919 189 PROPOSALS TRANSMITTED BY ORNL LTR DTD MAY 5, 1969

OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

PROGRAM: A-4797
189 No.

DATE: April 1969

PROGRAM AND BUDGET PROPOSAL

DESCRIPTIVE TITLE		AEC ACTIVITY NUMBER
NCI Viral Leukemogenesis		40 04 712343
PERSON IN CHARGE A.C. Upton, 1969; J.B. Storer, 1970-71	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
PRINCIPAL INVESTIGATOR R. L. Tyndall (25%)	unclassified	☐ NEW WORK BEGINNING FY ____ ☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 55,000	$ 60,000	$ 65,000
UNUSUAL COSTS	-0-	2,000	2,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 55,000	$ 62,000	$ 67,000
ORNL MAN-YEARS	1.5	1.5	1.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Tyndall, R. L. and J. A. Otten. Oncogenic effects of sheep erythrocytes in BALB/c mice. Proc. Amer. Soc. Cancer Res. (in press).

Allen, R. C., D. J. Moore and R. L. Tyndall. Quantitation of plasma and tissue esterase isoenzymes. Application to the study of physiological and pathological changes. Proc. Third Intern. Congr. Histo. and Cytochem., 1968, abstract.

Tyndall, R. L., R. C. Allen, E. Teeter, and D. J. Moore. Isozyme and protein profiles of normal and leukemic tissue from BALB/c mice (in preparation).

SCOPE:

The objective of this study is to elucidate some of the physiological and biochemical alterations in the pathogenesis of murine and human leukemias. The ultimate aim is to contribute to the control of leukemia in man. We are analyzing the qualitative and quantitative alterations in isozyme and protein profiles associated with leukemia virus infection both in vivo and in vitro. These studies include examination of BALB/c mice infected with the Rauscher leukemia virus and spontaneous leukemias in AKR mice. Radiation-induced leukemias in the RFM mouse are also being studied as is serum from human leukemia patients. The analyses are made by flat-bed gel electrophoresis and concentrate primarily, but not exclusively, on characterization of esterase isozyme and protein profiles. The alterations observed in infected cell cultures or animal

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tissues serve as guidelines in the search for preclinical markers and may also be indicative of the physiological relationship between various types of leukemic tissues and comparable normal or fetal tissues. The appearance of any "new" protein bands in infected cell cultures or leukemic mouse tissue may indicate the specific antigens associated with such tissue. Attempts will be made to separate various capsid proteins and antigens from large quantities of RLV propagated in cell cultures. The similarity in certain esterase isozyme alterations in sera of mice injected with either RLV or sheep erythrocytes has also prompted studies concerning the effects of repeated sheep erythrocyte injections in BALB/c mice.

RELATIONSHIP TO OTHER PROJECTS:

These studies are being carried out with the cooperation of Dr. R. C. Allen and D. J. Moore of the Oak Ridge Technical Equipment Company and with Dr. Frank Rauscher of the National Cancer Institutes.

TECHNICAL PROGRESS IN FY 1969:

Extensive studies were made of esterase isozyme and protein profiles of tissue from normal and leukemic BALB/c mice. The leukemias resulted from inoculation of either animal-passaged RLV or RLV propagated in the established JLS V5 cell cultures. The majority of leukemias examined were reticulum cell sarcomas (RCS) with a lesser number of thymic lymphomas.

The protein phenograms of splenic RCS differed from those of normal spleens as shown by increased amounts of proteins with electrophoretic mobilities similar to albumin, transferrin and erythropoietin. These changes were most marked in RCS induced by animal-passaged RLV in which there is concomitant erythroblastosis not associated with RCS induced by RLV propagated in cell cultures. Esterase isozyme profiles of leukemic spleens showed a marked increase in two isozymes and a diminution in pre-albumin esterase compared to normal spleen patterns. Both protein and esterase isozyme patterns of fetal and newborn spleen more closely resembled leukemic spleen than normal adult spleen patterns. Esterase isozyme patterns of thymic lymphoma tissue also showed increased activity in two isozymes and a diminished pre-albumin esterase activity, similar to the pattern seen in splenic RCS. Protein patterns of thymic lymphoma and normal thymus tissue did not differ markedly. As in the case of RCS, the sterase profiles of fetal and newborn thymus tissue more closely resembled patterns of thymic lymphoma than normal adult thymus tissue. Serum esterase patterns of leukemic mice did not differ markedly from normal serum patterns but protein phenograms of sera from mice infected with animal passaged RLV showed increased amounts of transferrin and albumin.

The diminution of pre-albumin esterases in sera of mice following inoculation of either RLV or sheep erythrocytes (SRBC) and the intimate involvement of both with germinal center cells led to studies aimed at determining the effects of repeated sheep RBC inoculations in BALB/c mice. In the first such experiment mice were injected twice weekly for 12 weeks. Fourteen of 19 animals surviving this treatment developed RCS within 30 weeks. These RCS were transmissible by inoculation of either live or irradiated (15,000 R) cells.

Serum protein profiles of normal and leukemic children were prepared and analyzed. These samples were obtained through the cooperation of Dr. Frank Rauscher of the National Institutes of Health. Protein phenograms of the normal control children showed typical patterns with the exception of a few samples which had several

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additional protein bands of limited electrophoretic mobility similar to haptoglobins. Conversely, the majority of sera from leukemic children showed these additional protein bands. Moreover, a number of sera from leukemic children lost these additional protein bands after the patients were treated with chemotherapeutic agents.

EXPECTED RESULTS IN FY 1970:

Studies of the isozyme and protein profiles of spontaneous AKR leukemia cells and of radiation-induced leukemia in RFM mice will be made. Extensive analysis of serum proteins and enzymes of mice during the first few days after infection with RLV will be made in attempts to detect specific pre-clinical markers of the impending leukemias. The relationship of leukemic tissue to fetal tissue both biochemically and antigenically will be explored. The significance of the protein changes in human leukemic sera, particularly the response to chemotherapy, will be studied. Studies of the effect of repeated antigen inoculation inBALB/c mice will be continued, with particular emphasis on the reproducibility of the oncogenic action of such treatment.

EXPECTED RESULTS IN FY 1971:

The protein and esterase alterations founds in various types of murine leukemia will be determined and pre-leukemic markers specific for the various types of leukemia will be defined. Viral antigens associated with the RLV will be purified by a combination of zonal centrifugation and gel electrophoresis. Specific protein or enzymatic markers associated with human leukemia will be detected and work will concentrate on the search for similar pre-leukemic markers. The physiological relationship between antigen challenge and RLV infection in BALB/c mice will be investigated and indigenous leukemia viruses associated with splenic germinal centers will be isolated.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

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OAK RIDGE NATIONAL LABORATORY
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UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Inhalation Co-Carcinogenesis	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
D. G. Doherty		☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR *See below	Unclassified	☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 825,000	$ 875,000	$ 905,000
UNUSUAL COSTS	15,000	15,000	10,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 840,000	$ 890,000	$ 915,000
ORNL MAN-YEARS	15.0	15.0	15.0

(SEE BACK PAGE FOR BUDGET COMMENTS)

*M. G. Hanna, Jr., P. Nettesheim, R. Newell (NIH), and C. T. Bahner (Consultant)

DATES AND TITLES OF PUBLICATIONS:

Bahner, C. T. and D. G. Doherty. Fate of 1-(4-Dimethylaminobenzylidine)indene in Rats. Submitted to Cancer Research, 1968.

Hanna, M. G., Jr., P. Nettesheim and Mary W. Francis. Requirement for Continuous Antigenic Stimulation in the Development and Differentiation of Antibody Forming Cells: The Effects of Passive Antibody on the Primary and Secondary Response. J. Exp. Med. (in press), 1969.

Hanna, M. G., Jr., A. K. Szakal and H. E. Walburg, Jr. The Relation of Antigen and Virus Localization to the Development and Growth of Lymphoid Germinal Centers. In: Lymphatic Tissue and Germinal Centers in the Immune Response. Advances in Experimental Biology and Medicine. L. Fiore-Donati and M. G. Hanna, Jr., Editors. New York: Plenum Press (in press), 1969.

Hanna, M. G., Jr., P. Nettesheim and H. E. Walburg, Jr. A Comparative Study of the Immune Reaction in Germfree and Conventional Mice. In: Germfree Biology, Clinical and Experimental Aspects, Vol. 3. Advances in Experimental Biology and Medicine. E. R. Mirand, Editor. New York: Plenum Press, 1969.

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Hanna, M. G., Jr., and A. K. Szakal. Localization of ^{125}I-Labeled Antigen in Germinal Centers of Mouse Spleen: Histologic and Ultrastructure Autoradiologic Studies of the Secondary Immune Reaction. J. Immunology 101:949-962, 1968.

L. Fiore-Donati and M. G. Hanna, Jr., Editors. Lymphatic Tissue and Germinal Centers in the Immune Response. Advances in Experimental Biology and Medicine. New York: Plenum Press (in press), 1969.

Buckton, Karin E., and P. Nettesheim. In vitro and in vivo Culture of Mouse Peripheral Blood for Chromosome Preparations. Proc. Soc. Exptl. Biol. Med. 128:1106-1110, 1968.

Nettesheim, P., and Anna S. Hammons. Lack of Recruitment of Antibody-Forming Cells during the Late Hemagglutinin Response. Nature 220:297-299, 1968.

Nettesheim, P., and M. G. Hanna, Jr. Radiosensitivity of the Antigen-Trapping Mechanism and Its Relation to the Suppression of Immune Response. In: Lymphatic Tissue and Germinal Centers in the Immune Response. Advances in Experimental Biology and Medicine. L. Fiore-Donati and M. G. Hanna, Jr., Editors. New York: Plenum Press (in press), 1969.

SCOPE:

The major purpose of this group is the study of the role of air pollutants via the inhalation route on the induction of pulmonary tumors in animals and to develop relevant model systems in which a wide variety of chemical and biological agents can be studied. In addition, programs allied to the central theme and in the scientific disciplines of the investigators such as: chemical carcinogenesis, immune response to viral and carcinogenic agents and viral oncogenesis will be pursued.

The initial inhalation study phase of the cooperative program with the National Cancer Institute and the Biology Division, Oak Ridge National Laboratory, on co-carcinogenesis is being undertaken to investigate the role of air pollutants, radiation and viruses in various combinations on the induction of lung tumors in mice. Considerable epidemiological evidence has accumulated over the past years implicating environmental factors in the rising incidence of pulmonary cancer in man. Carcinogenic agents as well as factors known to modify the host's absorption, retention and sensitivity have been shown to be present in atmospheric pollutants. Atmospheric irritants in addition may, by causing direct cellular injury, create an environment favorable to tumorigenic agents administered simultaneously or sequentially. Thus the lung cancer may be regarded as the final stage in a series of events involving the carcinogenic agent, modifying factors and the susceptibility of the host.

We are concluding the study of the induction of pulmonary tumors in mice exposed to radiation, viral and chemical insult. The radiation or viral insult or combination thereof has been administered and the animals placed in an inhalation chamber and subjected to a chemical aerosol atmosphere for the remainder of their life span. Complete serial autopsy and histologic study is being made of all animals in the experiment. Related factors that are being investigated include viral effects at the cellular and subcellular levels, response of immunologic tissue to viral challenge and distribution and biochemical effects of the chemical aerosols. The results obtained so far indicate the need for additional model systems; consequently we are initiating a program using the hamster as the test animal and a known carcinogenic chemical as the initiator and superimposing the air pollutant.

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Carcinogenesis - Pathology and Immunology (M. G. Hanna, Jr., and P. Nettesheim).

Carcinogenesis in general is characterized on the cellular level by proliferation of cells with partial or complete lack of differentiation resulting in unregulated malignant growth. Many viral, chemical and physical agents are known to be powerful inducers of cancerous growth. However, the nature of this induction, the neoplastic transformation of a cell or a cell population and the subsequent cytokinetic events of early carcinogenesis are poorly understood. A model of induced cell proliferation, cell differentiation and regulated growth of turnover cells is therefore of primary importance to obtain better insight into the induction and kinetics of nonregulated (malignant) growth. The induction of immunologically competent primitive lymphoid cells by a chemically defined agent (antigen) to undergo proliferation and differentiation is one of the best models to study these basic biological phenomena. In this system the pool of antigen inducible progenitor cells and the rate of cell proliferation and maturation can be assessed with great reproducibility and sensitivity.

There are reasons for believing that the host does exert some influence on the growth and development of malignant cells. Within highly inbred strains of experimental animals there is a remarkable variation in the response of individuals to a uniform carcinogenic stimulus. These differences justify investigations of the host's resistance to the cancer process. Because chemically-induced tumors are antigenically distinct, the reticuloendothelial system (RES) (and the immune system as an integral part of it) by virtue of its recognition and defense mechanism play an important role in protecting the individual (self) against clones of cancerous cells that are antigenically distinct (non-self). States of decreased immune competence (e.g., irradiation, immunosuppressive drugs, thymectomy, old age) may therefore permit establishment of cancerous cells in spite of their antigenicity. Enhancing the immunopotential may become an effective means to increase the bodies' defense against malignant growth. The effect of viral and chemical carcinogens and co-carcinogens also needs to be studied in terms of their immunogenicity and their effect on regional lymphatic tissues and overall immune capacity. It also seems important from the point of view of (a) the immune response to a virus, (b) the effect of viruses on the immune potential, and (c) tumor induction by a virus, to study the persistence of oncogenic, non-oncogenic live and killed viral antigens in lymphatic tissue. It is evident then that in order to define antigen induced proliferation as a model to study regulated and unregulated growth, and the possible roles of RES in defense against malignant clones we need to establish and study various parameters of the immune response; suppression and recovery of immune potential from physical, chemical and viral agents and alteration of the immune status in aging and thymectomized animals. This information will be highly relevant to our other studies concerning the influence of environmental factors on the occurrence of cancer.

The problem of chemical carcinogenesis (D. G. Doherty) is intimately concerned with the nature of the chemical insult that serves to initiate the sequence of biochemical events culminating in the formation of a cancerous growth. With certain classes of reactive organic compounds such as the epoxides, imines, and mustards, the injury produced is similar to that obtained with ionizing radiation, and in both instances free radical intermediates may play a significant role in initiating damage on the molecular level. A wide variety of chemicals affording protection against ionizing radiation have also been found to protect specific organ systems against the cytotoxic effects of these agents, and presumably should also protect against their carcinogenic effect. This approach should be capable of extension to other classes of compounds which may combine with and neutralize carcinogens in vivo, thus preventing the initial lesion. An additional fruitful area for exploration lies in the nature of the binding of carcinogens to specific biological sites. With sufficient information it should be

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feasible to devise compounds which would combine with these sites but be noncarcinogenic, and thus prevent the attack of the carcinogen. Such action would be similar to that of a competitive inhibitor in an enzyme system. The proposal is to explore these approaches as one avenue toward understanding the mechanisms of carcinogenesis.

RELATIONSHIP TO OTHER PROJECTS:

Certain phases of this work regarding the response of lymphatic tissue to viral agents and chemical carcinogenesis will be done in collaboration with the Mammalian Radiation Recovery Group. Some aspects of the cytokinetics of the immune system will be done in collaboration with the Radiation Immunology Group. In addition, we are providing mice and technical advice to a related asbestos dust inhalation project under Dr. Forde McIver at the Medical College of South Carolina, Charleston, South Carolina.

TECHNICAL PROGRESS IN FY 1969:

The first chamber exposure phase of the role of air pollutants on the induction of pulmonary tumors in mice that have been pretreated with either a radiation, viral or combined insult, is nearly complete. Eight of the treatment groups have terminated and the remaining six are down to small numbers of animals. The gasoline smog and chromic oxide treated groups have had 3,100 chamber hours exposure, the calcium chromate groups 2,200 hours chamber exposure, and the various control groups over 3,200 hours exposure. The median death times have ranged from as low as 18 months in the animals exposed to chemical insults to 31 months for the control untreated animals. All animals to date have remained free from pseudomonas, salmonella, internal and external parasites, the seven murine viruses originally typed, and there has been a minimal amount of respiratory disease. The stress of living on wire or daily in the chambers is reflected in the average median death times of those with no exposure to chemicals; e.g., males in pans 29 mos, wire 25 mos, chambers 20 mos; females in pans 26 mos, wire 22 mos, chambers 23 mos. In the male at least this can be directly correlated with the incidence of genitourinary disease which is low in those living in pans, fourfold higher in the wire group and sevenfold higher in the chamber group. There is an anomalous small increase in the median death time in the six irradiated female groups. The data from this study has been programmed for automatic record storage, retrieval and analysis and at present an examination of the various interactions between the experimental groups is in process. Preliminary indications are that with regard to the age specific incidence of lung adenomas, there is a consistent 100 day earlier incidence in the smog treated groups over the control and chromic oxide group. With regard to chemical treatment the gasoline smog is the most effective agent. The total number of tumors is 15 in the washed air chamber, 16 in the chromic oxide group and 30 in the smog treated group. Further analysis awaits termination of the other groups.

Morphologic and cytokinetic antigen induced lymphatic tissue changes (M. G. Hanna, Jr., and P. Nettesheim.

Based on the sequence of histologic and serologic events observed in our recent studies, we have developed the following concept: lymphatic tissue has two unique components, dispersed immunologically competent progenitor cells and defined areas of reticular cells capable of localizing antigen extracellularly and providing an excellent opportunity for surface contact of lymphoid cells with the antigen. After the initial antigenic stimulation, one phase of the proliferative expansion of immunologically competent cells occurs in the region of the antigen-retaining reticulum cells. This

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results in the accumulation of large pyroninophilic cells in this area and ultimately in the formation of a histologically well defined germinal center. At the same time specific antibody becomes detectable in the serum. The serum antibody response and the growth of the germinal centers simultaneously develop to peak levels. During this period the sensitized (memory) cell compartment is being established in the lymphatic nodules. Thus the proliferation of large pyroninophilic cells in germinal centers is probably related to the development of antibody producing cells as well as memory cells. Since the largest proportion of antibody producing and memory cells is originally found outside this region, the lymphoid cell population of the centers must be considered transitory. This concept is also supported by our previous kinetic studies of active germinal centers. The later decrease in the size of the centers probably is a manifestation of the diminishing growth rate of the antibody response and of memory formation. This may be a consequence of the elimination or loss of antigen from these areas.

A study with Rauscher virus demonstrated that upon injection of extraneous leukemia virus, localization of the virus occurred first in the reticular cells of these centers. This appeared to be an initial immune response to the virus. However, proliferation of the virus occurred in the parenchymal lymphoid cells of the germinal center and as a result of the normal dissemination of these cells during the immune response, the virus was transported to other target cell types. Ultimately this resulted in a fatal lymphoid leukemia in these mice. Thus, the effectiveness of the antigen trapping system appears to be functionally relevant to proliferating oncogenic virus.

The concept has also been supported by studies on alteration of the antigen trapping mechanism. These data raise the possibility that alteration of antigen capture and retention might be of major importance in modification or suppression of the immune response by various physical and chemical agents. It was found that the antigen capture and retention capacity of lymphatic tissue, in particular the germinal center stroma, is a radiosensitive process. The x-ray induced damage was maximum two weeks after x-irradiation with 600 R and its recovery was slow, taking several weeks to be complete.

The presence of persisting antigen in lymphatic germinal center required studies of the essential role of continuous antigenic stimulation in the development and differentiation of antibody forming cells. This was examined with the use of passively infused isologous antibody given at various intervals after antigenic stimulation. This resulted in suppression of the functional antibody producing cell compartment, and concommitant enhancement of the 19S and depression of the 7S memory cell pool. These data suggest that progeny of the antigen stimulated progenitor cell were forced into maturation arrest as a consequence of the lack of further antigenic stimulation. Our studies demonstrated further that isologous passive antibody also suppresses germinal center growth, which supports the notion that this histologic structure is the site of antigen stimulated proliferation of immunocompetent cells. We also obtained evidence suggesting that the germinal center growth is more closely related to the 7S than the 19S antibody production.

Our studies concerning suppression and recovery of immunocompetence from x-irradiation revealed (1) that long term immunological memory was probably not dependent on specific antigen, (2) that in the immune response recruitment of immune progenitor cells by antigen diminished while production of antibody continues. This indicates the existence of a long-lived population of antibody producing cells. (3) That the rate of recovery of the primary antibody forming potential from sublethal, as well as lethal, x-ray exposure is thymus dependent, and (4) that this recovery is not prevented by pre-irradiation thymectomy.

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Chemical Carcinogenesis (D. G. Doherty and C. T. Bahner).

1-(4-Dimethylaminobenzylidine)indene is effective against Walker-256 and Lymphoma 8 in rats. It is effective also in producing tumors in female inbred Lewis strain rats. The fate of this compound, dissolved in arachis oil and injected i.p. or administered orally, has been followed by its color, by ^{14}C tagged methyl group on the amino nitrogen, and by ^{3}H labeling of the benzene ring in the benzylidene portion of the molecule. A dose of 5 mg in 0.5 ml is largely absorbed within 2 or 3 days, but unchanged compound sometimes remains in the abdomen several weeks after large doses. About half of the administered ^{3}H was found in the fecal extracts in contrast with about 1/7 of the ^{14}C. Urinary excretion was much less than fecal excretion. Chromatograms of urine, fecal extracts, and tissue extracts support the hypothesis that demethylation is one of the chief metabolic changes, and that formation of two or more water soluble derivatives is significant, also. Autoradiograms indicating that much of the ^{3}H in the tissues is removed by the solvents in preparing specimens for paraffin sections, but such sections do retain radioactivity, especially in fat, spleen, liver, follicular area of ovary, epithelial lining of uterus and vagina, pancreas, adrenals, lung, kidney, and hair follicles. Walker 256 and Lymphoma 8 tumor cells were labeled.

EXPECTED RESULTS IN FY 1970:

The inhalation study in mice will be concluded and the data completely analyzed and recorded in this year. The shortcomings of the mouse system have indicated the need for additional model systems to study lung tumors. Toward this goal we are developing an experiment in hamsters using a specific carcinogen at low levels to induce a low incidence of tumors and superimposing on this exposure to gasoline smog and smog in combination with an inorganic dust. Additional studies will be made with the direct instillation of various carcinogenic agents directly into the hamster lung.

Our efforts to investigate the inter-relationship of the various immune progenitor cell pools and their dependence on humoral and/or cellular factors of the thymus are being continued. We expect that these investigations will also produce more information on the interaction of various cell types involved in the initiation of the immune response.

Studies concerning the regulation of antigen localization by 19S and 7S specific antibody will be conducted to obtain further insight into the role of antigen in the induction of the antibody response.

The effect of various immuno-suppressive agents other than x-ray on the antigen-retention mechanism are being studied. We hope that these studies will help to explain some of the differences in recovery observed after various kinds of immuno-suppression.

The possible role of an impaired antigen trapping mechanism in oncogenesis and leukemogenesis in particular will be investigated.

EXPECTED RESULTS IN FY 1971:

The hamster model system should be more suited to the study of lung carcinoma and lead to a further work on injury, repair and lung clearance mechanisms. These in depth studies will include the effects of various gases, chemicals and biological agents and involve histological, biochemical and radioactive tracer investigations.

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TITLE: NCI Inhalation Co-Carcinogenesis

ACTIVITY NO. 40 04
189 No.

1) Studies on the occurrence of tumors in animals with decreased and suppressed immune conditions. 2) Malignant transformations of somatic cells and alterations in antigenicity. 3) Antigen localization in regard to cellular manifestations of tolerance and unresponsiveness, especially as related to oncogenic viruses.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Enzymology of Chemical Carcinogenesis	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
G. David Novelli (33%)	unclassified	☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR: * see below		☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 202,000	$ 220,000	$ 250,000
UNUSUAL COSTS	3,000	3,000	3,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 205,000	$ 223,000	$ 253,000
ORNL MAN-YEARS	5.5	5.5	6.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Yang, Wen-Kuang, and G. David Novelli. Isoaccepting tRNA's in Mouse Plasma Cell Tumors that Synthesize Different Myeloma. Biochem. Biophys. Res. Commun., 31: 534-539, 1968.

Yang, Wen-Kuang, and G. David Novelli. Multiple Isoaccepting Transfer RNA's in a Mouse Plasma Cell Tumor. Proc. Natl. Acad. Sci., U. S., 59: 208-215, 1968.

Yang, Wen-Kuang and G. David Novelli. Studies on the Multiple Isoaccepting Transfer Ribonucleic Acids in Mouse Plasma Cell Tumors. Nucleic Acids in Immunology, eds. Otto J. Plescia and Werner Braun, Springer-Verlag.

Novelli, G. David. The Separation of Isoaccepting Transfer RNA's and the Possible Role of tRNA in Regulation. Symposium on Protein-Nucleic Acid Interaction, March 31-April 3, 1969.

Novelli, G. David. Action of Carcinogens on Nucleic Acids. Twenty-Third Annual Symposium on Fundamental Cancer Research, Houston, Texas, March 5-7, 1969 (in press).

Rosen, Lawrence. 2'-O-Ethylation of Rat-Liver tRNA by Ethionine. Southeastern Regional Meeting, ACS, Florida State University, Tallahassee, Florida, December 4-7. 1968.

* C. J. Wust (50%), A. N. Best (67%), L. Rosen (100%), L. C. Waters (100%), W. K. Yang (100%).

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Yang, W. K., K. M. Hilse and R. A. Popp. Isoaccepting tRNA's and in vitro Hemoglobin Synthesis in C_3H and $C_{57}BL$ Mice. Fed. Proc. 28, 1969 (in press).

Cumming, Robert B., K. B. Hellman, A. Hellman and G. David Novelli. ^{3}H-uridine Incorporation in LM Cells in vivo and after Return to Culture. 19th Annual Meeting of the Tissue Culture Association, San Juan, P. R., June 11-13, 1968.

Novelli, G. David. New Systems for Isolation of Individual Species of Iso-Accepting tRNAs and Their Analytical Use. International Symposium on Molecular Biology Entitled Transfer of Genetic Information in Protein Synthesis (Synthesis, Structure and Function of Transfer RNA), New York, N. Y., June 7-8, 1968.

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Rosen, Lawrence, E. L. Murray and G. David Novelli. Cell-free Incorporation of Amino Acids by a Trout-Liver System. Heat-Labile Aminoacyl Transferase Activity. Can. J. Biochem., 45: 2005-2014, 1967.

Wust, C. J. Binding of ^{125}I-Labeled Anti-L-Chain to Polysomes of Rat Spleen in vitro Arch. Biochem. Biophys. 126: 524-529, 1968.

Yang, W. K., G. David Novelli and C. J. Wust. Differences in Seryl-tRNAs in Mouse Plasma Cell Tumors. Federation Proc. 27:687, 1968.

Orthwerth, B. J., U. Del Monte, L. Rosen, and G. David Novelli. Changes in Rat Liver tRNA During Ethionine Feeding. Fed. Proc. 27: 803, 1968.

Hellman, K. B., A. Hellman, R. B. Cumming and G. David Novelli. Comparative Physiology of L-M Cells Propagated in vitro and in Male and Female Mice. J. Nat'l. Cancer Inst., 308, 653-664, 1968.

Waters, Larry C. and G. David Novelli. The Early Change in E. coli Leucine tRNA after Infection with Bacteriophage T2. Biochem. Biophys. Res. Communs., 32, 971-976, 1968.

SCOPE:

Our approach to the study of the biochemical mechanisms involved in the induction of cancer is based on the premise that one of the early events in carcinogenesis results in a change in the rate of protein synthesis and a qualitative change in the kinds of proteins that are synthesized. Therefore, the carcinogen (either chemical or viral) must invoke, either directly or indirectly, a change in DNA, RNA, or the protein synthesizing system, the polyribosome. Furthermore, the change produced in the cell must be heritable in the progeny of the initial cell.

Since relatively little is known about the factors that control the rate of synthesis of DNA, RNA and protein in normal cells. it is difficult to look for factors that upset this control in cancer cells. Therefore, we will study a number of model systems in which the rate of synthesis of DNA, RNA and protein can be altered in the hope of obtaining an understanding of the nature of the regulatory substances. Simultaneously similar studies will be made with livers from rats fed chemical carcinogens, with an L-M fibroblast in tissue culture, and with mouse plasma cell tumors.

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During recent years mounting evidence suggests that the amino acid specific transfer RNA's may participate in important regulatory functions at the translation level, i.e., during assembly of the growing polypeptide chain on the messenger RNA template. Transfer RNA is almost unique among the cellular nucleic acids in that many of the nucleotide bases are normally alkylated with methyl groups. Since many of the chemical carcinogens are alkylating agents or are metabolically converted to alkylating agents it is possible these carcinogens alkylate tRNA and result in a derangement of its normal control function.

We are exploiting several new column chromatographic procedures developed in these laboratories; they give the best resolution of individual tRNA's thus far obtained anywhere, to seek for changes that may occur in tRNA during carcinogenesis.

We are studying four model systems: (1) E. coli infected with phage T2. In this system, it is already known that phage infection results in an alteration of a leucine specific tRNA. We, ourselves, have found a new change in leucyl-tRNA that occurs late in the infectious process that results in the appearance of two new leucyl tRNA's not previously observed. (2) We are studying two mouse plasma cell tumors, one of which produces an excess of the L chains of gamma globulin. (3) We have been studying an L-M fibroblast growing in tissue culture. This cell is derived from NCTC clone 929 of Earl's L cell which was originally isolated from normal subcutaneous tissue of a 100 day old male C_3H/Andervont mouse. These cells can be grown in serum-free medium either in monolayer or in suspension and they will give rise to tumors when injected into C_3H mice. (4) As a fourth system, we are studying changes in tRNA in rat liver during ethionine carcinogenesis.

The procedures that we use are extremely simple, but very sensitive and discriminating. In general, tRNA is isolated from normal tissue or in the case of transplantable tumors from the normal tissue of origin. Transfer RNAs are also isolated from T_2 infected bacteria, from the tumor and in the case of ethionine carcinogenesis from the preneoplastic liver and the subsequent neoplastic nodule. Each pair of tRNA's are charged with the same amino acid but carrying a different radioactive label (^{14}C or ^{3}H). The tRNA's are mixed and separated on one or both of the reversed-phase systems (system I-isopentyl acetate is the organic phase: system II - utilizes a high boiling Freon as organic phase). Any lack of coincidence between a ^{14}C and ^{3}H peak indicates the presence of an altered tRNA.

If changes in tRNA are not readily apparent we will direct our attention to looking at changes in the synthesis of other specific RNA's and for changes in the information content of the cells.

There are several ways to assess changes in RNA synthesis in cells and to indirectly determine changes in information content of cells. One of the ways is to inject ^{32}P into rats as a 20 min pulse, isolate the nuclei and separate nuclear RNA into four fractions and determine the base composition of individual fractions. This technique will indicate whether any new kinds of RNA are being made. Comparable studies with normal rats and those on a carcinogenic diet may reveal some specific alterations that could be studied more directly. Another approach to the same problem involves the use of the DNA dependent RNA polymerase as an assay for changes in expressible information in DNA. The behavior of DNA in this system can be assessed in several ways: (1) yield of RNA, (2) the base sequence in the RNA product, and (3) the activity of the RNA to stimulate in vitro amino acid incorporation. We propose to study DNA and RNA nucleoprotein isolated from neoplasms, from livers of animals fed chemical carcinogens as primers in the RNA polymerase reaction to see if this reagent can distinguish any changes in potential information of these DNA's by the three criteria previously

mentioned. Hopefully, a last and best criterion may be applied. That is the ability of the DNA to direct the synthesis of a messenger RNA for a specific enzyme that has newly appeared in the liver as a consequence of the chemical carcinogen.

RELATIONSHIP TO OTHER PROJECTS:

Enzymology Group: This project is directly related to and will lend active support to the basic program of the Enzymology Group which is concerned with control mechanisms in protein and nucleic acid biosynthesis. Any effect of a chemical carcinogen on these processes may present a situation that will permit a better understanding of the normal control mechanism.

Nucleic Acid Enzymology Group: The project on changes in tRNA following T2 infection of *E. coli* is a collaborative effort between the two groups.

Mammalian Genetics Section: All studies dealing with morphology (including electron microscopy) and chromosomal characteristics in the L-M cell project are being conducted by R. B. Cumming of this group.

Pathology and Physiology Section: Observations on gross tumor morphology and examination of histological sections are being carried out by G. E. Cosgrove.

Co-Carcinogenesis Program: Viral, as well as certain biochemical studies, have been conducted in collaboration with A. Hellman. Even though Dr. Hellman has returned to NCI we expect to continue the collaboration. We are also collaborating with Dr. Stanfield Rogers on the use of viruses as carriers of genetic information.

TECHNICAL PROGRESS IN FY 1969:

Leucyl-tRNA from T2 Infected E. coli B

We have continued our studies on the changes in leucyl-tRNA that appear after infecting *E. coli* B with bacteriophage T2. We have made a careful study of the early change, discovered by Sueoka, who erroneously interpreted the change to be a conversion of some of the leucine in the first peak to become added to the leucine tRNA that chromatographs later on MAK columns. On such columns, essentially only two peaks of leucyl tRNA are apparent. However on our RPC-2 system we can separate 5 distinct species of leucyl-tRNA from normal cells of *E. coli* B. A careful study of the total leucine acceptor activity of tRNA from cells infected with T2 for 6 min compared with the acceptor activity for other amino acids revealed that leucine acceptor activity had decreased to about 75% of normal. If this fact is taken into consideration when the data are plotted, then the early change consists of the specific inactivation of one or perhaps two leucine tRNA's in peak 1. No further change in the 5 peaks of leucyl-tRNA occurs during the remainder of the infection, except that late in the infection one or more new peaks of leucine tRNA appear that elute from the column after the first 5 peaks have been eluted. ^{35}S labeling as well as hybridization studies with host or phage DNA are consistent with the idea that the new leucyl-tRNA's are synthesized *de novo* and are coded for by the phage genome. However, the labeling of tRNA with ^{35}S after phage infection is too great to be accounted for by the new leucine tRNA's, that is the specific activity of tRNA labeled after phage infection is 25% that of a sample from normal cells labeled for the same period of time. A recent report by Kennel presents evidence that a considerable quantity of host specific nucleic acid is synthesized after phage injection, although host protein synthesis is shut off immediately.

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Our current interpretation is that the early change is the cleavage of a specific leucine tRNA by an enzyme which is phage induced. Perhaps this is how the phage prevents host protein synthesis from taking place. If all E. coli messenger RNA's contain the codon for this species of leucine tRNA, then destruction of this tRNA would stop all host protein synthesis.

Recently we have been studying T4 amber mutants that are unable to grow in E. coli strain B. We obtained 3 mutants from Dr. Edgar of Cal. Tech., that are blocked in some late function associated with assembling the phage head. All three mutants caused the early change, but failed to bring about the late change in the leucine tRNA.

Studies on Mouse Plasma Cell Tumors

In order to determine whether the differences in seryl-tRNA's between plasmacytomas producing different immunoglobulins (i.e., IgA vs. IgG) were related to the synthesis of different proteins, it becomes necessary to attempt to set-up an in vitro immunoglobulin synthesizing system in order to cross species of tRNA's. We have found that unlike microbial ribosomes, mammalian ribosomes are not suitable for triplet coding experiments, so these will be delayed until we can get the ordered polynucleotides to work.

L-M Fibroblast Studies

As a continuation of the study of the tRNA's of L-M cells and L-M cell-induced tumors, a detailed chromatographic comparison of isoaccepting tRNA's has been carried out to determine the effect of drastic changes in the nutritional environment on the tRNA pattern in these cells. Transfer RNA's were isolated from washed L-M cells and from fresh solid tumor tissue. These were aminoacylated with homologous aminoacyl-tRNA synthetase preparations with the same amino acid, but carrying different labels (^{3}H and ^{14}C) and then co-chromatographed on the RPC-2 column. The comparison has been made for 16 amino acids. Similar chromatographic patterns were observed for leucyl-, arginyl-, lysyl-, methionyl-, glycyl-, valyl-, tryptophanyl-, and prolyl-tRNA's. Significant quantitative differences in isoaccepting tRNA peaks were observed in species accepting isoleucine, alanine, threonine, and serine. Marked differences were found for tyrosyl-, phenylalanyl-, aspartyl- and histidyl-tRNA's. A tyrosyl-tRNA migrating in front of the 4 tyrosyl-tRNA's which were resolved on the RPC-2 column, was very prominent in L-M tumors but was completely absent in the L-M cells. Of the two phenylalanyl-tRNA's detected, the first peak was about 60% in L-M cells and below 3% in L-M tumors; whereas the second peak was about 40% in L-M cells and more than 95% in L-M tumors. The L-M cells showed two aspartyl-tRNA's a major one and a minor one (80 and 20%); L-M tumors showed 4 aspartyl-tRNA's, two (8 and 2%) migrating at the same chromatographic positions as those of L-M cells, and two distinct peaks (75 and 15%) migrating at different positions where no aspartyl-tRNA's from L-M cells were observed. L-M tumors had three histidyl-tRNA peaks, of which the most proment one in front was detected as the most minor one in the L-M cells.

Further experiments showed the following facts: (1) Although tumors from the male and the female C_3H mice were found to have some differences in the growth and regression rate, no differences were detected in their tyrosyl aspartyl-tRNA's; (2) When the tumor tissue was trypsinized for dispersion, adapted again to in vitro culture by One passage in 19% horse serum-medium 199 and several passages in 0.5% Bacto-peptone-medium 100 as a monolayer, and finally grown in suspension culture to obtain enough material for isolating tRNA, the chromatographic patterns of tyrosyl-aspartyl-, and phenylalanyl-tRNA's of these in vitro grown L-M tumor cells showed those of the L-M cells instead of those of L-M tumors; (3) When 5% mouse serum or 5% horse serum was

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included in the culture medium in the place of 0.5% Bacto-peptone for three passages, no conversion of aspartyl- and tyrosyl-tRNA's into the tumor pattern was observed; (4) Co-chromatography of tyrosyl-, aspartyl-, seryl-, and phenylalanyl-tRNA's from C_3H mouse liver with those from L-M cells or L-M tumors revealed that the isoaccepting aminoacyl-tRNA patterns of L-M tumors and the liver were similar; (5) Since tRNA's were prepared from L-M cells which were in cell suspension and from L-M tumors which were solid tissue, the possibility arose that different physical treatment of the source of material before phenol extraction might alter the chromatographic properties of isoaccepting tRNA's. This possibility was excluded by the finding that isoleucyl-, valyl- and seryl-tRNA's prepared from the cell suspensions of C_3H mouse reticulocytes were similar to those of L-M tumors in chromatographic separation.

To summarize, the results showed that the multiple isoaccepting tRNA's undergo remarkable changes in L-M cells following the drastic change from the in vitro cell culture to the in vivo environment. Whether this phenomenon results from the selection of the cell clone population or to adaptation of the cells at the cytoplasmic level remains to be determined.

Ethionine Carcinogenesis

We have almost completed the comparison of alkylated bases in rat liver tRNA following their labeling with ^{14}C-methyl-methionine or with [ethyl-1-^{14}C]-L-ethionine. Different ethylpurines and pyrimidines were synthesized and characterized to make positive identifications. The results reported below demonstrate some qualitative similarities between ethionine and methionine but also show marked differences. This demonstrates that ethionine has a different specificity and does not randomly compete with methionine for the same sites in tRNA.

In the purine fraction, only guanine is ethylated, not adenine. There are 4 different ethylguanines, 3 of which have been identified. N^2-ethyl, N^2, N^2-diethyl-, and 7-ethylguanine account for 23, 2, and 7% of the total ethylation respectively; two-thirds of purine ethylation. An unidentified ethylpurine, probably another ethylguanine, based on electrophoretic studies accounts for 17% of the total ethylation. These results contrast with those obtained with methionine; in the latter case both guanine and adenine are methylated.

The major ethylated purine, N^2-ethylguanine, apparently has a shorter half-life than the other ethylated purines in tRNA. This also contrasts with methylated purines of which 7-methylguanine apparently has the shortest half-life.

Approximately 80% or more of the ethylation which occurs in the pyrimidine nucleotide fraction is accounted for by 2'-O-ethylation of the ribose moiety. Paper chromatography demonstrates there are not less than three different 2'-O-ethylpyrimidine nucleotides in rat liver tRNA and they appear to be uridylic acid derivatives as indicated by electrophoresis. One may be assumed to be 2'-O-ethyluridylic acid and the others could conceivably be 2-O-ethyl derivatives of minor bases related to uridylic acid such as ribothymidylic acid, pseudouridylic acid, etc. These compounds differ markedly from those obtained with the methylated pyrimidine nucleotide fraction. In the latter case, 2'-O-methylation accounts for 12% (maximum) of the total methylation found. The major methylated pyrimidine nucleotide are ribothymidylic and 5-methylcytidylic acids whereas only a small amount of 5-ethylcytidylic acid has been identified.

The above results indicate that in tRNA only guanine in the purine fraction and mainly uridylic acid (and/or its derivatives) in the pyrimidine fraction are ethylated to a major extent. For the former, only base ethylation occurs and for the latter, mainly

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2'-O-ethylation occurs. These findings indicate a high specificity for ethionine, different from methionine.

EXPECTED RESULTS IN FY 1970:

Leucyl-tRNA from Phage Infected Cells

We expect to do more work with T4 amber mutants that are blocked in certain known specific functions to see if we can come closer to pinpointing the function of the new species of leucyl-tRNA.

We are currently working on a procedure that should allow us to isolate all species of leucyl-tRNA in a high state of purity. The principle of the procedure is to aminoacylate a mixture of tRNA with leucine, then chemically phenoxyacetylate the amino group. When such a derivitized molecule is chromatographed over benzoylated-DEAE-cellulose, the phenoxyacetyl group will cause the derivatized tRNA to stick to the column, while all the other tRNA's will be eluted in a salt gradient. Then the derivatized molecules can be removed with salt in 10% ethanol. This will yield all the leucyl-tRNA's in a single fraction, but now separated from the other tRNA's. The mixture of leucyl tRNA's is then deacylated and the individual isoaccepting species can be separated from each other by RPC-2 chromatography. Having the separate species will then permit accurate coding and binding studies. Polyribosomes from uninfected bacteria should not bind the new leucine species, but they should bind to polysomes of infected cells. It will be interesting to do timing experiments to determine at which point in time the polyribosomes in infected cells become competent for binding the new leucyl-tRNA's. In a similar manner, the tRNA that is destroyed by phage infection, when isolated from uninfected cells, should bind to polyribosomes of such cells, but not to polyribosomes from infected cells.

Studies on Mouse Plasma Cell Tumors

We will continue to work with the *in vitro* amino acid incorporating system, working toward improving the efficiency of the system and looking for a chain initiation system for immunoglobulin synthesis.

L-M Fibroblast Studies

The results obtained by us to date with this system has alerted us to the possibility that environmental factors, such as nutritional status, hormonal influences, etc., could possibly have a profound affect on the aminoacyl-tRNA profile of a tissue. These factors will be kept in mind in planning future experiments. We would like to extend these studies to viral oncogenesis, where the idea of specifically induced changes in tRNA are particularly attractive. We would like to collaborate with people at the National Cancer Institute who are working on viral oncogenesis.

Ethionine Carcinogenesis

Since our calculations show that under saturating conditions, ethionine alkylation results in the alkylation, on the average, of less than 1 ethyl group/tRNA molecule. It becomes necessary to isolate a pure sample of ethylated tRNA and to demonstrate that the same molecule containing the ethyl group is also aminoacylated.

We expect to carry out coding experiments with ethylated tRNA to determine whether the alkylation of tRNA brings about changes in response to the normal codons for such tRNA molecules.

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We do not know whether the alkylation of tRNA by ethionine is part of the process leading to the resultant neoplasia. However, a working hypothesis that we like to consider is that some of the minor components of an isoaccepting set of tRNA species may function as repressors or derepressors of certain genomes. There are some indications in the literature suggesting that tRNA's may bind together with other proteins to make a stable enzyme-tRNA complex. The one or two known cases of this involve the binding of tRNA to proteins that are not involved in protein synthesis and the suggestion has been made that such protein-tRNA complexes together may act as repressors, or derepressors of genomes. We will explore the binding of normal rat liver tRNA to normal cytoplasmic proteins by the technique of Sephadex Gel-filtration. If positive results are obtained, we will repeat these experiments with tRNA that has been alkylated by ethionine to determine whether the alkylation has disturbed the protein binding reaction.

EXPECTED RESULTS IN FY 1971:

This is a long term program with several lines being investigated simultaneously and it is difficult to be specific about FY 1971. In general we will follow the outline given for FY 1970 with the exception that any new leads uncovered by current work will be vigorously pursued.

Intellectually we are prepared to expand our studies on changes in tRNA during carcinogenesis to encompass the inhalation carcinogenesis study by working directly with lung tissue. If sufficient funds could be added to the program to cover the cost of employing the needed technical assistants - we could quickly extend the study to lung tissue.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April, 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Mammalian Chemical Co-Carcinogenesis	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
A.C. Upton (10%), (1969); J.B. Storer (20%) (1970-71)	Unclassified	☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR: *See below		☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 65,000	$ 60,000	$ 65,000
UNUSUAL COSTS	-0-	2,000	2,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 65,000	$ 62,000	$ 67,000
ORNL MAN-YEARS	2.0	1.5	1.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Clapp, N.K., A.W. Craig, R.E. Toya, Sr. Oncogenicity by methyl methanesulfonate in male RF mice. Science 161: 913-914, 1968.

Clapp, N.K., A.W. Craig, R.E. Toya, Sr. Pulmonary and hepatic oncogenesis during treatment of male RF mice with dimethylnitrosamine. J. Natl. Cancer Inst. 41: 1213-1227, 1968.

Clapp, N.K. and A.W. Craig. Differences in tumor cell types and organ sites in RF mice treated with dimethylnitrosamine (DMN) and diethylnitrosamine (DEN). Proc. Am. Assoc. Cancer Res, 9: 14, 1968, abstract.

*R. C. Brown (35%), G. E. Cosgrove (10%), R. L. Tyndall (20%), and J. Yuhas (50%); A. W. Craig (collaborative work in England supplying purified compounds necessary for the current series of experiments).

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SCOPE:

Carcinogenic effects of chemicals. It has been postulated that the potent carcinogens dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) are metabolized to highly reactive by-products, such as the corresponding diazoalkanes, which, in turn cause alkylation of DNA and RNA, primarily at the 7-methyl position of guanine. The latter reactions have been implicated hypothetically in the carcinogenic activity of these compounds. In contrast, alkylation by the sulfonoxyalkane methyl methanesulfonate (MMS) also occurs at the 7-methyl position of guanine but has not been observed to be carcinogenic. Consequently, it is conceivable that competition between these two groups of alkylating agents for biologically important sites might occur, with MMS inhibiting the carcinogenic effects of DMN, DEN, and other like agents. The object of this investigation is to study the comparative and interactive effects of nitrosamines and sulfonoxyalkanes as a guide to their mode of action, with particular reference to carcinogenesis.

Combined effects of X rays and radiomimetic chemicals in carcinogenesis. Radiomimetic chemicals (triethylene melamine, nitrogen mustard, and myleran) have caused late somatic effects in RF mice similar to those resulting from X rays; i.e., shortening of life, carcinogenic effects on different organs, lens opacities, and various degenerative changes in tissues throughout the body. The objective of this study is to investigate whether the carcinogenic effects of these chemicals are mediated through mechanisms in common with those of radiation and to explore the combined effects such chemicals exert in conjunction with radiation.

Virus studies. Previous investigations have shown that Rauscher virus infection of mouse thymus-spleen cell cultures is associated with transformation, one manifestation of which is production of abundant mucin. Injection of such transformed cells into newborn BALB/c mice resulted in the occurrence of myxofibrosarcomas. Untransformed non-infected cell cultures did not produce mucin *in vitro* and on injection into newborn BALB/c mice produced fibrosarcomas. Our previous ultrastructural studies of subcutaneous myxofibrosarcoma showed that the tumor cells produced abundant protein-like material and contained numerous virus particles, contrasting with the tumor cells of the fibrosarcomas which produced little protein-like material and no detectable virus particles. The study of this and related aspects of the viral transformation are being pursued, with the objective of elucidating their mechanisms and significance in relation to the loss of contact inhibition and invasiveness characteristic of the neoplasms.

RELATIONSHIP TO OTHER PROJECTS:

This program will be closely coordinated with related projects in the Biology Division and the National Cancer Institute. It will also be closely coordinated with studies on the biological properties of the nitrosamines being carried out by Dr. Craig and his co-workers at the University of Manchester, England. To our knowledge the experiments are not duplicated in any other laboratory within the U.S. or elsewhere.

TECHNICAL PROGRESS IN FY 1969:

Carcinogenic effects of chemicals: (N. K. Clapp, A. W. Craig)

Since alkylation of nucleic acids has been postulated to explain the mutagenic and carcinogenic action of certain alkylating agents, such as dimethylnitrosamine (DMN) and diethylnitrosamine (DEN), we have studied methyl methane sulfonate (MMS), which

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methylates DNA and RNA but has not been reported to be carcinogenic hitherto. In a total of 63 11-week-old male mice from a noninbred subline of the RF/Un strain, which received 30 mg MMS/kg body weight daily in the drinking water throughout life, we found an increased incidence of lung tumors (70.2% compared to 38.9% in 162 controls) and thymic lymphomas (14.9% compared to 3.7% in controls). As measured by lung tumor induction, therefore, MMS resembles DEN and DMN. MMS also appears to resemble x-rays, nitrogen mustard, TEM, and myleran in inducing thymic lymphomas in RF mice. Since alkylation occurs primarily at the 7-position of guanine in both DNA and RNA after MMS, DMN, and DEN, differences in oncogenicity between MMS, DMN, and DEN suggest that a qualitative difference may exist between the site(s) of alkylation by these agents within nucleic acids, the precise location of the alkylation along the nucleic acid chain possibly being important in the oncogenic process. Studies to determine the co-carcinogenicity of MMS with DMN and DEN are now in progress.

Diethylnitrosamine (DEN) is a potent carcinogen in a number of species of animals, inducing a high incidence of liver hepatomas, lung adenomas, and squamous cell carcinomas of the forestomach in RF mice. To determine tumor incidence-cumulative dose relationships for DEN, eight-to-10-week-old male RF mice were given DEN in the drinking water in various daily doses for treatment periods of varied lengths; cumulative doses ranged from 57 to 943 mg/kg body weight. Mean survival times were markedly reduced in groups receiving high total doses and in groups where treatment periods (and drug toxicity) extended for several months. The incidence of liver tumors increased linearly with increasing cumulative dose throughout the range of doses used. In contrast, maximum incidences of lung and stomach tumors (∿80% and 100%, respectively) were reached at low cumulative doses, and low thresholds were suggested, while none was seen for liver oncogenesis. In three groups that received DEN at the same daily dose (6 mg/kg) but with varied treatment periods (1, 7, 22 weeks), the higher dose groups developed the highest incidences of tumors, despite decreased mean survival time. At the lowest dose, lung tissue appears to be most sensitive, stomach intermediate in sensitivity, and liver least sensitive to DEN carcinogenesis.

Combined effects of X rays and radiomimetic agents. (A. C. Upton, J. W. Conklin)

The radiomimetic alkylating agent triethylene melamine (TEM) has been found to resemble x-rays in certain late somatic effects on RF mice. These include shortening of the life span, leukemogenic effects on the thymus, induction of tumors in the lung, ovary, and other organs, and the formation of lens opacities. The present study was undertaken to explore the combined effects of TEM and X rays administered concurrently, with the aim of determining whether the effects of the two agents would be additive to a degree consistent with a common mode of action. Ten-week-old female RF/Un mice were subjected to: (1) I-P injection of TEM, 0.25-4.0 mg/K; (2) 25-500 R whole-body 300 kvp x-irradiation (80 R/min.); or (3) both TEM and x-irradiation (TEM injected 1-5 minutes before irradiation). Preliminary results 30 months after treatment show that each agent given alone shortened the life span but that the effect was greater in mice receiving both agents than in those receiving a comparable total dose of either agent alone, especially at high dose levels. Likewise, each agent alone induced thymic lymphomas and ovarian tumors, but when both agents were administered together, the incidence exceeded that induced by either agent alone, indicating that the two agents administered together exerted effects which were additive, if not synergistic, depending on the end-effect in question. Since, however, a few of the mice remain alive and the lesions induced by the two agents remain to be analyzed in full, the significance of their combined effects is yet to be determined.

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To explore possible mechanisms of synergistic effects of the two agents, the combined toxicity of the agents to thymus and hemopoietic cells is being analyzed with respect to effects on thymus weight and on killing of hemopoietic cells, as judged by the Till-McCulloch spleen nodule method. Preliminary results imply that X rays and TEM differ quantitatively, if not qualitatively, in their effects on blood-forming cells.

Myxofibrosarcomatous transformation by the Rauscher virus. (R.C. Brown, R.L. Tyndall)

Comparative ultrastructural examinations were made of non-infected, non-transformed and Rauscher-virus-infected transformed mouse thymus-spleen cell cultures, and of Rauscher-virus-infected and control cultures produced by cloning of the control spleen-thymus cultures and infection of the derived cloned cultures. In all four culture types, three kinds of cells were recognized: 1) a pale cell considered to be an immature cell showing sparse, small mitochondria, few ribosomes and very little endoplasmic reticulum; 2) a medium dense cell, morphologically like a fibroblast cell, showing a moderate amount of rough endoplasmic reticulum, moderate numbers of medium to large sized mitochondria, and a moderate number of ribosomes, and 3) a dark cell which contained very electron-dense cytoplasm containing moderate amounts of rough endoplasmic reticulum, a moderate number of mitochondria of moderate size, and a very electron-dense nucleus. Rauscher virus proliferated abundantly in cells of the cloned and uncloned infected cultures and was not seen in the control cultures. Virus replication was seen only in the light and medium electron-dense cells. One hundred twenty to 160 Å diameter extracellular fibrils were observed in the uncloned virus infected culture, but were not found in uninfected cultures or in the infected cloned cultures. Mucus also was identified only in the supernatant of infected uncloned cultures. Ultrastructural studies of myxofibrosarcomas resulting from injected infected cultures and fibrosarcomas resulting from injected uninfected cultures showed that the three kinds of cells observed in the injected cultures were present. In the fibrosarcoma as in the control cultures, the pale- and medium-electron-dense cells predominated and dark cells were rare. In the myxofibrosarcomas pale cells with marked secretory activity predominated, whereas medium-electron-dense cells and dark cells were rare. The cells of the myxofibrosarcoma produced extracellular fibrils like those seen in the infected uncloned culture as collagen fibers. Work thus far suggests that Rauscher virus has been responsible for phenotypic transformation of spleen-thymus mouse cells on at least two occasions.

EXPECTED RESULTS IN FY 1970:

Experiments aimed at determining the consistency with which Rauscher virus can induce transformation will be pursued. Ultrastructural and histochemical studies will be made to compare the phenotypic expression in tumors *in vivo* resulting from injection of Rauscher virus inoculated cultures that do or do not produce mucus *in vitro*.

The studies with nitrosamines and TEM will be completed.

EXPECTED RESULTS IN FY 1971:

Clonal experiments will be pursued to determine if the three kinds of cells observed in the thymus-spleen cell cultures and in fibrosarcomas and myxofibrosarcomas resulting from injection of control and infected cultures are varied forms of a single cell or are three different cell types. Studies will be directed toward detecting altered mucopolysaccharide metabolism in Rauscher virus-infected mice and its possible relationship to neoplastic and non-neoplastic diseases which occur in these infected mice.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION · NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April, 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Chromosomal Effects of Chemicals and Radiation	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
E. H. Y. Chu (50%)	Unclassified	☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR: E. H. Y. Chu, S. Bell (Consultant)		☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 80,000	$ 90,000	$ 125,000
UNUSUAL COSTS	5,000	5,000	5,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 85,000	$ 95,000	$ 130,000
ORNL MAN-YEARS	2.5	2.5	3.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Chu, E. H. Y. and H. V. Malling. Chemical mutagenesis in Chinese hamster cells *in vitro*. Proc. XII Internat. Congr. Genetics 1: 102, 1968.

Chu, E. H. Y., Patricia Brimer, K. B. Jacobson and E. Virginia Merriam. Mammalian cell genetics. I. Selection and characterization of mutations auxotrophic for L-glutamine or resistant to 8-azaguanine in Chinese hamster cells *in vitro*. Genetics (submitted).

Chu, E. H. Y. and H. V. Malling. Mammalian cell genetics. II. Chemical induction of specific locus mutations in Chinese hamster cells *in vitro*. Proc. Natl. Acad. Sci. U. S. 61: 1306-1312, 1968.

Buckton, K. E. and P. Nettehseim. *In vitro* and *in vivo* culture of mouse peripheral blood for chromosome preparations. Proc. Soc. Exper. Biol. Med. 128: 1106-1110, 1968.

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SCOPE:

This program is jointly sponsored by AEC and NIH. The work supported by the two agencies is so closely interrelated that parts of the report are the same for both agencies.

1017550

The program is concerned primarily with the cytogenetic effects of radiations and chemicals on mammalian cells, especially in vitro. The main objectives are threefold: (1) to estimate the biological sensitivity of human and other mammalian cells to radiation and chemical agents, including carcinogens, (2) to attempt to correlate the observed cytogenetic changes with biochemical modifications in the cell, and (3) to find the relationship between the chemically induced mutations (gene mutations and chromosome aberrations) and the neoplastic processes. This last approach is to test the idea of somatic mutation as a cause of carcinogenesis and the general relationships between carcinogens and mutagens. Experimental chemical carcinogenesis in mammalian cells in vitro will also be attempted in this connection.

The effects of radiation and chemicals on mammalian cells in culture can be measured by a variety of cytogenetic criteria which include chromosome aberration, mitotic inhibition, gene mutation and cell killing (colony formation). Our purpose is to establish the dose-effect relationship and then to correlate these phenomena with each other and with the concomitant modifications in the biosynthetic and metabolic processes in the treated cells.

In the past two years, we have developed techniques for the selection of mutant markers in Chinese hamster cells in culture. Furthermore, we have demonstrated chemical mutagenesis in these cells, thus opening new experimental avenues for mutation research in mammalian cells. We are continuing the efforts in refining technical procedures and in establishing a variety of new genetic markers. Furthermore, we have extended these studies to human cells in culture. The mammalian cell culture system offers opportunities for basic studies as well as for practical screening tests for possible mutagenic and carcinogenic agents in the human environment.

Chromosome aberration analysis has been and will continue to be one of our main efforts of the overall program. Our current interests center around several topics of which one, chromosome breakage and other cytological abnormalities induced by carcinogens, is especially pertinent to the present program. This study is basic to any consideration of the effects of chemicals as well as radiation on the genetic mechanisms of the cell.

RELATIONSHIP TO OTHER PROJECTS:

Among the laboratories where the effects of radiations and chemicals on mammalian chromosomes have been studied are the University of California Medical Center, University of Colorado Medical Center, University of Texas M. D. Anderson Hospital, Argonne National Laboratory, University of Aberdeen, Scotland, Western General Hospital, Edinburgh, Scotland, University of Lund, Sweden, and University of Uppsala, Sweden.

Relatively few laboratories are actively engaged in the genetic studies of somatic cells. Mention may be made of the University of Colorado Medical Center, Yale University, University of Michigan, University of Wisconsin and University of Washington. Within the Biology Division, ORNL, work is coordinated with the Fungal Genetics Group and the Mammalian Genetics and Development Section.

Coordination and possible cooperation is anticipated with other groups within the Biology Division that are participating in the Co-carcinogenesis Program.

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TECHNICAL PROGRESS IN FY 1969:

Selection and Characterization of Mutations in Mammalian Cells

Using various selection techniques analogous to those used in microbial genetics, we reported the isolation of mutants auxotrophic for L-glutamine or resistant to 8-azaguanine in an established aneuploid line of Chinese hamster lung cells. This type of endeavor has been continued, both in refining the different selective techniques and in building up a variety of new mutants.

With respect to selective methods, we have shown that cells whose purine and pyrimidine biosynthesis was blocked by aminopterin incorporated exogenous 5-bromodeoxyuridine (BUdR) much more effectively than cells not treated with BUdR. The heavily BUdR-substituted cells were very sensitive to visible light, as measured by colony forming ability. This modification greatly improves the effectiveness of selecting auxotrophic mutants, as first proposed by Puck and Kao (1967).

Applying the "thymidineless death" procedure, we have further isolated a group of auxotrophic mutants that were dependent on one or more of ten added metabolites. Their specific nutritional requirement as well as their genetic and biochemical nature are being investigated.

A mutant line was obtained which exhibited resistance to a high concentration (10^{-3} M) of BUdR. The cells failed to incorporate H^3-thymidine, probably lacking the thymidine kinase activity. Another group of mutants showed remarkable tolerance to 10^{-3} M thymidine whereas normal cells are repressed even with 10^{-5} M of the metabolite. The cells seemed to retain the normal permease activity, but probably had modified thymidine kinases which reacted variably with the substrate, thymidine.

Chemical Induction of Forward and Reverse Mutations

Evidence has been presented that in hamster cells ethyl methanesulfonate (EMS) significantly induces both forward and reverse mutations at the gene loci that control glutamine requirement or resistance to 8-azaguanine. These studies have been extended to include other chemical mutagens to define the conditions for mutation expression, and to test the mutagenic specificity.

A highly significant increase over the controls in the forward mutation frequency from azaguanine sensitivity to resistance can be induced with EMS, MMS (methyl methanesulfonate), MNNG (N-methyl-N'-nitro'N-nitrosoguanidine) and ICR-170 (an acridine mustard). In equimolar concentrations and under similar experimental conditions, the relative effectiveness of the four compounds used is as follows: ICR-170 > MNNG > MMS > EMS.

A large number of mutant clones have been isolated which were shown to fully retain the drug-resistant character after numerous cell generations in the absence of the drug. Reversion to drug sensitivity occurred either spontaneously or following mutagenic treatment. There appear to be qualitative differences in revertibility to sensitivity between the class of resistant mutants which originated spontaneously, those induced by EMS or MMS, and those induced by ICR-170. Some of the EMS- or MMS-induced mutants reverted either spontaneously, or very significantly after MNNG treatment. Some MNNG-induced mutants could only revert with EMS. Most of the ICR-170-induced mutants could not revert at all, probably representing multiple intragenic changes or deletions. The most interesting is the fact that 2 out of 3 ICR-170-induced mutants tested could be reverted only with the same mutagen that induced them. These results are consistent with the findings of mutagenic specificity in micro-organisms.

Photodynamic Effect of Carcinogenic Polycyclic Hydrocarbons in Mammalian Cells

We have thus far employed the following compounds for cell killing as measured by the colony forming ability of Chinese hamster cells in vitro: 1,2-benzpyrene, 3,4-benzpyrene, 1,2; 3,4-dibenzanthracene, 1,2; 5,6-dibenzanthracene, and 9,10-dimethyl 1,2-benzanthracene. Our results indicate a positive and dramatic correlation between carcinogeneic potency of the compound and the photodynamic effect.

Mutagenicity of Chemical Carcinogens in Mammalian Cells

With the availability of genetic markers and the demonstration of chemical mutagenesis in tissue culture mammalian cells, it becomes feasible to test in the same system both carcinogenecity and mutagenicity or a particular agent. We have studied the cytotoxicity and photodynamic effect of several carcinogenic and non-carcinogenic polycyclic hydrocarbons. These same series of compounds, as well as several aromatic amines, have been used for mutation induction in the hamster cells. The mutagenic change studied was from sensitivity to 8-azaguanine (wild type) to resistance.

Our results clearly show that 9,10 dimethyl-1,3-benzanthracene was highly mutagenic, whereas 1,2; 3,4-dibenzanthracene and 1,2; 5,6-dibenzanthracene caused slight but significant increase in the forward mutation frequency. On the other hand, neither 1,2-benzpyrene nor 3,4-benzpyrene was shown to be mutagenic.

Preliminary data indicate that 2-acetylaminofluorene (AAF) and N-hydroxy-AAF are slightly mutagenic whereas their metabolic derivative, N-acetoxy-AFF, is highly mutagenic in hamster cells. Further in vitro tests of this type, together with parallel studies of mouse cells in vivo, may throw light on the possibility that metabolic derivatives rather than the original compound per se is responsible for the final effect, whether it is a mutation or a cancer or both.

EXPECTED RESULTS IN FY 1970:

Somatic Cell Genetics

Selection and characterization of the available and additional genetic markers in hamster cells will be continued. The studies will involve cytological, genetical and biochemical analyses of mutants, revertants and somatic hybrids. Similar work will be extended to normal and neoplastic cell lines of human origin. Of special interest is the use of human cells derived from individuals with inborn error of metabolism. The first application will be the mapping of genes on human chromosomes through experimental hybridization of the various human cell strains with hamster or mouse cell lines carrying various genetic markers. The second use of the human mutant cell lines is the artificial induction of back mutations.

Chemical and Radiation Mutagenesis in Hamster Cells In Vitro

Considerable efforts will be devoted to and results are expected from mutagenesis experiments with this system. We plan to induce forward and reverse mutations at a few well-defined specific loci in Chinese hamster cells with various chemical mutagens and carcinogens, X-rays, ultraviolet light, etc. and compare the properties of mutants induced by different agents as well as the frequencies of mutations at different loci.

The molecular mechanism of mutagenesis, the problems of expression and repair of mutations, and the relationship between genic and chromosomal mutations are being investigated and certain results are expected.

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In Vivo Mouse Systems for Mutagenesis Studies

The Ehlich ascites tumor, strain ELD, has a bimodal chromosome numbers of 45 and 46. There are 5 morphologically distinct marker chromosomes, in addition to 40 or 41 telocentrics. These tumor cells are easily transplantable, rapidly dividing and amenable for chromosome analysis. The cells have an average generation time of 18 to 24 hours, depending on the host strain.

The tumor-bearing animals will be treated with chemicals through various routes. The tumor cells will then be harvested at desired times for chromosome preparation and analysis. The tumor cells can be treated in vitro in short term cultures within, for example, 24 hours after explantation. Moreover, we have also succeeded in maintaining the ELD cells continuously in vitro. The same treated, tumor-bearing animal will be utilized for chromosome preparations from its bone marrow, spleen, blood and, in case of males, the cells in spermatogenesis.

The obvious purpose of this study is to compare the cytogenetic effects of chemicals and their metabolic break-down products. Some preliminary evidence of chemically-induced chromosome aberrations in ELD cells in vivo and in vitro has already been obtained. Further information is expected in FY 1970 from experiments with this simple test system.

The second in vivo mouse system under consideration for somatic mutation studies is the lymphomas induced by virus or chemical carcinogens in inbred strains of mice. These tumor cells have been shown to retain the specific histocompatibility H-2 isoantigens characteristic of the host of origin. Rotman and Papermaster (1966) showed that lymphoma cells maintained in culture can hydrolyze nonfluorescent aliphatic acid esters of fluorescein, thus accumulating a fluorescent product (fluorescein) intracellularly. This property, referred to as "fluorochromasia", is easily visualized under a microscope using a light source which excites fluorescein.

Our planned experiments make use of this sensitive technique for a detection of induced mutations at the H-2 locus. The lymphoma-bearing animal will be treated with chemicals. The tumor cells are then harvested and treated in vitro with specific antisera plus complement. The survivors of this cytotoxic screening may turn out to be mutants.

Cytogenetic Effect of Carcinogens

Further results are expected on the effects of carcinogens on mammalian cells in culture. The criteria used are: (1) cell death (instant death and reproductive death), (2) mitotic inhibition, (3) gross cytological effect, (4) rupture of lysosomes, (5) frequency of chromosomal aberrations, and (6) induction of mutations. We expected certain conclusions from some of these studies, especially the cellular mechanisms leading to cell death and chromosome aberrations through combined treatment of carcinogenic compound and near visible light. Studies of lysosomal enzymes will be pursued in this respect.

The mutagenicity of chemical carcinogens will involve a somewhat larger scale of screening that requires time before any conclusions can be drawn. Nevertheless, since both mutants and methods for analysis are available, and since preliminary indications from mutagenesis experiments appear very encouraging, we are continuing studies in this direction with vigor and enthusiasm.

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EXPECTED RESULTS IN FY 1971:

FY 1971 will be devoted to continuation and in some cases, completion of the investigations listed in the above categories. The anticipated program will include the following main areas: (1) somatic cell genetics, (2) molecular basis of chromosomal, genic and cellular modifications by radiations and chemicals, (3) in vitro chemical carcinogenesis, (4) tumor cytology, (5) tumor antigens, and (6) mammalian cytogenetics.

The program has been planned to reach full size during FY 1969, but it now appears that it will not be possible until the FY 1971. The development of this research program is such that we must request additional staff in order to carry out the planned work fully and effectively. During FY 1971, we would like to add two staff scientists and one laboratory technician. One of the scientists should be a cell biologist with biochemical background and the other an immunogeneticist.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

1077555

OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Somatic Cell Genetics	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
R. F. Kimball		☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR: J. D. Regan (50%)	Unclassified	☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 50,000	$ 70,000	$ 90,000
UNUSUAL COSTS	10,000	7,000	8,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 60,000	$ 77,000	$ 98,000
ORNL MAN-YEARS	1.5	1.5	2.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Regan, James D., J. E. Trosko, and W. L. Carrier. Evidence for excision of ultraviolet-induced pyrimidine dimers from the DNA of human cells in vitro. Biophysical Jour. 8:319-325, 1968.

Regan, James D. and Susumo Takeda. Aspects of photoreactivation in animal cell cultures. Rad Res. (in press) (Abstract), 1968.

Regan, James D., M. M. Sigel, and W. H. Lee. Chromosome alteration in marine fish cells in vitro. Can. Jour. Gen. Cytol., 1968.

Regan, James D. The repair of ultraviolet-induced DNA lesions in cell cultures. Fla. State Univ. Alumni Studies Conference in Molecular Biology. (in press), 1968.

Regan, James D., J. S. Cook, and W. H. Lee. Photoreactivation of amphibian cell cultures. Journ. Cell. Physiol. 71:173-176, 1968.

Regan, James D., J. S. Cook, and Susumu Takeda. Reptilian cells in vitro exhibit photoreactivation. In Vitro 4: (in press) (Abstract), 1968.

Regan, James D. and J. S. Cook. Photoreactivation among the classes of vertebrates. Abstract for the V Internat. Cong. of Photobiology, Hanover, N. H., August 26-31, 1968.

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Regan, James D., H. Vopopick, S. Takeda, W. H. Lee, and F. M. Faulcon. Evidence for a serine requirement in leukemic and normal human blood cells. Science (in press), 1969.

Regan, James D. and Lewis I. Pizer. Molecular basis for the serine requirement in human blood cells: lack of 3-phosphoglycerate dehydrogenase. (to be submitted to Science), 1969.

SCOPE:

The major objective of this program has in the past been the development and utilization of genetic marker systems in animal cells in vitro. This led us into an examination of certain nutritional requirements in cell lines of the established epithelioid type and also in the more recently developed lymphoblastoid cell lines.

In connection with the above studies, we examined primary blood cells from leukemic patients and normal human bone marrow cells for nonessential amino acid requirements. These experiments indicated that while leukemic cells required asparagine (a finding previously indicated by work in other laboratories) normal bone marrow also required asparagine. However, another nonessential amino acid, namely serine, was found to be a more severe requirement in both types of cells. These findings have resulted in a shift in emphasis in the scope of these studies to the characterization of the nutritional requirements of malignant and normal human cells both in primary cells from patients and normal subjects and in established lymphoid cell lines.

RELATIONSHIP TO OTHER PROJECTS:

We are still engaged in extensive use of genetic markers in cell cultures. Thus the findings which derive from the nutritional studies indicated above will be of distinct value as markers in cell lines.

TECHNICAL PROGRESS IN FY 1969:

Our results with amino acid requirements in leukemic cells and in normal human bone marrow cells show that: (1) Asparagine is required by leukemic cells as suggested by other works but (2) asparagine is also required by normal bone marrow. (3) Serine is required much more than asparagine by both cell types.

In collaboration with Dr. Lewis I. Pizer, we have demonstrated a molecular basis for the serine requirement of these cells viz that they lack 3-phosphoglycerate dehydrogenase activity.

EXPECTED RESULTS IN FY 1970:

Further work on nutritional requirements in blood cells and other cell types should yield more information on the amino acid requirements of specific human tissues. In connection with nutritional experiments, work on the biosynthesis of these nutrilites and the enzymatic basis for the nutritional need will hopefully provide the molecular explanation for the requirements. In connection with these studies, we need a full time biochemist in this group.

1017557

TITLE: NCI Somatic Cell Genetics

ACTIVITY NO. 40 04
189 No.

EXPECTED RESULTS IN FY 1971:

In collaboration with area hospitals, we wish to secure an array of malignant and normal tissue types for characterization of their nutritional requirements.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April, 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE		AEC ACTIVITY NUMBER
NCI Biochemical Regulation		40 04
PERSON IN CHARGE F. T. Kenney (50%)	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION Unclassified	PROGRAM STATUS ☐ NEW WORK BEGINNING FY _____ ☒ ESTABLISHED PROGRAM
*PRINCIPAL INVESTIGATOR W. D. Wicks (50%); C. B. Hager (ACS) 25%;		

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER *(SPECIFY)*
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 82,000	$ 90,000	$ 95,000
UNUSUAL COSTS	3,000	5,000	5,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 85,000	$ 95,000	$ 100,000
ORNL MAN-YEARS	2.5	2.5	2.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

*Principal Investigators:

J. R. Reel (ACS) 30%; J. L. Wittliff (USPHS) 50%); K. L. Lee (Hoffman-LaRoche) 50%

DATES AND TITLES OF PUBLICATIONS:

Kenney, F. T., D. Holten and C. B. Hager. Hormonal regulation of enzyme synthesis. In: Gunma Symposia on Endocrinology, 5, 31 (1968).

Hager, C. B. Hormonal control of tyrosine transaminase in the isolated, perfused rat liver. Fed. Proc. 27, 641 (1968).

Reel, J. R. and F. T. Kenney. Regulation of tyrosine transaminase synthesis by hydrocortisone in hepatoma cell cultures. Fed. Proc. 27, 641 (1968).

Hager, C. B. and F. T. Kenney. Regulation of tyrosine transaminase in rat liver. VII. Hormonal effects on synthesis in the isolated, perfused liver. J. Biol. Chem. 243, 3296 (1968).

Reel, J. R. and F. T. Kenney. "Superinduction" of tyrosine transaminase in hepatoma cell cultures: differential inhibition of synthesis and turnover by actinomycin D. Proc. Natl. Acad. Sci. U. S. 61, 200 (1968).

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Kenney, F. T., J. R. Reel, C. B. Hager and J. L. Wittliff. Hormonal induction and repression. In: Regulatory Mechanisms for Protein Synthesis in Mammalian Cells. A. San Pietro, M. Lamborg and F. T. Kenney, eds. Academic Press, New York, 1968, p. 119.

Wicks, W. D. The possible role of cyclic adenylic acid in regulation of enzyme synthesis. In: Regulatory Mechanisms for Protein Synthesis in Mammalian Cells. A. San Pietro, M. Lamborg and F. T. Kenney, eds. Academic Press, New York, 1968, p. 143.

Wicks, W. D. Induction of tyrosine-α-ketoglutarate transaminase in fetal rat liver. J. Biol. Chem. 243, 900 (1968).

Wicks, W. D. Tyrosine-α-ketoglutarate transaminase: induction by epinephrine and adenosine-3',5'-cyclic phosphate. Science 160, 997 (1968).

Kenney, F. T. and J. R. Reel. Hormonal regulation of enzyme synthesis: transcriptional or translational control? In: Hormones in Development, to be published by the National Foundation.

Reel, J. R. and F. T. Kenney. Regulation of tyrosine transaminase in rat liver. VIII. Induction by hydrocortisone and template stability in cultured hepatoma cells. Submitted to J. Biol. Chem.

Lee, K. L. and F. T. Kenney. Regulation of tyrosine transaminase in rat liver. IX. Induction by glucagon and studies on mechanisms of hormonal induction in cultured hepatoma cells. Submitted to J. Biol. Chem.

Kenney, F. T., W. D. Wicks and K. L. Lee. Induction of hepatic enzyme synthesis *in vivo* by 3',5'-cyclic adenosine monophosphate. Submitted to J. Biol. Chem.

Wicks, W. D. Induction of hepatic enzymes by adenosine-3',5'-monophosphate in organ culture. Submitted to J. Biol. Chem.

Wicks, W. D. Induction of hepatic enzymes by adenosine-3',5'-monophosphate. Fed. Proc., in press.

Lee, K. L. Transcriptional and translational mechanisms in hormonal induction of enzyme synthesis. Fed. Proc., in press.

Wittliff, J. L. and F. T. Kenney. Regulation of synthesis of yolk proteins in amphibian liver by estrogen. Fed. Proc., in press.

Kenney, F. T. Hormonal regulation of synthesis of liver enzymes. In: Mammalian Protein Metabolism, Vol. IV, H. N. Munro, ed. Academic Press, New York, in press.

SCOPE:

The program of the Biochemical Regulation Group is directed towards understanding the operation of cellular control mechanisms in mammalian tissues, particularly those regulated by hormones, and the role of these mechanisms in normal and abnormal growth and differentiation. This program is predicated upon the following considerations: (1) that knowledge of these control mechanisms is essential to understanding the cellular changes induced by radiation and other agents of cellular damage; (2) that recognition of the mechanisms controlling growth is essential to understanding the phenomenon of uncontrolled growth; (3) that the complexities of the mammalian organism cannot always be bypassed in favor of the simpler microbial systems, indeed, that it is by

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virtue of these complexities (such as the imposition of hormones) that growth and differentiation of the mammalian organism is controlled; and (4) that the major mode of metabolic control, in mammalian as well as microbial systems, is through control of the activity levels of cellular enzymes.

The scope of this program encompasses all the means by which enzyme levels are regulated in mammalian tissues, with particular emphasis upon those mechanisms which alter the amount of enzyme protein. The proteins of mammalian tissues undergo metabolic turnover, and hence, the amount of a given protein can be changed by altering the rate of either the synthesis or the degradation of that protein. Work in this and other laboratories has established that the synthesis of enzyme proteins is controlled by hormones, and we are currently investigating several systems in an attempt to learn more of the mechanism of this control. We are also studying molecular mechanisms of enzyme degradation, in an attempt to analyze the basis for different turnover rates of various enzymes.

We are performing a study of the biochemical changes which occur in rat liver just before and just after birth. It has already been established that a specific sequence of changes in the level of certain enzymes is initiated in liver during the last few days of gestation. We are interested in the biochemical means by which these changes are brought about. Aside from an interest in developmental processes per se, an understanding of the mechanisms which produce alterations in the level of enzymes during gestation will be of great value in interpreting regulatory processes which occur in the adult. Furthermore, a better understanding of normal developmental changes may help to assess and perhaps to remedy developmental abnormalities. An organ culture technique has been developed and is being used for these studies.

Hormonal regulation of enzyme synthesis in neoplasia is being studied, utilizing cell cultures of a minimal deviation tumor developed from rat liver, the Reuber hepatoma. The availability of these cultures is of great significance in the study of both normal regulatory mechanisms and of neoplasia. Insofar as these cells respond to hormones in a normal fashion, they offer a system in which the role of various hormones in regulating enzyme synthesis can be studied individually and independently of the complex in vivo environment. On the other hand when instances are found in which the response of the hepatoma cells is lacking or abnormal, the possibility exists that an alteration peculiar to the neoplastic state has been uncovered.

A survey is being made of regulatory responses during the course of carcinogenesis brought about by the alkylating agent diethylnitrosamine. Hormonal induction of enzyme synthesis requires intact, fully functional nucleic acids and should therefore provide a sensitive assay for precancerous changes in the liver, if the capacity of DEN to alkylate nucleic acids is an integral part of its carcinogenetic effects.

RELATIONSHIP TO OTHER PROJECTS:

Work closely related to that described here is being done in several U. S. and foreign universities and in U.S.P.H.S. laboratories. Some duplication is inevitable but also necessary as it serves as a means of checking and confirming the results obtained in the various laboratories.

TECHNICAL PROGRESS IN FY 1969:

Hormonal regulation of hepatic enzyme synthesis

Much of our work on regulation involves the model system, tyrosine transaminase. In rat liver this enzyme undergoes rapid turnover, which renders the enzyme level unusually

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sensitive to changes in the rates of enzyme synthesis or degradation. Several years ago we purified this enzyme and prepared antisera to it, and we thus have a highly specific reagent with which to isolate the enzyme from crude liver extracts. Immunochemical isolation permits determination of the rate of transaminase labeling under different physiological conditions and thus provides us with a means of estimating rates of enzyme synthesis or degradation. This capacity is of paramount importance in understanding the regulatory phenomena being studied.

The bulk of our work on regulation of tyrosine transaminase and other liver enzymes is now being carried out in cell and organ culture and will be described under those categories in subsequent paragraphs. With the demonstration that glucagon induces tyrosine transaminase via the intermediate, cyclic AMP, it became of interest to determine if other hepatic enzymes can respond to this nucleotide. Some of these cannot yet be studied in our simplified experimental systems and hence an in vivo study was initiated. Tryptophan pyrrolase is induced by the steroid, hydrocortisone, but is completely insensitive to cyclic AMP. Serine dehydrase and phosphopyruvate carboxykinase show the reverse, being induced by cyclic AMP but not by hydrocortisone. Tyrosine transaminase, of course, responds to both inducers and when both are given together an additive or synergistic effect is obtained. We consider the studies on these enzymes to be representative of the two major modes of hormonal regulation of enzyme synthesis in liver, i.e., regulation by adrenal steroids and by cyclic AMP. Tyrosine transaminase appears to be unique in responding to both of these as well as to a third hormonal inducer, insulin.

Regulation in fetal liver in organ culture

The similarity and non-additivity of the response of tyrosine transaminase to insulin and glucagon in previous experimentation in vivo and in isolated, perfused livers led to the tentative conclusion that these two hormones induce enzyme synthesis by the same mechanism. Current studies with organ cultures of fetal rat liver have demonstrated important differences in the response to these hormones, and hence it can now be concluded that there exist three discrete mechanisms for inducing this enzyme. The failure of cyclic AMP (glucagon) and insulin to yield additive effects can now be attributed to an inhibition by insulin of the intracellular action of cyclic AMP. In other studies it was demonstrated that cyclic AMP actually stimulates synthesis of tyrosine transaminase in these cultures. This is the first proof that this ubiquitous mediator of hormone action accelerates the synthesis of specific proteins.

Cyclic AMP has also been found to effect a marked elevation in the activity of phosphopyruvate carboxykinase in these cultures. Hydrocortisone has no effect on this enzyme. Insulin also has no effect when added alone but it very effectively blocks the response to cyclic AMP. Other liver enzymes thought to be inducible from in vivo studies have been studied in the organ cultures, including glucokinase and serine dehydrase. These are present in barely detectable amounts and they do not respond to what are thought to be the appropriate hormonal inducers. Glucose-6-phosphatase is increased to some extent by cyclic AMP and glutamic-oxaloacetate transaminase may also be slightly increased. Pyruvic kinase was not altered by the hormones.

Regulation in neoplastic cells

Cell cultures of the Reuber (H-35) and HTC hepatoma lines respond much like the liver in vivo to the addition of inducing hormones and thus permit analysis of hormone action in a simplified test system. A thorough analysis of the phenomenon termed "superinduction" – a hyper elevation of enzyme brought about by addition of actinomycin to cells preinduced by hydrocortisone – was made, using our immunochemical method to measure rates of enzyme synthesis and degradation. This "paradoxical" effect of actinomycin was thought to indicate the action of cytoplasmic (translational) repressors which

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limit the response to hydrocortisone and which are in turn regulated by the steroid. Our data show clearly that this interpretation is incorrect; rather, the "paradoxical" increase in enzyme is due to inhibition of turnover of the enzyme. Synthesis of the enzyme is actually inhibited by actinomycin, but decays relatively slowly, thus permitting a period of time in which enzyme turnover is blocked and synthesis is proceeding at a high (but declining) rate, and in consequence the enzyme level rises.

These experiments also showed that the mRNA for tyrosine transaminase in these cells undergo first order decay with a half-life of about three hours. Armed with this information it was possible to show quite conclusively that the steroid inducer (hydrocortisone) acts on genetic transcription, effecting an increase in the mRNA content of the cells. Glucagon (cyclic AMP), on the other hand, acts at some post-transcriptional stage in enzyme synthesis, for induction by these agents does not increase the transaminase mRNA. With the cooperation of the ORNL Mathematics Division, we have developed a model for the DNA-mRNA-enzyme sequence, with known decay constants for the latter two components. With this model it is possible to predict the cellular content of enzyme under a wide variety of experimental manipulations. Our experimental data agree with the theoretical considerations, showing that the steroid hormone accelerates the first step (transcription) and cyclic AMP acts on the second (translation). This is the first time that it has been possible to state, with any certainty, the intracellular locus of action of hormones in regulating protein synthesis.

A study was made of responses to these regulators in vivo in rats exposed daily to the hepatic carcinogen, diethylnitrosamine. This compound is believed to act by alkylating nucleic acids. The feeding regimen employed (5 mg/kg/day) is known to result in 100% tumor yields; in our experiments the treated animals all developed massive hepatomas and were near death by the end of the experimental period. During the course of DEN treatment the response to hydrocortisone became progressively greater, while that to glucagon practically disappeared. Thus the capacity of the liver to synthesize enzyme in response to hormonal induction was not impaired, and the glucagon-sensitive (translational) pathway appeared to be open without hormonal stimulation. These experiments suggest that alkylation of nucleic acids may not be an essential component of DEN action, for this would predict a severe impairment of induced enzyme synthesis. They also suggest that the carcinogen may interfere with post-transcriptional events in the decoding of genetic information.

Hormonal control of RNA synthesis and transport

Several experimental approaches were made to test the possibility that hormones now known to act on post-transcriptional events in protein synthesis do so by altering the transport of mRNA from nucleus to cytoplasm. In vivo "chase" experiments proved not to be feasible, apparently due to the existence of a non-equilibrating pool of nucleic acid precursors in cell nuclei. The flow of RNA from labeled nuclei into unlabeled cytoplasm in vitro was not altered by any of the hormones tested or by cyclic AMP.

A study was initiated of the hepatic response of male toads to estrogen, in a follow-up of the demonstration of the Cell Growth and Differentiation Group that estrogens induce a massive synthesis of yolk proteins in these animals. The magnitude of this response suggests that this may be an ideal system in which to analyze the mechanism of steroid hormone action on genetic transcription. Anti-lipovitellin was prepared in rabbits and employed to study the time course of lipovitellin synthesis after estrogen treatment. The data are not yet complete but it appears that there is no synthesis of this protein until several hours after estrogen; if this observation is confirmed it will be the first demonstration of steroid activation of fully quiescent genes. Attempts are being made to perform a similar analysis of the second component of the yolk protein, phosvitin.

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Total RNA synthesis was stimulated at about the same time as that of lipovitellin. Base-composition analyses showed that the RNA being made at this time is markedly DNA-like, and may be mRNA. Later, about 12 hours after estrogen, there was a second burst in RNA synthesis which appeared to correlate with a shift in base composition towards the ribosomal type. If this is confirmed it will provide a much-needed explanation of previous indications that steroids induce specific enzymes but have a non-specific effect on synthesis of ribosomal RNA. It may be that the slow metabolism of the poikilothermic test animal used here has permitted a sequential definition of processes that occur almost simultaneously in mammals.

As the yolk proteins are very rich in phosphoserine, the possibility that their induction is effected by production of a new or altered species of seryl-tRNA was examined. Using the reversed-phase chromatography systems developed at ORNL, seryl-tRNAs were analyzed after charging in vitro and in vivo. Estrogen treatment effected no significant change in the control pattern, which showed there to be four isoaccepting species of seryl-tRNA in the liver of Xenopus laevis. Also there was no indication of a phosphoseryl-tRNA, in contrast to a published report from another laboratory.

Enzyme degradation

As mentioned above large doses of actinomycin inhibit the turnover of tyrosine transaminase in hepatoma cultures; in previous experiments this drug was also found to inhibit turnover in vivo, but the response was erratic. By adjusting the concentration of actinomycin in culture media, we have found that a level can be reached at which transaminase synthesis is blocked but turnover is not affected. Thus the possibility that turnover is obligatorily linked to synthesis can be discounted, and it appears that actinomycin's effect on turnover is an indication of toxicity rather than of selective inhibition of RNA synthesis.

An attempt was made to investigate the possibility that blocking protein synthesis allows the accumulation of a substance which stabilizes the transaminase against denaturation. No evidence was found for this, which may indicate only that denaturation studies in vitro do not constitute an adequate model for the process by which enzymes are degraded in vivo.

EXPECTED RESULTS IN FY 1970:

Hormonal regulation of hepatic enzyme synthesis

The multiple hormonal regulators of the synthesis of tyrosine transaminase and other enzymes will be studied further to delineate the detailed mechanisms involved. The intracellular mediator of one type of hormonal induction has been identified (cyclic AMP) and we know that it affects some post-transcriptional event in protein synthesis. Further, induction by this agent is rather non-specific and hence its effect, which results in accelerated synthesis of several enzymes, can be expected to be detectable. Most translational reactions are fairly well-understood and can be studied in appropriate cell-free systems. On these premises we plan to initiate a study of effects of cyclic AMP on protein synthesis in vitro, analyzing in terms of both total protein synthesis and selective synthesis of tyrosine transaminase, which we hope to detect as isotope incorporation into immunologically-precipitable protein. For example, the rate of release of completed transaminase chains from polysomes will be studied (chain termination) as will chain initiation, using synthetic messengers. Effects of cyclic AMP will be sought at the polysome level and on the recently-discovered mRNA-protein complexes that may represent partially active or inactive mRNA. But since our data indicate that no new mRNA is made available by cyclic AMP, attention will be focused

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primarily on the translation process per se. These studies will involve rat liver preparations but may also be carried out with the organ culture or cell culture experimental systems.

Regulation in fetal liver in organ culture

Phosphoenolpyruvate carboxykinase will be purified from adult liver in order to prepare antisera against it. Then it will be possible to determine directly by an isotopic immunochemical technique whether the rate of enzyme synthesis is indeed stimulated by cyclic AMP. Also the postulated role of this enzyme in gluconeogenesis will be examined in the organ culture system to see of there is a direct relationship between induction of the carboxykinase and stimulation of gluconeogenesis by cyclic AMP.

The role of insulin in blocking the action of cyclic AMP on the carboxykinase will be explored more critically. Studies on the effect of insulin on the synthesis and degradation of the cyclic nucleotide are to be performed to see if the inhibitory effect of the pancreatic hormone can be localized at one or both of these steps.

Attempts will be made, using these cultures, to identify the factors necessary to begin synthesis of those enzymes which are absent from the fetal liver but for which the genetic information is known to be present (e.g., tryptophan pyrrolase). It would appear that the steroid inducer of this enzyme cannot effect induction until synthesis of the enzyme begins, turned on by unknown regulatory mechanisms. It may be possible to identify these by supplementing the medium with appropriate hormones or other agents.

Regulation in neoplastic cells

Studies comparable to those described in this category above will be carried out to determine the locus of action of the third inducing hormone, insulin. Facilities are now available for the large-scale culture of both H-35 and HTC cell lines. Cells grown in this way may be employed in studies of cyclic AMP effects on protein synthesis discussed above but will primarily be used in analyses of hormonal regulation of RNA synthesis and transport. Induction by hydrocortisone in these cultures proceeds without the nonspecific burst in RNA synthesis that occurs in vivo and which complicates experimentation at the RNA level in animal experiments. Further, our kinetic experiments now show clearly that the steroid-mediated induction involves synthesis of new mRNA. An intensive effort will be made to detect this, and if possible, to isolate the RNA species synthesized in response to the steroid. Synthesis of RNA will be studied in cells, in isolated nuclei, and in subnuclear preparations (chromatin) in an effort to detect an effect of steroid which could explain its capacity to induce enzyme synthesis.

Hormonal control of RNA synthesis and transport

In addition to the hepatoma culture experiments on the role of glucocorticoids in enzyme induction, our studies on estrogen effects in amphibian liver will be continued. Our immediate aim will be to confirm the preliminary data described as tentative in the discussion of current results in this project. These are: (1) the indication that estrogens promote a sequential stimulation of mRNA, then ribosomal RNA synthesis; (2) the indication that the yolk proteins are not synthesized at all in the absence of estrogen. These important points will require a great deal of experimentation to be established unequivocally. We will continue our attempts to prepare antisera against phosvitin and, if successful, will perform an immunochemical analysis of synthesis of this component. Studies will be made of the phosphorylation of the yolk proteins, using extracts of liver from estrogen-treated and control males. Particular attention will be paid to the possibility of a role of cyclic AMP at this level, in analogy to the documented effect of this nucleotide on protein phosphorylation in other systems.

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We will also continue to seek a meaningful way to search for hormonal effects on the passage of RNA from nucleus to cytoplasm. That two-thirds of the messenger-type RNA synthesized in the nucleus never reaches the cytoplasmic site of protein synthesis is, we believe, a clear indication that transport of RNA must be carefully regulated.

Enzyme degradation

We expect to make a major effort in analyzing the mechanism by which tyrosine transaminase is degraded *in vivo* and the possibility that it is regulated by hormones. The effect of coenzyme, pyridoxal phosphate, will be determined in cell cultures which can be supplemented with or deprived of pyridoxine readily; pyridoxine-deficient rats may also be used. Using "chase" analyses to measure turnover rate, it should be possible to determine if the presence or absence of the coenzyme, which confers stability upon the apoenzyme *in vitro*, has any effect on turnover rate. Also, there are several hints from the current literature that hormones, possibly of pituitary origin, may influence the rate of turnover of liver enzymes. This will be investigated in both animal and cell culture experiments.

The rates at which both an enzyme and its mRNA are degraded *in vivo* are now known for two liver enzymes (tyrosine transaminase and amino levulinate synthetase). Curiously, in each instance both the enzyme and its mRNA decay at the same rate. We will attempt to determine if this relationship holds for other liver enzymes by measuring mRNA lifetimes for enzymes whose turnover rates are known. For example, an estimate can be made of the tryptophan pyrrolase mRNA lifetime by measuring the response of this enzyme to tryptophan in actinomycin-treated rats. Similar approaches can be made for several other liver enzymes. While no rational explanation can now be made for a relationship between enzyme and mRNA stability, the existence of such a relationship would surely be an important aspect of regulation in mammalian cells.

EXPECTED RESULTS IN FY 1971:

A biochemist should be added before or during FY 1971 to handle the anticipated increase in this program. Our analyses of the mechanism of hormonal control of RNA and enzyme synthesis will continue with increasing emphasis upon development of cell-free systems in which hormone action is manifested. This is a long range aim, and one that is only becoming feasible at the present time. Our tissue culture and organ culture programs represent an essential intermediate stage in this effort. Increasing attention will be paid the baffling problem of the basis of the vast differences in turnover rates of similar proteins occupying the same phase of the same cell, for regulation of turnover is of comparable significance to regulation of synthesis in the ultimate expression of genetic information.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

PROGRAM:
189 No.

DATE: April, 1969

PROGRAM AND BUDGET PROPOSAL

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Biochemistry of Co-Carcinogenesis	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
E. S. Rogers	Unclassified	☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR		☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 195,000	$ 215,000	$ 225,000
UNUSUAL COSTS	-0-	5,000	3,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 195,000	$ 220,000	$ 228,000
ORNL MAN-YEARS	5.5	5.5	5.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Rogers, Stanfield. Use of viruses as carriers of added genetic information. Nature 219: 749-751, 1968.

Rogers, Stanfield. Dialysis against enzymes: Its significance to the specific therapy of cancer and genetic deficiency diseases. Nature 220: 1321-1322, 1968.

SCOPE:

This work is divided roughly into three parts: studies toward the understanding of the mechanism of action of a tumor virus, the Shope rabbit papilloma virus; the development of the means to control the blood levels of various metabolites which methods are likely to prove useful in the therapy of tumors and a variety of other diseases; and the transmission of genetic information using virus vectors.

Studies of the mechanism of action of the Shope Papilloma virus

Previous work published from this laboratory has indicated that one important mechanism of the Shope virus is to induce the synthesis of a virus specific arginase. It was reported last year that a mutant line of the virus induced a similar arginase, though different enough in structure to be separated chromatographically from that induced with wild type virus, thus providing important evidence that the information for the synthesis of the enzyme is derived from the virus DNA. It is known that the protein coat of the virus is similarly derived. The molecular weight of the DNA is about

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4,000,000, double stranded. Efforts will be made to find whether the various messenger RNAs induced by the DNA can be isolated through the use of nitrocellulose-virus DNA columns. This should give us some estimate of the number of messages being coded by the virus. These RNA messengers are then to be chemically tied to the end of certain RNA viruses in efforts to see if they can be transmitted by the modified virus and perhaps give us some indication of their nature. For example, if the messenger for the virus induced arginase be attached and functions we should get the Shope virus specific enzyme induced in other cell types infected by the modified virus RNA.

Control of blood levels of individual metabolites through dialysis of the blood of the living animal against specific enzymes

We have just reported that the blood level of the amino acid arginine can be readily controlled by dialyzing rabbits against purified arginase. By keeping the blood level of arginine low through this means in rabbits carrying Shope rabbit papillomas for a period of a week, the tumors undergo marked regression. Because of this finding and other work previously done in this laboratory relating to the often marked variability in the metabolism of individual tumors in animals and man, together with the findings reported elsewhere that rat and certain human lymphomas can be made to retrogress (some permanently) through injection of the animal with the enzyme asparaginase thus specifically knocking out asparagine, now known to be critical to the responsive tumors, it appears that dialysis against specific enzymes may be the therapeutic method of choice in the therapy of cancer. This work is to be extended through the dialysis against other enzymes and to the therapy of spontaneous and transplantable rabbit cancers arising in the Shope papillomas. In addition, joint efforts are planned along these lines in the therapy of certain genetic deficiency diseases and cancers of other sorts in man in conjunction with laboratories elsewhere.

Concerning the transmission of genetic information using viruses as vectors

Since the discovery in this laboratory that the Shope papilloma virus has the systemic effect (because of its induced arginase) of lowering the blood arginine levels in domestic rabbits, wild rabbits, rats, mice and man, efforts have been made to use viruses as vectors of specific genetic information, either information innately carried by the virus type or added to the virus nucleic acid synthetically in vitro. The first successful synthetic addition was of poly-adenine to the tobacco mosaic virus RNA which in turn induced the synthesis of poly-lysine in systemically infected tobacco plants. Certain modified lines of this virus inducing single specific poly-lysines, such as decalysine, have now been cloned. Lederle Laboratories has kindly provided a large quantity of Sabin attenuated polio virus originally produced for vaccination. This virus type has an infective RNA like the tobacco mosaic does. Similar experiments to those just described are going to be done but using a mammalian tissue culture type system. Hopefully it can also be checked in monkeys to find if special physiologic effects are induced. We know for example that at one point in the purification of poly-lysine from plants infected with poly-A modified virus a difference in the coloration of our material from similar material extracted from wild type virus infected plants exists. Efforts have been started to find what accounts for this.

At this point we have become more interested in adding information of larger significance than that inducing a simple polypeptide. For example, researchers at the Los Alamos National Laboratory are making efforts to synthesize a double stranded DNA sequence for the small (9 amino acid) peptide bradykinin which when they succeed we plan to attach to virus nucleic acid. Perhaps of greater significance will be our efforts to add naturally occurring information to RNA or DNA viruses. One such effort was mentioned above, the addition to the specific Shope virus induced messenger RNAs to TMV or polio virus RNA and search both chemically and immunologically for the specific equivalent protein in modified virus infected cells. It is recognized that the chemical

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addition of large sequences to a large nucleic acid be it RNA or DNA will at best probably give a low yield. However, our detection system is quite sensitive and certainly should suffice. We have just received a simian adeno virus which has a linear infective DNA. This was the kind gift of Dr. Burnett of the Lilly Corp. This virus grows very well in culture and up to 1 mg virus per roller tube can be harvested. We plan to add both synthetic and naturally occurring sequences to the simian virus DNA. One particularly interesting possibility, to be done in conjunction with Dr. Ray Tennant of the Biology Division, is the addition of the Kilham rat virus DNA to the DNA of the simian adeno virus. Known immunologic markers for the rat virus will then be looked for in infected monkey cells. Naturally, a prerequisite not now known will be that the rat virus, when unattached to the simian virus, not infect monkey cells. Similar experiments are planned using fragmented Shope virus DNA and attaching them to the simian adeno DNA. The synthetic organic chemistry involved will be done with the help and hopefully the participation of Dr. B. C. Pal of the Biology Division.

RELATIONSHIP TO OTHER PROJECTS:

The large scale enzyme purification of Dr. David Novelli is closely if not critically related to the control of metabolite blood levels through dialysis against enzymes.

TECHNICAL PROGRESS IN FY 1969:

The use of an artificial kidney to dialyze rabbits against specific enzymes to control the blood levels of specific metabolites was proven effective. A collaborative program with the Department of Medical Genetics at Indiana University School of Medicine was set up to extend these findings to people.

Poly-A modified tobacco mosaic virus RNAs were cloned proving not only that the modified virus replicated the modification but that the virus complete with its protein coat was made in infected plants. The protein coat was found unchanged in its amino acid composition indicating that the information of the poly-lysine induction was not attached sequentially for that responsible for protein coat subunit synthesis. This is consistent with previously reported work of C. A. Knight using other methods indicating that the sequence for coat synthesis was not near the end of the virus RNA (PNAS 55: 1276, 1966).

On the order of 1/2 gram of Shope papilloma virus was purified to provide the material to do the specific messenger RNA isolations, and DNA for the purpose planned and outlined above.

It was found possibly to cause regression of the Shope papillomas through dialysis of the animals carrying the tumors against arginase. Similar dialysis but against inactive protein proved without effect.

EXPECTED RESULTS IN FY 1970:

The extension of dialysis against specific enzymes to the therapy of other tumors and other disease sorts such as genetic deficiency diseases should be well under way. The addition of synthetic and naturally occurring genetic information, both DNA and RNA, should be considerably advanced. In association with this, at least some additional information carried by the Shope virus and perhaps other virus nucleic acids should be known.

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TITLE: NCI Biochemistry of Co-Carcinogenesis

ACTIVITY NO. 40 04
189 No.

EXPECTED RESULTS IN FY 1971:

It is hoped that we will know enough about the metabolism of cancer and means to control metabolism through dialysis and other non-poisonous means to successfully treat a significant number in man. We should have enough proficiency provided we have adequate support to modify their metabolism through transmissible genetic information specifically built in the laboratory.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Fungal Genetics	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
F. J. de Serres	Unclassified	☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR: de Serres, 10%; Malling, 30%		☒ ESTABLISHED PROGRAM

METHOD OF REPORTING		WORK LOCATION	
☐ QUARTERLY	☐ TOPICAL	☐ X-10 SITE	☐ OTHER (SPECIFY)
☐ SEMIANNUAL	☒ OPEN LITERATURE	☒ Y-12 SITE	
☒ ANNUAL		☐ MELTON VALLEY	

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 107,000	$ 115,000	$ 130,000
UNUSUAL COSTS	3,000	3,000	5,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 110,000	$ 118,000	$ 135,000
ORNL MAN-YEARS	4.5	4.5	5.0

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Ehling, U. H., R. B. Cummings, and H. V. Malling. 1968: Induction of dominant lethal mutations by alkylating agents in male mice. Mutation Res. 5: 417-428.

Malling, H. V. and F. J. de Serres. 1968: Genetic analysis of hydroxylamine induced purple mutants (ad-3) in Neurospora crassa. Proc. XII Intern. Congr. of Genet. Vol. 1: 83.

Malling, H. V. and F. J. de Serres. 1968: Correlation between base-pair transition and complementation pattern in nitrous acid-induced ad-3B mutants of Neurospora crassa. Mutation Res. 5: 359-371.

Malling, H. V. and F. J. de Serres. 1968: Identification of genetic alterations induced by ethylmethanesulfonate in Neurospora crassa. Mutation Res. 6: 181-193.

Jolley, R. L., and H. V. Malling. 1968: Man or mouse? A comparison of urinary carbohydrate constituents using high resolution column chromatography. Abstract for the Regional Meeting of the Southeastern American Chemical Society, December 4-7, 1968, Tallahassee, Florida.

Chu, E.H.Y. and H. V. Malling. 1968: Chemical mutagenesis in Chinese hamster cells in vitro. Proc. XII Intern. Congr. of Genet., Vol. 1: 102.

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Ehling, U. H., D. G. Doherty and H. V. Malling. 1968: Differential mutagenic action of n- and iso-propyl methanesulfonate in mice. Proc. XII Intern. Congr. of Genet., Vol. 1: 103.

de Serres, F. J. and H. V. Malling. Identification of the genetic alterations in specific locus mutants at the molecular level. Proc. XII Intern. Congr. Genet., Vol. II: 129, 1968.

Malling, H. V., and F. J. de Serres. 1968: The relationship between carcinogenicity and mutagenicity of methylating compounds. Abstract. Genetics 60: 201.

Ehling, U. H. and H. V. Malling. 1968: 1.4-di(methanesulfonoxy) butane (Myleran) as a mutagenic agent in mice. Abstract. Genetics 60: 174.

Chu, E.H.Y. and H. V. Malling. 1968: Mammalian cell genetics. II Chemical induction of specific locus mutations in Chinese hamster cells in vitro. Proc. Natl. Acad. Sci. U.S. (in press).

Malling, H. V. and F. J. de Serres. Mutagenicity of alkylating carcinogens. Trans. N. Y. Acad. Sci. (in press).

SCOPE:

The principal aim in the Fungal Genetics Program is to obtain some understanding of the various types of genetic alterations leading to gene mutations at the molecular level. This is being accomplished by intensive study of the genetic effects of selected chemical mutagens. Our aim under the Co-Carcinogenesis Program is to determine the mechanism of nuclear and cytoplasmic inactivation and the mutation induction mechanism of chemicals with carcinogenic activity. The correlation between these activities is likely to give information about the mechanism of the carcinogenic activity of these compounds. This approach is especially significant with Neurospora since (a) the organization of the genetic material is typical of that found in all higher organisms, and (b) it is possible to mimic the diploid condition by using the dikaryon. Our test system consists of a genetically marked, balanced dikaryon which permits study of those events occurring either in the cytoplasm or the nucleus that leads to cellular inactivation in addition to those intragenic and extragenic alterations giving rise to forward mutations in the ad-3 region. A new dimension has been added to these studies by use of the host-mediated assay system. In this system Neurospora conidia are injected into the peritoneal cavity of mice and are later recovered and screened for presence of specific locus mutations in the ad-3 region of Neurospora crassa. This technique makes it possible to study the formation of mutagens during the metabolism of carcinogens. In the same system it is possible to isolate and study spontaneous or induced recessive lethal mutations occurring in the entire genome.

RELATIONSHIP TO OTHER PROJECTS:

No similar research is known.

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TECHNICAL PROGRESS IN FY 1969:

1. Mutagenesis of carcinogens.

(a) Proximate and ultimate carcinogens in vitro - Some compounds do not possess carcinogenic activity as such but are converted to potent carcinogens by enzymes in different organs of the body. A series of such compounds is represented by 2-acetylaminofluorene (AAF) as the precarcinogen, N-hydroxy-2-acetylaminofluorene (N-OH-AAF) as the proximate carcinogen and N-acetoxy-2-acetylaminofluorene (N-acetoxy-AAF) as the ultimate carcinogen. All three compounds were tested for their mutagenicity in the specific locus system in Neurospora crassa; N-acetoxy-AAF was the only compound which showed mutagenic effect. The mutants are in process of being characterized for their genotype and complementation pattern.

(b) Nitrosamides - The frequency of mutations in the ad-3 region (2 genes) after 240 min. treatment with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was 0.14% with 68% survival. Tests for genotype and allelic complmentation showed (a) that 82% of the ad-3B mutants showed allelic complementation with 81% nonpolarized and 1% polarized patterns and 18% noncomplementing and (b) that no chromosome deletion was induced.

(c) Hydroxylamine: Tests for genotype and allelic complementation showed (a) that no chromosome deletions were induced, (b) that 64% of the ad-3B mutants were complementing of which 52% had a nonpolarized and 12% a polarized complementation pattern; the remaining 36% were noncomplementing. Test of the induced mutants for revertibility of treatment with HA showed that 15% of the HA-induced mutants were able to revert.

(d) Polycyclic hydrocarbons - A positive correlation has been found between the photodynamic effect of a series of polycyclic hydrocarbons on Neurospora conidia and the same compounds carcinogenicity.

EXPECTED RESULTS IN FY 1970:

1. Mutagenesis of carcinogens - The host-mediated assay system with mice and Neurospora will be utilized to test the formation of mutagenic breakdown products after administration of carcinogens. The compounds will be fed to the animals or injected intravenously in the tail vein.

2. Polycyclic hydrocarbons - The mutagenic activity of polycyclic hydrocarbons will be tested with and without irradiation with black light.

EXPECTED RESULTS IN FY 1971:

The comparison of the mutagenicity of potent carcinogens as well as proximate carcinogens will be continued. The mutagenicity and the inactivation mechanism of carcinogens will be studied at different stages of the meiotic-mitotic cycles with special reference to changes in sensitivity with change in chromosome structure. The influence of potent carcinogens on the recovery process after UV and X-ray irradiation will be studied. The compounds chosen for these studies will be (1) those which might create a biological hazard through their general use and/or distribution, (2) those which will provide an experimental approach towards an understanding of the mechanism of induction of cancer, and (3) those which will be used in the program for the comparative mutagenesis with tissue culture and mice, and (4) the formation of mutagens among metabolic breakdown products of carcinogens will be studied in the host-mediated assay.

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OAK RIDGE NATIONAL LABORATORY
OPERATED BY
UNION CARBIDE CORPORATION • NUCLEAR DIVISION
Contract No. W7405-eng-26

DATE: April 1969

PROGRAM AND BUDGET PROPOSAL

PROGRAM:
189 No.

DESCRIPTIVE TITLE	AEC ACTIVITY NUMBER
NCI Biophysics	40 04

PERSON IN CHARGE	SECURITY CLASSIFICATION OF THIS PROGRAM DESCRIPTION	PROGRAM STATUS
R. B. Setlow (10%)	unclassified	☐ NEW WORK BEGINNING FY _____
PRINCIPAL INVESTIGATOR J.R. Einstein (90%), F. C. Hartman, J.W. Longworth (90%), C.H. Wei, J.A. Knopp (50%), J.J. ten Bosch (25%)		☒ ESTABLISHED PROGRAM

METHOD OF REPORTING

☐ QUARTERLY ☐ TOPICAL
☐ SEMIANNUAL ☒ OPEN LITERATURE
☒ ANNUAL

WORK LOCATION

☐ X-10 SITE ☐ OTHER (SPECIFY)
☒ Y-12 SITE
☐ MELTON VALLEY

PROPOSED BUDGET

	FY 1969	FY 1970	FY 1971
NORMAL PROGRAM COST	$ 168,000	$ 175,000	$ 185,000
UNUSUAL COSTS	2,000	3,000	2,000
TECHNICAL SERVICES			
SUBCONTRACT COSTS			
TOTAL PROGRAM COSTS	$ 170,000	$ 178,000	$ 187,000
ORNL MAN-YEARS	5.0	4.5	4.5

(SEE BACK PAGE FOR BUDGET COMMENTS)

DATES AND TITLES OF PUBLICATIONS:

Hartman, F. C. Irreversible inactivation of triose phosphate isomerase by 1-hydroxy-3-iodo-2-propanone phosphate. Biochem. Biophys. Res. Commun. 33 (6):888-894, 1968.

Longworth, J. W. (Editor). Basic Mechanisms in Photochemistry and Photobiology (Intern. Symp., Caracas, Venezuela, Dec. 4-8, 1967). Photochem. Photobiol. 7 (No. 6): 511-837, 1968.

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Longworth, J. W., J. A. Knopp, J. J. ten Bosch, and R. O. Rahn. Electronic energy transfer in oligomers and polymers of L-tyrosine. Proc. Intern. Conf. on Molecular Luminescence, Benjamin Press, 1968, p. xxx-xxx.

ten Bosch, J. J., R. O. Rahn, J. W. Longworth, and R. G. Shulman. Energy transfer in poly-L-tyrosine as a function of the degree of ionization of the phenolic hydroxyls. II. Electron spin resonance study of the triplet state. Proc. Natl. Acad. Sci. U.S. 59:1003-1008, 1968.

Wei, C. H., and L. F. Dahl. Triiron dodecacarbonyl: An analysis of its stereochemistry. J. Am. Chem. Soc. 91:xxx-xxx, 1969.

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Abstracts

Hartman, F. C. Active-site modification of triose phosphate isomerase. Federation of American Societies for Experimental Biology, 1969.

Knopp, J. A., J. W. Longworth, and R. O. Rahn. Optical properties of polynucleotides denatured by organic solvents. Biophysical Society, 1969.

Knopp, J. A., K. B. Jacobson, A. P. Pfuderer, and J. W. Longworth. Alcohol dehydrogenase from Drosophila: Characterization and interconversion of isoenzymes. American Chemical Society, 1968.

Longworth, J. W. The exciplex nature of endonuclease fluorescence. Biophysical Society, 1969.

Longworth, J. W. Techniques for measuring phosphorescence and fluorescence of biological molecules. Fifth International Congress on Photobiology, 1968.

Wei, C. H., and J. R. Einstein. Molecular structure of thymine (cis-syn) photodimer. American Crystallographic Association, 1968.

Articles in press

Einstein, J. R. On the analysis of intensity measurements of Bragg reflections with an equatorial-plane diffractometer. (Submitted to J. Appl. Cryst.)

Einstein, J. R. The combination of isomorphous replacement and anomalous scattering data in phase determination for protein crystals. (To be submitted to Acta Cryst.)

Knopp, J. A., and J. W. Longworth. The splitting of the phenolic acid dissociation constants for dityrosyl compounds in the ground state and the excited singlet state. Biochem. J. (In preparation)

Knopp, J. A., J. J. ten Bosch, and J. W. Longworth. Energy transfer in poly-L-tyrosine V. Fluorescence polarization studies. (Submitted to Biochim. Biophys. Acta)

SCOPE:

The research of this group is directed toward understanding the structures and functions of proteins and nucleic acids, the macromolecules which are of central importance in the normal and abnormal growth and functioning of living cells. X-ray diffraction is the only method presently available for providing complete three-dimensional structures of large molecules. Among the principal aims of the X-ray crystallography laboratory are a) the structure determinations of a transfer RNA, a tRNA-synthetase complex, and several small proteins, b) the development of improved methods for the structure determination of large molecules, and c) structure determinations of small molecules of biological interest, including pyrimidine and nucleotide photoproducts.

Chemical modification of biological macromolecules has provided more information pertinent to the correlation of structure and function than any other single technique. It has found application in such widely divergent disciplines as enzymology, immunology, X-ray crystallography, and cancer chemotherapy. Much of our present knowledge about the structure of the active-sites of enzymes and the chemical basis for

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antigenicity is the result of chemical modification studies. Thus the development and use of new chemical reagents that have specificities either for particular functional groups in proteins and nucleic acids or for the ligand binding sites of these molecules is a general goal of the chemical modification laboratory. The success of the endeavor will have an impact on many areas of biomedical science.

The aim of the research on the excited state chemistry and physics of macromolecules is to obtain information on their molecular conformations and on inter- and intramolecular interactions, such as between enzyme and substrate, or protein and polynucleotide. This will be accomplished by determinations of excitation and emission spectral data and observations of shifts in spectra for different conformations. To understand photochemical reactions such as thymine dimerization in DNA, a precise knowledge of quantum efficiencies of radiation and of conversion yield to the triplet states are necessary. Such data are obtained from combination of quantum yield determinations and fluorescence and phosphorescence lifetimes kinetics.

Electronic energy transfer processes are theoretically possible between the constituents of proteins and nucleic acids. The presence of such transfers will profoundly influence the luminescence properties, and moreover be sensitive to mutual orientations of constituents. A measure of transfer will be valuable in structural studies on these macromolecules. Before such an investigation is practical, it is necessary to have a detailed knowledge of transfer phenomena in simple, well-defined systems, such as dimers and oligomers, and homopolymers. To accomplish this aim, luminescence excitation spectra and polarization spectra of simple models will be investigated in detail.

Particular emphasis will be placed on an understanding of the intermolecular forces that are called into play when protein and nucleic acids specifically interact. The aminoacyl-tRNA synthetases form an ideal model system to investigate specific complexes down to atomic dimensions.

RELATIONSHIP TO OTHER PROJECTS:

X-ray diffraction studies are carried out in several other laboratories, but the Biology Division has a wealth of experimental material that previously was sent elsewhere. We anticipate excellent cooperation with the neutron diffraction group at ORNL. (1) X-ray crystallographic work on a transfer RNA would be directly related to existing and proposed biochemical research in this Division. Unique facilities exist at ORNL for producing and purifying the large amounts of transfer RNA that would be required for this work. (2) A possible method for the solution of large-molecule crystal structures involves the introduction into the crystal of heavy atoms giving large anomalous-scattering effects, such as mercury or uranium. Other facilities at ORNL will soon be able to supply large amounts of a stable plutonium isotope (Pu-244), which has X-ray properties preferable to uranium. Other members of the Division, F. C. Hartman and B. C. Pal, are pursuing projects in synthetic biochemistry which may provide reagents for the introduction of the required heavy atoms by synthetic modification of the large molecules. (3) For efficiency, the X-ray work depends on rapid automatic systems for data collection. ORNL is a world leader in the development and utilization of such automatic systems. A design is under study which should make possible a many-fold further increase in speed, and make more practical the investigation of molecules in the 100,000 molecular-weight range, such as (1) activating enzymes for the genetic translation process (which are being investigated in other groups in the Biology Division), and (2) complexes between activating enzymes and transfer RNA's. A multidisciplinary effort is now being

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organized at ORNL to coordinate the research in Biophysics, Biochemistry, Chemical Separations, Material Research and X-ray Crystallography, so that crystals may be grown of tRNA-synthetase complexes from E. coli, and their molecular structures determined using X-ray diffraction analysis.

There are many laboratories in which chemical modification of proteins, for a variety of reasons, is being employed; however, few investigators are actively seeking new protein reagents. It is hoped that reagents developed in this laboratory will find application in several disciplines of protein chemistry. With the increasing availability of homogeneous transfer RNA's and aminoacyl-RNA synthetases, it becomes tempting to employ chemical modification as a method of studying the interaction of transfer RNA's with aminoacyl-RNA synthetases and ribosomes. The possibility exists that some of the reagents currently being synthesized could be useful in this type of investigation.

The entire excited state emission program is coordinated with an independent ESR investigation of triplet states carried out by ORNL staff member, R. O. Rahn. Studies involving enzyme-coenzyme and enzyme-nucleic acid interactions are entirely dependent on cooperation with ORNL members M. P. Stulberg and K. B. Jacobson, who are able to provide a source of these unique and highly purified enzyme systems; this collaborative program involves the use of differential absorption and fluorescence spectroscopy, coordinated with fluorescence polarization studies and circular dichroism. The spectroscopic facilities which we have, and are in the process of establishing are in many ways unique, and most certainly not available commercially. Those facilities which provide similar performance that are available elsewhere, and oriented to biological problems are in the hands of investigators with whom we have previously collaborated -- at the Universities of Illinois and Minnesota and Bell Telephone Laboratories. Naturally there is extensive exchanging of ideas with these groups when interest overlaps, including the organization of joint symposia.

TECHNICAL PROGRESS IN FY 1969:

I. X-ray Diffraction (J. R. Einstein, C. H. Wei)

(1) Cis-syn photodimer of thymine: The molecular structure of the cis-syn photodimer of thymine has been determined by the crystal structure analysis of its monosodium and dipotassium salts. This photodimer is the primary one obtained on hydrolysis of UV-irradiated DNA. A complete refinement of the structure has been carried out for the monosodium salt, with final estimated standard deviations of about 0.02 A for bond lengths and 1.5° for bond angles. Comparison of the determined structure with the known structure of native DNA leads to a better understanding of the structural reasons for the denaturation of DNA by UV irradiation.

(2) Bromoacetyl-phenylalanyl-phenylalanine ethyl ester: The molecular structure of this dipeptide substrate of pepsin has been determined. Refinement of the structure is still in progress, but the general stereochemistry of the molecule has been clearly established. The determined configuration throws light on the stereochemical requirements for pepsin activity.

(3) Theory of diffractometry: Work has been completed on a graphical and mathematical method for the analysis of X-ray diffraction intensity measurements with an equatorial-plane diffractometer. Certain limitations common to all previous treatments have been removed. The method is useful in establishing proper experimental conditions for intensity measurements with crystals having very large unit cells

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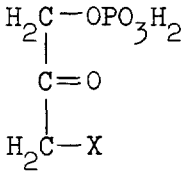
and/or large mosaic ranges.

(4) Cooling systems: A cooling system for the single-crystal orienter has been assembled. The temperature of gas flowing over a specimen crystal was controlled within narrow limits about a set point of 5°C.

II. Chemical Modification (F. C. Hartman)

(1) Haloacetolphosphates: Potential active-site reagents for aldolase, triose phosphate isomerase and glycerophosphate dehydrogenase. We have devised an efficient method of synthesizing the α-chloro (I), bromo (II), and iodo (III) derivatives of acetolphosphate. These reactive compounds are related to dihydroxyacetone phosphate (DHAP)(IV) -- a natural substrate of the above enzymes.



X = Cl(I), Br(II), I(III), and OH(IV).

Compound III inactivates aldolase by a complex mechanism as judged by chemical, radiochemical and physicochemical techniques. It results in the oxidation of sulfhydryl groups to intramolecular disulfides with changes in molecular conformation as well as other changes near the active site. Compounds I, II and III inactivate triose phosphate isomerase under mild conditions. The data suggest that a single, catalytically functional residue within the active site has been modified.

(2) Bifunctional protein reagents: It has been impossible to pursue the problem of the design and use of new bifunctional protein reagents as a method of studying protein-protein interactions and the conformation of proteins in solution due to the lack of supportive personnel.

III. Excited States of Molecules (J. A. Knopp, J. W. Longworth, J. J. ten Bosch)

(1) Energy transfer in proteins: The wavelength dependence of the quantum yields of fluorescence and phosphorescence of tryptophan residues in proteins, polypeptides and dipeptides has been used to demonstrate electronic intramolecular transfer of energy absorbed by tyrosine and then emitted by triptophan. The relative luminescence quantum yields at the various wavelengths were obtained from a detailed comparison of the luminescence excitation spectra and the absorption spectra.

(2) Exciplex formation in proteins: Exciplex formation dominates the tryptophanyl fluorescence observed at room temperatures from the vast majority of proteins so far investigated. At 77°K, in glassing solvents, this complex formation is inhibited, and the intrinsic fluorescence from tryptophan is observed. The fluorescence from an endonuclease of S. aureus has been analyzed in these terms.

(3) Emission from denatured DNA: The absorption spectrum of DNA in ethylene glucol indicates that it is a random coil. Such random coils have enhanced fluorescence and phosphorescence. Poly d(A-T) shows phosphorescence from T but DNA shows phosphorescence from A -- suggesting that phosphorescence depends strongly on the sequence of bases in random coils.

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(4) The polarization of fluorescence of adenine compounds has been measured in glycerol at 210°K. Under these conditions no evidence of singlet-singlet energy transfer in polyrA was obtained.

(5) The existing spectrofluorophosphorometer has been modified to permit "front face excitation" making possible luminescence studies on optically dense materials. A new temperature controlled cell holder has been constructed. It has interchangeable windows which include polarizing filters.

EXPECTED RESULTS IN FY 1970:

I. X-ray Diffraction Crystallography (J. R. Einstein, C. H. Wei)

(1) Work on the molecular structures of single purified tRNA's will be continued. Several tRNA's will be crystallized by known methods. Purer materials than those previously available will be used, the equilibrium conditions of crystallization will be altered, and the crystallization process improved, in attempts to produce crystals of better quality. Extensive collaboration is expected in the crystallization work with C. T. Butler of the Solid State Division, ORNL. Attempts will be made to obtain isomorphous heavy-atom derivatives (a) by soaking of crystals in various heavy-atom containing media, and (b) by synthetic modification of tRNA's (synthetic work by B. C. Pal and/or F. C. Hartman of this Division).

(2) Efforts will be made to grow good crystals for X-ray studies of desformyl gramicidin A as the soziodolate salt (already isolated), and of unmodified gramicidin B. The presence of two iodine atoms in the soziodolate salt should allow the structure to be solved. Vector search techniques would be employed to attempt to locate the four indoles in gramicidin B, as a key to solving the structure.

(3) The refinement of the crystal structure of bromoacetyl-phenylalanyl-phenylalanine ethyl ester will be completed. X-ray data will be collected, and the structure refined, for the isomorphous chloro-derivative.

(4) Equipment for microdensitometry of X-ray diffraction photographs will be installed and interfaced to the existing PDP-8 computer. Existing programs for automation of the densitometry will be modified as required.

The addition of another staff crystallographer will be necessary to complete the planned work.

II. Chemical Modification (F. C. Hartman)

(1) The identification of the amino acid at the active site of triose phosphate isomerase which is labeled by haloacetophosphates and the determination of the amino acid sequence adjacent to this residue will be attempted.

(2) The reaction between haloacetolphosphates and triose phosphate isomerase from species other than rabbit muscle will be studied with the goal of comparative sequence determinations of their active sites.

(3) Attempts to incorporate heavy atoms into proteins and nucleic acids for X-ray diffraction studies will continue.

(4) The effect of haloacetolphosphates on other enzymes which catalyze reactions

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of organic phosphomonoesters, particularly glycerophosphate dehydrogenase will be determined.

III. Excited States of Molecules (J. A. Knopp, J. W. Longworth)

Characterization of the relationship between excited state chemistry and the structures of proteins and protein substrate complexes will continue. The emphasis will be placed on proteins of established sequence and known crystal structure determined from X-ray diffraction; in particular, the proteins studied will be ribonuclease A, ribonuclease T_1, Staphylloccocal nuclease and lysozyme. The existing fluorescence polarization spectrometer will be modified to give results directly in degree of polarization with provisions for automatic scan of wavelength of excitation. In addition, choppers will be installed to enable the separation of fluorescence and phosphorescence and permit measurement of phosphorescence polarization.

EXPECTED RESULTS IN FY 1971:

I. X-ray Diffraction Crystallography (J. R. Einstein, C. H. Wei)

(1) Work on the molecular structure of single purified tRNA's will be continued and extended to structure analysis of complexes of tRNA and tRNA-synthetases.

(2) Efforts will be made to obtain suitable crystals and to solve the crystal structures for three dinucleoside monophosphates and diGDP, a dinucleoside tetraphosphate, and the analysis of the UV photoproduct $\overline{TpT}^1$ analysis will be initiated.

(3) The cooling system for the single-crystal orienter will be improved so that its temperature range extends to -50°C.

II. Chemical Modification (F. C. Hartman)

(1) Studies on the subunit structure of triose phosphate isomerase will be initiated.

(2) The effect of haloacetolphosphates on the rate of glycolysis *in vivo* will be studied.

(3) Attempts to incorporate heavy atoms into proteins and nucleic acids for X-ray diffraction studies will continue.

(4) A study of bifunctional protein reagents will be re-initiated if possible.

A reasonable rate of progress requires additional personnel.

III. Excited States of Molecules (J. A. Knopp, J. W. Longworth)

The luminescence properties of various nucleic acid bases as well as the different tRNA molecules will be studied with emphasis on relating this to structural information. The fluorescence polarization spectrometer will be used to determine the rotatory diffusion constants of protein substrate interactions complexes, especially the synthetase-tRNA system. Wavelength dependence of fluorescence polarization will be used to investigate electronic energy transfers in polypeptides, protein polynucleotides and nucleic acids. A phase-modulation fluorescence lifetime device, which is now largely constructed, will be established to measure lifetimes of fluorescence with high precision and resolution. At the same time a pulse modulation

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fluorimeter will be developed, using single photon timing techniques which will determine the extent of deviation from the exponentiallity of fluorescence decays, in particular, proteins such as lysozyme which are known to have tryptophan residues in distinct environments.

BUDGET COMMENTS:

The only unusual costs expected would be for capital equipment.

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