Glucuronyltransferases in DDT Resistant Mouse Leukemic 5178 Y Cells"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: G. W. Lucier, Ph.D.

OTHER INVESTIGATORS: J. Spalding, Ph.D.

COOPERATING UNIT: None

MAN YEARS:

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Mouse L 5178 Y cells have been shown to develop resistance to organochlorine pesticides such as DDT, chlordane and Kelthane. The mechanism of resistance remains unknown but might be directly or indirectly related to alterations in enzyme activities, such as the conjugative enzymes. The objectives of this study are (1) to detect and characterize conjugative enzymes in mouse L 5178 Y cells and (2) to compare enzyme activities between DDT resistant, chlordane resistant, and control cells.

METHODS EMPLOYED: A sensitive radioassay technique was used to measure UDP glucuronyltransferase. Standard colorometric assays were used for other enzymes.

MAJOR FINDINGS: 1-naphthol and testosterone UDP glucuronyltransferases were characterized with respect to kinetic data, magnesium requirements, pH optimum, temperature optimum, ionic strength optimum, centrifugal properties and stability. The results indicate that two different UDP glucuronyltransferases are operative.

1-Naphthol UDP glucuronyltransferase activity from DDT resistant mouse L 5178 Y cells was 15 times that of control or chlordane resistant cells. The difference was approximately 3-fold when using testosterone as the substrate. Apparently, the mechanism of resistance is at least indirectly different between chlordane and DDT. β-glucuronidase and aryl sulfatase activities were similar in DDT resistant, chlordane resistant, and control cells.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The mechanism by which organism and individual cells develop resistance to organochlorine insecticides is unclear. Enzymatic studies on the cellular level might lend insight to the mode of resistance as well as possible characterization of different types of resistance. The differences in UDP glucuronyltransferase activity between DDT resistant and control cells appears to be only marginally related to the development of resistance.

PROPOSED COURSE: The differences in UDP glucuronyltransferase between chlordane resistant, DDT resistant and control cells might be related to qualitative and/or quantitative differences in membrane synthesis. Attempts will be made to correlate membrane morphology and synthesis with alterations in activities of transferase enzymes.

Serial No.: NIEHS-CB-022
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Alterations of Fetal and Neonatal Development of Conjugative Enzyme Systems"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: G. W. Lucier, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.1
Professional:	0.1
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: (1) To determine qualitatively and quantitatively the fetal and neonatal development of UDP glucuronyltransferase, sulfokinase, and β-glucuronidase in liver, lung, and kidney, and (2) to determine effects of maternal exposures to various environmental agents (cadmium, mercury, and lead) on the development of these enzymes in a variety of species.

MAJOR FINDINGS: The fetal and neonatal development of rat liver and lung UDP glucuronyltransferase and β-glucuronidase are being studied. While the lung appears to have little UDP glucuronyltransferase up to 3 days after birth, the levels of this enzyme in the liver rise markedly just prior to birth, and decrease slightly after birth. β-glucuronidase activity appears prior to birth in both liver and lung.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: If the development of certain enzyme systems in the fetus and neonate is retarded by maternal exposures to compounds such as mercury, cadmium, and lead, the newborn will be unable to perform certain biochemical transformations involving detoxification of xenobiotics and metabolism of endogenous compounds.

PROPOSED COURSE: Studies will continue involving comparative maternal and fetal or neonatal development of conjugative enzyme systems following maternal exposures to various environmental agents. I plan to study developmental alterations using several different species; rat, guinea pig, mouse, and rabbit.

The studies will involve changes in fetal and neonatal metabolism of carbamate and organophosphate insecticides.

Serial No.: NIEHS-CB-023
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Teratogenic Responses to a Benzhydrylpiperazine Compound"

PREVIOUS SERIAL NUMBER: NIESH-PT-01

PRINCIPAL INVESTIGATOR: Herbert S. Posner, Ph.D.

OTHER INVESTIGATORS: Mrs. Joanne Wilson

COOPERATING UNITS: None

MAN YEARS:

Total:	0.8
Professional:	0.2
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: Chlorcyclizine produces a syndrome of malformations in rats representative for five benzhydrylpiperazine compounds. The syndrome includes cleft palate, receded mandible, small mouth, edema, some limb shortening and hypocalcification particularly of the spine. We found previously that the incidence of cleft palate was positively correlated with the degree of enlargement of the thorax and that enlargement of the thorax was the result of excess water in subcutaneous tissue. In addition, cleft palate and thorax enlargement remained correlated despite three tests designed to dissociate them. Presently, we tried to dissociate the events by two further means, one of which involved co-administration of a compound used as a diuretic in the human. The second means involved starting chlorcyclizine administration two days later in gestation. Finally, we began to measure physiological parameters in the fetus that might help to explain the mechanism of the edema formation.

METHODS EMPLOYED: Compounds were administered orally to the pregnant rats at doses which yield cleft palate in the fetus. Length, cross-sectional area of the thorax and status of the palate were determined as described previously. Hematocrit of the fetal blood was determined by centrifugation in capillary tubes and K^+ and Na^+ were determined by atomic absorption. Thin layer chromatograms identified and estimated the content of chlorcyclizine and its metabolite norchlorcyclizine in extracts from the fetus.

MAJOR FINDINGS: When chlorcyclizine was administered from days 15-17 of

gestation, visible edema but not cleft palate occurred on days 20 and 21. Enlargement of the thorax on the critical days 18 and 19 was half that found when chlorcyclizine was administered on the usual days 13-16. The palate, therefore, can close before edema develops sufficiently. This experiment indicates that cleft palate formation does not have to precede edema formation. It leaves the possibilities that the two events are coincidental and unrelated or that thorax enlargement (or perhaps edema at another site for which the thorax enlargement is a marker) is pathogenic for the formation of the cleft palate.

Triamterene resulted in both reduced incidence of cleft palate and a lesser enlargement of the thorax when co-administered with the chlorcyclizine. The triamterene did not block the metabolism of chlorcyclizine to norchlorcyclizine as did SKF-525A, and thus the ratio of chlorcyclizine to norchlorcyclizine in the fetus remained unchanged. It remains to be determined if triamterene is truly acting via its diuretic action.

Fetal heart rate, hematocrit and plasma K^+ values were similar to those of controls on days 17, 18, and 19. It can only be concluded from the latter at this stage that anemia is not present.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Cleft palate is a well-known congenital malformation in humans. It occurs alone or as part of a variety of syndromes. Cleft palate is also a frequent finding in teratologic investigations in animals when environmental or other agents are tested. These experiments will hopefully help to explain some cases of cleft palate formation. It may also aid in the extrapolation of findings from animal experiments to events in human gestation and help to develop knowledge of "equivalencies" of response between species.

PROPOSED COURSE: The project will continue with further study of the effects of diuretic agents and with measurement of physiological parameters in the fetus.

PUBLICATIONS

Posner, H.S. and Darr, A.: Fetal edema from benzhydrylpiperazines as a possible cause of oral-facial malformations in rats. Toxicol. Appl. Pharmacol. 17: 67-75, 1970.

Posner, H.S., Darr, A. and Barrow, M.V.: Anomalies of the internal organs and diminished calcification of vertebrae in fetuses after benzhydrylpiperazine treatment of pregnant rats. Toxicol. Appl. Pharmacol. 17: 76-82, 1970.

Posner, H.S.: Approaches to fetal pharmacology and toxicology. In Sunderman, F.W. and Sunderman Jr., F.W. (Eds.): Diagnosis of Diseases Caused by Toxic Agents. St. Louis, Mo., Warren H. Green, Inc., 1970, pp. 46-70.

Serial No.: NIEHS-CB-024
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Fetal Responses to a Variety of Environmental and Other Chemicals"

PREVIOUS SERIAL NUMBER: NIEHS-PT-02

PRINCIPAL INVESTIGATOR: Herbert S. Posner, Ph.D.

OTHER INVESTIGATORS: Mrs. Joanne Wilson

COOPERATING UNITS: None

MAN YEARS:

Total:	0.8
Professional:	0.4
Other:	0.4

PROJECT DESCRIPTION

OBJECTIVES: Cleft palate formation was studied in the rat in depth with chlorcyclizine (NIEHS-CB-023). Presently, we compared the findings with that obtained after administration of four other agents that are known to produce cleft palate in the rat or mouse. We also administered chlorcyclizine to the mouse. We were particularly interested in effects on fetal growth, enlargement of the thorax and the type of cleft palate that occurs.

METHODS EMPLOYED: Compounds were administered to rats or mice at doses that produce up to 100% cleft palate. Length, cross-sectional area of the thorax and status of the palate (vertical- or horizontal-type cleft palate or normal, closed palate) were determined. The compounds tested were: chlorcyclizine, β-aminopropionitrile (BAPN), triamcinolone acetonide, cortisone acetate, and N-nitroso-N-methylurea (NNMU).

MAJOR FINDINGS: Several patterns of fetal response were obtained. The glucocorticoids, triamcinolone and cortisone, resulted in rat and mouse fetuses that had almost exclusively horizontal-type cleft palates. Growth, as length, was retarded by as much as 17% on day 18 at the highest incidences of cleft palate. There was no evidence of catch-up growth by day 20. Neither compound produced thorax enlargement in the mouse. However, triamcinolone produced a 10% thorax enlargement in the rat on day 18. The enlargement was 8% on day 20. The degree of enlargement did not correlate with the incidence of cleft palate.

N-Nitroso-N-methylurea yielded only vertical-type cleft palates. Length of the fetus was reduced by up to 17% on day 18 and 21% on day 21. Area of the thorax was at first reduced by as much as 14% on day 18 but then it was normal for the reduced size of the fetus on day 21.

Chlorcyclizine in the rat and mouse and BAPN in the rat and mouse yielded both the horizontal- and vertical-type clefts. Length reduction with chlorcyclizine was, in general, 8% or less in both species. Length reduction after BAPN was not greater than after appropriate controls in either species. Chlorcyclizine in the rat produced a 17-25% enlargement of the thorax. In fetuses with low incidences of cleft palate, the enlargement partially subsided over 4 days. BAPN yielded a transient thorax enlargement in the mouse that was transient even at the highest incidences of cleft palate. The enlargement was 12% on day 18 in the 26-75% and 76-100% incidence of cleft palate groups, 6.5% on day 19 and only in the 76-100% incidence of cleft palate group, and it was absent on day 20.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Cleft palate can be produced in the two species with compounds of four chemical classes at incidences up to 100%. Comparison of the similarities and differences of fetal response associated with this malformation may make it possible to improve the predictive value for humans of safety testing in experimental animals. The events observed are shown to occur with drugs and environmental agents.

PROPOSED COURSE: Some additional environmental agents will be tested. When thorax enlargment occurs, its potential relationship to cleft palate formation will be explored. Likewise when cleft palate occurs evidence will be sought for the presence of a fluid imbalance. Attempts will be made to relieve or exacerbate fluid imbalances. In this and other ways we hope to learn if the imbalance is innocuous or detrimental.

Serial No.: NIEHS-CB-025
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Teratogenesis and Prenatal Toxicity Testing of Compounds From Fungi"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Herbert S. Posner, Ph.D.

OTHER INVESTIGATORS: Dr. Ronald W. Pero and Dr. Robert G. Owens

COOPERATING UNITS: None

MAN YEARS:

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVE: Purified components and in some cases crude extracts of fungi that are being studied by others in the Branch are tested for interference with prenatal development.

METHODS EMPLOYED: Compounds are administered to the pregnant animals at two time periods of gestation. The fetuses are then examined both fresh, grossly and after fixation in Bouin's solution.

MAJOR FINDINGS: No evidence for malformation or increased in utero death has been found so far for the combination of equal weight amounts of alternariol and alternariol methyl ether, two toxic metabolites from the fungus Alternaria tenuis, when administered from days 9-12 or 13-16 in Sprague-Dawley rats or DBA/2 mice. The compounds were administered subcutaneously, dissolved in DMSO. The methoxy compound was particularly insoluble in other solvents that might be used. Doses from 0.5 - 10 mg/kg of each were administered to the mouse and 1, 5, and 25 mg/kg to the rat. The combination was tested first because of evidence of potentiation in another test. The compounds are being tested now individually (growth retardation, malformation, death and resorption). Some fetal toxicity may now be occurring in the mouse at higher doses.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: In some cases fungi produce compounds during their growth that are toxic to mammalian species. Some of the compounds have been found to be extremely potent. This work is undertaken as part of a larger program involving the isolation, identification and toxicity testing of purified components from fungi often associated with food products.

PROPOSED COURSE: The project will continue with further testing of alternariol and alternariol methyl ether because of the potent toxicity found in several other tests. Additional fungal components will be tested as they become available and as evidence for their toxicity accumulates.

Serial No.: NIEHS-CB-026
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Comparative Placental Passage and Fetal Toxicity of Paraquat and Diquat"

PREVIOUS SERIAL NUMBER: NIEHS-PT-10 and NIEHS-PT-11

PRINCIPAL INVESTIGATORS: B. Clair Eliason, M.D. and Charles W. Sharp, Ph.D.

OTHER INVESTIGATORS: Herbert S. Posner, Ph.D.

COOPERATING UNITS: Animal Science and Technology and Biometry Branches

MAN YEARS:

Total:	0.5
Professional:	0.2
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: 1. To compare two chemically similar herbicides which are quarternary amines in their ability to cross the placenta at different gestational ages; and to determine their distribution in the fetus. 2. To determine and compare the fetal toxicity of the two compounds.

METHODS: Objective #1: A single dose of 15 mg/kg of ^{14}C-labeled paraquat or diquat is administered intravenously to pregnant rats at 13, 16 and 21 days of gestation. Whole fetus and selected fetal organs are analyzed for radioactivity at specific time intervals following the administration.

In order to determine whether the diquat or paraquat is metabolized, studies are underway using thin-layer chromatography.

Objective #2: Using a near-lethal dose (15 mg/kg, i.v.) for maternal rats, they are injected with either paraquat or diquat on days 7-21 of gestation. Following sacrifice on day 22, the number of fetal deaths and resorptions are determined. A pilot study was performed in order to determine the post-natal effects of the prenatal administration of paraquat.

MAJOR FINDINGS: Objective #1: More diquat than paraquat crossed the placenta at each stage of gestation. Paraquat, however, appears to persist longer than diquat. The concentrations of both paraquat and diquat per

gram of fetus increased significantly with increasing gestation.

The fetal distribution of paraquat and diquat differed. The fetal liver, kidney and amnoitic fluid had the highest levels of diquat, whereas the lung, kidney and amniotic fluid contained the most paraquat.

Objective #2: The fetal lethality of diquat was generally greater than paraquat. The adverse affect of paraquat on both the fetus and neonate appeared to parallel maternal toxicity.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: There has been little investigation of the placental passage and potential toxicities of these widely used herbicides. The results of placental passage of the quarternary amines, paraquat and diquat, will be compared with each other and the results obtained with C^{14}-dieldrin.

PROPOSED COURSE: We are currently adding to these data and determining if metabolites of paraquat or diquat are present in the fetus or mother. Dr. Eliason's work as principal investigator will terminate in June, 1971.

Serial No.: NIEHS-CB-027
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Placental Passage of Dieldrin Modified by Phenobarbital Pretreatment, Gestational Age, and Plasma Proteins"

PREVIOUS SERIAL NUMBER: NIEHS-PT-11

PRINCIPAL INVESTIGATOR: B. Clair Eliason, M.D.

OTHER INVESTIGATORS: Herbert S. Posner, Ph.D.

COOPERATING UNITS: Animal Science and Technology Branch, Biometry Branch

MAN YEARS:

Total:	1.2
Professional:	0.5
Other:	0.7

PROJECT DESCRIPTION

OBJECTIVES: In earlier work, we measured the placental passage of C^{14}-dieldrin at different gestational ages in the rat and tested nine common drugs for a modifying effect. Only phenobarbital produced a modifying effect, and it decreased the amount of dieldrin reaching the fetus. This work has been submitted for publication.

We noted that both maternal plasma and whole fetal levels of dieldrin increased considerably during the later stages of gestation. Since dieldrin is known to bind to plasma proteins we now seek to investigate the possible role of maternal plasma proteins in binding increased levels of C^{14}-dieldrin as gestation proceeds.

Since fetal plasma proteins are known to increase considerably during gestation, our objective in this study was to determine if the type and amount of protein on the fetal side of the placenta could alter the rate of maternal to fetal passage of dieldrin.

METHODS EMPLOYED: For an in vitro test, plasma is obtained from pregnant rats at days 13, 16, and 21 of gestation. C^{14}-dieldrin is added to the plasma in vitro, and mixed and electrophoresed. The electrophoresis gel is sliced and counted in a liquid scintillation counter.

An in vivo method for the perfusion of the fetal side of the pregnant guinea pig is used. The vessels leading to and from the fetus and placenta are cummulated and perfused. Various protein solutions are perfused, C^{14}-dieldrin is administered to the mother and the rate of dieldrin entering the perfusate is determined.

MAJOR FINDINGS: (Preliminary) Most of the C^{14}-dieldrin was located on the electrophoresis gel at the regions of albumin, beta-globulins and the origin. As gestation proceeded, the amount of C^{14} bound to albumin did not change whereas radioactivity at the origin decreased while the amount in the beta-globulin fraction increased.

Passage of C^{14}-dieldrin across the guinea pig placenta was found to be highly dependent on the quantity of plasma protein circulating on the fetal side. No dieldrin was detectable when saline was perfused. The rate of dieldrin passage increased in proportion to the protein concentration of the fetal perfusate.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The observation that there is increased binding of dieldrin with increasing gestation implies that the same phenomenon may occur with other similar chemicals that are protein bound. The overall effect on the health of the organism is not known. However, the possible modification of drug distribution due to increased protein binding during gestation could have important therapeutic implications.

Since fetal plasma proteins are normally quite low early in gestation and then increase, the dependency of dieldrin placental passage on the quantity and type of fetal plasma protein gives additional insight into a mechanism of placental transfer. Other chemicals are likely to be similarly affected.

PROPOSED COURSE: Additional data is being collected on the ability of five plasma protein fractions to alter maternal to fetal transport when perfused through the fetal placenta. As principal investigator, Dr. Eliason's work will terminate in June 1971.

HONORS AND AWARDS: Dr. Eliason is serving as a member of an NIH committee preparing a report on the NIH supported research covering the first five years of life.

Serial No.: NIEHS-CB-028
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Induction of Resistance to Pesticides in Cultured Lymphoma Cells."

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-7

PRINCIPAL INVESTIGATOR: Judson W. Spalding, Ph.D.

OTHER INVESTIGATORS: George W. Lucier, Ph.D.

COOPERATING UNITS: Analytical and Synthetic Chemistry Branch

MAN YEARS:

TOTAL:	1.5
PROFESSIONAL:	1.5
OTHER:	0.0

PROJECT DESCRIPTION

OBJECTIVES: The objective is to develop mammalian cell lines in culture which are resistant to selected drugs and environmental agents, to determine the mechanism by which the resistance is obtained, and to compare resistant cells with normal cells in terms of appropriate biochemical and metabolic parameters.

METHODS EMPLOYED: Mouse lymphoma cells of the L 5178 Y line, have been subjected to chronic treatment with gamma chlordane, p,p' DDT and carbaryl (Sevin). This treatment has resulted in the selection of cells which exhibit resistance to the particular pesticide to which they were chronically exposed. Cloning techniques have been employed to isolate resistant lines originating from single resistant cells.

MAJOR FINDINGS: The isolated clones have been subjected to toxicity studies with representative pesticides of different structual classes and presumably with different modes of action. DDT-resistant (DDT-R) cells continue to show a high degree of resistance to DDT and the DDT analogues. They also exhibit resistance to chlordane to about the same degree as the chlordane resistant (Chl-R) cells selected initially for resistance to chlordane. DDT-R cells showed no resistance to piperonyl butoxide, nor did piperonyl butoxide enhance DDT toxicity in either resistant or nonresistant cells. Extensive testing has indicated that DDT-R cells are resistant to other chlorinated hydrocarbons, e.g., lindane, dieldrin, and heptachlor.

Chlordane resistant (Chl-R) cells show the same degree of resistance to DDT as DDT-R cells.

It has been shown that resistance to DDT toxicity by DDT-R cells is not expressed via any detectable detoxification mechanism. Detoxification of DDT in mammals may be mediated by the microsomal mixed-function oxidases or by dehydrochlorination. Evidence for a mixed-function oxidase system could not be found in either the control or the DDT-R cell lines. The presence of either cytochrome P-450 or cytochrome b-5 was not detected.

The pathway of detoxification and excretion of many toxic compounds is mediated by conjugation via UDP-glucuronyltransferase (UDP-GTase) to the corresponding glucuronide. In collaboration with Dr. George Lucier, Cell Biology Branch, an attempt was made to measure levels of representative UDP-GTases in pesticide resistant and nonresistant cells. Testosterone and 1-naphthol were used as representative substrates for the assay. In DDT-R cells, levels of UDP-GTase activity were 3-fold and 15-fold greater than in the control for testosterone and 1-naphthol respectively. Levels of enzyme activity in chlordane resistant cells were similar to that in control cells. There was no significant difference in levels of β-glucuronidase or aryl sulfatase activity among DDT-R, Chl-R and control cells. However, there was evidence that 1-naphthol and testosterone are conjugated by different UDP-GTases.

The 15-fold higher level of UDP-GTase in DDT-R cells suggested that they might be more resistant to α-naphthol than control cells. However, α-naphthol was toxic to the same degree in both resistant and nonresistant cells.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The development of drug resistance in whole animals depends at least in part on the induction of microsomal enzyme systems in the liver. Modification of the toxic molecule is usually followed by conjugation and subsequent elimination.

Resistance exhibited by the DDT-resistant and chlordane resistant cells appears to depend on a different mechansim than that found in the microsomal enzyme system of liver and other tissues. Thus these cells, which do not have the usual microsomal detoxification system, are useful for studying alternative mechanisms of resistance.

PROPOSED COURSE: Efforts will be continued to determine and compare the structual and/or biochemical basis of pesticide resistance among the different resistant cell lines. The nature of resistance will also be studied in terms of how it may relate to any particular phase of the cell cycle. The toxicity of pesticide analogues will be compared in order to determine the structual requirements for cytotoxicity and the relationship between resistance and chemical structure. The toxicity of different classes of pesticides will be studied and compared in normal and resistant cell lines to determine the specificity of resistance and cross-resistance.

Serial No.: NIEHS-CB-029
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Cytotoxicity of Alternaria Toxins to Cultured Mammalian Cells"

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-20

PRINCIPAL INVESTIGATOR: Judson W. Spalding, Ph.D.

OTHER INVESTIGATORS: Ronald W. Pero, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	1.5
Professional:	1.0
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: To determine the degree of toxicity of Alternaria toxins to cultured mammalian cells and to determine the mechanism of action by which the toxicity is expressed.

METHODS EMPLOYED: Alternaria toxins are tested for toxicity to Hela cells and mouse lymphoma L 5178 Y cells in culture and the ID_{50} dose determined. Appropriate concentrations of the compound are selected to determine more specific effects on major metabolic systems e.g., DNA, RNA, protein and membrane synthesis, during defined phases of the cell replication cycle.

MAJOR FINDINGS: Two related toxins, isolated from Alternaria cultures, alternariol (A-OH) and alternariol methylether (A-ME), have been shown to be cytotoxic to both Hela cells and mouse L 5178 Y cells in culture. A-OH and A-ME are toxic to the same degree in both tissue culture systems at approximately 6 and 8 ppm (µgm/ml) respectively. A-OH has been used for most of the metabolic studies because it is much more soluble at concentrations (20 µgm/ml) that completely inhibit cell replication.

It has been established that A-OH has a selective effect on two phases of the cell cycle. The initiation of the G-2 phase of the cell cycle is especially sensitive to A-OH, though experimentally this inhibition can be reversed if A-OH is removed from the culture. Recent data suggest that A-OH may be expressing its effect in the G-2 phase of the cell cycle via the inhibition

of RNA synthesis. A-OH has also been shown to inhibit cytokinesis in cells which have been released from a state of metaphase arrest during mitosis. Again the toxic effect appears to be expressed through a dramatic inhibition of RNA synthesis. However, it should be emphasized that this effect of A-OH on RNA synthesis may be indirect, so that further investigations on the action of A-OH on other metabolic processes are being carried out to examine this possibility.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Toxigenic Alternaria species are commonly found on many foods available for human consumption as well as in feed products for domestic animals. Crude Alternaria extracts have been shown to be toxic to mice and rats as well as to bacterial and tissue culture cells. Several Alternaria toxins, A-OH and A-ME, are toxic to fungi, bacteria, tissue culture cells and mammals (Project No. NIEHS-CB- 034). The determination of the mechanism of toxicity in tissue culture cells is pertinent to the understanding of toxic responses in the intact animal.

PROPOSED COURSE: New toxins from Alternaria will be tested for biological activity as they become available.

Investigation will be continued to determine the mechanism of toxicity of alternariol (A-OH) and alternariol methylether (A-ME) in several unrelated mammalian tissue culture cell lines.

The different degrees of toxicity exhibited by alternariol, alternariol methylether and altenuene (another Alternaria toxin) will provide an opportunity to relate toxicity to functional groups on the alternariol molecule.

The synergistic characteristics of the Alternaria toxins will be examined in these same tissue culture systems.

Serial No.: NIEHS-CB-030
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Development of a Semi-Micro Assay for Mycotoxin Toxicity in Tissue Culture"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Judson W. Spalding, Ph.D.

OTHER INVESTIGATORS: Ronald W. Pero, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	0.5
Professional:	0.0
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: To develop a sensitive, semi-micro assay system for measuring the toxicity of purified mycotoxins in a mammalian tissue culture system.

METHODS EMPLOYED: Tissue culture cells are to be grown on a surface in 1.0 ± 0.5 ml cultures. Rates of macromolecular synthesis measured by the incorporation of the appropriate radioisotopically labeled precursor, will be used as an index of cell growth. The toxicity of any particular mycotoxin or growth inhibitor may then be reflected in the relative inhibition of radioisotope incorporation. In this way, mycotoxins may be compared with respect to their effect on DNA, RNA, protein, and lipid synthesis.

MAJOR FINDINGS: The development of a semi-micro tissue culture system for measuring mycotoxin toxicity has been nearly completed. At present, the assay system is undergoing preliminary testing.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: More often than not, pure compounds isolated from fungal extracts containing suspected mycotoxins are available in very limited amounts, 1 to 3 milligrams at most. A rapid and accurate assay for measuring toxicity in mammalian cells would provide information pertinent to confirming the importance of pursuing further study of any particular compound.

PROPOSED COURSE: The toxicities of known mycotoxins which have not been studied extensively in mammalian tissue culture will be compared in this system. In addition, the toxicity of these mycotoxins will be studied in combination to determine their synergistic characteristics.

PUBLICATIONS

Spalding, J.W., Pero, R.W., and Owens, R.G.: Inhibition of the G_2 phase of the mammalian cell cycle by the mycotoxin alternariol. J. Cell Biology 47: Number 2, Part 2, 1970.

Spalding, J.W., Ford, E., Lane, D., and Blois, M.: The character of DDT resistance in mouse L 5178 Y lymphoma cells. Biochemical Pharmacology (In Press).

Serial No.: NIEHS-CB-031
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Survey of Fungi for Presence of Mycotoxins."

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Miriam K. Slifkin, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

TOTAL:	.45
PROFESSIONAL:	.10
OTHER:	.35

PROJECT DESCRIPTION

OBJECTIVES: To survey food-contaminating fungi for the production of toxins.

METHOD EMPLOYED: Because the nature of possible toxins are not known, there is fear that any test using an extraction may give a false evaluation of potency. To avoid problems of this nature, five different bacteria - Sarcina lutea, Staphylococcus aureus, Bacillus mycoides, B. megaterium. and B. subtilis were tested against each fungus growing on Sabouraud's agar. The test fungus was inoculated in a line on one side of a large petri dish containing the agar. After 4 or 5 days of fungal growth the bacteria in their log phase were streaked horizontally and into the fungal mat. Clear zones of inhibition could be detected after 20 to 24 hours incubation when antibacterial substances were produced by the fungus.

Two percent methanol chloroform extracts were made of a selected group of fungi that ranged from very toxic to non-toxic for inhibition of bacterial growth. Small circles of filter paper were impregnated with 0.2, 0.1, and 0.05 mg of the extract in chloroform, air dried and placed on plates of nutrient agar inoculated with bacteria at their log phase of growth. The two methods of bacterial tests correlated well as to extent of toxicity and toxicity to different bacteria. Methanol-chloroform appeared to extract essentially all toxic components of the cultures and these extracts were used in animal tests.

CD1 Swiss albino female mice weighing 20-25 g were the test animals, and the extracts were administered in DMSO interperitoneally in duplicate at 200, 400, 800, 1200 mg/kg.

140 isolates in the Alternaria tenuis - tenuissima group were obtained from Dr. E.G. Simmons at the U. S. Army Natick Laboratories. Each isolate was tested against bacteria. Those showing greatest toxicity were tested or will be tested on mice.

MAJOR FINDINGS: All of the 140 isolates of Alternaria have been tested on bacteria. Of these, 39 were highly toxic. Thus far, 22 have been tested on mice. The LD_{50} ranges from less than 200 to 1200 mg/kg of crude extract for mice. As can be seen in Table 1, there is good correlation with the bacterial tests.

TABLE 1

Culture	mg crude extract/kg LD_{50} on Mice	Bacterial Index[1]	Min. crude extract inhibiting growth of S.a.	B.s.	S.l.	B.mg.	B.myc.
QM 1289	1200	.88	0[2]	.1	0	0	0
1354	1200	.77					
1355	800	1.16	.2	.1	.1	.2	.05
1362	400	1.75	.1	.05	.1	.2	.05
1366	800	1.24					
1377	600	1.20	.05	.1	.1	.2	.1
1516	600	1.50					
1714	800	1.06					
1883	200	2.02	.05	.05	.1	.1	.05
7158	1200	.80	.2	0	0	0	.1
7262	600	1.32	.1	.05	.1	.1	.1
7330	400	1.72	.05	.1	.1	.1	.1
7458	400	1.55					
7547	800	1.02	.1	.2	0	.2	.2
8305	800	1.10					
8308	800	1.04					
8312	320	1.87	.1	.1	.05	.1	.05
8396	600	1.30					
8477	600	1.30					
8680	1200	.92					
8761	400	1.56					
8764	800	1.22					

[1] The bacterial index is the average of the zones of inhibition induced by each living fungi on the 5 different bacteria.

[2] 0 = no inhibition

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Since Alternaria spp. are ubiquitous and occur as important contaminants of agricultural commodities it is important to evaluate the potential hazard of this genus to man and livestock. A number of strains in this genus are known to produce toxins which compare in potency to aflatoxins and other well-known fungal toxins.

PROPOSED COURSE: The organisms that are toxic to bacteria and not yet tested on mice will be examined. All tests will be repeated for confirmation. The toxic substances from toxicogenic strains will be isolated and identified, and the purified materials will be evaluated with regard to cytotoxicity and acute and long-term effects on mammals.

PUBLICATIONS

Slikfin, M.K. and Spalding, J.: Studies on the toxicity of Alternaria mali. Toxicology and Applied Pharmacology. 17: 375-386, 1970.

Serial No.: NIEHS-CB-032
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: Effects of fungicides on fungi and their toxicogenic properties.

PREVIOUS SERIAL NO.: None

PRINCIPAL INVESTIGATORS: Miriam K. Slifkin, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

TOTAL:	1.10
PROFESSIONAL:	.65
OTHERS:	.45

PROJECT DESCRIPTION

OBJECTIVES: Fungicides are used extensively to eliminate or reduce the growth of fungi in the production and storage of food products. In many cases, however, the fungi are not totally eliminated and may be affected by the presence of fungicides. There are several ways in which this can occur: (1) mutation, (2) induction of saltation, and (3) adaptation in which fungi gradually build up resistance to the chemical, but revert back to sensitivity after a few generations in the absence of the fungicides. In addition, forms grown in the presence of pesticides at sufficiently low doses may also be physiologically altered. The objective of this study is to demonstrate such changes and to determine the extent to which toxin production is enhanced or diminished in two fungi - Alternaria mali, which has been shown to be toxic, and Helminthosporium maydis, the causative agent of southern corn blight, which has been observed to be non-toxic to warm blooded animals.

METHODS EMPLOYED: The fungicides used are maneb, zineb, nabam, ferbam, captan and folpet.

Two systems were used to obtain variants: (1) an aqueous suspension of the pesticides is spread evenly over the surface of large petri dishes of Sabouraud and cornmeal agar media. After the water soaks into the agar, approximately 500 conidia per plate are introduced. (2) Conidia are spread over the surface of large Sabouraud agar plates. In the center of each dish is placed a 1 cm diameter filter paper covered with pesticides. In the first procedure any established colonies are recultured for testing. In the 2nd, any unusual colonies were used. Conidia or hyphal fragments from the new colonies were

inoculated onto cornmeal agar. Upon germination the tip of the germ tube was cut off and inoculated onto Sabouraud's agar. Colonies were considered variants if after 5 generations on 5 different media conidial and colony characters remained constant and were different from the original isolate.

For the growth of fungi in the presence of the fungicides, pilot tests were conducted to find concentrations of the compound at which the organisms would grow. Measured quantities of the pesticides were added to a medium containing rice, yeast extract and Czapeks broth. All test variants are grown in rice Czapeks media for 2 weeks. Crude extracts are made with 2% methanol in chloroform. Female swiss albino mice were the test animals.

MAJOR FINDINGS: For Alternaria mali (A3) wild type single spore isolate the LD_{50} of crude extract is 320 mg/kg for mice.

Five variants were obtained with maneb that behave like mutants. Because on recombination on minimal media (Czapeks) growth is greatly enhanced, two of these are definitely established as monokaryons. The LD_{50} of the extract from these forms vary from 400-1600 mg/kg. In addition one form upon being exposed to Maneb immediately acquired different cultural characteristics that remained as long as the chemical was present. Upon reinoculation in the presence of maneb it was resistant; however, after only two generations without the pesticide it lost its resistance. This form when grown in the presence of maneb was not toxic to mice.

Zineb induced 4 mutants, and one of these saltated twice to give 2 more forms. Preliminary tests reveal these to be highly toxic with one form more toxic than the wild type.

Ferbam induced 3 variants - 2 mutants and 1 saltation, Nabam - 4 variants, Thiram - 1 and captan - 2, one of which is a saltant. Toxicity tests have not yet been made of these forms.

Zineb is the only pesticide used thus far on Helminthosporium maydis. Neither the wild type nor the 3 variants so far derived are toxic to mice.

Adaptations. The cultures have been carried for 6 generations; although they are less susceptibel to the fungicides than originally they are still not as resistant as we would prefer.

Growth in the presence of fungicides. A. mali is being grown on rice in large flasks containing 1000 ppm captan and 100 ppm ferbam. A range of concentrations are being tested for maneb, nabam and folpet.

Studies with Helminthosporium maydis, obtained from NCSU, are just being initiated. Thus far only one different type isolate was obtained from H. maydis treated with zineb in the way described above. Neither the original culture nor the variant were toxic in mice except in extremely large doses (3600 mg/kg). These tests are being repeated for confirmation.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSITUTE: The more a pesticide is used the greater the probability that some organisms

survive and hence the greater the chance for selection of variants. Because of this selection process, there is a changing spectrum of fungi with unknown potential for toxin production. It is important therefore, to understand the frequency of changes and the pressures that bring them about in order to evaluate fungi as potential environmental hazards.

PROPOSED COURSE: The study will be continued with more emphasis on Helminthosporium maydis.

The changes involved in tolerance development will be clarified by physiological and biochemical studies in order to evaluate the role of chemical pressures on fungal metabolism in terms of changing populations with changing toxicogenic properties.

Serial No.: NIEHS-CB-033
1. Cell Biology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Toxin Production of Induced Mutants of Helminthosporium maydis."

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Miriam K. Slifkin, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	.45
Professional:	.25
Others:	.20

PROJECT DESCRIPTION

OBJECTIVES: The object of this study is to determine whether H. maydis, the causative agent of the southern corn blight, which has thus far been shown to be non-toxic to warm blooded animals, can produce mutations that are toxic.

METHODS EMPLOYED: Two isolates of H. maydis, one from N. C. and the other from Missouri, are used in this study. Cornmeal agar cultures of the organism at various stages of development have been exposud to mutagenic UV radiation of 2537 A°.

MAJOR FINDINGS: Nine mutations were induced by UV, of which one showed pronounced toxicity. The LD_{50} of a crude chloroform extract for white mice was 600 mg/kg. Two other variants showed slight toxicity. The potential conidial population exposed to UV was estimated at 500,000.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND PROGRAM OF THE INSTITUTE: Much of the crop affected by the corn blight was harvested early and stored for future use as fodder for livestock. Along with the corn stalks conidia from H. maydis too numerous to be imagined are being stored. Such a great concentration of conidia and mycelia is likely to result in numerous mutations that may pose problems in the future. Toxins may be produced that affect the livestock that feed upon the corn and ultimately affect human beings that eat the livestock. A knowledge of the possibility of toxin producing mutants should alert us to any potential danger of this nature.

PROPOSED COURSE: Other mutagenic agents will be tested in the same manner, and mutant strains will be assayed for toxicity as described before.

Serial No.: NIEHS-CB-034
1. Cell Biology Branch, NIEHS
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Toxicity, Purification, and Identification of Toxins Produced by Toxigenic Fungi"

PREVIOUS SERIAL NUMBER: NIEHS-CB-01-14

PRINCIPAL INVESTIGATORS: Ronald W. Pero, Ph.D. and Robert G. Owens, Ph.D.

OTHER INVESTIGATORS: Judson W. Spalding, Ph.D., Nancy Wilson, Ph.D., Louis Levy, Ph.D.

COOPERATING UNITS: Analytical and Synthetic Chemistry Branch

MAN YEARS:

Total:	1.1
Professional:	0.5
Other:	0.6

PROJECT DESCRIPTION

OBJECTIVES: The Alternaria, Penicillia, and Aspergilli are ubiquitous organisms, growing on food and other import crops both in storage and in the field. Crude extracts from a number of species and strains in these genera have been shown to be toxic to plants, bacteria, poultry, and mammals. The objectives of this study are (1) to isolate and identify potentially toxic fungal metabolites and (2) to determine their toxic activity in mammalian and other biological systems.

METHODS EMPLOYED: Rice cultures of toxigenic fungi were grown for two weeks and extracted with 70% acetone in water. The tetrahydrofuran (THF) soluble portion of this extract was fractionated by column chromatography using silica gel G as the sorbent and benzene with varying amounts of THF as the eluting solvent. Chemical purity of the column fractions was determined by thin-layer and gas chromatography. Toxicity was monitored by an antibacterial assay (disc method) using other Bacillus mycoides, Staphylococcus aureus, Sarcina lutea or Bacillus megaterium. Purified fungal metabolites were identified by use of IR, NMR, MS, and UV analyses.

No suitable method for evaluating antifungal activity with microquantities of test compound was available. Therefore, an assay was developed using spores and mycelium of Helminthosporium sativum. Experimentally, the test compound was dissolved in an appropriate solvent and dried onto microscope slides in

known concentrations. An agar disc containing spores and mycelium was then placed over the test compound and incubated in a moist chamber 2-4 hours after which inhibition of spore germination was determined by germination counts. This assay was also carried out on thin-layer plates with no interference from the silica gel. With water-insoluble compounds, the agar disc could be removed and the compound that did not dissolve into the agar could be easily recovered.

Purified toxins and crude extracts were evaluated for mammalian toxicity in random bred CD1 mice, hybrid DBA mice, and mammalian tissue culture (See Project No. NIEHS-CB-034).

MAJOR FINDINGS: The Alternaria have been studied most extensively and six fungal metabolites identified; e.g. alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT.), stearic acid, palmitic acid, and mannitol. Altenuene, a previously unknown metabolite, was identified and described in last year's report. The bacterial and fungal toxicity of these three toxins was discussed elsewhere. In tissue culture, AME and AOH are toxic, with ID_{50} levels of 15 and 5 ppm, respectively. Altenuene is considerably less toxic, with an ID_{25} value of 25 ppm. Mammalian toxicity was evaluated in mice by ip injection of the toxins according to the schedule: 100 mg;kg, 3/wk for 2 weeks. Under these conditions, 33% of the mice died with AME and Alt., and 66% lethality with AOH administration. The primary acute symptoms was severe hemorrhaging of the G.I. tract. Symptoms associated with chronic dosages were weight gain, spleen enlargement, liver enlargement, and either thymus enlargement or necrosis, depending on dosage and length of the exposure period. Histopathological examinations are incomplete but stem cell necrosis has been observed in the thymus and spleen.

Ten toxigenic isolates of Alternaria obtained from Dr. Mirocha at the Univ. of Minn. were compared with our isolates for toxicity and the content of AOH, Alt. and AME was determined for each isolate. Crude extracts from seven of the ten isolates contained these toxins in amounts from 0.5 to 25% of the weight of the total solids. The most toxic extracts contained no AOH, Alt. or AME, indicating the presence of one or more potent toxins that have not yet been identified. However, the importance of AOH, AME, and Alt. as toxins seems to be in low-dosage long-term chronic effects. This is indicated by the fact that these compounds can be recovered as unaltered AOH, AME, and Alt. 75 days after administration (ip) to mice and by long-term studies now in progress, where pathological changes are observed in some organs even at relatively low doses of the toxins.

Pencillic acid (verified by IR, NMR, MS analyses) was found to be produced by two fungi, Aspergillus ochraceous and Penicillium cyclopium, isolated from tobacco. This is an important carcinogen, but detailed toxicological studies have not been reported. Results in tissue culture indicate it to be very toxic with an ID_{50} of 2 to 3 ppm (see Project No. NIEHS-CB-034).

Patulin (verified by IR analysis) has been produced by two tobacco isolates of Aspergillus ochraceous and Aspergillus clavatus. The possible occurrence of penicillic acid and patulin in cigarette smoke is being studied.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: That toxigenic fungi associated with agricultural commodities can be a hazard to humans and food animals has been amply documented. The distribution of toxigenic fungi and the possible role of these fungi and toxic byproducts from their growth in food products has never been adequately evaluated. There is a growing conviction and an increasing body of supporting evidence that mycotoxins can and do constitute health hazards in some food products. Therefore, mycotoxins must be viewed as potential hazards in much the same manner as known potent toxic agents to which man is exposed through his environment.

PROPOSED COURSE: Efforts to isolate, purify, and identify toxins from selected isolates of the Alternaria, Penicillia, and Aspergilli that may be important to environmental health will continue. A search for high-toxin yielding strains will be made to facilitate isolation of quantities of toxins necessary for mammalian toxicity studies. Long-term, low-dosage effects of the toxins in test animals will be emphasized. Where possible, natural epidemics of dying livestock will be investigated for potential involvement of new mycotoxins or occurrences of those which are already known.

PUBLICATIONS

Pero, R. W., Owens, R. G., Dale, S. W., and Harvan, D. J.: Isolation and identification of a new toxin, altenuene, from the fungus alternaria tenuis. Biochemica et Biophysica Acta 230: 170-179, 1971

Pero, R. W., Owens, R. G., and Harvan, D.: Gas and Thin-layer chromatographic methods for the analysis of the mycotoxins altenuene, alternariol, and alternariol monomethyl ether. [In Press].

Pero, R. W. and Owens, R. G.: A simple micromethod for detecting antifungal activity. Applied Microbiology 21: 546-547, No. 3, 1971.

Spalding, J. W., Pero, R. W., and Owens, R. G.: Inhibition of the G_2 phase of the mammalian cell cycle by the mycotoxin, alternariol. J. Cell Biol. 47: 199a, 1970.

Pero, R. W. and Owens, R. G.: A micro-technique for evaluation of antifungal activity [Abstract] Phytopathology 61: 132, 1971.

Pero, R. W., Owens, R. G., Dale, S. W., and Harvan, D. J.: Isolation and identification of a new toxin, altenuene, from the fungus alternaria tenuis. ACS Southeast-Southwest Regional Meeting [Abstract] 122: 37-38, 1971.

Serial No.: NIEHS-CB-035
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Methodology for toxins isolated from toxigenic fungi"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Ronald W. Pero, Ph.D. and Robert G. Owens, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

TOTAL:	0.5
PROFESSIONAL:	0.3
OTHER:	0.2

PROJECT DESCRIPTION

OBJECTIVES: Qualitative and quantitative analytical methods are essential for detailed investigations of mycotoxicological problems. The objective of this study is to develop reliable sensitive methods for the detection of mycotoxins in naturally moldly agricultural commodities, and in mammalian tissues and cells.

METHODS EMPLOYED: Gas-liquid chromatography was evaluated for quantitative analysis of the mycotoxins, alternariol (AOH), alternariol monomethyl ether (AME), altenuene, and penicillic acid. Satisfactory GLC methods have been developed for patulin, terrein and kojic acid, and the feasibility of extention of these methods for simultaneous detection of all the toxins in mixtures was investigated. Liquid phases tested included: 3% OV-17, 3% OV-11, 3% Dexil 300, and 3% OV-101. The support was Gas-Chrom Q in all cases. The toxins were analyzed as trimethysilyl (TMS) derivatives prepared by reaction with N,O-bistrimethylsilyl acetamide (BSA) and trimethylchlorosilane (TMCS) in pyridine (3:1:9 v/v/v). Several non-toxic metabolites, e.g., succinic acid, stearic acid, palmitic acid, mannitol, and mesoerythritol, have been identified as common components of fungi and were also chromatographed as TMS derivatives to determine if they might interfere in toxin determinations in crude fungal or food product extracts.

MAJOR FINDINGS: Satisfactory GLC parameters have been worked out for simultaneous separation and quantitation of AOH, AME, Alt. and penicillic acid. AOH, AME, and Alt. were well resolved on 3% OV-17, 3% OV-25, 3% OV-11, and 3% OV-101. 3% OV-17 was the liquid phase of choice since it was the most stable with crude extracts of moldy rice. Sensitivity was 0.1 µg for each toxin. Penicillic acid chromatographed well on all the liquid phases tested

both silylated and unsilylated. Detection limits on 3% OV-17 were about 0.05 µg as the TMS derivative or 1 µg as underivatized penicillic acid. 3% OV-101 seemed the best phase for separating penicillic acid from components of a crude corn extract obtained from moldy silage. When the 7 mycotoxins were chromatographed with the 5 non-toxic fungal metabolites, difficulty was experienced in resolution of all components with some of the phases. However, all were completely separated by use of 3% OV-17. Satisfactory resolution of all metabolites except two (mesoerythitol and patulin) was accomplished with 3% OV-101 and 3% OV-11 (kojic acid and terrein). In crude corn extracts spiked with 1 µg of each of the fungal metabolites, resolution and quantitation was best achieved with, 3% OV-11. However, kojic acid was not separated from terrein even with this phase. Interference from components in the corn extract limited the usefulness of the OV-17 phase. These results indicate GLC is practical for simultaneous resolution and quantitation of at least the 12 fungal metabolites employed in these studies.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Contamination of food supplies by toxigenic fungi is widespread and evaluation of these fungi as health hazards is dependent upon adequate analytical methodology for the toxins they produce. With modern agricultural practices, acute levels of mycotoxins in the food chain occur only under unusual circumstances, but low levels may be present consistently in some products. Therefore, very sensitive quantitative methods are necessary for detection of trace amounts, as in the case of pesticides, in order to monitor and evaluate human and livestock intake of the toxins. Since fungi usually occur in nature as mixed populations, it is important also to develop methodology for simultaneous detection of numerous toxins produced by several fungi growing simultaneous in the same products.

PROPOSED COURSE: Efforts will continue toward development of sensitive quantitative methodology for toxins in cases where satisfactory methods are not now available. Efforts will be made to develop still more sensitive GLC methods by application of electron capture detection with Chloro-TMS derivatives. Application of GLC-MS techniques will be explored. Development of methods for simultaneous detection of mycotoxins in variety of foodstuffs will continue.

PUBLICATIONS

Pero, R. W., Owens, R. G. and Harvan, D.: Gas and thin-layer chromatographic methods for the mycotoxins, altenuene, alternariol and alternariol monomethyl ether. Analytical Biochemistry. (In Press)

Lucas, G. B., Pero, R. W., Snow, J. P., and Harvan, D.: Analysis of tobacco for the presence of the Alternaria toxins alternariol and alternariol monomethyl ether. Journal of Food and Agr. Chem. (In Press)

Serial No.: NIEHS-CB-036
1. Cell Biology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Synergism between Mycotoxins Per Se and between Mycotoxins and Other Compounds"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Ronald W. Pero, Ph.D. and Robert G. Owens, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.4
Professional:	0.2
Others:	0.2

PROJECT DESCRIPTION

OBJECTIVES: Some earlier tests on mycotoxins, which occur naturally in mixed fungal populations, gave striking evidence of synergism between alternariol and some related compounds. The objectives of this study are (1) to determine the occurrence and extent of synergism between fungal metabolites and between these metabolites and other environmental agents and (2) to determine the biochemical and/or physical basis of synergism.

METHODS EMPLOYED: Bacteria and fungi, which are used in our biological test systems show the greatest degree of synergistic response and will be used as experimental subjects. The mycotoxins used are Alternaria metabolites, altenuene (Alt.) alternariol (AOH), and alternariol monomethyl ether (AME).

Assay discs were impregnated with various known amounts of either Alt., AOH, AME, Alt. + AOH, AOH + AME, Alt. + AME, or Alt. + AOH + AME. They were then incubated for 24 hours on agar plates that have been inoculated with Bacillus mycoides. The various combinations of mycotoxins were added to bacterial broth cultures in these three ways: (1) separately or together on filter paper strips which were allowed to bathe in the culture medium, (2) first dissolved in a solvent and then added to the culture broth, (3) dissolved in an appropriate solvent, added to the tubes and then allowed to coat the tubes with solid by evaporation of the solvent before addition of the culture broth and test organism.

The effect of anaerobic and aerobic respiration on synergism were evaluated by incubating the bacterial culture plates in an atmosphere of CO_2 in a dessicator.

Spores and mycelium of Helminthosporium sativum were also tested for the synergistic response by use of the antifungal technique previously described (Project No. NIEHS-CB-035).

MAJOR FINDINGS: When Alt., AOH or AME were administered separately to assay discs, 500 ug of AOH was required to illicit a toxic response. However, when placed together with AME or Alt. on the assay disc, toxicity was observed with 0.25 ug of each toxin, indicating approximately a 2000-fold potentiation of toxicity. No synergism was observed in the absence of AOH, but either Alt. or AME at 1:1 ratios with AOH produce the effect. If the compounds were dissolved together in DMSO, or if they were added to culture broth simultaneously on separate paper discs, no synergism occurred. It appears, therefore, that drying the toxins together is essential for the response. This was borne out by the fact that synergism occurred if the toxins were merely dissolved together and then evaporated to dryness in a test tube before addition of the bacterial culture test solution. Thus, the data strongly suggests that physical factors may be critical in potentiation in this case.

Synergism was unaffected by incubation of the bacteria in a CO_2 atmosphere.

Synergism between AOH and AME against fungi was also evident but to a lesser degree. Singly, AME was inactive at 500 ug against fungi, and 250 ug of AOH was required for toxicity. However, toxicity was obtained at 62.5 of each of each compound under the conditions described for bacteria.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The phenomenon of synergism, while often observed in the action of drugs and pesticides, is not completely understood. Synergism between toxins, both synthetic and natural, can greatly increase long and short-term hazards of such materials. In some cases, it can render otherwise relatively innocuous materials hazardous, as in the case of the AOH discussed above. In the case of highly potent toxins, where the LD_{50} is of the order of 0.5 to mg/Kg., as in the case of patulin and a number of other natural and synthetic toxins, it is not unrealistic to expect to obtain chronic toxicity in nanogram or even picogram quantities where maximum degrees of interaction (synergism) occurs with other agents.

PROPOSED COURSE: Physical interactions between AOH, AME, and Alt. will be studied using IR, NMR and UV spectral analyses. C^{14} and H^3-labeled toxins will be used to study their physical interaction and their rates of penetration into cells, localization in cells and subcellular organelles, and their rates of metabolism. Studies are also being initiated to elucidate their biochemical modes of action separately and in combination.

Serial No.: NIEHS-CB-037
1. Cell Biology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Control of Biosynthesis of the Mycotoxins Altenuene and Alternariol and its Monomethyl Ether"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Ronald W. Pero, Ph.D. and Robert G. Owens, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

TOTAL:	0.5
PROFESSIONAL:	0.2
OTHER:	0.3

PROJECT DESCRIPTION

OBJECTIVES: The biosynthetic pathway for the Alternaria toxins is reported to be by way of a polyketide chain formation which undergoes cyclization and aromatization. Chain formation involves the polycondensation of malonyl-COA units and the participation of one actyl-COA unit. In fact, most of the mycotoxins known to man are produced by this pathway. The objective of this study is to elucidate biochemical control mechanisms which determine alternaria toxin production and, hence, concentrations in naturally moldy foodstuffs.

METHODS EMPLOYED: The test fungus was Alternaria tenuis (M1) which produces altenuene (Alt.), alternariol (AOH) and its monomethyl ether (AME) in 7 days when grown at 27°C on autoclaved rice fortified with Czapek's-Dox broth and yeast extract. Compounds expected to influence toxin production were added to the culture medium. Toxin production was determined by TLC and GLC analysis and compared with normal toxin production in standard culture after 7 days.

MAJOR FINDINGS: Toxin production was not altered by the addition of stearic or palmitic acids to the culture medium. This suggests that toxin formation is not subject to fatty acid feedback control. However, when glutamate at concentrations greater than 0.06 M were added to the medium, AOH, AME, and Alt. synthesis was completely inhibited. Inhibition occurred without alteration of fatty acid synthesis, since the amounts of stearic and palmitic acids in acetone extracts from glutamate treated and non-treated cultures were identical. Aspartate, on the other hand, stimulated toxin production by 50% over the control cultures. These results suggest that control of toxin production is involved with some aspect of metabolism of specific amino acids but not with nitrogen supply per se.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND PROGRAM OF THE INSTITUTE: Knowledge of the mechanism of control of mycotoxins synthesis can be expected to facilitate advances in at least two aspects of mycotoxicology relevant to hazards in food products: (1) identification of the kinds of food products where nutritional factors are favorable for toxin production, and (2) control of environmental and nutritional factors to minimize both fungal growth and toxin production.

PROPOSED COURSE: Efforts will continue to determine the basic mechanisms of glutamate inhibition and aspartate stimulation of toxin production and the control mechanisms in both cases. In addition, these effects will be studied in relation to environmental parameters as temperature, nutrition, oxygen tension, cohabitant organisms, etc. Other metabolites such as the Krebscycle acids and amino acid, and vitamins and cofactors will be evaluated for their effects on toxin production and as possible indicators of metabolic steps and/or pathways involved.

PATHOLOGIC PHYSIOLOGY BRANCH

PATHOLOGIC PHYSIOLOGY BRANCH
Summary Statement

The Pathologic Physiology Branch is concerned with the identification of environmental toxicants and the elucidation of their toxic manifestations and mechanisms at the organ, cellular, and subcellular levels. Correlations are being sought between toxicant induced changes in mammalian structure, as visualized by optical and electron microscopy, biochemistry and physiology. A major non-investigational, intramural role of the Branch is to provide pathologic, histologic, and photographic services to the Institute.

RESEARCH MISSION

A breeding colony of opossums with litter production adequate to support the work of the Cellular Control and Morphogenesis Section has now been established. At the mid point in the first full scale, full season use of the new opossum facility, the total number of litters born is already equal to that for the season last year. These results support the validity of the principles of opossum husbandry developed in this laboratory and embodied in the facility.

Studies with the objective of increasing opossum reproductive rate have revealed that factors which decrease reproductive efficiency are operative either at the time of mating (loss of synchrony between ovulation and estrus) or in the period post conception. These problems may be reduced by more stringent selection and culling of breeding stock.

The first phase in the development of a domestic opossum strain has been completed with the birth of several litters to a nucleus of animals born in the wild but raised in captivity. Breeding those animals born in captivity this year will hopefully result in the initiation of an inbred strain of laboratory opossums.

Further studies of cretinism in the opossum have been completed. In this model, which was developed in this laboratory, direct evaluation of thyroid pituitary functional development is possible in the absence of maternal hormone influence. The latest studies have revealed unique intracellular changes in the pituitary of animals which otherwise show typical alterations of the cretinoid state. The pituitary changes which include the intercellular accumulation of blood in a morphological setting compatible with TSH (thyroid-stimulating hormone) hypersecretion may provide a better insight into the normal mammalian pituitary secretory mechanism.

Additional experiments on iodide induced thyroiditis in the opossum have shown that the opossum, like a small portion of the human population, does not escape from the Wolf-Chaikoff effect and develops a goiter after prolonged potassium-iodide ingestion. In this regard, the animal appears to be the

only known model for this puzzling condition in man. It has also been found that the thyroid lesion in iodide-dosed animals is often associated with liver and gall bladder lesions of an as yet unknown etiology.

Related collaborative studies have revealed the lack of a major pre-albumin carrier for T_4 in the opossum. This is in contrast to the human and kangaroo which have distinct pre-albumin carriers of T_4. A polymorphism in the post-albumin binding proteins for T_4 was also found in a small number of animals; polymorphism has been reported to date only for the pre-albumin zone in the Rhesus monkey.

In a preliminary experiment to evaluate the susceptibility of the opossum neonate to tumor induction by ethyl nitrosourea, animals treated at birth developed micro tumors of the brain and liver and adenomas of the lung within three months of exposure suggesting that the opossum may be of value as a model in the study of carcinogenesis. In addition, the finding of a hamartoma-like lesion in the lung of one animal and renal cysts in association with brain lesions in a high percentage of animals implies that the young opossum may be a useful model in efforts to understand the association between congenital malformations and cancer. A large scale study now underway will explore the effect of age and sex on tumor type and incidence and the persistence of embryonic antigens in tumor bearing animals.

Further work on a seizure state induced in the opossum by a cholesterol biosynthesis inhibitor has revealed an apparent structure-activity relationship between the sterol configuration of the drug and seizure activity; seizures were not induced under comparable conditions with two cholesterol inhibitors without the androgen-like structure. Related studies underway suggest that age may be a factor in susceptibility to seizures induced by this drug and that a high cholesterol diet has no suppressive effect on seizure rate.

Repetition of the estrogen toxicity studies on a larger scale indicates that duodenal ulceration and liver damage are the major lesions produced in the opossum by high doses of estradiol. Preliminary studies show that the opossum differs from the rat and the human in that there is no affect of estrogen administration on bile excretion. This apparent difference may provide an explanation for the uniquely high susceptibility of the animal to toxic effects of estrogen.

Studies to explore the value of the developing opossum neonate as a model for respiratory toxicity have been initiated. The absence of true alveoli for approximately 70 days may make the young opossum of value in the evaluation of the effect of toxins on alveolar development and surfactant formation.

Similar base line studies on thyroid-pituitary morphogenesis are in progress. The post partum delay in functional maturation of the thyroid and pituitary of the opossum and the opportunity for direct manipulation of these organs during this period may make the opossum of value in studying the effects of alterations in these endocrine organs on morphogenesis and disease of environmental origin.

The problem of methyl mercury toxicity in mammalian systems received emphasis during this fiscal year. Mammalian pathology in kidney, liver, and the central nervous system specifically have been the focus of attention. Many of the findings confirm earlier reports on human toxicity, but new observations following acute and chronic exposure experiments have also been made. After exposure to 10 mg/kg of methyl mercury hydroxide given subcutaneously over a two-week period, necrosis of Bowman's capsule and of the glomeruli was observed in the kidney. In the central nervous system marked disturbances in sensory nerve conduction were observed, a condition that is reversible and presents one of the early diagnostic signs for organomercurial poisoning. Other organs were also found to be adversely affected by methyl mercury: Involution and fibrosis of the spleen and demyelinization of large peripheral nerve systems.

Biochemical studies on the subcellular level showed decreased activity of mixed function oxidases in the liver, and a decrease in glucuronyl transferases. These changes were observed before any clinical signs of methyl mercury poisoning were noted. Polyribosomal profiles showed alterations in liver and kidney in all alkyl mercury intoxicated animals. Future work will be focusing on correlation of biochemical changes with pathologic observations and clinical findings.

SERVICE MISSION

The Pathologic Physiology Branch plays a major service role at the Institute by providing histopathological and photographic support to individual scientists.

Optical Histology Laboratory

The histology laboratory aids in necropsy, prepares tissue for light microscopy and autoradiography. During the past fiscal year, the laboratory has processed and sectioned 12,743 blocks of tissue to yield 14,824 H&E stains and 289 autoradiograms. Interpretation of patho-anatomic tissue changes is provided by Dr. Bill Bullock, consultant to the Branch on routine histopathology.

Electron Microscopy

The electron microscopy laboratory provides ultrastructural support to the Institute. Tissues submitted are processed and sectioned and areas of interest are photographed for interpretation. During the past year the laboratory has processed and embedded 2,364 blocks of tissue to yield 654 thick sections and 473 thin sections. Services performed on the Philips electron microscope included 1,004 photographs for interpretation.

Photography Laboratory

The photography laboratory is responsible for all photographic services to the Institute including general photography and photomicrotomy. In addition to special projects, the laboratory has prepared 1,004 electron microscopic plates, 2,166 black and white transparencies, 1,232 color transparencies, 4,330 black and white prints, and 81 color prints during the past fiscal year.

Serial No.: NIEHS-PP-001
1. Pathologic Physiology Branch
2. Cellular Control and Morphogenesis Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "A Chemical and Morphological Study of the Neuropathy Induced by Anticholinesterase Pesticides"

PREVIOUS SERIAL NUMBER: NIEHS-PP-101

PRINCIPAL INVESTIGATOR: William Jurgelsky, Jr., Ph.D, M.D., William Brandt, M.D.

OTHER INVESTIGATORS: B. C. Bullock, D.V.M., F. Talley, and P. Hudson

COOPERATING UNITS: Bowman Gray School of Medicine
Department of Veterinary Medicine

MAN YEARS:

Total:	.8
Professional:	.2
Other:	.6

PROJECT DESCRIPTION

OBJECTIVES: The toxic manifestations of the majority of pesticides that inhibit acetylcholinesterase activity can be attributed to the accumulation of acetylcholine without associated morphologic change. However, it has long been known that the acute exposure of man and some species of animals to certain few of these agents produces, in addition to cholinergic effects a delayed and irreversible myelinopathy associated with a peripheral neuropathy. Those compounds with "demyelinating" potential are not distinguished by chemical structure nor can they be conclusively identified by biological test. The mechanism of the myelin breakdown is not known; whether the primary lesion is in the axon or the myelin has not been conclusively established. Recent work by Johnson, et al (Biochem. J. 114:711, 1969) implies that these compounds may act by inhibiting an esterase in the central nervous system. Another area which has not been explored is the chronic effect of these agents on the fetus and myelinating animal. Studies under way attempt to identify the primary neural lesion and to define the mechanism of nerve damage.

METHODS EMPLOYED: The neonatal marsupial opossum, dog, chicken, and the squirrel monkey are being used as models. The opossum neonate lends itself to this work because its development is characterized by a 14-day latent period between birth and myelination. Techniques of differential enzyme

inhibition, optical microscopy, electron microscopy, autoradiography, liquid scintillation, spectroscopy, ultra centrifugation, and gas chromatography are in use.

MAJOR FINDINGS: An initial attempt to identify the enzyme whose inhibition is responsible for the organophosphate-induced neuropathy by *in vivo* treatment with non-neurotoxic and radiolabeled neurotoxic organophosphates has revealed that this approach is not feasible.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The increasing levels of acute and chronic exposure of the general population to diverse cholinesterase inhibitors in the form of pesticides and petroleum additives and the relatively high incidence of poisonings attributed to these agents in children make imperative the acquisition of information on the neurotoxicity and mode of action of these pesticides. The curtailment in use of the chlorinated hydrocarbon pesticides will further increase usage and hence exposure to these agents. Knowledge of the mechanism of organophosphate-induced neuropathy may lead, minimally, to better criteria for identification of these agents, and, optimally, to an improved understanding of the toxic action of the cholinesterase inhibitors. A less direct, possible benefit of such studies is a better understanding of human neuropathies resulting from dysmyelination and demyelination.

PROPOSED COURSE: Dr. Brandt's transfer to the Cell Biology Branch in order to obtain a back ground in DNA studies has necessitated temporary discontinuance of this work until such time as a replacement for him can be found.

Serial No.: NIEHS-PP-002
1. Pathologic Physiology Branch
2. Cellular Control and Morphogenesis Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Effect of Excess Iodine and Iodine Deficiency on the Endocrine System of the Opossum (Didelphys marsupialis virginiana Kerr)"

PREVIOUS SERIAL NUMBER: NIEHS-PP-102

PRINCIPAL INVESTIGATOR: William Jurgelsky, Jr., Ph.D., M.D., William Brandt, M.D.

OTHER INVESTIGATORS: Paul J. Davis, M.D., Fred A. Talley, Patricia Thacker, and Pearlie Hudson

COOPERATING UNIT: Endocrinology Division, Baltimore City Hospitals, Baltimore, Maryland

MAN YEARS:

Total:	.5
Professional:	.4
Other:	.1

PROJECT DESCRIPTION

OBJECTIVES: Work performed elsewhere by members of this laboratory has revealed that the adult opossum develops a flagrant intrafollicular thyroiditis in response to the administration of potassium iodide at levels within the therapeutic range of humans. The implication from these studies, as well as from those of other workers who have demonstrated a low PBI in the opossum as well as a low thyroxine secretion rate, is that the marsupial has an iodine transport and storage mechanism that differs markedly from that of other mammals and is thus worthy of evaluation as a potential model for studies of the role of iodine in the endocrine system. The objective of these studies is to define the structural and chemical changes that take place within the hypothalamic-pituitary-thyroid axis of the opossum in response to iodine excess and iodine deficiency.

METHODS EMPLOYED: Through the use of column chromatography, thin-layer chromatography and scintillation spectroscopy, in vivo and in vitro radioiodine metabolism is being studied in adult opossums treated with potassium iodide. Histological changes are being documented by light and electron microscopy.

MAJOR FINDINGS: A definitive study of iodide-induced thyroiditis in the adult opossum is nearing completion. Findings to date are: (1) Opossums develop goiter when given 20 mg/kg of KI for one month. This dose has only transient effects on all other species tested. (2) Unlike other species, opossums do not escape from the Wolf Chaikoff effect; i.e., organic binding of iodide remains markedly suppressed. (3) In addition to the thyroiditis a hepatitis and cholescystitis develops in about 20% of exposed animals; at higher doses, all animals develop the lesions.

These results imply that iodide metabolism in the opossum differs from that in other mammals thus far investigated. The response of the thyroid to KI is reminiscent of that in the small proportion of the human population which develop goiter on prolonged KI ingestion (at comparable doses) for therapeutic purposes. To date there has been no animal model available for this puzzling human abnormality.

The liver and gall bladder lesions cannot be explained as yet; no similar lesons in response to KI have been reported in other species.

Collaborative studies have revealed the absence of a pre-albumin zone of radiothyroxine binding in the adult opossum. A polymorphism in the post-albumin binding proteins for T4 was found in one animal; the only other instance known of thyroxine binding protein polymorphism is in the pre-albumin of the Rhesus monkey. Screening of a larger population for this unique situation will be undertaken.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The intricacies of the role of iodine in the mammalian endocrine system are largely unknown. Such knowledge is essential to an understanding of many endocrinopathies, including thyroiditis, hyperthyroidism, and hypothroidism and for evaluation of the possible role of environmental contaminants in these pathologic processes. Study of iodine metabolism in an animal such as the opossum in which response to iodine appears anomalous could yield insight into mechanisms of iodination, iodine transport, and iodine storage.

PROPOSED COURSE: The thyroid, liver and gall bladder lesions are being studied at the morphological and metabolic (I^{131} distribution) level. The effect of thyroidectomy on the development of the liver and gall bladder lesions will be evaluated. The polymorphism of T4 binding will be explored on a collaborative basis in a larger opossum population.

Serial No.: NIEHS-PP-003
1. Pathologic Physiology Branch
2. Cellular Control and Morphogenesis Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Chemical and Morphologic Studies of the Role of Lipid Metabolism in CNS Irritability"

PREVIOUS SERIAL NUMBER: NIEHS-PP-103

PRINCIPAL INVESTIGATOR: William Jurgelsky, Jr., Ph.D., M.D., William Brandt, M.D.

OTHER INVESTIGATORS: Pearlie M. Hudson, Fred A. Talley and Patricia Thacker

COOPERATING UNITS: EEG Branch, NINDS

MAN YEARS

Total:	1.4
Professional:	0.5
Other:	0.9

PROJECT DESCRIPTION

OBJECTIVES: Disorders of steriod metabolism have been implicated among the causes of epilepsy. Previous studies, performed elsewhere by members of this laboratory, have established that an androgen-related, cholesterol-biosynthesis inhibitor, when administered once weekly to young marsupials (*Didelphys marsupialis virginiana Kerr*) in doses well below the toxic level, will induce a chronic major motor seizure state. Seizures may be induced at will by tactile stimulation. The opossum alone, among those standard laboratory species thus far tested, develops CNS hyperirritability in response to this drug. A number of organic phosphates and chlorinated hydrocarbon pesticides, which are known to influence steriod metabolism, also produce seizures at high levels of exposure. In the proposed studies, alterations in lipid metabolism produced by this drug and other agents, such as pesticides with seizure-producing potential, will be correlated with changes in CNS irritability.

METHODS EMPLOYED: Optical microscopy, electron microscopy, thin-layer chromatography, electroencephalography, ultra centrifugation, gas chromatography and scintillation spectroscopy are being used. Comparative studies are being performed in rats and opossums.

MAJOR FINDINGS: Comparative studies with two cholesterol biosynthesis inhibitors without sterol configurations have been negative implying that there may be a specificity for the sterol structure in the induction of

seizures in the opossum. Lipid analyses on neural tissues from these animals, which may provide an insight into the seizure mechanism, are not yet available.

An attempt to induce seizures in fully adult animals with the androgen-related drug has been completely negative during the usual period to induction. These results suggest that age may play a role in the development of the unknown lesion(s) which underlie the induced neural hyperirritability state.

A preliminary study has revealed that a high cholesterol diet has no suppressive effect on the established seizures. This is in contrast to the ameliorative effect of such diets on cholesterol biosynthesis inhibitor induced myotonia in rats.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The induction of seizures in the marsupial by administration of an inhibitor of cholesterol biosynthesis represents a previously unreported simply reproducible model for experimental epilepsy. The apparent direct implication of disordered steroid biosynthesis in the seizure state in these animals may offer a new window on neural irritability in general, and on epilepsy in particular.

PROPOSED COURSE: Lipid analysis of brain and brain subcellular fractions from animals with the fully developed seizure syndrome will continue in an attempt to explain the basis for the nervous system hyperirritability.

Characterization of the electroencephalographic pattern of animals with induced seizures will be done on a collaborative basis with the EEG Branch, NINDS.

The sterol turnover time will be determined in normal animals and animals with cholesterol biosynthesis inhibition and in animals from which the drug has been withdrawn to further explore the correlation between cholesterol-desmosterol alterations and CNS hyperirritability.

Serial No.: NIEHS-PP-004
1. Pathologic Physiology Branch
2. Cellular Control and Morphogenesis Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Marsupial Neonate as a Model for the Identification and Evaluation of Environmental Toxicants"

PREVIOUS SERIAL NUMBER: NIEHS-PP-104

PRINCIPAL INVESTIGATOR: William Jurgelsky, Jr., Ph.D., M.D.

OTHER INVESTIGATORS: Fred A. Talley, Patricia Thacker, and Pearlie M. Hudson

COOPERATING UNIT: Animal Science and Technology Branch

MAN YEARS:

Total:	1.2
Professional:	0.2
Other:	1.0

PROJECT DESCRIPTION

OBJECTIVES: The inframammalian position of the marsupial in the evolutionary scale endows the animals with characteristics not present in any other mammal. Some of these attributes are: (1) Gestation period of 12-3/4 days, (2) No placenta, (3) Intra-pouch development as an extension of intrauterine development, permitting experimental manipulation of young at birth at a stage of development comparable to that in utero in other mammals; i.e., equivalent to a four-week-old human and an eleven-day-old rat in utero, (4) Markedly slow development of the nervous and respiratory systems and hind limbs, (5) "Simplified" pituitary, thyroid, and brain, (6) Anomalous iodine transport and storage, (7) Extreme tolerance of developing young to high CO_2 levels, and (8) Marked growth rate during first year with a newborn to adult weight ratio of 1:40,000 (compared to 1:40 for the rat). These characteristics, many of which would appear to provide the basis for unique model systems for the study of environmental hazards, have not been exploited experimentally. With this objective, a colony of indigenous opossum (Didelphys marsupialis virginiana Kerr) has been established; and a preliminary evaluation of response of the neonatal opossum to selected toxicants is under way.

METHODS EMPLOYED: Special pharmacological techniques, light and electron microscopy, ultra centrifugation, thin-layer chromatography, and gas chromatography are being used.

MAJOR FINDINGS: The first full scale, full season usage of the semi outdoor opossum facility is now underway. The reproductive rate at mid-season is about 80% (49 litters from 59 females) in animals bred on the basis of changes in vaginal cytology and about 50% (19 litters from 35 females) in those bred without knowledge of the stage of the estrus cycle. The reproductive rate in breeding stock held over from last year is about 10% (2 litters from 18 females). The total number of litters (70) produced thus far at mid-season is equal to the total number produced during the entire past season. Thus a successful breeding colony of opossums maintained under controlled conditions with output of young adequate to meet experimental needs has been established at the Institute. A description of the husbandry, breeding and experimental techniques developed in this laboratory for the opossum and the results of the past two years operation of the colony will be submitted for publication at the end of the breeding season this year.

In continuing studies to improve the opossum reproductive rate, the complete estrus cycle has been followed at least once in 119 females. The results of this survey indicate that captivity under conditions in the new facility does not adversely influence initiation or maintenance of the estrus cycle in the female. However, the fact that coitus as indicated by the presence of sperm in the vaginal vault is correlated with successful pregnancy in only a moderate number of cases examined implies either a loss of synchrony between estrus and ovulation or the operation of unknown factors in the post conception period which are detrimental to establishment or maintenance of pregnancy or to survival of newborn. Future studies will explore these possibilities.

An attempt to develop a closed breeding colony of opossums has been initiated by the Animal Science and Technology Branch at the request of this laboratory. The nucleus of this colony is a group of young born in the wild but raised to maturity in captivity. The 83 young which have now been born to these animals represent the P_1 generation conceived and raised under laboratory conditions. Hopefully, these animals will be bred next year to produce the first inbred group. If a domestic strain of the opossum can be developed, many of the disadvantages of using the opossum in the laboratory which stem from use of wild-caught animals will be eliminated.

The technique developed in the laboratory for administration of test materials to neonatal opossums has been modified to permit more efficient intubation of neonates after three weeks of age. This technique has greatly reduced the time and effort involved in exposing animals on chronic studies to test materials.

Work has continued on the model for cretinism developed last year by treating opossums from birth with a herbicide (amino triazole) with propylthiouracil action. Repetition of this work on a larger scale has revealed that the typical cretinoid changes in behavior, growth and thyroid morphology and physiology (I^{131} uptake) are associated with a hitherto unreported hemorrhagic lesion in the pituitary. This condition, which consists of massive intercellular localization of blood, is seen in animals as early as two months after initiation of treatment and may be related along with several other intercellular alterations to a hypersecretory state for TSH (thyroid-stimulating hormone). The pituitary changes in the amitrol treated animals may provide new insights into the mechanism of TSH secretion in the mammal. An attempt

has been initiated to duplicate the pituitary lesion with radiothyroidectomy in order to determine the role of the goitrogen in the pituitary response. Possible neural changes in the cretinous animals are currently under investigation.

A preliminary investigation of the susceptability of the newborn opossum to tumor induction by ethyl nitrosourea has shown that the opossum like the rat develops brain tumors (mostly mixed, unclassified types) as early as three months after treatment. However, unlike the rat, the opossum also develops pulmonary adenomas and hepatomas. Of most interest, however, is the association between cortical cysts of the kidney and brain lesions in a high proportion of the animals and the presence in one animal of a pulmonary hamartoma associated with an adenoma. These latter findings imply that the neonatal opossum, because of its peculiar lack of uniformity in organ maturity at birth, may be a useful model for studies of the apparent assoiation between cancer and congenital malformations--a relationship for which an animal model does not exist.

Base-line studies of lung and thyroid-pituitary axis morphogenesis in the neonatal opossum have been initiated. The lung of the developing opossum is of interest because respiration in this animal is apparently at the level of the terminal bronchiole for approximately 70 days post partum. Since the time of appearance of surfactant and of functional alveolar components is not known, base-line information on lung development is a prerequisite for possible use of the animal as a model in studies of atmospheric pollutants on the developing mammalian lung.

In the opossum neonate the pituitary-thyroid axis is apparently non-functional for about one week post partum and can be manipulated directly without maternal intervention. Base-line information on these endocrine organs under the unique conditions present in the developing opossum is of interest in view of the major roles of the thyroid and pituitary in mammalian morphogenesis and in many disease states.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The marsupial neonate is in many respects a fetus that completes the final portion of its development in an extra-uterine environment. Only in the marsupial is it possible to expose embryonic tissue in vivo to toxic insult without maternal metabolic or placental intervention and without effect of the mother's own metabolism or the trauma of caesarian section. A systematic survey of the response of the marsupial to drugs and toxins with known pharmacologic or carcinogenic activity is not available. Such a study should indicate the value of the newborn marsupial for neonatal toxicology and experimental carcinogenesis and may yield new insight into the response of embryonic tissue to toxic insult. Preliminary findings indicate that the animal may have unique value as a model for studying mechanisms of carcinogenesis and endocrine development.

PROPOSED COURSE: Evaluation of the response of the opossum neonate at the chemical and morphologic level to toxins of known pharmacologic and/or carcinogenic potential will be continued with emphasis on carcinogens.

Attempts will be made to further define the mechanism in the herbicide-induced cretinism and associated pituitary lesions.

Experiments whose objectives are to obtain base-line information on morphogenesis of thyroid and lung have been initiated.

Studies in an attempt to improve breeding efficiency of the opossum will continue.

An attempt will be made this year to extend the six-month opossum breeding season by artificial extension of photoperiod. Early experiments in the literature imply that photoperiod is a prime factor in initiation and termination of the reproductive cycle in this species.

PUBLICATIONS

Jurgelsky, William, Jr.: Administration of test materials to the neonatal North American opossum (Didelphys marsupials virginiana Kerr). Laboratory Animal Science. (In Press)

Serial No.: NIEHS-PP-005
1. Pathologic Physiology Branch
2. Cellular Control and Morphogenesis Section
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Toxicity of Sex Hormones to the Opossum (Didelphys marsupialis virginiana Kerr)"

PREVIOUS SERIAL NUMBER: NIEHS-PP-105

PRINCIPAL INVESTIGATOR: William Jurgelsky, Jr., Ph.D., M.D.

OTHER INVESTIGATORS: Clyde W. Boyd, Fred A. Talley

COOPERATING UNIT: Pathology Department, Veterans Administration Hospital, Durham, North Carolina

MAN YEARS:

Total:	.50
Professional:	.25
Other:	.25

PROJECT DESCRIPTION

OBJECTIVES: Attempts to improve reproduction performance in the female opossum through the use of serum and pituitary gonadotropins to induce estrus, and estrogen and progesterone to insure receptivity to the male have resulted unexpectedly in a syndrome characterized by anorexia, elevated blood urea nitrogen, elevated serum potassium, gastric ulcers, and death within three weeks post treatment. Preliminary work indicates that the syndrome can be reproduced with estrogen alone. The response of the opossum to estrogen is apparently unique among mammalian species and implies either a supersensitivity or an inefficient or inadequate detoxification mechanism. The objective of this study is to define the mechanism of estrogen induced injury in the opossum.

METHODS EMPLOYED: Histologic study at the light and electron microscopic levels; clinical chemistry; hypophysectomy, oophorectomy, and adrenalectomy are being employed.

MAJOR FINDINGS: Attempts to explain the lethal effect on the opossum of levels of estrogen which produce only a decreased bile excretion and BSP elevation in other species including man, have revealed that BSP excretion in the opossum is unimpaired. This finding implies that estrogen metabolism and excretion in the opossum differs from that in other species studied to date and that these differences may be responsible for the low tolerance of the opossum to estrogen.

More definitive toxicity studies have confirmed the initial clinical findings with the exception that the loss of kidney function is not consistent. A more consistent effect of estrogen treatment is hepatic necrosis which may be a major factor in the cause of death.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Elucidation of the toxic and physiologic mechanisms of action of mammalian hormones is at an early stage. Further knowledge of the physiology and pathophysiology of the estrogens is particularly crucial in view of the large segment of the human female population currently exposed to estrogen derivatives chronically for contraceptive pruposes and acutely for treatment of infertility and menstrual dysfunction. A study of the atypcial response of the opossum to estrogen may give insight into both normal and abnormal estrogen metabolism and function in eutherian mammals.

PROPOSED COURSE: The metabolism of estrogen in the opossum will be studied using isotope techniques. An attempt will be made to reproduce the syndrome in the absence of several possible target organs, i.e., pituitary, adrenals, and ovaries.

Serial No.: NIEHS-PP-006
1. Pathologic Physiology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Pathology of Acute Methyl Mercury Intoxication in Rats"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Ronald Klein, M.D., and Sheldon Herman, M.D.

OTHER INVESTIGATORS: Martin Krigman, M.D., Consultant, University of North Carolina at Chapel Hill, Department of Pathology

COOPERATING UNITS: None

MAN YEARS:

Total	.3
Professional:	.3
Other:	.0

PROJECT DESCRIPTION

OBJECTIVE: A general study of gross and light microscopic pathology resulting from administration of methyl mercury hydroxide is basic for further investigations into alkyl mercurial toxicity.

METHODS EMPLOYED: Acute Minamata Disease was produced in adult male rats dosed with methyl mercury hydroxide, 10 mg/kg subcutaneously. All animals were sacrificed 15 days after the first dose.

MAJOR FINDINGS: Most of the pathologic findings confirm those already extensively reported in the literature. These include early CNS neuronal degenerative changes and slight gliosis, marked renal tubular destruction and degeneration, peripheral nerve swelling and inflammation, and normal appearing liver and muscle. New findings have been made including marked involution and fibrosis of the spleen, anterior horn cell degeneration, and segmental demyelinization of large peripheral nerve fibers (via teasing technique). In addition, hematrocrit and serum blood urea nitrogen and creatinine were measured on day 0 and day 14. Treated rats showed modest elevations of BUN and creatinine with stable hematocrit.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This project served as a springboard for further investigations into the pathophysiology of methyl mercury toxicity. We are presently involved in a combined histologic and electrophysiologic analysis of neuropathy seen in

these animals. A second experiment on long-term effects of methyl mercury on renal histology and function is in progress. Serum has been collected on treated animals for liver function tests. These results are not yet available.

PROPOSED COURSE: The plans are to complete present neurophysiologic analysis of methyl mercury neuropathy; analyze anterior horn cell function via neuropuncture electrode technique for transmembrane potentials; undertake therapeutic trials of several agents including multiple vitamins and aldactone for efficacy in methyl mercury intoxication, both acute and chronic; and establish a small colony of chronically exposed animals and observe basic clinical and pathologic changes.

Serial No.: NIEHS-PP-007
1. Pathologic Physiology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Neurotoxicity of Methyl Mercury Hydroxide in Adult Male Rats: Pathologic and Physiologic Correlations"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Sheldon Herman, M.D., and Ronald Klein, M.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: George Somjen, M.D., Ph.D., Professor of Physiology, Duke University; and Martin Krigman, M.D., Neuropathologist, University of North Carolina at Chapel Hill School of Medicine

MAN YEARS:

Total:	.6
Professional:	.6
Other:	.0

PROJECT DESCRIPTION

OBJECTIVES: The first objective is to characterize the functional properties of peripheral nerve and muscle of rats manifesting symptoms of subacute methyl mercury intoxication. Functional parameters include electrophysiologic profiles of nerve and nerve root, electromyograms, twitch tension and tetanic tensions of gastrocnemius muscles. The second objective is to describe the histologic changes in corresponding segments of the peripheral nervous system in these same animals. Light microscopy, nerve fiber teasing, histochemistry, and electron microscopy will be employed. The third objective is to correlate alterations in neurophysiology with neuropathology.

MAJOR FINDINGS: Preliminary data indicates marked distrubances in sensory nerve conduction properties. Muscle data has not been analyzed. Histologic review of several sciatic nerves reveals segmental demyelinization of large nerve fibers. Correlation of findings awaits completion of the experiment.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Peripheral neuropathy has been universally associated with the Hunter-Russell syndrome (organomercurialism). Neuropathy appears early in the course of the illness in man. The disease is reversible in rats if (diagnosed and) treated at this early stage. The literature is devoid of reports of neurophysiologic

analysis of this neuropathy. EMG and nerve conduction studies may provide early diagnostic evidence of organomercurialism at a stage when the disease is reversible provided further exposure is prevented.

PROPOSED COURSE: The present study will be completed. Characterization of the time course of physiologic and pathologic changes of peripheral nerves and therapeutic trial of antidotes with observations of their effects on nerve physiology and pathology will be undertaken.

Serial No.: NIEHS-PP-008
1. Pathologic Physiology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Methyl Mercury Hydroxide on Thyroid Function in the Rat"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: William Brandt, M.D., Ronald Klein, M.D., and Sheldon Herman, M.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	.05
Professional:	.05
Other:	.00

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to study thyroid function in rats intoxicated with methyl mercury hydroxide using changes in the total blood I^{131}, radioactive iodine uptake, and protein binding of intrathyroidal I^{131}.

MAJOR FINDINGS: The thyroid-serum I^{131} ratio was decreased in the rats receiving mercury. This change was attributed to the concurrent renal disease. There was no change in the other thyroid function parameters measured above. No gross or histopathological changes were noted.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Earlier studies had shown a correlation between chronic mercurialism and hyperthyroidism. The study was an attempt to find if a similar relationship existed in the rat.

PROPOSED COURSE: Further studies were discontinued since methyl mercury was shown not to affect thyroid function in the rat.

Serial No.: NIEHS-PP-009
1. Pathologic Physiology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Methyl Mercury Hydroxide on the Renal Function in the Adult Male Rat"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Ronald Klein, M.D., and Sheldon Herman, M.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	.5
Professional:	.4
Other:	.1

PROJECT DESCRIPTION

OBJECTIVES: Objectives of this project are to study the effect on renal function of the adult male rat treated with low doses of methyl mercury hydroxide by measuring changes in blood urea nitrogen, serum creatinine, creatinine clearance, and urine analysis; and to correlate these changes in renal function with changes in anatomical structure of kidney using light and electron microscopy.

MAJOR FINDINGS: Histologic sections of kidneys of adult male rats treated with 10 mg/kg methyl mercury hydroxide in subcutaneous doses over a two-week period were studied. Focal tubular degeneration was found in animals sacrificed during the first week of treatment. After two to three weeks, increased blood urea nitrogen was found as were regenerative tubular changes. The kidney sections of animals receiving 10 subcutaneous doses of 2.5 mg/kg over a two-week period showed mild focal tubular degeneration as well as reactive mesangial proliferation after one week of treatment. An interesting lesion has been noted in these animals when sacrificed 60 days after administration of the first dose. They developed necrosis of Bowman's capsule, evagination of the proximal tubules into the glomeruli and necrosis of the glomeruli. This lesion has not previously been reported in alkyl mercury intoxication, and its etiology is unknown.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The purpose of these experiments is to further characterize the toxicology of alkyl mercurials. Recent studies have revealed higher than allowable levels of methyl mercury in fish, fowl, foodstuffs, and water. The toxic effects of long-term exposure at these low levels are poorly understood. Most toxicologic studies on these organic mercurials have focused on the central nervous system. There are few studies about renal changes during methyl mercury intoxication.

PROPOSED COURSE: The nephrotoxicity of organomercurials will be further studied at the subcellular level by EM and studies on RNA and protein synthesis.

Serial No.: NIEHS-PP-010
1. Pathologic Physiology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Subcellular Ribosomal Changes in Various Organs in Methyl Mercury Poisoning"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: Ronald Klein, M.D., and Sheldon Herman, M.D.

OTHER INVESTIGATORS: Paul Brubaker, Ph.D., and George Lucier, Ph.D.

COOPERATING UNITS: Cellular Physiology Section, Cell Biology Branch

MAN YEARS:

Total:	.6
Professional:	.5
Other:	.1

PROJECT DESCRIPTION

OBJECTIVES: The objective of this study was to correlate the early gross clinical and pathologic, light microscopic, and EM changes in three organs of the rat--brain, kidney, and liver--with changes in protein synthesis. In addition, the effect of alkyl mercury compound on specific liver microsomal enzymes was studied.

MAJOR FINDINGS: Rats treated with daily doses of 10 mg/kg subcutaneously CH_3HgOH have the following clinical course: (1) Initial weight gain 1 day after administration of the mercurial (greater than experienced in control animals) followed by; (2) 2-5 days of increasing weight not significantly different from controls; (3) 6-12 days--weight loss, an initial clinical symptom; (4) 11-13 days--weakness, followed by ataxia of hind limbs; (5) 14-15 days--crossing of the hind limbs; and (6) 16-18 days--death.

Pathological Findings: Light Microscopic--Kidney: The kidneys of animals sacrificed during the first week showed focal tubular necorsis as well as mesangial proliferative changes. Regenerative changes were noted at the beginning of the second week. Liver: The liver changes remained essentially unremarkable. Central Nervous System: The brains showed slight cerebellar granular cell necrosis and sponginess in the cerebral neocortex. Most of the early change is manifest in section of peripheral nerve where segmental demyelinization and inflammatory response is seen by day seven. In addition, cell death and neuronophagia of the anterior motor horn cells and demyelinization of the dorsal column were noted.

Electron Microscopic--Increased ribosomes and rough endoplasmic reticulum were seen in sections of liver and kidney cells.

Biochemical Findings: The effects of methyl mercury hydroxide on microsomal enzyme activity were studied in the livers of rats treated with methyl mercury hydroxide. At day 2, before any clinical signs developed (during the latent period), decreasing activity of the mixed function oxidase enzymes was noted. A decrease in glucuronyl transferases activity was noted by day 5.

Total protein was decreased in the kidney and liver, but not in the brain of these rats. RNA levels remained unchanged in these organs. Polyribosomal profiles showed alteration in the various ribonucleoprotein particles in the liver and kidneys which correlated well with electron microscopic changes. This data suggests a block in translation step of protein synthesis.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Since the classic paper of Hunter and Russell in 1940, many investigators have described the toxicology of alkyl mercury compounds, particularly during and after the Japanese experience with Minamata Disease. Marked gross pathologic changes have been noted in the central nervous system of organic mercury intoxicated subjects, with lesser changes noted in other organs. At the biochemical level, specific enzyme inhibition was found in the brain of rats poisoned with methyl mercury. Ultrastructural changes in ribosomes appear early in the course of intoxication. Brown showed ribonucleoprotein particle swelling and endoplasmic reticulum dilatation occurring in the brain of baby chickens treated with methyl mercury nitrate. These ribosomal alterations implicate alkyl mercurials as inhibitors of protein synthesis. The object of these experiments was to determine the specific effects of an alkyl mercury compound upon protein synthesis and enzyme activities in order to further elaborate the chronological sequence of subcellular pathophysiology of mercury intoxication. This information is essential for further diagnostic and therapeutic advances.

PROPOSED COURSE: Using radioactive metabolic precursors, the rate of synthesis on both ribosomes and proteins will be studied in alkyl mercury intoxicated rats. An *in vitro* protein synthesizing system will be used to further establish the effects of organic mercury poisoning on protein synthesis. Further studies on rats intoxicated with methyl mercury will focus on the ability of these rats to detoxify various environmental agents, i.e., carbamate pesticides.

PHARMACOLOGY AND TOXICOLOGY BRANCH

PHARMACOLOGY AND TOXICOLOGY BRANCH
Summary Statement

The Pharmacology & Toxicology Branch carries on research in 3 major areas: (1) Developmental and Perinatal Biology, (2) Detoxication Mechanisms, and (3) Aerotoxicology. In all cases the goal is to assess the potential toxic effects of the environment or environmental constituents, or the interactions of these with other materials to which we are exposed, such as drugs. Any effects seen are then studied with regard to their specificity (species, age, etc. of animal), probable occurrence (reasonable concentrations or length of exposure), and mechanisms (especially as to whether this might give clues to modification of effect). For example: programs in developmental and perinatal biology include assessment of teratogenic effects of pesticides and heavy metals in mice and rats; programs on detoxication mechanisms include studies of detoxication systems in lung vs. liver and effects of pesticides and pollutants on these; and programs in aerotoxicology include studies of pesticide localizations in lung tissue and effects of drugs on the toxicity of pollutants administered by breathing. Since last year the major changes in programs involve the new sections on detoxication mechanisms and aerotoxicology. Changes now being implemented will add new programs in developmental biology and aerotoxicology. One major purpose of these new programs is to broaden the base of capability and expertise in the areas of pharmacology and toxicology so that more subtle kinds of toxic actions on heretofore less-studied organ systems can be assessed.

Sometime before the start of the next fiscal year new laboratory facilities will be available to the Branch. These are the rooms for and laboratories associated with the inhalation exposure facility. This facility will allow experiments not possible previously -- the exposure of large numbers of animals to controlled atmospheres of gases, aerosols, etc., or mixtures of these for varying periods of time, and the assessment of the effects of such exposures.

Program

Developmental and Perinatal Biology: The purposes of most of these studies are assessments of the teratogenic potential and effects of various pesticides or herbicides or heavy metals or NTA (nitrilotriacetic acid) or NTA plus heavy metals in rats and mice. Such studies resulted from interest on the part of our investigators plus requests from other agencies and the Scientific Directorates for certain specific pieces of information about effects of these materials in pregnant animals. So far the results indicate possibly important effects by 2,4,5-T and its associated dioxin (TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin), the heavy metals cadmium and mercury and the combination of one or more heavy metals with the detergent "builder" NTA. NTA alone and the polychlorinated biphenyls 1260 and 1254 were studied for effects in pregnant rats and mice but no significant teratologic effects were found. One investigator is studying some aspects of insecticides (especially carbamate pesticides) -- cholinesterase interactions in the fetal rat -- while another is starting some projects on chemical transport or transfer across the placenta. Such studies while not directly related to the assessment of the heavy metals or 2,4,5-T teratology may nevertheless be important in understanding chemical effects on the fetus.

A new program in perinatal biology will soon be initiated to supplement the projects mentioned above. It will be concerned with the study of the effects of chemicals on various processes of pre-natal life starting with fertilization and including implantation, cell division and differentiation and continuing beyond birth to events early in post-uterine life. Special attention will focus on the ability of various tests to detect the first signs of teratologic effect by a chemical--a continuing study of what can best be used to detect such actions by chronic, low-level exposures such as are typical of environmental pollutants. These studies were begun by one of our investigators several years ago, and have resulted in several changes in "accepted" routines used in assessing teratogenic effects of chemicals such as drugs.

Detoxication Mechanisms: These are new programs with several goals. The comparison of detoxication systems in organs other than liver are now being made. One group of scientists is working on detoxication systems in the lung. Methods of preparing these lung systems for study and comparison of these with the hepatic "drug metabolizing" systems in microsomes have already shown many similarities and a few differences. Studies are now beginning on response of lung vs. liver systems to inhibitors and stimulators of interests such as heavy metals, pesticides and polycyclic hydrocarbons. Changes in chemical toxicity as a result of changes in the detoxication of chemicals have been studied with a variety of materials used as drugs, but few studies have been made with other substances or when the detoxication systems are in organs other than liver. The results of these studies will be of particular interest to projects now being started in aerotoxicology where chemicals are administered by the inhalation route. It is believed that the toxicity of several inhaled materials will be markedly altered by changing the activity of lung detoxication mechanisms.

Another group of scientists is studying both liver and lung detoxication systems with respect to changes in these with age and effects of metals on these systems. The goal of these studies is to compare rate-limiting steps of the systems in the 2 organs at different ages and to see how metal ion effects on the systems are similar or different. Metal ions affect the detoxication systems in liver in at least two different ways...both stimulation and inhibition can be seen. Not only these direct effects but metal ion effects on other chemicals which stimulate or inhibit these systems can also be shown. Age-dependent changes in detoxication systems seem to be at least part of the reason for changing actions of chemicals and drugs in newborn vs. adult vs. old animals and man. Many interactions of chemicals or drugs and the effects of environment on chemical actions or toxicity seem to be mediated by changes in detoxication mechanisms--especially the effects of pesticides and air pollutants as inhibitors and stimulators of detoxication systems in the liver. The new studies will also evaluate the role of systems of analogous function in organs like the lung.

Aerotoxicology: One phase of a continuing program devoted to paraquat toxicity concerning the evaluation of paraquat ½ life in various tissues of the rat is now being completed using both chemical and radiologic assays. Such studies will complete the first stages of a project whose ultimate aim is the evaluation of the effects of selected pesticides on lung function. A comparison of paraquat with diquat and dipyridyl may help in understanding the mechanisms of localization and the role of concentration of these basic chemicals in their

toxic actions.

One researcher is involved in studies of systems involved in synthesis of lung phospholipids. These are most important to lung functions and the ultimate aim is to understand the effects of air pollutants on these systems and the mechanisms of modifying these systems either accidentally or on purpose. This program of phospholipid chemistry and enzymology finds interaction with most other groups in the Pharm-Tox Branch. The microsomal phospholipids seem to play a key role in the function of detoxication systems of these microsomes, at least in liver. Changes in hepatic microsomal phospholipid result from several environmental stresses which change chemical toxicities and detoxications.

Finally, some research is being started on the evaluation of the toxicity of chemicals, etc., as applied by the inhalation route. Eventually we will have the ability to expose animals to a variety of vapors, pesticides, aerosols, etc., either singly or in combinations for short or long periods of time. The major interests will focus at first on whether agents administered by the inhalation route can be made more or less toxic by prior exposure of the animals to pesticides, polycyclic hydrocarbons, etc. Later, we hope to study toxicity of "realistic" combinations of chemicals administered by the inhalation route--e.g., pesticides and volatile solvents, or, combinations of inhaled and ingested chemicals. Much of the effort in this project has thus far been devoted to getting the physical facilities for these kinds of experiments ready for use.

Facilities

Aerotoxicology and aerotoxicology research will occupy Building 14. The vapor exposure rooms will be made operational as the various chambers are installed and tested. Original plans called for several sizes and types of chambers so as to give us the capability of several types of exposure to quite different kinds of materials. Even the most toxic materials will be capable of study using the special "live-in" type isolation units. Both whole body and nose-only exposures for less toxic materials can be achieved. Personnel limitations more than equipment limitations presently restrict the kinds and number of experiments possible in the new facility. All people in the laboratory area adjacent to Building 14 will be exposing animals to materials in the inhalation units. A major bottleneck at this beginning seems to be manpower to maintain, monitor and operate the exposure units in the facility--especially since a modified barrier system will be used to minimize cross contamination from one exposure room and animal quarter to another. In the present fiscal year only minimal use of these quarters will be possible--due to the timing (occupancy in April or early May) and especially personnel restrictions. Dr. Drew is in charge of operating the vapor exposure facility. He now has 3 technicians to help him operate exposure units in 10-12 rooms (approximately 400 square feet each and having 2-5 units or chambers per room) and man the laboratories monitoring these rooms...3 labs.

Personnel

Personnel added to the Branch since the last progress report include Dr. Fouts, Branch Chief; Drs. Gram, Bend, Hook, Chhabra and Drew at the professional level; and technical support by Mrs. Pohl, Mrs. Devereux, Mrs. Schroeder and Mr. Riley. Secretarial additions were Mrs. Elliott (full time) and Mrs. Michael (part-time). Just before the end of this year Drs. Elliott and Staples will

join us in Developmental Biology.

Other Activities

Dr. Fouts is an adjunct professor in the Pharmacology Department at UNC, Chapel Hill and has lectured in graduate courses and the medical course in Pharmacology. He is also a member of the Dental Research Center's Scientific Review Committee (UNC, Chapel Hill). He is a member of the editorial boards of Chemico-Biological Interactions and Xenobiotica, and regularly reviews manuscripts for Toxicology and Applied Pharmacology, Science, etc. He is a member of the Education Committee of the Society of Toxicology, the American Society of Pharmacology and Experimental Therapeutics committees on Environmental Pharmacology and the Public Information Committee for the Fifth International Congress on Pharmacology. He is a member of the Committee on Anticonvulsant Drugs of the NINDS. At NIEHS, Dr. Fouts is chairman of the Safety Committee and a member of several other standing committees.

Dr. Gram is a member of the editorial board of Biochemical Pharmacology. Drs. Courtney and Chernoff have participated in numerous meetings, hearings, inter-and intragovernmental conferences on the teratology of 2,4,5-T, dioxins, 2,4 -D, NTA, mercury, etc. These myriad activities will be described in greater detail by the Associate Director for Laboratory Research. Dr. Courtney is also a member of the editorial committee at NIEHS and reviews papers for the NIEHS as well as "outside" journals.

Dr. Drew has devoted considerable time to travels to various installations in the U. S. whose vapor exposure facilities of different types are in operation. The decisions about types and number of exposure units for installation here at NIEHS were made after these visits (6 trips to over 9 installations). Dr. Drew is a member of the Toxicology Subcommittee of the Environmental Health Committee of the Society of Plastics Industries. He is also Radiation Safety Officer at NIEHS.

Serial No.: NIEHS-PT-001
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Enzymes Metabolizing Chemicals: Chemical Effectors of These Systems (Inducers, Inhibitors, Stimulators)"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: James R. Fouts, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	1.0
Professional:	0.2
Other:	0.8

PROJECT DESCRIPTION

OBJECTIVES: This is a general effort directed at classifying and understanding the actions of chemicals on body mechanisms for metabolizing "xenobiotics" and other substances which can be substrates of systems often called "drug metabolizing enzymes". Such enzyme systems can be found in most excretory organs of the body (liver, lung, kidney) and respond to a variety of chemicals to which man and animals are exposed in their environment (pesticides, heavy metals, etc.). Agents that affect these metabolizing systems may act differently at different doses, by different routes of administration or at different times after administration. Combinations of effectors may produce effects quite different from single agents used alone. This study concentrates on metal effects on these metabolizing systems as they are found in liver microsomes and on metal ion-inducer-xenobiotic substrate interactions.

METHODS EMPLOYED: Microsomes are isolated from livers of control animals or animals treated with chemicals which stimulate (induce) microsomal enzymes that metabolize xenobiotics. Various metal ions, at various concentrations, are added to these preparations of microsomes and the ability of the resulting mixture to metabolize various substrates is determined by classical methods. Metal ions providing effects on over-all metabolisms are further studied for effects on separate components of the metabolizing system, such as the flavoprotein and cytochromes of the microsomes. Special attention has been paid to the effects of metals on the rate of reduction of the microsomal cytochrome

P-450 by the cofactor NADPH--the so-called "P-450 reductase" activity of microsomes. This activity can be studied spectrophotometrically--i.e., in the Aminco-Chance using the stop-flow attachment.

MAJOR FINDINGS: Divalent cations usually affect hepatic microsomal metabolism of xenobiotics. Effects are either stimulatory or inhibitory. Typical stimulators are Ca, Mn, and Mg, while inhibitors are Pb, Hg,& Zn. Further study of components of the hepatic microsomal electron transport system showed that effects on overall metabolism of xenobiotics correlated well with metal ion effects on P-450 reductase--the rate of reduction of microsomal cytochrome P-450 by NADPH. As the first part of these studies we have chosen to investigate the ability of ions to stimulate this reaction. We have also chosen to concentrate on effects of Mg, since this is the ion most commonly added to *in vitro* mixtures used to study "drug metabolizing enzymes" and is an ion whose deficiency can affect *in vivo* metabolism of xenobiotics. Mg^{+2} was shown to stimulate the hepatic microsomal P-450 reductase in microsomes from control animals and animals pretreated with phenobarbital (a typical "inducer" of hepatic xenobiotic metabolizing enzymes). Animal pretreatment with the polycyclic hydrocarbon 3-methylcholanthrene (typical of the other major class of inducers of these enzymes; benzpyrene is a less potent member of this class) did *not* itself stimulate microsomal P-450 reductase and appeared to block some of the effects of metals on P-450 reductase--i.e., Mg^{+2} stimulation was much less in preparations from 3-MC-treated animals than from control animals. This deserves much more study in view of the documented interactions of metals and carcinogens and dependency of carcinogen action on carcinogen metabolism in many cases. Xenobiotics usually stimulate P-450 reductase, and metal ions can further add to these effects of substrates on this putative rate-limiting step of metabolism. Monovalent cations--i.e., KCl--can interfere with the stimulatory actions of Mg on P-450 reductase either in presence or absence of xenobiotics. This monovalent-divalent interaction also deserves more study for its possible role in regulation of xenobiotic metabolism *in vivo*. Certain concentrations of monovalent ions markedly affect the ability of small amounts of xenobiotics to affect P-450 reductase. *In vivo*, these ion-substrate interactions are likely to be very important to overall xenobiotic disposition by the liver.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The metabolism of xenobiotics by enzymes in excretory organs like the liver, lung, etc., can affect the toxicity, both qualitatively and quantitatively, of these chemicals. The fact that this metabolism is affected by materials in our environment (such as pesticides, polycyclic hydrocarbons, metals) gives the study of these systems relevance to the Institute's program. The specific study of metal effects on xenobiotic metabolism seeks to understand physiological control mechanisms as well as effects of exposures to contaminants that act by interference with or potentiation of these metal ion effects on xenobiotic metabolisms--e.g., lead, mercury as inhibitors and polycyclic hydrocarbon actions on metal ions as stimulators of xenobiotic metabolism.

PROPOSED COURSE: Further studies of metal ions, especially of alterations in their effects after animal exposure to pesticides or polycyclic hydrocarbons. Examination of effects of heavy metals on electron transport in hepatic (and other) microsomes from control and "induced" animals.

PUBLICATIONS

Fouts, J.R. and Pohl, R.J.: Further studies on the effects of metal ions on rat liver microsomal NADPH-cytochrome p-450 reductase. Journal of Pharmacology and Experimental Toxicology. (In Press)

Serial No.: NIEHS-PT-002
1. Pharmacology & Toxicology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Enzymes Metabolizing Chemicals; Physiological Effectors of these Systems (age, species, sex, nutrition)."

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: J. R. Fouts, Ph. D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.6
Professional:	0.1
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: This is a general effort directed at classifying and understanding various factors that can affect xenobiotic metabolisms. These studies will be devoted to understanding how such metabolizing systems vary as the age of the animal changes, or as one strain, sex, or species of animal is compared with another. Effects of nutrition on xenobiotic metabolism can involve a study of chemical effects, since various food substances or food additives may be responsible for or modify the effects seen. Current projects seek to understand how xenobiotic metabolism in liver and lung changes both qualitatively and quantitatively with age and how these systems may respond differently to environmental pressures as age changes.

METHODS EMPLOYED: Age dependent changes in hepatic and lung microsomal systems metabolizing xenobiotics are studied using rabbits of ages varying from 4 days to 6 months. Microsomes are isolated from lung and studied using technics assessed by Dr. Gram's group (optimal homogenizing, cofactors, substrate, tissue, temperature, etc.). Effects of animal pretreatment with various inducers on rates of development of the enzyme systems are being studied; pesticides such as DDT and chlordane are compared with phenobarbital, phenothiazine and polycyclic hydrocarbons (3-methylcholanthrene (3-MC) and dibenzanthracene). Liver enzymes are affected by all inducers, while lung enzymes have been studied in adult animals with phenothiazine and 3-MC or benzpyrene. Quantitative and qualitative changes in lung and liver xenobiotic metabolism are assessed from

overall rates of metabolism as well as by studies of individual components of the system, such as cytochrome P-450, NADPH cytochrome c reductase, P-450 reductase, substrate binding studies, etc.

MAJOR FINDINGS: This program has just started. We know quite a bit about rates of development and ways of affecting this for xenobiotic metabolism by liver. Little if anything is known about analogous systems in other tissues such as lung. We know that qualitative and quantitative changes appear to occur in the liver systems as the newly born animal matures. Available evidence might be used to suggest that the rate-limiting step in liver of the adult animal (apparently the rate of cycling of the P-450 substrate complex) may not be the rate-limiting step in the newborn for this xenobiotic metabolism. Virtually nothing is known about P-450 reductase in newborn liver or other tissues.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The fact that factors such as age, sex, diet, etc., may affect xenobiotic metabolism by various animal organs (and differently in different organs) has helped us understand different toxicities of materials, reactions to environment, etc., at different ages, with different sexes, etc. Studies such as these help define the factors most important to changing response to and interactions of the variety of materials, both chemical and otherwise, to which we are exposed. Studies on age effects on xenobiotic metabolism are immediately applicable to programs being started in the Developmental and Perinatal Biology group.

PROPOSED COURSE: We will study metal ion effects, substrate effects and response of this system to induction by pesticides, etc., in both liver and lung. Intramicrosomal distribution of the system (rough vs. smooth-surfaced microsomes) in tissues at different ages will also be studied.

PUBLICATIONS

Fouts, J. R.: Maturation and induction of microsomal drug-metabolizing enzymes. In Proceedings of the 13th International Congress for Pediatrics. Vienna, Austria, 1971

Serial No.: NIEHS-PT-003
1. Pharmacology & Toxicology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Effects of Chemicals on the Inhalation Toxicity of Chemical Solvents"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: R. T. Drew, Ph. D.

OTHER INVESTIGATORS: J. R. Fouts, Ph. D.

COOPERATING UNITS: Pathologic Physiology Branch (Histology), Biometry Branch

MAN YEARS:

Total:	1.5
Professional:	1.0
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: Certain chemicals are known to activate the liver microsomal enzyme systems in the rat. The object of this study is to determine if pretreatment by these chemicals affects the acute inhalation toxicity of common organic solvent.

METHODS EMPLOYED: Control rats or rats previously given 3 intraperitoneal injections of chemicals are given single 4 hour inhalation exposures to solvent at various levels. The LC_{50}'s for both treated and untreated rats are determined for the various solvents by conventional methods. Lungs and livers are saved for histological examination.

MAJOR FINDINGS: Neither sodium phenobarbitol (75 mg/kg) nor chlorpromazine (15 mg/kg) markedly affect the acute inhalation toxicity of benzene in rats.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Illicit drug use by the general public is increasing. Some of these compounds stimulate liver microsomal enzymes thus increasing the rate at which certain substrates are metabolized. This experiment is designed to determine if the toxicity of common solvents is altered as a result of enzyme activation by drugs.

PROPOSED COURSE: This study will be continued, studying the affects of chemicals on the inhalation toxicity of p-Xylene and possibly other solvents.

HONORS AND AWARDS: Dr. Drew is a member of Toxicology Subcommittee of the Environmental Health Committee of the Society of Plastics Industries.

Serial No.: NIEHS-PT-004
1. Pharmacology & Toxicology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Analytical Methodology - 1. Model Substrates of Mixed Function Oxidase in Liver and Lung"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: R. S. Chhabra, Ph. D., D.V.M.

OTHER INVESTIGATORS: T. E. Gram, Ph. D. and J. R. Fouts, Ph. D.

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	1.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: In vitro studies of hepatic microsomal detoxication of foreign compounds are carried out using various drug substrates; one of the commonly used substrates is aniline. The major metabolic product of this substrate can be 4-hydroxyaniline (p-aminophenol). There are two generally used methods available for the determination of pAP. One method involves extraction of the product into ether and its return to an alkaline aqueous phase. The second method employs trichloroacetic acid (TCA) to precipitate the proteins, and the p-aminophenol is analyzed in the supernatant. The latter method is simple and can be effectively used on large numbers of samples. With both technics, the final step--and the basis for the quantitative assay--is the same: the metabolite, pAP, is coupled with alkaline phenol to produce a phenol-indophenol chromophase with an Emax at about 620 nm. While adopting the TCA method for routine aniline metabolism studies we observed that the "recovery" of pAP standards was low compared with the ether extraction procedure. Therefore, a detailed study was undertaken to determine the reason for this discrepancy.

METHODS EMPLOYED: Spectrophotometry, dialysis, subcellular fractionation were employed.

MAJOR FINDINGS: In incubations of aniline with liver microsomes "recovery" of p-aminophenol depended on the type of NADPH generating system used. The use of an NADPH generating system containing liver "soluble fraction" (postmicro-

somal supernatant) as a source of glucose-6-phosphate dehydrogenase yielded poor "recovery" of p-aminophenol when compared with ether extraction procedure. Dialysis studies of liver soluble fraction from rat, rabbit, guinea pig and mice, revealed that endogenous substances present in hepatic soluble fraction interfered with the colorimetric determination of pAP. The presence of free sulfhydryl reacting groups in the soluble fraction might be responsible for the interference. Accordingly, the addition of small amounts of Hg++ blocked the interference. NADPH generating systems containing yeast glucose-6-phosphate-dehydrogenase gave approximately equal recoveries of p-aminophenol in both precipitation or ether extraction procedures.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: We use aniline routinely for drug detoxication studies for several reasons including its wide use in industries manufacturing dyes, medicinals, varnishes, etc. The study of such chemicals whose hazard to the health of human beings might be affected by metabolism is within the scope and program of this Institute.

PROPOSED COURSE: Problems of this nature--methodology for better assessment of the metabolism of poisons and environmental contaminants--will continue to occupy some fraction of the effort of this Section.

Serial No.: NIEHS-PT-005
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "A Comparative Study of the Mixed Function Oxidase Systems of Rabbit Lung and Liver with Special Emphasis on the Properties of the Pulmonary Mixed Function Oxidase Systems"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: J. R. Bend, Ph. D., G. E. R. Hook, Ph.D., and T. E. Gram, Ph. D.

OTHER INVESTIGATORS: William R. Jurgelsky, Ph. D., M. D.

COOPERATING UNITS: Pathologic Physiology

MAN YEARS

Total:	2.1
Professional:	1.8
Other:	0.3

PROJECT DESCRIPTION

OBJECTIVES: Although mammalian mixed function oxidase activity is most abundant in liver, detectable levels are also found in kidney, lung, placenta, skin, and other organs. Environmental contaminants, especially atmospheric pollutants, are most likely to gain entry into mammalian organisms via the lungs, gut, or skin. Despite extensive general knowledge about the MFO system, relatively little attention has been paid to this system in extrahepatic tissues. The objectives of the present investigation were: (1) To determine the optimum conditions for isolation and assay of the pulmonary and hepatic MFO systems. (2) Using this information, to fractionate liver and lung and to evaluate the subfractions with regard to MFO and a number of standard "marker" enzymes, biochemical components, and morphology (by electron microscopy).

METHODS EMPLOYED: Standard differential centrifugation and spectroscopic technics were employed.

MAJOR FINDINGS: Lungs of adult New Zealand rabbits were subjected to a preliminary passage through a meat grinder and homogenized in 0.25M sucrose in (1) a Virtis apparatus, (2) a Waring blendor, (3) a smooth-walled glass tube

(Potter-type) with a loose-fitting teflon pestle, or (4) a ground-glass tube (Potter type) with a tight-fitting ground glass pestle. Three different homogenization times were used for each of the four methods resulting in 12 tissue samples from a single pool (15-20 rabbits) of lung.

As expected, for any given technic, as one increased the duration of homogenization, the microsomal protein yield (mg/g tissue) increased as did the "contamination" of microsomes by mitochondria. Based primarily on the specific activity of the microsomal MFO components and secondarily on the extent of mitochondrial contamination of the microsomal fraction, the optimal results with lung and liver were obtained with the loose-fitting (Potter) glass tube-teflon pestle combination. Benzphetamine demethylase, aniline hydroxylase, biphenyl hydroxylase, cytochrome P-450, and NADPH cytochrome c reductase (all expressed per mg of protein) were highly concentrated in the microsomal fractions of liver and lung. In general, the specific activities in microsomes were 3-5 times those of the next highest fraction, usually mitochondria.

The microsomal "detoxifying" enzymes in liver and lung were NADPH- and oxygen-dependent and were inactivated by boiling. Substitution of NADPH by NADH,NADP, or NAD reduced activity 75-100%. NADPH could be replaced by a NADPH-generating system without loss of activity. Incubation under N_2 or CO markedly decreased or abolished activity.

Comparable sensitivity of the liver and lung detoxifying systems to inhibition by cytochrome c, CO, and SKF 525-A suggests that they may have similar electron transfer components.

Perfusion and washing experiments suggested organ differences in hemoglobin-binding to microsomes. Although hemoglobin can be removed from liver either by perfusion in situ with dilute salt solutions (about .15 Molar KCl) or by resuspension of the microsomal pellet in similar solutions and resedimentation (washing), only the former procedure was effective with lung.

Recently, subcellular fractions prepared from rabbit liver and lung have been evaluated against a somewhat broader base of marker enzymes (10) and biochemical components (5) (not necessarily involved in "detoxication").

In general, the subcellular distribution of microsomal, mitochondrial, and lysosomal markers in lung and liver were qualitatively similar. However, from a quantitative standpoint, aniline hydroxylase, cytochrome P-450, and monoamine oxidase (all expressed per mg of protein) were 2-5 times more active in liver than in lung. Glucose-6-phosphatase activity of liver microsomes was about 40 times that of lung.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Chemical environmental contaminants are likely to first contact man and animals via the lung, skin, or gut. These contaminants may stimulate or inhibit MFO activity, or be devoid of effect. It is possible that these effects may be exerted somewhat specifically at the portal of entry (e.g., lung) or throughout the organism. This project is the first step in an effort to systematically evaluate MFO systems in lung and other organs from the standpoint of biochemical control mechanisms.

PROPOSED COURSE: This project will be continued to investigate the possibility of preferential changes in certain target organs produced by environmental contaminants and the possible effects of such changes on gross response or toxicity, steroid homeostasis., etc.

Serial No.: NIEHS-PT-006
1. Pharmacology and Toxicology
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Evaluation of Carbamate-induced Anticholinesterase Effects in the Fetus"

PREVIOUS SERIAL NUMBER: NIEHS-PT-13

PRINCIPAL INVESTIGATOR: Neil Chernoff, Ph. D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS

Total:	0.2
Professional:	0.1
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: This is the continuation of a study begun to investigate possible changes in acetylcholinesterase levels in fetal tissues after maternal administration of an acetylcholinesterase inhibiting carbamate pesticide. Normal levels of acetylcholinesterase activity throughout development in different fetal organs are also required.

METHODS EMPLOYED: The enzyme is assayed by the radiometric microassay outlined by Potter. The method enables the determination of enzyme activity in tissue weighing 1 to 10 mg. The minute quantities of tissue, in turn, allow the testing of organs and portions of organs from young fetuses. Acetylcholinesterase activity is determined in fetuses of animals treated (orally) with carbaryl at differing times after intubation.

MAJOR FINDINGS: Experiments have shown that carbaryl inhibits acetylcholinesterase activity in fetal and maternal tissues after oral administration. The duration of inhibition is related to the dose administered with greater doses resulting in longer periods of inhibition. Initially, the fetal acetylcholinesterase activity is inhibited to a greater degree than the maternal enzyme. During the later periods of inhibition both systems are inhibited to the same degree. Normal fetal values for a variety of tissues were also obtained during the course of these experiments.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Use of

the carbamate group of pesticides may increase as they replace the chlorinated hydrocarbons which are to be phased out. The carbamate insecticides are all acetylcholinesterase inhibitors. Their widespread use could conceivably result in sufficient maternal intake to affect the fetus. This study will evaluate effects of the carbamates on fetal levels of acetylcholinesterase. The technique may provide a relatively easy screen for acetylcholinesterase-inhibiting effects of environmental agents, in general.

PROPOSED COURSE: The study will continue with the measurements of the duration and degree of fetal acetylcholinesterase inhibition after administration of differing amounts of carbaryl. The study will be extended to other carbamate pesticides. The ontogeny of acetylcholinesterase activity in different fetal organs will continue to be determined.

Serial No.: NIEHS-PT-007
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Effects of Cadmium on the Fetal Lung"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Neil Chernoff, Ph. D.

OTHER INVESTIGATOR: Donald Feldman, D.V.M.

COOPERATING UNITS: None

MAN YEARS:

Total:	.4
Professional:	.2
Other:	.2

PROJECT DESCRIPTION

OBJECTIVES: Work done by the principal investigator has shown that subcutaneous injections of $CdCl_2$ causes fetal death, reduced weights and teratogenic effects in the rat. This study was undertaken to elucidate the ontogeny of one of the defects commonly seen: small lungs.

METHODS EMPLOYED: Animals treated with $CdCl_2$ were sacrificed at varying times after treatment. Fetuses were weighed and examined, and kidneys, heart, and lungs removed and weighed. Histological sections of lung of varying ages were made.

MAJOR FINDINGS: Injections of 6 mg/kg $CdCl_2$ on days 14-17 of gestation result in lowered fetal weights when compared with controls as early as day 17. Lung/body weight ratios were lower in treated animals from day 19 on. A gross histological study of lungs of fetuses from day 15 to one day after birth has failed to reveal any significant differences between the controls and the treated fetuses.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Cadmium is a major by-product of numerous industrial processes and as such is a serious air pollutant. Large amounts of cadmium are also present in cigarette smoke. Cadmium is also present in quantity in many natural bodies of water. Cadmium has been implicated in an outbreak of osteomalacia in Japan and there have been indications that it may be a factor in hypertension in human populations.

These studies may help to determine the potential involvement of the fetal lung as a result of maternal cadmium intake.

PROPOSED COURSE: The studies will continue with emphasis on alternate routes of administrations.

Serial No.: NIEHS-PT-008
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Perinatal Toxicology of Cadmium"

PREVIOUS SERIAL NUMBER: NIEHS-PT-14

PRINCIPAL INVESTIGATOR: Neil Chernoff, Ph. D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	.3
Professional:	.2
Other:	.1

PROJECT DESCRIPTION

OBJECTIVES: Studies performed in this laboratory have indicated that subcutaneous injections of cadmium cause fetal mortality and a variety of teratogenic effects in the rat. The studies are continuing with emphasis on quantitating the types of defects in terms of dose and days of administration of the cadmium as well as specific organ effects.

METHODS EMPLOYED: Subcutaneous injections of 4 to 12 mg/kg $CdCl_2$ were administered on 4 consecutive days between the 13th and 18th days of pregnancy. Teratologic effects were determined by gross examination, necropsy and alizarin staining. Fetal weights and mortality were noted. Weights of selected fetal organs were obtained and organ/body weight ratios were calculated.

MAJOR FINDINGS: A dose-response was established for fetal death, fetal weight, and the percentage of anomalies. Fetal death rates and the percent of anomalies rose while the fetal weight dropped with increasing doses of cadmium administered. Anomalies produced were micrognathia, cleft palate, club foot, and small lungs. Micrognathia and small lungs were the most common. Administration of cadmium on different days of gestation indicates that the fetuses of mothers whose treatment was begun on day 13 were less sensitive than those of mothers whose treatment was begun on days 14, 15 or 16.

Cadmium retards the growth of fetal lungs. The lung/body weight ratios were significantly reduced in fetuses of animals treated with $CdCl_2$. This

retardation did not occur with either the fetal kidney or heart.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Cadmium is a major by-product of numerous industrial processes and as such is a serious air pollutant. Large amounts of cadmium are also present in cigarette smoke. This study will help to determine the extent of fetal involvement as a result of maternal cadmium intake in experimental animals.

PROPOSED COURSE: The study will continue with emphasis on the ontogeny of the anomalies. Levels of cadmium within treated fetuses will be determined.

Serial No.: NIEHS-PT-009
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Perinatal Toxicology of Nitrilotriacetate (NTA) and its Cadmium and Methylmercury Complexes"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: Neil Chernoff, Ph. D.

OTHER INVESTIGATORS: K. Diane Courtney, Ph. D.

COOPERATING UNITS: None

MAN YEARS:

Total:	3.0
Professional:	1.0
Other:	2.0

PROJECT DESCRIPTION

OBJECTIVES: Nitrilotriacetate (NTA) has been advanced as a possible substitute for phosphates in detergents. In its role as a builder it will chelate a variety of cations. NTA in the environment will probably chelate additional types of cations including some of the toxic heavy metals. This study was undertaken to determine effects of NTA, NTA-cadmium complex, NTA-methylmercury complex, and NTA-cadmium complex in conjunction with mineral deficient diets, on fetuses of the rat and mouse.

METHODS EMPLOYED: Teratological effects were determined by gross examination and necropsy. Fetal weights and mortality were noted. Maternal weights and liver/body weight ratios were recorded.

MAJOR FINDINGS: NTA does not appear to be teratogenic in the rat and mouse when administered either orally or subcutaneously. A number of encephalocoeles were seen in mouse fetuses whose mothers had been on zinc deficient and calcium deficient diets in conjunction with the administration of NTA. Cadmium administered subcutaneously as an NTA-complex was more lethal to pregnant rats than cadmium chloride. The administration of cadmium-NTA complex to rats on a zinc deficient diet resulted in a significant fetal weight loss. At this time no firm conclusion can be drawn as to the effects of NTA-methylmercury complex on the fetus.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: NTA may be used in quantities above 1 billion pounds per year. It appears desirable to have some knowledge of the toxicity of its chelates in mammalian systems in view of the fact that these chelates will exist in the environment.

PROPOSED COURSE: To complete the NTA-methylmercury complex portion of the study.

Serial No.: NIEHS-PT-010
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "The Teratogenic Evaluation of the Herbicide 2,4,5-T and Tetrachlorodibenzo-p-dioxin"

PREVIOUS SERIAL NUMBER: NIEHS-PT-05

PRINCIPAL INVESTIGATOR: K. Diane Courtney, Ph. D.

OTHER INVESTIGATORS: J. Moore, D.V.M.

COOPERATING UNITS: Biometry Branch

MAN YEARS:

Total:	1.0
Professional:	0.5
Other:	0.5

PROJECT DESCRIPTION

OBJECTIVES: Previous studies showed that earlier commercial grade 2,4,5-T containing 30ppm 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) was a teratogenic and fetotoxic agent in mice and rats. Therefore, studies were undertaken to establish whether 2,4,5-T or TCDD was the teratogenic and fetotoxic agent.

METHODS EMPLOYED: Mice of the CD-1, DBA/J2, and C57Bl/6 strains and rats of the CD strain were used. Compounds were administered during organogenesis and fetuses removed before birth. Fetal weight and mortality were recorded. Anomalies were detected by gross necropsy.

MAJOR FINDINGS: The herbicide 2,4,5-T as well as TCDD are teratogenic in all 3 strains of mice producing cleft palate and kidney anomalies. In the rat, 2,4,5-T was neither teratogenic nor fetotoxic. TCDD did produce some kidney anomalies in the rat.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The herbicide 2,4,5-T is one of the phenoxyacetic acid class that are used extensively. The impurity, TCDD, could be present in other compounds made from chlorinated phenols.

PROPOSED COURSE: Studies will be initiated to determine whether or not the effect on the kidney is transient or permanent and whether or not there are

secondary manifestations detectable postnatally.

PUBLICATIONS

Courtney, K. Diane and Moore, J.A.: Teratology studies with 2,4,5-T and tetrachlorodioxin. Tox. and Appld P'col. (In Press).

Serial No.: NIEHS-PT-011
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Metabolism and Distribution of 2,4,5-T and its Analogs in Pregnant and Nonpregnant Rats, Mice, and Guinea Pigs."

PREVIOUS SERIAL NUMBER: NIEHS-PT-06

PRINCIPAL INVESTIGATOR: K. Diane Courtney, Ph. D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	.4
Professional:	.2
Other:	.2

PROJECT DESCRIPTION

OBJECTIVES: Previous studies showed the herbicide 2,4,5-T to be teratogenic in mice. In the rat and guinea pig no adverse effects were produced. The species differences in the effects of 2,4,5-T may be related to the metabolism, excretion or placental transport of this compound.

METHODS EMPLOYED: Spectrophotometric, chromatographic and autoradiographic methods have been used. Urine and serum samples were obtained from pregnant and nonpregnant animals. Fetal tissue was obtained on various days of gestation.

MAJOR FINDINGS: The mouse appears to metabolize 2,4,5-T to a slight degree. Induction of metabolism in the mouse presents a different pattern from that obtained with the rat. These studies are still in progress.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The fetal effective dose of an environmental agent may be dependent upon maternal metabolism and distribution as well as transport of the compound across the placenta. Various species may achieve different concentrations of these agents in the fetus because of differences in rates of metabolism and excretion by the mother. In this way, some agents may be deleterious to the fetuses of some species and not others.

PROPOSED COURSE: The project will continue. In mice and rats the rates of metabolism, excretion and placental transport will be documented. These data will then be correlated with the teratogenesis study.

PUBLICATIONS

Courtney, K.D.: 2,4,5-T in the rat: Excretion pattern, serum levels, placental transport and metabolism. In Deichman, W. (Ed.): Pesticides Symposia. Inter-American Conference on Toxicology and Occupational Medicine. Halos Co. Fla, 1970.

Serial No.: NIEHS-PT-012
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Teratogenic Evaluation of Polychlorinated Biphenyls"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: K. Diane Courtney, Ph. D.

OTHER INVESTIGATOR: Neil Chernoff, Ph. D.

COOPERATING UNITS: None

MAN YEARS:

Total:	.8
Professional:	.2
Other:	.6

PROJECT DESCRIPTION

OBJECTIVES: Because polychlorinated biphenyls (PCB's) have recently been shown to present an environmental problem, it was decided to evaluate the teratogenic potential of the most commonly used PCB's.

METHODS EMPLOYED: Compounds were administered to CD rats or CD-1 mice during organogenesis and fetuses removed before birth. Fetal weight and mortality were recorded. Anomalies were detected by gross necropsy.

MAJOR FINDINGS: PCB 1260 and 1254 as well as the combination of PCB 1254 and p,p´-DDT were not teratogenic or fetotoxic in the rat. The mouse studies are currently in progress.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: PCB's have been implicated in a number of bird and fish kills. Their potential for mammalian toxicity is largely unknown at this time.

PROPOSED COURSE: The study with the mice will be completed.

Serial No.: NIEHS-PT-013
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Biosynthesis and Degradation of Lung Surfactant Phospholipids"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATOR: R. P. DiAugustine, Ph.D.

OTHER INVESTIGATOR: None

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	1.0
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: A surfactant system located at the lining of the lung appears to be essential for alveolar stability (patency). It is generally believed that this surfactant is chiefly composed of the phospholipid dipalmitoylphosphatidylcholine. The rapid rate of biosynthesis of this phospholipid by mammalian lung parenchyma is apparently vital for the replenishment of surfactant at the alveolar lining. The objectives of the proposed studies are to investigate the biosynthesis and degradation of lung surfactant phospholipids, especially dipalmitoylphosphatidylcholine, and to localize the site(s) for such biochemical events.

METHODS EMPLOYED: Chromatography (TLC and GLC) and liquid scintillation techniques were employed.

MAJOR FINDINGS: Time-course studies of the incorporation of 1-^{14}C-palmitate and ^{32}P revealed a rapid uptake of these isotopes into rat total lung phosphatidylethanolamine and phosphatidyl-N,N-dimethylethanolamine (PDME) and phosphatidylcholine. Although 1-^{14}C-oleate was rapidly incorporated into phosphatidylethanolamine, the appearance of radioactivity in PDME was relatively slower than that observed for ^{32}P and 1-^{14}C palmitate. These results suggest a disaturated pool of lung phosphatidylethanolamine that is rapidly N-methylated to form a corresponding acyl species of PDME. The latter compound has been found at the lung lining and shown to be highly surface-active. (^{14}C-palmitate)-PDME and (^{14}C-oleate)-PDME had half-lives of 5 and >20 hours, respectively. The rapid turnover of (^{14}C-palmitate)-PDME could be accounted for by PDME acting as

an intermediate in the biosynthesis of phosphatidylcholine or undergoing rapid degradation at the lining of the lung. The half-life of ^{14}C-palmitate-phosphatidylcholine in rat total lung is between 12 and 14 hours. Although $^{14}CH_3$-choline was abundantly and rapidly taken up into lung phosphatidylcholine no activity was observed in phosphatidylethanolamine or PDME within 20 hours after intravenous injection of this isotope. Thus, the recylcing of phosphatidylcholine methyl groups is of little quantitative significance in the lung.

Phospholipase A, an enzyme that cleaves acyl groups from phospholipids appears to be present in the lung and might account for the rapid disappearance of ^{14}C-palmitate from lung phospholipids: in rats sacrificed 5 min. after in vivo labelling with $^{14}CH_3$-choline, significant isotope untake was found in lung lysophosphatidylcholine.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: It is apparent that the lung as an organ for diffusion of gases with the external milieu must maintain alveolar surface stabilization. During normal deflation of the lung, the surface tension of the alveoli walls tend to promote alveolar collapse. However, the potent surface-active molecules of the lung surfactant system tend to counteract this collapse and surface tension falls to almost zero during deflation. Thus, the surface-tension lowering property of the acellular alveolar lining is vital for normal pulmonary function. Because of the significant role of phospholipids in the surfactant system, it is important to establish those factors which elaborate and dispose surfactant phospholipid. In turn, this information could serve in the evaluation of various environmental agents on lung integrity. Localized at the lining of the lung, the surfactant system is in direct contact with airborne agents.

PROPOSED COURSE: (1) To investigate using various fractions or components of the lung, including the acellular lining layer and alveolar macrophages, the disposition of in vivo labelled phosphatidylethanolamine and phosphatidyl-N, N-dimethylethanolamine. (2) To determine what factors are responsible for the rapid turnover of surfactant phospholipids. This proposed study shall examine at its outset alveolar macrophages as a source for catabolism of lining phospholipids. (3) To study the effects of various environmental agents on the uptake of various precursors into lung phospholipids and on lung morphology. (4) To investigate the transport of various phospholipid precursors into lung tissue.

PUBLICATIONS

DiAugustine, R. P.: Lung phospholipids I. In vivo studies of the incorporation of ^{32}P, [methyl-^{14}C] choline, 1-^{14}C-palmitic acid and 1-^{14}C-oleic acid into phosphatidylethanolamine, phosphatidyl-N,N-dimethylethanolamine and phosphatidylcholine. Biochem. Biophys. Res. Commun. (In press)

Serial No.: NIEHS-PT-014
1. Pharmacology and Toxicology Branch
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Pharmacology & Toxicology of Paraquat"

PREVIOUS SERIAL NUMBER: NIEHS-PT-08

PRINCIPAL INVESTIGATOR: Charles W. Sharp, Ph.D.

OTHER INVESTIGATOR: H.S. Posner, Ph.D., B. C. Eliason, M.D., T. E. Gram, Ph.D.

COOPERATING UNITS: Pathologic Physiolgy Branch (Histology)

MAN YEARS:

Total:	2.1
Professional:	0.6
Other:	1.5

PROJECT DESCRIPTION:

OBJECTIVES: Accidental or suicidal ingestion of Paraquat by humans has been reported to produce a delayed effect on the lung which can result in death. The herbicide is used extensively as a substitute cultivator or weed killer and is thereby made accessible to the lungs during spraying. The pathologic alterations resulting from a single dose of Paraquat is studied in the rat; attempts are made to correlate the dose-response relationship of Paraquat with the concentration of Paraquat in tissues and with the length of exposure.

METHODS EMPLOYED: Rats are administered Paraquat by intubation. Before or upon death, the tissues are extracted with sulfuric acid and analyzed photometrically for the concentration of the Paraquat free radical. In separate experiments, rats were administered Paraquat intravenously using C^{14}-labeled Paraquat. Tissues are analyzed for C^{14} before or after thin-layer chromatography. Improved chromatographic methods were developed for the separation of Paraquat from its metabolites and impurities.

MAJOR FINDINGS: C^{14}-Paraquat appears as one spot (96-98%) after paper or cellulose thin-layer chromatography using butanol, acetic acid and water (after adsorbent is coated with NaCl) or benzene, amyl alcohol, methanol and HCl as solvents. The same purity is observed after reflectance spectroscopy or after spraying with Dragendorff's reagent. Less than 0.5% of the parent compound, dipyridyl, is present. Diquat can be resolved from Paraquat by TLC, and other impurities are present in negligible amounts in either the

C^{14} or chemically pure material obtained from the supplier, Imperial Chemical Industries. The acetate and choloride salts of Paraquat have been resolved. Two spots on TLC when Paraquat is chromatographed may therefore indicate mixtures of these salts or others existing in tissue extracts.

The oral LD_{50} of Paraquat is 5-6 times the intravenous LD_{50}. Death usually occurs from 2-5 days after a single dose of Paraquat; occasionally, animals survive up to eleven days. Peribronchiolar lymphocytic and epithelial proliferation occurred only in the lungs of a few rats receiving a high dose of Paraquat by intubation.

Rapid weight loss (1-2 days) is common in animals that survive less than 4 days after oral Paraquat.

By chemical determination, Paraquat has been quantified in lungs (0.6-21 g) but not in livers, blood or kidneys several days after Paraquat administration. Paraquat has been detected up to 10 days in rats given C^{14} Paraquat I.V. The tissue level over a period of 10 days from the highest to the lowest level appears to be as follows: lung > adrenal $\geq$ thigh > kidney > heart > spleen > stomach > testes $\approx$ thymus $\approx$ liver >> blood. The half life of Paraquat in the blood is short but the half-life of Paraquat in lung and muscle is 1-4 days.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The dipyridyl herbicides are retained for long periods of time in several tissues. Low, multiple doses given orally, via inhalation or by other routes may, therefore, result in an accumulation, particularly in the lung, and produce toxic effects. A non-lethal dose of Paraquat might also render the lung susceptible to a variety of other environmental insults.

PROPOSED COURSE: Further studies will be conducted on the pharmacodynamics of Paraquat in rats; the pharmacology and toxicology of interactions of Paraquat with other agents will then be studied.

OFFICE OF THE ASSOCIATE DIRECTOR
FOR SCIENTIFIC INFORMATION AND COMMUNICATION

OFFICE OF THE ASSOCIATE DIRECTOR FOR
SCIENTIFIC INFORMATION AND COMMUNICATION
Summary Statement

Scientific Liaison with Categorical Environmental Programs

The transfer of the categorical environmental programs out of DHEW into the independent Environmental Protection Agency (EPA) has compounded the difficulties of formal liaison procedures reported regularly for the last four years. Attempts are being made to open new channels, but these will remain uncertain until the internal organization of EPA is settled. The direct relationships with operating elements, built over the last four years, is now paying dividends. Potential conflicts have been largely resolved in the course of joint input with EPA to documents on health policies, status of environmental health and proposals for the 1972 UN Conference on Human Environment. Two conferences on specific problems have been held with the Food and Drug Administration, and there has been close collaboration with the Bureau of Occupational Safety and Health (BOSH) in the preparation of documents and the organization of conferences on coal workers' pneumoconiosis. Most lacking is a set of formal project descriptions that can be used for semi-automatic retrieval. The NIEHS-BOSH interface will be changed somewhat by the passage of the Occupational Safety and Health Act of 1971 and the creation of the National Institute of Occupational Safety and Health (NIOSH) and the broadening of the scope of activities. The extent of the change will depend somewhat on the organizational location of NIOSH in HEW. The pending retirement of Mr. Sylvan Martin poses a severe operational problem. A replacement is being sought.

Quick Retrieval System for Scientific Information

The situation as reported last year is unchanged except for a greater storage of information and a sharpening of the procedures. The complete system in its Phase I form is now in operation. Information entry is from selected documents on to aperature cards in three forms: (a) typed citation, short abstract, key words, coded key words; (b) punched key words; and (c) microfilm of original document. Storage is in two forms: (a) manually in the cells of a coordinate (stressor x focus-of-interest) file; and (b) serially by entry number. Retrieval of small amounts of specialized information is manual from the coordinate file, of larger amounts by punch card collation from the serial file. At present, the latter is done remotely by telephone to N. C. Central University. Priority in entering material is given to topics of current interest, such as herbicides. A thesaurus permits use of standardized key words and their 5-letter code equivalents. A review by the Director and the Library Committee is planned for an early date.

Intramural Scientific Information Service (including Library)

This continues as a sectional activity directly responsible to the Associate Director. A Chief for an Information Branch which would have

inclusive responsibility for Storage and Retrieval, Information Services and Compilation has not been recruited. The scope of the Library has been expanded to meet new intramural scientific activities. An automatic title service to intramural scientists is now in partial operation. Inter-library "loans" continue at high levels.

Scientific Information Evaluation and Reporting Service

The compilation on "Coal Workers' Pneumoconiosis" will appear in the March 71 number of Journal of Occupational Medicine, and that on "Nitrates, Nitrites and Methemoglobinemia" in the final number of Environmental Research for 1970. The big effort this year has been in the preparation of book-length documents on four topics. Three are now in various stages of editing; the fourth is still in the planning stage. They should be ready for the printer by August. Considerable effort went into the preparation of a lengthy document on environmental health and health policies only to have it aborted at the Assistant Secretary's level. Some effort is currently being expended on input to a Report on the Health Aspects of Environmental Pollution that must be sent to Congress by the President in July. This Office has been assigned primary responsibility for the section on Research Needs and Accomplishments. The material is being coordinated by the Office of the Assistant Secretary. A Response to Inquiry File has been added which incorporates items received since July 1, 1970.

Beryllium Case Registry

This continues under contractural arrangements with Massachusetts General Hospital. A meeting with participating physicians is scheduled for May, 1971. (See NIH-69-77 under contracts)

International Programs

Under a bilateral agreement with France, arrangements have been made for Dr. J. Wyatt of Winnipeg to spend two months in Dr. Sadoul's laboratories at Nancy. Reciprocal arrangements to bring Dr. Sadoul to the U.S. are being made. Further developments in pursuit of the topic will depend on the outcome of these visits. Proposals have been developed for the consideration of the Preparatory Committee of the 1972 UN Conference on the Human Environment. Support has been offered to the New York Academy of Sciences for an international conference on Coal Workers' Pneumoconiosis.

Editorial Committee

The number of papers submitted for clearance has increased to 58 in the period since July 1, 1970. The Committee has been expanded and its procedures somewhat revised.

Serial No.: NIEHS-OSI-001
1. Office of Associate Director for Scientific Information & Communication
2. Liaison
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Scientific Liaison with Categorical Environmental Health Programs"

PREVIOUS SERIAL NUMBER: NIEHS-OSI-01

PRINCIPAL ADMINISTRATOR: Sylvan C. Martin

OTHER ADMINISTRATORS: None

COOPERATING UNITS: Categorical Environmental Health Programs

MAN YEARS:

Total:	1.0
Professional:	0.9
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The primary objective is to maintain awareness of relevant scientific and technical activities between the intramural operations of NIEHS and other Federal environmental programs. Formal processes support, but do not satisfy ultimate objectives of liaison --- meeting together, thinking together, working together.

BACKGROUND: One of the reasons advanced for creating NIEHS was the necessity for a facility which would provide the more basic, longer-term, multiple factor research necessary to the development of criteria, standards and control measures by the categorical environmental health programs. This necessitates continuing exchange of information on problems, needed research, investigative activities, and emergent scientific information between the research and categorical activities. The need was expressed in announcements by the Surgeon General on the implementation of NIEHS, and has been the subject of frequent inquiry by OMB and DHEW representatives.

MAJOR ACCOMPLISHMENTS: The removal of the control programs from DHEW into EPA, and the tendency in EPA to substitute a horizontal for a vertical organization, has broken up the formal lines of communication that were being reformed after the shift from CPEHS to EHS. However, the same individuals are doing much the same work at the operational level, and informal liaison proceeds even better than before. Close relationships were established with

EPA personnel in the preparation of the FIC-NIEHS environmental book-length documents, in input to a DHEW health policy document, to proposals for the 1972 UN Conference, and to the President's report on the health aspects of environment. BOSH (NIOSH) and NIEHS are collaborating in a book and an international conference on coal worker's pneumoconiosis. Tentative moves are being made to reestablish top-level formal communications.

PROPOSED COURSE: To reestablish formal liaison channels with EPA. To clarify research support needs by EPA. To further develop relationships with NIOSH and HLI.

Serial No.: NIEHS-OSI-002
1. Office of Associate Director for Scientific Information & Communication
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Quick Retrieval System for Scientific Information"

PREVIOUS SERIAL NUMBER: NIEHS-OSI-02

PRINCIPAL ADMINISTRATOR: Richard K. West

OTHER ADMINISTRATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.7
Professional:	0.6
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The objective is the development and operating of a system for quick retrieval of information on scientific topics relevant to NIEHS programs.

BACKGROUND: The relatively unstructured nature of information obtained from categorical programs (see Project OSI-001) and other sources imposes the need for some system of retrieving information on a particular subject with speed and precision. The preparation of position and other staff papers (see Project OSI-005) also demands quick access to important relevant information. Intramural scientists need assistance in securing references, abstracts and original documents relating to topics of immediate interest. It is desirable that one system be established for handling information acquired to meet these internal needs.

MAJOR ACCOMPLISHMENTS: The QRS system has been completed and tested. Information is now being stored in relation to topics of particular interest. Priority has been shifted away from liaison information to scientific topics of particular interest, such as herbicides and metals. Second priority is given to topics on which position papers have been compiled (nitrates, coal workers' pneumoconiosis).

PROPOSED COURSE: To bring storage up to a fully operating level for selected topics. To clarify relations with grant information and services to that office. To continue the approach to computerization.

Serial No.: NIEHS-OSI-003
1. Office of Associate Director for Scientific Information & Communication
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "NEHSC Library"

PREVIOUS SERIAL NUMBER: NIEHS-OSI-03

PRINCIPAL ADMINISTRATOR: Ralph Hester

OTHER ADMINISTRATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	0.1
Other:	0.9

PROJECT DESCRIPTION

OBJECTIVES: To provide necessary library holdings and services for the use of NEHSC personnel.

BACKGROUND: Although the three neighboring universities provide excellent backup library resources, and the services of both the NIH library and the National Library of Medicine are available to NIEHS, a moderate library facility is essential in the immediate vicinity to minimize the time spent by scientists in travel and absence from active work, and to facilitate consultation of relevant material.

MAJOR ACCOMPLISHMENTS: The Library functions well in the new quarters of Bldg. 10, but the space is already showing signs of saturation. The addition of new fields to the Institute staff has required expansion of coverage. There are 197 periodicals now regularly received. Demands for interlibrary loans average 440/month. The annual purchase budget is now $17,000.

PROPOSED COURSE: The initiation of culling procedures for periodicals of passing interest.

Serial No.: NIEHS-OSI-004
1. Office of Associate Director for Scientific Information & Communication
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Intramural Scientific Information Service"

PREVIOUS SERIAL NUMBER: NIEHS-OSI-04

PRINCIPAL ADMINISTRATOR: Ralph Hester

OTHER ADMINISTRATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	1.0
Professional:	0.8
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: The objective is to provide relevant scientific information by both routine and demand services to NEHSC personnel.

BACKGROUND: Both the preparation of position and other staff papers, and the consideration of relevant scientific literature by working intramural scientists, requires the acquisition, assembly, and referencing of documentary information from a wide range of sources. Both the Scientific Directorate and the intramural scientists require assistance in (a) using the Quick Retrieval System (Project NIEHS-OSI-002); and (b) going beyond the limited internal library resources (Project NIEHS-OSI-003) to external sources.

MAJOR ACCOMPLISHMENTS: Information Services now constitutes a Section in the functional organization of OSIC, charged with securing such scientific information as is required by Institute members for the various purposes. This includes normal library operations (Project NIEHS-OSI-003), personal services according to profiled requirements, and meeting demands for special purposes. It provides an essential input to the growing demand for position papers on a variety of subjects (Project NIEHS-OSI-005). Much of the information secured becomes potential input to the QRS (Project NIEHS-OSI-002). Staff of 3 full time.

PURPOSED COURSE: To develop services to a smoothly operating and responsive organization.

Serial No.: NIEHS-OSI-005
1. Office of Associate Director for Scientific Information & Communication
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Scientific Information Evaluation and Reporting Service"

PREVIOUS SERIAL NUMBER: NIEHS-OSI-05

PRINCIPAL ADMINISTRATOR: Douglas H. K. Lee, M.D., Ph.D.

OTHER ADMINISTRATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.6
Professional:	0.4
Other:	0.2

PROJECT DESCRIPTION

OBJECTIVES: The objective is to evaluate the relative significance of various environmental stresses and preparation of reports on selected topics in environmental health.

BACKGROUND: a. Efficient allocation of Institute resources to specific investigations presupposes a knowledge of the range of environmental situations that affect man, the relative seriousness of their effects, the feasibility of ameliorating the results, and the extent of investigative attention already being given to the significant situations.

b. The Directorate is frequently called upon to present position papers on various environmental health matters of national import.

c. One task the Institute has set itself is to arrange for the preparation of review articles on topics in environmental health which are important but which have not been adequately reviewed.

MAJOR ACCOMPLISHMENTS: The emphasis this year switched to the development, in conjunction with the Fogarty International Center, of four book-length documents on topics relevant to the UN Conference on the Human Environment (previously reported in OSI-09). That on Metallic contaminants is well along. That on Environmental Factors in Respiratory Disease is almost ready for systematic editing. That on Chemical Mutagens has been given to Dr. Eldon

Sutton (Texas) for editing. The fourth item on Multiple Factors is still in the planning stage. Contributions were made to a document on health policy for the Assistant Secretary, proposals for the UN Conference on the Human Environment, and the President's report on the health aspects of the environment. Extensive editing was done on a book, Pulmonary Responses to Coal Dust, in collaboration with BOSH. Addresses to professional and educational groups continue. There have been 24 items added since July 1, 1970 to the Response to Inquiry File. Investigators attending a Workshop on the Epidemiology of Heat Effects reviewed a draft report prepared at Yale for the Council on Environmental Quality, and examined suggested statistical methods for predicting heat death rates from meteorological data.

PROPOSED COURSE: To complete documents in conjunction with FIC. Participate in organization and conduct of NYAS Conference on Coal Workers' Pneumoconiosis. Development of "domestic" review documentation.

Serial No.: NIEHS-OSI-006
1. Office of Associate Director for Scientific Information & Communication
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Beryllium Case Registry"

PREVIOUS SERIAL NUMBER: NIEHS-OSI-06

CONTRACTOR'S PROJECT DIRECTOR: H. Kazemi, M.D., Chief, Pulmonary Unit

PROJECT OFFICER (NIEHS): Douglas H. K. Lee, M.D., Ph.D.

COOPERATING UNITS: NIOSH

MAN YEARS:

Total:	1.0
Professional:	0.9
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: The objective of this project is to maintain the Beryllium Case Registry originally set up in 1952 by Dr. Harriet Hardy, providing for: (a) periodic appeal to physicians to report all suspected cases of beryllium poisoning; (b) examination of reports by an expert panel to decide admission to the Registry; (c) acquisition of additional evidence of diagnostic significance; (d) diagnostic assistance to the reporting physician; (e) periodic follow-up on progress of cases.

The importance of maintaining the Registry as an information source lies in: (a) inadequate knowledge of late disease manifestations; (b) changing patterns of exposure to beryllium; and (c) uncertainities about the basic patho-physiological processes.

MAJOR ACCOMPLISHMENTS: The Registry is in full operation at the Massachusetts General Hospital. It consists of 806 cases, of which 275 are dead. Contact has been re-established with one quarter of the 531 presumed living cases. Attempts are being made to extend the contacts by direct communication with the patients. Cooperation has been established with the Pennsylvania State Department of Health and 60 living cases have been added to the Registry from this source. Another 60 dead cases not previously in the Registry are being added from this source. 6 new cases were added to the Registry in 1970, and 7 "doubtfuls" will probably be added. The radiographs are being systematically reviewed.

PROPOSED COURSE: To continue operation of Registry and standardization of records. A conference of participating physicians is arranged for May, 1971.

Serial No.: NIEHS-OSI-009
1. Office of Associate Director for Scientific Information & Communication
2.
3. Research Triangle Park, N.C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Participation in International Programs"

PREVIOUS SERIAL NUMBER: NIEHS-OSI-09

PRINCIPAL ADMINISTRATOR: Douglas H. K. Lee, M.D., Ph.D.

OTHER ADMINISTRATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.8
Professional:	0.4
Other:	0.4

PROJECT DESCRIPTION

OBJECTIVES: The objective is to participate in those aspects of international programs operating through DHEW that are relevant to the NIEHS mission.

BACKGROUND: With the transfer of the categorical environmental programs out of DHEW, NIEHS becomes a mainstay of DHEW interests in environmental health. This applied particularly to international programs, in which environmental aspects are getting increasing attention. Close cooperation is maintained in this connection with the Fogarty International Center and with the Office of International Health.

MAJOR ACCOMPLISHMENTS: Arrangements have been made for Dr. J. Wyatt, Winnipeg, to spend 2 months in the Groupe de Recherche de Physio-Pathologie Respiratoire at Nancy, under the bilateral agreement with INSERM.

Proposals have been prepared by request for consideration by the Preparatory Committee for the 1972 UN Conference on the Human Environment.

(Because of the wider readership involved, comment on the preparation of environmental books jointly with FIC has been transferred to Project OSI-05).

At a meeting with Japanese representatives in February, it was decided to restrict the proposed environmental project under the US-Japan Cooperative Medical Sciences Program to carcinogenesis.

PROPOSED COURSE: Arrangements for a reciprocal visit by Dr. Sadoul under INSERM arrangements. To continue cooperation with OIH and EPA on material for the 1972 UN Conference.

OFFICE OF THE ASSOCIATE DIRECTOR FOR EPIDEMIOLOGY AND BIOMETRY

OFFICE OF THE ASSOCIATE DIRECTOR FOR EPIDEMIOLOGY AND BIOMETRY
Summary Statement

Progress in development of program activities in the biometry-epidemiology area has been consistent with the availability of resources and the need to provide support to other segments of the Institute program. The primary function of the Biometry Branch has been and continues to be direct participation in the statistical design and analysis of laboratory experiments, construction of mathematical models and development of new analytic procedures appropriate to evaluation of multifactor experiments of interacting environmental agents. The development of probability models for analysis of competing mortality risks has been given priority consideration.

The increased complexity and volume of statistical analyses performed by the Biometry Branch in support of the intramural program has been sufficient to warrant installation of remote terminals which provide access to the computers of the Division of Computer Research and Technology, NIH, and the Triangle Universities Computation Center, Research Triangle Park, North Carolina. Biometry Branch staff members have provided a broad spectrum of advisory and consultative service to various expert committees and related Federal agencies; major effort has been devoted to design and analysis of studies for the Office of the Surgeon General and the Interagency Mining Review Group on the problem of lung cancer among uranium miners.

Program planning and development activities of the Epidemiology Branch included efforts to develop improved biomedical data resources at the national level, design large-scale studies of environmental determinants of congenital malformations and organize long-term prospective studies of occupational groups at high risk for exposure to pesticides and other environmental chemicals. Efforts to recruit epidemiologic staff have continued and two investigators will join the program during the next year.

During the past year the Occupational Studies Unit (Epidemiology Branch) has pursued investigations of environmental hazards among uranium miners, steel and cutting oil workers, chromate painters, and hardrock miners. Several studies have been completed and results published. During the year the Occupational Studies Unit was moved from Bethesda, Maryland, to the Research Triangle Park, North Carolina; several staff members and some occupational studies were transferred to the National Institute of Occupational Safety and Health.

BIOMETRY BRANCH

BIOMETRY BRANCH
Summary Statement

Due to the increase in the complexity and volume of statistical analyses performed by the Biometry Branch for the Intramural Research Program, remote computer terminal units have been installed. These provide access to the large IBM/360 computers of the Division of Computer Research and Technology, NIH, Bethesda, Maryland, and the Triangle Universities Computing Center, Research Triangle Park, North Carolina. Considerable effort is now being expended to assimilate procedures to make use of the enormous number of existing programs available at these two centers. In addition to scientific computing, applications of budgetary, personnel and research expenditures are being considered. One member of the Biometry Branch attended an NIH short course on programming and machine usage, including instruction on WYLBUR.

Histological data on uranium miner lung cancers were collected and classified by Dr. Geno Saccomanno, St. Mary's Hospital, Grand Junction, Colorado, and his associates. The materials were from lung cancer cases representing both uranium miners and non-miner controls matched for age and cigarette smoking habits. Under the auspices of the Interagency Mining Review Group and the Surgeon General, a new panel of three pathologists was formed to seek confirmation of the previous histological findings. A study was designed by the Biometry Branch employing rigorous statistical procedures of coding, randomness, and replication to support the validity of the findings of the panel of pathologists. Statistical analyses of the data indicated close agreement among the findings of the pathologists. The original conclusion that there was a statistically significant excess of small cell anaplastic carcinoma among cancer cases from uranium miners was confirmed. Also, among uranium miners with lung cancer there was a statistically significant positive relationship between cumulative working level months of exposure and an excess of small cell anaplastic carcinomas.

A large proportion of the services provided by the Biometry Branch continue to be in the analysis of teratogenic data. Therefore, some effort was expended to investigate and improve statistical methods used in teratology. In particular, the non-parametric Mann-Whitney U-test, for comparing two groups, requires an underlying continuous distribution. This condition appears to be approximately satisfied with most discrete teratological data with the possible exception of the large proportions of litters sometimes exhibiting no anomalies or mortality. The effect of these zeros on the U-test is being studied by development of probabilistic models and computer simulations. By treating percent anomalies (or mortality) as continuous data truncated at zero, it is possible to construct statistical tables for handling small sample sizes.

Studies are under way to develop efficient experimental designs for selecting the best of several experimental treatments. Data from laboratory or clinical trials are assumed to occur sequentially in time with dichotomous

responses. The emphasis of these designs is to minimize the number of trials using inferior treatments, which is critical for human trials. Several superior truncated selection procedures have been developed which use play-the-winner sampling.

Probability models and competing risks in mortality data are being studied to develop statistical techniques for assessing the effects of a treatment on specific diseases. A probabilistic model has been developed for describing occurrences of particular diseases in a competing risk framework. Non-parametric techniques are used to test and estimate the model giving both the disease incidence and cumulative mortality adjusted for competing causes of death. These methods are more efficient than classical interval techniques of mortality estimation which require large sample sizes.

Two long-term factorial experiments on rats were performed by General Foods to assess the interactive effects of piperonyl butoxide with DDT and with 2,7-fluoreuyl diacetamide. For various combinations of dose levels the quantities studied were total cumulative mortality, incidences of total malignant and total benign tumors. Also, incidences of tumors restricted to particular sites were considered. The analytical methods used were those developed under NIEHS-B-006 Probability Models and Competing Risks in the Study of Mortality Data.

The major emphasis of the Biometry Branch continues to be the provision of statistical analyses of laboratory data collected by scientists in other branches at NIEHS. Many of these results were subsequently submitted for publication by NIEHS scientists. Brief descriptions of several of these projects follow.

Statistical analyses were performed on a series of experiments with pregnant rats and mice to investigate the teratogenic and fetotoxic effects of NTA. Two metals were studied in combination with NTA, cadmium chloride and methyl mercury chloride. Also, NTA in combination with zinc, calcium, or iron deficient diets was investigated. Due to the replication of treatments obtained through the use of factorial experimental designs, it was possible to detect some effects which would have gone unnoticed by a simple comparison of a treated group with the control group. In other cases the use of statistical tests of hypotheses and statistical confidence limits indicated that the precision of the results was not sufficient to warrant firm conclusions.

Data were analyzed from a study investigating the teratogenic potential of cadmium in rats. When $CdCl_2$ was administered on days 14-17 of gestation, incidences of fetal anomalies (principally micrognathia, cleft palate and small lungs) were found to follow a dose-response relationship. A similar pattern emerged for fetal mortality and fetal weight.

A series of experiments were performed to evaluate the teratogenic and toxicologic effects of various esters of 2,4,5-T and of 2,4,5-Trichlorophenoxypropionic acid on the random bred CD-1 mouse. Although all of the esters produced a significant increase in fetal mortality and in the incidence of cleft palate in at least one experimental run, generalizations about the effects of the iso octyl ester could not be made since the experimental results were not

consistent over all replications. Animals treated with 2,4,5-Trichlorophenoxypropionic acid had no cleft palate, but there was some indication of a fetotoxic effect at the highest dose level studied. It was noted that all of the compounds under investigation caused a significant increase in maternal liver/body weight ratio and, in most instances, a reduction in fetal weight. A companion study of two different doses of Silvex in rats indicated that the treated animals displayed an increased liver/body weight ratio and a decreased maternal weight gain. In addition the average fetal weight in the treated litters was also reduced. Furthermore, these responses seemed to be dose related.

Tests were conducted to determine the teratogenic effects of the pesticides aldrin, dieldrin and endrin in the hamster and mouse. Non-parametric test procedures and analysis of variance techniques were used. It was found that all three compounds significantly increased cleft palate, webbed foot and open eye in the hamster. Increased fetal mortality and reduced fetal weight were also observed. In the mouse studies, all three pesticides significantly increased fetal anomalies, but had little effect on fetal mortality or fetal weight.

Various doses of chlorcyclizine, a known cleft palate inducer, were given to pregnant rats in order to determine to what extent cleft palate might be associated with fetal thorax size. In addition, the relationship between the incidence of cleft palate and the nature of the cleft produced (i.e., horizontal or vertical) was also investigated. Both within and between litter analyses were performed and standard regression techniques and certain non-parametric procedures were employed.

Statistical analyses were performed to determine the effect over time of DDT on enzyme induction in the liver of rats. Enzyme induction of acetylsalicylic acid, phenacetin, phenobarbital, stelazine, pyrene and diphenylhydantoin was compared with that of DDT and controls.

Data were analyzed from a study investigating mercury toxicity as reflected by fetal weight loss and tissue retention of mercury. Rats were examined two weeks after the last of five injections of methyl mercury hydroxide. It was found that the treated animals had gained significantly less weight and had greater concentrations of mercury in various body tissues than the controls.

In a study of the distribution and retention of paraquat in the liver, muscle, kidney, lung, and blood of rats, dose effects were assessed by use of a crossed analysis of variance with sub-sampling. Mathematical models to describe paraquat concentrations as a function of time were investigated. The numbers of tissues per animal and the number of animals per dose required to obtain various levels of precision of estimates were determined.

Statistical analyses were performed to estimate the levels of dieldrin deposition in rat fetuses at various time periods following pesticide administration. Analysis of variance techniques were used to compare three different days of gestation and to evaluate the effect of pre-treatment with phenobarbital. Similar analyses were performed in a study comparing the fetal and maternal plasma levels of diquat and paraquat.

A computer program was written to estimate the constants in the Michaelis-Menten equation for describing kinetic enzyme reactions. Four techniques (three linear and one non-linear) were employed. A computer program for plotting the data is being investigated. Other techniques for detecting departures from the simple first-order model are being investigated. The standard errors of estimates are being studied to determine the best estimation procedure. Also, the nature of the variance of velocity measurements is being considered to determine the best weighting scheme to use for kinetic enzyme reaction data.

An investigation is in progress on the methods of constructing isokinetic gradients for rate-zonal centrifugation. Very complicated computer techniques are required.

A statistical analysis was made to determine the relative effectiveness of several compounds in reducing the number of parasites (Physaloptera, Trematodes, Trichostrongyles, Whipworm and Cruzial) in opossums. L. Tetramisol, Parvex, Dichlorvos, Nemural, Thiabendazole and certain combinations of three agents were tested.

An investigation was conducted to determine the effects of high doses of inorganic iodide on opossum thyroid function. Groups of animals were sacrificed after one, two, three and four weeks of treatment. In each instance, the MIT, DIT, T_3 and T_4 levels and the percent of protein-bound iodide were ascertained. In addition, pituitary weight, thyroid weight and RAIU were determined.

Statistical analyses were performed to determine the precision of estimates and to test the effects of amitrol on body weight, pituitary growth and thyroid growth in a small study where amitrol was administered to opossums.

A study was made to determine how effectively the weight of a new-born opossum could be estimated from its age and/or length. When regression model building techniques were employed, it was found that a quadratic equation based on age gave an excellent fit, resulting in a maximum percent error of only 29%, for the 73 animals examined. A quadratic equation using length was found to be almost as good a predictor of weight. More complicated regression equations improved only slightly the fit obtained by these simple quadratics. Thus, weights can be estimated for dosage purposes without actually removing and thereby losing one or more newborn animals per litter.

Further analysis of data from a series of nine experiments performed for the purpose of evaluating the role of B. pertussis vaccine in ozone mortality led to a procedure for combining the original set of nine experiments into three larger subgroups, thereby increasing the sensitivity of the test procedures employed.

In response to a growing interest in studies based on human populations, various efforts are being undertaken to increase the activity of the Biometry Branch in the area of statistical epidemiology. Included in these efforts are the preparation of an annotated bibliography of the most recent papers in this area and the participation of two members of the Branch in a summer post-graduate short course in selected topics in epidemiology offered by the University of Minnesota.

A number of manuscripts prepared by NIEHS scientists for publication were reviewed by the Biometry Branch for adequacy of statistical analysis and interpretation. In addition, articles on statistical theory and techniques for evaluating biological effects from laboratory or epidemiological studies were reviewed.

Teaching services were provided by Dr. D. W. Gaylor for the Department of Experimental Statistics, North Carolina State University. He took part in presenting a graduate course on Advanced Topics in the Analysis of Variance and Components of Variance. Dr. Gaylor is serving as thesis advisor for a Ph.D. candidate.

PUBLICATIONS

Gaylor, D.W., Lucas, H.L., and Anderson, R.L.: Calculation of expected mean squares by the abbreviated Doolittle and square root methods. Biometrics 26:641-655, 1970.

Haseman, J.K. and Elston, R.C.: The estimation of genetic variance from twin data. Behavior Genetics, Vol. I, No. 1, 1970.

Haseman, J.K. and Elston, R.C.: The investigation of linkage between a quantitative trait and a marker locus. Behavior Genetics, Vol. I, No. 4, 1970.

Hoel, D.G.: A simple two-compartmental model applicable to enzyme regulation. J. Biol. Chem. 245:5811-5812, 1970.

Hoel, D.G. and Mitchell, T.J.: The simulation, fitting and testing of a stochastic cellular proliferation model. Biometrics 27:191-199, 1971.

Kastenbaum, M.A., Hoel, D.G., and Bowman, K.O.: Sample size requirements: one-way analysis of variance. Biometrika 57:421-430, 1970.

Kastenbaum, M.A., Hoel, D.G., and Bowman, K.O.: Sample size requirements: randomized block designs. Biometrika 57:573-577, 1970.

Crump, K.S. and Hoel, D.G.: Some applications of renewal theory on the whole line. J. Applied Probability 7:734-746, 1970.

Gaver Jr., D.P. and Hoel, D.G.: Comparison of certain small-sample Poisson probability estimates. Technometrics 12:835-850, 1970.

Hoel, D.G. A method for the construction of sequential selection procedures. Annals Math. Statistics 42:1971.

Serial No.: NIEHS-B-003
1. Biometry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Statistical Design and Analysis of a Pathological Study of Uranium Miner Lung Cancers"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: D. W. Gaylor, Ph.D.

OTHER INVESTIGATORS: M. D. Hogan, Ph.D. and J. K. Haseman, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	0.2
Professional:	0.2
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: Histological data on uranium miner lung cancers previously were collected and classified by Dr. Geno Saccomanno, St. Mary's Hospital, Grand Junction, Colorado, and his associates. Under the auspices of the Interagency Mining Review Group and the Surgeon General, a new panel of three pathologists was formed to seek confirmation of the previous histological findings.

METHODS EMPLOYED: A study was designed by the Biometry Branch employing rigorous procedures to insure the validity of the statistical findings of the new panel of pathologists. Tissue slides were coded so that it was unknown to the pathologists whether or not the cases were uranium miners or non-uranium miners. Slides were presented to the pathologists in a random order. For several cases, more than one slide was available. Second readings on some slides were obtained to check internal consistency. Approximately 300 histological slides were classified from 121 cases of bronchogenic carcinoma among American uranium miners and 118 cases from non-uranium miners. The control cases were selected from the same geographical region and had been matched with a miner of nearly the same age and cigarette smoking history.

MAJOR FINDINGS: Histological agreement between the two panels was achieved on more than 90% of the cases. Thus, the original conclusion that there was a statistically significant excess of small cell anaplastic carcinoma among lung cancers from uranium miners was confirmed. There was a statistically significant increase in the incidence of small cell anaplastic carcinomas from 20%

in the controls up to 80% in miners exposed to 700-1200 WLM of radon or more.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These results strengthen the establishment of higher lung cancer rates among uranium miners and will assist in the establishment of maximum permissible radon levels in uranium miners.

PROPOSED COURSE: This project has been completed.

Serial No.: NIEHS-B-004
1. Biometry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Statistical Methodology and Modeling in the Analysis of Teratogenic Data"

PREVIOUS SERIAL NUMBER: None

PRINCIPAL INVESTIGATORS: D. G. Hoel, Ph.D.

OTHER INVESTIGATORS: J. K. Haseman, Ph.D. and M. D. Hogan, Ph.D.

COOPERATING UNITS: None

MAN YEARS:

Total:	0.5
Professional:	0.5
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To develop probabilistic models and new statistical techniques for the analysis of teratogenic data. In particular, to obtain a procedure to compare two treatments when a large proportion of the animals in both groups exhibit no observed defects.

METHODS EMPLOYED: The Mann-Whitney U-test is a non-parametric procedure and therefore is rather versatile. This test has been used rather extensively to compare congenital malformations of two groups of animals. The U-test does require an underlying continuous distribution. This condition appears to be adequately satisfied with discrete (e.g., anomalies or mortality) teratological data with the exception of the large proportions of litters sometimes exhibiting no anomalies or mortality. By studying the underlying probabilistic structure of teratological data improved techniques for statistical analyses are being studied. These procedures are then evaluated by computer simulation studies.

MAJOR FINDINGS: By treating percent anomalies as truncated (at zero) continuous data, it can be shown that the usual non-parametric tests for censored observations yield the same statistic as the Mann-Whitney statistic corrected for ties. It is possible to construct statistical tables for handling small sample laboratory data on anomalies.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This research will determine the appropriateness of the Mann-Whitney U-test where

large proportions of negative responses are obtained and will also serve to indicate what modifications of the test are necessary. By the use of improved statistical techniques more efficient use of laboratory data is made. Through modeling some contribution to a better understanding of biological mechanisms may result.

PROPOSED COURSE: Because of extreme variation and many factors influencing teratological data a continuing effort is required for the improvement of statistical techniques.

Serial No.: NIEHS-B-005
1. Biometry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Sequential Selection Procedures"

PREVIOUS SERIAL NO.: None

PRINCIPAL INVESTIGATOR: D. G. Hoel, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.5
Professional:	0.5
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To develop efficient experimental designs for selecting the best of several experimental treatments.

METHODS EMPLOYED: Data from controlled laboratory or clinical trials are assumed to occur sequentially in time where the individual responses are dichotomous. Sequential regions have been developed for deciding when to terminate the experiment and which treatment to select as most effective.

MAJOR FINDINGS: Several new selection procedures have been developed and were shown to be superior to the existing ones. These new procedures use play-the-winner sampling and one of them is truncated which is of considerable importance in sequential sampling.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The emphasis of these sequential designs is to minimize the number of trials on inferior treatments. This has been overlooked in classical clinical trials and is a critical concern when dealing with human populations.

PROPOSED COURSE: Continued improvement in the efficient use of laboratory and clinical trial data is required. Also investigations into delayed clinical responses is needed.

Serial No.: NIEHS-B-006
1. Biometry Branch
2.
3. Research Triangle Park, N. C.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Probability Models and Competing Risks in the Study of Mortality Data"

PREVIOUS SERIAL NO.: None

PRINCIPAL INVESTIGATOR: D. G. Hoel, Ph.D.

OTHER INVESTIGATORS: None

COOPERATING UNITS: None

MAN YEARS:

Total:	0.5
Professional:	0.5
Other:	0.0

PROJECT DESCRIPTION

OBJECTIVES: To develop statistical techniques for assessing the effects of a treatment on specific diseases.

METHODS EMPLOYED: A probabilistic model has been developed for describing the occurrences of particular diseases in a competing risk framework. Non-parametric techniques are used to estimate the model giving both the disease incidence and cumulative mortality adjusted for competing causes of death. Non-parametric tests of significance are also employed.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These methods are much more efficient in their use of experimental data than the classical interval techniques of mortality estimation which require much larger sample sizes. Also a clearer picture of mortality patterns is produced.

PROPOSED COURSE: A detailed study of disease mechanisms is required with particular emphasis on interactions between diseases.

EPIDEMIOLOGY BRANCH

OCCUPATIONAL STUDIES UNIT
EPIDEMIOLOGY BRANCH
Summary Statement

During the fall of 1970 the decision was made to move the Occupational Studies Unit to the Research Triangle Park. Most of the personnel preferred to remain in the Washington, D. C. area and have obtained employment with other agencies. As a result of relocation, there was insufficient staff to continue the program. Consequently, the program of this Unit was transferred to the Bureau of Occupational Safety and Health (with the exception of Dr. Payne's study of Zinc Chromate painters).

The program of the Occupational Studies Unit during the past year has emphasized four major areas of activity. First was the continued collection, processing and analysis of data on groups of uranium miners, steelworkers, cutting oil workers, chromate painters, and hardrock miners. Second was the cataloging of information from the scientific literature which interrelates employment in specific occupations with known or suspected pathogenic agents and the incidence of chronic disease. A third activity, somewhat related to the second, was the establishment of efficient systems for computer storage and retrieval for reference material and tabular material required for analyses. The fourth activity of the Occupational Studies Unit was the maintenance and furhter development of the follow-up program for tracing industrial populations over long periods of time.

Because of limitations in professional staff and the need for meeting specified deadlines on publication of certain findings, our efforts in the latter two areas of activities have, of necessity, been in low key. The major in-house program during the year has again centered around the updating and analysis of uranium miner data for the Federal Radiation Coucil and other related activities; continued updating and analysis of steelworker mortality experience and overseeing of the University of Pittsburgh contract (see PH-43-62-147); establishment of a data file on cutting oil workers and overseeing of the Wayne State University contract (see PH-43-66-53); and limited efforts to complete the data files on chromate painters and hardrock miners.

A number of manuscripts were published or are in the process of publication. A very extensive analysis of uranium miner data was completed and reported for use in setting standards. The fourth and fifth papers of a serial presentation of steelworker findings, and three publications in other areas were completed during the year.

Other activities of the Unit during the year include service as members of scientific committees, as consultants to special research groups, and as faculty members of academic institutions; and affecting liaison with and obtaining cooperation of local, state, and federal agencies for obtaining vital statistics, death certificates, and for follow-up activities.

Because there is no central repository of information on chronic disease in relation to occupation, a key activity of the Occupational Studies Unit was the gathering and cataloging of reference material in this area. For that purpose, a senior staff member was assigned to review all pertinent

scientific literature, particularly as it relates to current studies, and to establish a reference system for rapid retrieval of information. This is currently a manual activity with the exception of occasional requests for MEDLARS bibliographies from the National Library of Medicine. This staff member attended a three day seminar in Abstracting and Indexing for ADP Systems and found that the manual method would be quite satisfactory for some time to come.

During the past year, our systems analyst has established efficient procedures for creation of data files on each of our study populations which allows for rapid input and correction of information on follow-up disease status and rapid output of tabular material required for analyses of these data. After some research and experimentation, a suitable way has been found to develop a computer-based Job and Exposure Index to aid in answering the following questions. What pathogenic agents are associated with employment in specific occupations? Which occupations are associated with exposure to specific pathogenic agents? What are the published sources of such information?

As part of the overall plan for an integrated data retrieval program, we have utilized a system whereby typewritten reports are produced immediately in response to specific inquiry by means of a remote terminal linked to the NIH central computer facility.

J. William Lloyd, Sc.D. was the NIEHS representative on the National Center for Health Statistics Panel of Advisors, and was lecturer at Georgetown University School of Medicine. Frank E. Lundin, Jr., M.D. was a faculty member of the Department of Epidemiology at Johns Hopkins School of Hygiene and Public Health and a consultant to the Cervical Cancer Morbidity Study at the University of Louisville. Hugh W. Rush served as the PHS representative of the Greater Pittsburgh Federal Executive Association. Winifred M. Mendez, R.N., B.S., served as a consultant to the Cervical Cancer Morbidity Study at the University of Louisville.

PUBLICATIONS

Christopherson, W. M., Mendez, W. M., Ahuga, E. M., Parker, J. E., and Lundin, F. E., Jr.: Cervix cancer control in Louisville, Kentucky. Cancer 26: 29-38, 1970.

Christopherson, W. M., Parker, J. E., Mendez, W. M., and Lundin, F. E., Jr.: Cervix cancer death rates and mass cytologic screening. Cancer 26: 808-811, 1970.

Christopherson, W. M., Mendez, W. M., Parker, J. E., Lundin, F. E., Jr.: Carcinoma of the Endometrium: A study of changing rates over a 15 year period. Cancer. (In press).

Serial No.: NIEHS-E-001
1. Epidemiology Branch, OASDD
2. Occupational Studies Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Mortality Among Steelworkers"

PREVIOUS SERIAL NUMBER: NIEHS-E-01

PRINCIPAL INVESTIGATOR: J. William Lloyd, Sc.D.

OTHER INVESTIGATORS: F. E. Lundin, Jr., M.D.; A. Ciocco, Sc.D.; E.M. Ahuja, M. Sc.; C. K. Redmond, Sc.D.; P. G. Geiser, M.S.; W. M. Mendez, B.S.; and H. W. Rush

COOPERATING UNITS: Graduate School of Public Health, University of Pittsburgh, and the American Iron and Steel Institute

MAN YEARS:

Total:	3.5
Professional:	2.4
Other:	1.1

PROJECT DESCRIPTION

OBJECTIVES: To determine which excesses and deficits in cause-specific mortality are associated with particular occupational subgroups in the steel industry and how this might be related to length of exposure. To determine the interrelationships among variables, not necessarily related to occupational exposures which have been previously associated with differential risks for specified causes of death. To develop methodological techniques for the analysis of long-term occupational studies and to develop mathematical models for assessment of latent effects and risk associated with particular occupational exposures at specified intervals and at various ages.

METHODS EMPLOYED: A study is being made of the mortality experience of approximately 59,000 steelworkers employed by three major firms in Allegheny County, Pennsylvania in 1953. Occupational histories and pertinent demographic information have been abstracted from employment records of the three firms. The vital status of each employee through 1961 has been determined by follow-up through Social Security records, postal inquiry, search of city directories, and reference to records of other governmental agencies. Extension of follow-up through 1966 was initiated during 1967.

A study of coke-oven worker mortality is being made through the cooperation of ten steel plants distributed throughout the United States and Canada. Employment records are collected on all men employed at the coke ovens between 1951 and 1955, and a two-for-one control group of other steelworkers matched for race and date of initial employment. Determination of vital status through 1966 is made by reference to Social Security records and other appropriate follow-up procedures.

Collaboration on the collection and analysis of steelworker and coke-oven worker data is provided by the University of Pittsburgh, Department of Biostatistics, under contract with the National Institute of Environmental Health Sciences (PH-43-62-147) and the American Iron and Steel Institute.

MAJOR FINDINGS: Analysis of Allegheny County data through December 1961, showed that non-white coke-oven workers employed at the "topside" of the ovens had experienced lung cancer mortality markedly in excess of that predicted by the mortality rates for other non-white steelworkers (14 observed vs. 2.2 expected). The level of risk for white "topside" workers could not be determined because of the small number of white men employed in that area. The mortality experience of men employed at other areas of the coke-ovens was also suggestive of a possible lung cancer excess for white (8 observed vs. 5.3 expected) and non-white (9 observed vs. 5.9 expected) employees.

The finding of such a striking mortality differential in the non-white "topside" workers could not be explained by any bias in selection or differences in demographic characteristics of coke-oven workers and other steelworkers in Allegheny County.

A number of other occupational groups within the steel industry may have experienced higher than expected mortality but because the numbers of years at risk through 1961 for men employed in these occupations was too small to allow for a firm conclusion of excess risk, the study period was extended through 1966.

The updated report on coke-oven workers shows that men employed in this area in steel plants outside of Allegheny County, Pennsylvania also experience an excess of lung cancer. The excess is seen in both non-white and white workers.

As a by-product of this investigation, the efficacy of various techniques for the follow-up of industrial populations has been explored and the results have been published.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: These studies will provide information regarding the pathogenesis and epidemiology of cancer and other chronic disease as related to certain occupational exposures. They are also of value in developing methodologies for field studies of chronic illness consequent to exposure in the occupational environment.

PROPOSED COURSE: Work histories for the Allegheny County study population have been up-dated, and follow-up to determine vital status through 1966 has been completed. Records of coke-oven workers and controls have been collected

from ten plants cooperating in the non-Allegheny County phase of the study. Records from two of the 12 plants initially contacted by the American Iron and Steel Institute, proved inadequate for our purposes. Follow-up of steelworker and coke-oven worker populations was delayed by the heavy work load at the Social Security Administration. Consequently, original deadlines were extended. A report of Allegheny County steelworker experience through 1966 and a report on the non-Allegheny County coke-oven worker experience have been completed. A fourth paper on mortality by work area and a fifth paper on mortality by work area within the coke plants have been published.

The University of Pittsburgh is completing its work for NIEHS under a "no-cost" extension to July 1, 1971. It is anticipated that this research will be continued through contract with the Bureau of Occupational Safety and Health.

HONORS AND AWARDS: At the Annual Meeting of the Industrial Medical Association on April 21, 1971, in Atlanta, Georgia, Dr. J. William Lloyd and his colleagues were awarded the Adolph G. Kammer Merit in Authorship Award for the two papers entitled, "Long-Term Mortality Study of Steelworkers, Part III Follow-Up" J. Occup. Med. 11: 513-521, Oct. 1969, and "Long-Term Mortality Study of Steelworkers, Part IV Mortality by Work Area" J. Occup. Med. 12: 151-157, May 1970. The award is for the best publication in the field of occupational health during the period September 1, 1969, to August 31, 1970.

PUBLICATIONS

Lloyd, J.W., Lundin, F.E., Redmond, C.K., and Geiser, P.B.: Long-term mortality study of steelworkers, IV: Mortality by work area. J. Occup. Med. 12: 151-157, 1970.

Lloyd, J.W.: Long-term mortality study of steelworkers, V: Respiratory cancer in coke plant workers. J. Occup. Med. 13: 53-68, 1971.

Serial No.: NIEHS-E-003
1. Epidemiology Branch, OASDD
2. Occupational Studies Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Epidemiological Study of Lung Cancer in Workers Exposed to the Inhalation of Zinc Chromate Paint in Airplane Construction and Maintenance Plants"

PREVIOUS SERIAL NUMBER: NIEHS-E-03

PRINCIPAL INVESTIGATOR: William W. Payne, Sc.D.

OTHER INVESTIGATORS: E. M. Ahuja, M.Sc.; M. F. Heid; and J. W. Lloyd, Sc.D.

COOPERATING UNITS: U. S. Air Force, Social Security Administration, and a private airplane manufacturing company

MAN YEARS:

Total:	0.3
Professional:	0.2
Other:	0.1

PROJECT DESCRIPTION

OBJECTIVES: Epidemiological studies have shown that workers in chromate-producing plants have an abnormally high incidence of cancer of the lung. Observations in Europe suggest that workers exposed to chromium pigments also experience a respiratory cancer hazard, but epidemiological evidence is lacking.

The airplane manufacturing and maintenance industry appears to offer the best opportunity for studying workers who have been exposed to the inhalation of zinc chromate paints and related compounds. This follow-up study of persons who have been occupationally exposed to chromium compounds was designed to determine whether exposure to zinc chromate paint by inhalation results in an increased risk to lung cancer.

METHODS EMPLOYED: Arrangements were made with the U. S. Air Force to obtain the names of painters, whose employment had been terminated during the ten year period prior to July 1959, together with identifying information and a brief summary of each worker's employment at the base. For controls, similar data were obtained for workers in the same plants who were presumably not exposed to chromium. Date of birth, sex, and length of employment were matched as closely as possible. Unfortunately, no smoking histories were

available for these workers. Similar information was obtained from a private airplane manufacturing company for both painters and a control group, but detailed employment history was not available in a form that could be readily used.

Mortality follow-up through 1960, on the entire study population was initiated in 1961. From records of the Social Security Administration, and federal retirement records, the place and date of death were determined for decedents. Copies of death certificates were obtained from the respective State Health Departments, and where any form of cancer or any pulmonary disease was specified as the cause of death, additional information on diagnosis was requested from the attending physician. An analysis of these data revealed an increased risk of respiratory cancer for painters as compared with controls. However, this difference was not significant.

In 1968, Social Security Administration records were reexamined to determine how many additional deaths had occurred since 1960. Approximately 450 additional deaths were identified. Of these, 134 death certificates could not be located in the state of claim. A request for verification of date and place of death from the Social Security Administration Payment Centers has been received on all but 26 individuals. After the remaining data have been received and additional certificates have been located, the mortality experience of painters will be reevaluated.

MAJOR FINDINGS: The preliminary results for the chromate exposed workers of the two Air Force bases for 1950 through 1960 revealed that total malignant neoplasms comprised 29.8 percent of all deaths, and respiratory cancers 8.3 percent; whereas for controls, total malignant neoplasms comprised 15.6 percent of all deaths and respiratory cancer 3.4 percent.

The revised results which do not reflect the unlocated death certificates are as follows:

Number of Deaths and Proportional Distribution
by Cause, Chromate Painters, Platers, and Controls 1950-1966

	Painters and Platers		Controls	
	Number	Percent	Number	Percent
All Causes	163	100.0	247	100.0
Total Malignant Neoplasms	39	23.9	42	17.0
Digestive Organs	9	5.5	12	4.9
Respiratory System	16	9.8	12	4.9
Genito-Urinary Organs	5	3.1	7	2.8
Brain and CNS	2	1.2	0	0.0
Leukemia and Lymphoma	3	1.8	3	1.2
Other Sites	4	2.5	8	3.2

These findings indicate that the difference for total malignant neoplasms may not be as great as previously suggested. However, the two-fold excess for respiratory neoplasms for painters is still apparent.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: This study provides information regarding the epidemiology of respiratory cancer relative to zinc chromate paint exposure.

PROPOSED COURSE: After death information is received the Social Security Administration and the outstanding death certificates are located and coded, further analysis of these data will be undertaken. If the findings warrant, the study will be enlarged to afford an examination of work histories for the entire study and control populations.

Serial No.: NIEHS-E-004
1. Epidemiology Branch, OASDD
2. Occupational Studies Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Epidemiological Study of Occupational Exposure to Cutting Oil Mists"

PREVIOUS SERIAL NUMBER: NIEHS-E-04

PRINCIPAL INVESTIGATOR: Frank E. Lundin, Jr., M.D.

OTHER INVESTIGATORS: T. L. Nghiem, M.D.; L. G. Salvin, A. B.; J. W. Lloyd, Sc.D.; and W. M. Mendez, R.N., B.S.

COOPERATING UNITS: Wayne State University (see PH-43-66-53), Detroit, Michigan, and the cooperating industry

MAN YEARS:

Total:	1.5
Professional:	0.8
Other:	0.7

PROJECT DESCRIPTION

OBJECTIVES: The original objective was to determine whether occupational exposure to cutting oil mists produces significant disease of the respiratory tract. In particular, evidence of impaired pulmonary function, chronic bronchitis, obstructive emphysema, chronic pneumonitis, pulmonary fibrosis, other distinctive pathologic changes, and respiratory cancer was sought. More recently the objective has been restricted to those diseases that can be studied by mortality alone, particularly evidence of lung cancer.

METHODS EMPLOYED: Mortality study is carried out for approximately 24,000 subjects who have been employed by the cooperating industry for one year or more prior to the end of 1967, and part of which was after 1936. Approximately 10,000 to 12,000 of these subjects were exposed to cutting oil mists, the length and time of which is determined from employment records. The relative intensity of oil exposure is determined from work area and job title of each period of employment. Death certificates are located through retirement and insurance records and the Social Security Administration. Using a life-table technique, person-years-at-risk by age, race, and year are determined. The cause-specific mortality is compared among subgroups of the study population classified by degree of exposure to cutting oil mists, if any.

Approximately 1300 employees currently exposed to cutting oil mists and an equal number of control subjects have completed questionnaires which include smoking histories, and have had chest x-rays, pulmonary function tests, complete medical histories, and physical examinations. (The participating industry provided the medical personnel for this aspect of the study.)

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: Carcinogenic hazards for man can be identified by epidemiologic studies of the effects of selected occupational exposures. In this way, preventive measures can be undertaken. This study is an example of the kind of epidemiologic study which could be initiated provided the following conditions are obtained: 1) experimental evidence of carcinogenic activity, 2) a sufficient number of employees exposed over a period of years, and 3) collaboration between PHS, industry, and academic institutions.

PROPOSED COURSE: As originally planned, the study was to require 3 to 5 years for completion. Delay in recruiting an epidemiologist, the tragic loss of the Project Director and of the epidemiologist has required additional time for completion. The scope of the investigation was changed significantly during the last quarter of FY 68 to include all blue-collar workers, increasing the total number of records to be surveyed from 12,000 to 24,000 in order to obtain intra-company controls rather than use rates for the state in which the industry is located.

Creation of a master file containing relevant personal data and complete work histories on each of the 24,000 subjects is essentially complete, except for corrections which will be indicated by computer edits which are being made now. Mortality follow-up is in progress. It is hoped that sufficient resources will be available to add the smoking histories to the master file for the currently employed subjects before final termination of the University's contract. This information is vital for estimation of the effects on lung cancer mortality of differences in smoking habits among the subgroups of the population classified by degree of exposure to cutting oil mists. The study is being transferred to the Bureau of Occupational Safety and Health.

Serial No.: NIEHS-E-005
1. Epidemiology Branch, OASDD
2. Occupational Studies Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Health Hazards of Uranium Miners of the Colorado Plateau"

PREVIOUS SERIAL NUMBER: NIEHS-E-05

PRINCIPAL INVESTIGATOR: Frank E. Lundin, Jr., M.D.

OTHER INVESTIGATORS: V. E. Archer, M.D.; J. K. Wagoner, Sc.D.; L. G. Salvin, B.A.; J. W. Lloyd, Sc.D.; and D. A. Holaday, M.A.

COOPERATING UNITS: Salt Lake City Field Station, Bureau of Occupational Safety and Health, PHS

MAN YEARS:

Total:	2.5
Professional:	1.6
Other:	0.9

PROJECT DESCRIPTION

OBJECTIVES: Objectives of this project are to study the cause-specific mortality experience of underground uranium miners in relation to a standard medical examination findings, cigarette smoking, and airborne radiation exposure data; to determine the quantitative relationship between airborne radiation exposure and the incidence of respiratory cancer; to delineate the role of a number of factors which might influence carcinogenic response to airborne radiation; and to develop realistic models for relating environmental exposure to cancer and other chronic diseases.

METHODS EMPLOYED: Study is made of miners and millers of the Colorado Plateau area who volunteered for at least one physical examination and provided social and occupational data and sufficient data to permit follow-up study of their health status. (Physical examinations were performed in 1950, 1951, 1953, 1954, 1957, and 1960.)

For the past several years, sputum collected annually from actively employed uranium miners has been examined cytologically.

Follow-up is maintained through an annual census of the uranium mining industry and by the collection of information on individuals by mail questionnaires and other methods. Deaths are ascertained by a variety of methods including review of local newspapers in the area and through records of the Social Security Administration. Death certificates are obtained for known decedents and are classified as to cause of death by a nosologist.

Medical information from autopsy, hospital, and physician reports is obtained and histopathologic studies of both malignant and non-malignant tissues are made.

Approximately 43,000 radon-daughter measurements were made from 1950 through 1968. Measurements continue to be made with increased frequency. These are used for quantitating the exposure of uranium miners to radiation.

MAJOR FINDINGS: A study of mortality experience among 3,366 white underground uranium miners during the period 1950 through September 1968, has shown an excessive occurrence of respiratory tract cancer (70 deaths observed as compared with 11.7 expected).

Study of the quantitative relationship between airborne radiation and respiratory cancer shows that age-standardized incidence tends to increase from the lowest cumulative exposure category to the highest. The dose-response relationship persists when confounding variables are taken into account, among them being time since onset of radiation exposure as well as cigarette consumption. An evaluation of variability in mine radiation data and the miners' histories of uranium mining has failed to account for the excess of respiratory cancer deaths which have occurred down to and including miners with 120 to 359 working level months of exposure.

The pathology of respiratory cancer in uranium miners is unlike that observed among an age-smoking-residence matched control group while being similar to that observed among factory workers exposed to a radiomimetic agent, mustard gas.

Mortality from respiratory tract malignancy increased from a four-fold excess in an earlier analysis to a nine-fold excess for the succeeding 45 month period. A relatively greater excess occurred after the age of 45 years. This age effect could not be explained by amount of radiation exposure and number of years after onset of underground uranium mining.

The great excess of lung cancer among U. S. uranium miners is largely limited to cigarette smokers. However, data are insufficient to determine whether non-cigarette smokers also have a significantly increased risk.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The findings of the present quantitative study emphasize the value of developing analytical methods which utilize retrospective work histories and exposure data to identify and study an etiologic agent in environmental carcinogenesis. These data are being used to study the role in human lung carcinogenesis of the following factors: age at first exposure, latency, antecedent respiratory disease, tobacco consumption, and radiation dosage. Such knowledge will be of value in three ways: 1) to improve our understanding of the mechanisms of carcinogenesis in man, 2) to form the basis for standards of airborne radiation in mines (see FRC Report No. 8, JCAE Hearings, May - August 1967, and March 1969), and 3) to suggest means of controlling the health hazards of uranium mining in addition to limitation of exposure. On the basis of the study with regard to cigarette smoking and lung cancer in uranium miners, smoking is now prohibited in uranium mines -- which, of course, does not

serve to protect the miner if he smokes after working hours.

PROPOSED COURSE: In January 1971, an extensive report was submitted to the National Academy of Sciences for review by its ad hoc committee on the epidemiology of uranium miners lung cancer. This report is being published as a joint monograph of NIEHS and the National Institute of Occupational Safety and Health. The NIEHS component of the Uranium Miners study will then be transferred to the National Institute of Occupational Safety and Health.

HONORS AND AWARDS: Dr. Lundin has been an attendee of all meetings of the National Academy of Sciences Epidemiology Committee on Health of Uranium Miners. Drs. Lloyd and Lundin were members of the Epidemiology Subgroup of the Interagency Uranium Mining Review Group, and Dr. Lundin was a member of the Subgroup on Diagnosis and Therapy of Lung Cancer.

PUBLICATIONS

Lundin, F.E., Jr., Wagoner, J.K., and Archer, V.E.: Radon daughter exposure and respiratory cancer quantitative and temporal aspects: Report from the epidemiological study of United States uranium miners. (National Institute of Environmental Health Sciences and National Institute of Occupational Safety and Health Monograph) U.S. Department of Health, Education and Welfare, Public Health Service, 1971.

Serial No.: NIEHS-E-006
1. Epidemiology Branch, OASDD
2. Occupational Studies Unit
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

PROJECT TITLE: "Study of the Cancer Experiences of Non-Uranium Hard Rock Miners in the Rocky Mountain Area"

PREVIOUS SERIAL NUMBER: NIEHS-E-06

PRINCIPAL INVESTIGATOR: Frank E. Lundin, Jr., M.D.

OTHER INVESTIGATORS: J. W. Lloyd, Sc.D., E.M. Ahuja, M. Sc., L. G. Salvin, A.B.

COOPERATING UNITS: Certain mining companies and the Salt Lake Field Station, Bureau of Occupational Safety and Health, ECA, CPEHS, PHS

MAN YEARS:

Total:	1.2
Professional:	0.4
Other:	0.8

PROJECT DESCRIPTION

OBJECTIVES: Objectives of this project are to delineate the factor(s) responsible for the excess cancer mortality previously reported among long-term metal miners; to determine the quantitative relationship between cancer and degrees of exposure as measured by length of underground employment and ventilation practices; to study cancer mortality among metal miners with regard to the interaction of age at beginning of exposure, changing ventilation, latency, and dosage; and to develop retrospective methods for use in future studies of occupational carcinogenesis.

METHODS EMPLOYED: Company records of 120,000 metal miners were reviewed and the occupational, medical, and vital statistics data were abstracted for 16,000 men who worked for the industry in 1937 or later, with initial employment prior to 1954 and with one or more years of underground service.

Deaths and reported disability are ascertained by review of company files and through records of claims from the Social Security Administration.

Death certificates are obtained from state health departments and classified by the staff nosologist.

The modified life-table technique will be used to compare the miners' cancer mortality experience by age at first exposure, latency, dosage, ventilation standards, calendar year, and age with comparable rates for white males living in the same area.

MAJOR FINDINGS: The life-table analysis has shown an excessive occurrence of pulmonary disease, both neoplastic and infectious, and of heart disease among long-term metal miners. The excess of respiratory tract cancer (47 deaths observed as compared with 16.1 expected) was not attributed to age, smoking, nativity, urbanization, heredity, socio-economic status, diagnostic accuracy, or silicosis.

SIGNIFICANCE TO BIO-MEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE: The findings of differential respiratory cancer risk for miners with specific ore exposures may help identify an environmental carcinogen under field conditions. These extensive data may also establish whether the factors age-at-first-exposure, latency, cessation of exposure, and dosage act in a manner comparable to carcinogenesis experiments. Intense interest in the results of this study has developed because low levels of airborne radiation are present in the mines. While radiation may not be the only carcinogen involved, it is unlikely that any greater risk of lung cancer can be attributed to the amount of radiation exposure in these mines.

PROPOSED COURSE: This study was transferred to the Bureau of Occupational Safety and Health for possible future coding of work histories, mortality follow-up, and data analysis.

OFFICE OF THE ASSOCIATE DIRECTOR FOR EXTRAMURAL PROGRAMS

INSTITUTE DIRECTORS SUMMARY ON EXTRAMURAL PROGRAMS

In early 1970 elements of the NIEHS Extramural Program were transferred from Bethesda, Maryland, to the Institute's North Carolina facility. Geographic relocation of the unit engendered extensive personnel changes at all levels of responsibility. During the past year the essentially new Extramural Program staff in North Carolina has become familiar with the grant policies and procedures of NIH and the Institute, has become oriented to the program and mission goals of NIEHS, and has gained the group experience requisite to efficient unit operation. This consolidation of NIEHS elements in their North Carolina headquarters has enhanced the coordination of Institute intramural and extramural functions and contributes significantly to program planning for optimal implementation of the Institute's mission objectives.

NIEHS during the past year made further progress in its efforts to bring extramural research and training protocols into closer alignment with the Institute's human-oriented mission. Grant commitments acquired by the developing Insitute through their transfer from the former Bureau of State Services were, in many instances, of marginal relevance to the research and training needs of NIEHS. In the ensuing period this Institute maintained the dual function of honoring support commitments to these grantees while endeavoring to phase them out either through orderly termination or by assisting in their reassignment to other, more appropriate support agencies.

Progress toward these objectives has been generally good in the individual research grant program. Progress in the training grant program is less advanced because of the relatively longer support periods involved and because of the inherent complexities of training in the environmental health field. A substantial number of active training grants are due for competing review during the present year and phase-out of the less appropriate awards may be expected to continue.

University-based Center grants constitute a large portion of the NIEHS research and training support activity. The integrated, multidisciplinary nature of these Centers provides a unique mechanism for the comprehensive training of promising new environmental health scientists in an active research setting. The Center Subcommittee of the Environmental Health Sciences Advisory Committee, through their liaison functions and their periodic visits to individual Centers, contribute significantly to the maintenance of health orientation in the research and training activities of these programs.

OFFICE OF THE ASSOCIATE DIRECTOR FOR EXTRAMURAL PROGRAMS
Summary Statement

RESEARCH GRANTS

Research grant applications received by NIEHS in FY-1971 increased in number by 16.8% over last year. Fiscal stringencies, however, permitted awarding of no more than 38% of the new and renewal applications approved for support by review groups. Even so, NIEHS Extramural Programs was able to make substantial increases this year in its support of individual research projects and multidisciplinary program projects. Approximately 100 grants were awarded with total support of $5.9 million. Among these grants were 23 for new research projects and 3 for establishment of new program projects. The program projects were funded, in turn, for integrated investigation of heavy metals pathology, air pollutant effects on lung, and food-borne toxicants.

Extramural Programs have continued to honor the prior commitments of support to projects transferred to the Institute from the former Bureau of State Services and at the same time have maintained efforts to phase-out those of marginal relevance in favor of those more directly focused on environmental health problems. These efforts generally have included discussion with investigators, transferal to more appropriate support agencies, or orderly termination of grant funds. Budget negotiations on all competing research grants continued to be stringent and considerations of mission relevance were weighed to assure optimal yield from available environmental health funds.

TRAINING PROGRAMS

Forty-two training program awards were made in FY-1971 with total funds of $3.1 million. These figures represent a slight decrease from levels of the previous year and reflect, in part, fiscal restrictions imposed on training support functions.

Total funds of $264,000 were awarded for support of thirteen postdoctoral and special fellowships and six Research Career Development Awards. Approximately half of these funds went to support the six RCDA's, one of them new this year. Postdoctoral fellowship and Career Development awards have become an important mechanism for meeting the unique requirements in the preparation of environmental specialists. Training in traditional departmental disciplines often has not provided the broad and comprehensive research background necessary for thorough understanding of environmental health complexities. Individual fellowship and Career awards provide advanced multidisciplinary exposures to environmental health problems and techniques, permit more immediate implementation of specific Institute programming, and respond more effectively to the expanding national needs for highly qualified environmental scientists.

UNIVERSITY-BASED CENTERS

Growing demand for an expanded national effort in the environmental health field has made increasingly evident the need for integrated multi-disciplinary facilities to attract and prepare new environmental scientists. University-based Environmental Health Centers continue to provide a unique mechanism for applying sustained effort against complex problems through multifaceted combinations of research and training. NIEHS supported six Centers in FY-1971 with total funds of $3.0 million. Preliminary plans were made for the establishment of a seventh Center, funded at about $500,000, in FY-1972.

The Environmental Health Sciences Advisory Committee's Subcommittee on University-based Centers continues to provide important liaison between the program needs of NIEHS and the research and training activities within the Center grants. Through periodic visits to individual Centers the Subcommittee has established effective means for the communication of mutual administrative and scientific concerns and has provided general assurance that the Center research and training programs maintain substantial relevance to the mission of NIEHS.

RESEARCH HIGHLIGHTS

The following descriptions of selected NIEHS grants are representative of the nature and scope of extramural research activities in FY-1971.

Air Pollutants

Nitrogen dioxide is an important air pollutant and a major precursor of photochemical smog. Inhalation of the gas produces in some biologic species pulmonary changes resembling human centrilobular emphysema. In order to learn more of the etiology and pathogenesis of this chronic lung disease, NIEHS is supporting research to determine in hamsters early pulmonary cell changes caused by long-term inhalation exposure to NO2, alone and in combination with the aromatic hydrocarbon, 3,4-benzpyrene. Exposures are maintained for periods of up to one year and normal regenerative processes to repair local tissue damage are being inhibited by injection of antimetabolites. The ultimate goal of this project is to find a satisfactory experimental model for study of human emphysema. Preliminary evidence has not yet demonstrated presence of the disease; however, valuable data on bronchial and alveolar effects of NO2 inhalation are being recorded.

Recent study has shown that an intermediate product of glucose metabolism in the human erythrocyte--2,3-diphosphoglycerate--acts to regulate the oxygen affinity of hemoglobin and promotes oxygen release to the tissues. NIEHS is supporting a research project to assess this phenomenon as a compensatory defense mechanism under conditions of oxygen deficiency such as might be imposed by inhalation of airborne pollutants. Carbon monoxide and cigarette smoke are the pollutants presently under test. Data obtained from this investigation are expected to provide additional information for a realistic assessment of carbon monoxide tolerance in man.

Food Toxicants

Food Additives

NIEHS is supporting a small research project to measure the capacity of drugs and food additives in common usage to induce chromosome breaks. The test system used is the sensitive onion root tip and results are obtained in terms of root tip growth, rate of mitosis, and anaphase aberrations. Investigation to date has included tests on caffeine, ethanol, Sucaryl, antioxidants, and combinations of these agents with each other and with commonly used drugs. Simple screening methods such as those used in this project may permit preliminary identification of environmental agents to be subjected to more elaborate and sophisticated testing.

Natural Products

Ingestion of moldy foods and feeds is known to cause a variety of serious toxicoses in humans and livestock. It is important to identify the associated food-borne toxins, to determine which mold species produce them, to measure environmental conditions favoring their production, to learn their toxic mechanisms, and to discover how these natural toxins may be detected in foodstuffs.

NIEHS supports a number of investigations into these and related food toxicity problems. The research of one such investigator includes an effort to isolate from moldy hay mycotoxins associated with abortion in dairy cattle. This scientist has extracted Ochratoxin-A from the feed and has shown that the toxin, when fed to pregnant rats and rabbits, induces fetal deaths and resorption.

Some three hundred or more "unusual" natural amino acids are known to exist in addition to the twenty-five or thirty amino acids common to all living organisms. When one of these "unusual" compounds is present in foodstuff and is a close analog of one of the common amino acids, the two in combination may compete at enzyme sites to disrupt the metabolism of normal organisms. An NIEHS-supported investigator is isolating, identifying, and testing the toxicity of "unusual" amino acids in legumes and cycad plants, and is attempting to establish the biochemical mechanisms of their toxicity in animals. The work is important to understanding and perhaps predicting food-borne toxicities in less-developed areas of the world.

Metals

The clinical manifestations of many overt heavy metal intoxications are well documented. There is much to be learned, however, concerning the subclinical toxicity of metals. In the past year NIEHS has established a program grant to investigate the biochemical correlates of molecular and subcellular disruption due to heavy metals. Lead and cadmium are the principle elements under study and their effects are being observed in the renal system, the nervous system, and the reproductive system. The investigators are working initially at high metal concentrations to determine their toxic mechanisms and to discover synergistic properties in other environmental stressors. Later studies will follow to assess the implications of long-term, low-level exposure to these and other heavy metals.

Lead at low concentrations has been shown to inhibit protein synthesis in the rabbit reticulocyte. If a comparable effect exists in human erythrocytes and bone marrow cells then persons exposed to high levels of lead pollution may suffer from inhibition of protein synthesis and embarrassed oxygen-carrying capacity. The precise point at which hemoglobin synthesis is sensitive to lead is not known. An NIEHS-supported scientist is attempting to measure the toxic effects of lead and to discover the specific mechanism by which low levels of lead inhibit hemoglobin and protein synthesis in human and

rabbit cells. From this study bases may emerge for effectively coping with acute and chronic lead toxicities.

Occupational Hazards

Dusts & Fibers

The broad utility of beryllium in the electronics, atomic, and aerospace industries makes complete elimination of this stressor from the environment unlikely. Beryllium compounds, dusts, and fuels are known to cause granulomatous pulmonary lesions in humans and have been implicated in carcinogenesis in animals. NIEHS supports several research projects on the toxic properties of beryllium, including one funded to study the sequence of effects leading to pulmonary berylliosis in guinea pigs. The investigator is testing the contention that the three major hypotheses of berylliosis pathogenesis--protein denaturation, enzyme inhibition, and autoimmune hypersensitivity--may not be mutually exclusive but rather interacting and dependent upon each other. Inhalation studies are proceeding and results of this project may suggest a basis for the variable responses seen in workers exposed to beryllium.

Synthetic Products

Industrial processing developments and technology multiply the risks of worker exposure to potentially hazardous levels and combinations of chemical compounds. Toxicological assays have not always kept pace with the introduction of new chemicals and mixtures. NIEHS supports a number of research projects exploring toxic chemical hazards in the industrial environment. Among them is a study to determine the toxicity of individual halogenated hydrocarbon solvents and to discover synergistic properties in combinations of them. The investigator has conducted initial studies on methylene chloride and perchlorethylene to evaluate their toxicity through dermal, oral, and pulmonary exposures. Using rabbits and rats, the investigator has tested these compounds individually at several concentrations and then in combinations with each other. He is tabulating data from these exposure tests and as potentiations are discovered he will examine the chemical and biological mechanisms involved.

Pesticides

Decreases in wild bird populations have been associated with the wide-spread application of pesticides. Laboratory studies, however, have not always demonstrated reduced viability of eggs and young at the pesticide levels usually found in nature. NIEHS is supporting a grant to test the postulate that mechanisms responsible for avian reproductive failures may include toxic alteration of steroid hormone levels and neural functions. In tests using chlorinated hydrocarbons, the investigator has measured pesticide-induced enzymatic breakdown of steroid hormones in the liver and is observing the effects of the reduced circulating hormone titer on behavioral and physiological parameters throughout the breeding cycle of wild birds. In tandem with his laboratory studies the investigator is conducting field tests to assure that

complex behavioral and physiological mechanisms associated with the birds' natural breeding cycles and habitat have not been disrupted by removal to the laboratory. Data obtained from these studies will enhance our understanding of pesticide toxicology and will provide a basis for reproductive studies in other animals at higher levels of biologic food chains.

Biochemical studies on biological detoxication systems have provided a basis for predicating enzyme induction or inhibition changes in response to exposure to combinations of drugs, pesticides, industrial and environmental chemicals. These studies, however, have often been conducted at dosage levels much higher than would be encountered under normal circumstances. NIEHS supports a research project to detect and measure the potential for interaction among combinations of environmental stressors at low concentration levels. The investigator has developed a method for biochemically screening simultaneous and sequential combinations of chemicals through measurement of their ability to alter the functions of detoxication enzymes. Data from this project will provide new information on environmental synergists and new insight into enzymatic detoxication mechanisms in animals and man.

The physiologic effects of acute and subacute insecticide exposures in fish have been extensively studied. There is a need, however, to examine the general and specific behavioral toxicology of sublethal concentrations of these compounds. NIEHS supports a project to determine the effects of low concentrations of DDT and parathion, alone and in combination, on behavioral control mechanisms in fish. The investigator is observing changes in their locomotion patterns and orientation behavior before and after exposure. He is also testing the presence and absence of thermal, acoustic, and olfactory stimuli to determine their roles in orientation and to detect any toxic changes in sensory mechanisms or their integration in the central nervous system. Results of this study, while not directly applicable to man, should reveal whether functions integrated by lower brain centers can be modified or disrupted by chlorohydrocarbons or organophosphates.

Physical Factors

Temperature and Humidity

The interaction of biological systems with their physical environment has been studied for centuries. The breadth of the subject is so great, however, that researchers have generally confined their efforts to one particular aspect of the whole; integration of the multifaceted data has not always been efficient. NIEHS supports a program grant devoted to a multidisciplinary study of physical factors in the environment and the complex responses to them in normal animals and man. Within this program scientists are investigating heat, cold, humidity, infrared radiation, and other environmental factors to detail their effects on biological systems and subsystems. They are measuring the effects of clothing, exercise, and other complicating parameters. Other scientists are studying neural, cardiovascular, respiratory, and endocrine responses evoked to counter these stresses and maintain body temperature. Another group has begun studies

on behavioral temperature control and comfort responses to temperature change. New exposure and measuring techniques developed under this grant will permit more accurate quantitation of thermal stresses and biological responses to them. Sophisticated data processing developments will facilitate the integration of these specific data into broader aspects of the total field. The importance of the program nature of this grant lies in its integrative function--physical, physiological, and psychophysical parameters of temperature regulation being investigated in one combined research effort.

NIEHS supports a project investigating correlates between the state of hydration of the animal body and its vulnerability to toxic chemicals. Initial studies in rats have shown that dehydration apparently reduces the number of protective free macrophages in the lung and may also affect the activity of microsomal enzymes in the liver. Either of these effects could seriously disrupt protective biologic mechanisms against toxic insults. Since men performing physical work in high temperatures may be expected to risk dehydration through heavy sweating, often in the presence of dusts and toxic fumes, this project has immediate application in industrial and community health. The investigator will continue to assess these hazards and to explore their specific mechanisms.

Radiation Hazards

NIEHS supports a coordinated program grant for research on the beneficial and malignant effects of environmental radiation on biological systems. Emphasis in the program is on ultraviolet radiation and visible light as they affect the skin and associated tissues; however, there is significant research on the effects of infrared and ionizing radiation. Studies underway within this program involve development and testing of radiation sources and radiation detection and measuring devices; development of animal models for environmental radiation research; photochemical studies on phototoxic and photoallergenic agents; natural history of possibly premalignant skin lesions in patients to follow their course; and correlation of the incidence of human skin cancer with sunlight exposures and genetic constitution. The multidisciplinary approach in this program involves aspects of physics, optics, photochemistry, radiochemistry, radiobiology, physiology, pathology, and clinical medicine.

Although substantial research has been conducted on the biological effects of laser radiation, there remains a need for more detail on laser interactions at the molecular level. NIEHS supports a research project concerned with the nature of specific photochemical effects caused by ruby laser radiation and leading to biological damage. The investigator has conducted tests on relatively simple compounds to provide a basis for more complicated studies on amino acids, purines, proteins, heme, flavins, DNA, and other biologically significant molecules. Information derived from this research is likely to have direct relevance to the growing use of lasers in biomedical and other applications.

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