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BIOLOGY DIVISION ANNUAL PROGRESS REPORT

Period Ending June 30, 1975

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J. B. Storer, Director
S. F. Carson, Deputy Director

NOVEMBER 1975

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37830
operated by
UNION CARBIDE CORPORATION
for the
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ORNL-969	Period Ending February 10, 1951
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ORNL-989	Period Ending August 10, 1951
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ORNL-1497	Period Ending February 10, 1953
ORNL-1614	Period Ending August 15, 1953
ORNL-1693	Period Ending February 15, 1954
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ORNL-2390	Period Ending August 15, 1957
ORNL-2481	Period Ending February 15, 1958
ORNL-2593	Period Ending August 15, 1958
ORNL-2702	Period Ending February 15, 1959
ORNL-2813	Period Ending August 15, 1959
ORNL-2913	Period Ending February 15, 1960
ORNL-2997	Period Ending August 15, 1960
ORNL-3095	Period Ending February 15, 1961
ORNL-3201	Period Ending August 15, 1961
ORNL-3267	Period Ending February 15, 1962
ORNL-3352	Period Ending August 15, 1962
ORNL-3427	Period Ending February 15, 1963
ORNL-3498	Period Ending August 15, 1963
ORNL-3601	Period Ending February 15, 1964
ORNL-3700	Period Ending August 15, 1964
ORNL-3768	Period Ending February 15, 1965
ORNL-3853	Period Ending July 31, 1965
ORNL-3922	Period Ending January 31, 1966
ORNL-3999	Period Ending July 31, 1966
ORNL-4100	Period Ending January 31, 1967
ORNL-4240	Period Ending December 31, 1967
ORNL-4412	Period Ending December 31, 1968
ORNL-4535	Period Ending December 31, 1969
ORNL-4740	Period Ending June 30, 1971
ORNL-4817	Period Ending June 30, 1972
ORNL-4915	Period Ending June 30, 1973
ORNL-4993	Period Ending June 30, 1974

CONTENTS

Section II

BIOPHYSICS AND CELL PHYSIOLOGY

Section III

GENETICS AND DEVELOPMENTAL BIOLOGY

Section IV

MAMMALIAN GENETICS

Section V

PATHOLOGY AND IMMUNOLOGY

Section VI

CARCINOGENESIS PROGRAM

INTRODUCTION

THE PAST YEAR was one of transition for the Biology Division. The Atomic Energy Commission, our primary sponsor for twenty-six years, passed into history and its functions were incorporated into the broader responsibilities of the new Energy Research and Development Administration. An early reflection of our response to this widened mission is the increased emphasis in this report on the biological effects of chemical agents. As usual, the individual reports consist of very brief summaries of principal research activities which will be reported in greater detail in the scientific literature.

John B. Storer
Director, Biology Division

5/246

SECTION I

BIOCHEMISTRY

Elliot Volkin

Chemical Protection and Enzyme Catalysis

D. G. Doherty
J. R. Totter

Biochemistry of Cell Differentiation

John Papaconstantinou
C. L. Parker[a]

Protein Synthesis

G. David Novelli
Audrey N. Best
Jan Dijk[b]
G. D. Griffin[a]
Beth C. Mullin[a]
E. F. Phares
Helen G. Sellin
Audrey L. Stevens
L. C. Waters
Wen-Kuang Yang

Enzymatic Mechanisms in Protein Synthesis

M. P. Stulberg
L. R. Shugart

Enzyme Regulation

K. Bruce Jacobson
Michael Litt[c]
J. E. Tobler[a]

Nucleic Acid Chemistry

W. E. Cohn
B. C. Pal
Diane G. Schmidt
Mayo Uziel

Nucleic Acid Enzymology

Elliot Volkin
W. E. Barnett
J. W. Bynum[a]
A. K. Datta[a]
R. K. Fujimura
J. X. Khym
C. G. Mead
Sankar Mitra
S. K. Niyogi
Ann C. Olson[d]
S. D. Schwartzbach[a]

Nucleotide Metabolism

F. D. Hamilton

Developmental Biochemistry

F. J. Finamore
Peter Pfuderer
Alden Warner[c]
W. F. Zapisek[c]

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[b]Visiting investigator from abroad.
[c]Visiting investigator.
[d]Consultant.

ELECTRON SPIN RESONANCE STUDIES OF PHOTOLYZED PEPTIDE SOLUTIONS

D. G. Doherty, Ralph Livingston,* Margaret A. Turner, and Henry Zeldes*

Livingston and Zeldes found that when two Bence-Jones proteins were photolyzed and the radicals formed were examined by microwave spectroscopy, characteristic ESR (electron spin resonance) signals were obtained that could not be interpreted by direct analysis of the spectra. With the expectation of discovering systematic trends in the spectra of simpler peptides that would be applicable to more complex systems, we began a cooperative program involving the synthesis and examination of specific di- and polypeptides.

The initial study involved simple dipeptides of glycine, L-alanine and β-alanine. Photolyses of an aqueous solution of the peptides in the zwitterion form containing 1% hydrogen peroxide produced radicals by the abstraction of hydrogen by ·OH radical attack. The results with the first five dipeptides were given in the last annual report.[1] These dipeptides along with two additional ones, β-alanyl-L-alanine and β-alanyl-β-alanine, have been reported elsewhere.[2] In all cases the nature of the ·OH attack is highly systematic; hydrogen is abstracted from the α carbon between the peptide nitrogen and the carboxylate group. When β-alanine is in the C-terminal position, a mixture of radicals is obtained by abstraction of H from both the α and β methylene groups. All these peptides were found to be in the transconfiguration. Hyperfine couplings and g values were measured, and spin density for the carbon from which abstraction took place was measured.

The study has been extended to dipeptides containing more complex amino acids. Radicals from L-prolylglycine and β-alanyl-L-proline have been identified, and in both cases hydrogen abstraction follows the previously observed systematic trends.

In the former case the spectrum lines are quite broad, presumably due to the presence of *cis* and *trans* isomers which have slightly different (unresolved) values for the hyperfine coupling constants. In the latter case the spectrum shows additional complexity which is thought to arise from a "flip-flop" of the proline ring. In the case of β-alanylsarcosine, where the peptide hydrogen is replaced by a methyl group, the radical attack is quite different; in this case the hydrogen is abstracted from the *N*-methyl group of the sarcosine residue, thereby giving rise to a mixture of two radicals which are *cis* and *trans* isomers. In the case of β-alanyl-L-valine and glycyl-L-valine, the ·OH attack is not at the α carbon of the valine residue but at the isopropyl side chain, giving rise to a mixture of two radicals.

Four tripeptides have also been studied: β-alanylglycyl-L-proline, β-alanyl-L-prolylglycine, β-alanylglycylglycine, and β-alanylglycyl-L-alanine. The β-alanyl residue was used in the *N*-terminal position, since the work with the dipeptides indicated that more easily analyzed spectra were obtained. Much to our surprise, essentially only one radical is formed in each case. Hydrogen abstraction takes place almost exclusively from the α carbon of the C-terminal amino acid. Results for two of these tripeptides along with results from the corresponding dipeptides are given in Fig. 1. The coupling

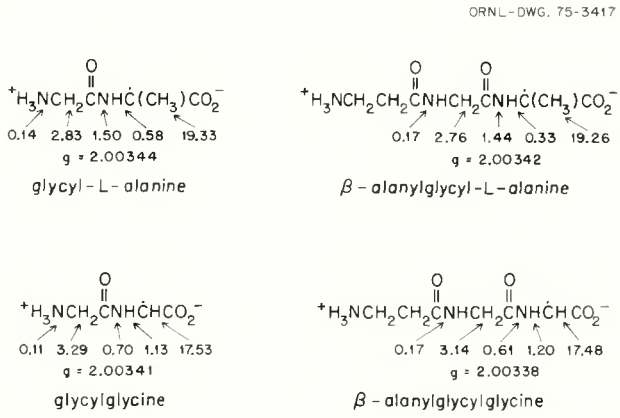

Figure 1.

constants and g values are very little changed in going from dipeptides to tripeptides. At present we do not know if the simplicity will be exhibited by longer peptides with other side-chain R groups. If this is the case, the formation of the radical is not only significant for pinpointing the region of ·OH attack but also for labeling a peptide with an unpaired electron, which is sensitive (at least in principle) to conformational effects.

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*Chemistry Division.

1. R. Livingston, D. G. Doherty, M. A. Turner, and H. Zeldes, *Biol. Div. Annu. Prog. Rep. June 30, 1974*, ORNL-4993, p. 4.

2. R. Livingston, D. G. Doherty, and H. Zeldes, *J. Am. Chem. Soc.* 97, 3198–3204 (1975).

MECHANISMS OF CHEMILUMINESCENT OXIDATION-REDUCTION REACTIONS

J. R. Totter and D. G. Doherty

The conversion of 5-aminophthalazine 1,4-dione (luminol) in aqueous solution to 3-aminophthalic acid with the emission of light can be brought about by hydrogen peroxide and a catalyst, by peroxydisulfate alone or in the presence of H_2O_2, by peroxidases and H_2O_2, and by xanthine oxidase and its substrates or by xanthine dehydrogenase, one of its substrates and a reversibly reducible quinone. In the absence of H_2O_2, free O_2 is required for light emission, and the quantum yield based on luminol is much reduced.

The peroxydisulfate chemiluminescence in the presence and absence of H_2O_2 has been studied to elucidate the mechanism step-by-step. One current theory postulates a two-electron oxidation of luminol anion by peroxydisulfate followed by addition of H_2O_2 (when it is present), and there is a concerted decomposition leading to activated aminophthalate dianion which emits in going to the ground state.

These steps may be formulated as follows:

$$C_8H_6O_2N_3^- + S_2O_8 \rightarrow C_8H_5O_2N_3 + SO_4^{2-} + HSO_4^- ,$$

$$C_8H_5O_2N_3 + H_2O_2 \rightarrow C_8H_5O_4N_2^{2-} + 2H^+ ,$$

$$C_8H_5O_4N_3^{2-} \rightarrow C_8H_5O_4N^{2-}{}^* + N_2 ,$$

$$C_8H_5O_4N^{2-}{}^* \rightarrow C_8H_5O_4N^{2-} + \text{light} .$$

This scheme requires the production of three H^+ per molecule of luminol destroyed. Experiments in which production of H^+ was measured did not agree with this stoichiometry. In fact, the H^+ production was quite variable: from zero to seven or eight H^+ per molecule of luminol in either the presence or absence of H_2O_2 at pH 12.0.

Experiments still in progress indicate that the 5-amino group may be oxidized to a radical which can combine with itself in various ways (an "aniline black" type of reaction), but many of the combinations are again reduced to luminol by any H_2O_2 present. This reversible system may then act as a catalyst for the decomposition of H_2O_2 by peroxydisulfate.

Experiments have also been conducted on the chemiluminescent compound dimethylbiacridylium nitrate (lucigenine). Kinetic studies have been made on the reaction between lucigenine and fructose, hydroxylamine, creatinine, or hydrogen peroxide in alkaline aqueous solution. As with luminol, light emission in the absence of H_2O_2 requires free O_2. With fructose, light emission follows the kinetics expected from a reaction of the type:

$$A \rightarrow 2B \rightarrow \text{end products} .$$

The end products were found to be about ½ methylacridone and ½ dimethyl biacridylidene. The oxygen uptake rate was found to be independent of the lucigenine concentration above about 2×10^{-6} *M*. With hydroxylamine the oxygen uptake rate was directly related to the square root of the lucigenine concentration over a relatively large range (5×10^{-6} to 2×10^{-4} *M*). These data are presently interpreted to indicate that the lucigenine undergoes a reversible decomposition to two identical radicals which then undergo disproportionation with unchanged lucigenine base.

A small fraction of the radicals formed initiates auto-oxidation of the substrate.

SYNTHESIS AND EVALUATION OF SERINE ANTIMETABOLITES

D. G. Doherty, Margaret A. Turner, and James D. Regan

The initial discovery by J. D. Regan that human granulocytic leukemia cells had an abnormal requirement for serine stimulated a search for serine antimetabolites. A series of compounds selected to the structure of serine was prepared, and one of them, 1-(hydroxyamino)cyclohexane carboxylic acid, inhibited DNA and RNA synthesis but had a minimal effect on protein synthesis.[1] The results suggested that one carbon donation from serine to purine biosynthesis via the enzyme serine transhydroxymethylase was greatly reduced. The structures of those compounds already tested indicated fairly strict requirements for inhibition. In consequence we have synthesized a series of compounds isosteric to serine, *e.g.*, with a phosphorus atom replacing the carboxyl carbon, and various combinations of amino and hydroxyl groups. At this time these compounds are awaiting testing in the tissue culture system. To have an alternate means of testing enzyme inhibitors we have isolated serine transhydroxymethylase from rabbit and rat liver and are currently developing a spectrophotometric assay.

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1. N. H. Pazmiño, D. G. Doherty, and J. D. Regan, *J. Natl. Cancer Inst.* **51**, 761–765 (1973).

SEPARATION OF BASES, RIBONUCLEOSIDES, AND DEOXYRIBONUCLEOSIDES BY ANION-EXCLUSION AND PARTITION CHROMATOGRAPHY ON CATION-EXCHANGE RESIN. APPLICATION TO THE ASSAY OF RIBONUCLEOTIDE REDUCTASE, DEAMINASE, AND NUCLEOSIDASE

Bimal C. Pal, James D. Regan, and F. D. Hamilton

[5-^{3}H] CDP (cytidine 5′-diphosphate) and CTP (cytidine 5′-triphosphate) are used as substrates in the assay of ribonucleotide reductase, deaminase, and nucleosidase activity in crude enzyme preparations. After incubation the nucleotides are hydrolyzed to nucleosides by sequential treatment with potato apyrase and alkaline phosphatase. An aliquot is then chromatographed on a cation-exchange column at 50°C with 0.1 *M* boric acid, adjusted to pH 7.4 with ammonia, used as eluent. The pyrimidines Ura, Urd, dUrd, Cyt, Cyd, and dCyd are separated and eluted in about 50 min in small volumes. Assays by this procedure of CTP reductase activity in crude fractions of ribonucleotide reductase from *Euglena gracilis* gave results comparable to those obtained by the standard method. The new procedure is also applicable when adenine or guanine nucleotides are used as substrates. The adenine derivatives Ade, Ado, dAdo, Hyp, Ino, and dIno, as well as the guanine derivatives Gua, Guo, dGuo, Xan, and Xao, are separated from each other in this chromatographic system in about an hour.

IN VITRO TRANSFORMATION OF 4-THIOURIDINE TO 4-SELENOURIDINE IN *E. COLI* MIXED tRNA

Bimal C. Pal and Diane G. Schmidt

Selenium is present in our environment. The element is an essential micronutrient for man and other species. On the other hand, many selenium compounds are toxic at higher dose level. Sodium selenite has been recently reported to retard the growth of various experimental tumors in rats and mice.[1] For these reasons the metabolism of selenium compounds is of wide interest in nutrition and toxicology as well as in biochemistry. It has recently been reported from two laboratories that *E. coli* grown in a medium containing ^{75}Se-labeled sodium selenite or sodium selenosulfate incorporate selenium as 4-selenouridine in their tRNAs.[2,3] Biological studies on such selenated tRNAs are hindered by poor yields from *in vivo* experiments. This led us to explore the possibility of chemical conversion of 4-thiouridine to 4-selenouridine in tRNA. We have already reported the route to *in vitro* labeling of tRNA with ^{35}S: tRNA(SH) $\xrightarrow{CNBr}$ tRNA-(SCN) $\xrightarrow{^{35}SH}$ tRNA(^{35}SH).[4] The same technique can be used for partial conversion (*ca.* 60%) of 4-thiouridine to 4-selenouridine in *E. coli* mixed tRNA using SeH^- in the last step.

1. G. B. Abdullaev, G. G. Gasanov, R. N. Ragimov, G. V. Toplyakova, M. A. Mekhtiev, and A. I. Dzhafarov, *Dokl. Akad. Nauk Az. SSR* **29**, 18 (1973).
2. J. L. Hoffman and K. P. McKonnell, *Biochim. Biophys. Acta* **336**, 109 (1974).
3. Y. S. Prasada Rao and J. D. Cherayil, *Life Sci.* **14**, 2051 (1974).
4. B. C. Pal and D. G. Schmidt, *J. Am. Chem. Soc.* **96**, 5493 (1974).

REGULATION OF DIMETHYLSULFOXIDE-INDUCED HEMOGLOBIN SYNTHESIS IN FRIEND LEUKEMIA CELLS. I. NONCOMMITTED AND COMMITTED STATES OF HEMOGLOBIN SYNTHESIS DURING THE INDUCTION PERIOD

P. R. McClintock* and John Papaconstantinou

When Friend leukemia cells (FLC) are grown in 2% Me_2SO medium for only 24 hr, hemoglobin synthesis begins on schedule at 48 hr, *i.e.*, after two replications. The induction, however, is short-lived, and data indicate that synthesis soon stops. With a 48-hr exposure to Me_2SO, the induction is initiated, but again synthesis ceases within a short period after removal of the inducer. A 72-hr exposure to Me_2SO is sufficient to give a rate of hemoglobin synthesis identical to the controls, where Me_2SO is present throughout the experiment. These results seem to indicate that a specific amount of exposure time to Me_2SO is required before the FLC is committed or stabilized in the pathway of differentiation characterized by the appearance of hemoglobin. If the inducer is removed before the stabilization has taken place, then the reactions which commit the cell to this line of differentiation are arrested. In addition these experiments suggest that the stabilization takes place between the second and third generations (48–72 hr).

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REGULATION OF DIMETHYLSULFOXIDE-INDUCED HEMOGLOBIN SYNTHESIS IN FRIEND LEUKEMIA CELLS. II. INHIBITION OF HEMOGLOBIN SYNTHESIS BY HYDROCORTISONE

P. R. McClintock* and John Papaconstantinou

It has been shown in other laboratories that the Me_2SO-mediated induction of hemoglobin synthesis in FLC is accompanied by an increase in the production of Friend virus particles. On the basis of this observation the question arises of whether the induction of hemoglobin synthesis and the stimulation of virus production are closely linked. Since hydrocortisone (HC) is one of the glucocorticoids known to stimulate oncornavirus synthesis in cells, experiments were done to determine whether this steroid hormone will stimulate the synthesis of hemoglobin. Our preliminary experiments indicated that 10^{-6} *M* HC is *in fact* a potent inhibitor of hemoglobin synthesis. There is little effect on the growth and viability of the cells in concentrations of HC ranging from 10^{-5} to 10^{-8} *M*. The specificity of the inhibition is indicated by the fact that a related steroid, tetrahydrocortisone, has no effect on hemoglobin systhesis. Furthermore, since growth and replication are not affected, the site of inhibition seems to be at the level of transcription.

With the development of this system, *i.e.*, the Me_2SO-mediated induction and HC-mediated repression of hemoglobin synthesis, we now propose to extend our studies to the analysis of the molecular mechanisms of gene expression.

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REGULATION OF DIMETHYLSULFOXIDE-INDUCED HEMOGLOBIN SYNTHESIS IN FRIEND LEUKEMIA CELLS. III. DENSITY-DEPENDENT INDUCTION OF HEMOGLOBIN SYNTHESIS IN THE ABSENCE OF INDUCER

John Papaconstantinou

In the course of studies on the Me_2SO-mediated induction of hemoglobin synthesis in Friend leukemia cells, it was noticed that under certain culture conditions a large number of these cells which normally grow in suspension attach to the surface of the culture flask and form colonies. By selecting for these "attached forms," cultures were established which have the following characteristics: (*a*) cells form confluent, dense layers, (*b*) cells cannot be shaken loose from the surface of the flask, (*c*) cells can be resuspended by proteolytic enzyme treatment and will reattach to the surface when subcultured, (*d*) cells have a cloning efficiency of about 20%, (*e*) cells have an epithelial structure and will pile on top of each other in their growth pattern, and (*f*) a population density of 0.5–1 $\times 10^6$ cells/cm^2 is attained in stationary phase. At this density many of the cells which are piling up are released and will grow in suspension. In true suspension culture the highest population density achieved in this laboratory is 4–5 $\times 10^6$ cells/cm^3.

The establishment of the "attached cell" cultures was undertaken to facilitate studies on the possible role of cell density, cell:cell contact, and cell membrane changes on the induction of hemoglobin synthesis. Preliminary studies clearly show that when these cells form a heavy monolayer (stationary phase), hemoglobin synthesis is initiated *without the mediation of Me_2SO.* Treatment of these cells with Me_2SO further enhances hemoglobin synthesis. I conclude from these preliminary studies that the cell membrane, and/or cell:cell contact may indeed play an important role in the pathway of erythroid cell differentiation and in the induction of hemoglobin synthesis in the polychromatophilic erythroblast. Whether the membrane is the primary site of action of Me_2SO, or whether chromatin is the main site of action remains to be shown experimentally.

REGULATION OF ALBUMIN AND α-FETOPROTEIN SYNTHESIS IN SYNCHRONIZED MOUSE HEPATOMA CELLS

C. L. Parker* and John Papaconstantinou

Mouse hepatoma cells derived from the solid tumor BW7756 were synchronized by the isoleucine-deficiency method and by double-thymidine block. These synchronized cells were used to study the characteristics of albumin and α-fetoprotein (αFP) synthesis throughout the cell cycle. Our studies have shown that the incorporation of [^{3}H]leucine is linear and continuous throughout the cell cycle and that each protein shows a sharp increase (critical point) in the rate of synthesis at a specific phase of the cell cycle. The critical point for albumin synthesis is at late G_2–early mitosis; the critical point for αFP synthesis is in mid-G_2. Pool analyses have shown that the critical point cannot be due to an increase in the specific activity of the intracellular leucine pool. Amino acid analyses of immunoprecipitated [^{3}H]albumin, isolated several hours after the critical point, have shown that

99% of the radioactivity is in leucine, indicating that the increase in rate of synthesis cannot be attributed to a generalized metabolic distribution of [^{3}H]leucine into other amino acids. We conclude that the critical point for albumin and αFP synthesis is due to the activation of new mRNA for these proteins. On the basis of these observations, we used the *critical point* as an assay to detect any interference with the transcription of a specific mRNA. To determine whether the transcription of albumin mRNA is discontinous and to localize the phase in the cell cycle where transcription occurs, we treated synchronized cells with inhibitors of transcription (actinomycin D, cordycepin) at specific stages during the cell cycle. These experiments were designed to localize an inhibitor-sensitive phase for albumin and αFP. Our assay for the detection of an inhibitor-sensitive phase is the loss of the critical point. Treatment with actinomycin showed that the critical point for albumin and αFP is abolished when the inhibitor is present during late S-phase and early G_2. One objection to the use of actinomycin is its toxicity and inhibition of mitosis. This problem was solved by using cordycepin in concentrations that abolish the critical point but have no effect on the cell replication. Under these conditions, the drug-sensitive period for albumin and αFP was found in late S–early G_2. We conclude from these studies that transcription of mRNA for albumin and αFP is discontinuous; the period of transcription is in late S and early G_2, and the period of processing and transport of mRNA extends from early G_2 to the critical point (late G_2); after the mRNA enters the cytoplasm because of its stability, it is transcribed continuously throughout the cell cycle.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

REGULATION OF DIMETHYLSULFOXIDE-INDUCED HEMOGLOBIN SYNTHESIS IN FRIEND LEUKEMIA CELLS. IV. EFFECT OF PROFLAVIN ON THE INDUCTION OF HEMOGLOBIN SYNTHESIS

A. W. Wiens,* P. R. McClintock,† and John Papaconstantinou

It has recently been shown by one of us (A.W.W.) that proflavin is a potent inhibitor of the hydrocortisone-mediated induction of glutamine synthesis in embryonic chick retina cells. This study indicated that proflavin inhibits the transcription of glutamine synthetase mRNA templates. To expand our understanding of the mechanism of induction of hemoglobin synthesis in FLC, studies were done to determine whether proflavin will inhibit this induction. Our results have shown that (*a*) proflavine is a potent inhibitor of cellular replication, (*b*) induction of hemoglobin synthesis by Me_2SO *is not* inhibited by proflavine and the rate of accumulation of hemoglobin is not affected, and (*c*) induction of hemoglobin synthesis is delayed by proflavine. This delay is linked to the effect of proflavine on the generation time of these cells. Thus, 1 μM proflavine will extend the generation time of FLC from 24 to 60 hr, and hemoglobin induction is delayed from 48 to 120 hr. In both cases, however, the induction and synthesis of hemoglobin is the same. We conclude from these studies that (*a*) the mechanism of induction of hemoglobin synthesis by Me_2SO is basically different from the mechanism of induction of glutamine synthetase by HC – this conclusion is based on the fact that proflavine has a specific inhibitory effect on glutamine synthetase induction, and (*b*) proflavine, an intercalating agent, has a potent inhibitory effect on the replication of FLC. Thus, this potentially mutagenic and carcinogenic compound shows a strong inhibitory effect on the replication of the cell but shows no effect on the expression of a gene which is activated during a specific pathway of cellular differentiation.

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ISOLATION OF ALBUMIN POLYSOMES AND ALBUMIN mRNA

Peter Brown* and John Papaconstantinou

To study the regulation of transcription and translation of albumin mRNA in synchronized mouse hepatoma cells, experiments were initiated to establish techniques for detection and isolation of albumin polysomes and for detection and isolation of albumin mRNA. The detection and isolation of albumin polysomes have been accomplished through the use of radioimmunoassay and immunoprecipitation techniques. Highly purified albumin was prepared and coupled to Sepharose (by CNBr treatment) for use in the affinity chromatographic preparation of monospecific albumin antibody. This highly specific antibody was iodinated with ^{125}I, and the [^{125}I]antiserum was reacted with liver polysomes. The polysomes were spun

in a sucrose gradient and the fractions were collected and counted. The radioactive profile showed a peak on the heavy side of the polysome (A_{260}) profile. This peak was sensitive to ribonuclease treatment and to competition with cold albumin antibody. We now propose to use this procedure to determine whether there is any change in size of albumin polysomes in synchronized hepatoma cells. This information is important for the distinction between a translational stimulation of protein synthesis (more ribosomes/mRNA) or a transcriptional stimulation of albumin synthesis in synchronized cells. In addition we propose to use the immunoprecipitation of albumin polysomes for the isolation of albumin mRNA. Preliminary experiments on the mRNA isolation have been done. Immunoprecipitated polysomes have been phenol extracted and the RNA fractionated on an oligo-dT column. The polyadenylated RNA fraction shows a strong stimulation of *in vitro* protein synthesis in a wheat-germ system. The purity of the product of this *in vitro* synthesis is being determined by radioimmunoassay.

*Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

PROGRESS IN PURIFICATION OF TUMOR-SPECIFIC TRANSFER FACTOR

G. D. Griffin,* Helen G. Sellin, Den-Mei Yang,† and G. David Novelli

The effectiveness of immunotherapy of cancer by transfer factor could probably be greatly increased by using preparations of purified transfer factor which contain only the molecules involved in transferring tumor immunity. Purified preparations would also allow quantification of effectiveness of various dose schedules. We are currently attempting to purify the active components from transfer factors specific for osteogenic sarcoma and mammary carcinoma.

Initial experiments with mammary carcinoma transfer factor used chromatography on Aminex A-28. Separation into 15 UV absorption peaks occurred, and all of these peaks were found to possess transfer factor activity when assayed in an *in vitro* cytotoxicity assay.[1] The activity of some of the fractions could be demonstrated only upon extensive dilution; this suggested the presence of inhibitors of transfer factor activity.

The presence of inhibitors of transfer factor activity had gone unrecognized previously, and we felt that a significant purification step would be accomplished simply by separating the inhibitory from the active components. Sephadex G-25 chromatography was used for this separation, and eight UV absorption peaks were resolved. Cytotoxicity assay of these components again indicated varying levels of transfer factor activity in all peak components. Sephadex G-10 chromatography was also used to try to separate inhibitory components from the active material. Assay of pooled material from this chromatography indicated that transfer factor activity was spread throughout the run and that considerable activity could be found in non-UV-absorbing material, indicating that transfer factor activity may not necessarily be associated with UV-absorbing molecules.

The separation of transfer factor into a number of active components by two quite different processes (ion-exchange chromatography and molecular filtration) may indicate that the classic methods of transfer factor preparations produce various sized active components of a larger molecule, and this large molecule may be resynthesized by lymphocytes during the course of the assay.

In addition to these chromatographic methods of purification, we are also engaged actively in a study of the cell type from which transfer factor originates, since it might then be possible to separate this particular cell type from other contaminating cells before preparing transfer factor. The procedure could result in significantly less degradation of the active molecules in transfer factor. Preliminary experiments have indicated that a transfer factor preparation from "T" lymphocytes is active, and this activity resides in a larger molecular species than the activity of dialyzable transfer factor.

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TUBERCULIN TRANSFER FACTOR: A MODEL FOR CERTAIN ASPECTS OF CELL-MEDIATED IMMUNITY

G. D. Griffin,* Helen G. Sellin, and G. David Novelli

We are attempting to develop the tuberculin transfer factor system in animals and in human lymphocytes *in vitro* to obtain information regarding basic aspects of this immunological transfer phenomenon, such as which cell type is the source of transfer factor, which cell type responds to transfer factor, and the molecular species involved in transferring ability to react to antigen by

blastogenesis and/or cytotoxicity. Current research efforts have been directed at developing new methods for preparation of molecules able to transfer immunological specificity, separation of these active molecules from endogenous inhibitor, and methods of assaying these activities which unambiguously measure only one aspect of cellular immunity (*i.e.*, cytotoxicity, blastogenesis, etc.).

A method for preparing tuberculin transfer factor from guinea pigs immunized with live BCG is being developed which involves extraction of leukocytes from blood and spleens, homogenization, and Sephadex G-25 chromatography. Probably, this procedure will avoid much of the enzymatic degradation inherent in the standard procedures used for transfer factor preparation. At least two systems will be used to assay the material from G-25 chromatography. One is the cytotoxicity assay involving destruction of antigen-coated target cells by lymphocytes activated by transfer factor,[1] and the other is a blastogenesis assay involving measurement of proliferation of transfer-activated lymphocytes in the presence of specific antigen.

We have experienced difficulty in carrying out the blastogenesis assay *in vitro* and have found that this is at least partially due to the presence of numerous inhibitors of blastogenesis which are present even in transfer factor fractions which have been separated by Aminex A-28 chromatography. Unfractionated ("crude") dialyzable transfer factor inhibited phytohemagglutinin blastogenesis of lymphocytes even when diluted 1:10,000. Fractions of Aminex-separated transfer factor shown to be active in a cytotoxicity assay were found to inhibit phytohemagglutinin blastogenesis, although considerable dilution abolished the inhibitory activity. This inhibitory effect could also be seen using concanavalin A or pokeweed-mitogen-induced blastogenesis. We are currently attempting to develop ways to separate these inhibitory factors from molecules which are active in inducing blastogenesis.

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1. G. D. Griffin, H. G. Sellin, Sister M. P. Nevins, and G. D. Novelli, this report.

PROGRESS ON FRACTIONATION OF TRANSFER FACTOR FROM BEAGLES*

Helen G. Sellin, G. D. Griffin,† and G. David Novelli

Demonstration of *in vivo* transfer of cellular immune competence in humans can be shown by injection of white blood cells (WBC) or by injection of a low-molecular-weight dialysate, called transfer factor, from ruptured, WBC from sensitized individuals. A practical application of transfer factor has been found clinically in the treatment of various human diseases where a patient's cellular immune competence can be greatly improved by administration of appropriate transfer factor. Progress in the purification and study of the chemical nature of transfer factor has been slow due to the lack of an adequate animal model and to the lack of a rapid, reproducible, *in vitro* assay.

We are attempting to use beagles for this purpose and would like to compare preparations from the same animal before and after immunization. Unimmunized beagles (TB^-) have been bled and WBC separated. After bleeding, the same dog was immunized with BCG so the animal would be TB^+. The bleeding was repeated and WBCs and lymphocytes were separated. Separation of these WBC crude preparations into constituent fractions was attempted chromatographically on Sephadex G-25 followed by chromatography of the G-25 included material on Aminex A-28 using a volatile salt gradient. The profiles from TB^- WBCs and TB^+ WBCs were similar in that eight UV absorption peaks were obtained. However, between 0.7 and 0.8 *M* salt a shift in peaks occurred. A large A_{280} absorption peak at approximately 0.8 *M* salt in the unimmunized WBCs disappeared in the immunized WBCs, and a new, much smaller peak with $A_{260}/A_{280} = 1$ appeared at a lower salt concentration (0.73 *M*). If this change can be shown to be due to immunization and if TB reactivity can be demonstrated in this portion of the profile, transfer factor purification will have made progress. Further work is concentrating on development of a reproducible, *in vitro* assay for our chromatographic profiles.

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DEVELOPMENT OF A GENERALIZED LYMPHOCYTE CYTOTOXICITY ASSAY USING SOLUBLE ANTIGENS COUPLED TO CELL MEMBRANES

G. D. Griffin,* Helen G. Sellin,
Sister Mary Paul Nevins,† and G. David Novelli

The successful isolation of the active components in transfer factor, a task in which we are currently involved, requires a reliable method of measuring some aspect of transfer factor activity following various fractiona-

tion procedures, and this measurement must be of some aspect of activity which is unequivocally important in the function of transfer factor. This last point is essential, since there may be a number of different active molecules in transfer factor preparations which produce differing immunological and biochemical expressions, not all of which may be relevant in the fundamental expression of cell-mediated immunity. Since a lysis of tumor cells by lymphocytes that have been activated by transfer factor is clearly of paramount importance in the immunotherapy of cancer by transfer factor, we have chosen to try to develop a cytotoxic assay, in which transfer-factor-activated lymphocytes are exposed to cells which are tagged in some manner with an antigen which the lymphocytes specifically recognize.

For this purpose, we have been using chicken or sheep erythrocytes labeled with ^{51}Cr or [^{3}H]adenosine. By a process of chemical coupling, PPD (purified protein derivative) or KLH (keyhole limpet hemocyanin) are then attached to these erythrocyte membranes, and this now antigenically tagged cell is incubated with lymphocytes from animals previously sensitized against BCG or KLH. The sensitized lymphocytes, if they are able to recognize the modified erythrocyte, should lyse the cell, and the radioactive label will be released. A simple counting procedure allows one to calculate the extent of such lysis.

Two problems have arisen in the development of this assay. One of these is that it is difficult to label the erythrocytes to a high enough level so that small numbers of erythrocytes may be used in an assay (this latter condition is necessary because the ratio of lymphocytes to erythrocytes needs to be large, and numbers of lymphocytes are the limiting factor). ^{51}Cr, [^{3}H]deoxyglucose, which enters the cell and is phosphorylated but not further metabolized, and [^{3}H]adenosine have all been used as labels. Of these, ^{51}Cr and [^{3}H]adenosine both show promise, although adenosine may be preferable, since the cellular constituents labeled by ^{51}Cr are ill-defined.

The other problem encountered involved the procedure for coupling antigen to the erythrocyte membrane. It was necessary to obtain an adequate level of substitution and yet maintain the integrity of the membrane. Initial attempts using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide to couple the antigen resulted in excessive leakage of label from the erythrocytes. A procedure using a buffered $CrCl_3$ solution has produced better results. If these difficulties can be overcome, this assay should be of real value in development of purification procedures for transfer factor.

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TRANSFER RNA ALTERATIONS OCCURRING IN HUMAN LEUKEMIC LYMPHOCYTES

G. D. Griffin,* G. David Novelli, and Wen-Kuang Yang

Whether tRNA has a regulatory role in cellular metabolism, in addition to its well-known importance in protein synthesis, is a question which is still largely unanswered. Various investigators have shown that alterations in chromatographic behavior of various tRNA species can be found in various tumor tissues. These chromatographic differences could either be the result of changes in primary structure or alterations in degree of modification.

We have been studying the chromatographic profiles of tRNA species from human lymphocytes before and after stimulation with various mitogens (mainly phytohemagglutinin), using the RPC-5 chromatographic system. We have not detected any large differences in total cellular tRNA species when resting lymphocytes were compared with stimulated lymphocytes. Individual species of tRNA, specifically, aspartyl, tyrosyl, and phenylalanyl tRNAs, were found to display the same chromatographic profiles, whether isolated from resting or stimulated lymphocytes.

When tRNA from two leukemic lymphocyte cell lines was used, differences in aspartyl and tyrosyl tRNAs in comparison with noncancerous lymphocyte tRNA could be seen. In the case of tyrosyl tRNA, the leukemic profile was shifted as a whole to elute at lower NaCl concentrations. The aspartyl tRNA from leukemic cells showed a new isoaccepting species eluting at a higher salt concentration than any normal peaks. The two peaks seen in normal lymphocyte tRNA were also seen in leukemic profiles in the case of aspartyl tRNA.

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EXAMINATION OF URINE FROM TUMOR-BEARING RATS FOR INCREASED NUCLEOSIDE CONTENT

Audrey N. Best and G. David Novelli

There have been several reports of increased excretion of certain nucleosides in human urine in patients with various forms of cancer. One study[1] involving investigators from ORNL and NCI using various advanced analytical procedures revealed increased excretion of

the nucleosides N^2,N^2-dimethylguanosine, 1-methyl inosine, and pseudouridine, presumably derived from tRNA, in the urine of some patients with various types of malignant tumors. Increased methylase activity has been reported for tumor cells,[2] but the examination of certain tumors has not always revealed the expected hypermethylation.[3,4]

We are interested in a collaborative program with the Respiratory Carcinogenesis Group involving the determination of the nucleoside level of rat urine from animals that have developed squamous carcinoma in tracheal grafts.[5] It is possible that with this animal model system we might see changes in the levels of several nucleosides at various stages of carcinogenesis. If these nucleosides are predictive of the appearance of carcinoma, it is possible that their concentrations in the urine would decrease as a function of therapeutic improvement. Such a study might give useful information in following the progress of patients on transfer factor therapy.

The initial portion of the program will be trial experiments in methodology for the separation of the nucleosides and also their purine and pyrimidine derivatives from other components in urine; these experiments are now in progress.

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STUDIES ON THE tRNA OF METHANE-PRODUCING BACTERIA

Audrey N. Best

In the past three years the Protein Synthesis Group under the direction of G. David Novelli has been interested in the formation of methane from crude carbohydrates (cellulose). While the main impetus of this complicated project has diminished due to uncertain funding, I have been investigating the metabolism of the tRNA of the methane-generating bacteria in conjunction with our other research on tRNA. At present I am completing these experiments and do not plan to continue this project unless adequate funding becomes available.

The nucleoside content of the enzymic digest of purified, bulk tRNA from a fast-growing methanogenic coccus using formate for its carbon source has been examined by ion exchange and thin-layer chromatography, showing differences from the intensively investigated tRNA from *E. coli.* The methylation pattern differs with respect to adenosine and guanosine, and there is a lack of ribothymidine and 7-methyl guanosine, usually present in purified *E. coli* tRNAs. The tRNA preparations contain the modified nucleosides pseudouridine, dihydrouridine, thiolated uridine and cytidine, and 2-*O*-methyl cytidine as well as several unidentified nucleosides. The apparent lack of hypermodified nucleosides found in tRNAs from *E. coli* and various eukaryotic cells is also found in the few tRNA species purified from some primitive mycoplasma organisms. The tRNA from another species of methanogenic bacteria (carbon source, acetate, or methanol) has even lower methylation and also lacks ribothymidine.

Despite the absence of ribothymidine, *in vitro* methylation using *E. coli* methylases and *S*-adenosyl methionine (SAM) has not shown these tRNA preparations to accept methyl groups as completely as a sample of undermethylated tRNA from *E. coli.*[1] Enzyme extracts from the formate-utilizing organism can incorporate methyl groups from SAM into the undermethylated *E. coli* tRNA. The positions of methylation have not been determined.

The tRNA preparations can be aminoacylated, and at least 80% of the 3′-terminal groups have been identified as adenosine. Since the organism lyses so easily, RNA obtained by phenol extraction consists of a mixture of small oligonucleotides, tRNA, and larger-molecular-weight fractions. It is difficult to remove other nucleic acid fragments from the tRNA. I plan to aminoacylate the tRNA (purified twice by G-100 chromatography) with [^{3}H]methionine and chromatograph on RPC-5 columns under conditions which would separate intact tRNA from oligonucleotides. The analyses of nucleoside content can be checked on this purified material as well as other properties of tRNA. Low acceptance of methylation can occur for a number of reasons, but we must rule out the presence of degraded tRNA.

Several recent reports have demonstrated a folate derivative rather than SAM as a donor or intermediate for the methyl moity of ribothymidine in tRNA of *S. faecalis, B. subtilis,* and *M. lysodeikticus.*[2-5] It is of interest that two organisms, growing on different carbon sources, generating methane via incompletely described pathways in which a folate derivate is strongly implicated,[6] have been found to lack ribothymidine in their tRNAs. Possibly the lack of methylation

may be due to a control mechanism preventing the loss of needed intermediates for energy-generating metabolism. Although this has not been tested, it is possible that, like *S. faecalis* grown in the absence of folate,[7] the $tRNA_f^{Met}$ of methanogenic bacteria is not formylated during cell growth due to lack of suitable substrate.

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INHIBITION OF DNA-DEPENDENT RNA POLYMERASE OF *E. COLI* BY PHOSPHOLIPIDS

Audrey L. Stevens

After labeling normal and T4-phage-infected *E. coli* with ^{32}P we found that DNA-dependent RNA polymerase contains small amounts of phospholipids. The three major phospholipids of *E. coli* are phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin, and they are found in the ratio 10:3:1 respectively. Because of the lipid content of RNA polymerase, we investigated the effect of micelle preparations of the three phospholipids on the activity of the enzyme. The results were as follows: The enzyme is strongly inhibited by phosphatidylglycerol and cardiolipin at concentrations of 5 to 8 $\times$ 10^{-5} *M*. The two lipids inhibit the formation of rifampicin-resistant RNA polymerase–DNA preinitiation complexes. Phosphatidylethanolamine is not inhibitory itself, and at a tenfold higher concentration it partially overcomes inhibition by the other two lipids.

A SALT-PROMOTED INHIBITOR OF RNA POLYMERASE ISOLATED FROM T4-PHAGE-INFECTED *E. COLI*

Audrey L. Stevens and Joyce C. Rhoton

Upon infection of *E. coli* with T4 phage, unique species of phage-specific messenger RNA are formed at specific times during the phage multiplication cycle. To accommodate the new program of transcription, DNA-dependent RNA polymerase of *E. coli* is modified in several ways. The modifications apparently change its promoter specificity. We have studied RNA polymerase from *E. coli* infected with a T4 phage gene $42^- \times 47^-$ mutant, examining the activity of the sigma subunit of the enzyme. The infected polymerase contains about 0.2 equivalent of the sigma subunit. Purification of sigma fractions by phosphocellulose chromatography followed by gradient centrifugation or agarose gel chromatography shows that the fractions that contain sigma also contain the T4-specific polymerase-binding protein of 10,000 mol wt.[1] These sigma fractions are poorly stimulatory toward core enzyme fractions except in the presence of Triton. With T4 DNA as a template, these sigma fractions inhibit the activity of core enzyme plus host sigma fractions, particularly in the presence of salt. The 10,000 mol wt protein can be separated from sigma by including 6 *M* urea in the gradient centrifugation or chromatography solutions. The sigma thus purified is then highly stimulatory, whereas the fractions containing the 10,000 mol wt protein have the salt-promoted inhibitory activity toward both host and T4 sigma-stimulated core enzyme fractions. The inhibition occurs at the stage of initiation, since formation of rifampicin-resistant RNA polymerase–DNA complexes is inhibited by the protein. The inhibition is overcome by Triton. Increasing the concentration of T4 sigma purified with 6 *M* urea or host sigma also overcomes the inhibition by the 10,000 mol wt protein.

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ABNORMALITIES IN PROTEIN-SYNTHESIS COMPONENTS AS DETECTED WITH AN *IN VITRO* PROTEIN-SYNTHESIS SYSTEM

K. R. Isham and M. P. Stulberg

Previously[1] we described the preparation of an *in vitro* protein-synthesis system from ascites tumor cells. This system is completely dependent on exogeneous mRNA and almost completely dependent on exogenous tRNA. We have completed the detection system by perfecting a discontinuous slab-gel electrophoresis system to analyze specific polypeptide formation. Thus we are now in a position to test, at least, mRNAs and tRNAs from aberrant sources including aged, cancerous, mutated, and irradiated animals. Dividing and nondividing organs from young and aged animals have been collected and stored. We will solicit mouse organs from mutation and irradiation experiments and also isolated murine cancers. Components of protein syn-

thesis will be isolated from these sources and tested in our system.

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TRANSLATION OF mRNAs IN AN *IN VITRO* PROTEIN SYNTHESIS SYSTEM

M. P. Stulberg, K. R. Isham, Beth C. Mullin,* and L. C. Waters

In this report[1] we have described progress in constructing and optimizing an *in vitro* protein synthesis system prepared from ascites tumor cells. We have successfully used the system to translate RNAs isolated from encephalomyocarditis (EMC) virus and reticulocytes (globin mRNA).

In this collaborative effort we are attempting to translate RNAs isolated from AKR murine leukemia virus (MLV) and avian myoblastosis virus (AMV). Both 70S and 35S components of the viral RNA have been tested without significant translation. Conceivably, translation of heterologous messengers requires specific factors that reside only in the tissue of origin of the mRNA. In fact, we found very little translation of globin mRNA without an addition of factors extracted from reticulocyte polysomes. However, the addition of these factors allowed for only a small amount of translation of AMV and MLV RNA. We will attempt to increase the amount of translation by preparing more factors from reticulocytes to allow for optimizing procedures.

Concomitantly, we discovered that the reticulocyte factors inhibited translation of homologous EMC viral RNA; therefore heterologous factors may, in contrast, inhibit translation of homologous mRNAs. Accordingly, we prepared polysome-extracted factors from AMV-infected myeloblasts and tested them for activity in the translation of AMV RNA; however, no stimulation was found. In fact, this factor preparation markedly inhibited the translation of all mRNAs tested. We are now investigating the nature of this inhibition.

*Postdoctoral investigator supported by Subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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THE CONTENT OF MODIFIED BASES IN UNDERMETHYLATED PHENYLALANINE tRNA

K. R. Isham, Millicent Sutton,* and M. P. Stulberg

Previously[1] we have reported on the content of modified bases in undermethylated $tRNA^{Phe}$ from an *rel*$^-$ Met$^-$ mutant of *E. coli* and interpreted the results in relation to the function of $tRNA^{Phe}$.

We have now determined the effect of these undermodifications in a complete, purified mixture of undermethylated tRNA (UU-tRNA) on their capability to support protein synthesis. We prepared an *in vitro* protein synthesis system from *E. coli* Q 13, depleted it of endogenous mRNA and tRNA, and tested UU-tRNA for stimulation of protein synthesis in comparison with normal tRNA. Translation was directed with phage RNA as messenger. When the system was supplemented with UU-tRNA, little, if any, stimulation was observed beyond that of controls lacking added tRNA, whereas the addition of normal tRNA doubled protein synthesis.

Clearly, undermethylated tRNA (also lacking other modifications, especially methylthio-isopentenyl adenosine) lacks the capability for stimulating protein synthesis in a tRNA-depleted system.

*Student trainee, Carnegie Corporation Program.

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SELECTIVE METHYLATION OF NEWLY SYNTHESIZED tRNA FROM AN *rel*$^-$ MUTANT OF *ESCHERICHIA COLI* DURING METHIONINE STARVATION

Lee R. Shugart

Transfer RNA obtained from an *rel*$^-$ mutant of *E. coli*, strain χ402, cultured under conditions of methionine starvation, is not completely undermethylated. This observation, based on a comparison of the methylated nucleoside content of tRNA obtained from cells grown under normal (plus methionine) culture conditions and under methionine-starved (minus methionine) culture conditions, was interpreted to mean that selective methylation occurs during the latter condition.[1]

Selective methylation was postulated to take place as a result of a loss of certain tRNA methyltransferase activities by the organism and/or was a reflection of the affinity of these enzymes for the precursors of trans-

methylation, coupled with the differential inhibitory property of *S*-adenosylhomocysteine, a product of transmethylation.[2]

Recent experiments with both viable, permeabilized cells and cell-free extracts of the *rel*⁻ mutant suggest that selective methylation cannot be attributed either to the absence of certain tRNA methyltransferase activities or to the presence of *S*-adenosylhomocysteine in methionine-starved cells. Therefore, analytical techniques for the estimation of the nucleoside content of tRNA are being reexamined and reevaluated.

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PURIFICATION OF tRNA METHYLTRANSFERASES FROM *ESCHERICHIA COLI*

Lee R. Shugart and Barbara H. Chastain

Because of the difficulties encountered during purification, several different methods for the separation of proteins from nucleic acids are being investigated in an effort to obtain a satisfactory procedure for the isolation of the various tRNA methytransferases from *E. coli.*[1] Two techniques are currently being tried: (*a*) precipitation of nucleic acids from microbial extracts with lysozyme, which involves removing nucleic acids and acidic proteins after complex formation with a basic protein, and (*b*) affinity chromatography, in which *S*-adenosylhomocysteine, an inhibitor of transmethylation, is attached to agarose gels through a hydrocarbon extension that terminates with an active carboxy *N*-hydroxysuccinimide ester. It is anticipated that the tRNA methyltransferases will form a protein-ligand complex with this material. Initial experiments indicate an increase in purity of the tRNA methyltransferases after treatment by either technique.

In addition, the levels of these enzymes are being studied and compared in an *rel*⁻ mutant of *E. coli* grown under different nutritional culture conditions. In methionine-starved cells (as compared to cells cultured in a complete growth medium) there is a twofold increase in the tRNA(adenosine-6 and -(guanosine-1)methyltransferase activities, but a fourfold decrease in the tRNA(guanosine-7)methyltransferase activity.

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S-ADENOSYLHOMOCYSTEINE NUCLEOSIDASE

Lee R. Shugart

Enzymatic transmethylation of nucleic acids is one of the major pathways in the metabolism of *S*-adenosylmethionine by most living organisms. *S*-adenosylhomocysteine, a product of this reaction, is a potent inhibitor of transmethylation and, in *E. coli,* this compound is degraded enzymatically by an *S*-adenosylhomocysteine nucleosidase.[1] This activity has been found in partially purified tRNA(uridine-5)methyltransferase preparations[2] and continues to copurify during subsequent isolation procedures. Therefore, the purification of this enzyme according to a published procedure,[1] is being investigated to answer two questions: (*a*) Can this procedure be used to further purify the tRNAmethyltransferase activities? (*b*) Is there more than casual correlation between the copurification of the nucleosidase and the tRNAmethyltransferases activities?

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TYROSYL-tRNA LIGASE OF *DROSOPHILA:* ITS ABILITY TO DISCRIMINATE $tRNA^{Tyr}$ FROM A SUPPRESSOR MUTANT AND WILD TYPE

K. Bruce Jacobson and J. B. Murphy

Earlier studies showed that the suppressor mutant, *su(s)*[2], had only the first of two isoacceptors for $tRNA^{Tyr}$ (ref. 1). However, subsequent studies revealed *su(s)*[2] did indeed possess the second isoacceptor, $tRNA^{Tyr}_{su(s)\ B}$, but demonstrated that it was different from that obtained from wild-type flies, $tRNA^{Tyr}_{SAM\ II}$ (ref. 2). The latter study showed further that the variation in results between the two studies was in the method of preparation of the tyrosyl-tRNA ligase. Efforts to define conditions that would allow reproducible production of the discriminating form of this enzyme were unsuccessful.[3] Discrimination is defined as the inability to charge $tRNA^{Tyr}_{B}$ but ability to charge $tRNA^{Tyr}_{II}$. We are continuing to study this problem through several approaches.

Enzyme preparations made in the past year have produced discrimination in the majority of cases, whereas in the previous two years the majority was nondiscriminating. Storage of the discriminating enzyme at −20°C for 1–3 weeks results in loss of discrimination, although activity remains; at −196°C the

discrimination is retained for 6–7 weeks, but, again, the enzyme then becomes nondiscriminating.

These two forms were examined to determine if one affected the specificity of the other. Mixed together, they charge $tRNA^{Tyr}_{su(s)\ B}$, thus showing that the discriminating form does not prevent the activity of the nondiscriminating form. Incubation of $tRNA^{Tyr}_{su(s)\ B}$ and the discriminating form of the enzyme prior to adding the nondiscriminating form does not interfere with the latter enzyme's activity; thus the former enzyme is not inactivating the tRNA. Each form charges $tRNA^{Tyr}_{SAM\ I}$ or $tRNA^{Tyr}_{su(s)\ A}$ equally. These experiments have been done by charging mixtures of $tRNA^{Tyr}_{su(s)\ A}$ and $tRNA^{Tyr}_{su(s)\ B}$ and also by charging $tRNA^{Tyr}_{su(s)\ B}$ that was separated from $tRNA^{Tyr}_{su(s)\ A}$. From these experiments we conclude that (*a*) there is not a freely dissociating factor that controls the isoacceptor specificity of the two enzyme forms, and (*b*) there is no interconversion of the isoacceptor forms of $tRNA^{Tyr}$.

The difference between $tRNA^{Tyr}$ wild-type and $su(s)^2$ flies is the difference in the second isoacceptor; this is detected by means of the discriminating form of tyrosyl-tRNA ligase. To learn how the enzyme distinguishes $tRNA^{Tyr}_{SAM\ II}$ and $tRNA^{Tyr}_{su(s)\ B}$, as well as how the enzyme loses this specificity while retaining its catalytic activity, seems of paramount importance.

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GUANYLATION OF *DROSOPHILA* tRNA AND THE GUANYLATING ENZYME OF *DROSOPHILA*

W. R. Farkas* and K. Bruce Jacobson

Transfer RNA has a variety of substituted and modified forms of the four major nucleotides, all of which are thought to occur after transcription. In previous work[1] a novel reaction in rabbit reticulocytes resulted in the incorporation of guanine into an internal nucleotide of reticulocyte tRNA, accompanied, presumably, by the displacement of a purine or pyrimidine. We have examined tRNA and enzyme extracts to determine if a similar phenomenon occurs in *Drosophila melanogaster*.

Using the guanylating enzyme of rabbit reticulocytes, we find that *Drosophila* tRNA is an effective substrate. [^{14}C]guanine is incorporated into four chromatographic forms (RPC-5 chromatography). Transfer RNAs from wild-type and $su(s)^2$ flies are equally effective substrates, though the distribution of [^{14}C]guanine among the four peaks differs.

Enzyme preparations from adult flies are active in causing guanine incorporation into yeast tRNA. For a partially purified preparation, the guanylating enzyme from *Drosophila* shows a linear relation of rate to enzyme concentration and has a K_m for yeast tRNA of 0.18 A_{260}/ml, a K_m for guanine of 1×10^{-8} *M*, and is unstable to dialysis. These K_ms are lower than those of the rabbit reticulocyte enzyme.

We are continuing our study by examining methods of fractionation and stabilization of the enzyme and by comparing developmental stages of *Drosophila* for enzyme content. The physiological role of the enzyme is unknown.

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QUANTITATION OF PTERIDINES BY DIRECT MEASUREMENTS OF FLUORESCENCE ON THIN-LAYER CHROMATOGRAPHY SHEETS

Marilyn A. Sutton,* T. G. Wilson,† and K. Bruce Jacobson

As one approach to studying the biochemical block of the purple (*pr*) mutant of *Drosophila melanogaster* and the mechanism of suppression by the suppressor-of-sable [*su(s)*] mutant, it is necessary to measure the quantity of pteridines in these mutants. The suspected site of the affected enzymatic reaction in *pr* is in the pteridine biosynthetic pathway leading to diosopterin biosynthesis.

Methods of quantitating pteridines after separation by thin-layer chromatography (TLC) usually involve eluting the pteridine(s) of interest in an aqueous solvent and measuring the fluorescence in solution. We found several of these methods unsatisfactory owing to the instability of several of the pteridines in solution.

We therefore examined the quantitation of *Drosophila* pteridines directly on the TLC sheets after drying. Using a Turner fluorometer with a TLC door attachment, we are able to quantitate pteridines of interest to us. Sepiapterin is quantitated with Turner filter 58 as the secondary filter; drosopterins are measured with a 23A filter; and filter 47B is used to quantitate biopterin, pterin, and isoxanthopterin.

This method can be used to quantitate pteridines in small amounts; for example, we can easily measure biopterin in quantities as small as 10^{-11} moles and as large as 5×10^{-10}. The fluorescence is proportional to the amount of material from *Drosophila* heads applied to TLC.

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STUDIES ON THE BIOCHEMISTRY OF THE PURPLE MUTANT IN *DROSOPHILA MELANOGASTER*

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As a prelude to studying the mechanism of suppression of the purple (*pr*) mutant by the suppressor-of-sable [*su(s)*] mutant in *Drosophila melanogaster,* we have been investigating the site of the affected enzymatic reaction in *pr.* Previous studies in this laboratory have shown that several pteridines are deficient in *pr* relative to wild-type and $su(s)^2$; *pr.* Specifically, we noted sepiapterin, drosopterins, and an unidentified presumed pteridine which quenches 365-nm UV light to be reduced in *pr.*

We have obtained a clearer understanding of the affected enzymatic reaction in *pr* by studying the behavior of eye pteridines in double mutants constructed from *pr* and other eye-color mutants. In *pr; se* and *pr;* Hn^{r3} the level of sepiapterin, which accumulates to high titers in *se* and Hn^{r3}, is greatly decreased. Likewise, the quenching substance, elevated in Hn^{r3}, is depressed in *pr;* Hn^{r3}. Biopterin, which accumulates in *pn, pd,* and *se,* is depressed in the corresponding double mutants containing *pr;* however, for mutants which have wild-type levels of biopterin, the corresponding double mutants with *pr* also have wild-type titers of this pteridine. Since sepiapterin and perhaps biopterin are thought to be early precursors of drosopterin, these results indicate that the defective enzyme in *pr* is prior to sepiapterin formation and therefore is early in the pathway.

Since the precursor molecule for pteridine biosynthesis is GTP (guanosine triphosphate), nucleotide pools in head extracts of *pr* flies were examined to localize further the affected enzymatic reaction. We found no significant difference in GTP and ATP titers in extracts of *pr,* $su(s)^2$; *pr,* and wild-type flies. This indicates that *pr* flies are not deficient in GTP with which to biosynthesize eye pteridines. This narrows the position of the affected enzymatic reaction in *pr* to either the reaction in which GTP forms dihydroneopterin triphosphate or to a reaction soon thereafter.

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ALTERED FORMS OF PHENYLALANINE tRNA IN A YEAST MUTANT

K. Bruce Jacobson, J. B. Bell,* Lee R. Shugart, and J. F. Lemontt

The yeast *Saccharomyces cerevisiae* has a single isoacceptor for $tRNA^{Phe}$ and $tRNA^{Tyr}$, such as that of the mutant XB109-5B if the latter is grown without agitation.[1] When XB109-5B is grown with vigorous aeration, these tRNAs then occur in 3–5 isoacceptor forms. We propose the hypothesis that one or more of the minor bases typical of these tRNAs is not produced during aerated and rapid growth. Evidence is being sought by chemical and genetic experiments to test this hypothesis.

The mutant XB109-5B was grown in the pilot plant in 250 liters of medium with vigorous aeration. The $tRNA^{Phe}$ was obtained in five isoacceptor forms by RPC-5 chromatography. Peaks A, B, and C are the major forms. tRNA was extracted and chromatographed on benzoylated DEAE-cellulose and then on RPC-5. All three major forms are retained on BD-cellulose during the NaCl gradient but are eluted with 20% ethanol and 1 *M* NaCl; this indicates that the Y-base is present, at least in part, in all three isoacceptors. The resolution of the uncharged $tRNA^{Phe}$ isoacceptors on RPC-5 is more difficult than the charged forms, so several stages of rechromatography were necessary to obtain peaks A, B, and C of $tRNA^{Phe}$ in purified form. The specific acceptances of peaks A, B, and C of $tRNA^{Phe}$ were 0.99, 0.95, and 1.19 moles phenylalanine per mole of nucleoside respectively.

The $tRNA^{Phe}$ in each peak was hydrolyzed (*a*) by NaOH and phosphatase, and (*b*) enzymatically to obtain nucleosides; the minor base composition was analyzed quantitatively by ion-exchange chromatography (Aminex A6). The three isoacceptors of $tRNA^{Phe}$ do not differ in m_2^2Guo, m^6Ado, m^5Cyd, rThd, ψ, m^2G, m^6Ado (m^1Ado), m^5Cyd, rT, ψ, and OMeG. The peaks A and C have the same amount of *O*-methyl cytidine (OMe-Cyd), but peak B is devoid of the nucleoside.

The nucleosides found at the 3′-end group are as follows in nanomoles per A_{260}: peak A – 1.19 Ado, 0.41 Cyd, 0.10 Guo, 0 Urd; peak B – 1.07 Ado, 0.24 Cyd, 0.20 Guo, 0.17 Urd; peak C – 1.11 Ado, 0 Cyd, 0.10 Guo, 0 Urd. The variety of end-group nucleosides indicates more or less removal of the CCA end groups – the appearance of Guo and Urd indicates that the three terminal nucleosides are all missing. This cannot account for the different chromatographic forms (A, B, and C), since they are observed after charging with phenylalanine; those tRNAs that are irreparably damaged at the 3′-terminus will simply not be charged.

Experiments are under way to prepare an active set of tRNA methylating enzymes from yeast and to test the different peaks of $tRNA^{Phe}$ as methyl acceptors. Of special interest, of course, is the OMe-Cyd at position 32 in the anticodon loop, since peak B is devoid of this nucleoside.

To explain the multiple peaks, we have assumed that certain minor bases are not produced. Alternatively, we could propose that the $tRNA^{Phe}$ is altered after it is produced in the normal form. One experiment followed the forms of $tRNA^{Phe}$ produced out to 50 hr of growth, whereas we normally harvest at 16 hr. No peak appeared greatly altered during this extended growth period.

Genetic control of multiple-peak pattern of $tRNA^{Phe}$ is not a simple one-gene type. A genetic cross of XB109-5B with S288C (wild type) would give spore tetrads, each containing two spores of multiple-peak type and two spores with single peaks of $tRNA^{Phe}$ (indicated as 2:2) if a single gene is responsible for the multiple-peak pattern. Of three tetrads analyzed, one each of 4:0, 1:3, and 2:2 was observed. These observations indicate either that multiple genes are controlling the $tRNA^{Phe}$ pattern or that the inheritance is not through nuclear genes. The diploids that gave rise to these spores all have single peaks of $tRNA^{Phe}$, which would be expected from a recessive nuclear mutation. Further experiments are planned to resolve these possibilities. The tetrad that gave the 4:0 ratio was additionally unusual in that the multiple-peak pattern was also obtained when the cells were grown without agitation; in every other case only a single form of $tRNA^{Phe}$ was obtained from anaerobically grown yeast.

α-GALACTOSIDASE IN *MUS MUSCULUS*

J. E. Tobler* and K. Bruce Jacobson

A mosaic genetic system in which one can induce cancer tissue and study its development is being sought. The project basically involves locating and characterizing a biochemically defined X-linked gene. Since only one X chromosome is active in somatic cells, this gene can then be used to tag the X chromosome and determine whether the carcinogenic event originated in a single cell or in many cells in heterozygous female mice. The system requires an X-linked gene that produces an enzyme highly active, histochemically discernable in most tissue, nonessential enough to allow mutant genotypes to exist, and conveniently screenable in mutagenized mice. Experimental results so far indicate that α-galactosidase may provide such a tool.

We find α-galactosidase is present in spleen, kidney, ovary, testes, liver, lung, small intestine, and brain tissue of the mouse. Although a complete developmental profile has not been done, enzyme activities have been observed in very young and very old animals. Electrophoretic studies show a slow and fast band on starch gels. These two forms of α-galactosidase show tissue specificity; some organs have the slow form, some the fast, and some have both. Heat-stability studies also show two forms of the enzyme. After 15–20 min at 55°C, one form is inactivated. The heat-labile form is the slow form on starch gels. Studies with crude extract show that $HgCl_2$, $FeCl_3$, *p*-nitrophenol, *p*-nitrosophenol, myoinositol, and D-galactose inhibit the enzyme(s) to various degrees. D-galactose, the most effective inhibitor, does so competitively. However, both electrophoretic forms of the isozyme seem to be inhibited equally. We are currently purifying the two α-galactosidases to study the interrelationships, the inhibitor sensitivities, and production of specific antibodies.

Other experiments in progress are designed to confirm X-linkage of α-galactosidase in two ways: (*a*) by determining inheritance patterns in electrophoretic variants if available, and (*b*) by quantitating α-galactosidase in eggs from X/O and X/X females. We have developed a simple α-galactosidase mutant screen with urine as the enzyme source; an intensive hunt for induced mutants will soon be under way.

Successful completion of the project will provide a genetic mosaic system to study neoplasm development and other basic developmental phenomena, such as cell lineage relationships and chromosomal differentiation. Another possible benefit will be a mouse model for

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1. J. B. Bell and K. B. Jacobson, *Biol. Div. Annu. Prog. Rep. June 30, 1974*. ORNL 4993, p. 19.

examining Fabry's disease. Such a model could lead to treatment and possible control of the disease.

*Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

SYNTHESIS AND DEGRADATION OF SPECIFIC tRNAs IN MAMMALIAN CELLS

Michael Litt*

In eukaryotic cells the levels of various tRNAs reflect the amino acid composition of the proteins being synthesized. We are trying to answer five questions about this phenomenon: When cells differentiate, how are specific tRNA levels adjusted? Is the adjustment made by varying the rate of synthesis or the rate of degradation of specific tRNAs? What characteristics of the intracellular environment influence rates of degradation and synthesis of specific tRNAs? Specifically, does the extent of aminoacylation of a tRNA species play a role? Is the rate of synthesis or degradation of a tRNA species influenced by the intracellular concentration of that species?

Using Friend-virus-infected mouse leukemia cells (FL cells) as a model system, I have examined the degree of heterogeneity of turnover rates of 4S RNAs resolved by RPC-5 chromatography. In exponentially growing FL cells, the most labile 4S RNAs turn over at least 1.75 times more rapidly than the most stable ones.

To measure rates of synthesis and degradation of specific tRNAs, I have been attempting to purify tRNAs by a one-step micromethod published by Klyde and Bernfield.[1] This method involves specific aminoacylation of a tRNA mixture with a single amino acid, followed by complex formation with EFTu-GTP and separation of the ternary complex of aatRNA-EFTu-GTP from uncharged tRNA on 10% acrylamide gels. The aminoacylation of tRNA by an amino acid other than the one added has been observed repeatedly and causes the production of complexes with unwanted tRNA. Apparently, proteases in the synthetase preparations generate unwanted amino acids. Sephadexing the synthetase preparations immediately before use reduces, but does not eliminate, this problem. I am presently trying to overcome this difficulty by purifying an aa-tRNA synthetase complex from reticulocytes, such as is described by Som and Hardesty.[2]

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AUTOMATED SEQUENTIAL DEGRADATION OF RNA

Mayo Uziel and A. J. Weinberger*

The automated apparatus for sequential removal of nucleoside residues from RNA is the first operating instrument of its kind.[1] Its major potential uses are twofold: the preparation of truncated RNA molecules for further research in the relationship of RNA structure to its function, and the sequencing of oligonucleotides larger than 10 to 15 residues.

We have tested the instrument with four different tRNA preparations and several different preparations of poly(A) (length ~80 or 1000 residues per chain). As indicated in the previous report,[1] the removal of nucleosides ceases when about one-fourth of the molecule is removed. This is primarily due to the peculiar structure of tRNA in this region of the molecule, not to the chemistry of nucleoside removal. The three-dimensional structure of tRNA (determined by A. Rich at MIT) is a special sandwich-like arrangement with hydrogen bonding between nonadjacent regions of the tRNA molecule; such a structure could stabilize the region to prevent further degradation. Poly(A) does not have this structure, and we have gone through more than 30 cycles without observing cessation of degradation such as is found in tRNA. In addition, the sequences in the four tRNAs are quite different in this region. The only common features (indicated below) are the presence of pseudouridine and cytidine.

E. coli	$tRNA^{Glu}_{1,2}$	T ψ C G A A
Wheat Germ	$tRNA^{Glu}$	U ψ C G mA U
E. coli	$tRNA^{fMet}$	T ψ C A A A
E. coli	$tRNA^{Phe}$	T ψ C G C C

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1. M. Uziel, A. J. Weinberger, and A. J. Bandy, *Biol. Div. Annu. Prog. Rep. June 30, 1974*, ORNL-4993, pp. 20–21.

ANALYSIS OF MODIFIED BASES IN tRNA FROM EUKARYOTE[1] AND PROKARYOTE SOURCES

Mayo Uziel, L. H. Smith, and A. Jeannine Bandy

The modified bases in RNA can be grouped into two categories: those involved in the recognition of messenger codons and those involved in tRNA structure. This

creates the possibility of translational control of protein synthesis through the degree of modification of the anticodon bases. Increasing evidence indicates that the levels of enzymes catalyzing these modifications differ in different cell types. The increase of methylase activity in tumor cells is of particular interest. The presence of the modified bases and the variable levels of modifying enzymes suggests that the level of these bases in the bulk tRNA may be characteristic for the individual cells. In a number of tRNA preparations, by use of our high resolution columns,[2] we find a wide range in the level and structure of these bases. We have chosen to compare levels by calculating the number of nanomoles present per A_{260} unit of RNA (~50 μg). Several observations are worth noting: under conditions where *E. coli* and yeast tRNAs are completely hydrolyzed enzymatically, mammalian tRNAs are not; repeated digestions do not alter the level of unhydrolyzed tRNA. This must reflect on the structures of the RNA present. In addition, this obviously puts limits on the interpretation of levels of modified bases. We are devising procedures to analyze the unhydrolyzed portion.

On comparison of the qualitative distribution of modified bases in the various tRNA preparations, we find (as others have) that the number and amount of these modified bases are greater in the more highly differentiated cells – *E. coli* and yeast tRNA have less modifications than the thymic liver and tumor tRNA. In addition, several qualitative differences are apparent between C57BL normal thymus and the radiation-induced thymic lymphoma[3] in the same animal. The base V appears to be absent (or very low) compared to the levels in the tumor. A comparison of the bases found in different strains of mice (C57BL and AKR) also shows qualitative differences.

These observations justify further examination of the modified bases to see if they can be considered as cell markers or as diagnostic for tumor formation.

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HIGH-RESOLUTION–HIGH-SPEED SEPARATION OF 35 MODIFIED NUCLEOSIDES FOUND IN RNA

Mayo Uziel and A. Jeannine Bandy

Our earlier systems for separating the modified nucleosides[1,2] have been considerably improved, and charge neutralization by pH changes and borate addition[3] has been introduced, for the first time, to increase the rate and sensitivity of the assays (Figs. 2A, B and Table 1). This system (col. X, refs. 1 and 3) allows detection of 20 to 60 pmoles of nucleoside; thus it is possible to detect one nucleoside out of 2000 to 5000 added to the column; quantitative analysis requires about 100 to 250 pmoles of nucleoside on the column. Column XII (refs. 1 and 3) is about three times more sensitive.

The modified nucleosides used in these experiments either were purchased, or were gifts, except for two that were synthesized: N^4-acetyl cytidine was prepared in a one-step reaction using acetic anhydride and cytidine in an ethanol solvent. The yield was 80% and the purity greater than 99%. Nuclear magnetic resonance (NMR) analysis and UV spectral data were consistent with the formation of N^4-acetyl cytidine. The second compound, 5-carboxymethyloxyuridine (Vm), was prepared by esterification of 5-oxycarbonylmethyloxyuridine (V) with methanol and a carbodiimide. The yield was 40%. Spectral data and chromatographic position characterized the product.

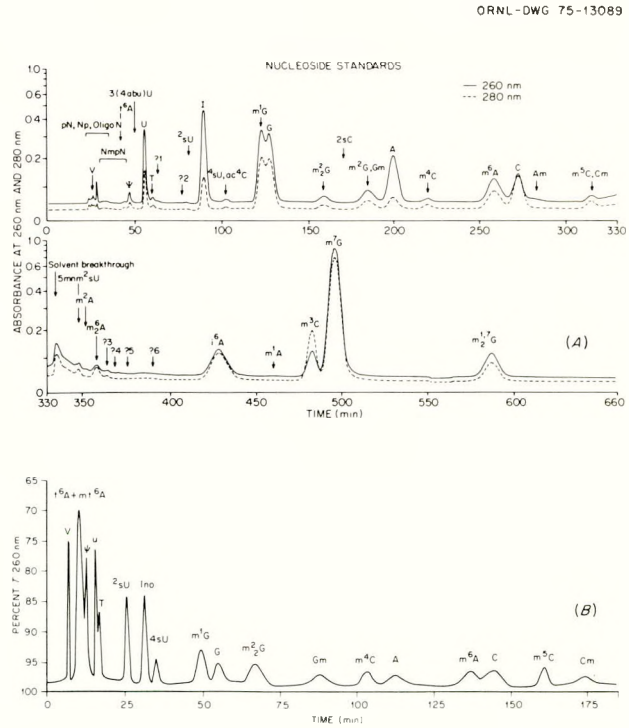

Fig. 2. (A) A group of standard nucleosides have been chromatographed with a solvent change near 300 min. The first solvent is from ref. 1, and the second solvent is 0.1 *M* borate, 0.8 *M* NH_4 formate (pH 7.0) and **(B) the speed of the analysis has been increased by use of a 9-μ resin.** The group of nucleosides separated by solvent 1 only are shown.

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Table 1. Column X positions for modified nucleosides

Time (min)	Substance	Time (min)	Substance
25	V	175	m^2G
29	$4(SO_3^-)U$	176	Gm
36	t^6A	200	A
39	mt^6A	220	m^4C
48	ψ	259	m^6A
50	$(4abu)^3U$	272	C
56	U	283	AM
		buffer change	
61	T	318	Cm
71	Vm	318	m^5C
75	X	346	mam^5s^2U
81	s^2U	350	m^2A
90	Ino	356	m_2^6A
103	s^4U	462	i^6A
104	ac^4C	520	m^1A
123	m^1G	547	m^3C
127	G	567	m^7G
159	m_2^2Guo	667	$m_2^{1,7}G$
		buffer change	
171	s^2C	760	ms^2i^6A

AFFINITY CHROMATOGRAPHY OF RNA DERIVATIVES OF DIHYDROXYBORYL COLUMNS

S. A. Taylor* and Mayo Uziel

The isolation of small quantities of purified tRNAs can be facilitated by the use of the boronate affinity column that selectively excludes charged tRNAs. Thus a group of isoacceptors may be collectively purified free of other uncharged tRNAs. These can subsequently be separated into the individual acceptors for sequence or other studies.

The boronate affinity columns are not as yet very well characterized. To establish their usefulness in isolation of thymic tRNA, we have prepared a packing material from a highly cross-linked polyacrylhydrazide substituted with phenylboronate. Preliminary tests with adenosine illustrate that the complex is fully reversible at all pH values from 7 to 10.5, and that the effective capacity (equilibrium binding of adenosine) is considerably less than 1% of the maximum theoretical binding.

The boronate content was 0.5 meq/ml of packed gel, and the room-temperature equilibrium association constants for adenosine ranged from 20 to 500 M^{-1}, depending upon the pH and solvent.

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A NOVEL OLIGORIBONUCLEASE OF *ESCHERICHIA COLI*. I. ISOLATION AND PROPERTIES

S. K. Niyogi, Brenda H. Underwood, and A. K. Datta*

A new ribonuclease has been isolated from *Escherichia coli*. The enzyme is present in the 100,000 *g* supernatant fraction and has been purified over 200-fold. Studies of the enzyme reveal that:

(*a*) The enzyme shows a marked preference for oligoribonucleotides; indeed, the reaction rate is inversely proportional to the chain length of the substrate. The enzyme does not attack polynucleotides even at high concentrations of enzyme and has no detectable DNase activity.

(*b*) The enzyme is stimulated strongly by Mn^{2+}, less strongly by Mg^{2+}, and not at all by Ca^{2+} and monovalent cations.

(*c*) The enzyme is purified free of RNase I, RNase II, RNase III, polynucleotide phosphorylase, and other known ribonucleases of *E. coli*. The enzyme displays identical properties when isolated from mutants of *E. coli* that are deficient in the above ribonucleases.

(*d*) The enzyme has a marked thermostability, a point of further distinction from RNase II.

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A NOVEL OLIGORIBONUCLEASE OF *ESCHERICHIA COLI*. II. MECHANISM OF ACTION

A. K. Datta* and S. K. Niyogi

Detailed studies of the mechanism of action of the novel oligoribonuclease of *Escherichia coli* described in the previous report[1] led to the following conclusions:

(*a*) The enzyme prefers a free 3′-hydroxyl group for its action.

(*b*) The enzyme attacks the oligoribonucleotide substrate in a sequential manner from the 3′-end producing 5′-ribonucleotides.

(*c*) The mode of attack appears to be processive: the enzyme acts by degrading one oligoribonucleotide chain to completion before proceeding to the hydrolysis of another chain.

(*d*) The reaction rate is inversely proportional to the chain length of the substrate; however, the enzyme has a higher affinity for longer chains.

(*e*) The enzyme activity is markedly inhibited by secondary structure; oligoribonucleotides combined with complementary polyribonucleotides are attacked poorly below the melting temperature of the complex and efficiently above the melting temperature.

(*f*) The enzyme is inhibited by 5′-nucleotides of adenine and guanine; those of cytosine and uracil have a much smaller effect. The enzyme is not inhibited by 3′-nucleotides.

(*g*) Studies with dinucleoside monophosphates show highest reaction rates with pyrimidine sequences in the order: CpC > UpU > CpU > UpC. The presence of guanine at the 3′-end is strongly inhibitory, reaction rates being CpG > UpG ≅ ApG > GpG.

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*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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PROMOTER REGION(S) OF SINGLE-STRANDED M13 DNA

S. K. Niyogi and Sankar Mitra

Certain specific regions in the genome play a crucial role in biological regulatory mechanisms. One such region, called the "promoter," is involved in the specific binding of RNA polymerase and subsequent accurate initiation of RNA synthesis. Previous work (Arthur Kornberg, Stanford University) suggests that there may be one unique site (promoter) on single-stranded M13 DNA that is involved in the synthesis of the RNA primer needed for initiation of DNA replication. We are attempting to isolate and characterize the promoter region(s) with respect to size, secondary structure, base composition and sequence, and location in the M13 genome. RNA polymerase of *E. coli* was allowed to bind to labeled single-stranded M13 DNA. The unbound and weakly bound polymerase molecules were removed by adding an excess of poly(I), which has a high affinity for the enzyme. After digestion of the unbound DNA regions with pancreatic DNase and snake venom phosphodiesterase, the protected DNA-RNA polymerase complexes were isolated by Sephadex G-200 column chromatography. The protected DNA sites were then isolated by phenol extraction and alcohol precipitation. Studies of the DNA recognition regions reveal: (*a*) Very little binding is observed in the absence of the sigma subunit – the protein factor in *E. Coli* RNA polymerase that dictates specific binding and accurate initiation of RNA synthesis. (*b*) The amount of protection varies from 1.0 to 1.8% of single-stranded M13 DNA. In the absence of poly(I), substantially higher protections are observed. (*c*) The protected regions display two size classes as determined by polyacrylamide gel electrophoresis (about 50 and 100 nucleotides in length). (*d*) The two peaks have somewhat different base compositions. (*e*) Hybridization experiments are in progress to determine whether these protected fragments are "unique" and whether they arise from the same region of the M13 genome.

THE ROLE OF *ESCHERICHIA COLI dnaA* FUNCTION AND ITS INTEGRATIVE SUPPRESSION IN M13 COLIPHAGE DNA SYNTHESIS

Sankar Mitra and D. R. Stallions

F^+ derivatives of *E. coli* E508 thermosensitive in *dnaA* locus (involved in DNA synthesis initiation function), its revertant, and an Hfr derivative of E508(ts) in which the temperature-sensitive phenotype is suppressed by integrative suppression have been compared for their ability to support M13 phage DNA synthesis at the nonpermissive temperature. Upon infection at nonpermissive temperature (42°C), both the revertant and the Hfr strain support normal phage replication, while the temperature-sensitive mutant does not. However, when infection is carried out at permissive temperature and the temperature is shifted up late after infection, phage synthesis occurs in the temperature-sensitive mutant also, although in lesser quantity than in the revertant strain. Ultracentrifugal analysis of intracellular labeled phage DNA indicates (*a*) parental replicative form (RF) synthesis is not dependent on *dnaA* function, (*b*) progeny RF synthesis is strongly inhibited in the temperature-sensitive *dnaA* mutant but not in the other two, (*c*) progeny single-stranded (SS) DNA synthesis does not absolutely

require *dnaA* function, and (*d*) progeny SS DNA is present in the circular form. These results are in contradiction to the earlier observations of Bouvier and Zinder, who suggested that *dnaA* function is involved in the circularization of progeny SS DNA. Our results also suggest that either (*a*) M13 RF, for replication, gets associated with the host chromosome, since during integrative suppression the initiator function of the integrated episome is believed to act in *cis*, or (*b*) phage RF replication requires the synthesis of certain host protein(s) whose synthesis itself is dependent on initiation of host DNA synthesis.

THE ROLE OF *ESCHERICHIA COLI dnaG* FUNCTION IN M13 COLIPHAGE DNA SYNTHESIS

Santanu Dasgupta* and Sankar Mitra

Escherichia coli dnaG function is known to be involved in the initiation of formation of Okazaki DNA fragments during DNA synthesis. Kornberg and his collaborators have shown that *dnaG* protein is a new RNA polymerase that synthesizes the primer RNA during replication of single-stranded (SS) DNA of coliphages ϕXI74 and G4 to the corresponding replicative forms (RF). It was also shown by workers in the laboratories of Kornberg and Hurwitz that *in vitro* systems for replication of M13 phage SS DNA to RF do not require *dnaG* protein.

Mitra and Stallions[1] have shown, using a temperature-sensitive *dnaG* mutant of *E. coli*, that *dnaG* function is required for M13 phage synthesis. In this report we summarize our results on the role of *dnaG* function in various stages of M13 DNA synthesis:

(*a*) In confirmation of the *in vitro* experiments, M13 parental RF synthesis does not require *dnaG* function.

(*b*) M13 progeny RF synthesis is strongly inhibited in the *dnaG* mutant at the nonpermissive temperature as compared with the synthesis at the permissive temperature or when compared with the synthesis in the temperature-resistant wild-type *E. coli*. Similar results were obtained both with wild-type phage, where progeny SS DNA synthesis occurs, and with M13 gene 5 mutant, where SS DNA synthesis is absent.

(*c*) M13 progeny SS DNA synthesis does not require *dnaG* function. The reduced synthesis of M13 DNA at 42°C in the mutant host is presumably due to inherent temperature sensitivity of the M13 SS DNA synthesis itself. The intracellular SS DNA synthesized at the nonpermissive temperature is circular, and the pulse label can be chased into mature extracellular phage at the nonpermissive temperature.

(*d*) Although there is no significant RF synthesis in the *dnaG* mutant at the restrictive temperature in the absence of SS DNA synthesis, there is a significant amount of label in the RF when SS DNA synthesis does occur at the nonpermissive temperature. Analysis of these RF molecules in alkaline CsCl equilibrium centrifugation suggests that these molecules arise from the progeny SS and not by the normal mechanism of RF replication.

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*Postdoctoral investigator supported by Subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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CHARACTERIZATION OF BACTERIOPHAGE-T5-INDUCED DNA POLYMERASE WITH DNA CONTAINING SINGLE-STRAND BREAKS

R. K. Fujimura, Barbara C. Roop, and D. P. Allison

Earlier studies by Steuart *et al.*[1] have shown that, like other bacteriophage-induced DNA polymerases, T5 DNA polymerase prefers the templates that are single-stranded with either 3′-OH ends looped back to form primers or oligonucleotides annealed to the denatured DNA to serve as primers. It was shown clearly with T4 polymerase that the polymerase by itself is not capable of synthesis from a single-strand break in a duplex molecule accompanied by strand displacement.[2]

Our studies with temperature-sensitive DNA polymerase induced by bacteriophage T5 revealed that DNA synthesis with nicked DNA as a template is more temperature stable than the synthesis with denatured DNA.[3] This observation induced us to investigate more rigorously the nature of DNA synthesis from single-strand breaks using wild-type T5 DNA polymerase. Bacteriophage-T5-induced DNA polymerase was characterized using mainly circular duplex DNA of bacteriophage PM2 with single-strand breaks formed by DNase I action. The purified polymerase preparation had synthetic activities with both denatured DNA and nicked DNA. In polyacrylamide gel electrophoresis of native protein, the polymerase activities with denatured DNA and nicked DNA superimposed on each other. In SDS gel electrophoresis there was only one band. Both polymerase activities were shown to be phage-induced. Thus both polymerase activities are very likely due to one phage-induced protein.

DNA synthesis with nicked DNA as a primer template increased with increase in number of single-strand

breaks. Most of the characterization was carried out with PM2 DNA with one or less than one single-strand break per strand. Essentially all such breaks were repairable by ligase. Alkaline sucrose gradient centrifugation showed that synthesis occurs with the strand with a single-strand break as a primer yielding DNA longer than one phage DNA unit long. Newly synthesized DNA is covalently linked to the primer strand. Thus the synthesis is occurring very likely by strand displacement. This is supported by electron micrographs.

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EXONUCLEASE ASSOCIATED WITH BACTERIOPHAGE-T5-INDUCED DNA POLYMERASE

S. K. Das* and R. K. Fujimura

Upon infection of its host *Escherichia coli,* bacteriophage T5 induces synthesis of a DNA polymerase. The DNA polymerase is presumably involved in T5 DNA replication. Studies with ts mutants, which map in the structural gene of this protein, indicate that this enzyme might be an essential component of phage replication machinery. This enzyme has been purified to homogeneity, in our laboratory, as indicated by the presence of a single band in native and SDS gel electrophoresis.

Other groups have shown that bacterial- and phage-induced polymerases also have exonuclease activity. We have found an exonuclease activity in the purified DNA polymerase preparations. The activity was shown to be associated with the same polypeptide which has the polymerase activity. The enzyme degrades both single- and double-stranded DNA. Single-stranded DNA is degraded at a faster rate. The enzyme attacks the DNA at the 3′-OH end and produces 5′-deoxynucleotides. The rate of degradation was found to depend on the number of available 3′-OH ends when excess enzyme was used. The pH optima of the enzyme coincide with the pH optima of the polymerase activity. The enzyme showed an essential requirement of Mg^{2+} for its activity. *N*-Ethylmaleimide inactivated the enzyme, which indicated the presence of an —SH group crucial for its activity. For optimal activity during all purification steps the presence of DTT was found to be desirable.

The exonuclease activity associated with the polymerases has been implicated in the editing function by various investigators. Therefore it was of interest to see what happens to the template and the newly synthesized strand under synthetic conditions. We followed the degradation of the newly synthesized strand by following template-dependent conversion of dNTP to dNMP. Under synthetic conditions, *i.e.,* in the presence of 4dNTP, the template was totally protected when nicked double-stranded DNA was used as the template. However, the newly synthesized strand was degraded, as indicated by dNMP production. These results indicate the T5-polymerase-associated exonuclease might be crucial for a faithful replication of T5 DNA.

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A DNA-BINDING PROTEIN FROM BACTERIOPHAGE-T5-INFECTED *ESCHERICHIA COLI*

S. K. Das* and R. K. Fujimura

Even in the case of simple bacteriophages, current evidence indicates that the replication might be a complex process involving many components. In the T4-infected *E. coli* system, gene 32 protein has been shown to stimulate the rate of DNA synthesis *in vitro*. A DNA-binding protein, which is presumably coded by T5, has been detected and purified from T5-infected *E. coli* cells. DNA-cellulose affinity column and DEAE-Sephadex columns were used in the purification procedure. Its effect on T5 DNA polymerase has been studied. Our preliminary observations indicate that, unlike the gene 32 protein, this DNA-binding protein inhibits DNA polymerase even at low concentrations. The inhibition was seen with nicked DNA as template. The inhibitory capacity of the protein was found to be very strong in that only microgram quantities were needed to produce 50% inhibition under our assay conditions.

This protein may have some role in recombination and probably protects the 3′-OH at the nicks and gaps on DNA template. Currently we are investigating a number of DNA synthesis mutants to learn more about the protein.

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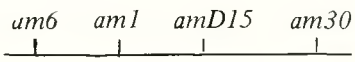
INTRACISTRONIC MAPPING OF BACTERIOPHAGE T5 DNA POLYMERASE MUTANTS

W. M. Ardrey* and R. K. Fujimura

Three amber mutants of structural gene for T5 DNA polymerase, designated *am1, am6,* and *am30,* were obtained from Y. Lanni. Two factor crosses were carried out between these and one additional mutant, designated *amD15* (obtained from McCorquodale), known to be adjacent to the polymerase gene but outside of the polymerase cistron. To our surprise, the map was



Thus the results indicated that *am30* is outside of the DNA polymerase cistron. To confirm these results, cell extracts obtained from amber-mutant-infected *su*$^-$ cells were analyzed for DNA polymerase activities. The results were consistent with the above results in that only *am6*- and *am1*-infected *su*$^-$ cell extracts did not have DNA polymerase activities; therefore these are the only mutants in the polymerase cistron.

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A MICROEVOLUTIONARY STUDY ON THE ORIGIN OF CHLOROPLASTIC AMINOACYL-tRNA SYNTHETASES

J. G. Farrelly, L. I. Hecker, and W. E. Barnett

We are continuing our efforts to elucidate the evolutionary origin of chloroplasts. Two theories exist concerning their origin: (*a*) that chloroplasts evolved from a prokaryotic endosymbiont such as a blue-green alga and (*b*) that organelles simply arose from subcellular compartmentalization. We are approaching this problem in two ways: The first involves the use of an amino acid sequenator to compare the sequence of the first 50–60 amino acids of a typical chloroplastic tRNA synthetase (Phe-tRNA synthetase) with that of the Phe-tRNA synthetase from the cytoplasm of the same organism (*Euglena*) and with the synthetase purified from a blue-green alga. The second approach involves a comparison of the degree of homology of different immunological domains (such as the ATP binding site, the tRNA binding site) within the three enzymes. Both approaches require that a substantial quantity of all three enzymes be purified to homogeneity.

We have recently applied three techniques which are new to this laboratory toward the purification of these three enzymes: preparative acrylamide electrophoresis, acrylamide-agarose gel filtration, and affinity chromatography. The first two have been incorporated into our scheme for purification of the *Euglena* chloroplastic enzyme. Using these methods in conjunction with hydroxyapatite and DEAE chromatography, small-scale preparations indicate that homogeneous enzyme will be available soon. We are presently purifying a large preparation of this enzyme. Since the *Euglena* cytoplasmic enzyme is somewhat unstable, we are using affinity chromatography for its purification. This entails binding the enzyme to a Sepharose column to which $tRNA^{Phe}$ has been attached, followed by elution and rechromatography through a Sepharose column to which all the other tRNAs have been attached. These studies are now in progress. We have also prepared the enzyme from a blue-green alga, but at present it is not homogeneous. We are now attempting to purify this enzyme to homogeneity using the above techniques.

PURIFICATION AND NUCLEOTIDE COMPOSITION OF PHENYLALANYL-tRNAs FROM THE CHLOROPLASTS AND CYTOPLASM OF *EUGLENA*

L. I. Hecker, Mayo Uziel, Arlee P. Teasley, and W. E. Barnett

Euglena gracilis var. *bacillarius* contain two sets of tRNAs; the cytoplasmic tRNAs are constitutive, while the chloroplast tRNAs are light-inducible, are present only at very low levels in dark-grown cells, and are absent from cells containing no chloroplast DNA. To investigate any possible evolutionary relationship between organelle and cytoplasmic tRNAs, Phe-tRNAs from chloroplasts and cytoplasm have been purified and analyzed for minor components. Both species of Phe-tRNA were purified by a combination of BD-cellulose and RPC-5 column chromatography. tRNAs were enzymatically digested and applied to a cation-exchange column for nucleotide analysis. Cytoplasmic Phe-tRNA contains many minor bases, including base Y, and its general composition closely resembles those of Phe-tRNAs from baker's yeast and wheat. Chloroplastic Phe-tRNA, with a composition different from cytoplasmic Phe-tRNA, more closely resembles *E. coli* Phe-tRNA. Chloroplastic Phe-tRNA has few minor

bases but does have ms^2-i^6-A (2-methylthio-N^6-isopentyladenosine) and an unknown base (which is different from the X base found in *E. coli* Phe-tRNA).

THE TRANSCRIPTIONAL ORIGIN OF *EUGLENA* CHLOROPLAST tRNA

S. D. Schwartzbach,* L. I. Hecker,
Arlee P. Teasley, and W. E. Barnett

Chloroplast (Chl) tRNA has been iodinated *in vitro* and hybridized to Chl DNA. At 70°C in 2 × SSC, total Chl tRNA hybridizes to approximately 1.0% of the Chl DNA, while purified Chl Phe-tRNA hybridizes to approximately 0.15% of the Chl DNA. The T_ms of the hybrids are 62 and 63°C, respectively, in 0.1 × SSC. From the known base composition of Chl Phe-tRNA, it can be estimated that less than 10% of the bases in the hybrids are mismatched. BD-cellulose chromatography of Chl tRNAs indicates that most amino acids are accepted by a single tRNA species. Thus it is concluded that the Chl genome codes for 40–41 tRNA species, *i.e.*, two structural genes for each amino acid. Hybridization experiments with purified chloroplastic Phe-tRNA do indeed show two structural genes for this species. Total tRNA isolated from light-grown cells competes with Chl tRNA to virtually the same extent in the hybridization reaction as total Chl tRNA, while total tRNA from dark-grown cells is not an effective competitor. Thus the data probably indicate that the light-induced appearance of Chl tRNA represents *de novo* synthesis rather than posttranscriptional modification of precursor tRNA and that the structural genes for chloroplastic tRNAs reside within the chloroplastic genome.

*American Cancer Society Postdoctoral Fellow (Fellowship PF-1055).

SEQUENCE HOMOLOGY STUDIES WITH ORGANELLE AND CYTOPLASMIC tRNAs

L. I. Hecker, S. D. Schwartzbach,*
Arlee P. Teasley, and W. E. Barnett

It has been postulated that mitochondria and chloroplasts evolved from free-living prokaryotes which formed endosymbiotic relationships with ancestral (proto)eukaryotic cells. As an experimental approach to testing this hypothesis, we are comparing the primary sequences of tRNAs from the organelles and cytoplasms of several organisms. Previously we had purified the Phe-tRNAs from the cytoplasm and chloroplasts of *Euglena*, and more recently the Phe-tRNAs from the cytoplasm and mitochondria of *Neurospora crassa* have been purified. These tRNAs are being sequenced in collaboration with Dr. U. Rajbhandary of MIT; the sequences will then be compared with each other and with known sequences for Phe-tRNAs from various prokaryotes and eukaryotes.

Neurospora crassa contains four species of Met-tRNA. We have purified the formylatable species from both the mitochondria and cytoplasm. These are also being sequenced in collaboration with Dr. Rajbhandary. Preliminary fingerprints indicate a marked similarity between the cytoplasmic Met-tRNA species from *Neurospora* and other eukaryotic organisms.

These experiments represent the first purification of organelle tRNAs, and sequence homology comparisons between these molecules, tRNAs from prokaryotes, and the eukaryotic cytoplasmic tRNAs should provide considerable insight into the evolutionary origin of organelle tRNA structural genes.

*American Cancer Society Postdoctoral Fellow (Fellowship PF-1055).

THE DIFFERENTIAL EXTRACTION AND CHARACTERIZATION OF NEWLY SYNTHESIZED HETEROGENEOUS NUCLEAR RNA FROM HUMAN NEOPLASTIC LYMPHOCYTES

J. W. Bynum,* M. Helen Jones, and Elliot Volkin

Human neoplastic lymphocytes labeled with [^{3}H]uridine contain three RNA fractions (A, B, and C) which are differentially extracted. The soluble and nuclear fractions isolated from RPMI-4265 human lymphoblastoid cells labeled for 1 and 24 hr revealed that fractions B and C were mainly associated with the nucleus and were similar to comparable fractions isolated from the organelles. The heterogeneity indicated that fractions B and C constituted nuclear heterogeneous RNA and possibly cytoplasmic messenger RNA. Fraction A from subcellular sources contained stable cytoplasmic RNA species such as 28, 18, 5, and 4S RNA and their precursors. All three RNA fractions were further evaluated by pulse-labeling RPMI-8226 human myeloma cells for 1 hr and analyzing the RNA at various periods during a 6-hr chase. Fraction A constituted most of the extractable RNA and contained stable rRNA and tRNA species in addition to rRNA precursors; fraction B was heterogeneous with no major low-molecular-weight com-

ponent; fraction C was heterogeneous with a major peak in the 4S region. Nucleotide analysis suggested that fractions B and C contained newly synthesized RNA.

Poly(U) Sepharose chromatography of the three fractions showed that fractions B and C contained a more stable percentage of poly(A)-containing RNA than did fraction A. The elution profiles showed that high-molecular-weight poly(A)-containing RNA with long poly(A) regions was processed in fraction A 2 hr before it was processed in fractions B and C.

*Postdoctoral investigator supported by PHS Research in Aging Training Grant, HD 00296 from the NICHHD.

THE SYNTHESIS OF THREE CLASSES OF POLY(A)-CONTAINING RNA IN HUMAN MYELOMA CELLS

J. W. Bynum* and Elliot Volkin

[^{3}H] Uridine-labeled RNA was extracted from human myeloma cells (RPMI-8226), applied to a poly(U) Sepharose column in 0.8 *M* KCl, then washed with 20 volumes of 1 *M* KCl. Stepwise gradient elution of the bound RNA revealed three distinct peaks of poly(A)-containing RNA. Peak I was eluted with 0.5 *M* KCl and presumably has the shortest poly(A) segment; peak II was eluted with 0.25 *M* KCl; peak III was eluted with 0.1 *M* KCl and had the longest poly(A) segment. Further reduction of the KCl concentration resulted in no additional peaks. Human myeloma cells were labeled for 1 hr with [^{3}H] uridine and chased for 6 hr in fresh medium. At the beginning of the chase, 1.7% of the RNA applied to the column was eluted in peak I, 5.4% in peak II, and 3% in peak III. In 6 hr these values decreased to 0.96% in peak I, 1.7% in peak II, and 0.85% in peak III. Intermediate time points showed that peak III decreased at a faster rate than peaks I and II. Electrophoresis indicated that the decrease in peak III coincided with the disappearance of high-molecular-weight heterogeneous RNA. After 24-hr labeling, peak III was found in the chromatin subcellular fraction and not in the polysomes. This suggested that peak III was newly synthesized poly(A)-containing RNA and possibly mRNA precursor.

*Postdoctoral investigator supported by PHS Research in Aging Training Grant HD 00296 from the NICHHD.

RNA SYNTHESIS AND PROCESSING IN NEOPLASMA OF SLOW AND NONDIVIDING CELLS

J. W. Bynum,* Elliot Volkin, and James D. Regan

Under normal physiological conditions, neurons and lymphocytes possess an unusual longevity and can exist for prolonged periods in an intermitotic state. In the intermitotic state the lymphocyte regulates its ribosome content by selectively degrading newly synthesized 18S rRNA. Consequently, in the lymphocyte, RNA processing can be related to proliferation. Earlier studies from this laboratory showed that neoplastic lymphocytes retain some of the regulatory traits of the untransformed cell. Cell lines with different generation times were used to evaluate the relationship between RNA processing and proliferation. The mouse neuroblastoma C-1300 has an average doubling time of 33 hr; the human myeloma RPMI-8226, 24 hr; and HeLa HCAAT, 17 hr. When grown in suspension, the mouse neuroblastoma maintains an undifferentiated state, but when grown in monolayer, the cells extend neurites and assume the morphology of the differentiated neuron.

The different cell lines were pulsed for 1 hr with [^{3}H] uridine, then transferred to fresh medium having unlabeled uridine and chased for 6 hr. Aliquots of cells were removed after a 15-min labeling and after chase periods of 0, 1, 3, and 6 hr. Chromatin and extra-chromatin RNA were differentially extracted from whole cells. In RPMI-8226 and HeLa cells, the labeling of extra-chromatin RNA as measured by specific activity leveled off after a 3-hr chase, while in the neuroblastoma labeling was still increasing at 6 hr into the chase. The percentage of labeled chromatin RNA in RPMI-8226 reached a maximum of 10% at 0 hr, then leveled off. HeLa cells showed maximum labeling of 16% after 15 min, and then labeling declined steeply to 10% by 3 hr. Neuroblastoma cells resembled RPMI-8226 in that labeling of the chromatin RNA fraction reached a maximum early in the chase and then leveled off. However, the differentiated neuroblastoma had a two- to threefold higher percentage of label in the chromatin RNA than did the undifferentiated neuroblastoma.

After the pulse, the processing of 45S rRNA precursor into 28S and 18S rRNA was followed by agarose-acrylamide gel electrophoresis. Cells having the largest percentage of rRNA precursor after 6 hr were as follows: HeLa > RPMI-8226 > undifferentiated neuroblastoma > differentiated neuroblastoma. This order is in direct agreement with the average doubling time of each cell line. Poly(U) Sepharose chromatography indicated that the percentage of poly(A) RNA in the

extra-chromatin and chromatin RNA fractions decreased during the chase, but in RPMI-8226 the percentage of poly(A) RNA in the chromatin fraction remained constant. This cell line synthesizes and secretes lambda light chain into the medium.

*Postdoctoral investigator supported by PHS Research in Aging Training Grant HD 00296 from the NICHHD.

SEPARATION OF NUCLEIC ACID COMPONENTS ON POLYACRYLAMIDE GEL COLUMNS

J. X. Khym

The exclusion property of the polyacrylamide gel Bio-Gel P-2 for low-molecular-weight anionic species allows separations of nucleic acid components to be effected in dilute alkaline borate buffer at pH 8.8. The degree of ionization, determined by pH, fixes the elution position of the compounds chromatographed in this system. Optimum conditions have been developed for making group separations as well as many other useful separations of nucleic acid components and related compounds.

AN ANALYTICAL SYSTEM FOR THE RAPID SEPARATION OF TISSUE NUCLEOTIDES AT LOW PRESSURES ON CONVENTIONAL ANION EXCHANGERS

J. X. Khym

An analytical anion-exchange procedure has been developed for the rapid separation of acid-soluble nucleotides (the so-called "free" or tissue nucleotides). It permits assay at low pressures (40–60 psi) in less than 1 hr on 10-cm columns of Aminex resins (conventional styrene-type anion exchangers) with alkaline citrate solutions as the eluent. Separation parameters have been investigated to determine optimum conditions for the routine analysis of samples containing tissue nucleotides. A simple solvent extraction procedure has also been developed to remove $HClO_4$ or CCl_3CO_2H quantitatively from cell extracts containing acid-soluble nucleotides. This procedure involves the removal of aqueous acid solutions with a water-insoluble amine dissolved in a water-immiscible solvent.

EVALUATIONS OF MAMMALIAN RIBONUCLEOTIDE POOLS BY COMBINED POLYACRYLAMIDE AND ANION-EXCHANGE CHROMATOGRAPHY

J. X. Khym, Elliot Volkin, and J. W. Bynum*

Interference from co-eluting nonnucleotide components has been largely ignored in the newer chromatographic systems devised for the rapid quantitative determination of acid-soluble pool components. In our studies involving the metabolic functions of free nucleotides in mammalian cells, we have found that certain less-common nucleoside phosphates and nonnucleotide components such as the nucleosides and bases co-elute with nucleotides such as AMP, GMP, CDP, ADP, etc., in our analytical anion-chromatographic procedure. This type of interference was discovered by the rigorous comparison of not only the retention time but also the width of solute bands in unknown mixtures with the values for authentic compounds chromatographed under identical conditions. To alleviate this interference, we carry out a prior fractionation of acid-soluble components via chromatography on polyacrylamide gel columns. This preliminary step allows nucleotides to be separated as a group from bases and nucleosides in about 10 min and thus does not impede rapid determinations of acid-soluble nucleotide by ensuing anion-exchange chromatographic procedures. This combined chromatography is now being used for quantitative evaluation of mammalian ribonucleotide pools.

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CHARACTERIZATION OF CHROMOSOMAL PROTEINS

C. G. Mead and E. A. Hiss

Chromosomal proteins have been isolated and purified from Chinese hamster ovarian tissue culture cells blocked at metaphase with colcimid. The isolated proteins were found to be in such small quantities that it was necessary to label them with ^{125}I in order to demonstrate different classes of separable proteins. Gradient sievorptive chromatography has been successfully used to separate the chromosomal proteins from each other and from the chromosomal nucleic acids.

Proteins from unlabeled chromosomal preparations have been separated and assayed for DNA-exonuclease and DNA-endonuclease activity in the presence of either Mg^{2+} or Ca^{2+}. No nuclease activity in these separated protein fractions has yet been detected. Other DNA-involved enzymatic activities are being examined. Some effort has been spent on increasing the amount of metaphase cells used as starting material and the yield of metaphase chromosomes from these cells, but further improvements are needed.

CROSS-REACTION OF THIOREDOXIN REDUCTASE AND RIBONUCLEOTIDE REDUCTASE IN *ESCHERICHIA COLI* AND *EUGLENA GRACILIS*

R. G. Holt* and F. D. Hamilton

Thioredoxin reductase and thioredoxin, proteins that couple NADPH oxidation to ribonucleotide reduction by ribonucleotide, have been identified in a number of prokaryotic organisms. The thioredoxin reductase described for *Euglena*[1] is the first such system reported for a eukaryotic cell. To determine if the prokaryotic and eukaryotic systems would function together, the *Euglena* thioredoxin reductase system was tested with *E. coli* ribonucleotide reductase. Tests were also made of the reaction of the *Euglena* ribonucleotide reductase with partially purified *E. coli* thioredoxin and thioredoxin reductase. In both of the heterologous reactions we observed 45–73% of the activity obtained with the homologous system.

*Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

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THE RIBONUCLEOTIDE REDUCTASE SYSTEM IN *EUGLENA GRACILIS*

S. Munavalli,* Dorothea V. Parker, and F. D. Hamilton

Studies have shown that *Euglena* contain a protein system which can utilize the reducing power of NADPH in the ribonucleotide-reductase-catalyzed reduction of CTP. The proteins required for this reaction are thioredoxin reductase, a flavoprotein of approximately 185,000 mol. wt, and a second protein (Protein I) whose function in the reaction sequence is unknown. This new protein has a molecular weight of 240,000 daltons, contains two subunits, and does not appear to contain a prosthetic group. In addition, our studies have shown that the ribonucleotide reductase that is active in the *Euglena* NADPH-thioredoxin reductase system is more complex than the protein reported in a previous publication.[1] The more complex enzyme contains four different types of polypeptide chains.

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ISOLATION OF RIBONUCLEOTIDE REDUCTASE IN *PSEUDOMONAS STUTZERI*

B. D. Mehrotra* and F. D. Hamilton

P. stutzeri contains a ribonucleoside triphosphate reductase which requires added dithiol and adenosylcobalamin for activity. The enzyme has been purified 40-fold by acid pH precipitation, ammonium sulfate fractionation, and Biogel chromatography. CTP reduction is activated only by dATP. CTP is not reduced to a significant extent if other dNTPs are used. No activity has been obtained with ATP, GTP, or UTP as substrates. We have not determined if the lack of activity with these triphosphates results from the lack of an essential ingredient for the assay of the enzyme or the need of another enzyme for these compounds.

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STUDIES ON THE FUNCTION OF ASCORBATE SULFATE

F. J. Finamore and Rose P. Feldman

The natural occurrence of a sulfated derivative of vitamin C was first demonstrated by this laboratory in extracts of *Artemia salina* embryos; more recently it has been identified as a metabolite in rats, guinea pigs, fish, rabbits, and man. At present we are actively engaged in studies of the function of this compound and its relation to vitamin C.

To determine whether ascorbate sulfate is a storage form of vitamin C, we have embarked on a search for the existence of an ascorbate sulfohydrolase that has the ability to convert the sulfated derivative to the active vitamin. We have succeeded in isolating and

partially purifying an enzyme with this property from extracts of digestive glands of *Helix pomatia.* The purification procedure involves gel filtration, DEAE-cellulose column chromatography, and isoelectric focusing. The enzyme exhibits arylsulfatase activity when *p*-nitrocatechol sulfate is used as substrate, and so far we have been unable to separate this activity from ascorbate sulfohydrolase activity. We have observed that upon isoelectric focusing the enzyme shows considerable heterogeneity, suggesting the presence of isozymes. Occasionally during focusing, aggregation of the molecules occurs, resulting in the formation of two major peaks of activity: one that focuses with an isoelectric point between pH 3.9 and 4.5 and another that focuses between pH 6.2 and 6.9. The latter activity can readily be converted to the former by dialysis and refocusing.

The enzyme has a molecular weight of 80,000, and the abscorbate sulfohydrolase activity shows a pH optimum of 4.0 and a K_m of 9.2 m*M* and is inhibited by SO_4^{2-}, SO_3^{2-}, PO_4^{3-}, and F^-. The arylsulfatase activity, using 4-nitrocatechol sulfate as substrate, has a pH optimum of 7.5 and a K_m of 3.2 m*M* and is inhibited by SO_3^{2-} but not by ascorbate sulfate. We believe that both activities reside on the same protein (a Type I arylsulfatase), but there are two active sites: one for the hydrolysis of nitrocatechol sulfate, and the other for the hydrolysis of ascorbate sulfate.

Another aspect of our work involves a comparison of the efficacy of ascorbate sulfate and ascorbic acid in preventing atherosclerosis in rabbits. We previously observed[1] that, under oxidative conditions, ascorbate sulfate transfers its sulfate moiety to suitable acceptor molecules such as *p*-nitrophenol. Furthermore, there is suggestive evidence[2] that, *in vivo,* ascorbate sulfate may directly or indirectly transfer its sulfate to cholesterol to form cholesterol sulfate. These observations along with those indicating ascorbic acid is effective in preventing hypercholesterolemia[3,4] prompted us to investigate the possibility that ascorbate sulfate is an effective agent in the prevention of atherosclerosis. Our results indicate that neither ascorbic acid nor ascorbate sulfate prevents hypercholesterolemia in rabbits maintained on a high-cholesterol diet. However, these compounds do appear to be effective in minimizing plaque formation in the aorta. At the present time we are in the process of gathering other biochemical, histochemical, and pathological data in this experiment.

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TRANSLATIONAL CONTROL OF PROTEIN SYNTHESIS IN BRINE SHRIMP EMBRYOS

A. H. Warner*

Encysted embryos of the brine shrimp, *Artemia salina,* contain an inhibitor of protein synthesis that appears to be important in translational control. The inhibitor has been isolated from the postribosomal fraction of embryo homogenates and purified about 40-fold by chromatography on DEAE-cellulose, hydroxyapatite, and G-150 Sephadex. The inhibitor is both heat labile and trypsin sensitive and appears to be a protein of molecular weight of about 100,000. The inhibitor has chromatographic properties very similar to elongation factor G (EF-2), but it doesn't promote the binding of GTP to 80S ribosomes or catalyze peptide bond formation in the presence of elongation factor T (EF-1). In a cell-free protein-synthesizing system derived from brine shrimp embryos and using poly(U) as messenger, the inhibitor appears to function at an early step in the elongation process. Preliminary data indicate that the inhibitor effects the binding of GTP and aa-tRNA by elongation factor EF-1 and, therefore, inhibits formation of the ternary complex, 80S ribosome · poly(U) · aa-tRNA, required for chain elongation to begin. Work is continuing to further characterize the mode of action of the inhibitor in the translational process and to elucidate its relationship, if any, with elongation factor EF-2.

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LIPID REGULATION OF CELL FUNCTION

Peter Pfuderer

We are finding increasing evidence that there are lipids which function as potent hormone-like regulators in vetebrate systems, but which are not prostaglandins or steroids. We have been studying three closely related lipid regulating factors. The first was isolated from crowded goldfish.

When fish are overcrowded, as a survival mechanism they secrete lipids which depress their growth, reproduction, and heart rate and increase their mortality.[1] We have used a heart rate depression assay to isolate the lipid responsible for crowding in goldfish, and found it to have the chromatographic behavior of a diglyceride and the spectrum of a conjugated trienoic fatty acid.[2] It exerted its effect through the central nervous system[1] and was found in brain and spinal cord tissue as well as in the fish water.

We have also isolated an analogue to the heart rate depressor from commercial tung seed oil. Tung seed oil contains one of the few natural conjugated trienoic oils. This analogue is physiologically active and is chromatographically similar to and has a similar spectrum to the goldfish factor. This active analogue is a minor constituent of the tung oil preparation, probably an oxidation product, and appears to rearrange on prolonged contact with protonating solvents such as ethanol. One of the rearranged products is a potent growth inhibitor to *Xenopus laevis* tadpoles, which led us to investigate crowding in *Xenopus*.

We have found that *Xenopus* tadpoles also secrete a lipid when crowded, which inhibits their growth. This *Xenopus* growth assay is easy and convenient and is currently being used to isolate the active analogue from tung oil.

Oxidized tung oil has been used by several workers in the past to treat patients with renal hypertension, because of its similarity to a natural lipid antihypertension agent (ANRL) secreted by the mammalian kidney. The active antihypertension fraction isolated by us from rat kidney and assayed on a renally hypertense rat showed activity separating on the trailing edge of the triglyceride peak from a silica gel column. Again, this fraction had the UV spectrum of a conjugated trienoic fatty acid and also gave a positive thiobarbituric acid test.

It appears that lipids containing these unusual conjugated fatty acids are present in mammalian tissues and may be physiologically active in mammals. A novel lipoxidase has recently been found in human blood platelets capable of forming conjugated fatty acids from methylene-interrupted unsaturated fatty acids through an endoperoxide compound.[3]

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SECTION II

BIOPHYSICS AND CELL PHYSIOLOGY

Peter Mazur

Cell Physiology

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J. F. Brandts[a]
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Nicholas Rigopoulos
Hiroshi Souzu[b]
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Chromosome Chemistry

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Marilyn B. Senior[c]

Growth and Regeneration

D. M. Skinner
Kiah Edwards III[c]
P. F. Sanders[c]
L. H. Yamaoka[c]

Microbial Radiation Biology and Cell Division

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H. I. Adler
D. P. Allison
D. R. Stallions

Radiation Biophysics

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X-Ray Diffraction

J. R. Einstein
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Protein Chemistry

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Excited-State Biophysics

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Photosynthesis and Solid-State Biophysics

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MICROSCOPIC OBSERVATION OF INTRACELLULAR ICE FORMATION IN MOUSE OVA AS A FUNCTION OF COOLING RATE

S. P. Leibo,* J. J. McGrath,† and E. G. Cravalho†

A physical-chemical analysis of water loss from cells at subzero temperatures showed that the likelihood of intracellular ice formation increased with increasing cooling rate.[1] We have now used a modified version of a unique conduction-cooled cryomicroscope stage[2] to observe the freezing of mouse ova suspended in dimethyl sulfoxide (DMSO). Previously published data[3] showed that the respective survivals of mouse ova were about 50, 40, 25, and 0% when they were cooled at rates of 0.3, 0.6, 2, and 6°C/min. Direct microscopic observation of mouse ova during freezing has now shown that the respective fractions of cells that froze intracellularly were about 12, 50, 72, and 100% when they were cooled at rates of 1.3, 2.0, 3.0, and 5°C/min or faster. These values agree approximately with those predicted from the physical-chemical analysis for cells the size of mouse ova. The microscopic observations have also shown that intracellular freezing occurred at about −40 to −45°C. We had previously observed that mouse embryos must be cooled slowly to −50°C or below if they are to survive subsequent rapid cooling to −196°C.[4] The observation of intracellular ice formation at −45°C supports the interpretation that at temperatures above −50°C the embryos still contain water capable of freezing intracellularly.

*Work performed while Visiting Scientist at the Harvard-MIT Program in Health Sciences.

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PERMEABILITY OF MOUSE EMBRYOS TO GLYCEROL

Peter Mazur, S. P. Leibo, and Nicholas Rigopoulos

Preliminary evidence indicates that glycerol and DMSO can protect mouse embryos from freezing damage without permeating them prior to freezing. On the other hand, there is evidence from rabbit embryos that survivals are superior if protective additives are allowed to permeate.[1] However, when permeation occurs, it can cause osmotic problems during thawing and subsequent dilution. Furthermore, injury during freezing appears in some instances to be related to the volume of cells prior to freezing.[2]

An understanding of the contributions of these various factors can be greatly facilitated by knowing the permeability coefficients of the solute in question and the temperature coefficient of the permeability coefficient. One can, for example, use the permeability coefficient to estimate the intracellular concentration of the permeating solute and the volume of the cell as a function of time.

Methods have been developed[3] to estimate permeability coefficients by determining the volume of a cell or embryo vs time in a permeating additive. We have begun to apply these methods to early mouse embryos (two-cell to blastocyst) suspended in 1 *M* glycerol at 2 and 22°C. By measuring the cross-sectional area of the photographed embryos, and using the simplifying assumption that the embryo is a sphere, one can calculate the embryos' volume as a function of time in glycerol. The preliminary results show that, at a given temperature, the rate of glycerol entry as evidenced by the return to original volume increases with increasing embryo stage. For example, the blastocyst recovers its original volume in about 5 min at 22°C whereas a two-cell embryo requires about 120 min. At 2°C, the results are similar except that the rate of entry is somewhat slower.

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SURVIVAL OF FROZEN-THAWED MOUSE EMBRYOS AS A FUNCTION OF GLYCEROL PERMEATION

S. P. Leibo

The protective effect of glycerol and dimethyl sulfoxide has been ascribed to their ability to reduce the electrolyte concentration at any given temperature during the freezing of complex solutions. A keystone of this explanation is the assumption that these compounds must permeate cells to protect them, for if they did not, they would be unable to reduce the *intra-*

cellular electrolyte concentration during freezing. This assumption has been tested on mouse embryos using glycerol. The approach is based on the fact that glycerol permeation is time- and temperature-dependent. Several observations indicate that permeation of glycerol is not required for protection. (*a*) About 75% of eight-cell embryos survive freezing after only a 20-sec exposure to glycerol at 0°C prior to freezing, the same percentage that survives after a 60-min exposure. (*b*) A 20-sec exposure to glycerol prior to freezing confers the same protection whether the embryos are exposed at 0, 10, 20, or 37°C. (*c*) A 20-sec exposure to 0.5 *M* glycerol at 0°C confers almost the same protection as 1.0, 2.0, or even 4.0 *M* glycerol. We have also found that embryos exposed to glycerol for varying times prior to freezing exhibit a dramatic but transient decrease in survival, in a fashion analogous to that displayed by erythrocytes.[1]

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PHYSIOLOGY AND LOW-TEMPERATURE BIOLOGY OF BOVINE EMBRYOS

Peter Mazur, S. P. Leibo, B. H. Erickson,* and J. C. Daniel, Jr.†

To be agriculturally significant, the procedure of superovulation and transfer of bovine embryos requires the development of five techniques: (*a*) endocrinological techniques for superovulation, (*b*) techniques for the recovery of embryos, (*c*) *in vitro* techniques for determining the viability of treated embryos, (*d*) freezing procedures that will yield a high proportion of embryos that are viable on the basis of the *in vitro* assay, and (*e*) surgical techniques for reimplanting embryos into foster mothers. The weak link in the sequence is the inability so far to perform step (*d*), the freezing.

We have developed competence in steps *a*, *b*, and *c*. As an example, the normal cow ovulates a single egg per reproductive cycle and produces about six calves in her lifetime. We have succeeded in developing reliable procedures for the superovulation and collection of embryos, obtaining an average of 15, and as many as 40, fertilized ova from one cow at a single estrous cycle. We have also modified two published procedures to assay the viability of treated bovine embryos by *in vitro* culture and by fluorescence. The culture procedure is usable for embryos between the two- and eight-cell stage, but promotes cleavage of only about 50% of one-cell ova, and supports only very limited development beyond the eight-cell stage. Because of the limitations of the culture system, we have also begun to use a fluorescence assay of viability, developed first for use with tissue-culture cells. We have shown that bovine embryos will take up nonfluorescent fluorescein diacetate and convert it to the fluorescent form, fluorescein. Preliminary evidence indicates that one can distinguish between viable and damaged or dead embryos on the basis of their fluorescence. Preliminary attempts to freeze bovine embryos using procedures that we had developed to freeze mouse embryos have succeeded only to a very limited extent – 6 out of approximately 160 frozen and thawed embryos were viable on the basis of the *in vitro* culture assay.

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EFFECTS OF SUBZERO TEMPERATURES ON EGGS AND EMBRYOS OF *ARBACIA PUNCTULATA*

Suzanne C. Jackowski* and R. A. Wallace

There is increasing evidence that a physical-chemical model[1] of the response of a cell to freezing is both qualitatively *and* quantitatively correct. For example, it has been shown that several cell types freeze intracellularly at those cooling rates predicted by the model.[2–4] Since the original model included an analysis of the response of sea urchin eggs to freezing, we have begun an experimental test of the model with these cells. Eggs and early embryos of *Arbacia punctulata* were exposed to subzero temperatures and assayed for normal development by examination under the light microscope. Samples were suspended in seawater and cooled to −5.8°C, then seeded with ice. Ten minutes later samples were cooled to temperatures ranging from −10.5 to −27.0°C at a rate of 0.7°C/min and were immediately warmed at a rate of 2.1°C/min. Survival was assayed by fertilizing the eggs and culturing them as well as the treated embryos in seawater at 22°C. The fertilized eggs were scored for viability at the four-cell stage, and the embryos at the blastula stage. Only 70% of the unfertilized eggs survived cooling alone to −5.8°C, whereas 95% of the fertilized eggs (5 to 115 min after fertilization) survived that treatment. After seeding and cooling to −10.5°C, only 2% of the unfertilized eggs survived. The survival of fertilized eggs

and embryos was greater, depending on embryo stage and state. That is, survival of fertilized one-cell eggs, two-cell embryos, and four-cell embryos was 57, 24, and 78%, respectively, after cooling to −15.0°C, whereas survival of embryos during first and second cleavage division was only 15 and 10% respectively. Similar patterns of susceptibility were obtained after cooling to −17.0°C. Regardless of embryo stage or state, no more than 5% of the embryos survived cooling to −27.0°C. These preliminary studies demonstrate that there is a cell-cycle dependency of sensitivity to freezing damage. But they also suggest that the quantitative model can be tested using this cell system.

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PHASE DIAGRAMS OF SOLUTIONS OF DIMETHYL SULFOXIDE IN HANKS' BALANCED SALT SOLUTION

W. F. Rall* and Peter Mazur

One theory of freezing damage suggests that slowly cooled cells are killed by being exposed to increasing concentrations of electrolytes as the suspending medium freezes. A corollary to this view is that cryoprotective additives such as dimethyl sulfoxide (DMSO) protec' cells by acting colligatively to reduce the electrolyte concentration at any subzero temperature. Previous data from this laboratory suggest that this interpretation may not be correct. However, to test this interpretation rigorously requires precise information as to the electrolyte concentration in partially frozen solutions previously used for cell survival experiments. To do this, phase diagrams of these solutions are being constructed. Two methods have been used: (*a*) a temperature-rebound method to calculate the freezing point of solutions of high weight-percent water, and (*b*) differential thermal analysis to measure the freezing point and other phase properties for solutions of lower weight-percent water.

The three series of solutions observed were 0.4, 0.7, and 1.0 *M* DMSO in isotonic Hanks' balanced salt solution (HBSS), which correspond to weight ratios of DMSO to salts of 3.61, 6.46, and 9.43 respectively. The phase-diagram data obtained for these solutions have been found to be virtually identical to previously published phase-diagram data obtained for DMSO-NaCl-H_2O solutions of the same weight ratios. Since NaCl comprises ~80% by weight of the total salts present in HBSS, the data indicate that the other minor salts have little or no effect on the solution's phase properties. Preliminary data analysis suggests that the mechanism of DMSO's cryoprotective ability cannot be explained entirely by its colligative properties.

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SURVIVAL OF SLOWLY FROZEN HUMAN RED CELLS AS A FUNCTION OF WARMING RATE

Peter Mazur and R. H. Miller

We reported last year that when human red cells equilibrated in 2 *M* glycerol were frozen slowly to −196°C, survivals were considerably higher when warming was *slow* (0.5 or 1°C/min) than when it was 24, 160, or 525°C/min. This response is analogous to that observed recently in mouse embryos[1,2] and in higher plant tissue culture cells[3] and to that observed for many years in higher plants. It confirms previous observations by Meryman on human red cells.[4] It may reflect osmotic shock from rapid dilution, but if so, the basis of the osmotic shock is uncertain. We have since determined (*a*) that the damage from rapid thawing occurs chiefly between −30 and −20°C, and (*b*) that the differential effect of warming rate is maximum in 2 *M* glycerol. In concentrations of 1.7 *M* glycerol or less, few cells survive regardless of warming rate. In 2.5 *M* glycerol or higher, most cells survive regardless of warming rate. Cells frozen slowly in 1 or 2 *M* solutions of the rapidly permeating solute, dimethyl sulfoxide, behave entirely differently. Slow warming is somewhat more damaging than rapid. The explanation of these effects of warming rate and its highly nonlinear dependence on glycerol concentration remains obscure.

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SURVIVAL OF FROZEN-THAWED HUMAN RED CELLS AS A FUNCTION OF MINIMUM TEMPERATURE OF EXPOSURE, GLYCEROL CONCENTRATION, AND WARMING RATE

Hiroshi Souzu* and Peter Mazur

One widely accepted explanation of slow freezing injury is that damage results when the concentration of electrolyte reaches a critical level in partly frozen solutions during freezing. We have conducted experiments on human red cells to test this hypothesis. Cells were suspended in saline or phosphate-buffered saline containing 0 to 3 *M* glycerol, held for 30 min at 20°C to permit solute permeation, and cooled at a rate of 0.5 or 1.7°C/min to various temperatures between 0 and −100°C. Upon reaching the desired minimum temperature, the samples were warmed at rates ranging from 1 to 550°C/min and the percent hemolysis was determined. The results for a cooling rate of 1.7°C/min (Fig. 3) indicate the following:

(*a*) Between 0.5 *M* and 1.8 *M* glycerol the temperature yielding 50% hemolysis (LT_{50}) drops slowly from −20 to −35°C.

(*b*) The LT_{50}s over this range of concentrations are relatively independent of warming rate.

(*c*) With glycerol concentrations of 1.95 *M* and 2.0 *M*, the LT_{50} drops abruptly from −55 to −100°C, and becomes dependent on warming rate. The LT_{50} is lower with slow warming than with rapid.

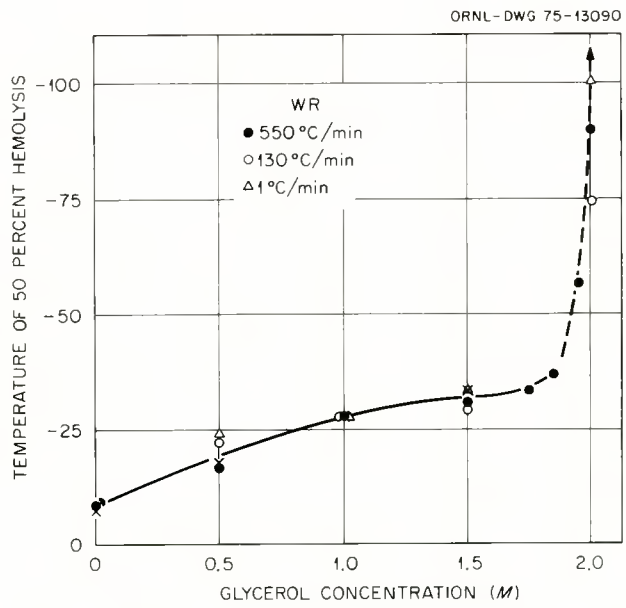

Fig. 3. Relationship between glycerol concentration and hemolysis temperature in 0.15 *M* S13. Cooling rate 1.7°C/min.

With still higher concentrations (2.5 and 3.0 *M*), there is no LT_{50}; that is, more than 50% of the cells survive freezing to −100°C, and survival again becomes independent of warming rate. The next step will be to compare the percentage survivals with the concentrations of sodium chloride present at each temperature. These concentrations will be obtained from published phase diagrams for glycerol-NaCl-water.

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COMPARISON OF THE RESPONSE OF MAMMALIAN TISSUE-CULTURE CELLS TO FREEZING

Nicholas Rigopoulos, S. P. Leibo, and Peter Mazur

This laboratory was the first to recognize and to offer a rational explanation of the fact that the response of a wide variety of cell types to freezing was similar in that their survival is a biphasic function of cooling rate.[1,2] Those analyses also led to the concept of an optimum cooling rate for maximum survival. To collect further examples of this phenomenon as well as to develop practical procedures for low-temperature preservation of cell lines, we have been conducting a survey of the response of a variety of mammalian tissue-culture cell types to freezing and thawing. The procedure is straightforward. Small samples of cells are suspended in dimethyl sulfoxide, seeded with ice to induce crystallization, cooled to −196°C at rates from about 1 to 800°C/min, and then warmed rapidly. Survival is assayed by procedures appropriate for a particular cell type. Thus far, the following cell lines have been tested and found to exhibit maximum survival at an optimum cooling rate or range of rates: two mouse lymphoma lines, L5178Y/TK$^-$ and L5178Y/ASN$^-$, and a mouse hepatoma line, BW7756. None of these cells survive cooling at rates in excess of 60°C/min.

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ISOLATION AND PROPERTIES OF CHROMATIN *ν* BODIES*

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There is now considerable electron microscopic and biochemical evidence that the first level of DNA folding

in eukaryotic chromosomes is the spheroid chromatin ν body. It appears that the association of histones with DNA results in a six- to sevenfold compacting of DNA within the ν body.

ν bodies can be isolated by two methods: (*a*) sonication of formaldehyde-fixed water-swollen nuclei and subsequent fractionation by sucrose-gradient ultracentrifugation; (*b*) nuclease digestion of intact unfixed nuclei followed by sucrose gradient ultracentrifugation. The properties of monomer ν bodies isolated by the two procedures may be compared:

	Sonicated monomers	Nuclease monomers
DNA length (base pairs)	~200	~125
Histone content	All present	All present
Electron microscopy	ν bodies with tails	ν bodies without tails
Low-angle X-ray reflections	Present	Present

From these and other data it appears that sonication produces double-strand breaks on the connecting DNA strands between the ν bodies; the resulting monomer particles represent the upper limit of the fundamental repeat unit in chromatin (*i.e.*, ~200 np). Nuclease digestion appears to result in the rapid destruction of the connecting strand followed by slower degradation of internal DNA. Preliminary studies reveal that the early stage of nuclease cleavage produces ν bodies with tails, suggesting that early digestion resembles breakage by sonication.

High-resolution microscopy studies now in progress suggest that ν bodies may have a characteristic internal structure, possibly possessing a hole or crevice in the middle of the particle.

Computations of the low-angle X-ray scattering properties of various close-packed arrays of spherical particles, assuming different models for the internal electron density distribution, have shown that: (*a*) the ~110-Å reflection could arise from the particle lattice; (*b*) the reflections at ~55, 37, 27, and 22 Å arise principally as a consequence of the spherical particle shape; and (*c*) the relative peak intensities are sensitive to the exact choice of internal electron density distribution. Measurements are in progress to determine the proper choice of a radial electron-density distribution.

The universality of ν body occurrence is now well documented; evidence demonstrates their presence in plants and fungi and in various animal tissues. Preliminary studies from our group indicate that ν bodies can be visualized in synchronized CHO cells sampled at different stages of the cell cycle.

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¶Postdoctoral investigator supported by Subcontract 3322 from the Biology Division, ORNL, to The University of Tennessee, Knoxville.

THE FORMATION OF TRIPLE-STRANDED COMPLEXES BETWEEN SATELLITE DNAs AND DOUBLE-STRANDED DNAs

C. A. Chambers and D. M. Skinner

The interaction of purines or synthetic simple sequence ribo- or deoxyribopolymers with complementary polynucleotides to form double- (ds) or triple-strand (ts) helices was reported some years ago (reviewed in ref. 1). We find a similar formation of ts complexes by the G-rich (50% G) or C-rich (50% C) single strands of two satellite DNAs with ds main component DNA from the same organism as the satellite or with DNA from heterologous sources (*i.e.*, the main component DNA from other organisms including bacteria). The satellites studied were one of very simple sequence – Satellite I[2] of the hermit crab, *Pagurus pollicaris*, whose sequence is pure $(-T-A-G-G-)_n$: $(-A-T-C-C-)_n$ (ref. 3) and the more complex α-satellite of the guinea pig, *Cavea porcellus*, 50% of whose residues are $(-T-T-A-G-G-G-)_n$: $(A-A-T-C-C-C-)_n$, with the remaining sequences variations theron. The ss DNAs have been labeled *in vitro* by ^{125}iodination or by ^{3}H-substrates using DNA polymerase I. Control experiments were performed with [^{3}H]Thd or ^{3}H-C-labeled guinea pig α-satellite isolated from tissue culture cells (GPBC). The satellite DNAs have been purified by centrifugation in Hg^{2+} + Ag^{+}–Cs_2SO_4 (ref. 4) or Ag^{+}–Cs_2SO_4 gradients. Their single strands have been recovered from alkaline CsCl gradients.

We find that: (*a*) Centrifuged in CsCl gradients without ds DNAs, either single strand from either satellite forms a very broad band (width at half height extends over a 50-mg density range). (*b*) In contrast, each ss DNA forms a narrow band (width at half height extends over a 15-mg density range) with any one of the different carrier DNAs, either main component DNA (homologous or heterologous) or DNA from either of two bacterial sources [*M. luteus* (71% G + C, ρ = 1.727 g/cm^3) or *E. coli* (50% G + C, ρ = 1.7035 g/cm^3)]. The ts complexes form bands at characteristic

positions in a CsCl density gradient 5 to 7 mg/cm^3 more dense than the peak of the carrier DNAs. Thus the density of the ts complex is 1.732 g/cm^3 with *M. luteus* and 1.707 g/cm^3 with *E. coli*, crab, or guinea pig main component DNAs, all of whose densities are ~1.700 g/cm^3 in neutral CsCl. (*c*) The complexes formed by the G-rich strands are more stable than those formed by the C-rich strands as shown by their dissociation from the ds carrier DNAs. In these experiments, the ts complexes recovered from CsCl gradients were adsorbed on hydroxyapatite (HAP) in 0.02 *M* phosphate buffer from which they were dissociated by heating. C-rich strand ts complexes do not bind to HAP while G-rich strand complexes bind and are eluted at 55 to 60°C. Since the transition of the melt is quite broad, the complex is probably heterogeneous. In previous studies of synthetic polymers, it was concluded that the third strand of a ts nucleic acid structure is positioned in the minor groove of the ds helix. Therefore, such a broad transition might be expected, particularly in view of the relatively higher charge density of the ts complex.[1]

To determine whether the ss satellite DNA associates over much of its length with the ds DNA, we have treated the ts complexes with S_1 nuclease, specific for single-stranded DNAs. After nuclease treatment, more than 96% of the radioactive DNA (*i.e.*, the single strand) does not bind to HAP. From these results we conclude that the association is one in which many single-stranded tails of the satellite protrude from the minor groove.

The biological implications of the associations reported here are highly speculative. It has been postulated that middle repetitive DNAs may participate in the regulation of transcription; the highly repetitive DNAs may participate similarly. Studies on the transcription of main-band DNA in a ds (native) or ts (complexed) state may answer that question. Others have reported[5] that ts polymers involving two DNA strands plus one ribonucleotide are less effective templates for RNA polymerase. In that instance, the mechanism of product inhibition was invoked: the ribopolymers are likened to RNA transcripts. In the case of ts DNA complexes described here, a different mechanism would pertain.

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MOLT-CYCLE-CORRELATED MOLECULAR CHANGES IN MUSCLE AND HEMOLYMPH IN THE LAND CRAB, *GECARCINUS LATERALIS*

L. H. Yamaoka* and D. M. Skinner

The claw muscle of the land crab, *Gecarcinus lateralis*, undergoes breakdown and reformation during the molt cycle. Up to 40% of the claw muscle mass is lost during the premolt period and is rapidly re-formed after ecdysis; the magnitude of the muscle mass lost suggests that major structural proteins are involved.[1,2]

Estimations of protein synthesis and turnover based on the uptake and loss of isotope from a protein require (*a*) isolation and identification of the protein and (*b*) information on the free pool of amino acids in the hemolymph and cell water.

(*a*) *Isolation and identification of crustacean myosin.* We have isolated and purified *Gecarcinus* myosin to study its synthesis and turnover during the period of muscle breakdown and reformation. As in most nonmammalian skeletal muscle, including other arthropods, *Gecarcinus* myosin is isolated as an actomyosin complex. When displayed by SDS–acrylamide gel electrophoresis, our preparations show myosin as well as actin and other proteins. The latter migrate with molecular weights characteristic of troponin and tropomyosin. *Gecarcinus* actomyosin possesses an adenosine triphosphatase (ATPase) activity with the following characteristics: activation by Ca^{2+} with a broad optimum ranging from 10^{-3} to 10^{-1} *M*; a double pH optimum (5.3 and 8.6); inhibition by Mg^{2+}, which is characteristic of other arthropod actomyosins. The ATPase activity is not inhibited by ouabain or azide, indicating that it is not Na^+-K^+ ATPases or mitochondrial ATPases.

Gecarcinus myosin has been partially purified from this actomyosin complex using the Weber method[3] of high-speed centrifugation in the presence of MgATP. Under these conditions, myosin and actin are dissociated, and the actin sediments while myosin is the predominant product in the supernatant.

During the postmolt period when the muscle is reconstituted, the incorporation of radioactive amino acids is markedly elevated in soluble proteins. Others[4] have recently shown that precursor myofibrils are found in a similar soluble fraction of mammalian muscle. We are investigating whether the crustacean

proteins being rapidly synthesized at this stage are similar precursor myofibrillar proteins.

(*b*) *The free amino acid pools of muscle and hemolymph during the molt cycle.* The total free amino acids in muscle water of the land crab decrease almost threefold during the premolt period in comparison to the intermolt period (193 μM/g during intermolt and 67 μM/g during late premolt). This decrease is accounted for primarily by changes in the nonessential amino acids proline, glycine, and alanine. Proline decreases six- to tenfold immediately prior to ecdysis and is maintained at this low level during the postmolt period. Glycine shows a similar molt-stage-related decrease of a lesser magnitude. Simultaneous with the decrease in proline and glycine, several essential amino acids (lysine, methionine, and tyrosine) increase two- to fourfold.

Although the total free amino acid levels in hemolymph were about 30-fold lower and were more variable than those in muscle, the same amino acids showed similar molt-stage-related changes. The decreases in glycine and proline may be associated with the synthesis of the new exoskeleton and connective tissue elements during the premolt period.

Groups of amino acids (*e.g.*, glycine and alanine; and leucine, isoleucine, and valine) appear to rise and fall in concert. These amino acids are known in mammalian cells to be transported via common transport systems. It is possible that in Crustacea the change in muscle amino acid concentrations is a reflection of a changing amino acid transport system(s).

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INTERACTING CONTROLS ON MOLTING AND REGENERATION IN CRUSTACEA

C. A. Holland* and D. M. Skinner

Prior to the experiments discussed in this section, it was assumed that two hormones control molting in crustaceans. The molting hormone, secreted by the Y-organs, is believed to be β-ecdysone, a steroid that induces molting in crustaceans as well as insects. Secretion of the molting hormone is presumably controlled by the molt-inhibitory hormone (MIH), the hormonal product of the X-organ–sinus gland complex located in the crustacean eyestalk. The results of our experiments with animals lacking eyestalks indicate a more complex set of interactions, including inhibition of molting in animals presumably unable to produce MIH.

This laboratory has recently demonstrated an interaction between molting and regeneration in Crustacea.[1,2] In the land crab, *Gecarcinus lateralis,* regeneration of more than five missing appendages induces a precocious molt. Loss of many limbs has the same effect on numerous marine crustaceans, as shown by others using our method.[3–7] Our recent results show that the loss of one or more partially regenerated appendages (regenerates) before a critical time in the premolt period significantly delays the time to ecdysis. When partial regenerates are removed early in the premolt period, growth and DNA synthesis in the remaining regenerates decrease markedly or cease while new blastemas form at the base of each missing limb. Such a premolt pause appears to be a generalized physiological response, since the epidermis also ceases its normal molt-correlated cytological changes.

The nature of the controlling mechanism is not understood. The mechanism could be humoral and/or neural. Two types of humoral factors are possible: an inhibitory factor that retards growth may be present in the hemolymph, or a stimulatory factor necessary for growth may be absent from the hemolymph. Studies have been started on the effect of crab serum placed *in vitro* with autotomized regenerates. Serum (10%) from an animal rapidly regenerating limbs (and presumably producing any stimulatory factor) was placed *in vitro* with partial regenerates in which DNA synthesis had been inhibited by the previous autotomy of other partial regenerates from the animal. The relative rate of DNA synthesis of the regenerates maintained *in vitro* with crab serum was the same as that of other regenerates removed from the same animal at the same time but maintained under standard culture conditions (*viz.*, with calf rather than crab serum). Serum removed from an animal 48 hr after the autotomy of partial regenerates (and presumably containing any inhibitory factor) did not inhibit the rate of DNA synthesis in rapidly growing regenerates removed from another animal.

Since these results could be explained by an ineffective concentration of the factor under the culture conditions used or by a requirement of the regenerates for prolonged exposure to a factor(s), we have begun experiments to join the circulatory systems of pairs of animals in parabiosis. Pericardial chambers were exposed by removing the exoskeleton and the underlying

epidermis. Pairs of animals were joined and sealed with dental cement. To test the effectiveness of this co-joining, [^{3}H]Tid was injected into one animal and the hemolymph of its partner sampled. Within 1 hr, the specific activity of the hemolymph of the non-injected animal equaled that of the injected animal. Experiments with such animals should indicate whether the factor(s) controlling growth of regenerates and molting is hemolymph borne.

The data described thus far could possibly be accounted for by the minimal hypothesis that the inhibition of growth and DNA synthesis after autotomy of partial regenerates is induced by secretion of MIH. To test this hypothesis, experiments were performed on animals whose eyestalks had been removed. In such animals, presumably unable to produce MIH, growth and DNA synthesis of the regenerates remaining after autotomy of their partners showed stage-dependent inhibition comparable with that observed in intact animals still capable of producing the hormone. As in animals with eyestalks, during the early stages of regeneration, autotomy results in an induced premolt pause accompanied by a decrease in DNA synthesis in the regenerates remaining while re-regeneration of the missing limbs occurs. Ecdysis is delayed, making the premolt period in animals that regenerate two sets of limbs in sequence as much as twice as long as in animals that regenerate only one set. Since molting can be significantly delayed in animals without eyestalks, we conclude that either another site of synthesis and/or storage of the molt inhibitory hormone exists or another factor(s) is involved in the control of molting and regeneration. The latter may be an enzyme that destroys the molting hormone, ecdysone. In summary, it is necessary to postulate a third biological factor which at a minimum compartmentalizes the activity of the known factors so that the growth of some limbs is stimulated concomitantly with the inhibition of growth of others in the same animal.

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POLYAMINO-ACID-STIMULATED UPTAKE OF EXTRACELLULAR FLUID MARKERS INTO HeLa CELLS

Emily Tate Brake and J. S. Cook

As part of our program to investigate factors affecting membrane turnover, we have begun a study of pinocytosis as induced by polyamino acids[1] in HeLa cells. Apart from observations with an electron microscope, our quantitative measurement is the uptake of [^{14}C]sucrose as a marker for the volume of medium imbibed by the cells. We find that under all conditions, there is a fast component of uptake completed in about 1 hr, followed by a slow uptake that is the same for both control and stimulated cells. We therefore use the 1-hr uptake as a measure of the effectiveness of any compound. Following uptake, if the cells are returned to normal growth medium, a fraction of the radioactivity is rapidly lost, while the remainder leaves the cells very slowly. As a preliminary conclusion we assume that the fast component is weakly bound while the slow component represents medium that has been taken into the cell.

Controls take up a volume of medium equal to 3–5% of the cell volume per hour. Poly-D-lysine is more effective than poly-L-ornithine in stimulating uptake, the maximum enhancement of the rate being upwards of sixfold. The cells grow at their normal rate in either compound at 2×10^{-8} *M*, which is an effective stimulating concentration, but are growth-inhibited at 6×10^{-8} *M*. The toxicities of these compounds are in the same order as their abilities to stimulate uptake. At the higher concentration of poly-D-lysine, the leakiness of the membrane to ^{86}Rb is enhanced, cell potassium decreases, and protein synthesis is depressed about 25–30% at 3 hr, although DNA synthesis is not affected.

[^{14}C]sucrose uptake is not stimulated at subtoxic levels by either compound in attached cells but may be readily observed in suspension cells. The stimulated uptake does not result in the loss of measurable [^{3}H]ouabain-binding sites on the cell surface. This latter result suggests that either the polyamino acid is stimulating membrane turnover or that the ouabain-binding sites are excluded from the area of membrane involved in the pinocytosis. This problem is still under investigation.

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TURNOVER OF HeLa MEMBRANE PROTEINS

J. S. Cook, P. C. Will,* and Emily Tate Brake

Continuing with the double-isotope, two-pulse technique,[1] the use of which in our membrane preparations we validated last year, we have measured turnover of membrane proteins in logarithmically growing HeLa cells. On SDS-gel electrophoresis of purified membranes we find, confirming similar observations on other systems from Schimke's laboratory, that, in general, the larger molecular weight proteins turn over faster than the smaller proteins. There are approximately 30 identifiable bands per gel. The range for all proteins is 0.5 to 1.5 turnovers per generation. In the vicinity of 125,000 mol wt, which is the approximate size of the known ouabain-binding subunits of Na^+-K^+-ATPases, the turnover is about 1.5 per generation. This value is not greatly different from the value of 2 per generation derived from ouabain-binding studies. Thus the ATPase appears to turn over more rapidly than the mean value for membrane proteins (1.1 turnovers per generation) but not significantly more rapidly than proteins of similar molecular weight.

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RADIOSENSITIZATION OF *ESCHERICHIA COLI* BY THE FREE RADICAL TMPN

R. J. Brake*

The water-soluble, stable free radical 2,2,6,6-tetramethylpiperidinol-*N*-oxyl (TMPN) sensitizes bacterial and mammalian cells to ionizing radiation at otherwise nontoxic concentrations.[1,2] TMPN competes with oxygen for transient, radiation-induced radicals in DNA, leading to covalent TMPN-DNA adducts or oxidation of DNA radicals.[3] In contrast to the oxygen effect, TMPN sensitization occurs with little if any increase in the yield of DNA single-strand breaks.[4] The radiochemical basis of sensitization is not known for either oxygen or TMPN, but probably involves both the number and repairability of damaged nucleotides in the cellular DNA. I have examined the sensitization to gamma rays of strains of *E. coli* deficient in the prereplicative excision (*uvrA*) and postreplicative (*recA*) pathways for repair of UV-induced pyrimidine dimers, and a doubly deficient (*uvrA recA*) mutant. Dose reduction factors for gamma irradiation of bacterial suspensions under air or anoxically in 1 m*M* TMPN have been calculated, with irradiation under nitrogen as the standard in both cases (Table 2). These data agree with earlier results.[5]

The sensitivity of the strains to gamma rays increases noticeably as repair functions are removed (reading down the columns), with the greatest effect at removal of the *recA*-dependent pathway. Sensitization with TMPN emphasizes this effect, inducing a maximal 50-fold greater sensitivity of the double mutant as compared with the wild type. The excision-repair function, absent in the *uvrA* mutants, appears to be unimportant for survival after irradiation in the presence of oxygen (except in the double mutant), but responsible for repairing about 30% of the anoxic lesion and 50% of the anoxic TMPN-sensitized lesions. The enhancement ratios (columns 5 and 6) show that as the repair functions are removed the capacity of TMPN to sensitize increases, whereas that of oxygen decreases. This further supports the greater dependence on *uvr*- and *rec*-dependent pathways for the repair of lesions produced in the presence of TMPN. It is interesting to

Table 2. Sensitization by TMPN and oxygen of *E. coli* repair mutants

E. coli strain	Gamma-ray dose (kilorads) at 10% surviving fraction				Dose modification factor[a]	
	N_2[b]	N_2 + TMPN[c]	Oxygen[b]	Oxygen + TMPN	TMPN	Oxygen
AB1157 wild-type	40	20	12	12	2.0	3.3
AB1886 *uvrA*	27	9.5	12	12	2.8	2.3
AB2463 *recA*	4.6	0.7	2.5	2.5	6.6	1.8
AB2480 *uvrA recA*	2.7	0.4	1.6	1.6	6.7	1.7

[a] Dose modification factors are D_{10}(sensitized)/D_{10}(nitrogen).
[b] Oxygenation created by bubbling air, anoxia by bubbling nitrogen.
[c] TMPN concentration was 1 m*M*.

note (compare columns 2 and 4) that oxygen competes against the sensitizing effect of TMPN and essentially "protects" the repair-deficient mutants. These data are consistent with a model where TMPN enhances the non-strand-break, "UV-like" fraction of gamma-ray damage (subject to *uvr* and *rec* repair), whereas oxygen enhances a "non-UV-like," perhaps strand-break-associated, fraction, at least partly repairable, independent of the *uvrA* and *recA* functions.

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THE NUMBER AND KINDS OF CYCLOBUTYL PYRIMIDINE DIMERS IN HUMAN DNA

W. L. Carrier, W. H. Lee, and James D. Regan

Dimers are produced in DNA between adjacent pyrimidines by UV light. The most convenient way to estimate the amount of dimers is to incorporate radioactive thymidine into the DNA, irradiate, hydrolyze with formic acid, separate the acid-stable thymine-containing photoproducts from the thymine by paper chromatography, and finally compare the amount of label in the photoproducts (T<>T, T<>C) with the amount in thymine. Recently Unrau *et al.*[1] used [^{14}C]uracil as a general precursor of pyrimidines in DNA and RNA. They were able to determine the number and kind of dimers in the DNA of several microorganisms irradiated with UV light. We used this method to measure dimers formed in cultured human fibroblast cells exposed to 254-nm UV light. The number and kind of dimer formed are shown in Table 3. Thymine-containing dimers are more readily formed at this wavelength of UV light than cytosine-containing dimers. After 50 J/m^2 the ratio of C<>C, C<>T, T<>T is 14:20:66. In contrast, this ratio is 16:38:38 in *E. coli*. At lower doses of UV, thymine makes up about 75% of the bases involved in dimer formation; this figure for *E. coli* is about 60%. The ratio changes with increasing doses of UV. The production of cytosine-containing dimers levels off before thymine-containing dimers due to the difference in absorption characteristics of these bases. We can now make a reasonable estimate of the total number of dimers simply by knowing the percent radioactivity in the thymine-containing dimers. These numbers provide a refinement in the estimation of the amount of radiation damage and repair. For example, we can now correlate the number of dimer-specific endonuclease-sensitive sites in irradiated DNA with the actual number of pyrimidine dimers present.

Table 3. Cyclobutyl pyrimidine dimers in human DNA by 254-nm UV irradiation

Fluence (J/m^2)	Dimers/pyrimidines × 100			
	C<>C	C<>T	T<>T	Total
50	0.036	0.052	0.18	0.27
100	0.063	0.11	0.24	0.41
200	0.066	0.15	0.42	0.64

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A SENSITIVE METHOD FOR THE DETECTION OF ULTRAVIOLET PHOTOPRODUCTS IN *SACCHAROMYCES CEREVISIAE* DNA

R. J. Reynolds*

UV irradiation of *S. cerevisiae* is known both to induce mutations and to reduce viability. Ultimately, the lesions most probably responsible for these effects, either directly or indirectly, are cyclobutyl pyrimidine dimers. We have now developed a technique which is capable of detecting dimers in yeast DNA after fluences of less than 5 J/m^2. This is 10- to 100-fold more sensitive than methods normally used for the detection of dimers in yeast. The increase in sensitivity is made possible by the use of a dimer-specific endonuclease found in extracts of *Micrococcus luteus*, an endonuclease previously shown to incise near pyrimidine dimers.[1] Measurement of the resulting single-strand breaks may be used to quantitate the number of dimers.

Briefly, the DNA to be assayed, prelabeled with [6-^{3}H]uridine, is released from yeast spheroplasts and gently extracted with phenol. The latter step eliminates nonspecific nucleases found in yeast lysates. The DNA

is treated with the *M. luteus* extract and sedimented on calibrated alkaline sucrose gradients, from which the number of single-strand breaks can be determined. We have shown that the number of breaks is linear with fluence and that no breaks are induced by the enzymatic treatment of DNA from unirradiated cells.

This technique has been used to follow the loss of UV-endonuclease-sensitive sites in the wild-type haploid strain s288c, a strain from which a number of UV-sensitive mutants have been derived. After 30 J/m^2, a fluence resulting in about 70% survival, 80% of the sites are removed within 2 hr at 28°C in potassium phosphate buffer, pH 7.0. After 24 hr, about 95% of the sites are gone. Studies are now being extended to the radiation-sensitive mutants.

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IN VITRO STUDIES WITH BACTERIOPHAGE T7

W. E. Masker

Recent sutides[1] have shown that cell-free extracts of *E. coli* infected with bacteriophage T7 can support extensive *in vitro* DNA synthesis. Although the product synthesized by extracts made from *polA* hosts (deficient in DNA polymerase I) consists of short (11S) fragments, extracts prepared from wild-type hosts or *polA* hosts supplemented with exogenous DNA polymerase I synthesize larger DNA molecules, many of which have the same molecular weight as intact T7 chromosomes.[2] These large molecules, which are synthesized semiconservatively, are able to infect *E. coli* spheroplasts and produce phage particles, thus demonstrating that the fidelity of *in vitro* replication can be sufficiently accurate to produce biologically active DNA.[2] In the process of establishing this *in vitro* system in this laboratory, we have encountered a previously undetected nuclease activity. This endonuclease, which is present in extracts of phage-infected cells, attacks exogenous T7 DNA provided as template and makes two to three double-strand breaks per DNA molecule. The activity is present in hosts deficient in endonuclease I and in exonuclease V and is not inhibited by either tRNA or EDTA. The nuclease activity is reduced by using reaction mixtures with high ionic strength – a condition which also reduces the rate of *in vitro* DNA synthesis. Efforts are now being made to identify the source of this nuclease activity and, in particular, to determine whether the activity persists in extracts made from hosts lacking the K-12 restriction enzyme.

We have also begun to examine the response of this *in vitro* system to UV radiation. *In vivo* T7 is quite sensitive to UV and has a biphasic survival curve which shows dependence upon the *E. coli uvrA uvrB* genes. However, the rate of *in vitro* DNA synthesis seems to be relatively unaffected by UV. Doses of 50 J/m^2 applied to the exogenous template reduce the rate and extent of *in vitro* DNA synthesis by only 50%. Also, we have as yet found no evidence for *in vitro* excision of pyrimidine dimers in this system. Moreover, although extensive semiconservative replication synthesis takes place, we have thus far been unable to detect any UV-stimulated nonconservative DNA synthesis characteristic of repair resynthesis. These observations suggest that T7 may deal with UV damage primarily through postreplicational repair rather than by a "cut and patch" excision scheme. Further experiments will be needed to test this interesting possibility.

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RADIATION-INDUCED CESSATION OF RESPIRATION IN *ESCHERICHIA COLI* AND ITS ROLE IN RADIATION CELL DEATH

P. A. Swenson

We have previously presented evidence that *E. coli* B/r cells which are nonviable after UV irradiation (254 nm) have had their respiration turned off within an hour after exposure. The cause-and-effect relationship between cessation of respiration and lethality is not clear, but the respiratory response is dependent upon the presence of the $recA^+$ and lex^+ genes.[1] A number of other responses to UV by this bacterium require these genes and are thought to be induced in the sense of derepression of an operon.[2] This thought is consistent with much of our published data, and, in fact, we have previously advanced a hypothesis which states that UV induces an operon and causes the excess synthesis of a protein involved in the cessation of respiration.[3] During the past year our work has dealt with the induction problem, the biochemistry of cessation of respiration, development of a method

for separation of viable and nonviable cells from irradiated cultures, and the repair capabilities of the two classes of cells.

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THE ROLE OF ADENOSINE CYCLIC 3′:5′-MONOPHOSPHATE IN THE ULTRAVIOLET-INDUCED CESSATION OF RESPIRATION IN *ESCHERICHIA COLI* B/r

P. A. Swenson, R. L. Schenley, and J. G. Joshi*

An important piece of evidence that cessation of respiration after UV irradiation of *E. coli* B/r is an induced phenomenon is that adenosine cyclic 3′:5′-monophosphate (cAMP) causes more glucose-grown cells to shut off respiration and to become nonviable than in its absence.[1] The intracellular concentration of cAMP in glucose-grown cells is quite low, and our interpretation is that the increased level of this cyclic nucleotide stimulates the induced synthesis of the respiratory control protein in the same manner that it stimulates the synthesis of β-galactosidase in glucose-grown cells that are induced by a β-galactoside such as lactose. For maximum induced formation of β-galactosidase, a complex of cAMP and a cAMP-binding protein must be present, presumably to enable the RNA polymerase to transcribe the β-galactosidase gene.[2] We infer that cAMP exerts its inhibitory effect on respiration by combining with a cAMP-binding protein and allowing transcription of a gene coding for a respiratory control protein that is involved in the cessation of respiration.

During the past year, cAMP has been used by us in combination with two treatments, 5-fluorouracil (FUra) and elevated temperature (42°C), each of which maintains respiration in many of the cells where it would normally cease after UV.[3] The cAMP has only a small effect on the respiration maintained by FUra but almost completely prevents respiration in thermally treated cells. Our interpretation is that FUra treatment causes production of a fraudulent messenger RNA so that, even though cAMP causes more mRNA to be formed, this mRNA is not active. Thermal treatment was formerly thought by us to inactivate the respiratory control protein, but the above results suggest that this thermal treatment acts on the control system. Our hypothesis is that the cAMP binding protein is thermolabile in that its binding constant is altered. An alteration in the binding constant would prevent transcription and subsequent respiratory failure in UV-induced cells, but exogenous cAMP would enhance the possibility of binding and favor transcription.

We have detected significant differences in elution patterns of radioactive cAMP binding proteins from unirradiated and irradiated cells using agarose gels to which cAMP is covalently bound (affinity chromatography). We are now in a position to test our hypothesis of altered cAMP binding properties by thermal (42°C) treatment with *in vivo* and *in vitro* experiments.

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CHANGES IN BINDING PROPERTIES OF PYRIDINE NUCLEOTIDE BINDING PROTEINS IN ULTRAVIOLET-IRRADIATED *ESCHERICHIA COLI* B/r CELLS

J. G. Joshi* and P. A. Swenson

Associated with the cessation of respiration in UV-irradiated *E. coli* B/r cells is the selective loss of pyridine nucleotides. This loss might be caused by changes in the binding properties of pyridine nucleotide binding proteins. In affinity chromatography experiments, we have used agarose columns to which NAD was bound covalently and have found very different elution patterns for radioactive proteins from unirradiated and irradiated cells. Certain peak activities in extracts from unirradiated cells are absent in irradiated ones and vice versa. We have begun the process of isolating, purifying, and analyzing the composition of binding proteins to determine how radiation causes these changes. One possible way that the changes occur is by selective proteolytic cleavage of amino acids or short peptide chains from pyridine nucleotide binding proteins. We are conducting a search for such a radiation-induced enzyme.

Our plans are to study the biochemistry of the irradiated cell, and large quantities of cells in which

radiation-induced changes have taken place will be needed. We have designed and constructed a quartz flow cell for large-scale irradiations. Two flow cells are in series and sandwiched between UV germicidal lamps. In collaboration with E. F. Phares of the Biochemistry Section we have pumped 2½ liters/min of log-phase cells from a fermenter through the irradiation system and have been able to duplicate respiratory and lethal responses obtained in our small-scale experiments. We expect to accumulate a kilogram or more of irradiated cells for biochemical studies.

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SEPARATION OF VIABLE AND NONVIABLE CELLS FROM ULTRAVIOLET-IRRADIATED *ESCHERICHIA COLI* B/r CULTURES

W. D. Fisher, R. L. Schenley, and P. A. Swenson

Although the repair of DNA in UV-irradiated bacterial cells has been extensively studied, no method has existed which could distinguish between the events taking place in the viable and nonviable components of the culture. We have developed a centrifugal method for the preparative separation of viable and nonviable cells from a UV-irradiated culture. When a UV-irradiated (20 J/m^2, approximately 10% survival) culture of *E. coli,* grown on minimal medium containing glycerol, is incubated in the presence of the nonionic detergent Triton X-100, the nonviable cells, which have stopped respiring, lose RNA and protein and become smaller within 120 min after irradiation. Viable, respiring cells are not vulnerable to the detergent. To separate the classes of cells, the culture is mixed into a 0 to 5% neutral sucrose gradient on a 5% cushion and subjected to differential centrifugation. After three cycles of centrifugation, the pellet contains cells which range in size from unit length to short filaments. All of these cells are viable. The supernatant contains small cell entities with very low viability (0.001%). Cell counts, viability measurements by plate colony count, and size distribution studies show that the recovery of the viable cells is nearly quantitative (80–100%). This method also works for separation of viable and nonviable cells from UV-irradiated cultures grown on glucose and from gamma-irradiated cultures.

EXCISION OF PYRIMIDINE DIMERS FROM VIABLE AND NONVIABLE CELLS IN ULTRAVIOLET-IRRADIATED *ESCHERICHIA COLI* B/r CULTURES

R. L. Schenley, W. D. Fisher, and P. A. Swenson

We have measured the pyrimidine dimer excision in separated viable and nonviable cells from UV-irradiated glycerol-grown cultures. Within 120 min after UV (20 J/m^2), in the presence or absence of detergent, 60–70% of the dimers were excised from an irradiated culture; little or no excision occurred during an additional 2 hr of incubation in growth medium. When the viable and nonviable cells were separated at 120 min, the number of dimers in each population was identical to that of the whole culture. No further excision was seen in the viable culture when it was incubated in growth medium for an additional 3 hr. Reirradiation of viable cells containing residual dimers showed two interesting features. Dimers were once more excised, and the cells were no more sensitive than log-phase cells that had not been previously irradiated. These results show that survival is not well correlated with the extent of dimer removal and suggest the possibility of selective repair of dimers in UV-irradiated cultures.

AN IMPROVED PROCEDURE OF SEPARATION AND PURIFICATION OF TWO PRINCIPAL TOXINS (RICINS V-3 AND III_L) AND A HEMAGGLUTININ FROM SEEDS OF *RICINUS COMMUNIS*, AND CRYSTALLIZATION OF RICIN III_L

C. H. Wei and ChongKun Koh

Two principal toxins and a lectin present in the seeds of *Ricinus communis* have previously been separated and purified to homogeneity by a procedure, the final stage of which is CM-cellulose chromatography[1] with gradient elution using 0.02–0.2 *M* sodium phosphate buffer (pH 6.5). In order to obtain better resolution, we have employed shallower gradients, 5–50 m*M* followed by 50–100 m*M* buffer. Although the overall profile appeared similar to that obtained before, characterization of the products by gel filtration, polyacrylamide disc gel electrophoresis, and isoelectric point determination revealed that toxic ricin III_L now preceded rather than followed nontoxic agglutinin III_H. Furthermore, the III_L fraction was resolved into two com-

ponent proteins, which have the same isoelectric point, 7.4, and are also indistinguishable with respect to SDS electrophoresis patterns and to electrophoretic mobilities at various pH values (4.5, 7.0, and 9.5). Resolution of a preceding shoulder from the main peak of ricin V-3[1] was not achieved.

After intensive trials, toxin III_L has finally been crystallized in the form of fine needles. A modified microdiffusion cell[2] was used for slow equilibrium dialysis against 0.05 *M* Tris-H_3PO_4 buffer (pH 7.2) to which small increments of 3 *M* ammonium sulfate in the same buffer were added daily. The crystals started to appear at an ammonium sulfate concentration of 1.25 *M*, and their growth appeared complete after one month, at 1.41 *M* ammonium sulfate concentration. Because of the limited size and unfavorable shape of the III_L crystals, they do not appear to be useful for X-ray structural investigation.

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FURTHER PURIFICATION OF THE MOST TOXIC RICIN V-3 FROM SEEDS OF *RICINUS COMMUNIS*

ChongKun Koh and C. H. Wei

In previous studies on the crystallization of ricin V-3,[1] the most toxic component obtained in CM-cellulose chromatographic separation, the crystal growth was occasionally hindered by slight amounts of a contaminant, which usually precipitated as white amorphous material from the crystallizing medium. Previous attempts to remove the impurity, by the use of a DEAE-cellulose column with sodium phosphate buffers (pH 7.5) as elutants, were not fully successful. However, the impurity can be removed by gradient elution of a CM-cellulose column using 10–50 m*M* sodium phosphate buffer, pH 7.2. The toxic ricin V-3 elutes as a single symmetrical peak, which on crystallization has yielded cleaner crystals, which can be used in our current X-ray investigation.

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AMINO-TERMINAL SEQUENCES OF TWO PEPTIDE CHAINS FOR RICIN V-3[1]

S. S.-L. Li,* C. H. Wei, J.-Y. Lin,† and T.-C. Tung†

After careful screening of five different protein crystals first obtained in our laboratory from seeds of *Abrus precatorius* and *Ricinus communis*, preliminary X-ray data for four of which have been reported,[2–4] we chose to concentrate our X-ray structural studies on ricin V-3, the most toxic protein present in the latter seeds. Knowledge of the amino acid sequence would be of utmost importance in the X-ray structural determination. The toxin consists of two peptide chains (A and B) of slightly different molecular sizes. Collaborative research has so far resulted in the amino-terminal sequence of 28 residues of the A chain and 14 residues of the B chain as shown below.

```
          1                   5                  10      12
A-chain   Ile-Phe-Pro-Lys-Met-Tyr-Pro-Ile-Ile-Ile-Val-Thr-
         13    15                  20                  24
          Ile-Ala-Gly-Ala-Thr-Val-Leu-Ser-Tyr-Asn-Val-Phe-
         25      28
          Ile-Gly-Ala-Val-

          1                   5                  10
B-chain   Ala-Asp-Val-Thr-Gln-Asp(Pro)Glu-Pro(Ile)
         11      14
          Phe-Arg-Thr-Val-
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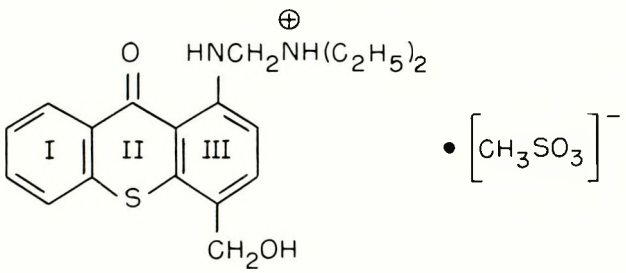
X-RAY STRUCTURAL ANALYSIS OF HYCANTHONE METHANESULFONATE, A HIGHLY ACTIVE SCHISTOSOMICIDAL AGENT

C. H. Wei and J. R. Einstein

Hycanthone, 1-[2-(diethylaminoethyl)amino]-4-hydroxylthioxanthene-9-one, has been developed by Sterling-Winthrop Research Institute for the treatment

of schistosomiasis, a parasitic-worm disease estimated to affect more than 200 million people in the tropics. Although this drug is a potent frameshift mutagen, so that its safety as a drug is questionable, it was estimated (as of July 1974) that at least 700,000 people infected with *Schistosoma hematobium* and *S. mansoni* have been treated with hycanthone during the past six years in Brazil, Africa, and the Middle East.[1] The structural investigation of hycanthone was undertaken as part of the minor portion of our work devoted to the structure determination of "small" molecules of biological interest and as a contribution to the eventual explanation of hycanthone activity.

The compound (supplied by Dr. F. J. de Serres of NIEHS, Research Triangle Park, N.C.) crystallizes with



four molecules in a monoclinic unit cell of symmetry $P2_1/a$ and parameters a = 26.1935(13), b = 8.7997(4), c = 10.6373(5) Å, and β = 116.420(7)°. The structure was solved by the heavy-atom method and was refined by full-matrix least squares to a discrepancy index, $R(F)$, of 3.3%, based on 3538 independent observed counter data. The average estimated standard deviation for bond distances between nonhydrogen atoms is 0.002 Å and for bond angles, 0.2°. The molecular parameters, including bond distances and angles, are all within the range of normally expected values.

The structural results revealed that hycanthone base is protonated at the nitrogen atom of the $-CH_2N(C_2H_5)_2$ group and that this proton and the hydroxyl hydrogen atom are hydrogen-bonded to oxygen atoms of two neighboring methanesulfonate ions, thus forming an infinite chain of hydrogen-bonded molecules around the crystallographic two-fold screw axes. The carbon atoms of rings I and III are planar to within 0.02 and 0.01 Å, respectively, and these planes intersect along the C(9) . . . S(10) line to form a slightly bent configuration with a dihedral angle of 167.7°. The methanesulfonate anion has a tetrahedral configuration of D_{2d} symmetry. Its charge is delocalized, and all S-O bonds are equal to within ±0.01 Å.

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PRELIMINARY X-RAY DATA FOR SOME COMPOUNDS OF BIOLOGICAL INTEREST

C. H. Wei

Sodium salt derivative of thymidylyl-(3′-5′)-2′-deoxythymidine. – $Ca_{0.5}TpT \cdot 2H_2O$, generously supplied to us by Dr. F. N. Hayes of Los Alamos Scientific Laboratory, was further purified and converted into a sodium salt by passing the aqueous solution through a sodium-converted Dowex 50 column. Single crystals were obtained from water–ethanol solution at 4°C over a period of months. The crystals seem to be extremely sensitive to X radiation. Preliminary Weissenberg and precession photographs indicate that the crystals possess the following data: a = 12.70, b = 8.98, c = 13.47 Å, β = 122.5°; space group $C2/c$ or Cc; Z = 8 or 4; V = 1294 Å^3. As judged from the size of the unit cell, the derived compound could be a nucleoside degradation product, rather than a dinucleoside monophosphate.

Derivatives of di-GDP (P^1,P^4-diguanosine-5′-tetraphosphate). – A sample of di-GDP was kindly supplied by Dr. F. J. Finamore of this Division. The compound was converted into sodium and potassium salts by the use of Dowex 50 columns. After storage at 4°C for a few months, the water–ethanol solution of the sodium-converted compound gave rise to apparently single crystals whose cell parameters are yet to be determined. The H-form of di-GDP was also treated with 48% HBr and evaporated to dryness. From the HBr-containing aqueous solution, platelike crystals were obtained. This material crystallizes in a monoclinic unit cell of symmetry $P2_1/c$ and parameters a = 4.63, b = 10.01, c = 19.11 Å, and β = 99.4°. A comparison of the crystal data with those for guanine and guanine–HCl derivatives suggests that the derived compound is likely guanine hydrobromide monohydrate, the structure of which has not yet been reported.

THE pK_a OF THE ACTIVE-SITE CARBOXYL GROUP OF TRIOSE PHOSPHATE ISOMERASE

F. C. Hartman and G. M. LaMuraglia*

3-Haloacetol phosphates and glycidol phosphate inactivate triose phosphate isomerase by a highly selective esterification of an essential glutamyl residue.[1,2] Attempts to determine the pK_a of the active-site carboxyl group from the pH dependence of the inactivation rate have been complicated by a variable affinity of the reagents for the enzyme as a result of changes in the ionization states of the reagents over the pH range examined. To circumvent this problem, we have synthesized chloroacetol sulfate, a monoprotic acid that exists only as a monoanion over the entire pH range at which protein carboxyl groups ionize. This new reagent esterifies the same glutamyl residue of triose phosphate isomerase as do the previously described active-site-specific reagents, and therefore the pH dependence of the inactivation rate by chloroacetol sulfate provides a measurement of the pK_a of the essential carboxyl group. For the yeast enzyme, the apparent pK_a calculated from the pH dependence of the inactivation rate is 3.9 (±0.1). We were unable to determine the pK_a of the corresponding group in the rabbit muscle enzyme because of its instability at low pH. However, since the inactivation rate was virtually the same at pH 5.0 as at pH 8.0, the pK_a must be well below 5.0.

The interconversion of D-glyceraldehyde phosphate and dihydroxyacetone phosphate as catalyzed by triose phosphate isomerase involves proton abstraction from C-3 of the ketonic substrate by an acid-base group of the enzyme to generate an enediol intermediate followed by proton transfer from the acid-base group to C-2 of the enediol. From k_{cat} and the ratio of intramolecular proton transfer to proton exchange with solvent during the isomerase-catalyzed conversion of dihydroxyacetone phosphate, stereospecifically tritiated at C-3, to glyceraldehyde 3-phosphate, Plaut and Knowles[3] have concluded that the dissociation rate of the protonated essential base must be at least 2×10^4 sec^{-1} and therefore must have a pK_a not greater than 6. Thus, the active-site carboxyl group with an apparent $pK_a < 4.0$ is a likely candidate as the effector of proton transfer.

*ORAU Undergraduate Research Participant, Summer 1974, from Georgetown University, Washington, D.C. 20001.

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AFFINITY LABELING OF RIBULOSE BISPHOSPHATE CARBOXYLASE BY 3-BROMO-1,4-DIHYDROXY-2-BUTANONE 1,4-BISPHOSPHATE

J. V. Schloss* and F. C. Hartman

3-Bromo-1,4-dihydroxy-2-butanone 1,4-bisphosphate, a reactive analogue of the substrate ribulose 1,5-bisphosphate, has previously been synthesized as a potential affinity label for ribulose bisphosphate carboxylase.[1] This reagent inactivates the carboxylase from spinach as a consequence of alkylation of two essential lysyl residues.[2] If these residues play a crucial role in catalysis, analogous residues should be present in ribulose bisphosphate carboxylases from diverse species, and therefore the same reagent should also inactivate these enzymes by alkylation of lysyl residues. Thus, we have inspected the effects of bromobutanone bisphosphate on ribulose bisphosphate carboxylase from *Rhodospirillum rubrum*, a purple nonsulfur bacterium. This organism is among the most primitive known to contain the carboxylase. In contrast to the enzyme from spinach (which has a molecular weight of 560,000 and contains eight protomeric units, each with a 56,000- and 14,000-dalton subunit), the enzyme from *R. rubrum* has a molecular weight of 114,000 and is composed of two identical subunits of 57,000 daltons.[3]

The purified carboxylase from *R. rubrum* is inactivated by bromobutanone bisphosphate, and ribulose bisphosphate affords protection against inactivation. To determine the kind(s) of amino acid residues modified, the inactivated enzyme was treated with sodium [^{3}H]borohydride, which reduces the carbonyl group of the protein-bound reagent and thereby incorporates a stable tritium label. The enzyme was then subjected to total acid hydrolysis, and the hydrolysate was chromatographed on the amino acid analyzer. From the elution positions of the radioactive components as compared with synthetically prepared standards, we conclude that inactivation of the carboxylase is at least partially due to alkylation of lysyl residues.

*Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

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EVIDENCE OF A PREVIOUSLY UNDETECTED LYSYL RESIDUE AT THE ACTIVE SITE OF ALDOLASE

J. P. Brown* and F. C. Hartman

His-359, adjacent to the penultimate residue, of rabbit muscle aldolase has been implicated as a component of the active site by its preferential alkylation at pH 6.5 with the affinity label *N*-bromoacetylethanolamine phosphate.[1] We now find that the specificity of the reagent is pH-dependent. At pH 8.5, alkylation with [^{3}H] BrAcNHEtO*P* abolishes both Fru-1,6-P_2 cleavage activity and transaldolase activity. The stoichiometry of incorporation, kinetics of inactivation (pseudo first-order and a rate-saturation effect), and the protection against inactivation afforded by competitive inhibitors are consistent with the involvement of an active-site residue. A comparison of ^{3}H profiles obtained from chromatography on the amino acid analyzer of acid hydrolysates of inactivated and protected samples reveals that inactivation results from the alkylation of lysyl residues. The major labeled peptide in tryptic digests of the inactivated enzyme has been isolated. Based on the amino acid composition of this peptide (Trp_1, CM-Lys_1, Arg_1, Asp_2, Gly_1, Phe_1) and the known sequence of aldolase,[2] Lys-146 is the residue preferentially alkylated by the reagent. This residue could be one of the two cationic groups that function in electrostatic binding of the substrate's phosphate groups.

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*University of Tennessee–NIGMS Postdoctoral Fellow supported by Grant GM 1974 from NIGMS.

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AFFINITY LABELING OF PHOSPHOGLYCERATE MUTASE FROM RABBIT MUSCLE

I. Lucile Norton, C. D. Stringer, and F. C. Hartman

N-Bromoacetylethanolamine phosphate was devised previously in this laboratory as an active-site-specific reagent for aldolase. In surveying other enzymes with an affinity for phosphate esters, we find that this same reagent can be used to label the active site of phosphoglycerate mutase. The enzyme is rapidly and totally inactivated, and inactivation is subject to substrate protection. The kinetics of inactivation are consistent with the initial formation of a reversible enzyme-reagent complex preceding the irreversible inactivation step. Also, from kinetic studies in the presence of substrate, we conclude that the initial binding of reagent is at the substrate binding site. An additional observation consistent with a highly selective modification of an essential residue is that one mole of reagent is covalently incorporated per mole of catalytic subunit inactivated. Further studies are in progress to elucidate the kind of amino acid residue alkylated by the reagent.

DATA-ACQUISITION SYSTEM

Margarita K. Churchich, S. S. Stevens, and M. L. Randolph

Since the automated data acquisition and processing system of the Biology Division was operating at near capacity, more processing capability has been added to meet user requirements expected over the next several years. New equipment is now in final stages of testing; the required software is being developed.

The new equipment includes a PDP-11/40 processor, additional core memory, two DEC-tape units, and a cartridge disk memory. The hardware is organized into two independent, linked systems, each of which includes a processor, bulk storage device, and input/output devices.

The data acquisition unit (primary system), organized around the old PDP11/20 processor, includes a console keyboard-printer, the analog-to-digital and digital-to-digital data acquisition circuitry, the cartridge disk, special data interfaces described in previous reports, and an interface to the other processor system.

The data processing unit (secondary system), based on the PDP11/40 processor, includes three graphics terminals, several Teletypes, magnetic tape and disk storage, and an interface to the PDP11/20 system.

The primary system will run under the DEC-supplied RSX11-M monitor, an event-driven executive program system optimized for real-time operation. Drivers and data-collection tasks, which are being written by the Division's system manager, will accomplish a range of automated-data-collection and apparatus-supervision functions as specified by system users.

The secondary system is operating under RSTS/E-V-06, a DEC-supplied time-sharing monitor. This system functions primarily as a user-interactive BASIC system, accepting user instructions, translating them and trans-

mitting them to the primary system, and fetching data from the primary system for user inspection and manipulation.

Software for the linking of the two processors will be written by personnel from the Computer Sciences Division at X-10. There is every expectation that the interlinked, two-processor system will be fully functional within calendar year 1975.

TRANSFER OF TRIPLET ELECTRONIC ENERGY IN DINUCLEOTIDES

J. W. Longworth* and S. K. Das†

The energy of the triplet level of nucleic acid bases differs from base to base. Studies of dinucleotides in neutral aqueous salt solutions made equivolume with polyalcohol and cooled to form glasses at the temperature of boiling nitrogen (77.4°K) have shown that phosphorescence only derives from the species with the lowest energy for the triplet level. Triplet electronic energy transfer was suggested to account for this phenomenon. We have chosen to study the dinucleotide ApG. Here G is more energetic than A – all phosphorescence is from A – characterized by the nature of the fine structure, the energy, and the decay time. In ApG in ethylene glycol:water mixture at pH 7.0, only A phosphorescence is detected. In the neutral organic solvent ethylene glycol, where the dinucleotide is known to be "unstacked," a contribution from G residues to the phosphorescence can be detected. Moreover this phosphorescence contribution from G is associated with a nonexponential and shortened decay time for G.

All these observations are consistent with electronic energy transfer at the triplet level in denatured dinucleotide (unstacked conformers) from G to A. This provides the first direct support for the existence of interbase electronic energy transfer in dinucleotides.

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DEPENDENCE OF FLUORESCENCE AND PHOSPHORESCENCE OF BENCE-JONES PROTEINS ON TEMPERATURE*

J. W. Longworth and A. Solomon†

The fluorescence of proteins, including Bence-Jones proteins, depends upon temperature according to a simple model. There are in competition with fluorescence two nonradiative processes – one independent of temperature, the other dependent upon temperature. Phosphorescence also depends upon temperature. A process independent of temperature must be considered to account for the observation since linear Arrhenius behavior is not found. An initial approximation shows phosphorescence derives from fluorescence in proteins; *i.e.*, only a single temperature-activated process, the one at singlet level, exists.

Phosphorescence spectral properties – width, energy, and lifetime – depend upon temperature. At higher temperatures a broadening and red shift of spectrum and reduction in lifetime occur. This is specific to the individual Bence-Jones protein in its influence. GENNLS, a general nonlinear least-squares program that allows errors in both the independent (temperature) and the dependent (intensity) variables of a nonlinear function, is now being used to optimize fit parameters required to compare fluorescence intensity and lifetime dependence on temperature and likewise for phosphorescence lifetime and intensity and in addition their respective dependence upon transition energy (*i.e.*, spectral location). Preliminary studies suggest that meaningful correlations may prevail consistent with generalized interactions with polar media by excited states as they vary with temperature. Differences in behavior from the general pattern may be associable with specific chemical interactions brought about by the unique tertiary structure of individual Bence-Jones proteins.

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ANALYSIS OF FLUORESCENCE LIFETIME HISTOGRAMS*

J. W. Longworth, S. S. Stevens, M. K. Churchich, and Jonas Haldeman†

The decay of fluorescence following a brief flash of UV light is detected by a delayed coincidence and stored as a histogram in a multichannel analyzer. The digital data can be transferred to a PDP11 system either by a high-speed highway or by paper tape. To analyze for a single exponential, we used a procedure based on FRANTIC where successive linearization of a convoluted model was fitted to the data. Efficient initial estimates are obtained from the minicomputer for 1 K of data points. The problem of optimal model analysis of the convolution of lamp and excited-state

decay function involving a sum of exponentials is complex because more than one model will fit the problem. We have chosen to make operational many of the popular procedures and compare their performance. This is in part to indicate to users the dependence of the optimal parameters on the procedures used and the sensitivity to noise from the ill-conditioning of the problem.

The method of moments (FLORTRAN), numerical approximation of Laplace integral, least-squares fitting of Lorentzians to a Fourier transform, and nonlinear optimization of the fast Fourier transfer of convolution integral are examples of direct solutions. Analytical substitution of the lamp profile with a sum of exponentials, sum of Gaussians, or series of modulation functions effects a deconvolution, and the fluorescence function is then modeled by nonlinear least-squares procedure. A direct solution of the normal equations by using constraints written by Toby Mitchell[†] and Richard Hemenger[‡] also carries out a deconvolution. Nonlinear optimization of numerical integration of the lamp with a trial model function has been utilizing MINUIT to permit a study of the goodness of the model fit. Many of the programs required extensive modification to operate on the ORNL IBM 360 system. For a single lifetime, reasonable consistency in optimal parameter values and error estimates is provided by the various forms of analysis. This is not true for two exponentials. To further study the difficulties, nonlinear constrained optimization programs will be needed, and generalized nonlinear optimization used.

Our studies indicate that much caution must be taken in analyzing fluorescence lifetime histograms. Different model parameters may reflect more the ill-conditioning of the problem rather than different samples or different experimental procedures between laboratories. Use of linear statistical estimates for these nonlinear models must also be interpreted with great caution. We recognize that this is a difficult conclusion to present, and more experience is required before general procedures can be adopted by all workers.

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[†]Computer Sciences Division.
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FITTING MODELS TO DATA*

J. W. Longworth and M. K. Churchich

Several FORTRAN IV programs have been made operational on the ORNL IBM 360 system that can implicitly optimize parameters of nonlinear model functions to experimental data. MINUIT, a suite of programs from European Organization for Nuclear Research (CERN), performs an initial simplex fit, and then uses a variable metric optimization. The particular feature of this program is its capability to perform error contour analysis and simulate the dependence of the function at the minimum upon parameter values. A second suite performs an initial parameter estimation, using Marquardt's version of the Newton-Raphson implicit optimization. Then the general least-squares procedure of Powell and MacDonald (GENNLS) carries out an optimization which permits variation in the independent parameter as well as the dependent parameter. The goodness of the fit is assessed by Pearson's chi-square test, sign test, run test, Wallis's conjoint test, and Kolmogorov-Smirnov test, and the cumulative frequency of the residuals is graphically displayed. A third suite of programs (CRIF) uses Newton-Raphson optimization to solve coupled differential equation systems after numerical integration.

MINUIT has been used to study the reliability of solutions for the sum of two exponentials. GENNLS is being used to analyze Arrhenius activated quenching of fluorescence, since there is uncertainty in the data for both fluorescence intensity and temperature. CRIF has been used to solve a system of equations describing the binding of drugs by human cell lines. MEETER-WOOD, a Marquardt-type nonlinear least-squares program which calculates numerical derivatives, was used to analyze the fluorescence quenching of amino acyl synthetase by cognate tRNA. There are now available for users several general programs which can find values for the optimum parameters of nonlinear models. Applications are in kinetic studies, and in binding studies, as common examples. The programs are simple to use, general in application.

The preceding programs are of the class known as unconstrained optimization analysis. A more complex problem is that of constrained nonlinear model optimization. GPM, a gradient-projection-method program, is now operational and is being used to study weak and strong binding of ligands to proteins. A very general program, OPTIME, is also largely operational, and this permits linear and nonlinear constraints and performs extensive analysis of the goodness of fit and can use several stepping procedures. GPM and OPTIME allow, for example, one to set the number of binding sites to lie between 0.8 and 1.2, or the value of the fast lifetime in a sum of exponentials, never to be less than a particular value. There is frequently available independent evidence that the constraints are applicable.

*Research supported in part by NCI Grant CA 15173.

DNA DEGRADATION KINETICS AFTER X RAYS

M. L. Randolph

Previous work on DNA degradation in *Hemophilus influenzae* cells which have been X irradiated has been extended.[1,2] The uncertainty which we find in statistical resolution between models depending on unilateral degradation vs models depending on bilateral degradation at equal rates is qualitatively explicable if one assumes that degradation is generally bilateral (which implies at least two exonucleases) acting at unequal rates in the two directions. In this framework our results are consistent with the conclusions that: (*a*) all degradation is bilateral; (*b*) in log-phase DB117 cells, degradation is much faster in one direction than the other, but in log-phase DB112 the two rates are nearly equal; and (*c*) in stationary-phase cells the degradation rate in the slower direction is greater than in log-phase cells, so that the rates become nearly equal for wild-type cells. Since the interruption rates are nearly equal for log- and stationary-phase cells, the measured degradation is faster and greater in stationary phase. The similarity of results of measurements of degradation with a temperature-sensitive strain, *dna* 9, vs wild type provides evidence that the bulk of what we measure is truly remaining DNA and not a reflection of an intracellular pool of DNA precursor or reincorporation of degraded radioactively labeled fragments. Preliminary results indicate that there is an X-ray-inducible inhibitor of degradation. If so, what we have called an "interruption (of DNA degradation) constant" probably represents the growth rate of inhibitor during incubation and simultaneous, in our work, with degradation.

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ENERGY DEPOSITION BY NEUTRONS

M. L. Randolph

When materials are irradiated by ionizing photons, each electron looks pretty much like the others. Hence for measurements of energy deposition by ionizing photons, little care need be taken to match exactly the elementary composition of the detector to that of biological tissues of interest. However, for neutron irradiations the energy deposition is entirely by a variety of nuclear interactions and is strongly dependent on the target nuclei and neutron energy. Hence for neutron irradiation work, calculation of kerma factors (essentially dose per unit neutron fluence) is necessary. Results of such calculations (done at the National Bureau of Standards by R. S. Caswell and J. J. Coyne with collaboration from here) based on 17 nuclei most important in tissue composition and the latest nuclear cross-section compilation ENDF/B-4 and covering the energy range from thermal neutrons to 20 MeV have been reported.[1] Separately a survey of variations in kerma factors at various neutron energies with variations in elemental composition of mammalian tissues reveals that the hydrogen concentration is the dominant consideration at neutron energies greater than about 1 keV and nitrogen (or perhaps ^{10}B) at energies less than about 5 eV. In the higher energy domain the kerma factor differs by less than 10% from that for standard tissue, but at low energies the factors may be considerably greater (3.5 times for kidney and 1.6 times for ox lens) or considerably less (0.35 times for liver or ovaries) than that for standard tissue. At least by implication, kerma factors are for ionizing radiation, yet at neutron energies of less than perhaps 30 keV, elastic scattering, which accounts for most of the total kerma, results in such low-energy recoil nuclei that they cannot directly cause ionization. In such cases only exothermic interactions, such as $^{14}N(n,p)^{14}C$, $^{10}Be(n,\alpha)^{7}Li$, and $H(n,\gamma)D$, can produce ionizing secondaries. In these cases, calculation of an "ionizing kerma factor" rather than the conventional "total kerma factor" may be the meaningful calculation for radiobiology.

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ADENINE PHOTOCHEMISTRY

R. O. Rahn

UV irradiation of poly dA results in the formation of one or more photoproducts.[1] It is of interest as to whether similar products are formed in DNA. The

photoproducts can be followed in poly dA by measuring the increase in absorbance at 310 nm as well as by measuring the increase in fluorescence at 430 nm. Acid hydrolysis of irradiated poly dA gives stable products as indicated by an increase in absorbance at 310 nm and a new fluorescence maximum at 388 nm. Paper chromatograms of the hydrolysates using a butanol:water:acetic acid (80:30:12) solvent showed products migrating with an R_f of 0.21–0.29. The fluorescence associated with these products was measured directly on the chromatogram. The profile obtained agreed well with that obtained using radioactive labeling procedures. A comparison of native and denatured DNA irradiated with 50,000 J/m^2 at 254 nm showed that only the latter had an appreciable fluorescence resembling that of poly dA. This result is consistent with the observation that hydrogen bonding of poly dA to poly rU quenches photoproduct formation.

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PLATINUM BINDING TO DNA

Linda L. Munchausen* and R. O. Rahn

The amount of *cis*-dichlorodiammineplatinum(II) bound to DNAs of varying (dA + dT) content was assayed by both UV absorbance spectrophotometry and the use of the radioisotope ^{195}Pt. Radioisotope labeling indicates twice as much bound platinum as do optical measurements. The molar ratio of bound platinum r at saturation is approximately half the sum of the nearest-neighbor frequencies of all base pairs that do not contain thymine. We therefore conclude that platinum does not bind to thymine in DNA. Chromatographic studies with [^{14}C]purine-labeled DNA indicate preferential binding of platinum to guanine, followed by binding to adenine. The luminescence properties of DNA and of homopolynucleotides are strongly affected by bound platinum as a result of a heavy-atom effect. A plot of the fluorescence-to-phosphorescence ratio as a function of r gives a saturation binding curve similar to that obtained using ^{195}Pt. UV irradiation of DNA treated with the platinum compound results in a 30% increase in the rate of thymine <> thymine formation. When acetophenone sensitization is employed, platinum binding enhances cystosine dimerization tenfold, presumably because the triplet level of cytosine complexed with platinum is lowered below that of acetophenone. The viscosity of DNA decreases sharply upon binding platinum, with half the change occurring when less than 6% of the bases are complexed. From the rate of reaction with formaldehyde, we conclude that binding of the platinum compound to DNA induces small denatured regions that unwind in the presence of formaldehyde with a rate about 40 times slower than that of a single-strand chain break.

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IN VITRO LABELING OF DNA WITH ^{125}I

R. S. Stafford and R. O. Rahn

Early difficulties associated with ^{125}I reaction with filter paper discs seem no longer a problem. Some material of a nonspecific nature seems to be introduced into the DNA-labeling reaction along with the addition of ^{125}I. Much of the nonspecific labeling can be reduced (without reducing the specific DNA labeling) by going to long reaction times near 24 hr. It is now possible to label ~25 ng of DNA and detect it with a tolerable "signal-to-noise ratio." Some of the problems associated with precipitating small amounts of DNA from alkaline sucrose gradient fractions have been solved. A sucrose gradient profile has been obtained for ^{125}I-labeled DNA which agrees exactly with the known distribution of DNA as determined by an intrinsic [^{3}H] thymine label.

PHOTOSYNTHESIS

W. A. Arnold* and J. R. Azzi

Joliot and Kok[1] have shown that oxygen production in photosynthesis involves a four-step process. Each step is photochemical. The delayed light that we have been studying shows this same "four-step behavior." We are trying to understand the relationship between the delayed light and oxygen production.

When chemical reactions take place, they show volume changes of ±0–20 cc/mole. Preliminary experiments are being made to see whether volume change(s) in photosynthesis can be measured. For this purpose we are experimenting with small pressure-sensitive piezo-

electric ceramic cells, which were originally developed for naval underwater sound detection research. (These materials allow the reversible conversion of mechanical and electric power.) If these experiments are successful, they will provide an entirely different method for studying the individual steps in oxygen production.

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*Consultant.

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SINGLET EXCITATION TRANSFER FROM POLY rA TO BOUND DYE AT 77°K

R. M. Pearlstein* and F. Van Nostrand†

Both singlet and triplet electronic excitation energy can be transferred from nucleic acids to bound moieties. The most efficient transfer reported is that of triplet excitation in adenosine systems at 77°K or less. The phosphorescence of polyriboadenylic acid (poly rA) in glycol-water glasses is half-quenched by paramagnetic metal ions at fractional concentrations (metal ion/adenosine), $r_{1/2} \leqslant 0.01$. Similar low values of $r_{1/2}$ are obtained for metal-ion quenching of the phosphorescence of stacked adenosine aggregates in frozen aqueous solutions. Significantly smaller values of $r_{1/2}$ are observed for such aggregates when the phosphorescence is quenched by the dye proflavine.

Singlet excitation transfer from nucleic acids to bound moieties appears to be less efficient. Studies done with nucleic acid-bound dye systems at room temperature suggest values of $r_{1/2} \sim 0.1$. Metal-ion quenching of nucleic acid fluorescence at 77°K also has been observed, the most efficient case again being stacked adenosine in ice, for which quenching by Cu^{2+} gives $r_{1/2} \sim 0.02$. No report of fluorescence quenching of covalently linked nucleic acid polymers by dyes at 77°K has yet been published, although it has been recently noted that the increased (unquenched) lifetime of the nucleic acid singlet at low temperatures should greatly increase the efficiency of singlet transfer at 77°K as compared with room temperature.

Using a frequency-quadrupled Nd:YAG laser as excitation source ($\lambda_{ex} = 266$ nm), we have observed at 77°K the quenching by bound dye of the fluorescence of poly rA frozen in an aqueous medium containing sugar. Previous experiments on excitation transfer from nucleic acids to bound dyes, done at room temperature, used aqueous solvents. Nucleic acids frozen in most aqueous media tend to aggregate, a condition we wanted to avoid in our experiments. Nucleic acids are commonly dissolved in mixtures of water with either ethylene or propylene glycol, solvents in which they do not aggregate, for low-temperature spectroscopy; however, very poor binding of ethidium bromide and proflavine to poly rA occurred in a 50% propylene glycol:water mixture. Kleinwachter and collaborators[1] obtained extensive spectroscopic evidence at 77°K that poly rA frozen in an aqueous solution with 10 m*M* acetate and 0.25% glucose is not aggregated. All our work was done with such glucose-acetate solutions (room temperature pH ≅ 7.0).

Poly rA fluorescence is half-quenched by bound proflavine or ethidium bromide at a fractional dye concentration of about 0.01 – smaller by an order of magnitude than that required to sensitize the room-temperature fluorescence of dyes bound to nucleic acid polymers. No quenching occurs of the fluorescence of similarly frozen adenosine solutions having the same absolute concentrations of base and dye.

From these results we draw the following conclusions:

(*a*) The fluorescence of adenosine is not quenched by dyes in glucose-acetate solution at 77°K, thus verifying that adenosine does not form aggregates to which dyes can bind in that frozen solution.

(*b*) Poly rA fluorescence in frozen aqueous solution at 77°K is half-quenched by either proflavine or ethidium bromide at 0.1–0.2 the *r* value required to sensitize the fluorescence of dyes bound to nucleic acids at room temperature, and less than 0.5 the *r* value required to half-quench the fluorescence of stacked adenosine aggregates at 77°K. This represents the most efficient transfer of nucleic acid singlet excitation yet demonstrated.

(*c*) Unless three-dimensional aggregation of adenosine in frozen glucose-acetate solution is facilitated by the presence of phosphodiester bonds (an unlikely possibility), the transfer of singlet excitation from poly rA to dye is *intramolecular.*

Although we have shown that poly rA can efficiently transfer its singlet excitation to bound dye and that the transfer is most likely intramolecular, we have not shown that the transfer is entirely, or even mostly, in-stack, *i.e.,* essentially one-dimensional. If it is in-stack, this certainly proves the existence of singlet exciton transfer in poly rA, because a one-dimensional transfer range of about 50 bases of approximately 150 Å is far too great to be attributed to direct Förster

transfer from adenosine to dye. (We calculated R_0 of about 20 Å for transfer to either proflavine or ethidium bromide, using the poly rA fluorescence spectrum and the measured absorbance spectra of the bound dyes.) We have not, however, ruled out the possibility that poly rA is highly folded, *i.e.*, has relatively short stacked regions, in frozen glucose-acetate solution, and thus that three-dimensional direct Förster transfer to bound dye is an important, if not principal, pathway for singlet excitation transfer in that system. Further work to distinguish these two possibilities is in progress.

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THEORY OF EXCITED-STATE INTERACTIONS IN BIOMOLECULAR AGGREGATES

R. P. Hemenger,* K. Lindenberg,† and R. M. Pearlstein‡

Biophysical information in such diverse areas as photosynthesis, radiation damage to nucleic acids, and biomolecular structure is derived from optical absorption and luminescence measurements. The optical properties of many biological systems depend on the interactions of electronically excited chromophores. If meaningful information is to be obtained from optical measurements, it is important to eliminate gaps in the understanding of the optical phenomena themselves. These gaps arise because, in the usual theoretical description of absorption phenomena, approximations are made that preclude the application of such descriptions to the subsequent time evolution of the excited state; similarly, theories that approximately describe the time-dependent behavior of excitation are inappropriate for absorption. However, both sets of phenomena are aspects of the history of the same excited state. It is therefore essential to develop a theory that unifies the description of all optical properties from absorption through radiationless processes, including especially excitation transfer, to emission. This requires the explicit inclusion of thermal effects as part of the dynamical evolution of the excited state, rather than the use of thermal relaxation as a point of demarcation between absorption- and emission-related phenomena.

We are attempting to develop such a unified theory in four stages:

1. In chromophore aggregates of biological interest, the individual chromophore spectra display no sharp features. By taking advantage of the continuum nature of these broad structureless spectra, and by initially neglecting interactions with the heat bath, we have constructed a model Hamiltonian for a dimer. Such a Hamiltonian is more tractable than those previously developed, and solutions derived from it are simultaneously applicable to absorption and to the immediately subsequent time evolution of this optically prepared state.

It has been experimentally established that there are substantial alterations of chromophore absorption spectra upon aggregation. We expect our theory at this stage of development to explain spectral changes of dimers at long wavelengths, presently not well understood. Also it should be possible to predict whether or not prerelaxation transfer occurs in aggregates for which the spectral properties of the individual chromophores are well known.

2. The relaxation process, particularly in a solution, is a very complicated one and not at all well understood. As a simple first approximation, we have introduced the heat bath phenomenologically as the inducer of transitions among the stationary states. These transitions are characterized by a single thermal relaxation rate parameter, the constraint that a Boltzmann distribution be attained at long times, and by other physical constraints. Further developments might include more complicated features of the relaxation process, such as the possible existence of microscopic "hot" regions containing optically excited chromophore and solvent whose effective temperature is higher than the equilibrium heat bath temperature.

We plan to investigate features of the transitions induced by the heat bath that may have new experimental consequences. In particular, these transitions provide interchromophore excitation transfer pathways whose consequences have not been investigated.

3. It would of course be desirable to introduce the heat bath dynamically, *i.e.*, as part of the model Hamiltonian, rather than phenomenologically. We plan to explore the feasibility of this more general approach along the lines of the model Hamiltonian used by Grover and Silbey.[1] As new quantitative experimental information arises, it becomes increasingly important to develop a model theoretical approach to the problem of vibrational relaxation.

4. We plan to extend our general theory to larger aggregates in ways specifically suited to comparison with experimental measurements.

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ANALYSIS OF FLUORESCENCE DECAY DATA

R. P. Hemenger* and T. J. Mitchell†

A fundamental problem in extracting useful information from measurements of the time course of molecular fluorescence is removing the influence of the detailed shape of the exciting light. These quantities are connected by the convolution integral

$$p(t) = \int_0^t dt'\, q(t - t')\, f(t') . \qquad (1)$$

In Eq. (1) $p(t)$ is the measured time course of molecular fluorescence, $q(t)$ is the measured time course of the exciting light, and $f(t)$ is the delta function response, *i.e.*, it is the fluorescence which would occur if the exciting light were a pulse of zero width occurring at time zero. The function $f(t)$ contains all the interesting physics of the system without any distortion due to details of the actual exciting flash function.

Formal solutions of Eq. (1) for the delta function response $f(t)$, using raw data for $p(t)$ and $q(t)$, are readily found. However, these solutions have not been useful, because it is in the nature of Eq. (1) that small distortions in $p(t)$ and $q(t)$ (*i.e.*, experimental error) lead to enormous distortions in $f(t)$. Therefore, a large number of more-or-less indirect methods of extracting information about the delta function response of fluorescent systems have been proposed.

In most of these methods $f(t)$ is taken to be an explicit function of a small number of parameters of more-or-less direct physical interest. The problem remains of using Eq. (1), with $p(t)$ and $q(t)$ determined experimentally, to find the best values of the parameters. Thus, for example, it is usually assumed for fluorescence decay experiments that $f(t)$ is a sum of a small number of decaying exponentials. The coefficients and decay constants of these exponentials are the parameters of physical interest.

Several more general deconvolution methods have been proposed. In every case restrictions of some sort, such as smoothness conditions, must be placed on allowed solutions. The difficulty with these methods is that the class of allowed solutions is not well defined.

We have developed a fairly general deconvolution method and have applied it to a number of sets of real fluorescence decay data taken on systems of biological interest. Our procedure determines the best solution to Eq. (1) in a least-squares sense, subject to a system of linear constraints. This system of constraints defines precisely the set of possible solutions (delta function responses). This set is just the set of all (finite or infinite) sums of decaying exponentials with positive coefficients. This class of functions contains a wide range of physically interesting fluorescence decay processes.

This general deconvolution method has three advantages over the more common procedure of fitting the data directly to a specific model:

(*a*) It is applicable to situations for which no simple model is available.

(*b*) In practice this method is useful in revealing systematic errors in the data due, for example, to alterations in lamp characteristics.

(*c*) Fluorescence decay data fitted to a definite model, such as a sum of two exponentially decaying terms, may be used to estimate the lifetimes involved. However, it is usually difficult to decide from the raw data whether such a model is appropriate. The results of our deconvolution method using real data have shown immediately in each case whether a one- or two-decaying exponential model is appropriate, or whether a more complicated model is required. Moreover, our method has been useful in finding initial model parameters, *i.e.*, lifetimes, to be used in further attempts to fit the data to some definite model.

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APPLICATIONS OF MULTIPLE SCATTERING METHODS TO BIOLOGY

R. P. Hemenger*

Several results have been obtained in the general theory of multiple light scattering which have potential usefulness in biology:

(*a*) The leaf is an example of an intact biological structure in which structural irregularities strongly affect optical properties. The primary observation is that an intact leaf is a much more efficient absorber of

sunlight than a suspension of its extracted pigments.[1] The absorption spectrum of a leaf, as well as its other optical properties, can be explained very well by a simple two-layer model. The upper layer of this model contains most of the pigments, has an index of refraction greater than unity, and has no scattering properties. The lower layer contains the remaining pigments and scatters light intensely, *i.e.*, the photons entering this layer are scattered several times before being either reflected, transmitted, or absorbed. This model agrees in its general features with the actual structure of a typical leaf whose upper layer (palisade layer) is a fairly regular arrangement of elongated cells and whose lower layer (spongy tissue) consists of irregularly arranged cells separated by large air spaces.

(*b*) An interesting feature of the light reflected diffusely from a surface of a scattering medium is that the type of information to be gained from measuring this light depends on how much of this light is collected. Let R be that distance in the scattering medium over which a photon "forgets" its original direction because of being scattered repeatedly. If the diffusely reflected light is collected over an area of the reflecting surface with linear dimensions much greater than R, as is usually done, then the reflectance is a sensitive function of the absorption properties of the medium, as the reflectance oximeter shows. If, on the other hand, reflected photons are collected only within a distance R of where they originally entered the surface, one finds a very simple and potentially useful result. The intensity of reflected light is then proportional to the concentration of scattering centers in the medium times the mean cross section for scattering of these centers. This accounts, for example, for the ability of a simple fiber optic instrument to measure accurately the concentration of bacterial cells in suspension over a very wide range of optical densities.[2]

(*c*) Measurements of light transmitted through a slab of intensely scattering material may also yield useful results. For example, using Beer's law to find the absorption spectrum of pigment-containing cells in dilute suspension leads to results severely distorted by scattering by the cells. However, if the absorbing cells are mixed with a suspension of nonabsorbing but intensely scattering particles, then the transmittance of this mixture can be used, at least in principle, to obtain the undistorted absorption spectrum of the cells.

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LOCALIZED SYNTHESIS OF ZEIN IN MAIZE ENDOSPERM

Benjamin Burr and Frances A. Burr*

Electron micrographs show that protein bodies, distributed throughout the internal layers of the developing endosperm, are entirely limited by a single membrane and have polyribosomes at their surface. Protein bodies isolated from other cytoplasmic organelles and from matrix protein contain zein as their major protein component and continue to be associated with RNA. The polyribosomes can be separated from the protein bodies by detergent treatment. They are active as the particulate component in *in vitro* protein synthesis but will not incorporate radioactive amino acids in the absence of added soluble factors or in the presence of the inhibitor puromycin. Zein is the only protein formed because (1) lysine and tryptophan, absent from zein, are not incorporated, and (2) completed chains migrate with zein in SDS gel electrophoresis.

This work demonstrates a specialized apparatus for the synthesis of the largely insoluble zein protein close to its site of deposition in the protein bodies. Because zein, which lacks the above-mentioned amino acids essential to health, makes up 50% of the seed protein, regulation of its synthesis is important for nutritional reasons. There are probably a number of processes peculiar to the zein protein body system. Conceivably, mutations blocking any one of the zein synthesis steps could prevent the accumulation of zein and thus explain why a number of loci condition the *opaque* phenotype.

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MUTAGENESIS FOR PROTEIN COMPOSITION IN SOYBEANS

D. E. Foard, Wen-Kuang Yang, L. L. Triplett, and C. D. Stringer

Methionine is present in soybean storage proteins in limiting amounts for human nutrition; free methionine is absent in mature seeds. We are trying to induce, by radiation and chemical treatment, mutants which have higher levels of protein-bound methionine. The greatest problem continues to be the lack of a reliable and rapid quantitative screening assay for methionine. Since other methods have thus far been of limited usefulness, we are trying to develop an immunological assay. This

presupposes that in the seed one protein, higher in methionine than any other, is present in suitable concentration. It is believed that such a protein does exist and that antibodies against it can be prepared.

Sephadex G-100 column chromatography has been used initially to attempt the isolation and characterization of such a protein from extracts of defatted soybean meal. Five peaks were resolved by this method. The pooled fractions of each peak were dialyzed, lyophilized, and hydrolyzed in preparation for quantitative amino acid analysis. Methionine was present in the first three peaks only and in the greatest relative amount in the third of these peaks.

IMMUNOLOGICAL STUDIES OF SOYBEAN LECTINS

D. E. Foard, Frances M. Tate,*
and Wen-Kuang Yang

Neither the role of lectins in legume seeds nor their fate during seed germination is known. Using soybeans, we have begun a study of these problems. Our aims are to develop immunologic methods to detect lectins in soybean seeds and seedlings and to use this knowledge to determine the role and fate of lectins. Such information might be used to test the postulate[1,2] that lectins are the binding mechanism for the nitrogen-fixing bacterium *Rhizobium,* which invades and forms nodules on the soybean's roots.

The two lectin fractions used in this study are peaks IV and VIII isolated on a DEAE-cellulose column, as has been previously described.[3] Two different crude extracts of lectins from seeds were obtained – one with a pH 4.7 buffer, the other with a pH 7.5 buffer. Extracts of lectins were made from germinating seedlings 1 to 12 days after moistening the seeds.

Solutions of lectin fractions were injected subcutaneously into rabbits at intervals of two weeks over a period of eight weeks; final injections were intravenous. Antiserum was prepared from blood taken periodically from the rabbits' ears.

Double diffusion on Ouchterlony plates was used to characterize the lectin antigens and antibodies. The antigen-antibody interaction is specific, *i.e.,* there was no cross-reaction between antilectin IV and lectin VIII or 11S globulin. The appearance of diffuse precipitin bands indicated that the lectin preparation either was not completely pure or was a mixture of more than one component. Although the double diffusion test is highly sensitive, it is not able to resolve a mixture of antigens. For this reason, immunoelectrophoresis was used to test the purity of the lectin preparations.

Immunoelectrophoresis of lectin IV produced one major and one minor precipitin arc, indicating that the preparation was a mixture. To determine whether the two components of fraction IV are both lectins, rabbit red blood cells were mixed with fraction IV, washed extensively, and subsequently eluted with pH 4.5 buffer to release the bound lectins. Immunoelectrophoretic analyses of the eluate revealed the same – one major and one minor precipitin arc – indicating that both are isolectins.

Hemagglutination was used as a functional assay of lectins for samples extracted from the seedlings. Positive results were obtained only with extracts of cotyledons and roots; epicotyl and hypocotyl extracts showed no hemagglutinating activity. A marked decrease in hemagglutination activity in cotyledons occurred as the seedling aged. Twelve days after the seed was moistened, no hemagglutination activity was evident in extracts of cotyledons; however, the root extract, which was detected at low titers in the early stages of germination, remained active.

The presence of lectin fractions IV and VIII in seedlings has been assayed using the radial immunodiffusion assay, in which antilectin sera are incorporated in agar into which wells are punched to hold antigen preparations. A circular precipitin spot forms around the well where antigen and antibody combine. The area of the spot is proportional to the concentration of the antigen. Comparing the areas of these spots with those formed by known concentrations of lectins, a quantitative measure of lectin concentrations in the extracts was obtained. Positive results were found only in extracts of cotyledons. Fraction IV lectin was present at a concentration of 0.218 mg per 100 mg of wet weight on the first day after soaking the seed, and at 0.200 mg per 100 mg of wet weight on the third day. It was undetectable thereafter. Fraction VIII lectin was present in a concentration of 1.383, 2.050, 0.966, 0.533, and 0.266 mg per 100 mg of wet weight on 1, 3, 6, 9, and 12 days, respectively, after soaking the seed.

Immunofluorescence has been employed in a preliminary manner using thin sections of different organs of the seedlings. After adding fraction IV antiserum to the sections, coat antirabbit immunoglobulin labeled with fluorescein was applied. Sections examined under the UV microscope showed especially strong fluorescence in the cotyledons and the epidermis of the roots. We need to refine our methods before drawing firm conclusions from our initial observations.

Some of the results obtained in this study indicate that further detailed experiments would be useful in testing the postulated binding of *Rhizobium* to the root by means of lectins.

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EXCISION REPAIR OF UV-INDUCED PYRIMIDINE DIMERS IN THE DNA OF PLANT CELLS

G. P. Howland

Last year we reported that isolated protoplasts of cultured wild carrot cells could efficiently repair DNA strand breaks induced by ionizing radiation.[1] We also reported the first evidence of DNA repair replication in plant cells following UV irradiation; however, direct assays of pyrimidine dimer excision gave equivocal results.[2] Difficulties with the dimer analyses were overcome with a more effective DNA labeling procedure.[3]

In the wild carrot protoplasts, thymine-containing dimers are initially induced at a rate of 0.002% for a UV dose of 1 J/m^2. The 24-hr excision data fit a curve showing 100% dimer removal after low doses (*e.g.*, 14 J/m^2), but the proportion of dimers excised is reduced to about 2% at the higher UV doses.[4] The efficiency of dimer excision after low UV doses is comparable to that observed by Regan and Carrier[5] in cultured human cells. Previous failures to detect dimer excision in plant cells possibly resulted from the high UV doses employed in those studies (cf. ref. 4).

Although the disappearance in the dark of dimers from the acid-insoluble cell fraction is generally accepted as evidence of excision repair, the clearest proof lies in the simultaneous demonstration of excised dimers appearing in the acid-soluble cell fraction (Fig. 4). These data also indicate that, after a UV dose of 42 J/m^2, dimer excision in these cells begins with a high initial rate and proceeds with decelerating kinetics. Under the conditions of our experiments, no further excision is detected after 24 hr, even though many dimers remain in the cells' DNA (Fig. 4 and ref. 4), and

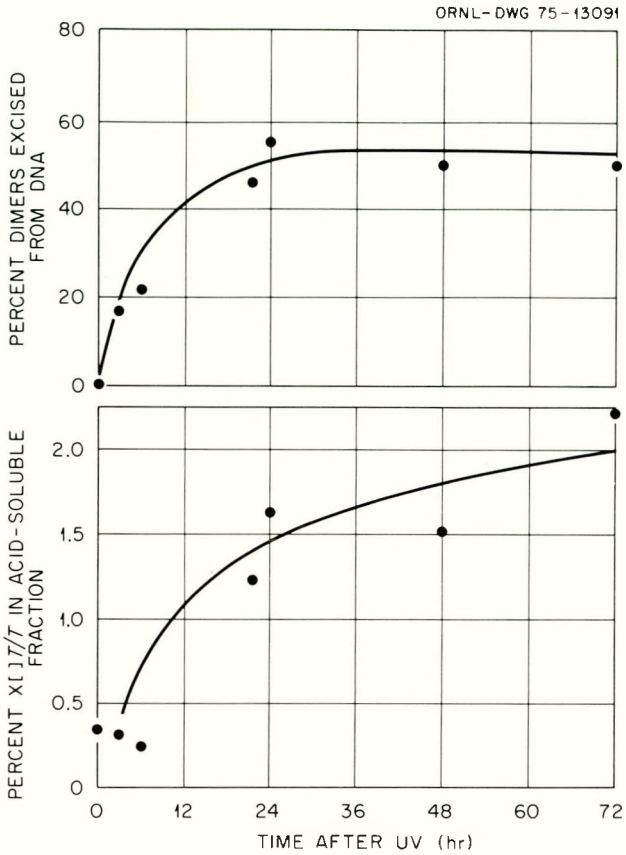

Fig. 4. Time course of pyrimidine dimer excision (UV fluence = 42 J/m^2) from the DNA of wild carrot protoplasts (top) and appearance of excised dimers in the cold-acid-soluble cell fraction (bottom).

$$\%X[\,]T/T = \frac{\text{radioactivity in thymine-containing dimers}}{\text{radioactivity in thymine}} \times 100$$

the protoplasts continue to give a positive vital stain reaction.

A wide range in excision repair capacity has been observed among different animal species,[6] so a similar variation might be expected among plant species. We have begun to examine a variety of cultured plant cells for the ability to excise UV-induced dimers. Substantial dimer excision has been found in cells of *Haplopappus* and tobacco, and in isolated Petunia protoplasts.

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FLUORODEOXYURIDINE INHIBITS THYMIDINE DEGRADATION AND STIMULATES ITS INCORPORATION INTO PLANT CELL DNA

G. P. Howland and Margaret L. Yette

Cultured plant cells degrade exogenously supplied thymidine (dThd). 5-Fluorodeoxyuridine (FdUrd) can inhibit this degradation while at the same time enhancing dThd incorporation into DNA.[1] Since reporting on this observation last year, we have characterized the FdUrd inhibition of [2-^{14}C]dThd degradation by polyacrylamide gel chromatography. (J. X. Khym helped to set up these analyses.) After a 20-hr incubation in the presence of 10^{-5} *M* FdUrd and [2-^{14}C]dThd, 93% of the ^{14}C activity in the acid-soluble cell fraction of cultured wild carrot cells is chromatographically identical with [2-^{14}C]dThd; while in the absence of added FdUrd, less than 10% is. These results are discussed more fully elsewhere.[2]

Routine utilization of 10^{-5} *M* FdUrd during a 20–24-hr incorporation by cultured cells of [*Me*-^{3}H]dThd (5 μCi/ml) has yielded wild carrot DNA with a calculated specific radioactivity of 2×10^6 ($\pm 5 \times 10^5$) cpm per μg of DNA. This level of incorporation has permitted the application of sensitive assays for DNA repair in plant cells.[3]

As an extension of our studies on pyrimidine metabolism, we will determine the extent of uridine and bromodeoxyuridine degradation by plant cells.

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PREFERENTIAL SYNTHESIS OF CYTOPLASMIC DNA IN ISOLATED WILD CARROT PROTOPLASTS

G. P. Howland and Margaret L. Yette

Cytoplasmic DNA (*i.e.,* chloroplast and mitochondrial) is a minor component of the total cellular DNA and is labeled along with nuclear DNA when [^{3}H]thymidine is supplied to cultured cells of wild carrot (*Daucus carota*). In protoplasts isolated from these cells, DNA synthesis is initially reduced to less than $^{1}/_{10}$ the rate in growing cells, increasing to the normal rate by 24 hr after isolation. When protoplasts are isolated and cultured in the presence of certain drugs (*e.g.,* gentamycin, 50 μg/ml; amphotericin B, 2.5 μg/ml), [^{3}H]dThd incorporation into nuclear DNA (single-strand weight-average mol wt *ca.* 150×10^6 daltons) is inhibited, while synthesis of cytoplasmic DNA (30×10^6–50×10^6 daltons) is apparently unaffected. This conclusion is based on analyses of [^{3}H]dThd-labeled DNA on alkaline sucrose gradients, on CsCl gradients, and by autoradiography (autoradiographs prepared by Stella A. Perdue).

Blamire *et al.*[1] have also reported that a variety of drugs, thought to have some degree of specificity in inhibiting chloroplast RNA or protein synthesis, allow preferential labeling of chloroplast DNA in *Chlamydomonas* by reducing incorporation into nuclear DNA.

We wish to use this technique in probing the fate of mutagen-induced lesions in chloroplast DNA.

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ETHYLENE AND CO_2 CONTROL OF FERN-SPORE GERMINATION

M. E. Edwards*

(Sponsored by G. P. Howland)

Regulation of cell division by ethylene, a gaseous plant hormone, is being studied in axenic cultures of sensitive fern (*Onoclea sensibilis* L.) gametophytes. Unicellular spores are inhibited from germinating by the presence of ethylene. In several multicellular plant systems, carbon dioxide reverses ethylene inhibition and is thought to act competitively with ethylene. Using sterile fern spores, the effects of carbon dioxide *per se* and the combined effects of carbon dioxide and ethylene are under investigation.

Spores are sown aseptically on 10 ml of Knops solution in culture tubes, which are sealed individually in 2000-ml desiccators and cultured at 25°C under *ca.* 400 ft-c of white fluorescent light. Desiccators may be partially evacuated through a side-arm port where given amounts of ethylene or carbon dioxide may be introduced by syringes. If desired, beakers of aqueous 10%

mercuric perchlorate or 10% potassium hydroxide may be set inside a desiccator to remove atmospheric ethylene or carbon dioxide respectively. Germination is scored after 50 hr with the aid of an acetocarmine chloral hydrate stain.

To determine the effect of carbon dioxide *per se* on germination, spore cultures are placed in desiccators containing beakers with the ethylene-absorbant mercuric perchlorate. Results from such conditions indicate that spores tolerate carbon dioxide atmospheres of 2.0% without inhibition of germination. Higher concentrations are progressively inhibitory. Less than one-fourth of the spores germinate in 10% carbon dioxide, and none germinate in carbon dioxide atmospheres greater than 15%. Inhibition by carbon dioxide is reversible: If spores are removed from a carbon dioxide-rich environment and placed in normal atmospheric conditions of a growth chamber, complete recovery from carbon dioxide inhibition of germination results within the following 48 hr.

The combined effects of carbon dioxide and ethylene on the initial cell division of fern spores are also under investigation. To date, results have been obtained with a single ethylene concentration, 1 ppm, which I reported earlier inhibits spore germination *ca.* 50%. Preliminary data indicate that the ethylene effect can be influenced by carbon dioxide. In carbon dioxide-reduced atmospheres, ethylene inhibition is enhanced; in carbon dioxide-enriched atmospheres, ethylene inhibition may be partially reversed. Increasing atmospheric carbon dioxide concentrations from zero (CO_2 absorbed with KOH) to 1.0% (carbon dioxide added) brings about a decrease of ethylene effectiveness. However, above 1.0% carbon dioxide, reversal of ethylene inhibition does not occur, and increased inhibition of germination results. Thus, the effect on germination of 1 ppm exogenously added ethylene depends upon the concentration of carbon dioxide in the surrounding environment.

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SECTION III

GENETICS AND DEVELOPMENTAL BIOLOGY

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Mutagenesis and Cytochemistry

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Drosophila Recombination and Chromosomal Behavior

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Structure and Function of Aromatic Multienzyme Systems

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Electron Microscopy and Cell Biology

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Reproductive Biology

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HYDRAZINE MUTAGENESIS IN *HAEMOPHILUS INFLUENZAE*

R. F. Kimball and Bernice F. Hirsch

Our studies on hydrazine mutagenesis, part of which were reported last year,[1] have been continued. Further work on mutation "fixation," using lysates made at various times after mutagen treatment and measuring mutation in bacteria transformed with these lysates, has failed to confirm the existence of the mutation maximum at intermediate times mentioned in the previous report. Instead, mutation in the transformants increases to a maximum as the time between treatment and lysis increases just as in the earlier work with nitrosocarbaryl.[2]

Almost all the fixation seems to be associated DNA replication, since in a temperature-sensitive DNA synthesis mutant very little fixation occurs at the restrictive temperature. However, in experiments designed to avoid any residual replicative synthesis, no fixation was found. Thus the only fixation is at replication.

We have been unable to detect any single-strand breaks or alkaline labile sites in the DNA of bacteria treated with hydrazine. We have also been unable to find any evidence for postreplication gaps. In both respects, hydrazine differs markedly from alkylating agents such as nitroguanidine and nitrosocarbaryl. We have also been unsuccessful in finding any evidence for excision repair despite the evidence for loss of premutational damage when DNA replication is delayed. However, it is not clear how well one would detect very short stretches of repair replication in this system.

The evidence all suggests that hydrazine acts to make base analogues within the DNA and that these produce mutations directly by mispairing at replication rather than by errors in postreplication repair.

1. R. F. Kimball and B. F. Hirsch, *Biol. Div. Annu. Prog. Rep. June 30, 1974,* ORNL-4993, pp. 69–70.
2. K. L. Beattie and R. F. Kimball, *Biol. Div. Annu. Prog. Rep. June 30, 1974,* ORNL-4993, pp. 67–68.

DEVELOPMENT OF AN AUXOTROPHIC MUTATION SYSTEM FOR *HAEMOPHILUS INFLUENZAE* AND ITS USE TO COMPARE THE MUTAGENIC EFFECTS OF HYDRAZINE AND *N*-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE

R. F. Kimball

Last year[1] it was reported that two proline auxotrophs had been isolated, both of which were closely linked to a locus for thymine requirement. It has now been shown that the two mutants are very closely linked to each other, give a detectable but very low rate of proline independent recombinants, and are probably mutants at different sites in a proline locus.

As reported previously, one of the two auxotrophs has a low (*proB1*) and one a high (*proB2*) rate of both spontaneous and induced reversion. It has now been shown that *proB2* is a suppressible mutant and that about 90% of the reversions are unlinked suppressors. The frequency of true revertants is, however, quite high enough to allow detection by linkage to *thy*$^+$ in a transformation assay. Thus the system allows simultaneous and separate assay for forward mutations at suppressor loci and revertants at the *proB2* site. The *proB1* mutant gives a low but usable frequency of both spontaneous and induced reversion, entirely by reversion at the locus.

Comparison of the induction of mutation by MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) and HZ (hydrazine) shows that the *proB2* systems, both true revertants and suppressors, give very similar kinetics to the previously studied mutations to novobiocin resistance. With MNNG, the mutations increase as a power of the dose between two and three and reach a high maximum at a few percent survival. HZ gives slowly rising curves that reach a low maximum extending over a wide exposure range within which no cell killing occurs. The *proB1* system gives similar kinetics for HZ. With MNNG, however, the mutations increase linearly with dose, and a relatively low maximum is reached at about 30% survival. These results show (*a*) that the peculiar features of HZ mutagenesis are not confined to one mutation system but are general and (*b*) that MNNG can induce mutation with different kinetics and so probably by different mechanisms in different mutation systems.

1. R. F. Kimball, *Biol. Div. Annu. Prog. Rep. June 30, 1974,* ORNL-4993, p. 70.

MUTAGENESIS WITH NITROSO COMPOUNDS

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and Jane K. Setlow†

Mutagenesis with nitroso compounds is being studied as a function of chemical structure, experimental conditions, and genetic background in two species of bacteria, *Escherichia coli* and *Haemophilus influenzae*.

Structure-activity relationships. – (*a*) Binding experiments: Three nitroso compounds of differing biological activity (nitrosomethylurea, NMU; nitrosomethylnitroguanidine, MNNG; nitrosocarbaryl, NC) have been found to bind differentially to both species of bacteria. The order of binding of radioactive compound follows the order of mutagenicity in both species, but only in *E. coli* does the relative binding correlate well with the biological results. In *H. influenzae* the differences in binding are not great enough to account for the differences in mutagenicity, nor do the binding results reflect the differences in mutability between the two bacteria. For example, whereas the binding of nitrosomethylurea to the two species of bacteria is very similar, *E. coli* is mutagenized at a concentration tenfold lower than that required to mutate *H. influenzae*. From these experiments we conclude that the structure-activity relationships seen for mutagenesis by these compounds are due only in part to differential uptake of the compounds. Other factors such as metabolism and reactivity of the compounds at different genetic loci must also be important.

(*b*) Isotope effect: The mutagenicity of deuterium-substituted (CD_3-methyl) nitroso compounds has been compared with that of the nondeuterated analogues. No differences were seen in *E. coli* or *H. influenzae* under several different sets of conditions. We conclude that transfer of the intact methyl group without loss of a proton is involved in the mechanism of mutagenesis. These results are interesting in light of the deuterium isotope effect found for tumor induction with nitroso compounds, suggesting that the mechanisms for mutagenesis and carcinogenesis are different.

(*c*) Test of carbamate insecticides: Nitroso derivatives of six carbamate insecticides were tested for mutagenicity in *E. coli*. All were mutagenic at concentrations between 10^{-4} and 10^{-5} *M*. Some of the compounds were more potent than nitrosocarbaryl and some were less so, the differences being within about one order of magnitude of concentration. *H. influenzae* is considerably more sensitive to nitroso carbamates than is *E. coli*. These results are relevant to the consideration of new pesticides as substitutes for DDT.

Genetic background. – The role of genetic background in mutagenesis by nitroso compounds is being studied in various repair-deficient mutants of *E. coli*. No mutant has been tested in which nitroso compounds are not mutagenic, in contrast to other mutagenic chemicals and radiations. Some evidence has been obtained that two polymerase-defective mutants are less mutable with nitroso compounds and that the dose response follows different kinetics. Experiments with a temperature-sensitive DNA synthesis mutant have not provided evidence of repair under the conditions used.

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STUDIES ON MUTATION INDUCTION IN PHAGES OF *HAEMOPHILUS INFLUENZAE*

M. E. Boling and R. F. Kimball

During the past year, mutation experiments were initiated with two *Haemophilus* phages, HP1c1 and S2. The basic goal was to compare results with the phages with those obtained in the whole cells. Both phages are lysogenic; consequently it is, in principle, possible to compare the results when the phages are treated outside the cell and then replicate vegetatively inside the cell after infection, when they are treated inside the cell and multiply vegetatively, and when they are treated as lysogens and replicate subsequently as part of the bacterial chromosome. For both phages, three temperature-sensitive mutants were available and have been used to measure mutation by reversion to temperature insensitivity. Attempts to isolate suppressible mutants are in progress.

So far, four potential mutagens – hydrazine, ethyl methanesulfonate (EMS), MNNG, and UV – have been tested in one or more systems. The tests with hydrazine are incomplete but so far are negative, in contrast to the positive results with the bacteria. The tests with EMS were also negative even at fairly high exposures to extracellular phages and to lysogens. MNNG produced a high level of mutation when lysogens or infective centers were treated but not when the phages were treated extracellularly. UV produced no mutations when lysogens or extracellular phages were irradiated. This negative result is especially interesting because *H. influenzae* itself is not mutated by UV. One possible interpretation of these negative results is that this

bacterium lacks the inducible error-prone repair system that seems to be responsible for mutation induction in *E. coli* by UV.

It would be desirable to distinguish between mutation fixation during replication of the prophage as opposed to fixation during vegetative replication of the phage, since these two kinds of replication employ different polymerases. Lysogens were treated with MNNG, which induces some prophage, and the reversion rate was measured at intervals thereafter. As phage production increases sharply and then returns to the spontaneous induction level, the phages appearing in the medium represent, at first, prophages which were induced immediately and replicated in the cytoplasm and, later, prophages that replicated with the cell genome first before being induced. The initial data suggest that the reversion rate with MNNG may be much the same for phages produced over this whole period. In other words the mode of replication may have little effect on fixation.

REPAIR AND UV MUTAGENESIS IN *ESCHERICHIA COLI*

Donna L. George*

The *mfd* mutant of *Escherichia coli* has a markedly reduced ability to exhibit mutation frequency decline (MFD), the rapid, irreversible loss of potential suppressor mutations which occurs when protein synthesis is briefly inhibited after UV irradiation. This strain is 4–10 times more UV mutable than its *mfd*$^+$ parent strain, although it has the same UV resistance. In an attempt to better understand the processes involved in mutagenesis initiated by UV irradiation, we have studied the properties of the *mfd* strain. We have found that after a UV dose of 40 J/m^2, resulting in a 1–5% survival level, the rate of pyrimidine dimer excision in the *mfd* mutant is only about one-third that of its *mfd*$^+$ parent strain. Therefore, elevated UV mutability in this strain is associated with a slow rate of dimer excision.

Another mutant of *E. coli*, designated *polA107*, is deficient in the 5′-3′ exonuclease activity associated with DNA polymerase I and also has a slow rate of dimer excision. To compare the effects of the slow dimer excision resulting from the *polA107* mutation with those associated with the *mfd* mutation, we have introduced the *polA107* allele into the isogenic *mfd*$^+$ and *mfd* strains by P1 transduction. In contrast to the *mfd* mutant, the *polA107* strain is slightly more UV sensitive than the *mfd*$^+$ *polA107*$^+$ strain, but it exhibits a normal level of UV mutability and has a normal rate of MFD. The *mfd polA107* double mutant exhibits a level of UV mutability characteristic of the *mfd* allele and a UV sensitivity characteristic of the *polA107* allele.

However, when these various strains are used to assay the survival of UV-irradiated bacteriophage T1, the sensitivity of T1 increases in the order *mfd*$^+$ *polA107*$^+$, *polA107*, *mfd*, *mfd polA107*. Therefore, the T1 system may be a sensitive assay to further characterize the nature of the defect in the *mfd* strain and in the *polA107* strain and may provide some insight into the problem of why elevated UV mutability accompanies the slow dimer excision in the *mfd* strain, but does not accompany the slow dimer excision caused by the *polA107* mutation.

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MUTAGENIC EFFECTS OF *cis*-DICHLORODIAMMINEPLATINUM IN REPAIR-DEFICIENT MUTANTS OF *ESCHERICHIA COLI*

Donna L. George* and Linda L. Munchausen†

cis-Dichlorodiammineplatinum II (*cis*-DDP) was among the first inorganic antitumor drugs submitted for clinical trials. We have attempted to better understand the biological activity of this antitumor agent by analyzing its lethal and mutagenic effects on a number of repair-deficient mutants of *Escherichia coli* (*uvrA*, *exrA*, *polA1*, *polA107*).

E. coli uvrA cells (deficient in UV-endonuclease) and *exrA* cells are much more sensitive to the lethal effects of both UV irradiation and *cis*-DDP treatment than are wild-type cells. Comparable to the situation found after UV irradiation, *cis*-DDP induces more mutants (Tyr$^+$ revertants) per survivor at lower concentrations in *uvrA* cells than in wild-type cells, and induces no detectable mutants in *exrA* cells. These results suggest that *cis*-DDP-induced lesions are repairable, that the UV-endonuclease coded for by the *uvrA*$^+$ gene is involved in the repair of at least some of these lesions, and that mutagenesis initiated by *cis*-DDP has some features in common with UV mutagenesis.

However, there are important differences between the actions of *cis*-DDP and UV irradiation in *E. coli*. The mutants *polA1* and *polA107* are deficient in the polymerase and 5′-3′ exonuclease activities, respectively, associated with DNA polymerase I, and are 3–5 times more UV sensitive than *pol*$^+$ cells. In contrast, the

lethal and mutagenic effects of *cis*-DDP on the mutants and on *pol*$^+$ cells are very similar.

The level of mutability induced by several concentrations of *cis*-DDP in a number of strains of *E. coli* was about 20-fold lower, at comparable survival levels, than after exposure of the cells to UV irradiation. Also, whereas over 85% of the UV-induced Tyr$^+$ reversions in wild-type cells are due to second-site suppressor mutations (based on their ability to support growth of amber and ochre mutants of bacteriophage T4), only 40–50% of the *cis*-DDP-induced Tyr$^+$ reversions are suppressors.

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AUTORADIOGRAPHIC STUDIES OF THE CELL CYCLE IN CONFLUENT CULTURES OF CHINESE HAMSTER OVARY CELLS

R. F. Kimball, Stella W. Perdue, Patricia A. Brimer, and A. W. Hsie

This research is a continuation of our effort to get as complete an understanding as possible of the composition of the cell cycle of mammalian cell populations that have reached confluency and are increasing in numbers, at most, very slowly. Among the questions to be answered is whether such cultures contain a fairly uniform population of cells undergoing a prolonged cell cycle or whether these cultures are a mixed population with a fraction of the cells undergoing a reasonably short cell cycle and the remainder noncycling. For this purpose, it would be desirable to label separately those cells that synthesize DNA in each of two or more successive periods, *e.g.*, one day. If most cells that label at all do so in both periods, then some cells are probably cycling, some not. If only a fraction of the cells are labeled in both periods and if the fraction of labeled cells increases with time, then it is likely that many or all cells are undergoing a cell cycle that is long compared with the labeling period.

A new procedure for autoradiographic distinction of such labeling classes was developed after unsatisfactory trials with published double labeling procedures. This new procedure is described by Stella Perdue elsewhere in this report. It has been tried with CHO cells grown with daily medium renewal to confluency. In such cultures a sufficiently large fraction of the cells label each day so that estimation of labeling classes can be made from the total frequency of labeled cells as well as from counts of labeled classes in autoradiographs, thus providing a check on the method. The general conclusions were that the procedure worked reasonably well, though some corrections are needed to allow for errors in classification, that the great majority if not all cells in such cultures are undergoing a prolonged cell cycle, and that not only G_1 but S and G_2 as well are prolonged.

COMBINED TRITIUM AND CARBON-14 AUTORADIOGRAPHY

Stella W. Perdue

The distinction between ^{3}H and ^{14}C radioisotopes in autoradiographs would allow a wide range of applications in biological research where comparisons of the same precursor under different conditions or different precursors are required. Published procedures require (*a*) double dipping of autoradiographs and exposure times of several months to a year, (*b*) excessive ^{3}H label in comparison to ^{14}C label, and (*c*) use of nuclear track emulsions that are not readily available. Our experimental design requires equal ^{3}H- and ^{14}C-thymidine label indexes for cell cycle analysis of confluent CHO tissue cultures. Therefore, a more satisfactory method was developed.

Double-dipping autoradiographic procedures are dependent upon recording the short-track ^{3}H decay events in the first film layer with a minimum of ^{14}C decay events, followed by a second film layer with a long exposure time to record the longer-track ^{14}C decay events. A single expanded-film autoradiographic technique which would permit classification of ^{3}H- only, ^{14}C- only, and ^{3}H- plus ^{14}C-labeled cells would require less exposure time and dependence upon low-level ^{14}C label. Kodak NTB nuclear track emulsion was chosen for use, since it has a low background and is readily available. Film depth lost during development and the usual dehydration procedures can be restored by soaking the autoradiographs in 20% glycerol and mounting the cover glasses with glycogel. Optical sectioning to distinguish grain depth in a single film layer is done with oil-immersion microscopic examination. Silver grains from ^{3}H decay events are randomly arranged in the first three focal depths directly above the nucleus. Silver grains from ^{14}C decay events occur as clusters and tracks, with some random grains through several focal depths surrounding the cell. The differences in the horizontal and vertical distribution of silver grain patterns produced by the ^{3}H and ^{14}C decay events are sufficient to readily distinguish ^{3}H from ^{14}C

label. Double-labeled cells can also be classified, but with greater difficulty, especially in the cases of low ^{3}H label and a high ^{14}C label. This technique greatly reduces the time required for completion of experimental results and is less restrictive of ^{3}H and ^{14}C label levels, thus permitting wider experimental applications.

PRODUCTION BY CHEMICALS AND TRANSMISSION OF CHROMOSOMAL ABERRATIONS IN MAMMALIAN GERM CELLS

H. E. Luippold, P. Carolyn Gooch, and J. G. Brewen

Studies have been carried out on the induction of chromosomal aberrations in several tissue systems by the alkylating agent triethylenemelamine (TEM). The systems studied were human leukocytes *in vitro* and mouse bone marrow, spermatogonia, and primary spermatocytes.

Human leukocytes were treated with various doses of TEM 34–36 hr after stimulation. This time was chosen because most alkylating agents have maximum clastogenic effects during DNA synthesis, and, in our hands, this time is the mid-S phase for leukocytes. In addition, one TEM dose was used to treat G_0 cells to confirm the specificity of time of action. Table 4 summarizes the results. The data show a clear-cut time of action and dose effect.

The mouse experiments consisted in injecting young adult male mice intraveneously with various doses of TEM in Hanks' BSS. The doses used were 0.05, 0.10, 0.20, 1.0, and 2.0 mg per kg of body weight. Beginning 4 hr after TEM treatment, groups of four animals were given an ip injection of colchicine and 2 hr later were killed; slides were made of bone marrow and differentiating spermatogonia. This procedure was carried out in such a manner that samples were collected at 6-, 12-, 18-, 24-, 30-, and 36-hr intervals after TEM treatment. Parallel groups of eight mice were injected and held for 8–10 weeks, at which time slides were made of diplotene-diakinesis of the primary spermatocytes. In addition, a third group of mice was treated with TEM plus [^{3}H]TdR to study cell population kinetics. Although the slides of this latter experiment are made, we have not collected all of the data.

Table 5 summarizes the complete data from the cytogenetic experiments. At the doses of 1.0 and 2.0 mg/kg, no dividing cells were obtained in the spermatogonia preparations from 24 through 42 hr after TEM treatment. We interpret this to represent those cells that were in the process of replicating their DNA and were killed because of their high sensitivity to the compound. No similar complete lack of cell division was seen in the bone marrow. At the lower doses, where no massive delay, or killing, occurred in the spermatogonia, the level of chromosomal damage was essentially the same in both tissues. Thus it appears that the bone marrow may be a suitable indicator of chromosome damage induced in germ cells at low doses.

When all the symmetrical chromatid exchanges seen in the spermatogonia were totaled at 0.1 and 0.2 mg/kg, there were 19 in 3100 cells. This is a frequency of 0.613%. Correcting for random segregation, a frequency of 0.15% reciprocal translocations would be expected in the primary spermatocytes at these two doses. We actually observed three translocations in 1200 cells for a frequency of 0.25% (see Table 6).

We conclude that the reason so few chromosome aberrations are recovered from spermatogonial cells after chemical treatment is that the most sensitive cells are killed at higher doses, and at the lower doses where they survive, very few aberrations are induced.

Table 4. Human leukocyte cultures treated with TEM

TEM (conc.)	Time of treatment (hr)	Total cells scored	Normal cells	CD	Iso	Interchanges		Intrachanges	Triradial	Shattered
						Sym.	Asym.			
Control		200	198	2	0	0	0	0	0	0
5×10^{-7} *M*	34–36	100	96	3	1				0	0
1×10^{-6} *M*	34–36	200	179	15	9				0	0
2.5×10^{-6} *M*	34–36	200	180	16	9			2	0	0
5×10^{-6} *M*	34–36	200	140	49	29	3	5		3	0
1×10^{-5} *M*	34–36	200	82	194	35	22	29	5	7	10
1×10^{-5} *M*[a]	0–2	200	169	23	9	3	1			0

[a]PHA stimulated after TEM treatment.

Table 5. Chromosomal aberration frequency in TEM-treated spermatogonia and bone marrow

Interval (hr)	Spermatogonia					Bone marrow				
	No. of cells	Dels. (%)	Sym. (%)	Asym. (%)	Intra. (%)	No. of cells	Dels. (%)	Sym. (%)	Asym. (%)	Intra. (%)
					0.1 mg/kg TEM					
6	300	2.0	0	0	0	300	4.0	0	0	0
12	300	8.33	0	0	0	300	6.33	0.33	0.66	0
18	300	14.3	0.66	1.00	0.33	300	10.33	0.33	0	0
24	300	4.0	0.33	0.33	0	300	9.66	0.33	0	0
30	300	3.33	0.66	0	0	300	1.66	0	0	0
36	300	6.66	0.33	0.33	0					
					0.2 mg/kg TEM					
6	200	1.00	0	0	0	100	3.0	0	0	0
12	300	7.0	0.33	0	0	300	6.66	0	0.33	0
18	200	16.00	3.5	0	0	300	10.66	0.33	0.66	0
24	300	6.33	1.33	0.33	0.33	300	11.0	0	0.66	0
30	300	4.66	0.33	0	0	300	4.66	0.33	0	0
					1.0 mg/kg TEM					
6	300	3.0	0	0	0	300	8.0	0.66	0	0
12	300	18.33	1.33	0	0	300	36.33	3.66	2.0	0
18	200	8.0	1.0	1.5	0					
24			No divisions[a]			300	62.66	12.0	16.0	0
30			No divisions							
44			No divisions			300	25.0	6.33	8.33	1.0
					2.0 mg/kg TEM					
6	300	2.33	0	0	0	100	6.0	0	1.0	0
12										
18	300	4.0	0.33	0	0	300	20.6	2.0	2.0	0
24			No divisions							
30			No divisions			300	98.66	31.66	44.66	1.33
36			No divisions							

[a]This part of the experiment was done twice.

Table 6. Reciprocal translocations in primary spermatocytes after TEM treatment

Dose (mg/kg)	No. of cells	No. of translocations
Control	600	0
0.05	600	1
0.10	600	0
0.20	600	2
1.00	300	1
2.00	300	1

CYTOGENETIC ANALYSIS OF DOMINANT LETHALITY INDUCED IN POSTMEIOTIC MALE GERM CELLS

J. G. Brewen, Helen S. Payne, and R. J. Preston

In an attempt to better understand the mechanisms of chemically induced genetic effects on male germ cells, we have undertaken the study of the cytogenetic consequences of treating postmeiotic male germ cells. The experiments are done by treating young adult male mice with the mutagen of choice and then mating them, at varying intervals, to superovulated females. The resultant fertilized ova are then collected and cultured, *in vitro,* to the first cleavage mitosis, when preparations are made of the metaphase chromosomes. Since the male and female pronuclei are slightly out of synchrony during the first cleavage division, it is usually possible to distinguish between the male and female chromosome complements.

We have finished a study on the effects of methyl methanesulfonate (MMS). In these experiments, males were injected with 25, 50, or 100 mg of MMS per kg of body weight. On varying days after the treatment, single pair matings were made, the plugged females killed, and the ova collected. Usually 25–30 successful matings occurred at each dose and interval combination

studied. The precise intervals used at each dose are summarized in Table 7. The intervals studied encompass the spermatogenic stages of early-spermatid through spermatozoa.

Two basic types of chromosomal damage were observed in these studies. The one type was simple chromatid aberrations, usually isochromatid deletions and chromatid interchanges (Figs. 5*A* and 5*B*). The second predominant cytogenetic effect observed was the shattering of the male complement (Fig. 5*C*), and this occurred most frequently at the highest MMS dose and the period of peak sensitivity. The complete data are summarized in Table 7.

The aberration data were used to calculate "predicted" levels of lethality. In making these calculations it was assumed that any aberration that resulted in loss of a visible piece of chromosome material would cause a lethal event. Thus total dominant lethality is that proportion of cells that contained at least one aberration. The level of predicted preimplantation loss was arbitrarily adjudged to be that proportion of cells with more than five aberrations, and the predicted level of dead implants was calculated by taking the proportion of cells with 1–5 aberrations of the total with 0–5 aberrations.

The levels of predicted dominant lethality for the 50- and 100-mg/kg doses as a function of time are plotted in Fig. 6. Actual dominant lethal data from a study with EMS by Generoso and Russell[1] are also presented. These data were chosen because of their completeness and the similarity of action of MMS and EMS. An interesting aspect of the present data is the very high

Table 7. Distribution of chromosomal aberrations in male pronuclei treated in spermatid and spermatozoa stages with MMS

Dose (mg/kg)	Interval (days)	Cells with aberrations							Shattered	Total cells	Predicted dominant lethality[a] (%)	Predicted preimplantation loss[b] (%)	Predicted dead implants[c] (%)
		0	1	2	3	4	5	>5					
Control		99	1	0	0	0	0	0	0	100	1	0	1
100	2½	24	6	10	2	3	2	3	0	50	52	6	49
	5½ + 6½	6	0	1	2	1	2	4	45	61	90	80	50
	9½	0	0	2	2	8	5	6	27	50	100	66	100
	11½ + 12½	19	7	11	3	5	1	5	9	60	68	23	58
	14½	45	4	0	0	1	0	0	0	50	10	0	10
	15½	45	3	1	1	0	0	0	0	50	10	0	10
	17½	45	4	1	0	0	0	0	0	50	10	0	10
	18½	49	1	0	0	0	0	0	0	50	2	0	2
	22½	50	0	0	0	0	0	0	0	50	0	0	0
50	2½	25	0	0	0	0	0	0	0	25	0	0	0
	3½	54	9	0	0	0	0	0	0	63	14	0	14
	5½	27	14	5	1	1	0	0	2	50	46	4	44
	9½	23	12	12	1	1	0	0	1	50	54	2	52
	10½	29	11	8	1	1	0	0	0	50	42	0	42
	11½ + 12½	32	12	7	1	2	1	1	2	58	45	5	42
	13½	41	6	1	1	0	0	0	1	50	18	2	16
	15½	69	5	1	0	0	0	0	0	75	9	0	9
	16½	49	1	0	0	0	0	0	0	50	2	0	2
	23½	50	0	0	0	0	0	0	0	50	0	0	0
25	6½	44	5	1	0	0	0	0	0	50	12	0	12
	7½	46	3	0	0	1	0	0	0	50	8	0	8
	8½ + 9½	80	8	1	0	0	0	0	0	89	10	0	10

[a] Total percentage of cells carrying at least one chromosomal aberration.
[b] Proportion of cells with more than five chromosomal aberrations.
[c] Proportion of cells with five or less chromosomal aberrations that had an aberration, *i.e.*, (1–5)/(0–5).

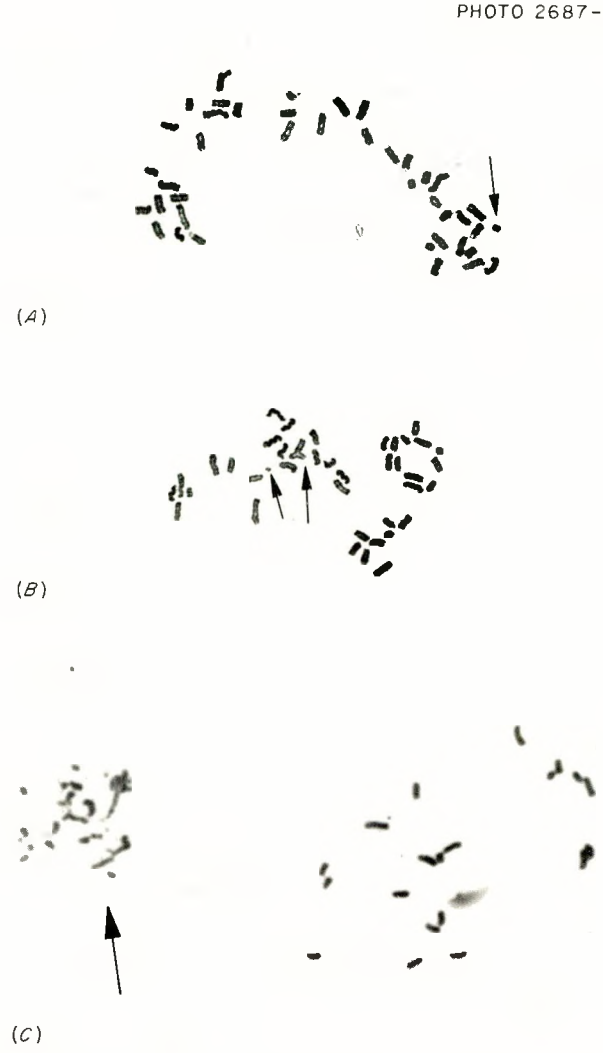

Fig. 5. Examples of first cleavage mitosis metaphase figures in cultured mouse ova after treatment of spermatozoa with MMS. (*A*) Single-double fragment (arrow), (*B*) triradial chromatid interchange with accompanying fragment (arrows), (*C*) shattered male chromosomes (large arrow).

frequency of shattered chromosomes on days $5\frac{1}{2}$ through $9\frac{1}{2}$ after a dose of 100 mg/kg. This time corresponds to when a high frequency of "infertile matings" occur. We propose that the "infertile matings" result from very early death of the blastomeres as a consequence of the massive chromosome damage.

1. W. M. Generoso and W. L. Russell, *Mutat. Res.* 8, 589–98 (1969).

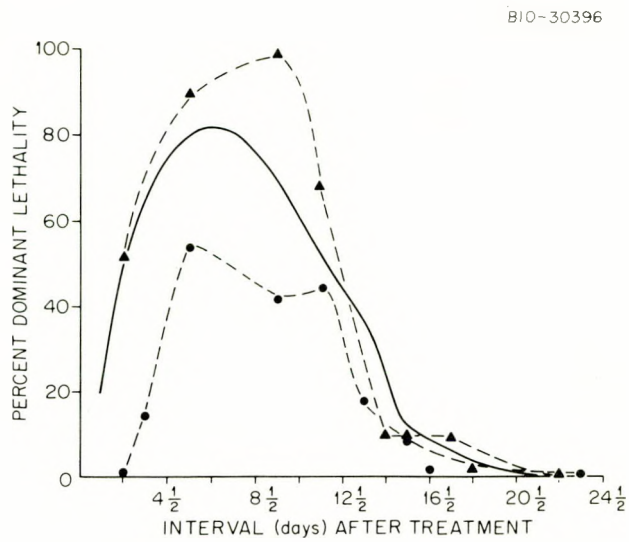

Fig. 6. Yield of dominant lethals, with time, after injection of male mice with either EMS or MMS. Solid line is a generalized representation of the 300 mg/kg EMS data of Generoso and Russell. ▲----▲, calculated from aberration data after 100 mg/kg; ●----● calculated from aberration data after 50 mg/kg MMS.

CYTOGENETIC CONSEQUENCES OF IRRADIATION OF DICTYATE OOCYTES

J. G. Brewen, Helen S. Payne, and R. J. Preston

In the past ten years a great deal of information has become available on the cytogenetic effects of irradiation of male germ cells. This advance in knowledge is due principally to the simple techniques developed to make preparations of the meiotic chromosomes. Until recently, however, similarly simple techniques were not available for studying the meiotic chromosomes of oocytes. We have recently begun studying the types and frequencies of chromosome aberrations produced in dictyate mouse oocytes by X rays.

To date, two experiments have been done. One was to determine what, if any, variation in aberration yield might exist with time between irradiation and ovulation. Young adult female mice were irradiated with 200 R at various intervals before they were induced to superovulate. The intervals used were 1, 3, 5, 7, 14, and 21 days. Metaphase I oocytes were analyzed for chromosome aberrations. Since the dictyate oocytes have completed DNA synthesis, the aberrations observed were chromatid-type. The results of the experiment are presented in Table 8.

Once it was established that an interval of 14 days between irradiation resulted in the highest yield of aberrations, a dose-response curve was done using this

interval. The doses employed and the aberration frequencies obtained are presented in Fig. 7, which is a plot of log aberrations vs log dose. The slope of the curve for translocations is 1.7, indicating the presence of a significant two-track component. This is unlike the data reported by several investigators for the male, where the frequency of translocations seen at MI increases approximately linearly with dose.

Table 8. Frequencies of chromatid aberrations in MI oocytes at various times after an acute X-ray dose of 200 R

Interval (days)	No. of cells scored	Deletions (%)	Interchanges (%)
1	100	1.0 ± 1.0	1.0 ± 1.0
3	100	4.0 ± 2.0	1.0 ± 1.0
5	125	4.8 ± 2.0	0.8 ± 0.8
7	100	6.0 ± 2.5	6.0 ± 2.5
14	125	10.4 ± 2.9	12.8 ± 3.2
21	100	6.0 ± 2.5	6.0 ± 2.5

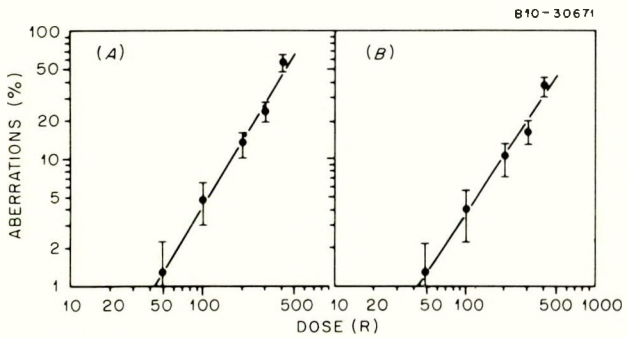

Fig. 7. Visual log yield vs log dose plot of aberrations observed in metaphase I oocytes. (*A*) interchanges; (*B*) pooled deletions.

In early studies[1,2] on the recovery of reciprocal translocations in the F_1 of irradiated mice, it was noted that very few translocations were recovered after doses of 300 and 400 R. This seems surprising in light of the fact that we observe 23% and 56% interchanges at these doses respectively. The actual cytological data were used to calculate an expected recovery frequency of reciprocal translocations. In making these calculations, four assumptions were made: (*a*) based on C-band staining, symmetrical and asymmetrical interchanges occur at approximately equal frequencies; (*b*) there is no preferential segregation of exchange, or nonexchange, chromatids into polar nuclei; (*c*) inclusion of any deleted chromosome, dicentric chromosome, or duplication deficiency in the mature ova will result in dominant lethality; (*d*) chiasmata location and frequency in reference to the exchange point would not influence segregation (although some effects may occur). The types and relative proportions of ova expected from these 100 oocytes are summarized (Table 9). A simple example of possible segregation patterns is diagrammed in Fig. 8. The possible segregants conform to the ratio 9 normal:6 duplication deficiencies:1 balanced translocation. In the case of a symmetrical exchange plus a deletion, the probability of both exchange chromosomes and the undeleted chromosome going to the same pole is $\frac{1}{2} \times \frac{1}{2}$; this then must be multiplied by the probability ($\frac{1}{2}$) of that pole not forming the polar nucleus; at AII the probability of the two exchange chromatids segregating together is $\frac{1}{2}$ multiplied by the probability ($\frac{1}{2}$) that

Table 9. Expected proportions of ova genotypes recovered from the data at 400 R 14 days before ovulation

Aberration class	No. of cells	Expected proportions of:				
		Normal	Deleted	Duplication and deficiency	1 balanced translocation	2 balanced translocations
None	36	1				
1 deletion	18	1/2	1/2			
1 symmetrical exchange	10	9/16		6/16	1/16	
1 asymmetrical exchange	9	1/4	3/4			
1 deletion + 1 symmetrical exchange	3	9/32	1/2	6/32	1/32	
1 deletion + 1 asymmetrical exchange	3	1/8	7/8			
2 symmetrical exchanges	4	81/256		156/256	18/256	1/256
2 asymmetrical exchanges	4	1/16	15/16			
Multiples	13			0.13[a]		
Proportion of total		0.556	0.236[a]	0.068[a]	0.010	⩾0.001

[a]Presumed to be dominant lethals.

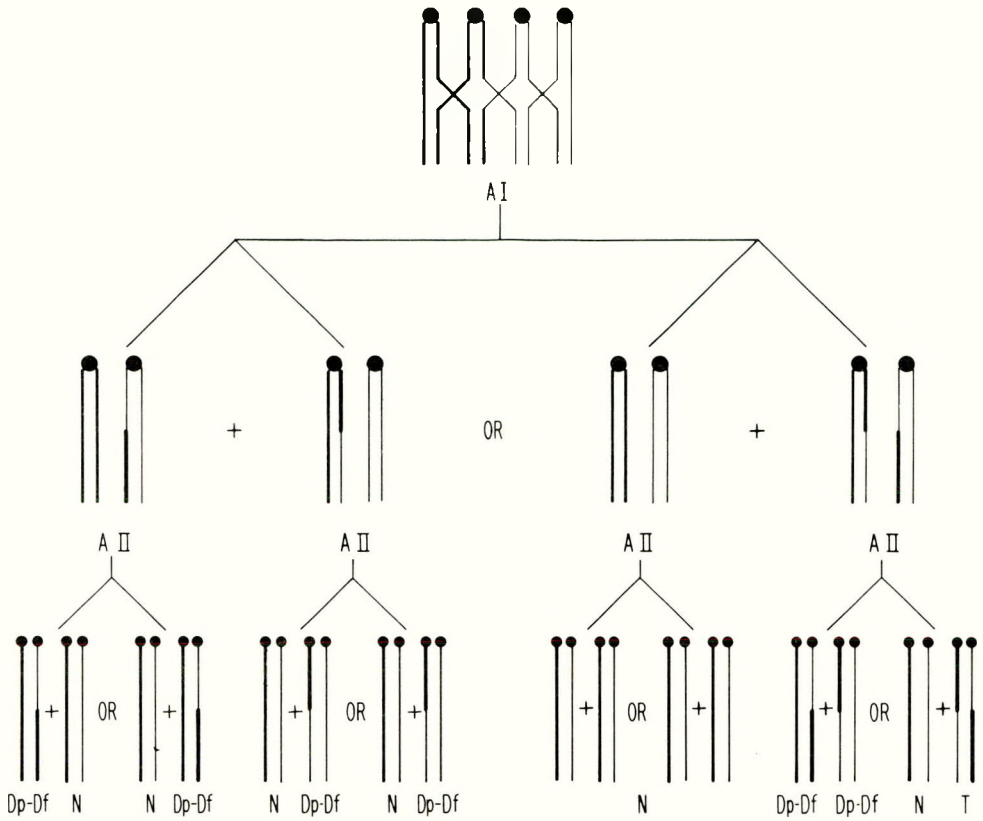

Fig. 8. Schematic representation of the possible segregants, and their genotypes, from a symmetrical chromatid interchange multivalent.

they will not form the polar nucleus. Thus the chance of recovery of the translocation in a balanced form is $^1/_{32}$.

The calculated frequency of expected translocation heterozygotes in the live F_1 of females irradiated with 400 R of X rays 14 days prior to ovulation is 1.8% (0.01/0.566, Table 9). If the same calculation is made for our 300-R data, the predicted recovery rate is 0.6% compared with Searle and Beechy's confirmed frequency of 0.4% at the same dose.[2] Searle and Beechy reported eight semisterile offspring in 680 tested F_1 from females given 300 R from 1 to 42 days prior to conception. All of the semisterile were females, and only three of them were cytologically confirmed as translocation heterozygotes. The chromosome analysis was done on somatic cells, however, and it is possible that some of the other five were indeed translocation heterozygotes.

One other interesting aspect of our data is the calculated frequency of presumed lethality in Table 9 of approximately 43%. This agrees well with Searle and Beechy's frequency of 35% dominant lethality at 400 R given 21 days prior to conception.

1. L. B. Russell and L. Wickham, *Genetics* **143,** 392–93 (1957).
2. A. G. Searle and C. V. Beechy, *Mutat. Res.* **24,** 171–86 (1974).

X-RAY-INDUCED TRANSLOCATIONS IN MOUSE SPERMATOGONIA

J. G. Brewen and R. J. Preston

In previous reports on this study[1,2] we presented data on the yields of reciprocal translocations induced in spermatogonial stem cells of mice and observed in primary spermatocytes following single or split doses of X rays. It was clear that the dose-response curve following single acute exposures was hump-shaped, with a maximum at about 600 R. It was argued that this was

due to a differential sensitivity of cells in the different stages of the cell cycle to both cell killing and chromosome aberration induction. The split dose experiments described below were designed to determine whether or not this was a feasible suggestion. The rationale behind the experiments was that cells should show a cyclic response to translocation induction as the cells surviving the first dose, that is, the ones more resistant to killing, pass into sensitive or resistant stages of the cell cycle at the time the second exposure is given.

If doses which gave a yield on the ascending part of the dose-response curve were split into two fractions, the yield decreased when compared with the yield from the same dose given in a single exposure for intervals of 90 min or more between the fractions, as is observed in many other cell systems from *Tradescantia* to mammalian cells. However, for total doses which gave a yield on the descending part of the dose-response curve, the split-dose response was somewhat different (Fig. 9, Table 10). A dose of 1000 R gave a yield of 5.4% translocations, and a single dose of 500 R gave about

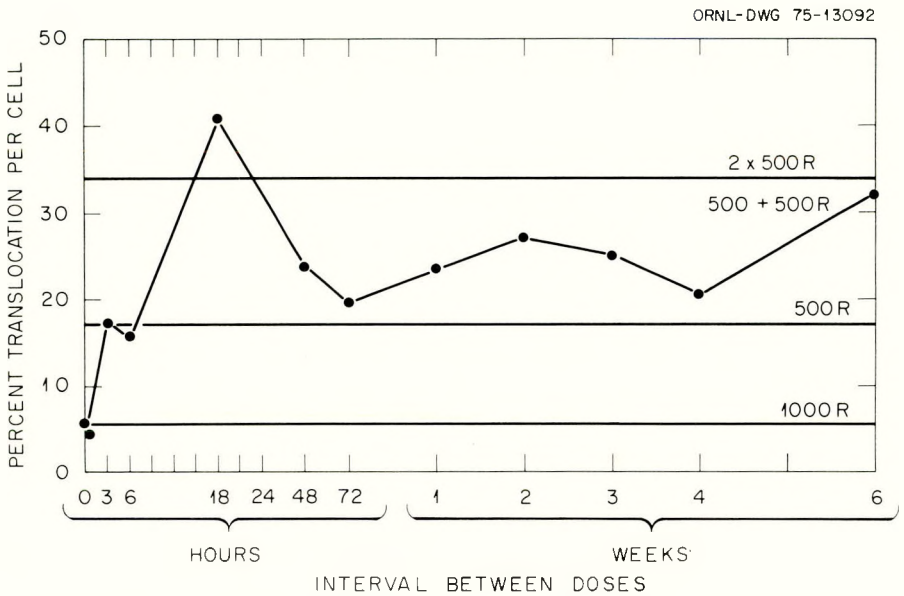

Fig. 9. The yield of translocations following 1000 R or 500 R + 500 R separated by various time intervals.

Table 10. Yields of reciprocal translocations induced by a 1000-R dose, a 500-R dose, or 500-R + 500-R doses separated by various time intervals

Dose (R)	Time interval between doses	No. of cells scored	Translocations (%)
1000	–	500	5.4 ± 1.0
500	–	800	17.0 ± 1.5
500 + 500	30 min	600	4.3 ± 1.0
500 + 500	3 hr	700	17.1 ± 1.6
500 + 500	6 hr	650	15.7 ± 1.5
500 + 500	18 hr	600	40.7 ± 2.6
500 + 500	48 hr	600	23.8 ± 2.0
500 + 500	72 hr	600	19.5 ± 1.8
500 + 500	1 week	600	23.5 ± 2.0
500 + 500	2 weeks	600	27.0 ± 2.1
500 + 500	3 weeks	600	25.0 ± 2.0
500 + 500	4 weeks	800	20.5 ± 1.6
500 + 500	6 weeks	600	32.0 ± 2.3

17.0% translocations. The translocation yield increased as the interval between the doses was increased, up to a maximum of 40.2% for an interval of 18 hr. The yield decreased to 23.8% for a 48-hr interval and still lower to 19.5% for a 72-hr interval, which is close to the yield for a single dose of 500 R. The translocation yields for fractionation intervals of 1, 2, 3, and 4 weeks were 23.5, 27.0, 25.0, and 20.5% respectively, which was between the values for 500 R alone and 2 × 500 R (additivity). When the interval between the doses was six weeks, the translocation yield was 32.0%, or the same as that for additivity. For intervals greater than six weeks the yield would be expected to remain the same, as indicated by some data reported earlier for repeated 400-R doses, when additivity was always observed for intervals of eight weeks between the doses.

This result is that which would be predicted from the suggestion that the spermatogonial stem cells do have differential sensitivities in the different stages of the cell cycle to both cell killing and translocation induction. This is particularly exemplified by the cyclic response of cells to translocation induction for different fractionation intervals, and by the observation that the yield increased to greater than additivity for the 18-hr fractionation interval. These observations indicate that the first 500-R dose kills the most sensitive cells and leaves a partially synchronous population of cells which pass first into a cycle stage when they are sensitive as shown by the increased translocation yield, the decrease in yield indicates a progression into a more resistant stage, and by six weeks after the first dose the cells have reassorted into a normal ("nonsynchronous") population. The fact that it took such a long time for the cells to return to a stage having the same sensitivity as the normal and previously unirradiated population is somewhat surprising. It could be that the cells repopulating the testis after the first 500-R dose have a very altered cell cycle compared with a normal stem cell, particularly a much shorter overall cycle time, such that the cells are in a more resistant stage for a greater proportion of the cell cycle. This could be the case, for example, if the S phase were the resistant one, and repopulating cells had a total cycle time considerably shorter than that of a normal cell. Then, because the S phase is likely to be rather constant for any cell type, it would occupy a much greater proportion of the cycle in the repopulating cell. This might be a more reasonable explanation than one which assumes that it does indeed take some six weeks for the cells to regain their asynchronous state, particularly as the fractionation response would require that the cells exposed to a second dose from 1–4 weeks after the first would by chance have needed to be in stages of the cycle with similar sensitivities, because of the relative uniformity of response of cells over this range of fractionation intervals.

1. R. J. Preston and J. G. Brewen, *Mutat. Res.* **19**, 215–23 (1973).
2. J. G. Brewen and R. J. Preston, *Biol. Div. Annu. Prog. Rep. June 30, 1974*, ORNL-4993, p. 71.

CYTOGENETIC EFFECTS OF OZONE: INHALATION OR *IN VITRO* EXPOSURES

P. Carolyn Gooch, D. A. Creasia, and J. G. Brewen

This project was designed specifically to determine if ozone, at concentrations in the general range of the ambient level in metropolitan areas, caused chromosome aberrations. This was prompted by the report of Zelac *et al.*[1] that a 40-hr exposure to ozone at the maximum recommended industrial concentration resulted in a high level of chromosome damage. A more recent paper by Merz *et al.*[2] showed that inhalation of ozone by humans in a study where the ozone concentration was controlled resulted in an increase in chromatid deletions in their leukocytes. Because of the large difference in the degree of effects and types of aberrations described in these two papers, it was decided to conduct a thorough investigation using several systems to clarify the situation.

Ozone was produced by exposing a stream of pure oxygen to ultraviolet radiation in a Mast ozone generator and then mixing it with dilution air to give different ozone concentrations, which were determined by the neutral potassium iodide method of Byers and Saltzman.[3]

Bone marrow samples were taken from Chinese hamsters which had been exposed 2, 6, or 12 hr earlier to ozone of different concentrations for different periods of time. The results are presented in Table 11, and it can be seen that there were no significant differences in aberration yields between treated and control groups. Only data for chromatid aberrations are presented because there were very few chromosome-type aberrations and no significant differences between treated and control groups.

Blood cultures were established from mice which had been exposed to different ozone levels, either immediately after exposure, or 12 hr, 1 week, or 2 weeks later. Preparations were made from these cultures 52 hr after PHA stimulation. The results are given in Table 12, and

Table 11. Chromosomal aberration frequencies in Chinese hamster bone marrow after ozone inhalation

Average O_3 exposure (ppm)	Length of exposure (hr)	Postexposure sacrifice time (hr)	No. of cells analyzed	Chromatid aberrations (%)		
				Achromatic lesions	Deletions	Exchanges
Control			400	1.00 ± 0.50	0.50 ± 0.35	0.00
0.23 (1.15 ppm hr)	5	2	300	0.67 ± 0.47	1.33 ± 0.67	0.00
		6	300	0.33 ± 0.33	0.33 ± 0.33	0.00
		12	300	1.33 ± 0.67	0.67 ± 0.47	0.00
5.2 (31.2 ppm hr)	6	2	300	1.00 ± 0.58	1.33 ± 0.67	0.00
		6	300	2.00 ± 0.82	0.67 ± 0.47	0.33 ± 0.33[a]
		12	300	1.00 ± 0.58	1.67 ± 0.75	0.00

[a]Two dicentrics, 1 chromatid exchange in the same cell.

Table 12. Chromosomal aberration frequencies in mouse leukocytes after ozone inhalation

Average O_3 exposure (ppm hr)	Postexposure blood withdrawal time	No. of cells analyzed	Chromatid aberrations (%)		
			Achromatic lesions	Deletions	Exchanges
Control		400	1.75 ± 0.66	1.00 ± 0.50	0.00
0.75	Immediate	250	4.40 ± 1.33	0.40 ± 0.40	0.00
	12 hr	200	3.00 ± 1.22	2.00 ± 1.00	0.00
	1 week	300	2.00 ± 0.82	3.33 ± 1.05	0.33 ± 0.33
	2 weeks	300	2.67 ± 0.94	1.00 ± 0.58	0.00
1.05	Immediate	105	0.95 ± 0.95	3.81 ± 1.90	0.00
	2 weeks	200	1.50 ± 0.87	3.50 ± 1.32	0.00
1.98	Immediate	300	3.33 ± 1.05	1.00 ± 0.58	0.00
	1 week	300	1.67 ± 0.75	2.00 ± 0.82	0.00
	2 weeks	300	2.33 ± 0.88	2.33 ± 0.88	0.00

again it can be seen that there is no increase in aberration frequency in the treated group compared with the controls. Spermatocyte preparations were also made from these mice eight weeks after exposure to determine if there was any increase in reciprocal translocation frequency. No reciprocal translocations were observed in the 1500 cells analyzed, indicating no effect of ozone at the exposures used.

In addition to these inhalation experiments, several *in vitro* exposures of human leukocytes were performed. There were basically two methods of exposure. The first was to bubble ozone, of a known concentration, through leukocyte cultures, which had been stimulated with PHA 12 or 36 hr previously. Aliquots were removed at different times, so that a range of exposure levels was given. The complete results for the cultures exposed 12 or 36 hr after PHA stimulation are given in Table 13. There appears to be a significant difference in the frequency of chromatid deletions between treated and control groups for exposures above 7.23 ppm hr in the cultures exposed 36 hr after stimulation.

The second method of exposure was to put leukocytes, which had been stimulated 12 or 36 hr earlier, into ozone-saturated solutions. The exposure times,

together with the aberration data, are given in Table 14. There was no difference between the treated and control groups for cells in different stages of the cell cycle (G_1 for cultures 12 hr poststimulation, S for 24 hr) or for different levels of exposure.

These results suggest that ozone at levels similar to or in excess of an anticipated human environmental exposure level does not significantly increase the frequency of chromosome aberrations, except in the one instance. This is the case whether exposure is by inhalation or directly to the cells under study, or whether the experimental animal is the mouse, Chinese hamster, or human. It is difficult to reconcile the one significant result, *i.e.*, an increase in chromatid deletions

Table 13. Chromosomal aberration frequencies in human leukocyte cultures exposed to ozone at 12 and 36 hr after PHA stimulation

Ozone exposure (ppm hr)	No. of cells analyzed	Chromatid aberrations (%)		
		Achromatic lesions	Deletions	Exchanges
12 hr				
Control	900	4.0 ± 0.67	3.44 ± 0.62	0.00
1.3	300	1.67 ± 0.75	2.00 ± 0.82	0.00
2.4	300	2.67 ± 0.94	4.00 ± 1.15	0.00
2.6	300	3.00 ± 1.00	3.00 ± 1.00	0.00
4.8	300	2.67 ± 0.94	3.30 ± 1.05	0.00
7.5	250	5.60 ± 1.50	5.20 ± 1.44	0.40 ± 0.40
36 hr				
Control	900	3.78 ± 0.65	3.67 ± 0.64	0.22 ± 0.16
1.65	300	1.67 ± 0.75	3.67 ± 1.11	0.00
2.50	300	4.00 ± 1.15	5.33 ± 1.33	0.33 ± 0.33
4.06	300	1.67 ± 0.75	4.00 ± 1.15	0.00
5.20	300	3.67 ± 1.11	7.00 ± 1.53	0.00
5.73	231	0.87 ± 0.61	7.36 ± 1.78	0.00
7.00	300	4.33 ± 1.20	4.67 ± 1.25	0.00
7.23	120	10.00 ± 2.89	14.17 ± 3.44	0.00
7.95	250	5.20 ± 1.44	12.00 ± 2.19	0.00
14.2	150	4.00 ± 1.63	8.00 ± 2.31	0.00

Table 14. Human leukocyte cultures exposed to an ozone-saturated solution at 12 and 36 hr after PHA stimulation

Exposure time (min)	No. of cells analyzed	Chromatid aberrations (%)		
		Achromatic lesions	Deletions	Exchanges
12 hr				
Control	900	4.00 ± 0.67	3.44 ± 0.62	0.00
30	300	3.00 ± 1.00	4.00 ± 1.15	0.00
60	250	3.60 ± 1.20	6.80 ± 1.65	0.00
90	160	5.63 ± 1.88	5.00 ± 1.77	0.00
36 hr				
Control	900	3.78 ± 0.65	3.67 ± 0.64	0.22 ± 0.16
5	300	8.67 ± 1.70	4.33 ± 1.20	0.00
10	300	7.00 ± 1.53	7.00 ± 1.53	0.00
15	300	6.00 ± 1.41	5.67 ± 1.37	0.00
30	300	6.00 ± 1.41	5.67 ± 1.15	0.67 ± 0.47
60	250	2.40 ± 0.98	2.80 ± 1.06	0.00
90	150	2.00 ± 1.15	6.00 ± 2.00	0.00

for exposures above 7.23 ppm hr to cultures 36 hr after PHA stimulation, when there was no similar increase in similar cells exposed to saturated solutions of ozone. No really satisfactory explanation can be offered for the one significant increase in aberration frequency.

These results are very different from those of Zelac *et al.*, and somewhat different from those of Merz *et al.* Ozone is clearly, from the description here, a very weak clastogen.

1. R. E. Zelac, H. L. Cromroy, W. E. Bolch, B. G. Dunavant, and B. A. Bevis, *Environ. Res.* 4, 262–82 (1971).
2. T. Merz, M. A. Bender, H. D. Kerr, and T. J. Kulle, *Mutat. Res.*, in press.
3. D. H. Byers and B. E. Saltzman, *Adv. Chem. Ser.* 21, 251–57 (1958).

N-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE-INDUCED REVERSION OF GLY D MUTANTS IN CHINESE HAMSTER OVARY CELLS

E. A. Hiss and R. M. Wallace, Jr.

K_1 18 is a group D, glycine-requiring mutant of line CHO-K_1. It was induced by EMS and has been shown to revert spontaneously 3×10^{-7}/generation. Induction of reversion has been achieved with X-ray doses up to 400 R. The induction is linear and 10^{-8} revertant per locus per rad. Reversion can also be induced by MNNG. The kinetics appear to be linear up to 1 μg/ml, and the slope is 9×10^{-6} revertant μg per ml of MNNG. Using a rad equivalent unit as a means of standardizing mutagens, we can calculate a rad equivalent for MNNG of 1×10^{-3} μg of MNNG per ml per rad. This value is similar to one which we calculated from Puck's data for the induction of forward mutations in CHO.

A thioguanine-resistant line isolated from a population irradiated with 800 R has been shown to be three times as sensitive to killing by MNNG as its wild-type parent. A spontaneous mutant resistant to thioguanine is also highly sensitive. This differential sensitivity is not true for X rays or UV. The respective values for D_0 of the two lines are identical. Since it is argued that chromosomal aberrations are the cause of cell death, we investigated whether the presence of the thioguanine-resistant mutation led to an increased frequency of chromosomal aberrations and mutation. We concluded from a comparative study of K_1D *vs* TG^r-2 that there was no significant difference in induction of aberrations with MNNG as assayed from 6 to 54 hr (at 6-hr intervals) after administration of 0.1 μg/ml MNNG to an asynchronous population. However, the number of mitotic figures was reduced severalfold in TG^r-2. At present we are investigating the effect of TG^r on reversion of gly D locus, using a spontaneous TG^r mutant derived from K_1 18. It is possible that the TG^r mutation alters the metabolism of the cell in such a way that a defective repair process in MNNG-treated cells results in increased lethality and possibly an increased point-mutation rate.

X-RAY-INDUCED FORWARD MUTATION RATES IN CULTURED MAMMALIAN CELLS

E. A. Hiss and R. M. Wallace, Jr.

One approach to estimating the mutational hazard of radiation to man is through the use of cultured mammalian cells. Our initial objective was to evaluate a human-hamster hybrid cell for its potential in assaying forward and reverse X-ray-induced mutation rates of human genes. We chose thioguanine resistance, since the selective system has been well researched by a number of investigators. In addition, the hypoxanthine-guanine phosphoribosyl transfer gene (the loss of whose function confers TG^r phenotype) is hemizygous in humans, as well as in transformed CHO cells, and also in a hybrid of TG^r CHO cell and a normal human cell. Published data of other investigators using V-79 hamster cells and human fibroblasts using resistance to 8-azaguanine as a marker suggested that the induced hamster rate was 30-fold greater than that of normal human cells. The dose power function was approximately 2 in both cases, suggesting that a two-hit event was required for most, if not all, recoverable mutations. We therefore presumed that a human gene in the hybrid cell would mutate similarly to the hamster "host" cell if repair function was the major factor governing mutation rates. If the induced rate of the hybrid was similar to that of the human, we could argue that the hybrid was a valid system for measuring X-ray mutation rates of other or all human genes not easily assayed in the normal diploid cell.

To date, our investigations strongly suggest that the mutation rates of the hamster cells and the human cells are the same. The kinetics of induction are linear with a rate of 1.4×10^{-7} mutant per survivor per rad. The time of maximum mutant expression in the population of irradiated cells is about five generations. The apparent spontaneous rate is 4×10^{-7} mutant/generation. The doubling dose of 3 R fits well with published data estimating human risk.

GAMMA-RAY-INDUCED UV-LIKE REPAIR IN NORMAL CELLS AND DEFECTIVE REPAIR IN XERODERMA PIGMENTOSUM (XP) CELLS DETECTED AT LONG TIMES AFTER IRRADIATION

James D. Regan, F. M. Faulcon, and R. B. Setlow*

Ultraviolet light induces dimers between adjacent pyrimidine residues in DNA. In normal human cells these dimers are excised, and the lesion is repaired in a typical prereplication mode which results in "long-patch" repair. The typical long patch after a dose of 200 ergs/mm^2 of 254-nm UV is approximately 100 nucleotides. This repair takes 20 or more hours to complete in normal cells. In cells from the UV-sensitive human mutants, XP long-patch repair is defective.

Ionizing radiation induces single-strand breaks in DNA which are repaired in human cells in the short-repair mode. For example, after 10 kilorads of ^{60}Co gamma rays, the average repaired region is approximately 3 to 4 nucleotides. This repair process is completed within 60 min.

It is well known, however, that ionizing radiation can induce a number of DNA damages other than single- or double-strand breaks. Some of these may be base damages which result in a local distortion of the helix and resemble distortions induced by pyrimidine dimers.

We have investigated the repair of ionizing-radiation-induced lesions in normal and XP cells both at short times after irradiation and at long times (*i.e.*, 20 hr) after irradiation. If we modify the γ-irradiation conditions, making the cells anoxic, increase the dose, and assay the damages induced and their repair by bromodeoxyuridine photolysis at long times (20 hr) after irradiation, repair events very similar to those seen in UV-irradiated cells are seen in normal cells (Fig. 10). The same experiment with xeroderma cells results in defective repair (Fig. 10). Thus it appears that ionizing radiation can induce UV-like damages in human DNA that are repaired in the long, UV-like mode. Furthermore, these damages in xeroderma cells are defectively repaired.

The above results led us to reexamine one of the chemical mutagens that we had previously classified as a short-repair-type chemical mutagen. Utilizing this chemical mutagen with normal and XP cells assayed at long times after treatment, we obtained the results shown in Fig. 11. Ethyl methanesulfonate, when assayed at long times after the insult, produced results comparable to those seen with chemical agents we previously classified as UV-type agents. This result indicates that EMS has a UV-type capability in terms of DNA damage and repair along with its ionizing or short-repair-type function.

It is probable that many DNA-damaging agents such as ionizing radiation and certain chemical carcinogens make several types of changes in DNA. They may induce ionizing-type damage, the repair of which is rapid and is optimally observed at short times after treatment and at the same rate in normal and XP cells; they may also induce UV-type damage, the repair of which is observed only at long times after treatment

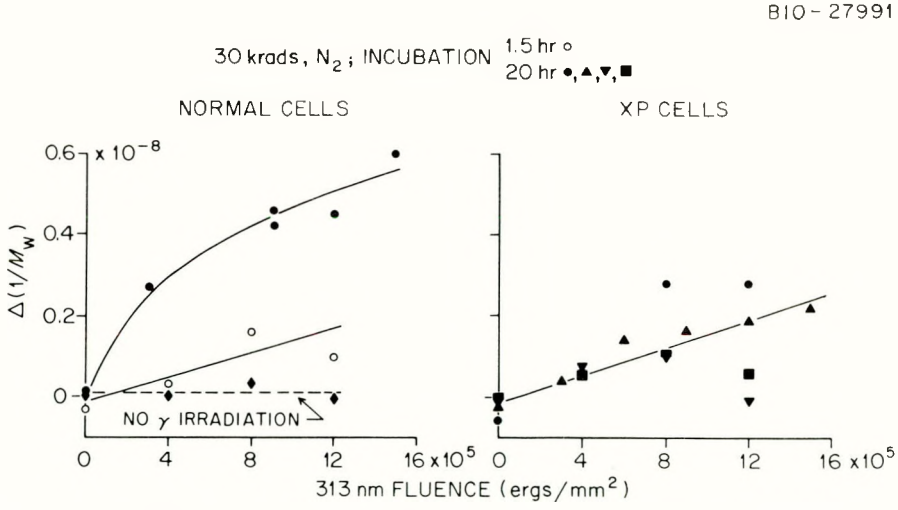

Fig. 10. Relationship of the difference in the reciprocal of the weight-average molecular weight of either normal cells or XP cells irradiated with 30 krads of ^{60}Co γ-rays under nitrogen and incubated either at long times or short times before assay. The different symbols refer to different experiments.

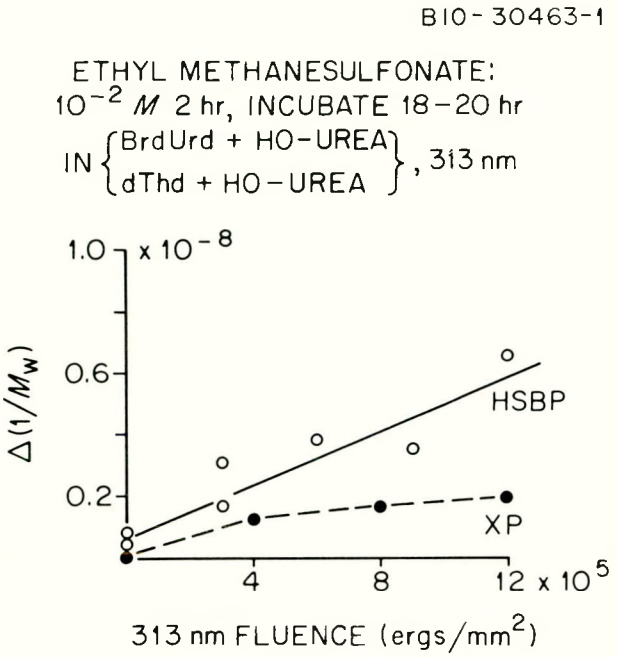

Fig. 11. Results of an analysis of normal and XP cells after treatment with 2×10^{-5} *M* EMS and a BrdUrd photolysis assay done at 20 hr after insult. Note that the XP cells under these conditions appear to display defective repair of lesions induced by this chemical agent when assayed at long times after the delivery of the original insult, and normal cells appear to exhibit an UV-type repair response, contrasting with the result previously found at short times after treatment with this chemical agent. [J. D. Regan and R. B. Setlow, *Cancer Res.* **34**, 3318–25 (1974).]

and which may appear to be rather differently repaired in normal and XP cells. DNA-damaging agents may induce several types of damages; these damages will be repaired according to the structural configuration of the damage and the suites of enzymes available and evolved for dealing with these damages in human cells.

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POSTREPLICATION REPAIR IN NORMAL HUMAN CELLS AND IN XERODERMA PIGMENTOSUM COMPLEMENTATION GROUPS

Raymond Waters* and James D. Regan

Initial studies on the prereplication repair of UV-induced pyrimidine dimers [pyr()pyr] have shown that in normal human skin cells these photoproducts are removed via excision-resynthesis, whereas cells from patients with XP exhibit varying abilities to perform this process.[1,2] Complementation tests have revealed groups A, B, C, D, E, and the XP variants, these being able to excise <2, 3–7, 25–55, 60, and 100% of UV-induced pyr()pyr respectively.[3]

The existence of a postreplication repair process in human cells has been shown more recently.[4] UV irradiation results in DNA synthesis being blocked by a pyr()pyr, leaving a gap of $\sim 10^3$ nucleotides. This gap is then filled in to join the shorter segments, resulting in high-molecular-weight DNA.

Buhl *et al.*[5] found no difference with regard to this ability in normal cells, in XP cells unable to excise pyr()pyr, or in XP variant cells. More recent studies by Lehmann *et al.*[6] on similar cell lines at earlier times after UV irradiation resulted in the statement that XP variants are considerably deficient in postreplication repair, and XP A-group cells are inexplicably intermediate between normal and variant cells with regard to this ability. Studies in our laboratory have shown that XP B- and D-group cells are also intermediate, resembling group A. However, a closer evaluation of both our data and Lehmann's reveals that the XP variant, and to a lesser degree XPs of groups A, B, and D, although synthesizing DNA of a smaller size than normal cells at a set time after UV irradiation, increase the molecular weight of their DNA/unit time after UV, the same as in normals, XPs, and variants (see the slopes of Fig. 3 in ref. 6). Secondly, although Lehmann *et al.* state that in all strains the DNA becomes "big" after 5–8 hr, their gradients have a limit of resolution at a molecular weight of 150×10^6, thus making difficult accurate estimates of their size of "big" DNA. We have shown that such "big" DNA is larger at 5–8 hr and longer in normal cells as compared with XP and variant cell lines. Control experiments are presently being conducted to determine whether the rates of DNA replication, both with and without UV, vary considerably in all the cell lines. If variations occur, both pulse and chase times will require adjustments for accurate comparison.

Finally, some differences in the sizes of newly replicated, chased DNA may tentatively be attributable to a phenomenon reported by Buhl and Regan.[7] These authors found that in an XP line unable to excise pyr()pyr, for 1 in 30 of the pyr()pyr "by-passed," the newly replicated DNA contained an endonuclease-sensitive site. No such site was observed in the "by-passed" regions opposite pyr()pyr induced in normal cells. An extension of this study to include groups A, B, C, D, E, and XP variants has been undertaken. It is possible that although postreplication repair makes the DNA larger, attempts (defective attempts?) to repair endonuclease-

sensitive sites in the newly synthesized DNA opposite pyr()pyr make the DNA slightly smaller in the XP and variant cell lines.

Considerable work will be required to clarify the relationship between postreplication repair and the XP phenotype both in XP and variants. It is hoped that these experiments will eliminate some of the possible artifacts and clarify the significance of what is inserted opposite the pyr()pyr in these various XP cell lines.

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LACK OF PHOTOREVERSAL OF UV-INDUCED PYRIMIDINE DIMERS IN NORMAL HUMAN AND XERODERMA PIGMENTOSUM SKIN CELLS

W. L. Carrier, W. H. Lee, and
James D. Regan

We first reported in 1968 that normal human cells are capable of the excision of UV-induced pyrimidine dimers.[1] We subsequently showed that XP cells were essentially incapable of excision of these dimers.[2] Recently we showed that, while in earlier reports we found only 50% excision, with improved methods and lower doses we could not demonstrate complete excision of dimers in normal human cells.[3]

Sutherland *et al.*[4] have recently reported that human cells are capable of the photoreversal of UV-induced pyrimidine dimers and possess photoreactiving enzyme activity. Sutherland *et al.*[4] further showed that XP cells have lowered levels of photoreactivating enzyme. We have therefore investigated (using Sutherland's experimental conditions) the photoreversal of UV-induced pyrimidine dimers in normal human cells and two different XP fibroblast strains. The data of these experiments are shown in Table 15. As can be seen from these data, there is no photoreversal of UV-induced pyrimidine dimers after 60 min of white-light treatment under Sutherland's experimental conditions. We therefore concluded that neither normal cells nor xeroderma cells are capable of *in vivo* photoreversal of dimers. Although, it has been clearly shown[4] that these cells possess an activity which appears to be photoreactivation enzyme, this enzyme apparently does not perform its proposed function *in vivo*.

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Table 15. Lack of photoreversal of UV-induced pyrimidine dimers in normal human and xeroderma pigmentosum skin cells

Cells	UV dose at 254 nm (J/m^2)	Dimers at 0 time (%)	Dimers after 60 min of white light (%)
Normal	13	0.055	0.058
(HSBP)	75	0.270	0.320
XP12BE	10	0.041	0.043
(Jay Tim)	75	0.34	0.30
	100	0.43	0.42
XPSGOR			
(SoGre)	10	0.045	0.045

EFFECT OF PUTATIVE REPAIR INHIBITORS ON DNA REPAIR IN NORMAL HUMAN SKIN CELLS AFTER ULTRAVIOLET RADIATION

James D. Regan and W. C. Dunn, Jr.

Using the BrdUrd photolysis assay for DNA repair, we have examined a number of possible inhibitors of DNA repair. Some of these chemicals are produced by coal conversion processes. Certain others of these agents have been suggested as possible repair inhibitors or are inhibitors of semiconservative synthesis. The data from these experiments are summarized in Table 16.

Quinacrine hydrochloride is an effective inhibitor of DNA single-strand-break rejoining after gamma radiation in human cells. We found it had no effect on repair in human cells after ultraviolet radiation. 8-Aminoquinoline at 10^{-3} *M* is a very effective inhibitor of repair, inhibiting by approximately 50%. Lower concentrations also inhibit to a lesser degree. 2,4-Dinitrophenylhydrazine at 5×10^{-4} *M* is also an effective inhibitor of repair. It also, upon radiation of the cells with 313-nm light, produces a photodynamic effect resulting in further breakage of the DNA. However, by the methods of our analysis this photodynamic effect is subtracted out from the repair-inhibitory effect (Table 16). Hydrazine sulfate at 10^{-3} *M* was also found to be an effective inhibitor of repair but had no photodynamic action. Three other compounds (1-naphthylamine, furylfuramide, and nalidixic acid) had significant photodynamic effects, in combination with the 313-nm light, on the cells, but did not inhibit DNA repair. Further studies of the mechanism of the compounds found to be inhibtory to DNA repair are in progress.

NITROSOCARBARYL AND NITROSOCARBARYL-LIKE COMPOUNDS: THEIR EFFECTS ON HUMAN DNA

R. D. Blevins, William Lijinsky, A. A. Francis, and James D. Regan

Human skin cells (normal and xeroderma pigmentosum) were treated with carbaryl (*N*-methyl-1-

Table 16. Effect of putative repair inhibitors on DNA repair in normal human skin cells after 200 ergs/mm² of 254-nm radiation

Agent	Dose[a]	DNA damage	UV repair inhibition	Δ 1/mol. wt × 10^{-8}		Photodynamic action
				Control	Expt.[b]	
Quinacrine hydrochloride	2×10^{-6} *M*	–	–	3.38	3.42	None
	1×10^{-6} *M*	–	–	3.58	3.62	
8-Aminoquinoline	1×10^{-3} *M*	–	++ (~50%)	3.53	1.55	None
	5×10^{-4} *M*	–	+ (<40%)		2.21	
	2×10^{-4} *M*	–	+		2.51	
8-Hydroxyquinoline	1×10^{-6} *M*	–	–	3.53	4.68	None
1-Naphthylamine	5×10^{-4} *M*	–	–	2.93	2.42	Photodynamic mol. wt reduction by 313 nm equal in dThd and BrdUrd incubated cells
2-Methylnapthalene	5×10^{-4} *M*	–	–	2.93	2.48	None
2,4-Dinitrophenylhydrazine	5×10^{-4} *M*	–	++	2.75	1.38	Photodynamic mol. wt reduction by 313 nm unequal in dThd and BrdUrd incubated cells
	1×10^{-4} *M*	–	–		3.00	
Hydrazine sulfate	1×10^{-3} *M*	–	++		1.54	None
Furylfuramide (AF-2)	5×10^{-5} *M*	–	–	1.98	2.38	Photodynamic mol. wt reduction by 313 nm equal in dThd and BrdUrd incubated cells
	1×10^{-5} *M*	–	–		2.33	
Deoxycholate	5×10^{-5} *M*	–	–	4.04	3.76	None
Nalidixic acid	1×10^{-3} *M*	–	–		2.57	Photodynamic mol. wt reduction by 313 nm equal in dThD and BrdUrd incubated cells
	1×10^{-4} *M*	–	–		2.87	

[a]Exposure time = repair period (20 hr) for UV-repair studies
= 90 min for DNA damage studies.

[b]Δ 1/mol. wt is a measure of the magnitude of repair. A lower Δ 1/mol. wt means less BrdUrd insertion, hence less repair.

Table 17. Weight-average molecular weights of DNA in normal human skin observed in alkaline sucrose gradients after treatment for 1 hr with the indicated compound at a concentration of 10^{-5} *M*

Insecticide	(10^6 daltons) at three assay times:					
	Zero time		2 hr		20 hr	
	Treated with insecticide	Treated with *N*-nitroso derivative of insecticide	Treated with insecticide	Treated with *N*-nitroso derivative of insecticide	Treated with insecticide	Treated with *N*-nitroso derivative of insecticide
Aldicarb	294	138	272	122	267	135
Baygon	276	111	228	80	263	90
BUX	266	151	279	185	274	149
Carbofuran	289	168	276	172	276	176
Landrin	268	186	316	218	285	180
Methomyl	256	139	268	176	322	270
Control	~300		~300		~300	

napthyl-carbamate), a common agricultural pesticide, or its *N*-nitrosoderivative, nitrosocarbaryl, and the DNA of the cells was sedimented in alkaline sucrose gradients at several times after treatment. Numerous single-strand breaks were apparent in the DNA of the nitrosocarbaryl-treated cells but not in the DNA of those treated with carbaryl. The nitrosocarbaryl effect on the DNA could be observed up to 20 hr after removal of the chemical from the culture. The DNA of human cells treated with nitroso[^{3}H]carbaryl was isolated and banded in cesium chloride density gradients. The peak of radioactivity coincided with the optical density peak of the human DNA from these gradients. There were approximately ten bound nitrosocarbaryl molecules per observed single-strand break. If the human DNA was first isolated and then treated *in vitro* with nitroso-[^{3}H]carbaryl, no association with the DNA was seen. These observations suggest that nitrosocarbaryl can be activated *in vivo* and can form an irreversible association with human cellular DNA, resulting in chemical changes observable as alkaline-sensitive bonds.

In addition to nitrosocarbaryl, we have also conducted similar studies with six other insecticidal derivatives of *N*-methyl-carbamic acid and of their *N*-nitroso derivative. These insecticides (*i.e.*, the parent compounds employed) were Aldicarb, Baygon, BUX, Carbofuran, Landrin, and Methomyl. With all these compounds, after treatment of human cellular DNA, numerous single-strand breaks were apparent in the DNA of all the nitroso-derivative-treated cells but not in DNA of those treated with the parent compound (see Table 17). Since the effect of the nitroso derivative on the DNA could be observed up to 20 hr after removal of the chemical from the culture, the DNA-repairing events normally occurring in human cells after UV- or ionizing-type damage did not occur. These observations suggest that these six nitroso derivatives of *N*-methyl-carbamic acid also cause numerous alkaline-sensitive bonds in the DNA of human cells as reported above with nitrosocarbaryl. The genetic and possible carcinogenic effects of these compounds are currently under study.

INHIBITION OF SERINE TRANSHYDROXYMETHYLASE BY A SERINE ANTIMETABOLITE

A. A. Francis, D. G. Doherty, and James D. Regan

Leukocytes from patients with chronic granulocytic leukemia have been observed to exhibit a requirement for the amino acid serine. This requirement can be observed as an inhibition of incorporation of radioactive precursors into DNA and RNA[1] and should make chronic granulocytic leukemia cells especially sensitive to serine antimetabolites. A cyclic serine antimetabolite, cyclohexylserine [1-(hydroxylamino)cyclohexane carboxylic acid], has been synthesized and shown to be effective as an inhibitor of DNA and RNA synthesis in leukemia cells.[2]

The enzyme serine transhydroxymethylase, which is involved in the donation of one-carbon fragments from serine to nucleic acid precursors, has been tested for inhibition by cyclohexylserine. The enzyme was partially purified from mouse liver by centrifugation and ammonium sulfate precipitation and was assayed by the

method of Taylor and Weissbach.[3] The enzyme activity of our preparation increased with enzyme concentration, giving first-order kinetics; inhibition of the enzyme with known inhibitors, 2-aminoethylphosphonic acid and aminomethylphosphonic acid, gave results predictable from published data. We have demonstrated that cyclohexylserine is a weak inhibitor of serine transhydroxymethylase. The inhibitor reduced the enzyme activity by 30% when the substrate concentration:inhibitor concentration ratio was about 10^{-2}. The kinetics of this inhibition are not straightforward and are now being investigated, as well as additional serine analogues for their properties as inhibitors of this enzyme.

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COMPARATIVE MUTAGENESIS

J. L. Epler, Alice A. Hardigree, Ti Ho,
Ruby D. Wilkerson, and William Winton

The major goal of the comparative mutagenesis group is to provide a means of mutagenicity testing of those compounds produced by various existing or proposed methods of energy generation. These compounds include the primary effluents of existing fossil fuel sources such as sulfur dioxide, the oxides of nitrogen, ozone, hydrocarbons, and heavy metals, as well as products of newly proposed methodology such as coal liquefaction and of auxiliary methodology such as cooling-tower additives.

The work is divided into two phases: one dealing with known compounds expected to occur in the environment through energy production, conversion, or use; and another dealing with actual samples from existing or experimental processes.

To approach the problems of dealing with and the testing of large numbers of compounds, we set up a form of the "tier system." Operating units utilizing *Salmonella, E. coli,* yeast, human leukocytes, mammalian cells, and *Drosophila* have been initiated. Coordinated work has begun in whole-mammal systems. Compounds giving positive results in the "lower tier" (microbial) assays will be tested further in the higher organisms.

During the past year the bulk of the experimental results was obtained with the *Salmonella* reversion system. Approximately 150 compounds were preliminarily tested. At least two previously undetected "frame-shift" mutagens were uncovered — one a phenylhydrazine and the other a heterocyclic quinoline derivative. As a working list we have looked to those compounds expected to be used in fossil fuel production or conversion.

Many of these compounds are polycyclic hydrocarbons and require metabolic activation with mammalian extracts. The comparative role of assays with activation in microbial systems and the mutagenic response in higher organisms is being investigated.

MUTAGENICITY OF COAL CONVERSION FRACTIONS

J. L. Epler, Alice A. Hardigree, and Ti Ho
with
M. R. Guerin,* Hisashi Kubota,* and I. B. Rubin*

In a preliminary effort to examine energy conversion processes, a collaboration with the Analytical Chemistry Division was begun. The chemists furnished partially purified and identified fractions from the crude product of the coal conversion process. Preliminary screening for mutation with "Ames" strains (*Salmonella*) was carried out. A number of the fractions produced genetic damage. A few were extended to the human leukocyte system for cytogenetic analyses.

The microbial assays involved the determination of a crude inactivation curve and, subsequently, reversion of four different histidine-requiring mutants. The four "Ames" strains utilized differ in their mutational origin: TA 1535, a base substitution; and TA 1536, 1537, and 1538 frame shifts.

Results are preliminary and are obviously obtained using impure fractions. Attempts will be made to ascertain the specific compound(s) responsible for the mutagenic action. The assays will be extended to fractions obtained by liquid-liquid chromatography.

These investigations, especially the incidence of frame-shift mutagenesis and its correlation with carcinogenesis, are the initial attempts to monitor environmentally important processes for genetic damage.

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GENETIC SCREENING: HIGH-RESOLUTION CHROMATOGRAPHY AND IDENTIFICATION OF METABOLITES

J. L. Epler, C. D. Stringer, and William Winton

The research of the Biochemical Analysis of Genetic Variants group consists of two general efforts: (*a*) the

screening and chromatographic analyses of selected genetic variants, and (*b*) the isolation and identification of metabolites presumably involved in genetic lesions.

The primary materials investigated have been human body fluids and plant extracts. None of the human samples investigated thus far has pointed to a metabolic lesion under genetic control that was not suspect or already known to exist. With respect to plant materials, a preliminary collaborative effort was set up with Dr. Peter Carlson* in order to look at an array of corn mutants from the point of view of detecting biochemical parameters that might be indicative of the basis for the genetic lesion.

In addition, the collaborative effort with the Plant Sciences group was continued, and considerable effort was used in setting up automated amino acid analyses to rapidly quantitate methionine in plant extracts.

A new phase of metabolic screening was initiated using mammalian systems treated with potential mutagens. An attempt will be made to fractionate and identify metabolically activated forms of chemical mutagens through high-resolution chromatography correlated with bioassays.

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PRELIMINARY STUDIES ON THE DEVELOPMENT OF A HOST-MEDIATED MAMMALIAN CELL MUTATIONAL ASSAY FOR SCREENING POTENTIAL CHEMICAL MUTAGENS

A. W. Hsie, J. M. Holland, Richard Machanoff, and Jerry W. Hall

Using a variety of well-defined microbial tester strains, host-mediated assays have assumed an important place in defining potential genetic risk to mammals associated with the accidental or intentional exposure to a variety of chemicals and drugs. These mutagenicity assays combine the economy, simplicity, and speed of *in vitro* systems with the metabolic activation and detoxication reactions that occur in the intact organism. A critical assumption, essential to the rational interpretation of results of these assays, is that the test microorganism is equivalent, in the sense of genetic risk, to mammalian cells. It is by no means clear that this assumption is necessarily valid. Certainly, the simplest assumptions of similar accessibility, efficacy of repair enzymes, competing detoxication reactions, and cytotoxic effects are so seldom satisfied that the results of the host-mediated assay must be interpreted with caution, especially in the absence of response data obtained by more classical mammalian genetic assays.

Recently, we have developed a CHO mutational assay suitable for quantitating induced mutations at the HGPRT locus by various physical and chemical agents.[1] Because of their fibroblast origin, the cells lack the activation systems for converting some mutagens to their active forms. To broaden the applicability of mutagenicity testing of our CHO cell system, we have undertaken an approach that combines many advantages of the host-mediated assay but without the uncertainties concerning qualitative dissimilarity between the host and indicator genomes. In these preliminary studies, genetically defined CHO cells, clone K_1-BH_4 (generally referred to as CHO-K_1BH_4 cells), which have proven suitable for quantitative mutagenesis at the HGPRT locus *in vitro*,[1] are injected subcutaneously into genetically athymic nude mice. Within seven days, palpable tumors are observed at the injection site. At this time compounds of known mutagenicity and of unknown mutagenicity are injected intraperitoneally into the tumor bearers. After allowing 24–48 hr for metabolism of the drug and exposure of the growing tumor to the drug and metabolites, the animals are killed and tumors are removed, dispersed, and cultured in medium F12 supplemented with 10% heat-inactivated (56°C for 30 min) extensively dialyzed fetal calf serum [medium F12FCM(10)]. These cells were allowed to express the mutant phenotype in medium F12FCM(10) for seven days. Routine subculture (1:4) was performed whenever cell confluency was reached during the expression period. Then 1.4×10^5 cells were plated in a 100-mm dish which contained 10 ml of medium F12 minus hypoxanthine but with 10 μM 6-thioguanine added as selective agent. A total of ten plates for each treatment was used. The selective medium was renewed after two days, and drug-resistant colonies developed within 7–8 days. The colonies were then fixed, stained, and counted. Mutation frequency was calculated based on the number of the drug-resistant colonies developed per survivor at the end of the expression period. Preliminary experiments have shown that benzo(α)pyrene and 3-methylcholanthrene, which require metabolic activation to be effective mutagens, caused an approximately threefold increase of mutation frequency in this system. We are currently investigating whether the mutagenic efficacy of these agents can be improved by varying the routes of mutagen injection during different stages of the tumor growth.

Tumor growth characteristics were ideal in nude mice in that the tumors grew locally, did not infiltrate, and were easily removed without host-cell contamination. Furthermore, minor contaminations (if any) of mouse

cells could be eliminated because medium F12FCM(10) did not favor the growth of primary mouse cells. On reculture, the indicator CHO cells derived from the tumor had similar cell morphology to the original cell line and karyotypically were exclusively CHO cells. Histological sections of a series of "CHO-tumors" growing in nude mice revealed a tumor that was well circumscribed but nonencapsulated. The tumor cells were elongate and grew in parallel bundles and anastomosing bands. The tumors were divided into poorly defined lobules by a thin vascular stroma. Although the general characteristics of the tumor approximated a fibrosarcoma, overt collagen synthesis was not in evidence, which is consistent with our previous biochemical and electron-microscopic study of CHO cells in culture.[2,3] Thus the "CHO-tumor" growing in nude mice has tentatively been classified as an undifferentiated sarcoma of low-grade malignancy. The architecture and behavior of separate tumors in the same mouse and different tumors in different mice were identical.

If the "nude mouse-CHO cell mutational assay" system can be perfected, a broader spectrum of environmental agents can be screened for potential mutagenicity. Nude mice of various genetic backgrounds can be produced and maintained quickly and relatively inexpensively for the short time necessary for the assay. A variety of target tissues can be challenged *in vivo*, including human diploid lines, the only requirement being that suitable *in vitro* mutational systems for the cells be available. Thus, when perfected, we will have available an assay which is done exclusively in mammals and which may eventually provide an opportunity to challenge human cells under controlled conditions with potential environmental mutagens. Since virtually all carcinogens have also been shown to be mutagenic, this mutagenicity system should serve as a valid prescreen for potential carcinogens.

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DOSE RESPONSE OF MUTATIONS AT THE HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE LOCUS BY ALKYLATING AGENTS IN CHINESE HAMSTER OVARY CELLS[1]

A. W. Hsie, Patricia A. Brimer, J. P. O'Neill,* and D. B. Couch†

As a first step in attempting to study the possible involvement of modification of cellular DNA in mutagenesis, we have undertaken detailed analyses of mutations at the HGPRT locus by various alkylating agents in CHO cells. The frequencies of EMS-induced mutations to 6-thioguanine resistance were studied at many doses, including the minimally lethal range (0–100 μg/ml) as well as the exponential killing portion (100–800 μg/ml) of the survival curve. (For all chemically induced mutagenesis, cells were treated with mutagen for 16 hr.) We observed that the mutation frequency increases proportionally with increasing EMS concentration at fixed treatment time. The pooled data for the observed mutation frequency, $f(X)$, as a function of EMS dose, X, over the entire dose range, fitted by weighted regression analysis, could be described by $f(X) = 10^{-6}(8.73 + 3.45X)$. One interpretation of the linear fit is that, as a result of EMS treatment, ethylation of cellular constituents occurs, which is directly responsible for the mutation. That the induced mutations to 6-thioguanine resistance affect the HGPRT locus is supported by the observation that nearly all randomly isolated drug-resistant colonies contained highly reduced or undetectable HGPRT activity. The dose response of induced mutations at this locus was also studied in other alkylating agents which differ in their ability to alkylate cellular DNA. Treatment of cells with MMS at doses up to 1.25 μg/ml did not significantly affect cell survival. An exponential cell killing was observed at dose range 12.5–30 μg/ml. The MMS-induced mutation frequency for the dose range 0–20 μg/ml also fits a linear dose-response line. Compared with EMS, MMS is a much less effective mutagen, but is more toxic to the cells. MNNG causes exponential cell killing from a dose of 1 ng/ml to 1 μg/ml. A linear dose response of MNNG-induced mutations was observed for the same dose range. Similar

experiments are being carried out using *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea. Results from these studies will establish the role of the chemical structure and the reactivity of alkylating agents on two biological effects: cell survival and induced mutations.

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*NIH Postdoctoral Fellow.

†Postdoctoral investigator, Carcinogenesis Training Grant CA 05296 from NCI.

QUANTITATIVE ANALYSES OF MUTATIONS AT THE HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE LOCUS BY PHYSICAL AGENTS IN CHINESE HAMSTER OVARY CELLS[1]

A. W. Hsie, Patricia A. Brimer, Richard Machanoff, J. C. Riddle,* and A. P. Li*

Exposure of CHO cells to UV doses up to 86 ergs/mm^2 did not significantly reduce cell survival. UV doses of 86–648 ergs/mm^2 produced an exponential cell killing. The observed mutation frequency to 6-thioguanine resistance induced by UV increases in proportion to increasing doses up to 260 ergs/mm^2 in the range of 5–648 ergs/mm^2 examined. The pooled data of mutation frequency, $f(X)$, as a function of dose X from 0–260 ergs/mm^2 is adequately described by $f(X) = 10^{-6}$ (13.6 + 2.04X). One interpretation of the linear dose response is that the UV-induced premutational lesions, presumably the unrepaired pyrimidine dimers and other photoproducts, increase with increasing UV irradiation, which is directly responsible for the induced mutations. The possible cell-cycle phase specificity of UV-induced mutations to 6-thioguanine resistance was studied using synchronized cells obtained by the mitotic shake-off procedure. The results indicate the highest increase of mutation frequency in early S over that observed in G_1, with a subsequent drop again as the cells progress through S and G_2. Late mitotic cells appear to yield a relatively low mutation frequency. Using an exposure distance of 36 in., as recommended by the manufacturer, sunlamp exposure of up to 1 min did not affect cell survival appreciably; an exponential cell killing was observed when cells were exposed to the sunlamp from 1.5–6 min. Sunlamp-induced mutations to 6-thioguanine resistance could be observed for an exposure time as low as 15 sec, and increased proportionally with exposure times up to 4.5 min. The shapes of sunlamp-induced cell survivals and mutations to 6-thioguanine were similar to those produced by UV. Analyses of the rate of mutation frequency per unit of sunlamp dose demonstrated that 1 min of sunlamp exposure yielded an "equivalent" UV dose of 70 ergs/mm^2. X ray at doses up to 100 rads did not significantly affect the survival of CHO-K_1 BH_4 cells. However, doses of 200–800 rads produced an exponential loss of cellular reproductive capacity. X ray at doses lower than 200 rads did not appear to induce mutations to 6-thioguanine resistance appreciably. A linear increase of X-ray-induced mutations was observed from 200–800 rads. The nonlinear, cumulative type of dose-response curve suggests that there could conceivably be a threshold effect of X-ray-induced mutations.

1. In part in *Somatic Cell Genetics*, **1**, 247–61 (1975).

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ORIGIN OF VARIANTS RESISTANT TO PURINE ANALOGUES IN CHINESE HAMSTER OVARY CELLS

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Studies from many laboratories have established that the enzyme HGPRT is responsible for the sensitivity of mammalian cells to purine analogues such as 8-azaguanine and 6-thioguanine. Whether variants resistant to purine analogues arise as the result of gene mutation or of epigenetic changes in diploid (1S) and tetraploid (2S) CHO cell lines was examined by investigating: (*a*) the difference of the dose-response relationship for EMS-induced cell survival and mutations to 6-thioguanine resistance, which are known to affect primarily if not exclusively the HGPRT locus; (*b*) the differential effects of 8-azaguanine vs 6-thioguanine on the spontaneous and EMS-induced mutation frequency; (*c*) the physiologic-biochemical analysis of the HGPRT activity of the drug-resistant clones. We observed that EMS induced higher cell killing in 1S than in 2S cells at every dose examined. Both D_g and D_0 are higher in 2S than in 1S cells. In the 1S cells, EMS at 25 μg/ml induced mutations to 6-thioguanine at a frequency 5 times the spontaneous frequency. The induced mutation frequency increases with increasing doses up to 200 μg/ml, whereas in the 2S cells, EMS is ineffective in inducing any appreciable increase in mutation frequency. The virtual absence of induced mutation at this recessive locus in the 2S cells is consistent with the notion that the linear dose response to 6-thioguanine resistance induced in the 1S cells arose as a result of mutation at the gene or chromosome level. Furthermore, the spon-

taneous and EMS-induced mutation frequency in both cell types was compared by varying the concentrations of 8-azaguanine (1.2, 3.0, 7.5, and 30.0 μg/ml) and 6-thioguanine (1.7 and 6.0 μg/ml). In both cell types, both the spontaneous and the induced mutation frequencies decrease with increasing concentration of purine analogues. When 8-azaguanine at 30 μg/ml was used, no resistant colonies were observed in spontaneous or EMS-treated cultures of either cell type. Preliminary physiologic-biochemical characterization of the HGPRT activity of the resistant clones indicates that a high proportion of those arising at lower 8-azaguanine concentrations (1.2 and 3.0 μg/ml) contain high levels of HGPRT activity, suggesting that variants unaffected at the HGPRT locus were selected under less stringent selective conditions.

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AUTORADIOGRAPHIC ANALYSIS OF MUTATIONS AT THE HYPOXANTHINE-GUANINE PHOSPHORIBOSYL TRANSFERASE LOCUS IN CHINESE HAMSTER OVARY CELLS

G. P. Hirsch and A. W. Hsie

Autoradiographic and staining techniques have been applied to CHO colonies surviving 6-thioguanine selection for cells which have lost activity for HGPRT. Control cells which have normal HGPRT activity incorporated tritiated hypoxanthine into macromolecules, especially RNA. The level of accumulation of hypoxanthine was 100-fold higher in control cells than in several mutant lines recovered from 6-thioguanine selection. The number of mutant colonies which had normal activity (false positives) was low (2%), while the majority of mutant colonies had very low levels of hypoxanthine accumulation. Spontaneous reversions were examined autoradiographically, but only one of approximately 20 sublines contained revertant cells. Preliminary experiments indicated that HGPRT autoradiography and staining for linked (X-chromosome) glucose-6-phosphate dehydrogenase (G6PDH) can be conducted on the same plate containing mutant clones. Routine evaluation of induced mutations by autoradiography and staining should provide specific information about the relative frequency of different classes of mutations. Small deletions, frameshifts, and some point mutations would appear as zero HGPRT activity mutants, while some point mutations would have low but significant levels of HGPRT. Colonies which are both HGPRT and G6PDH negative would be classified as large deletions.

CELL-CYCLE PHASE SPECIFICITY OF UV-LIGHT-INDUCED MUTATIONS TO 6-THIOGUANINE RESISTANCE IN CHINESE HAMSTER OVARY CELLS

J. C. Riddle,* Richard Machanoff, and A. W. Hsie

Studies from microbial systems have shown that some chemical mutagens act primarily on replicating DNA. In order to determine whether mammalian cells might also show a preferential S-phase sensitivity to mutagens, we are investigating the mutagenic effects of UV irradiation at different time points during the cell cycle of CHO cells in culture. The genetic marker followed is 6-thioguanine resistance, which is known to result from alterations in the HGPRT locus in CHO cells.

The procedure used involves obtaining synchronous populations of CHO cells by a mitotic shake-off method, then irradiating replicate plates with varying doses of UV at different time points during the cell-cycle progression. Irradiated cells are transferred to hypoxanthine-free medium after the fourth day of a seven-day expression period. Subsequently, cultures are exposed to a seven-day selection regimen using hypoxanthine-free medium containing 6-thioguanine. Surviving colonies are scored as 6-thioguanine-resistant mutants. The mutation frequency is expressed as the number of mutants per survival at the end of the expression period.

Results indicate that, with a UV dose of 100–120 ergs/mm^2 (at a dose rate of 1.2 ergs per mm^2 per sec), induction of mutation is 10- to 20-fold greater during early S than that observed during G_1. Mutation frequency declines as the cells progress through the remainder of S and G_2, with late mitotic cells yielding a relatively low mutation frequency. Lower doses give less pronounced cell-cycle effects. Using the mutation frequencies obtained over the cell cycle, one can estimate a value for an asynchronous population which compares favorably with that found experimentally. As has been previously found, cell survival is lowest when irradiation is performed in S, especially early S. Experiments to explore the role of UV dose rate, DNA synthesis and repair mechanisms, and the UV action spectrum in this mutational system are progressing.

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A VARIANT OF CHINESE HAMSTER OVARY CELLS WITH ALTERED TRAVERSE OF CELL CYCLE

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CHO cells in culture have a cell cycle of 12–13 hr which can be divided approximately into G_1 (4.5–5.0 hr), S (4–4.5 hr), G_2 (2–2.5 hr), and M (1 hr). Variants with a longer cell cycle time were isolated by treating cells with EMS followed by incubating in the presence of 5-bromodeoxyuridine (BrdUrd) for 12–14 hr. This time is sufficient for all wild-type cells to complete one cell cycle. The cells were then exposed to visible light which causes the photolysis of DNA, thus killing all cells which had incorporated BrdUrd into their DNA. Only cells which had not entered the S phase during the incubation with BrdUrd survive this selection procedure. We are analyzing one such survivor which has a generation time of 19–21 hr. Cell-cycle analysis indicated that this mutant has a normal S and G_2 phase but is altered in its M to G_1 transition. The cells' entrance into S is delayed 7–8 hr, in comparison with wild-type CHO-K_1 cells. Time-lapse photomicrographic studies demonstrated that this delay is at least partially due to an alteration in the process responsible for cytokinesis and subsequent spreading of the cells onto the substratum, suggesting that the variant might be anomalous in its microtubule-microfilament system. Chromosome studies show an apparent deletion and translocation, resulting in the net loss of one chromosome and an increase in the size of another. Further analyses of the chromosome and cell-cycle alterations are proceeding.

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BIOCHEMICAL CHARACTERIZATION OF A TEMPERATURE-SENSITIVE AUXOTROPHIC VARIANT OF CHINESE HAMSTER OVARY CELLS[1]

C. H. Schröder* and A. W. Hsie

We used a conventional procedure involving treatment with BrdUrd and visible light to isolate a stable, temperature-sensitive, auxotrophic variant, TsNd-6, from its parental CHO-K_1 cells. At the nonpermissive temperature of 39.5°C, TsNd-6 requires thymidine, hypoxanthine, and glycine for growth. Folinic acid can be substituted for hypoxanthine and glycine at the elevated temperature.

The observed ability of folinic acid to replace hypoxanthine and glycine in the culture medium suggests that this variant may be deficient in the formation of tetrahydrofolic acid (THF) and its derivatives, which play a role in the formation of glycine from serine and in the conversion of deoxyuridine monophosphate to deoxythymidine monophosphate, as well as in the biosynthesis of purines, including hypoxanthosine. It has been reported that folinic acid can be substituted for glycine in several primary cultures and in "glycine B mutants" derived from CHO-K_1 cells. In addition, the "glycine B mutants" have been shown to possess dihydrofolate (DHF)-reductase activity comparable to that of parental cells.

Our finding that in the case of the variant cell, TsNd-6, folinic acid could be substituted not only for glycine but also for hypoxanthine led us to compare the DHF-reductase activities of the variant and CHO-K_1 cells. To determine the temperature sensitivity of the DHF-reductase activity, cell extracts were prepared from both CHO-K_1 and TsNd-6 cells grown in the enriched medium at 34°C, and the enzyme activity was measured in a temperature range from 27–45°C. At each temperature (27, 36, 40, and 45°C) measured, there was no appreciable difference in DHF-reductase activity between the two cell types. Extracts were also prepared from both cell types grown in enriched and minimal medium (both at 34°C) as well as from cells grown in minimal medium at 34°C and then shifted for 6 hr to 40°C, which reduces the colony-forming ability of TsNd-6 cells by about 20%. When the DHF-reductase activity was measured at 27°C, there were no significant differences in DHF-reductase activity among the various extracts.

The variant TsNd-6 cell line described here appears to furnish a tool for studying the nature of the defect in thymidine-, hypoxanthine-, and glycine-requiring cells. This type of auxotrophy has been reported to be common in mutagen-induced auxotropic cells. In the case of TsNd-6, it was observed that folinic acid, a potential source of THF, was able to overcome the need for glycine and hypoxanthine. This suggests that the auxotrophy is caused by the inability of the cells to form THF. However, we were unable to demonstrate an obvious change in the DHF-reductase activity, a major enzyme in the synthesis of THF.

1. C. H. Schroder and A. W. Hsie, *Exp. Cell Res.* **91**, 170–74 (1975).

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CATABOLISM OF THE N^6,$O^{2'}$-DIBUTYRYL CYCLIC ADENOSINE 3′:5′-PHOSPHATE AND THE MORPHOLOGICAL TRANSFORMATION OF CHINESE HAMSTER OVARY CELLS[1,2]

J. P. O'Neill,* C. H. Schröder,† Kohtaro Kawashima,‡ and A. W. Hsie

Treatment of CHO cells in culture with N^6,$O^{2'}$-dibutyryl cyclic adenosine 3′:5′-phosphate (Bt_2cAMP) converts the normally epithelial-like cells to a fibroblast-like form. This morphological change cannot be induced by unsubstituted cAMP. The inactivity of cAMP is explained by the observation that, although CHO cells take up cAMP rapidly, it is immediately degraded to 5′-AMP by the cAMP phosphodiesterase. Incubation of CHO cells with Bt_2cAMP results in the steady-state accumulation of N^6-monobutyryl cAMP which inhibits the cAMP phosphodiesterase activity with a low K_m and thus causes an increase in intracellular cAMP. We also observed what appeared to be additional catabolism of the Bt_2cAMP, which was further studied through the use of cell-free extracts. Bt_2cAMP is rapidly deacylated to N^6-monobutyryl cAMP and more slowly to $O^{2'}$-monobutyryl cAMP. The $O^{2'}$-monobutyryl cAMP is further rapidly degraded through two pathways: deacylation to cAMP and hydrolysis to 5′-AMP as well as hydrolysis to $O^{2'}$-monobutyryl 5′-AMP and deacylation to 5′-AMP. The N^6-monobutyryl cAMP is slightly deacylated to cAMP (and then hydrolyzed to 5′-AMP) and also hydrolyzed to N^6-monobutyryl 5′-AMP. In general, the N^6-monobutyryl side chain is fairly stable. These cell extract studies are confirmed by the whole-cell uptake studies, in which N^6-monobutyryl cAMP and N^6-monobutyryl 5′-AMP were found to accumulate within the cells.

This study demonstrates that the butyryl derivatives of cAMP are degraded *per se* by CHO cell cAMP phosphodiesterase. This observation, combined with the uptake studies reported previously, point out the problems which may complicate the interpretation of experiments employing Bt_2cAMP as "second messenger." Intracellular metabolism is not usually considered, although we have demonstrated such metabolism in CHO cells resulting in the accumulation of N^6-monobutyryl 5′-AMP as well as 5′-AMP, ADP, and ATP. There is also an accumulation of N^6-monobutyryl cAMP as would be expected from earlier studies. The effect of incubation of CHO cells in culture with Bt_2cAMP is envisioned as follows: (*a*) partial hydrolysis of dibutyryl cAMP to both N^6- and $O^{2'}$-monobutyryl cAMP in the medium; (*b*) uptake of all three-butyryl derivatives, presumably at different rates; (*c*) rapid phosphodiester bond hydrolysis as well as deacylation of intracellular $O^{2'}$-monobutyryl cAMP; (*d*) slow phosphodiester bond hydrolysis and deacylation of intracellular N^6-monobutyryl cAMP; (*e*) deacylation of intracellular Bt_2cAMP, primarily to N^6-monobutyryl cAMP; (*f*) metabolism of the 5′-AMP and adenosine which is formed as a result of the above degradation; (*g*) accumulation of N^6-monobutyryl 5′-AMP which the cell appears not to metabolize further; (*h*) accumulation of N^6-monobutyryl cAMP which inhibits cAMP phosphodiesterase and results in an increase of the intracellular level of cAMP.

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1. A. W. Hsie, K. Kawashima, J. P. O'Neill, and C. H. Schröder, *J. Biol. Chem.* **250**, 984–89 (1975).
2. J. P. O'Neill, C. H. Schröder, and A. W. Hsie, *J. Biol. Chem.* **250** 990–95 (1975).

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CELL-CYCLE PHASE-SPECIFIC MORPHOLOGICAL TRANSFORMATION OF CHINESE HAMSTER OVARY CELLS BY N^6,$O^{2'}$-DIBUTYRYL CYCLIC ADENOSINE 3′:5′-PHOSPHATE[1]

J. P. O'Neill,* C. H. Schroder,† J. C. Riddle,‡ and A. W. Hsie

We have demonstrated previously that in CHO cells a high concentration of Bt_2cAMP alone or a low concentration of Bt_2cAMP plus hormones such as testosterone or prostaglandin E_1 has a remarkable morphogenetic effect in converting cells from a compact epithelial-like morphology to the spindle-shaped fibroblast form. Under our standard growth conditions, the generation time of these cells is approximately 13 hr. Bt_2cAMP treatment increases the generation time slightly. The possible cell-cycle phase-specific susceptibility of the cells to morphological transformation by these agents was studied using a synchronized cell population prepared by the conventional mitotic shake-off technique, which yields over 95% mitotic cells. Bt_2cAMP at 1 m*M* was added every hour for various time intervals over two cell cycles. This protocol permits analysis of the responsive phase (S), since the morphogenetic effect of Bt_2cAMP is clearly recognizable after 1 hr. Only cells in the first or second early G_1 phases converted to the fibroblast-like form within 2 hr after Bt_2cAMP addition. Cells in other phases were not affected morphologically, even after long treatment periods. It can

therefore be concluded that elements responsible for the cell elongation process are responsive to Bt_2cAMP only during the early G_1 phase of growth. Studies on the possible changes of cellular cyclic nucleotide systems upon Bt_2cAMP exposure and of cyclic AMP receptors during the cell cycle are in process.

1. J. P. O'Neill, C. H. Schröder, J. C. Riddle, and A. W. Hsie, *Exp. Cell Res.*, in press.

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IN VIVO ACTIVATION OF CYCLIC ADENOSINE 3′:5′-PHOSPHATE-DEPENDENT PROTEIN KINASE IN CHINESE HAMSTER OVARY CELLS TREATED WITH N^6,$O^{2'}$-DIBUTYRYL CYCLIC ADENOSINE 3′:5′-PHOSPHATE[1]

A. P. Li,* Kohtaro Kawashima,† and A. W. Hsie

Treatment of CHO cells with Bt_2-cAMP which resulted in a net increase of the intracellular cAMP level converts the compact, epithelial-like cells to a fibroblast-like shape. Kinetic studies of protein kinase activity from the 1 m*M* Bt_2cAMP-treated cells showed a threefold increase in V_{max} but no appreciable changes in the apparent K_m ($\sim 5 \times 10^{-5}$ *M*) for ATP. A time-course study demonstrated that upon treatment of CHO cells with Bt_2cAMP for ½, 1, 6, and 24 hr, approximately 50, 60, 75, and 75%, respectively, of the total cAMP-stimulable protein kinase was converted to the cAMP-independent catalytic subunits, as demonstrated by Sephadex G-100 gel filtration. Concomitantly, there is a decrease of total activity in the enzyme complex peak. These experiments demonstrate the activation of the cAMP-dependent protein kinase *in vivo*. Similarly, activation of protein kinase activity by cAMP *in vitro* (threefold over basal activity) and the similar dissociation profile to form catalytic subunits were also demonstrated in the extracts of the untreated control cells. It is possible that such *in vivo* activation, which leads to subsequent phosphorylation of certain cellular constituents, may be involved in the Bt_2cAMP-mediated morphological transformation of CHO cells.

1. A. P. Li, K. Kawashima, and A. W. Hsie, *Biochem. Biophys. Res. Commun.* **64**, 507–13 (1975).

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IN VIVO ACTIVATION OF CYCLIC ADENOSINE 3′:5′-PHOSPHATE-DEPENDENT PROTEIN KINASE IN CHINESE HAMSTER OVARY CELLS TREATED WITH PURIFIED CHOLERA TOXIN

A. P. Li,* Kohtaro Kawashima,† and A. W. Hsie

Enterotoxins are known to stimulate adenylate cyclase, resulting in the elevation of intracellular cAMP level in several mammalian tissues and cell lines. It has been demonstrated that upon exposure of CHO cells to preparations of cholera toxin, the cells convert from an epithelial-like morphology to an elongated form similar to that produced by the exogenous addition of Bt_2cAMP. Our dose-response study shows that a minimum of 1 ng of protein per ml of purified Finkelstein cholera toxin causes cell elongation within 3 hr when the cells are grown in medium F12 supplemented with 10% fetal calf serum. Time-course studies establish a positive correlation between elevation of intracellular cAMP level (threefold over the control), activation of protein kinase *in vivo* (threefold over the control), and cell elongation. Using a Sephadex G-100 gel filtration procedure, we observed a time-dependent conversion of the cAMP-stimulable protein kinase to the cAMP-independent catalytic subunits when cells were treated with cholera toxin. Such studies suggest the possible involvement of phosphorylation of certain cellular constituents by cAMP-dependent protein kinase in the cholera toxin-mediated morphological transformation of CHO cells.

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CHARACTERISTICS OF THE MICROTUBULE SYSTEM OF THE CHINESE HAMSTER OVARY CELLS AND THEIR MORPHOLOGICAL VARIANTS TREATED WITH N^6,$O^{2'}$-DIBUTYRYL ADENOSINE CYCLIC 3′:5′-PHOSPHATE

Linda S. Borman* and A. W. Hsie

The morphological response of CHO-K_1 cells to Bt_2cAMP treatment involves polymerization of microtubules and their alignment into arrays parallel to the new long axis of the cell.[1] Using several morphological variants,[1] isolated from CHO-K_1 cells after ethyl methanesulfonate treatment, the colchicine-binding capacity of cell fractions has been studied as a probe for

understanding the structure of tubulin and its alterations during morphological alteration. Two morphological variants have been examined: an epithelial-like variant M7 which polymerizes its microtubules in response to Bt_2cAMP treatment, but does not align them, and variant M6, which is always fibroblastic, even without Bt_2cAMP treatment. Since alignment of microtubules may involve the cell membrane, cells for assay were homogenized and separated by centrifugation into a membrane fraction and a supernatant fraction, both of which were assayed for colchicine-binding activity.

In general, tubulin comprises approximately 1% of total cell protein of all the cell types. Upon treatment, there does not appear to be a marked increase in the amount of tubulin present, although there is an increase of about 20% tubulin in the supernatant fraction. In all cell types, colchicine binding in the presence or absence of GTP increases sigmoidally with respect to protein concentration. The effect of increasing colchicine concentration on colchicine-binding activity reveals a difference between CHO-K_1 cells and the morphological variants when membrane fractions are assayed. In all cases, supernatant fractions from untreated or treated cells show a linear increase in amount of colchicine bound as the colchicine concentration increases. However, in the membrane fraction of CHO-K_1 cells the effect of colchicine concentration on colchicine binding changes from a sigmoidal one in untreated cell extracts to a linear one in treated cell extracts. This change is dependent on the presence of GTP in the homogenizing buffer. There is no change in colchicine binding of the membrane fraction of M7 variant with or without Bt_2cAMP treatment: the binding kinetics remain sigmoidal with respect to colchicine concentration. This observation is consistent with the hypothesis that tubulin associated with the membrane is involved in microtubule alignment. The change in binding properties observed in the CHO-K_1 cells' membrane fractions does not occur in the M7 variant which does not align its microtubules as CHO-K_1 cells do. Variant M6, which is always fibroblastic, shows a linear colchicine-binding profile with respect to colchicine concentration with or without Bt_2cAMP treatment. In untreated cells this profile is dependent on GTP, but in treated cells it is not. The presence of a linear response to increasing colchicine concentrations in a cell line which always has polymerized and aligned microtubules is consistent with the possible involvement of this membrane-associated, colchicine-binding activity with microtubule alignment and cell shape.

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1. L. S. Borman, J. N. Dumont, and A. W. Hsie, *Exp. Cell Res.* **91**, 422–28 (1975).

RADIATION-INDUCED HEMOGLOBIN VARIANTS IN THE MOUSE

R. A. Popp, Carolyn M. Vaughan, Liane B. Russell, W. L. Russell, and K. Bruce Jacobson

One mouse, $SPBB_b$ ♂72, with a radiation-induced hemoglobin difference,[1] was sterile, and its anomaly could not be studied by breeding tests. The electrophoretic and solubility properties of the hemoglobin from ♂72 suggested that its α chains were different from the α chains of its siblings. Hemoglobin from ♂72 was analyzed chemically to determine the nature of the radiation-induced hemoglobin anomaly. The α and β chains were separated by chromatography over carboxymethyl cellulose. Each chain was digested by trypsin, and aliquots of the tryptic digests were analyzed by two-dimensional chromatography and electrophoresis (fingerprinting). Soluble peptides in the α-chain tryptic digest were separated initially by chromatography over Dowex 50-X2 and further purified by paper chromatography and/or paper electrophoresis. Amino acid analyses were made for each purified tryptic peptide. Alpha-chain tryptic peptide 9 (αT-9) did not contain threonine, which is present at position 68 in one of the two α chains (the other chain has serine at position 68) in the hemoglobin of ♂72's sire. More complete analysis of αT-9 revealed that asparagine was the only amino acid at position 68; thus, neither of the duplicated and adjacent α-chain genes of the irradiated strain SEC ♂ parent of ♂72 were expressed. Whether the deficiency of the expression of the SEC α-chain genes resulted from X-ray-induced gene inactivation without absolute loss of the gene or through deletion of a region of the chromosome bearing the duplicated α-chain genes was not established.

Similar methods were used to analyze the hemoglobin in another variant, $SPBB_b$ ♂352. Chemical analyses showed that this mouse also has an α-chain deficiency indistinguishable from that in ♂72.

Mice identified as $HGB_{Fo\text{-}a}$ ♂81 and $SPBB_b$ ♂122 each were anemic when originally tested, and they continued to be anemic when tested many months later. Although a few progeny of ♂81 had high reticulocyte counts, most of its offspring had phenotypically normal blood. The hemoglobin in all progeny of ♂122 appeared to be normal. Hemoglobin from each of these anemic mice was analyzed following procedures outlined for ♂72. Fingerprints of the tryptic peptides of both the α and β chains appeared normal.

Nevertheless, many of the larger tryptic peptides were analyzed in search for neutrally charged amino acid substitutions, but none was found. Thus, the anemia in ♂81 and ♂122 does not appear to be caused by structural alterations of hemoglobin.

Chemical analyses of the hemoglobin from a variant identified as 300 $FHGB_{as}$ ♀86 are in progress; analyses have not begun on the hemoglobins from $SPBB_c$ ♀27 and $SPBB_c$ ♀732.

1. *Biol. Div. Annu. Prog. Rep. June 30, 1974,* ORNL-4993, pp. 116–17.

AMINO ACID SUBSTITUTION FREQUENCY IN HUMAN HEMOGLOBIN

R. A. Popp, G. P. Hirsch, and E. G. Bailiff

Hemoglobin from some Marshallese who were accidentally exposed to fallout from an atomic test blast in March 1954 was found to contain significantly higher amounts of the noncoded amino acid isoleucine than age- and sex-matched controls.[1] This past April we received 27 samples; next year samples from the remainder of the exposed individuals will be obtained for similar analyses. Some of this year's samples also show an isoleucine content higher than expected for controls; nine samples remain to be analyzed before data on the sex, age, and exposure of these 27 individuals will be forwarded by Dr. Robert Conard of Brookhaven National Laboratory, who also provided the samples.

During the past year, blood was obtained (from Dr. Gino Zanolli) from four of the eight persons exposed to a mixture of gamma rays and neutrons in the 1958 Y-12 accident. The isoleucine substitution frequency in hemoglobin of each of these four samples was less than the upper control range of 5 parts of isoleucine per 100,000 amino acid residues.

The higher isoleucine content in hemoglobin of persons exposed to radiation could have resulted from base-substitution point mutations that changed nonisoleucine into isoleucine codons in erythroid stem cells. Efforts are being made to prove this in an experimental animal. Another possible cause for an elevation in the quantity of isoleucine in the highly purified hemoglobin of persons exposed to irradiation might be the presence of a protein that contains isoleucine and is not completely resolved by chromatography from hemoglobin A. Fetal hemoglobin is such a protein, and it could be elevated in exposed individuals owing to radiation-induced inactivation of the β-chain gene in a few erythroid stem cells; as a consequence, HbF might be synthesized in these erythrocytes as it is in individuals with hereditary persistence of high fetal hemoglobin. For this reason, we have reinvestigated the efficiency of the rechromatographic methods to remove HbF specifically.

Human cord blood was incubated in medium containing [^{3}H]isoleucine to label the gamma chain of HbF, which contains four residues of isoleucine. Mixtures of 1 and 12% HbF and HbA were rechromatographed on carboxymethyl cellulose, and removal of HbF was determined at each purification step. Data showed that approximately 15% of the HbF was not removed by procedures employed. Thus, persons with an initial 5% level of HbF in their whole blood might have more than 10 parts of isoleucine per 100,000 amino acid residues in their hemoglobin as a result of HbF even after extensive efforts to remove it by rechromatography. Some of the hemoglobin samples we are analyzing for HbF content are also being analyzed by Dr. Samuel Boyer (Johns Hopkins University) to determine whether or not HbF levels in whole blood are correlated with our chemical analyses of isoleucine in highly purified HbA.

1. *Biol. Div. Annu. Prog. Rep. June 30, 1974,* ORNL-4993, pp. 87–89.

FIDELITY OF PROTEIN SYNTHESIS IN DIFFERENTIATED MOUSE TISSUES *IN VIVO*

G. P. Hirsch, J. M. Holland, and R. A. Popp

The frequent turnover of intracellular components and the protein synthetic apparatus in mammalian tissues is thought to require the maintenance of a high fidelity of protein synthesis. To estimate the fidelity of protein synthesis *in vivo*, the level of incorporation of the amino acid analogue α-aminoisobutyric acid (iAbu) into protein was measured. The amino acyl tRNA ligases discriminate against this analogue effectively, allowing its incorporation only 80 times per million events as an average substitution frequency for other amino acids in the hemoglobin of mouse reticulocytes. To control for potential differences in protein synthesis rates and pool sizes, the incorporation of the analogue was compared with the incorporation of leucine. Isotopic iAbu was recovered, after protein hydrolysis of liver and kidney tissues, representing 30% of the total radioactivity. Separation of tissue proteins by molecular weight revealed that the analogue was incorporated in proteins of all sizes.

In mouse strain C57BL/6Cum, analogue incorporation rate (iAbu/Leu) was 25% higher in kidney tissue

from 25-month-old mice than from 10-month-old mice; about the same in liver, heart, and serum proteins; but lower in brain. The absolute level of analogue incorporation at 24 hr agreed with the ratio data, except that the kidney differences were reduced. Statistical analysis of these data set an upper limit of 50% for the error rate increase with aging.

In strain C3H mice, iAbu incorporation relative to leucine was 47% higher in liver of 33-month-old mice than in mice three months of age. Old mice which had been treated chronically from four weeks of age with 20 mg per kg per day of methyl methanesulfonate and old mice which had received 300 rads of X ray at five weeks of age both showed protein synthesis with a fidelity comparable to untreated aged controls. Several mice with liver tumors had more iAbu in their liver tissues than did normal mice. In addition, the fidelity of protein synthesis was less in the tumor mass than in the adjacent normal tissue from the same liver. The significance of these observations rests on further characterization of the kinetics of incorporation and on the effect of experimental manipulation on the fidelity of protein synthesis. BALB/c mice which were maintained for more than two weeks on water containing 5% dimethyl sulfoxide (DMSO) showed increased errors in brain, kidney, and liver tissues with ratios of incorporation being 35, 19, and 21% higher, respectively, at 24 hr. At 48 and 72 hr the DMSO-treated mice showed less dramatic changes relative to control mice, suggesting that selective degradation and increased turnover occurs in animals with higher levels of errors. Another interesting observation was that the iAbu/Leu ratios were sometimes different for the same tissue in two different strains of mice.

SPERMATOGONIAL ENRICHMENT FOR THE PRODUCTION OF LESCH-NYHAN (HYPOXANTHINE-GUANOSINE PHOSPHORIBOSYL TRANSFERASE DEFICIENT) MUTANT MICE

G. P. Hirsch, Diana M. Popp, and R. A. Popp

Preliminary experiments were undertaken to evaluate the potential production of mutant offspring of expected genotype by enriching the population for spermatogonia of the selected phenotype prior to mating. Spermatogonial enrichment would reduce the cost of germinal-mutagen screening in the male since the frequency of recovery of mutant offspring would be increased. Mice were treated with the selective agent 6-thioguanine, which kills normal cells in culture but does not kill cells which lack the title enzyme. *In vivo*, the drug kills normal dividing cells in the gut and testis, and it causes a reduction in the number of colony-forming units in bone marrow. Female offspring from males treated twice with 6-thioguanine were challenged after weaning with the same compound. Heterozygous female carriers for this X-linked locus should express the enzyme activity in only half of their somatic cells and are therefore expected to be resistant to high levels of the drug. None of 124 female offspring has as yet demonstrated the expected phenotype. A limitation on the level of enrichment was encountered when permanent sterility was induced in males treated three times in succession.

AN ASSAY SYSTEM FOR STEM-CELL SENSITIVITY TO DRUGS

Diana M. Popp, G. P. Hirsch, and R. A. Popp

In extensive studies being done to establish fundamental knowledge in the field of environmental mutagenesis, many compounds are being studied that have multiple effects. Depending upon the method of assay, they are mutagenic, carcinogenic, and/or immunosuppressive. The multiple effects produced by a drug may result from one or more mechanisms of action. That is, a direct effect upon stem cells resulting in mutations may also cause malignancy and/or a decreased immune response. On the other hand, killing of more differentiated cells may contribute to a reduced immune response without affecting stem cells. Therefore, a study was initiated to compare the effect of various compounds on stem cells *vs* differentiated cells. When hematopoietic stem cells from bone marrow are infused into an irradiated mouse, some of the cells settle in the spleen and in nine days develop into discrete clones of cells described as spleen colonies. The number of stem cells in a bone marrow pool can be determined by assaying the number of spleen colonies developing from a prescribed inoculum of bone marrow cells. The effect of a drug on stem cells can be determined by assaying the number of colony-forming cells in the marrow of treated and untreated animals. The effect of a drug on the more-differentiated cells can be determined from nucleated-cell counts of bone marrow in treated *vs* normal mice. Compounds presently being tested are cyclophosphamide, a mutagen, immunosuppressant, and anticancer drug; butyl nitrosourea, a carcinogen; 6-thioguanine, an immunosuppressant and potent cell killer used to select for HGPRT$^-$ cells in tissue culture;

cortisone acetate, an immunosuppressant that destroys lymphocytes; and 1-(hydroxylamino)cyclohexanecarboxylic acid, an amino acid analogue synthesized by D. G. Doherty in this laboratory and suspected of being a serine antimetabolite.

The drugs were administered in regimens which the literature suggested would furnish a maximum nonlethal dose or yield a maximum effect either by killing cells or inducing mutations. Femurs and tibias were removed from the mice after treatment; the marrow was flushed from the marrow cavity and suspended in Tyrode's solution. Nucleated cell counts were made, and 10^6 and 10^5 bone marrow cells were injected into isologous, lethally irradiated mice. Nine days after bone marrow injection the mice were killed, the spleens were removed and placed in Bouin's fixative, and the number of spleen colonies was counted.

As can be seen in Table 18, the drugs can be classified into groups that affect differentiated cells only (cortisone acetate), affect differentiated cells and stem cells alike (cyclophosphamide), and affect primarily stem cells (6-thioguanine). The compound butyl nitrosourea affects stem cells to a greater degree than more-differentiated cells. 1-(Hydroxylamino)cyclohexanecarboxylic acid at the concentrations tested has no effect on cellularity or stem-cell pool; if anything this compound appears to stimulate the marrow. This study will be extended to include other compounds and broader treatments. This preliminary study already demonstrates the potential value of the colony-forming-unit assay in determining the effectiveness of a drug on the stem-cell pool; it shows that 0.1 m*M* 6-thioguanine in the drinking water for five days reduces the hematopoietic stem-cell pool to 4% of that of normal bone marrow.

Table 18. Effects of drugs on bone marrow cellularity and stem-cell pool

Compound[a]	Dose	Time (days)	No. of nucleated cells/mouse (% of normal)	CFU/mouse (% of normal)
CA	2.5 mg	2	74	115
	2.5 mg	4	58	93
CPP	3 mg	1	38	58
	5 mg	1	16	15
6TG	0.1 m*M*	3	64	18
	0.1 m*M*	5	25	4
BNU	8.0 mg	1	51	22
	8.0 mg	2	48	31
1-HACC	0.5 m*M*	3	102	125
	0.5 m*M*	5	94	133
	1.0 m*M*	3	110	127
	1.0 m*M*	5	127	132

[a]CA = Cortisone acetate, suspended in saline, injected ip; CPP = cyclophosphamide, diluted in saline, injected ip; 6TG = 6-thioguanine, diluted in H_2O, fed continuously in water bottle; BNU = butylnitrosourea, dissolved in 20% ethyl alcohol and phosphate buffer pH 4.5, injected subcutaneously; 1-HACC = 1-(hydroxylamino)cyclohexanecarboxylic acid, dissolved in H_2O and fed continuously in water bottle.

EFFECTS OF 1-(HYDROXYLAMINO)-CYCLOHEXANECARBOXYLIC ACID ON THE IMMUNE RESPONSE TO SHEEP RED BLOOD CELLS (SRBC)

Diana M. Popp

Pazmino *et al.* have shown that a cyclic serine antimetabolite restricts the growth of human granulocytic leukemia cells in tissue culture.[1] These cells have a high serine requirement in culture, and the compound, 1-(hydroxylamino)cyclohexanecarboxylic acid, appears to function as a serine antimetabolite. It inhibits DNA synthesis but not protein synthesis. Since anticancer compounds are antiproliferative, in general, they also have the corresponding effect of being immunosuppressive, which is an undesirable result of cancer drug therapy. Therefore, a study was undertaken to determine if 1-(hydroxylamino)cyclohexanecarboxylic acid is immunosuppressive.

The compound was synthesized by and obtained from D. G. Doherty. Mice were fed 2- or 4-m*M* concentrations of the compound continuously in their water for seven days. Water consumption was normal, and no ill effects were observed except that mice became hyperreactive. SRBC (1/2 ml of a 2% suspension, iv) were injected, and the mice were kept on the compound. Spleens were removed five days later and assayed for plaque-forming cells to SRBC by a modified slide Jerne plaque technique. Control mice had a normal value of 157,000 ± 18,000 plaques/spleen and 570 ± 71 plaques/10^6 nucleated spleen cells. Mice fed 2 m*M* 1-(hydroxylamino)cyclohexanecarboxylic acid had 120,000 ± 19,000 plaques/spleen (24% reduction) and 403 ± 64 plaques/10^6 cells (29% reduction). When given 4 m*M* 1-(hydroxylamino)cyclohexanecarboxylic acid there were 92,000 ± 12,000 plaques/spleen (41% reduction) and 345 ± 55 plaques/10^6 nucleated spleen cells (39% reduction).

When highly immunosuppressive drugs such as cyclophosphamide and 6-mercaptopurine are given to mice prior to antigen administration, the immune response can be completely abrogated.[2] In addition, the colony-forming stem cells of the bone marrow are severely reduced. However, administration of 0.5 and 1.0 m*M* 1-(hydroxylamino)cyclohexanecarboxylic acid has no effect on the stem cells of the bone marrow (see "An Assay System for Stem-Cell Sensitivity to Drugs," this report), and 2 and 4 m*M* 1-(hydroxylamino)cyclohexanecarboxylic acid fed for seven days has a limited effect on the immune response to SRBC. If this compound can effectively control (granulocytic leukemia) cells *in vivo*, it may be a welcome addition to the cancer drug therapy arsenal since the effect on stem cells and immune cells is not as severe as other anticancer agents.

1. N. Pazmino, D. G. Doherty, and J. D. Regan, *J. Natl. Cancer Inst.* **51**, 761 (1973).
2. A. C. Aisonberg, *Adv. Pharmacol. Chemother.* **8**, 31 (1970).

POSSIBLE EXPLANATION FOR THE REDUCED PRIMARY RESPONSE TO SHEEP RED BLOOD CELLS IN AGED MICE

Diana M. Popp

Immunocompetent cells from aged mice respond less well to a primary stimulus of sheep red blood cells than immunocompetent cells from young mice. It has been suggested that the decreased response is due to an absolute decrease in responsive cells. An alternative

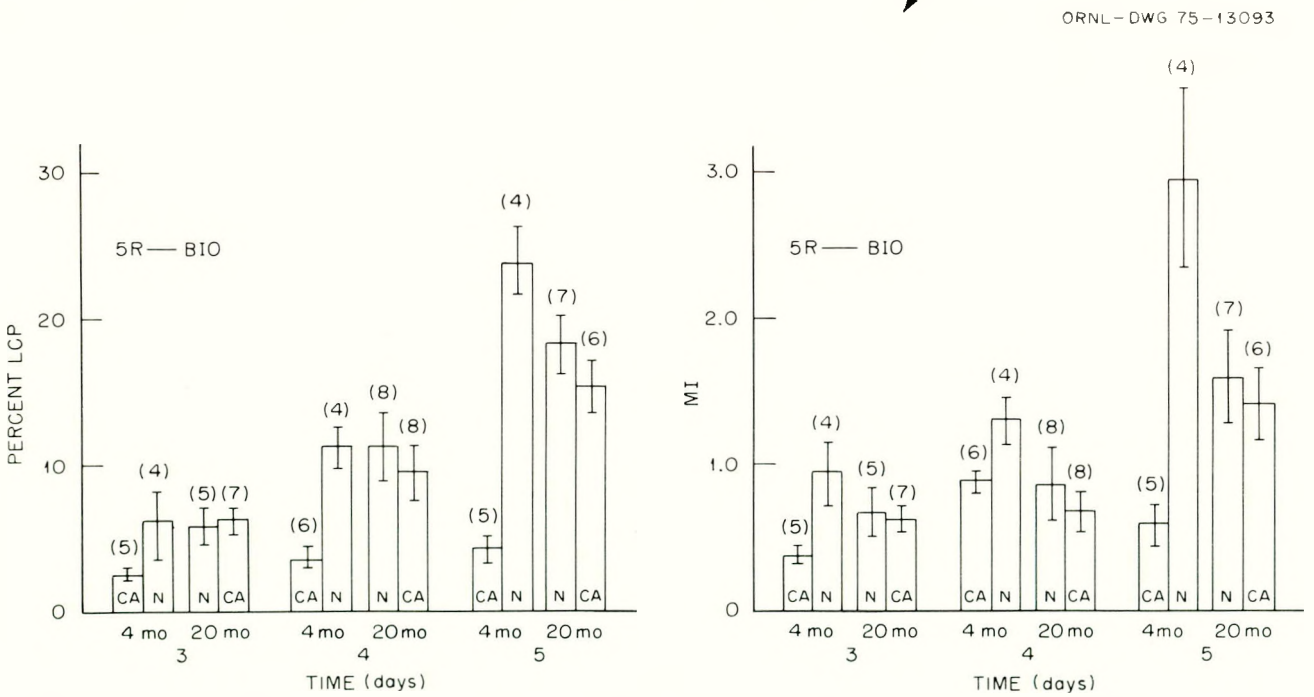

Fig. 12. The appearance of LPC in the spleen of irradiated B10 recipients of B10.A → 5R lymph node lymphocytes from young (4 month) and aged (20 month) donors 3, 4, and 5 days after injection. CA = cortisone acetate-treated donors. N = normal donors. Numbers in parentheses = number of animals.

explanation is that the cells capable of responding to a primary stimulus decrease in number with age and exposure to environmental antigens and are replaced with a population of antigen-reactive but educated cells. Such cells would no longer be capable of a primary response as defined in a young animal. The second alternative is testable since the primary immune response is sensitive to cortisone acetate, whereas cortisone acetate has little effect on the immune response when given after a priming dose of antigen. Therefore, the cortisone acetate sensitivity of lymphocytes from 20- to 23-month-old donors was tested. Strain B10.A(5R) donors of 3–4 and 20–23 months of age were injected with 2.5 mg of cortisone acetate ip two days before their lymphocytes were removed for iv injection into lethally irradiated H-2D allogenic (strain B10) recipients. The immune response of normal cells of young and old donors to H-2D antigen results in the appearance of many large pyroninophilic cells (LPC) in the spleens of the irradiated recipients. Untreated, young and aged mice show no detectable difference in the number or appearance of the LPC. When the young donors have been treated with cortisone acetate prior to lymphocyte harvest, the LPC response is abrogated. However, as seen in Fig. 12, prior treatment of the aged donors has little effect upon the appearance of LPC in the spleens of the irradiated recipients. These data suggest strongly that the lymphocyte population of aged mice consists of a large number of cells that can respond to a new antigen but behave as though they have been exposed to antigen before, presumably a cross-reacting environmental antigen such as *E. coli.*

SEPARATION OF H-2 ANTIGEN BY AFFINITY CHROMATOGRAPHY

B. S. Bradshaw,* R. A. Popp, and E. L. Candler†

Various means of extraction and solubilization of cell-surface antigens have been used, including autolysis, papain hydrolysis, sonication, hypertonic salt extraction, and exposure to detergents. One of the more successful methods of removing cell-surface antigens has been extraction with 3 *M* KCl solution. When selective immunoglobulins are attached to an insoluble polymer or gel, specific antigens, even those present in extraordinarily small quantities, can be removed by immunoabsorption from a heterogeneous population. The purpose of this study was to determine whether H-2 antigen could be isolated by 3 *M* KCl extraction in conjunction with affinity chromatography.

Spleens and thymuses from 64 RFM mice were homogenized to form a single-cell suspension of 10×10^9 cells. The cells were suspended for 20 hr in 20 ml of 3 *M* KCl in Hanks' MEM. After centrifugation at 30,000 rpm for 3 hr, the supernatant was dialyzed extensively against 0.01 *M* phosphate-buffered saline (pH 7.0). The dialyzed suspension was recentrifuged as before, and the supernatant was tested for H-2 activity by hemagglutination inhibition.

Immunoglobulin for the affinity chromatography column was prepared from C3H anti-RFM H-2 serum. Twenty-nine milliliters of dialyzed antiserum (0.01 *M* sodium phosphate buffer, pH 8.0) was placed on a DEAE-cellulose column. The column was eluted in a stepwise fashion with each of the following buffers: 0.01 *M* sodium phosphate (pH 8.0), 0.02 *M* sodium phosphate (pH 8.0), 0.03 *M* sodium phosphate (pH 8.0), and 1.0 *M* NaCl in 0.01 *M* sodium phosphate (pH 8.0). The protein eluted by 0.02 and 0.03 *M* sodium phosphate buffer (pH 8.0) was found to contain anti-RFM H-2 antibody, and it was attached to CNBr-activated Sepharose 4B; 29.3 ml of the activated Sepharose contained 3.2 mg of protein per milliliter of Sepharose. After washing twice with 10 ml of 3 *M* thiocyanate, 14.8 ml of 3 *M* KCl extract containing 1 mg/ml of protein was placed on the column and allowed to sit overnight at 4°C. The following day, 1.4 mg of protein was eluted in the absorbed fraction. This protein was found to be H-2 positive by the hemagglutination inhibition assay, but it has not yet been analyzed any further. However, this same affinity chromatography column can be used over again several times to collect more antigen.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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ROSETTE CELL FORMATION IN RESPONSE TO H-2 ANTIGEN STIMULATION

B. S. Bradshaw,* Diana M. Popp, and R. A. Popp

When donor lymph-node lymphocytes (LNL) are transferred into irradiated allogeneic recipients, some of the donor lymphocytes respond to the antigens of the host by transforming into LPC by day 1 and subsequently by proliferating. Twelve hours after the appearance of the LPC, rosettes involving the LPC and small lymphocytes develop in the spleen. The rosette can be described as an LPC surrounded by many small lymphocytes, and it presumably represents a histological visualization of an early phase of the immune response. The purpose of this study was to determine the origin

of the LPC, whether the LPC are interacting with donor or recipient lymphocytes, and whether the LPC and surrounding small lymphocytes are pure or mixed populations of T- and B-cells.

Donor lymph node lymphocytes were suspended in a mixture of 10% minimal essential medium minus Ca^{2+} and Mg^{2+}, 10% dialyzed fetal calf serum, and a pool of amino acids containing 1 μCi of [^{3}H]isoleucine. After labeling the donor cells by incubation for 30 min at 37°C, the unincorporated label was removed by washing the cells several times. Fifty million viable cells were injected iv into recipient mice that had been irradiated, 850 R, three days previously. Spleens were removed 36 hr after the infusion of labeled LNL, and sections of spleen were prepared for autoradiography. Autoradiograms showed that both the LPC and small lymphocytes of the rosettes were labeled; therefore, both are of donor origin. When the donor LNL were treated with anti-ϕ serum to remove T-cell lymphocytes, the numbers of LPC and rosettes were reduced.

The LPC seems to be a donor T-cell. The surrounding small lymphocytes are also donor cells but it has not been proven whether they are T- or B-cells.

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*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

IONIZING-RADIATION-INDUCED MUTAGENESIS: INDUCTION AND EXPRESSION IN VIABLE AND NONVIABLE CELLS

J. F. Lemontt

The *in vivo* enzymatic assay sensitive to induced reversion of *nar1-m* in *Ustilago maydis*, described in last year's report,[1] has been utilized to study gamma-ray-induced mutagenesis at doses corresponding to considerable inactivation of colony formation. The results clearly indicate that mutation induction and expression occur in nonviable cells with the same frequency as in the surviving cells.

Since *NAR1* is the structural gene for nitrate reductase (NR) enzyme, reversions of a mutant but revertible allele, *nar1-m*, leading to an active enzyme are detected by observing increased production of nitrite, the enzymatic product, from nitrate present at very large concentrations. This is achieved in a *nir1-1* background, that is, genetically defective nitrite reductase, such that nitrite accumulates in cells and in the medium. Under these conditions the rate of formation of nitrite is porportional to the concentration of active enzyme, which in turn is determined by the frequency of revertant structural genes.

Induced reversion frequencies of a single culture of *nar1-m nir1-1* cells were determined in two different ways:

(a) *Among surviving clones.* – Irradiated and unirradiated control cells were diluted and plated to ammonium nitrate minimal agar immediately after treatment. This medium contains enough NH_4^+ (0.03%) to supplement the *nar1-m* and *nir1-1* blocks. Yet, there is not enough to repress NR enzyme induction, effected by the 0.3% nitrate present. Colonies were first counted to score surviving fraction, then overlaid with soft agar containing the reagents sulfanilamide (S) and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED). These produce a red color reaction (absorbance maximum at 540 nm) visible to the naked eye in regions of high nitrite concentrations. With this technique *nar*$^+$ revertant colonies having active NR enzyme are readily identifiable. Induced reversion frequency (revertants per survivor) was calculated as the proportion of revertants after irradiation less the spontaneous level.

(*b*) *In suspension without clonal growth.* – Immediately after irradiation, treated and control cells were suspended in nitrate minimal medium and incubated with shaking at the usual optimal 32°C. Since nitrate is the sole nitrogen source, cell division is not possible because of the doubly blocked pathway of assimilation. It should be noted that *nar*$^+$ revertants do not grow either, because they still carry the nonleaky, nonrevertible *nir1-1* allele. At appropriate time intervals, samples were removed and filtered on Millipore GS membrane (0.22 μm) discs, and the clear medium was assayed for nitrite concentration using previously calibrated S and NED reagents. One unit of *in vivo* NR enzymatic activity is defined as 1 nanomole of nitrite produced by 10^8 cells per hour. Since the activity is a function of the proportion of cells that have reverted and produced active enzyme, enzymatically determined reversion frequencies were calculated by dividing this activity by the average *in vivo* activity exhibited by a collection of isogenic revertants. This assumption that all revertants lead to a single constant activity may not be the case. For example, if base substitutions are involved, a number of different base-pair changes may prove acceptable for full or partial function. Moreover, reversion frequencies so calculated must be corrected by a factor that indicates how much direct radiation damage has been suffered by the *NAR1* structural gene. Exposure of wild-type or revertant *NAR1* cells to radiation causes a dose-dependent lag in the first appearance of NR, much the same as previously

observed with UV treatment. The final level of enzyme, however, is the same as without radiation exposure unless the dose is too great. Increasingly higher doses presumably inflict increasingly more DNA damage to *NAR1*, resulting in increasingly less *in vivo* activity. Yet, although the kinetics of this inactivation are similar to those of whole cell killing, the structural gene survives far better than the entire cell itself, which indicates that all cells in the culture (those able to form colonies as well as those that are not) can and do synthesize enzyme. For example, it takes approximately 500 krads to inactivate cells of this very radioresistant fungus to 2% survival, while at this dose NR activity is reduced to only 30% of the unirradiated level. The possibility that the surviving 2% of the cells are making 15 times the normal amount of NR seems unlikely enough to be excluded.

Mutation frequencies estimated among surviving clones after gamma-ray treatment of this *nar1-m nir1-1* strain compare reasonably well with those obtained previously in the original *nar1-m nir*$^+$ isolate.[1] Enzymatically determined mutation frequencies, corrected for structural gene inactivation (*i.e.*, per functional *NAR1* cistron), are very close to those obtained among whole surviving cells. The most reasonable interpretation of these results is that expressible induced reversion is occurring, and with the same frequency in both viable and nonviable subpopulations of cells. If, on the other hand, it is assumed that mutagenesis may only occur in cells that will form colonies (survivors), the enzymatic reversion frequencies would be expected to reach a maximum and then decrease, matching the reversion-induction curve plotted as revertants per cell plated. This latter kind of result has been obtained previously by Holliday,[2] who first employed the *NAR1*-NR gene-enzyme system to monitor gamma-ray-induced allelic recombination in diploids. It was suggested that recombinational repair enzymes are themselves inducible, so that recombination could occur *only* in cells that had undergone successful repair, namely the survivors. Although very little is known about pathways of induced mutagenesis in *Ustilago*, a similar argument consistent with the current data would suggest that, if gamma-ray-induced revertants of *nar1-m* are generated by an error-prone repair system, then this system is probably not itself inducible by the radiation treatment.

1. J. F. Lemontt, *Biol. Div. Annu. Prog. Rep. June 30, 1974*, ORNL-4993, pp. 92–94.
2. R. Holliday, *Nature New Biol.* **232**, 233 (1971).

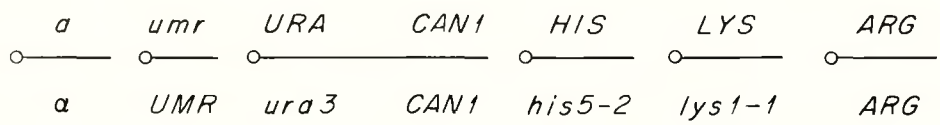
GENETIC ANALYSIS OF *umr* MUTANTS IN YEAST

J. F. Lemontt and Elizabeth L. Galyan*

Forty UV-mutation-resistant (*umr*) strains had previously been selected for varying degrees of defective UV-induced forward mutation to recessive *can1*, which confers resistance to the arginine analog, canavanine, by virtue of a faulty arginine permease enzyme. Seven of these had originally been classed as slow growing; 17 were ρ^- (petite); 16 were neither slow growing nor petite; and 3 mutants from each of the 3 above classes would not mate. Of the 16, 6 were actually self-diploidized variants; 2 were very UV sensitive, not mutation-deficient, and subsequently were found to be alleles of *rad2* and *rad4*.

All putative mutants not already excluded were tested for complementation with *rev1*, *rev2*, and *rev3*. Since all such *umr/rev* hybrids also carried the very UV-revertible *arg4-17* in heterozygous condition, mitotic recombinants leading to homozygous *arg4* were selected at first. Then, a positive complementation response was indicated in these derivatives by normal UV revertibility of *arg4-17*, that is, a reversion frequency comparable with that expected from +/+, *umr*/+, or *rev*/+ controls. Only 3 *umr* isolates exhibited noncomplementation to *rev1*, *rev2*, or *rev3* (one allele at each locus).

The remaining ten isolates were each crossed to a suitably marked *umr*$^+$ strain to yield diploids of the following type:



The petite strains were not considered at this time. After meiosis and sporulation, individual tetrads were dissected with the use of a micromanipulator to yield, following incubation and germination, pure clones of haploids derived from single meiotic cells. Routine tetrad analysis demonstrated that:

(*a*) Single nuclear mutations are responsible for defective UV mutability to *can1* in nine out of ten crosses.

(*b*) These single gene segregations cannot be explained by a disomic chromosome V because heterozygous *ura3* segregated 2:2 and not like a triploid +/+/*ura3*. *CAN1* is linked to *ura3* on chromosome V so that haploids disomic for V would not be expected to express recessive *can1* mutations, whether they be induced or spontaneous, unless in rare instances mutations were followed by a mitotic crossover or gene conversion event leading to *can1* homozygosis.

(*c*) The *umr* mutations are neither centromere-linked nor linked to other markers in the cross (α, *his5, lys1, ura3*).

Only three of the ten *umr* mutants were found to confer increased UV sensitivity compared with wild type (DRF ~ 2). Two of these, *umr-130* and *umr-338*, have no significant effect on UV-induced reversion frequencies, while the others have not yet been tested. Unlike *rad6* and *rev3*, which are relatively nonspecific blocks to UV mutagenesis, these two *umr* mutants may represent steps in error-prone repair pathways having specificity for certain kinds of DNA alterations.

Another mutant, *umr-101*, does not lead to UV sensitivity, yet it has a rather strong effect on UV mutability to *can1*. In the cross to the *umr*$^+$ strain of α mating type it was discovered that although *umr-101* and the mating type locus did not exhibit linkage, segregants carrying α and *umr-101* could not mate to *a* strains, whereas *a umr-101* segregants had normal mating ability like the original *a umr-101* parent. Assays for the production of α hormone were performed. Alpha hormone, produced by α but not *a* cells, is a diffusible peptide factor thought to be involved in the conjugation process. It inhibits initiation of DNA synthesis in *a* cells and causes them to elongate or "schmoo." It was found that α *umr-101* meiotic segregants failed to produce α hormone. Such sterile mutants have been observed before in yeast, yet this apparent relation between α hormone production and mutability is difficult to understand. More experiments are needed to exclude trivial explanations such as aneuploidy and to investigate the nature of the *umr-101* mutational deficiency.

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ERROR-PRONE REPAIR PATHWAYS IN YEAST

Gail P. Wright* and J. F. Lemontt

In previous studies of UV-induced mutation in fungi, UV-sensitive strains have been selected on the assumption that UV mutagenesis might be related to dark repair of lethal damage. In some of these UV-sensitive strains, the frequency of UV mutation is reduced compared with wild type at equal UV doses, whereas in others these frequencies are enhanced. On the other hand, in other studies strains isolated for mutational resistance were also UV sensitive.

Mutants defining an error-free repair pathway in yeast include the *rad1, rad2, rad3, rad4,* and *rad22* loci, which confer UV sensitivity and enhanced UV mutability. Mutants defining an error-prone repair pathway in yeast are not involved in excision repair and confer defective mutability: *rad6, rev1, rev2, rev3, rad9, rad18,* and others, including *umr-130* and *umr-338* (UV-mutation resistant).

To look more closely at error-prone repair pathways, various haploid double mutants were constructed and tested for UV sensitivity and UV mutability. Since all the loci are unlinked, the double mutants, *umr-338 rev3, umr-130 rad6, umr-130 rev3,* and *umr-130 umr-338,* were isolated after dissection of asci by micromanipulation followed by tetrad analyses in each respective cross. Nonparental ditype asci arise by random assortment and contain two wild-type and two double mutants. Tetratype asci could be used, but each spore would have to be outcrossed to distinguish between the double and single mutants.

UV sensitivity was tested for each double mutant at 20, 40, and 60 J/m^2, but in strains carrying *rad6* the doses were between 5 and 14 J/m^2. Single haploid mutants and a wild-type strain were also tested. Forward mutations to canavanine resistance can be induced vigorously by UV in wild-type yeast. These recessive mutations occur at a single locus (*can1*) and result from alterations of a gene coding for an arginine-specific permease. If the cells can effect arginine-deficient biosynthesis, *can1* mutants may be selected on an arginine-deficient synthetic agar medium containing canavanine. UV-induced revertibility of *his5-2* and *lys1-1* was also monitored in all the double mutants. Both these alleles are revertible and nonsense-suppressible by the same suppressor gene. This made it possible to screen for true or site revertants by replica plating. For example, the Lys$^+$ revertants at each respective dose were replica-plated onto histidine-deficient media. Colonies that do not grow are assumed to be site revertants. Of course, some of the less-common classes of suppressors will be erroneously scored as site revertants by this method.

If two loci control different steps in a single linear DNA repair pathway, then a doubly mutant strain should have the phenotype of a strain mutant at only one of the two loci. However, if the two mutants affect

independent parallel pathways, then double mutants should be more sensitive than either single-mutant strain. The interaction could be additive or synergistic. Mutants are assumed to be nonleaky.

The *umr-130 umr-338* double mutant was found to be more UV sensitive than either of the single mutants. This suggests that each mutation is in a different pathway leading to the repair of UV-damaged DNA.

Unlike the UV sensitivity, the forward mutability data show that the *umr-130 umr-338* double mutant is more mutable than either of the single mutants, not less mutable as would be expected from an interaction. The basis for this is not understood. If both single mutants were in the same linear pathway, the double mutant should be no more mutable than the *umr-338* strain, which is the most defective in mutability.

The *umr-338 rev3* double mutant was found to be just as sensitive as the most sensitive single mutant, *rev3*. This suggests that the single mutants affect the same linear repair pathway. With regard to forward mutability, this double mutant was not as defective as the most defective single mutant, *umr-338,* but its spontaneous mutation frequency was very low.

The double mutant *umr-130 rev3* remains a questionable true double mutant. It is almost as UV sensitive as the most sensitive single mutant, *rev3*. Complementation tests were later performed, however, by crossing this strain to known *umr-130* and *rev3* single mutants. Failure to complement in either case supports the existence of the two genes; yet independent segregation of each in a cross to wild type would be unequivocal.

The *rad6 umr-130* strain was found to be more sensitive than either of the *rad6* or *umr-130* single parental strains. Therefore, the two single mutants involved are probably affecting different independent pathways in the repair of UV-damaged DNA. Regarding UV-induced forward mutations, the double mutant was not as defective as the *umr-130*. Unfortunately, the *rad6* culture had a high spontaneous forward mutation frequency compared with the wild type, thereby confounding accurate measurements of induced mutability.

The results suggest a tentative pathway scheme whereby *rev3* and *umr-338* block steps in a single pathway parallel to that blocked by *umr-130*. Failure to isolate other double mutants from this collection of four single mutants makes it impossible at this time to assign a place for *rad6*.

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EFFECT OF *rev3* ON DARK-HOLDING RECOVERY AND PHOTOREACTIVATION IN YEAST

Kendra L. Caldwell* and J. F. Lemontt

The *rev3* mutation is believed to be involved in an error-prone repair pathway acting on DNA damage inflicted by UV light. Haploids (*rev3* vs *REV3*) and diploids (*rev3/rev3* vs *REV3/rev3*) with similar genetic backgrounds were constructed by use of standard yeast-genetics techniques involving microdissection of spore tetrads and induced mitotic recombination. The strains were examined for viability by plating on yeast extract–peptone–dextrose agar after the following post-UV treatments: (*a*) none, that is, immediate plating, (*b*) maximum photoreactivation with black light (specific for pyrimidine dimer reversal to undimerized form *in situ*), (*c*) holding in buffer for six days in the dark, and (*d*) six-day dark holding followed by black-light exposure. The *rev3* strains (haploids and diploids) were found to exhibit photoreactivation and dark-holding recovery. Moreover, photoreactivability was lost when preceded by the period of dark-holding. Similar results were obtained in the *REV* controls, as expected. The data suggest that (*a*) pyrimidine dimers are responsible, either directly or indirectly, for at least part of the UV-induced lethality seen in *rev3* mutants; (*b*) a dark recovery system acting on UV damage is functional despite the *rev3* defect; and (*c*) this dark recovery mechanism is one that is able to remove or excise dimers. Alternatively, it may render a certain proportion nonlethal, or else possibly it may repair nondimer damage while at the same time rendering dimers nonphotoreactivable. The data do lend further support to the hypothesis that *rev3* blocks an error-prone repair of UV damage, different from the excision system.

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GENETICS OF A DNase IN *DROSOPHILA MELANOGASTER*

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The DNases of *Drosophila* are of interest because they may have important functions in development, gametogenesis, and digestion. We are particularly interested in their role in processes of genetic importance such as crossing-over and repair of DNA damaged by mutagen.

The first step is a knowledge of the basic genetics of DNases. For this purpose we have adapted the tech-

niques of Boyd and Mitchell[1] for detecting DNase activity in polyacrylamide gels which have been electrophoresed. Most of our success has been with the DNases that are active at a pH of 4.5. In larvae and adults there are two components which migrate at different rates to the anode and there is considerable activity at the origin. In pupae one or sometimes two more components migrate to the anode on polyacrylamide gel.

D. melanogaster is polymorphic for at least three alleles of the locus that we called DNase 1. It appears that all forms of the activity that we observe are specified by this locus with the exception of the nonmigrating form. This locus is on the right arm of chromosome 3 at a genetic map position of 62.0. The locus of stripe (*sr*) is also at 62.0. DNase 1 must be less than 0.1 map unit from *sr*, but a recombinant has not been obtained, so the map order is not known at present. A deficiency of a segment of 3R bands 90D2 through 90F7 causes a loss of the DNase locus. This cytological location is consistent with the genetic locus.

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LATE CROSSOVER RESPONSE OF THE HISTONE REGION

Rhoda F. Grell

Thermal studies of the *Drosophila* oocyte have demonstrated a coincidence between the S-phase (24–30 hr in duration) and the sensitive period for increasing recombination throughout the genome.[1] During the sensitive period, different regions display characteristic peaks of response, possibly correlated with the temporal sequence of DNA replication.[2] We should like to know if the coincidence could be extended to a small region housing only a few genes. An approach to this question seemed possible with the histone genes.

In another system the transcriptional activity of the histone genes has been shown to be closely coupled to DNA replication.[3] Termination of histone transcription precedes termination of S by several hours, and presumably it is during the late, uncoupled phase that histone genes replicate. In *Drosophila,* the histone genes have been localized to region 39D,E in the salivary gland chromosome, representing a maximum of 12 bands in the proximal portion of chromosome 2.[4]

To determine if the crossover response to heat is late in this region, closely flanking markers were introduced on either side of the histone genes. Crossing-over in the demarcated region measured 0.041% (7 co/17,229 flies) in the untreated control (25 ± 1°C). Heat treatment (35 ± 1°C) of 12 hr, given before, during, or following S, gave a peak response late in S of 1.43% (180 co/12,603 flies), corresponding to a 36-fold increase in recombination (Fig. 13). This peak is in sharp contrast to that for the entire genome, which occurs ~12 hr earlier.

Labeling studies have shown that the proximal region of 2L is late labeling.[5] If labeling of the histone bands conforms to the overall pattern of this region, coincidence in replication and recombination response would be extended to a specific region.

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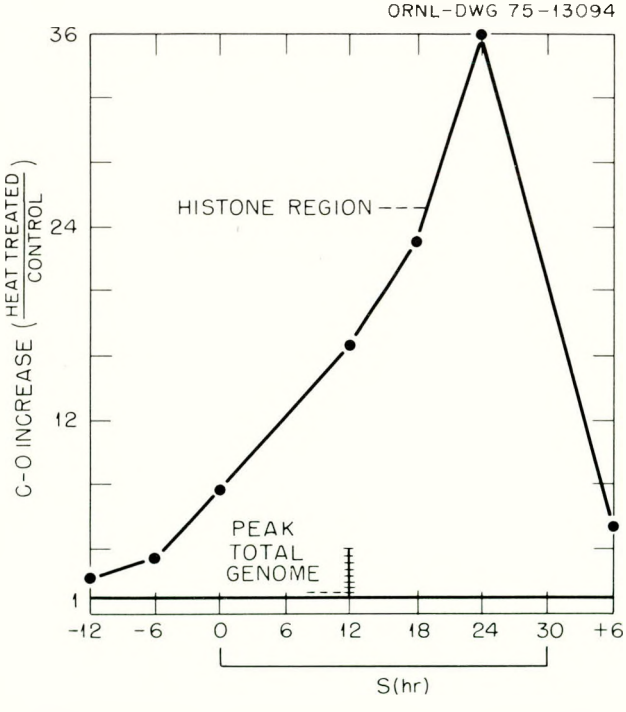

Fig. 13. Late crossing-over peak of histone region at 24h of S contrasted with peak for total genome at 12h of S.

Rec⁻ MUTANTS IN DROSOPHILA

Estela E. Generoso, Rhoda F. Grell,
and J. W. Day*

A program to recover and characterize EMS-induced *rec*⁻ mutants is continuing. Such mutants, including the temperature-sensitive variety, should serve as useful tools for analyzing the recombination process in *Drosophila*. Availability of a temperature-sensitive *rec* mutant should permit selective control of recombination for regions whose responses are temporally distinct from one another, and at the same time should furnish an independent criterion for the time of meiotic exchange.

Rather than using nondisjunction as an indicator of reduced recombination, our system measures crossover frequency directly and selects for mutants which eliminate or drastically reduce exchange. Attention thus far has focused on a chromosomal region of 18 bands within which the *rec* gene *c(3)G* lies. Newly induced mutants in this segment are characterized by genetic studies including complementation tests, temperature sensitivity, and segregation behavior, as well as by electron microscopic examination.

A total of 20,000 EMS-treated third chromosomes have now been screened, and 17 new mutants have been recovered. Among these, 14 are recessive alleles of *c(3)G* which completely eliminate meiotic exchange; 2

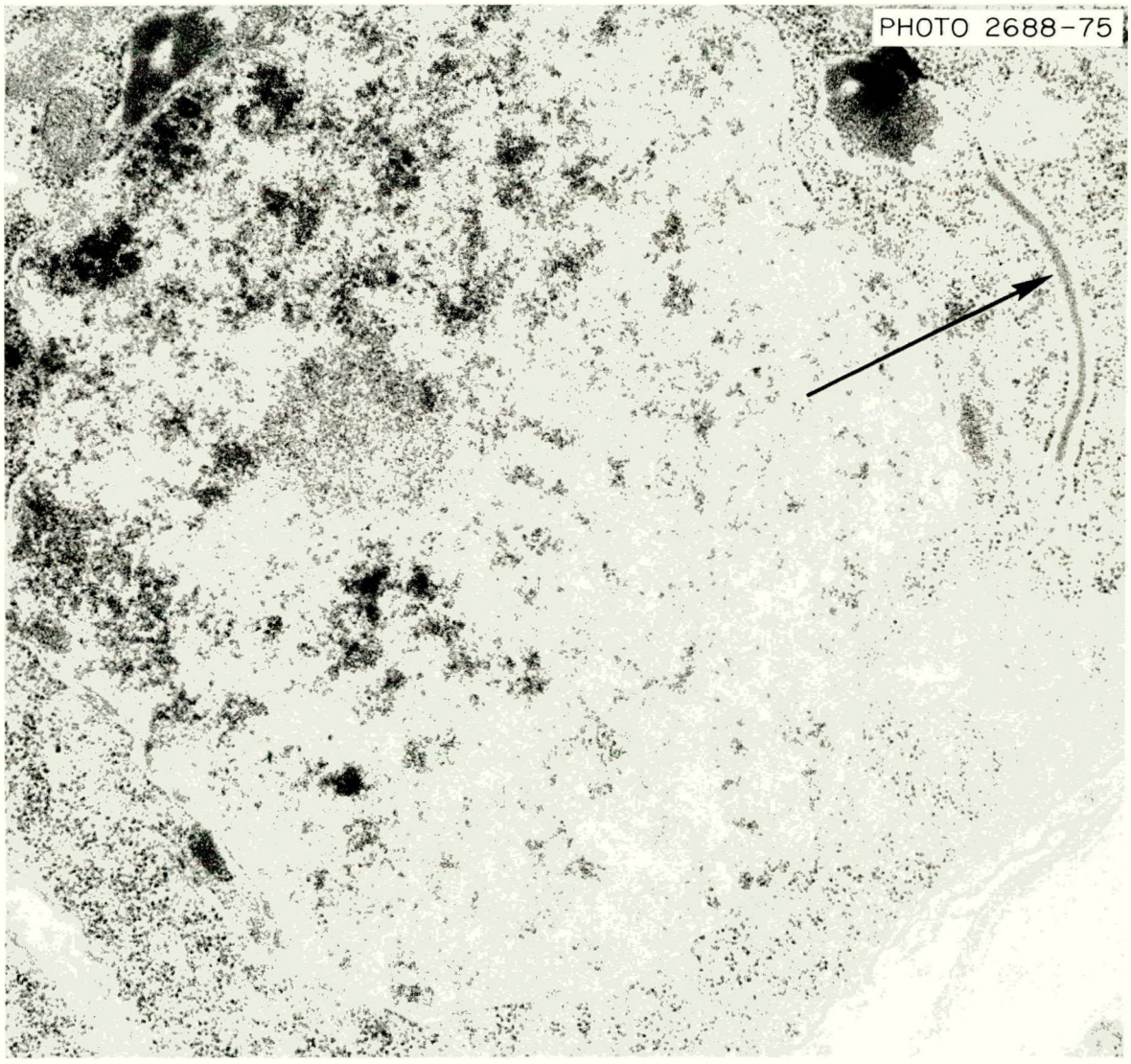

Fig. 14. Putative synaptonemal complex in cytoplasm of *c(3)G* mutant.

are recessive alleles at a new locus which reduce meiotic exchange to ~5% of normal; and 1 is either a third allele at the new locus or a third locus within the 18-band segment.

Electron microscopic examinations of 5 of the 14 new *c(3)G* alleles have revealed the absence of complexes within the nucleus, but in two cases they showed the presence of putative complexes or putative polycomplexes in the cytoplasm in the neighborhood of the nuclear membrane (Fig. 14). This is an anomalous, and to our knowledge hitherto unreported, location for the complex and warrants further study.

Electron microscopic examinations of the two alleles at the new *rec* locus show that complexes are present despite the drastic reduction in exchange. Genetic studies show that the heterozygote of the two alleles possesses the unique property of reducing exchange in the X chromosome in a polarized fashion such that the greatest reduction occurs proximally and the least reduction occurs distally. This polarity is the reverse of that hitherto observed and generalized to be the rule for polar meiotic mutants.[1]

Thus far a satisfactory temperature-sensitive *rec* mutant has not been isolated.

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HEAT AND INTERCHROMOSOMAL EFFECTS ON CROSSING-OVER

Rhoda F. Grell

The interchromosomal effect on crossing-over refers to the ability of structural alterations in one part of the genome to enhance recombination elsewhere in the genome. One goal of the temperature studies has been to determine if the pattern of heat response bears any resemblance to the pattern of the interchromosomal effect. Maps for both effects for the five chromosomal arms have now been completed, and in each case they show a striking similarity. Both produce maximal increases proximally, lesser increases distally, and minimal effects in the medial portions of the chromosome arms. The net effect of both is to cause the genetic map to more closely resemble the physical map of the chromosomes. At this point a common mechanism seemed to be a reasonable assumption.

Recently, the temperature studies have been extended to two very short chromosomal segments, one located adjacent to proximal heterochromatin and the other spanning a centromere region. In these segments the responses to heat have been found to be much greater than any encountered previously, and show a 30- to 40-fold increase over the control. By contrast, tests of the interchromosomal effect on the same two regions have produced only a four- to fivefold increase. In view of the similarity in gross response, the difference encountered at a finer level may be confined to proximal regions and may be indicative of a different type of recombinational event.

A CRYOBIOLOGICAL METHOD FOR THE ENRICHMENT OF FUNGAL MUTANTS

J. L. Leef* and F. H. Gaertner

The process of recovering auxotrophic mutants of fungi can be extremely laborious and time-consuming. For example, a common enrichment procedure used for isolating such mutants in *Neurospora crassa* involves repeated filtration of conidia through cheesecloth over a period of four to five days. Although this method has been used with considerable success in the isolation of various amino acid auxotrophs, it is not only tedious but, notably, has not been successful for the enrichment of vitamin auxotrophs such as those requiring niacin. Another method, commonly referred to as "inositol-less death," is less difficult but also appears to be limited in its capacity to enrich for niacin auxotrophs. Presumably, both methods fail to recover such mutants because of the incubation times required for each during the enrichment process. With a long incubation time, vitamin auxotrophs in particular can be lost by cross-feeding.

As a result of these difficulties we have begun to examine alternative enrichment techniques. Here we report an extremely simple cryobiological method which requires a very short incubation time (5–7 hr).

The method, in its simplest form, simply entails (*a*) treating the conidia with a mutagen such as UV or nitrosoguanidine, (*b*) incubating the condidia in minimal medium for 5–7 hr to allow wild-type spores to germinate, (*c*) freezing 0.1-ml aliquots of 10^7 to 10^8 incubated conidia per ml in liquid nitrogen, (*d*) thawing the frozen aliquots at 37°C, and (*e*) plating the spores on the appropriate supplemented medium. For example, tryptophan auxotrophs may be enriched at least 1000-fold by this procedure.

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HYDROXYANTHRANILATE AND RELATED TRYPTOPHAN METABOLITES AND THEIR ROLE IN CARCINOGENESIS AND MUTAGENESIS

A. S. Shetty,* J. L. Epler, and F. H. Gaertner

Chromatographic, metabolic, and kinetic studies revealed two distinct kynureninase-type enzymes in many microorganisms. In these studies[1,2] a single constitutive hydroxykynureninase was observed in *Saccharomyces cerevisiae*. Due to the activity of this enzyme, *S. cerevisiae* excretes hydroxyanthranilate (HA). The latter observation led to an analysis for the presence of HA (a putative bladder carcinogen) in fermentation products such as wine and beer. Although HA was not detected by paper or gas chromatography, some aromatic compounds with fluorescent and chromatographic characteristics similar to HA were found.

Since most carcinogens are known to be mutagens, we are testing the mutagenicity of several tryptophan metabolites, as well as ethyl acetate extracts of beer and wine, with the *Salmonella* strains of B. Ames *et al.*[3] Our preliminary results indicate that hydroxykynurenine, or an oxidized form of it, may be a frame-shift mutagen of strain TA 1537. We are also interested in the mechanism of detoxification of HA. Our experiments with mouse-liver preparations show the presence of a highly active hydroxyanthranilate oxygenase (HAO). Rapid metabolism of HA is further evidenced by the lack of HA in urine of mice, collected after injections of this compound either singly or in combination with reported inhibitors of HAO such as salicylate or *p*-aminobenzoic acid.

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KYNURENINASE-TYPE ENZYMES OF *PENICILLIUM ROQUEFORTI, ASPERGILLUS NIGER, RHIZOPUS STOLONIFER*, AND *PSEUDOMONAS FLUORESCENS:* FURTHER EVIDENCE FOR DISTINCT KYNURENINASE AND HYDROXYKYNURENINASE ACTIVITIES

A. S. Shetty* and F. H. Gaertner

The kynureninase-type enzymes of three fungi and one bacterium were isolated and examined kinetically for their ability to catalyze the hydrolysis of L-kynurenine and L-3-hydroxykynurenine. The phycomycete *Rhizopus stolonifer* was found to contain a single, constitutive enzyme with K_m for L-3-hydroxykynurenine and L-kynurenine of 6.67×10^{-6} and 2.5×10^{-4} *M* respectively. The ascomycetes *Aspergillus niger* and *Penicillium roqueforti* each contain an enzyme, induced by L-tryptophan, with similar K_m for L-3-hydroxykynurenine and L-kynurenine ranging from 5.9×10^{-5} to 14.3×10^{-5} *M*, as well as a constitutive enzyme with K_m for the two substrates of 4×10^{-6} and 10^{-4} *M*. The bacterium *Pseudomonas fluorescens* has a single, inducible enzyme with K_m for L-3-hydroxykynurenine and L-kynurenine of 5×10^{-4} and 7×10^{-5} *M*. In addition, significant differences in maximal velocities (V_{max}) were observed in two cases. The V_{max} of the inducible activity from *P. fluorescens* was 4.5 times greater for L-kynurenine than for L-3-hydroxykynurenine, whereas the V_{max} of the constitutive activity from *R. stolonifer* was 2.5 times greater for L-3-hydroxykynurenine. It is concluded (*a*) that the constitutive activities are hydroxykynureninases involved in the biosynthesis of nicotinamide adenine dinucleotide from L-tryptophan, (*b*) that the inducibile activities are kynureninases involved in the catabolism of L-tryptophan to anthranilate, and (*c*) that *R. stolonifer* and *P. fluorescens*, respectively, carry the most specific examples of each type of enzyme.

*Supported by a Carnegie Corporation Grant to the University of Tennessee, an NIH Grant RR 08111 to Florida A & M University, and the U.S. Energy Research and Development Administration. Permanent address: Florida A & M University, Tallahassee 32307.

COORDINATE ACTIVATION OF A MULTIENZYME COMPLEX BY THE FIRST SUBSTRATE. EVIDENCE FOR A NOVEL REGULATORY MECHANISM IN THE POLYAROMATIC PATHWAY OF *NEUROSPORA CRASSA*

G. R. Welch* and F. H. Gaertner

The catalytic constants of four of the five enzymes of the aromatic complex of *Neurospora crassa* were enhanced significantly when incubated with the first substrate, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). Activation with DAHP was accomplished independently of catalysis by incubating the purified enzyme system in a mixture devoid of requisite cofactors and intermediate substrates. The activity of each enzyme in the complex was subsequently assayed in appropriate complete reaction mixtures. Double reciprocal plots of the kinetic data were used to determine the effect of DAHP on the catalytic constants of each enzyme. The results for the five enzymes – dehydroquinate synthase, dehydroquinase, dehydroshikimate reductase, shikimate kinase, and *enol*-pyruvylshikimate synthase – were as follows. Incubated in the absence of DAHP (*i.e.*, unactivated) the maximal velocities (V) in relative units were 1, 20, 4, 2, and 5, respectively, and the k_m's were 0.06, 0.1, 0.04, 0.1, and 0.1 mM. In direct comparison, when the complex was incubated with DAHP (*i.e.*, activated), the V values were 2, 20, 4, 2, and 5, and the k_m's were 0.01, 0.02, 0.02, 0.1, and 0.01 mM. This suggests but does not prove that a single site, distinct from the catalytic site, is responsible for the coordinate activation. We propose that the physiological importance of the activation involves a novel regulatory device which provides a means for directing the flow of aromatic intermediates from the anabolic polyaromatic route to a catabolic one in response to the energy charge of the cell. In support of this view are the facts that shikimate kinase was found to be inhibited by ADP and that, as a result of the activation of the other four enzymes in the complex, shikimate kinase catalyzes a rate-limiting nonequilibrium reaction.

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INFLUENCE OF AN AGGREGATED MULTIENZYME SYSTEM ON TRANSIENT TIME: KINETIC EVIDENCE FOR COMPARTMENTATION BY THE AROMATIC COMPLEX OF *NEUROSPORA CRASSA*

G. R. Welch* and F. H. Gaertner

The aromatic complex of *Neurospora crassa* is an aggregated multienzyme system which catalyzes five consecutive reactions in the central pathway leading to the biosynthesis of the aromatic amino acids. In an attempt to understand the physiological importance of this complex in particular, as well as the importance of cellular organization of enzyme systems in general, we have isolated the complex and have begun to characterize its catalytic properties. Optimum conditions for the assay of the overall five-step reaction catalyzed by the partially purified complex have been determined. An analogue computer was programmed to represent an unaggregated system of five enzymes with rate constants identical to those found for the constituent enzymes of the complex. By direct comparison, it was shown that the lags (transient times) obtained for the overall reaction were 10 to 15 times longer for the hypothetical unaggregated system than for the complex. We conclude from these data that the unaggregated multienzyme system compartmentalizes intermediate substrates during the course of the overall reaction. We suggest that, in addition to "channeling" intermediates of competing pathways, reduction of the transient time may be an important consequence of the containment of intermediates within a physically associated enzyme sequence. The fact that the aromatic complex exhibits a second catalytic property unique to aggregated enzyme systems, "coordinate activation,"[1] indicates that the physical association of these enzymes may have more than one physiological function.

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PHOSPHOCELLULOSE, AN AFFINITY CHROMATOGRAPHIC SYSTEM FOR CHORISMATE SYNTHASE AND THE AROMATIC COMPLEX OF *NEUROSPORA CRASSA*

K. W. Cole and F. H. Gaertner

In previous studies on the aromatic multienzyme system of *N. crassa,* we have observed that 11 of 13 enzymes catalyzing the conversion of erythrose-4-phosphate and phospho*enol*pyruvate to L-tryptophan bind to DEAE-cellulose and elute in approximately the same concentration range (0.08–0.12 *M*) with a linear phosphate gradient. In particular, the five-enzyme aromatic complex and the three-enzyme anthranilate synthase complex elute nearly in coincidence.

Recently, a very active and highly stable protease activity was demonstrated in *N. crassa* which, coincidently, has some of the same molecular properties as the aromatic enzymes. Fortunately, this protease is inactive during early stages of purification because of a highly efficient and specific natural inhibitor.

In our own studies on this protease and its inhibitor (unpublished data), we have found that the protease does not bind to DEAE-cellulose, and the inhibitor binds only slightly (which again fortuitously protects the aromatic enzymes from proteolytic attack). However, some proteolytic activity may be unmasked at later stages in purification which could lead to artifactual results in studies concerning the structure and function of the aromatic enzymes.

As a consequence of the above observations and conclusions, we have attempted to eliminate the protease from our preparations at an early purification stage by taking advantage of the wide difference in the affinity of the aromatic enzymes and the protease in ion-exchange chromatography. Since the protease does not bind to DEAE-cellulose (an anion-exchange column), we anticipated that it would bind tightly to a cation-exchange column such as phosphocellulose. On the other hand, since the aromatic enzymes bind to DEAE-cellulose, we expected them to simply wash through the phosphocellulose, free of the protease.

Here we report the surprising result that two of the aromatic enzyme components, chorismate synthase and the aromatic complex, bind tightly to phosphocellulose. Our results indicate that this cation exchanger may serve as an affinity chromatographic system for these enzymes as well as other similar types of enzymes.

SCANNING ELECTRON MICROSCOPY (SEM) OF THE OOCYTE FOLLICLE

J. N. Dumont

SEM provides a new dimension to the study of the morphology of cells and tissues. In our studies of developing amphibian oocytes it has provided new data regarding the relationships of the cells and tissues comprising the oocyte follicle. Such information is important if we are to understand, for example, the influence of certain cells (*i.e.*, follicle cells) on gamete development and maturation or the transport of material to the oocyte surface. The follicle which surrounds developing oocytes is composed of several discrete tissue components. Covering the external surface of the follicle is a continuum of extremely flat squamous cells referred to as the superficial epithelium. The margins of adjacent cells form close junctions with each other so that individual cells are almost indistinguishable from each other. The surface of these epithelial cells is studded with short microvilli. Beneath the superficial epithelium is a layer of connective tissue composed of collagen fibers, a few fibroblast cells, and capillaries. When examined with SEM it is revealed that the collagen fibers are aggregated into rather discrete loosely packed bundles and that these bundles are randomly oriented throughout this stratum. The capillaries are embedded in the connective tissue. Underlying the connective tissue layer is another stratum of cells, the follicle cells. These are relatively large stellate squamous-like cells, but, unlike the cells of the superficial epithelium, they are not as closely associated with each other. Although they form a continuous layer, associations between adjacent cells are made via radiating pseudopodial processes. There are, therefore, many spaces between adjacent cells. This feature is important when one considers that oocyte nutrients and yolk protein precursor (which is synthesized in the liver) must be transported from the capillaries through this layer of cells in order to reach the developing oocyte. Separating the follicle cells from the oocyte is an acellular, finely filamentous layer referred to as the vitelline envelope.

SEM has revealed features of this envelope which were previously undetected. Randomly dispersed throughout the vitelline envelope are relatively large pores or channels. Macrovillar processes from the follicle cells traverse some of these channels to make contact with the membrane of the developing oocyte.

Other channels appear empty. The porosity of the vitelline envelope indicates that this component of the follicle does not provide a substantial barrier to the passage of nutrients and yolk precursors from the blood to the developing oocyte. The surface of the oocyte itself is studded with microvilli which extend upward and make contact with the vitelline envelope.

A CULTURE MEDIUM FOR DEVELOPING OOCYTES

J. J. Eppig* and J. N. Dumont

A supplemented oocyte maintenance medium has been developed which is capable of maintaining developing oocytes for periods up to 15 days. The medium contains a mixture of amino acids, vitamins, polyvinylpyrrolidone, salts, and oxaloacetate as an exogenous energy source. Four parameters of normal oocyte development have been measured to determine the condition of the oocytes after various lengths of time of culture in a supplemented oocyte maintenance medium. In all experiments oocytes manually dissected from their follicles were used. First, the ability of the developing oocytes to respond to progesterone was examined by measuring the ability of this hormone to induce germinal vesicle breakdown. Oocytes cultured for 15 days are capable of this response. Second, it was determined that RNA and protein synthesis continues throughout the duration of culture. Third, the ability of the oocytes to endocytose labeled yolk proteins was measured. Incorporation is reduced 50% in the first three days of culture but thereafter remains at a relatively constant rate. Oocytes cultured in a previously developed saline medium do not maintain their ability to incorporate proteins beyond about three days. Finally, the morphological integrity of cultured oocytes was examined by electron microscopy. The results indicate that cellular organelles appear normal and that endocytotic activity at the oocyte surface is maintained. The development of a supplemental oocyte maintenance medium will aid our studies of hormonal control of membrane activity, the role of membrane in the adsorption of macromolecules at the cell surface and their entry and transport within the cell, and the energy and synthetic requirements of the cell during development.

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INHIBITION OF PROTEIN INCORPORATION IN ISOLATED AMPHIBIAN OOCYTES

R. A. Wallace, J. N. Dumont, and A. W. Schuetz*

In vitro effects of steroids (deoxycorticosterone, progesterone, estrone) on uptake of radioactively labeled blood protein (vitellogenin) by isolated *Rana pipiens* oocytes were measured. Oocytes were preincubated in steroid for approximately 20 hr and then transferred to medium containing vitellogenin and no steroid. Groups of oocytes were exposed to various concentrations of steroids (0.01–1.0 μg/ml). Radioactivity was measured in oocytes after 12 hr of incubation. Estrone at all doses neither stimulated maturation nor inhibited vitellogenin uptake. Deoxycorticosterone and progesterone markedly suppressed vitellogenin incorporation, and this was closely linked to the induction of nuclear breakdown. Partial suppression of protein incorporation was observed at doses of steroid insufficient to induce maturation. Electron microscopic studies revealed a close relationship between inhibition of vitellogenin incorporation and inhibition of micropinocytosis and other structural alterations at the oocyte cortex. The results suggest a high degree of steroid structural specificity for inhibition of vitellogenin incorporation mechanisms.

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DIETHYLAMINOETHANOL AS AN ANTIFERTILITY AGENT

Mary Lou Anderson* and J. N. Dumont

DEAE has been shown to be an effective oral antifertility agent in mice at a concentration of 0.05 *M*. The compound acts as a postcoital contraceptive, which is effective during the preimplantation stages of pregnancy (days 1 through 3). All evidence indicates that DEAE prevents early embryonic development with no obvious effects on the mother. Cleavage of embryos from DEAE mothers is repressed. These embryos never consist of more than four or five blastomeres at day 3, while those from control mothers are undergoing blastulation. Culture of embryos in media containing DEAE at a concentration of 10^{-3} *M* also resulted in inhibition of cleavage of the embryos.

Investigation of embryos from DEAE-treated mothers by electron microscopy revealed changes in the ultrastructure of the blastomeres. Nuclear changes were the most obvious. Nucleoli were quite abnormal, and there was clumping of chromatin. Changes in cytoplasmic structures included a reduction in ribosomal clusters and formation of large crystalloids. Mitochondria appeared normal. It is known that protein synthesis is necessary for cell division in these embryos. It seems that protein synthesis may be affected by DEAE; however, DEAE at 10^{-2} *M* concentration had only a slight effect on protein synthesis in HEB-1A cells as demonstrated by the incorporation of [^{3}H]leucine.

Scanning electron microscopy demonstrated that the blastomeres of embryos from DEAE mothers appeared to range from normal to quite infolded or wrinkled. It was also observed that microvilli of some blastomeres either were not evenly distributed or were sparse.

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DNA POLYMERASE ACTIVITY IN *XENOPUS LAEVIS* OOCYTES DURING OOGENESIS, MATURATION, AND EMBRYOGENESIS

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During oogenesis, the amphibian oocyte accumulates and stores many different materials, such as yolk, mitochondria, glycogen, and special types of RNAs and proteins. Following a short period of hormone-induced maturation, the egg, after being fertilized, begins to divide rapidly and experiences the complex and finely controlled interactions necessary for embryogenesis. Understanding the nature of the oocyte's storage products is essential for clarifying ideas on the control of developmental processes.

We found enough DNA polymerizing activity in a single oocyte to incorporate 100–200 pmoles of [^{3}H]dAMP when assayed with poly(rA · poly(dT) primer-template. Incorporation was linear for at least 30 min in the presence of artificial template, though some deviation was seen when activated DNA was used. The addition of equal amounts of cold rATP to the [^{3}H]dATP in the assay mix did not reduce radioactive incorporation. No incorporation was obtained in the absence of primer-template or when poly(rA) was used in place of the poly(rA) · poly(dT). This activity is similar to that reported for DNA polymerase-α from chickens.

Another activity, similar to DNA polymerase-β from chickens, is also seen, although at reduced levels. The two activities can be separated on phosphocellulose columns and in glycerol gradients. Both activities appear to be concentrated in the germinal vesicle.

The amounts of DNA polymerase activity increase during oogenesis, a time period when little DNA synthesis occurs. The highest levels of enzyme activity are seen in full-grown oocytes. Although full-grown oocytes do not synthesize DNA, they have orders of magnitude more DNA polymerase activity than somatic cells with about the same DNA content. Levels of both activities remain relatively constant during steroid-induced maturation and early cleavage.

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CHROMOSOMAL-INDUCED DEVELOPMENTAL ABNORMALITIES IN EMBRYOS OF *XENOPUS LAEVIS*

T. G. Hollinger* and Katherine Luby†

It has been known for some time that many human abnormalities are caused by chromosomal aberrations. Several well-known examples are Down's, Klinefelter's, and Turner's syndromes. Convincing arguments can be made to show that chromosomal abnormalities may arise through overripeness of the oocyte. That is to say, oocytes remaining too long in either the ovary or uterus may develop chromosomal or cytoplasmic abnormalities.

Much work has been done on overripeness in amphibians. These early studies predominantly involved two means of generating overripe oocytes. First, oocytes in *Xenopus laevis* were allowed to remain in the ovary for months or even years prior to ovulation and fertilization. Second, in *Rana pipiens* it is possible to ovulate females and allow mature (*i.e.*, second metaphase) oocytes to remain in the uterus for a number of days prior to fertilizing *in vitro*. In both cases it appeared likely that aging oocytes had chromosomal abnormalities and "typical" overripeness syndromes. However, aging of oocytes either in the ovary or in the uterus does not provide exposure of the oocytes to experimental manipulation. Due to the hardiness and large size of the amphibian oocyte, many useful experimental manipulations are possible. A method for aging oocytes in a manner such that experimental access is possible

would provide a powerful tool for investigating oocyte aging and associated phenomena such as chromosomal and cytoplasmic abnormalities.

We have developed a method for aging *Xenopus* oocytes *in vitro* and testing various experimental manipulations as to effect on chromosomal abnormalities. Because techniques for fertilizing *Xenopus* eggs *in vitro* have recently been published, it is now possible to fertilize oocytes at various times following their removal from the uterus. We found that oocytes could be stripped and, if the proper culture media was used, stored *in vitro*. After changing the oocytes from storage to growth media, fertilization occurs with the addition of sperm obtained from *Xenopus* testes. When oocytes were fertilized at later times following stripping, the following results were obtained. First, eggs could be fertilized more than a day after being stripped. Second, as eggs were fertilized later, a higher percentage of embryos showed developmental abnormalities similar to those previously reported for oocytes aged *in vivo*. Third, chromosomal abnormalities similar to those reported for *in vivo* oocytes were seen.

Because of these three results, it was reasonable to conclude that aging *in vitro* in our system corresponded to and resulted in similar abnormalities when compared with *in vivo* results obtained by others.

Further experiments to clarify the cause of these abnormalities were undertaken. Recent advances in understanding meiotic and mitotic spindle function both *in vivo* and *in vitro* indicated several experimental situations that either increased or decreased the presence of spindle microtubules (the component generally thought to be responsible for chromosomal movement). Since the chromosomal abnormalities seen in overripeness ovopathy could reasonably be hypothesized to result from precocious movement of one or more chromosomes, various treatments known to affect the meiotic apparatus were tried. In particular, we tried cold treatment, since this was known to rapidly decrease microtubule organization of the spindle. When oocytes placed *in vitro* were exposed to cold (4°C), the developmental abnormalities characteristic of oocyte aging appeared much sooner. This experiment tended to confirm the hypothesis given above.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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SPECIFICITY FOR VITELLOGENIN INCORPORATION BY ISOLATED AMPHIBIAN OOCYTES

R. A. Wallace and D. W. Jared

Vitellogenin is a female-specific protein sequestered from the bloodstream by growing oocytes; it serves as the macromolecular precursor in *Xenopus laevis* for the yolk proteins lipovitellin and phosvitin. Previous experiments have indicated that vitellogenin is more rapidly sequestered than other serum proteins *in vivo*. We have now labeled a variety of proteins by reductive alkylation with [^{14}C]formaldehyde and incubated growing *X. laevis* oocytes in their presence *in vitro*. Macromolecules of vitellogenin were sequestered by oocytes 20–50 times (on a molar basis) more rapidly than other proteins tested. Selectivity for vitellogenin did not appear to involve molecular size or charge. The K_m for vitellogenin incorporation was at least several orders of magnitude less than that for bovine serum albumin. At concentrations less than 10 mg/ml, bovine serum albumin did not measurably compete with vitellogenin. Conversely, above a concentration of 2 mg/ml, vitellogenin promoted bovine serum albumin incorporation by about 40%. We have developed a model to explain these results which involves binding of vitellogenin to specific clustered receptor sites on the oocyte membrane followed by micropinocytosis of the membrane complex.

A KINETIC MODEL FOR DETERMINING AMINO ACID POOL SIZE AND RATE OF PROTEIN SYNTHESIS IN RAPIDLY SYNTHESIZING CELLS

R. E. Ecker* and R. A. Wallace

If certain constraints in experimental design are followed, mathematical relationships can be derived which will describe the kinetics of incorporation of labeled amino acid into systems with expandable amino acid pools. This kinetic model can be used to derive, from the incorporation data, the amino acid pool size and the absolute rate of protein synthesis in the system. The basic equation we have derived for the incorporation of label into protein as a function of time is

$$P = Kf_2 t - \frac{Kf_2 V}{f_3}\left(1 - e^{-f_3 t/V}\right) , \qquad (1)$$

where P is the amount of label in protein at any time t, K is the specific activity of the amino acid in the extracellular environment, f_2 and f_3 are the rates of amino acid flow into the pool from the environment and out of the pool for protein synthesis, respectively, and V is the equilibrium size of the pool. This expression describes a curve which approaches a linear function with slope of Kf_2 and intercept of Kf_2V/f_3 (thus V/f_3 can be evaluated). A further extension of the model has provided the relationship

$$1/f_2 = (k_1 k_2 f_2^2)\ (1/C) + 1/f_3 \quad , \tag{2}$$

where k_1 and k_2 are proportionality constants, and C is the concentration of amino acid in the medium. Using this expression, f_3 can be determined as that value which f_2 approaches as C approaches infinity ($1/C \to 0$). Thus, by incubating systems in different concentrations of labeled amino acid and measuring P at various times, f_3 and V can be evaluated. Finally, since

$$V = V_0 + k_1 C \quad , \tag{3}$$

where V_0 is the pool size when the external concentration of amino acid is zero, values of V can be plotted as a function of C to obtain both V_0 and a measure of pool expandability. This model has now been tested with three actively synthesizing systems: (*a*) sea urchin blastulas, (*b*) mouse cleavage embryos, and (*c*) amphibian oocytes. Values for rates of protein synthesis have been derived which agree reasonably well with published data obtained by more cumbersome methods.

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MEMBRANE POTENTIALS IN LARGE OOCYTES OF *XENOPUS LAEVIS*

R. A. Wallace and R. A. Steinhardt*

Large oocytes (1.2–1.3 mm) within their follicles have an average resting potential of −25 mV. When manually dissected out of their follicles, these oocytes undergo a hyperpolarization over the next 30 min to values around −70 mV. Small increases in external $[K^+]$ hyperpolarize further. The hyperpolarization is prevented by maintaining oocytes in the follicle at 20°C, keeping dissected oocytes at 5°C, or incubating dissected oocytes at 20°C in 10^{-5} *M* ouabain or K^+-free medium. Dissected oocytes at 20°C also accumulate K^+ and lose Na^+ over an 8-hr period. The evidence thus indicates that the hyperpolarization represents the activation of an Na^+,K^+ transport system. Further pronounced changes in membrane potential do not occur in the presence of progesterone, which induces germinal vesicle breakdown and the other events associated with maturation. Ouabain and K^+-free medium inhibit the transport system and facilitate progesterone-induced germinal vesicle breakdown. The generation of the transport system or its consequences thus appears detrimental to oocyte maturation. The very largest oocytes (>1.3 mm) do not hyperpolarize when dissected from their follicles, do not accumulate K^+ and lose Na^+, but do respond to progesterone maximally, and the response is not facilitated further by ouabain or K^+-free medium. High membrane potentials observed by other workers for dissected oocytes thus appear to be a preparation artifact. Instead, one of the terminal events of oogenesis is a suppression of the tendency to generate an Na^+,K^+ transport process when oocytes are ovulated artifically (by dissection) or naturally (which occurs approximately at the same time as germinal vesicle breakdown). Apparently, little in the way of membrane potential changes occurs during the normal process of oocyte maturation.

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ROLE OF DNA LIGASE IN DNA POLYMERASE-I-DEPENDENT REPAIR SYNTHESIS IN TOLUENE-TREATED *ESCHERICHIA COLI*

Daniel Billen, G. R. Hellermann,* and D. R. Stallions

In toluene-treated bacteria, both replicative and repair synthesis occur in the presence of all four deoxyribonucleotide triphosphates. In such bacteria many small precursor molecules are lost from within the cells, and therefore their role in DNA metabolism may be measured.

In toluene-treated *Bacillus subtilis* and *E. coli* possessing normal DNA ligase activity, nicotinamide adenine dinucleotide (NAD) reduces and nicotinamide mononucleotide (NMN) stimulates a DNA polymerase-I-dependent DNA repair synthesis following X-ray exposure.[1] Since NAD is the cofactor for DNA ligase in bacteria and NMN is an end-product inhibitor of DNA ligase, the tentative conclusion was drawn that DNA ligase is involved in a coordinated control of DNA polymerase-I-directed repair synthesis.

For direct evidence of the role of DNA ligase in controlling the extent of nucleotide insertion during repair synthesis, we constructed a double mutant of *E. coli* K-12 with a conditionally lethal mutation in the structural gene for DNA ligase conferring temperature sensitivity and a mutation in the *end A* gene.

If NAD acts solely through its role as cofactor for DNA ligase, then at the nonpermissive temperature of 42°C the presence or absence of NAD should have little effect on the extensive repair synthesis observed in irradiated toluene-treated cells from which NAD has been removed by washing. It was observed that at 30°C the double mutant exhibited an NAD-sensitive X-ray-induced repair synthesis similar to that seen in toluene-treated *B. subtilis* and other *E. coli* strains that possess DNA polymerase I and DNA ligase activity. At the nonpermissive temperature of 42°C, NAD no longer reduced repair synthesis in the X-irradiated cells.

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ROLE OF DNA POLYMERASES I, II, AND III IN DNA REPAIR SYNTHESIS IN X-IRRADIATED, TOLUENE-TREATED BACTERIA

Daniel Billen and G. R. Hellermann*

We have previously reported that in *Bacillus subtilis* made permeable to nucleotides by toluene treatment, a DNA polymerase I repair activity was markedly stimulated by X-ray exposure of the permeabilized cells.[1] In a mutant lacking DNA polymerase I activity, repair synthesis was observed, but on a more limited scale. DNA ligase activity was found to reduce the DNA polymerase-I-directed repair synthesis, but had little influence on DNA synthesis in polI⁻ mutants. Because many more mutants of DNA metabolism have been isolated and characterized in *E. coli*, extensive study of DNA repair and replicative synthesis in *E. coli* is under way.

The results to date show that all three DNA polymerases, namely I, II, and III, are capable of carrying out X-ray-induced repair synthesis in toluene-treated cells. However, there are several striking differences of action among the three DNA polymerases. X-ray-induced repair synthesis carried out by DNA polymerase II or III is totally dependent on the presence of ATP, unlike that of DNA polymerase-I-directed repair synthesis. Only DNA polymerase-I-directed repair is coordinated with DNA ligase activity. As observed in *B. subtilis*, there is an exaggerated repair synthesis in cells containing polymerase I when DNA ligase is inactive, whereas in mutants lacking DNA polymerase I but possessing polymerase II and/or III activity, the extent of repair synthesis is not influenced by DNA ligase. The same relationships hold for X-ray-induced strand breakage and repair.

Using mutants in which the polymerase I defect resides in its 5′→3′ exonuclease activity, we have ascertained that this activity (nick translation) is necessary for the exaggerated repair synthesis observed in X-ray-induced synthesis carried out by DNA polymerase I.

In addition, as we observed in *B. subtilis*, replicative synthesis in X-irradiated *E. coli* is dramatically reduced unless DNA ligase and DNA polymerase I activities are expressed.

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RECOVERY FROM UV-LIGHT-INDUCED DAMAGE IN *BACILLUS SUBTILIS uvr-1*

C. T. Hadden* and Daniel Billen

A mutant (*uvr-1*) of *Bacillus subtilis* which is deficient in excision of UV-induced pyrimidine dimers from DNA shows a marked increase in ability to survive UV irradiation when plated on amino-acid-supplemented agar medium compared with survival ability when plated on nutrient agar. The extent of killing depends on the richness of the plating medium (D_0 = 2.2 J/m² for nutrient agar, and 5.6 J/m² for synthetic medium), so the effect is considered to be one of growth-dependent lethality. Irradiated stationary-phase *uvr-1* cells incubated in liquid medium lacking amino acids required for growth recover from this sensitivity to rich medium within 3–4 hr after irradiation. Recovery is greatly reduced in the absence of glucose or in the presence of NaCN, although not completely eliminated. Exponentially growing cells have only a limited ability to recover from sensitivity to rich medium.

Growth-dependent lethality can also occur in liquid medium: in nutrient broth the ability of irradiated stationary-phase *uvr-1* cells to form colonies on defined agar medium decreases during postirradiation incubation, but treatment with chloramphenicol inhibits the loss of this ability. Recovery from sensitivity to rich

media is inhibited by caffeine but not by 6-(*p*-hydroxyphenylazo)-uracil, an inhibitor of DNA replication.

Alkaline-sucrose-gradient profiles show that conditions allowing recovery also favor maintaining intact DNA strands, while DNA strand breakage or degradation is associated with loss of viability.

Recovery from sensitivity to rich medium has not been observed in the Uvr$^+$ parent or in strains carrying the mutations *uvs-42* (another deficiency in dimer excision), *recA1*, or *polA59*. A *uvr-1, recA1* mutant shows a higher level of recovery than does the *recA1* single mutant, but a much lower level than the *uvr-1* single mutant.

Apparently both the *uvr-1* defect and Rec$^+$ and Poll$^+$ functions are essential for recovery from sensitivity to rich medium. For optimal recovery, growth immediately after irradiation must be delayed. The process requires energy, apparently involves recombination, and probably results in rejoining of DNA strands in which incision but not excision has occurred. Since most stationary-phase cells of this strain contain partially replicated chromosomes, it seems likely that recovery depends on recombination between sister chromosomes before replication resumes.

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DEFECTIVE EXCISION REPAIR IN *BACILLUS SUBTILIS uvr-1*

C. T. Hadden* and Daniel Billen

Excision repair of pyrimidine dimers, a major lesion in DNA following UV irradiation, can be summarized by the following steps: (*a*) endonucleolytic incision of DNA at the site of the lesion (nicking), (*b*) exonucleolytic removal of the dimer and an additional small amount of DNA (excision), (*c*) resynthesis of DNA to fill the resulting gap (repair synthesis), and (*d*) ligation to seal the remaining nick. Repair synthesis can be studied in gram-positive bacteria by using 6-(*p*-hydroxyphenylazo)-uracil to inhibit replicative DNA synthesis. In this way it has been possible to show that UV irradiation induces a defective repair synthesis in *uvr-1* mutants of *B. subtilis*.

Two mutants which are defective in excision of pyrimidine dimers were studied (*uvr-1* and *uvs-42*). Although the mutants are nearly equal in sensitivity to UV irradiation (D_{37} for Uvr$^+$ = 19.2 J/m^2, for *uvr-1* = 2.25 J/m^2, and for *uvs-42* = 1.8 J/m^2), their responses at the macromolecular level are different. At low UV fluences, *uvr-1* cells carry out repair synthesis at fluence-dependent rates, about 30% of the rate in Uvr$^+$ cells. In contrast, *uvs-42* cells show hardly any UV-induced repair synthesis, even after fluences leading to a surviving fraction of less than 10^{-4}.

Alkaline sucrose gradient analysis of DNA shows that *uvs-42* cells are deficient in the incision step of dimer removal. The *uvr-1* strain, however, carries out incision at about 20% of the rate in Uvr$^+$ cells. This incision provides primer sites for repair synthesis. However, the defect in excision results in faulty termination of repair patches. Isopycnic analysis of DNA made by repair synthesis following a density shift shows that stretches of 300 nucleotides or greater are inserted by the *uvr-1* mutant, while the repair patches are probably considerably less than 50 nucleotides in length in the Uvr$^+$ strain.

The sensitivity of *uvs-42* cells thus results from their inability to initiate excision repair, while the *uvr-1* mutant is sensitive because it cannot complete the process once it is initiated.

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DNA METABOLISM IN TWEEN 80 PERMEABILIZED CHINESE HAMSTER OVARY CELLS

Daniel Billen and Ann C. Olson*

We have determined that 1% Tween 80 pretreatment will allow nucleotides to penetrate Chinese hamster ovary cells. As with permeabilized bacteria, this system presents an opportunity to study DNA metabolism in the "normal" or pathological state as might be found after irradiation or chemical exposure.

In our system, DNA synthesis requires the presence of all four deoxyribonucleoside triphosphates and ATP. All four deoxyribonucleoside triphosphates are incorporated. Synthesis continues for approximately 1 hr, with the highest rate of precursor incorporation between 10 and 45 min. The quantity of ^{3}HTTP incorporated is not unbearably far from that expected if only initiated replicons complete synthesis during the reaction, using current estimates for numbers and size of replicons in mammalian cells.

Prelabeled DNA is not degraded during exposure to Tween 80 or during the subsequent incubation of cells in the reaction mixture.

Scanning electron micrographs (carried out by J. N. Dumont) indicate that the treated cells remain intact in terms of gross morphology.

This system is being used to study DNA metabolism in cells exposed to ionizing radiation and should be useful in other investigations, including studies of RNA metabolism using direct precursors.

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*Consultant.

SECTION IV

MAMMALIAN GENETICS

W. L. Russell

[a] Postdoctoral investigator.
[b] Visiting investigator from abroad.

RELATIVE INDUCIBILITY OF HERITABLE TRANSLOCATIONS AND HERITABLE INVERSIONS IN POSTMEIOTIC GERM CELLS OF MALE MICE*

W. M. Generoso, Katherine T. Cain, and Sandra W. Huff

It is generally known that the various alkylating chemicals effective in inducing chromosome breakage in mammalian male germ cells induce their highest effect on postmeiotic stages (spermatozoa and spermatids) and that the most sensitive period may vary with the chemical. In addition to radiation, a number of alkylating chemicals that are effective inducers of dominant-lethal mutations have also been found to induce heritable translocations and heritable inversions in the same male postmeiotic germ-cell stages as those in which dominant lethals are induced. Thus, it is now possible to compare the relative usefulness of dominant lethals, heritable translocations, and heritable inversions as indicators of induced chromosome breakage at these germ-cell stages. We have already established that, for EMS and TEM, heritable translocations are a much more sensitive and definitive end point of chromosome breakage induced in the male postmeiotic stages than dominant-lethal mutations.[1,2]

EMS, TEM, and X rays, which are effective in inducing heritable translocations in the male postmeiotic stages, have been studied also for induction of heritable inversions in similar germ-cell stages by Roderick and Hawes.[3] Comparison of the relative inducibility of the two heritable end points can be made (Table 19) even though there are some differences in the conditions under which the respective frequencies were obtained. First, in the inversion studies, males from inbred strain DBA/2J received the mutagenic treatment, while in the translocation studies, hybrid (101 × C3H)F_1 males were used. Second, in the inversion studies the animals scored were conceived during the first two weeks after treatment. In the translocation studies the X-ray data were also based on progeny conceived during the first two weeks after irradiation, but for EMS and TEM the progeny tested were conceived during the few days when dominant-lethal, and presumably also translocation, effects are known to be maximum. And third, while the TEM dose is the same in the two studies, higher average doses of EMS and X rays were used in the inversion study. Thus, higher frequencies of translocations could be expected at the doses of EMS and X rays used for the inversion studies.

In the translocation experiments, translocation heterozygosity among male progeny was determined by fertility test and cytological examination of diakinesis-metaphase I spermatocytes.[4] The method used by Roderick and Hawes for detecting inversion heterozygotes is essentially a search for high frequencies of bridges in the first meiotic anaphase in male progeny. One of the testes is removed and histologically prepared for bridge analysis. The animal is kept alive and bred for further studies if the incidence of anaphase bridges is at least 15% (the "background" frequency is about 4%).

Table 19. Inducibility of heritable inversions and heritable translocations in the postmeiotic stages of male mice

Treatment	Dose	No. of offspring tested	No. of progeny with aberration
		Inversions[a]	
Control	–	44	0
X ray	700–1000 R	1557	15 (0.96%)
EMS	200–400 mg/kg	156	1 (0.64%)
TEM	0.2 mg/kg	97	3 (3.09%)
		Translocations	
Control	–	2633	3 (0.11%)
X ray	700 R	151	41 (27.15%)
EMS	200 mg/kg	246	79 (32.11%)
TEM	0.2 mg/kg	204	59 (28.92%)

[a] All data on heritable inversions are those of T. H. Roderick and N. L. Hawes, *Genetics* **76**, 109–17 (1974).

Despite the differences in experimental conditions under which the two studies were conducted, there seems to be, on the whole, little doubt that heritable translocations are a considerably more sensitive end point than heritable inversions for detection of chromosome breakage induced in male postmeiotic germ cells. Such a large difference in the induction rates is worth some comment. Both translocations and inversions are products of two breakage events, and on the assumption that the occurrence of a break is random within and between chromosomes, it is possible that the difference lies in the rates by which translocations and inversion exchanges are formed in mammalian male postmeiotic germ cells. On the other hand, this difference may be nonexistent or certainly not as large in mammalian oocytes.[5] Another possible explanation may lie in the methods used for detecting the two types of aberrations. It is likely that the great majority of translocations confer partial or complete sterility in heterozygous males, and detection of such reduction in fertility is rather simple. The method used for detecting inversion heterozygotes, on the other hand, not only involved considerable effort but is capable of detecting only large inversions; on the assumption that the incidence of bridges is directly proportional to the length of inversion, the minimum length of the inverted segment is equivalent to 7.5 centimorgans. Detection of shorter inversions is difficult.

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INEFFECTIVENESS OF ALKYLATING CHEMICALS IN INDUCING HERITABLE TRANSLOCATIONS IN MOUSE SPERMATOGONIA*

W. M. Generoso, Katherine T. Cain, and Sandra W. Huff

One of the most striking differences between radiation and chemical mutagens studied so far is the fact that while radiation is effective in inducing chromosome breakage in spermatogonia stem cells, alkylating chemicals are virtually not. This is a paradox because some of these chemicals, like X rays, are potent chromosome breakers in the postmeiotic stages and affect spermatogonia stem cell survival. This difference became quite clear from the cytological studies at the diakinesis–metaphase I stage done by a number of workers who found that radiation is effective in inducing reciprocal translocations on treated spermatogonia stem cells but the alkylating chemicals studied so far were virtually ineffective. It is well known, however, that very often a reciprocal translocation does not manifest itself at the diakinesis–metaphase I stage, and it is possible that certain chemicals may produce predominantly such translocations in spermatogonia stem cells. Thus there remains the possibility that chemically induced translocations not detectable cytologically can be passed through some of the progeny. To check on this possibility and to have a thorough understanding of the chemical hazard to spermatogonia stem cells, we studied the inducibility of heritable reciprocal translocations at this cell stage with chemicals cyclophosphamide, TEPA, and TEM – doses (in mg/kg) are 350 and 400; 20, 25, and 30; and 3.0 and 4.0 respectively. These chemicals were chosen because, like radiation, they are known to be potent chromosome breakers in male postmeiotic germ cells and they affect spermatogonial survival. In this study, chemically treated males were caged individually with untreated females 42 days after injection.

In all experimental groups, the treated males were sterile at the time of caging with females. The length of the sterile period among cyclophosphamide-treated males was short, 3 to 4 days for the two doses, while for TEM and TEPA doses the sterile period was long, ranging from 17 to 54 days. Combining all doses for each chemical, the incidences of partially sterile and sterile male progeny were 2 and 4 in 1633 tested for TEM, 1 and 4 in 1148 tested for cyclophosphamide, and 0 and 7 in 1031 for TEPA. The sterile ones are currently being studied cytologically by N. L. A. Cacheiro, M. S. Swartout, and L. B. Russell, but from the results so far it already appears that, unlike sterility induced in postmeiotic stages,[1] sterility induced in spermatogonia may have causes other than translocation heterozygosity.[2] The three partially sterile males (two from TEM and one from cyclophosphamide) were all confirmed cytologically as translocation heterozygotes. Nevertheless, if the rates in the experimental groups are compared with the spontaneous level of 4 in 4423 tested (pooled from controls of various experiments), none of the chemicals studied significantly

induced transmissible translocations in mouse spermatogonia. On the other hand, X rays, when given at optimum conditions, induced unequivocal increases in heritable translocations at this cell stage,[3–5] although the rates are lower than would be expected on the basis of the cytological frequency determined at diakinesis–metaphase I.

*Research sponsored jointly by the National Center for Toxicological Research and by the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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INDUCTION OF HERITABLE TRANSLOCATIONS IN MOUSE OOCYTES WITH ISOPROPYL METHANESULFONATE*

W. M. Generoso and Katherine T. Cain

A number of chemicals that are known to be effective dominant-lethal inducers in male postmeiotic germ cells have been found to induce dominant-lethal effects in dictyate oocytes of mice. While it is generally known that one of the consequences of chromosome breakage in male postmeiotic stages is the formation of reciprocal translocations, no information for such chemical effects on oocytes has been available until now. We studied the inducibility by isopropyl methanesulfonate (IMS) of heritable translocations in dictyate oocytes of mice that were in various stages in follicular development. IMS was chosen because it is an effective inducer of dominant-lethal mutations both in male postmeiotic cells and in dictyate oocytes.[1,2]

(SEC × C57BL)F_1 female mice (10–12 weeks old) were injected intraperitoneally with a dose of 75 mg/kg of IMS. This dose, in addition to dominant-lethal effects on oocytes in advanced stages of follicular development, also affects survival of young oocytes.[3] However, the degree of cytotoxicity with this dose still permits production of progeny from oocytes treated at the early stage. Treated females were caged individually with untreated males carrying the sex-linked gene *Greasy* (*Gs*) immediately after injection. A total of 1464 male progeny were tested for translocation heterozygosity. Of these, 549 were conceived within 24 days after treatment and 915 at later periods. It should be noted that there is considerable dominant-lethal effect on oocytes ovulated within 24 days after treatment. Two sterile and no partially sterile males were found among progeny conceived at the early period, and one sterile and one partially sterile male were found among progeny conceived in the later period. The partially sterile male was confirmed cytologically as a translocation heterozygote. One of the sterile males died before cytological analysis was done, and the other two that were analyzed (testes weights were normal) did not show cytological evidence of translocation heterozygosity. Thus, the results indicate that IMS, which is an effective inducer of dominant-lethal mutations in dictyate oocytes, does not induce a significant increase in the incidence of translocations in those cells that were transmissible to male offspring.

Our results are similar to those of Russell and Wickham[4] and Gilliavod and Léonard[5] with X ray in that no translocations were recovered when male progeny were scored. Searle and Beechey,[6] on the other hand, claimed that while they also did not detect transmissible translocations among male progeny from X-irradiated oocytes, induced translocations were recovered in female progeny (three confirmed translocations in 294 female progeny tested). This finding awaits verification because the number of female progeny scored is rather small and because unlike with X ray, they did not find such an effect with fission neutrons.

*Research sponsored jointly by the National Center for Toxicological Research and by the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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COMPARATIVE INDUCIBILITY BY 6-MERCAPTOPURINE OF DOMINANT-LETHAL MUTATIONS AND HERITABLE TRANSLOCATIONS IN EARLY MEIOTIC MALE GERM CELLS AND DIFFERENTIATING SPERMATOGONIA OF MICE*

W. M. Generoso, Sandra W. Huff, and Katherine T. Cain

Recent studies in male mice with two mutagenic chemicals, mitomycin C and 6-mercaptopurine (6-MP), have contributed significantly to our present knowledge of the overall picture of chromosome breakage effects of chemicals in male germ cells. Until results with these two compounds became available, the chemicals which clearly induced chromosome breakage in male germ cells either were alkylating agents or were known to be transformed *in vivo* into alkylating forms, and clear-cut chromosome breakage effects of such chemicals in males were found only when postmeiotic germ cells or late spermatocytes were treated. The antileukemic purine analogue 6-MP is an exception in that it is not an alkylating chemical and is not known to be transformed into one and in that the chromosome breakage effects were induced only in germ cells that were presumably in late differentiating spermatogonial and early meiotic spermatocyte stages. The chromosome breakage effect of this compound was first detected as clear-cut increases in induced dominant-lethal mutations.[1,2] Subsequent cytological analysis of the germ cells in the diakinesis–metaphase I stage revealed that chromatid deletions were the likely cause of dominant lethality.[2] The cytological study also revealed, unexpectedly, that the relative yield of chromatid interchanges was very low.

Results on induction of heritable translocations with 6-MP at the same germ-cell stages are as follows. Out of 215 male progeny tested from male parents treated with 196 mg/kg of 6-MP, 1 partially sterile translocation was recovered. Four hundred additional male progeny from male parents treated with 150 mg/kg were tested for translocation heterozygosity. Of these, no translocation heterozygotes were observed, but there were four sterile male progeny. Cytological analysis of these sterile males[3] revealed the presence of XYY condition in three of them. The fourth one was normal. All three XYY males had small testes and spermatogenic arrest.

These findings clearly demonstrate a marked difference between postmeiotic stages on one hand and the early meiotic and differentiating gonial stages on the other in the relative rates by which dominant-lethal mutations and heritable translocations are produced. While heritable translocations are a more sensitive end point in postmeiotic stages, the reverse seems to be true in the early meiotic and differentiating gonial stages. However, it should be noted that the comparative inducibility of dominant lethals and heritable translocations in the early meiotic and differentiating gonial stages was studied only for the compound 6-MP. More information on these cell stages is certainly needed.

*Research sponsored jointly by the National Center for Toxicological Research and by the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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SENSITIVITY OF DIFFERENT POSTCOPULATION GERM-CELL STAGES OF MICE TO INDUCTION OF DOMINANT LETHALS WITH THREE ALKYLATING CHEMICALS*

K. E. Suter† and W. M. Generoso

In an earlier study[1] we found that IMS treatment of male and female germ cells after mating but prior to sperm entry yielded frequencies of presumed dominant lethals that were much higher than the added effects on most sensitive dictyate stage and sperm in vas and epididymis. In this study, the oocytes were predominantly at metaphase II at the time of treatment. This postmating–precleavage stage is still several hours prior to pronuclear formation and DNA synthesis and may not be the most sensitive period. There are already good indications from work on mammalian cells *in vitro* that certain alkylating chemicals are most effective during S-phase.[2] And studies in mice with X rays[3,4] and fission neutrons[5] showed that early pronuclear stages were most sensitive to induction of dominant-lethal effects and sex-chromosome loss. Thus, the present report is an investigation of the relative sensitivity of postmating–precleavage germ cells when treated before fertilization, at early pronuclear stage, or during DNA synthesis. In addition to IMS, this study included EMS and TEM.

Females used were either from (C3H × 101)F_1 or from a mixed stock obtained by crossing Gs/Y males with (SEC × C57BL)F_1 females. These two stocks will be referred to as JH and SBGS respectively. In all

experiments, males were from (C3H × C57BL)F_1 stock. All animals were put in a room with a 5-hr dark period daily (2:00–7:00 a.m.) at least 4 weeks prior to the start of an experiment.

Presumed dominant-lethal effects of the three compounds on postmating–precleavage germ cells when treated at various times during the period before fertilization through first DNA synthesis – that is, 4.5–17.5 hr after the midpoint of the dark period – are summarized in Table 20. Only data obtained with JH females were included in this table, as results obtained with SBGS females were generally similar. IMS given at 25 mg/kg induced clear-cut dominant-lethal effects on all stages studied. The sensitivity clearly increased progressively with germ-cell maturation. The highest effect, found on DNA-synthesizing zygotes, was about three times that observed with postcopulation-prefertilization germ cells (4.5 hr after the midpoint of the dark period). Dominant-lethal effects at all periods studied were exhibited by reductions in the number of living embryos with corresponding increases in the incidence of dead implantations. The number of total implantations was not affected.

As with 25 mg/kg of IMS, 300 mg/kg of EMS induced dominant-lethal effects at all postmating–precleavage periods studied. However, the sensitivity pattern of EMS is diametrically opposite that of IMS. Treatment at 4.5 or 11.5 hr after the midpoint of the dark period (*i.e.*, prior to sperm entry or early pronuclear development respectively) resulted in markedly higher dominant-lethal effects than that at 17 hr. There seems to be an increasing resistance with maturation, but the difference between the 4.5- and 11.5-hr periods is not significant. Unlike IMS, significant increases in preimplantation losses were observed at all these periods.

The pattern of sensitivity for TEM is still different from the ones found for either EMS or IMS. TEM treatment (0.6 mg/kg) at the early pronuclear stages (11.5 hr after the midpoint of the dark period) resulted in considerably higher dominant-lethal effects than treatment during prefertilization (4.5 hr) or during DNA synthesis (17 hr), these stages having similar degrees of sensitivity. Like EMS, TEM induced preimplantation losses at all postmating–precleavage stages.

Thus, EMS and TEM, in contrast to IMS, are similar to X rays[3,4] and fission neutrons[5] in that zygotes undergoing DNA synthesis are more resistant than the early pronuclear stages.

*Research sponsored jointly by the National Center for Toxicological Research and by the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

Table 20. Stage sensitivity of postcopulation germ cells[a]

Treatment	Hours after midpoint of dark period[b]	No. of copulated females	No. of fertile females	Total implants per fertile female	Living implants per fertile female	Dead implants (%)	Dominant lethals (%)[c]
Control	4.5	44	42	7.2	6.8	6	–
IMS (25 mg/kg)	4.5	42	38	7.2	5.8	19	14
	11.5	46	44	7.4	5.3	27	22
	17.5	45	40	7.1	4.1	42	40
Control	4.5	46	45	7.6	7.3	4	–
EMS (300 mg/kg)	4.5	43	39	4.7	1.6	65	78
	11.5	33	29	5.0	2.2	56	70
	17	42	39	6.1	3.0	50	58
TEM (0.6 mg/kg)	4.5	44	43	6.0	4.2	30	43
	11.5	46	40	4.3	2.7	37	63
	17	44	39	6.6	4.1	39	44

[a]Only results with JH females are included in this table.

[b]The preponderant germ-cell stages for 4.5, 11.5, and 17 hr after the midpoint of dark period are, respectively, ovulated eggs in metaphase II with sperm in oviduct, male and female pronuclei formation, and male and female pronuclei undergoing DNA synthesis.

[c] $$\%\ \mathrm{DL} = 1 - \left\{ \frac{\text{Living implants per pregnant female in experimental group}}{\text{Living implants per pregnant female in control group}} \right\} \times 100 .$$

†Postdoctoral investigator supported partly by subcontract No. 3322 from the Biology Division, ORNL, to the University of Tennessee and partly by the government of the Canton of Zurich (Switzerland).

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LACK OF DOMINANT-LETHAL EFFECTS OF ETHYL ALCOHOL IN MALE MICE*

W. M. Generoso, Katherine T. Cain,
Sandra W. Huff, and W. L. Russell

A recent report in *Nature* by F. M. Badr and R. S. Badr[1] compelled us to conduct our own investigation on the mutagenic effectiveness of ethyl alcohol in male mice. In this report they presented a seemingly unequivocal, high dominant-lethal effect of ethyl alcohol in spermatozoa and spermatids of CBA mice when 0.1 ml of either 40 or 60% w/w alcohol was given once a day for three consecutive days. Effects ranging from 31 to 67% dominant lethals were observed in their study.[2]

We conducted our study on two hybrid stocks of male mice, $(101 \times C3H)F_1$ and $(SEC \times C57BL)F_1$. These mice were 3 to 4 months old at the time of treatment. Sixteen males of each stock weighing 22 to 27 g were given, by intubation, 0.1 ml of 55% w/w ethyl alcohol once a day for three consecutive days. This dose is equivalent to 156–191 ml of pure alcohol given all at once to a 70-kg person each day for three consecutive days. Twelve control mice from each stock were handled similarly and given the same volume of distilled water. Each experimental and control mouse was caged with two virgin $(101 \times C3H)F_1$ females immediately after administering the last dose. Females were examined for presence of vaginal plugs every morning (for 21 days thereafter), and plugged females were replaced by fresh ones. The procedures for our study are generally similar to those used by Badr and Badr.

Contrary to the clear-cut dominant-lethal effects found by Badr and Badr, ethyl alcohol did not induce a detectable increase in dominant-lethal mutations in the two stocks of mice in our study. We killed for uterine analysis a total of 73 experimental and 53 control females that were mated at the time when Badr and Badr found dominant-lethal effects of ethanol (4–13 days after the last intubation). With these numbers of mated females, high dominant-lethal effects such as that observed by Badr and Badr should have been easily detected. Because we followed their procedure closely, the only explanation possible for the remarkably different results is that the strain of mice they used (CBA) is highly sensitive to dominant-lethal induction with ethyl alcohol.

*Research sponsored jointly by National Center for Toxicological Research and the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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2. Percent dominant lethals was calculated as

$$1 - \left[\frac{\text{Live implants of experimental group/female}}{\text{Live implants of control group/female}} \right] \times 100.$$

RESULTS FROM A SPECIFIC-LOCUS TEST OF THE MUTAGENICITY OF SULFUR DIOXIDE IN MICE

W. L. Russell and Elizabeth M. Kelly

Since large human populations are exposed to appreciable concentrations of sulfur dioxide from energy production and other sources and since sodium bisulfite has shown mutagenic properties in microorganisms, it seemed desirable to test whether any mutagenic effect of sulfur dioxide, or, more specifically, of sodium bisulfite, could be detected in mammalian systems. Human exposure to bisulfite and sulfite as food additives was an additional reason for mammalian mutagenicity testing.

Still another argument for conducting this investigation appeared after the work had started. Shapiro and Louis[1] presented new evidence on the nature of the deamination action of bisulfite which led them to conclude that bisulfite might be mutagenic at concentrations much lower than had previously been supposed. They argued that "the presence of sulfite oxidase is not a sufficient guarantee against the possibility of bisulfite mutagenesis," and they recommended extensive testing in mammalian systems.

After a dominant-lethal test in mice by Generoso[2] had shown no mutagenic effect of injected sodium bisulfite, we proceeded to a specific-locus test, using some of the same males that had been employed in the dominant-lethal study. These had been injected peritoneally with 400 mg/kg of sodium bisulfite daily, 5 days/week, for a total of 20 treatments. At $7\frac{1}{2}$ weeks after the completion of the treatments, these wild-type

males were mated to females homozygous for the seven marker genes used in our standard specific-locus test. The offspring were scored for any mutations at the seven loci that could have occurred as a result of treatment in spermatogonial stages.

To date, in a total of 13,568 offspring scored, no mutations have been observed. Until further information becomes available on the metabolic fate under various conditions, it is difficult to make a quantitative comparison between the exposure level in the testes of mice that had received a total of 8000 mg/kg of sodium sulfite, injected over 20 days, and the exposure level in the gonads of humans breathing sulfur dioxide-polluted air for a generation. However, we have attempted an estimate of the possible upper limit of the genetic risk in man from sulfur dioxide in the atmosphere. Taking the upper 95% confidence limit of the observed zero mutation frequency in the mouse and assuming what are probably ridiculously high values for the exposure levels in human gonads, we have concluded that, if there is any genetic hazard in man, it must be much less than a doubling of the spontaneous mutation rate. Although we are not yet able to come close to calculating a precise quantitative answer, we have, at least, excluded the possibility of a catastrophic genetic risk.

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SPECIFIC-LOCUS MUTATION FREQUENCIES INDUCED IN MOUSE SPERMATOGONIA AT VERY LOW RADIATION DOSE RATES

W. L. Russell and Elizabeth M. Kelly

The current set of experiments on this subject has entered the productive phase now that the long radiation exposure times necessary have ended. One purpose of these experiments is to help settle a current controversy on what happens at low radiation levels. An investigator at Harwell, citing the slightly, but not statistically significant, higher mutation frequency observed by us in mouse spermatogonia at the lowest dose rate tested, compared with the next lowest, concludes that, at these very low levels of radiation, mutation frequency may actually increase as dose rate is lowered.[1] Another view, from Chalk River, holds that a low level of radiation may stimulate the repair process and result in a mutation frequency lower than the spontaneous mutation rate in the unirradiated control.[2] A third view, the one we have taken, is that, below a dose rate of 0.8 R/min, induced mutation frequency in mouse spermatogonia is independent of dose rate. This was proposed as the most reasonable hypothesis to fit the observed lack of statistically significant change in mutation frequency as dose rate was lowered over an 800-fold range, from 0.8 R/min through 0.009 R/min to 0.001 R/min.

In our new experiments, the lowest dose rate used was 0.0007 R/min. The accumulated dose of 300 R used in this experiment required a continuous exposure of over 10 months to our low-level ^{137}Cs source. The results obtained at this dose rate are being compared with those from mice exposed to the same total dose accumulated in 38 days at a dose rate of 0.005 R/min.

Two factors that were not considered in earlier experiments have been controlled in the current series. In the earlier work, animals in all groups entered the experiment at about the same age. Thus, those that required the longest exposure time were irradiated over a more advanced age than the rest. Second, their matings did not start until they were considerably older. In the new experiments, the animals exposed at 0.005 R/min for 38 days were divided into four groups. The exposure periods of these four groups were scattered at approximately 3-month intervals of age to cover the age range involved in the 0.0007 R/min group that was exposed for over 10 months. All groups, including controls, were not mated until the 0.0007 R/min group had completed its exposure. Thus, if there is any possibility that the difference in exposure times could have affected mutation frequencies in the earlier experiments as a result of the germ cells in older animals being more mutable or having lower capacity for repair, then that factor is largely eliminated. Similarly, any influence of interval between irradiation and fertilization will be more equally matched in the two parts of the current experimental series.

It is hoped that a comparison of the results at the two different dose rates will throw more light on whether or not mutation frequency is independent of dose rate at these levels. In any case, the data will increase the precision of our figures for mutation frequencies at the lowest dose rates tested. This alone is clearly a desirable goal, since Oakberg and Palatinus have shown[3] that at the lowest dose rate used, 0.0007 R/min, there is no evidence of killing of spermatogonial stem cells and, therefore, no chance for differential cell killing to have affected the mutation rate. Since most of the genetic risk from radiation in man presumably involves doses or dose rates that are too low to cause spermatogonial stem cell killing, the data in the mouse that are obtained at dose rates low enough to avoid cell killing

are likely to be the most suitable for estimating human genetic hazards.

More than 40,000 offspring have, so far, been scored in the combined data from the two experimental groups and the control. Since all of the several presumed mutants obtained are still being tested, it is too early to draw firm conclusions. However, if the current estimates are taken at face value, there is, so far, no significant difference between the mutation frequencies at the two radiation dose rates.

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CRITICISM OF A CURRENT MODEL FOR ESTIMATING GENETIC RISKS OF RADIATION

W. L. Russell

Since our discovery of the effect of radiation dose rate on mutation frequency in the mouse,[1] we have repeatedly discussed the question of whether the phenomenon involves only the repair of two-track mutational events or primarily the repair of one-track events.[2–5] As was pointed out in our 1958 paper,[1] single-track events can show a dose-rate effect (and a nonlinear, concave-upward dose curve at high dose rates) if the repair system is damaged or saturated at high doses and high dose rate. For various reasons that we have enumerated, we favor this latter hypothesis.[2–5]

In a paper by Abrahamson *et al.*,[6] the alternative hypothesis is accepted, and the authors attempt to fit our mouse data to the quadratic

$$Y = C + \alpha D + \beta D^2 ,$$

where Y is the expected yield of mutations, C is the control rate, D is the dose, and α and β are the coefficients of induction. Separate curves are fitted to the male and female data, and the authors, working as a Task Group for the International Commission on Radiological Protection, claim that the values for α and β have proved useful in making risk estimates, the α term (mutations/locus/R) representing the genetic risk for low-dose-rate irradiation.

It should be recognized that mathematical models for both hypotheses can be made to fit the observed data. Therefore, the fit of a model for the hypothesis preferred by Abrahamson *et al.* does not prove that hypothesis to be superior. Other criteria, which we have discussed elsewhere, provide more cogent arguments on the relative merits of the two hypotheses.[2–5] Here, however, we shall consider only the validity of the risk estimates obtained from the curves fitted by Abrahamson *et al.*

Risk estimates in the male. – Consider their fit to the male data first. Here the α term (6.6×10^{-8}), as the authors themselves state, was not derived by them from the curve fitting; it was taken directly, unchanged, from the observed results with chronic irradiation. Since this is the source that has been used, ever since we obtained the data, for estimating the risk from low levels of radiation to the male, the Abrahamson *et al.* report adds absolutely nothing to risk estimation for chronic irradiation of the male.

Does it contribute anything to the relatively less-important estimation of risk from high acute doses to the male? Taking the α, as described above, from the chronic irradiation results, the authors fit their quadratic to go through the observed 0 and 300-R points. (It is not surprising, since only two points are fitted, that this gives a perfect fit!) It is clear that this procedure adds nothing to the prediction of what would be expected from a 300-R exposure. How about the prediction for higher doses? The fitted curve rapidly departs from the observed results for 600 and 1000 R, overestimating them by factors of more than 2 and 8 respectively. The authors accept my view that the 1000-R observed mutation frequency is low as a result of differential cell killing, and invoke this explanation also to account for the departure of their predicted value from the observed results at 600 R. Now in the human male, the effect of differential cell killing in the testis is likely to be at least as great as in the mouse, judging by comparative effects of radiation on fertility. So the actual observed results in the mouse at 600 and 1000 R seem likely to give a better estimation of the risk in man than is obtained by a curve which makes no allowance for the effect of differential cell killing, and consequently grossly overestimates the observed mutation frequencies.

One must conclude that, so far as the male is concerned, the quadratic model of Abrahamson *et al.*, which adds nothing to risk estimation for chronic irradiation, gives misleading estimates for high-dose acute irradiation.

Risk estimates in the female. – Now consider the fit to the female data. Here the authors did the opposite of what was done with the male data. Instead of taking α from the observed results for chronic irradiation and fitting the curve to the data for acute irradiation, they fitted the curve for females to the acute irradiation data

and thereby derived an α (2.45×10^{-7}) which they used to predict the risks from chronic irradiation. (Incidentally, they used an incorrect estimate of the spontaneous mutation rate in females, having biased the calculation by an erroneous treatment of a cluster. We have given the correct treatment elsewhere.[7]

For acute irradiation, it is highly questionable whether the fitted curve (which is based on a questionable hypothesis as to the mechanism of the mutational events) adds any precision to risk estimates based on the actual data or on other fitted curves. So we are left with the question of whether the α derived from the quadratic fit is of any help in estimating the risk from chronic irradiation.

The authors discuss three sets of data on chronic irradiation of maturing oocytes and compare the observed mutation frequencies with what would be expected on the basis of the α derived from their fitted quadratic.

The first set of data was obtained by Carter[8] with a 600-R exposure. The mutation frequency predicted by Abrahamson *et al.* exceeds this observed frequency by a factor of 11.

The second and third sets of data are taken from our results with 258- and 400-R exposures.[7] Here the values predicted by Abrahamson *et al.* exceed the observed by factors of 3.9 and 12.0 respectively. The authors try to discount these large discrepancies by claiming that the observed mutation frequencies are low, because the results are not based solely on maturing oocytes but on an admixture of immature oocytes which have low mutability. There is such admixture, but the authors made no attempt to find out *how much* this factor affected the results, and they ignored the fact that such adjustments had been made in earlier risk estimates. When, for example, a correct adjustment is made to estimate the induced mutation frequency for maturing oocytes in our 400-R data, the result is still far below the Abrahamson *et al.* prediction. Their predicted value is at least 9 times higher than the observed. The discrepancy would come out still greater if certain other complications not considered by them were taken into account. There was no admixture of immature oocytes in the Carter 600-R experiment, and so the mutation frequency at this dose, predicted by Abrahamson *et al.*, stands at 11 times greater than that observed.

How does the Abrahamson *et al.* prediction compare with earlier estimates of risk in females? The α of 2.45×10^{-7} now proposed by them for maturing oocytes is approximately 10 times greater than that published in the BEIR[9] and UNSCEAR reports.[10] In my opinion, those reports gave values which correctly reflected the observed results available then and which are still approximately in line with new, unpublished data of ours obtained since.

There is another aspect of the Abrahamson *et al.* report which is misleading. So far, in the female, we have been discussing only the mutation frequencies in maturing oocytes, which for present purposes may be defined as those oocytes which are within 6 weeks or less of ovulation. In his presentation, Abrahamson specifically stated that the authors consider the human immature resting oocyte (which is the stage of primary concern in human hazards) to be more comparable to the mouse maturing oocyte than to any other mouse oocyte stage, so far as mutability is concerned. There is no evidence for this assumption, and there are several reasons for questioning it. To us, it seems more reasonable, on the basis of metabolic activities, to expect that the immature resting oocytes in humans may correspond, in mutability, to the immature resting stage in the mouse, or perhaps, on cytological grounds, to a slightly earlier stage in the mouse. In any case, the assumption that the maturing oocyte in the mouse is mutagenically comparable to the immature resting oocyte in the human is an extreme view, because the maturing oocyte is by far the most mutable stage in the mouse. Earlier reports have taken this into consideration, and have concluded that estimates of risk based solely on this oocyte stage are likely to be overestimates.

In short, the authors have taken the maturing oocyte in the mouse, the most mutable of all stages, as representing the immature resting oocyte in the human, and have then proposed a risk estimate based on a mathematical model which produces a figure one order of magnitude higher than the observed mutation frequency of the mouse's most mutable stage. The final result of this ignoring of data is that, whereas other groups have considered the risk from chronic irradiation of the female to be small or negligible compared with that in the male, Abrahamson *et al.* estimate the risk in females to be 3.7 times that in males.

Summary. — The quadratic fit proposed by Abrahamson *et al.* adds nothing to the risk estimates for chronic irradiation of males and acute irradiation of females. For large-dose acute irradiation of males and for chronic irradiation of females, it gives highly misleading risk estimates. Since it has already been presented, at national and international meetings, as the work of a Task Group for the International Commission on Radiological Protection, we felt that it was particularly important to point out to regulatory and other concerned agencies that it manifestly overestimates the

genetic risk from low-dose-rate irradiation of females by at least one order of magnitude, but probably more, and overestimates the average risk for both sexes by approximately 5 times.

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*Oftedal and Sankaranarayanan removed their names from a later version of this paper.

EFFECT OF HYCANTHONE ON X-CHROMOSOME LOSS IN FEMALE MICE

W. L. Russell, Patricia R. Hunsicker, Elizabeth M. Kelly, Carolyn M. Vaughan, and Georgia M. Guinn

In a preliminary report[1] on a test of the effect of the antischistosomal drug hycanthone on X-chromosome loss in mice, data were presented which supported the tentative conclusion that there is such an effect of the drug in matings made shortly after treatment. This is now confirmed, and much additional information has been obtained which is illuminating in the general understanding of chemical mutagenesis and will clearly prove useful in developing adequate methodology for mammalian mutagenicity testing.

Mature females are injected intraperitoneally with hycanthone methanesulfonate and, on the day following treatment, are mated to males carrying the sex-linked gene "greasy" (*Gs*). The offspring are scored for presumed *Gs*/0 females, which are checked by breeding tests and chromosome counts in cultured ear tissue. Chromosome counts of the mothers of these females are also made, to exclude any cases where the parent is already X0.

In the earlier paper,[1] 6 X0s were reported in 4110 classified female offspring of hycanthone-treated females. However, 3 of these 6 X0s came from matings made in the first week following injection, and in a total of only 420 female offspring. This frequency, although not significantly different from that in the limited sample of contemporary controls, was significantly above the frequency in the total controls ($P < 0.01$).

The frequency now obtained in the first-week matings of females injected with 150 mg/kg of hycanthone is 19 X0s in 1351 classified female offspring. This is highly significantly above the contemporary control value (Table 21). Note, however, that the effect is limited to the first week: the offspring from treated females mated in later weeks show no increase in X0 frequency over that in the controls.

This pattern of sensitivity parallels the results of Generoso *et al.*[2] who found that large doses of hycanthone injected intraperitoneally into female mice cause a reduction in litter size, but only from matings made within the first week after treatment. Subsequent analysis showed that the fertility effect was attributable to an increase in the frequency of dead implantations. In collecting the X0 data we have also confirmed the effect on litter size discovered by Generoso *et al.*[2] In 691 litters conceived in the first week after injection of hycanthone-treated females, the mean litter size was 3.85. In 634 control litters conceived in the first week after mating, the mean litter size was 6.36.

An interesting, and perhaps basic, point emerging from the results on X0 induction by hycanthone is that the distribution of the effect with time after treatment is diametrically opposite to that obtained with X rays. With X rays, we find a highly significant increase in X0 frequency in matings made 2–6 weeks after irradiation, as compared with matings made in the first week. One

Table 21. Induction by hycanthone of X-chromosome loss, scored as *Gs*/0 individuals in the offspring of treated female mice

Treatment	Mating period after treatment	Offspring	
		Gs/0	Total females
Contemporary control	1st week	1	2214
	Later weeks	6	6740
150 mg/kg (ip) hycanthone	1st week	19	1351
	Later weeks	8	9068

possibility suggested by this difference in response to hycanthone and radiation is that different mechanisms may be involved in the induction of the X-chromosome loss, perhaps one agent involving an effect on the spindle and the other on chromosome breakage. However, an alternative explanation is suggested by our recent finding[3] that intraperitoneal injection is the only route of administration, when compared with gavage and intramuscular and subcutaneous injection, by which 150 mg/kg has a significant effect on litter size. This may indicate that, at this dose level, only the exposure received by mature oocytes, in ripe follicles on the surface of the ovary, to the hycanthone directly injected into the peritoneum is adequate to produce an effect. If so, then the absence of X0 induction in offspring from later matings could be due to the lower exposure level received by the less mature oocytes. As is pointed out in the accompanying paper,[3] it is important for an understanding of chemical mutagenesis and for the development of methodology of mammalian mutagenesis testing to find out if either of the above possible explanations for the difference in response to hycanthone and X rays is correct.

Having obtained a clear-cut induction of X-chromosome loss, as shown in offspring conceived in the first week after intraperitoneal injection of females with 150 mg/kg of hycanthone, we decided to explore the dose-effect relationship. Preliminary results with intraperitoneal injection of 75 mg/kg show 4 X0s in 1820 female offspring. Although the sample is still small, the frequency at 75 mg/kg already shows a drop significantly below that expected on a linear interpolation between 150 mg/kg and zero dose ($P = 0.01$, relative likelihood test). The induced frequency (*i.e.*, with control subtracted) at 75 mg/kg, instead of being one-half of that at 150 mg/kg, is only one-tenth. The effect on litter size also showed a drop below linearity. The practical conclusion seems clear: that linear interpolation between 150 mg/kg and zero dose would have grossly overestimated the effect of the therapeutic dose of 3 mg/kg. This may also be true for linear interpolation between zero dose and 75 mg/kg, which is still 25 times the therapeutic dose.

If, however, we calculate the effect expected from the therapeutic dose, assuming linearity between the 75 mg/kg intraperitoneal dose and zero and allowing for the fact that very few of the total conceptions in the population of treated females are likely to occur in the first week after treatment, then we arrive at a risk of approximately one X0 per 16,000,000 conceptions. Even this low frequency is presumably an overestimate, because we have assumed a linear response with dose and we have based our calculation on the effect from intraperitoneal injection. In humans, the drug is administered intramuscularly, a route which, in the mouse, when litter size is used as an end point, has much less effect.

Most human X0s terminate as early abortions. Other chromosome losses do too, and are, therefore, not a very serious hazard. If, at worst, the X0 induction seen is only the top of an iceberg of other chromosomal types of damage, it could still be said that even a total damage 100-fold greater than that actually observed would surely rate as an acceptable risk in the use of an effective antischistosomal drug.

Since we are still in the stage of knowing very little about the mutagenic effect of chemicals, particularly with regard to transmitted genetic effects in mammals, our present and earlier results with hycanthone, in addition to their immediate practical value for decisions on chemotherapy of the world's second most prevalent disease, are also illuminating and fascinating in many general respects. Some of these seem worth emphasizing:

(*a*) The conclusion from work on *Salmonella* that hycanthone is a frame-shift mutagen, operating only on cells in synthesis, has proved of no predictive value in mice. The only genetic effect we have seen is in mature oocytes in which no synthesis is occurring.

(*b*) Tests on males alone are not adequate to detect mutagenesis. The only effect of hycanthone we have seen is in females, despite extensive testing in males.

(*c*) The effect of hycanthone on X-chromosome loss shows a curious limitation to offspring from conceptions occurring in the first week after treatment.

(*d*) This was unexpected, being opposite to the effect of radiation, which is lower in offspring conceived in the first week after exposure than it is in immediately subsequent weeks. It will be important to find out whether different mechanisms of induction of X-chromosome loss are involved, or whether the intraperitoneal route of administration of the chemical is responsible for the difference.

(*e*) After finding no genetic effect of hycanthone in extensive tests with treated male mice, the magnitude of the effect seen in females was surprising. For conceptions occurring in the first week after treatment with 150 mg/kg, the X-chromosome-loss frequency is approximately 4 times as great as that obtained with 400 R of acute X rays and 14 times as large as that from 400 R of gamma rays delivered over a time period similar to that over which the chemical is probably acting.

(*f*) However, the effect drops to one-tenth of the above when the dose is halved; and, in offspring from conceptions occurring in all later weeks after the first, no effect at all has been detected.

(*g*) There will also probably be a large drop in XO incidence when injection is intramuscular instead of intraperitoneal, judging by the drop in effect on litter size.

(*h*) In spite of the large effect with 150 mg/kg, the human risk is estimated to be very small indeed, if not zero, from the therapeutic dose. Thus the test has turned out to have a fantastically fine resolution. Once one knew where to look, it was easy to get not only a detectable effect, but a quantitatively measurable one which permitted the estimation of the human risk to be probably negligible.

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EFFECT OF ROUTE OF ADMINISTRATION OF HYCANTHONE ON LITTER SIZE IN THE OFFSPRING OF TREATED FEMALE MICE

Patricia R. Hunsicker and W. L. Russell

Since a transmitted genetic effect of hycanthone in mice had been detected only in treated females, despite extensive testing on males, and only in litters conceived in the first week after treatment,[1,2] the question arose as to whether the route of administration might be responsible, or partly responsible, for the sex difference in response and possibly also for the limitation of the effect to early matings. Intraperitoneal injection had been used throughout all experiments, and it seemed conceivable that the surface of the ovary, where mature follicles are located, might be exposed to a higher concentration of an intraperitoneally injected chemical than would the testis, or perhaps immature follicles in deeper regions of the ovary. Accordingly, a test has been run on different routes of administration.

The genetic effects that have been detected in our earlier studies on treated females are litter size reduction,[2] increase in dead implantations, and X-chromosome loss.[1] For a preliminary screening of the possible effect of different routes of administration, litter size was chosen as the easiest end point to measure.

The results obtained are shown in Table 22. Although the number of litters scored is not large, it is clear that, of the routes of administration tested, intraperitoneal injection is the only one that has a clear-cut effect on litter size. It is questionable whether any effect at all will be detected with the other routes of administration.

It is obviously important to test whether the effect of hycanthone on X-chromosome loss will also be dependent on the route of administration. Up to now, we have estimated genetic risks from treatment of human females on the basis of results of intraperitoneal injections in mice. The findings reported here indicate that such calculations may greatly overestimate the risk from intramuscular injection, the procedure used in humans. It should be pointed out that even the human risks estimated on the basis of mouse intraperitoneal injection are minimal at the therapeutic dose. So the practical result of applying the new finding, if it also proves valid for X-chromosome-loss induction, may simply be the addition of an extra safety factor.

The present results could have an important influence on the methodology of mammalian chemical mutagenicity testing. If mature oocytes on the surface of the ovary can be exposed, via intraperitoneal injection, to a much higher concentration of a chemical than can any other germ-cell stage in either sex, then screening for possible mutagenicity by litter-size reduction in early matings and confirmation by the X-chromosome-loss test may prove to be a sensitive, as well as relatively easy and quick, way of detecting some classes of mutagens.

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Table 22. Effect of various routes of administration of 150 mg/kg of hycanthone on litter size in offspring conceived in the first week after treatment of female mice

Treatment	No. of litters	Mean litter size
Control	17	6.1
Injection (ip)	13	3.0
Injection (im)	16	6.3
Injection (sc)	14	6.1
Gavage	12	6.5

RESULTS FROM SCREENING FOR RADIATION-INDUCED HEMOGLOBIN VARIANTS IN THE MOUSE

W. L. Russell, Carolyn M. Vaughan, R. A. Popp, Liane B. Russell, and K. Bruce Jacobson

Continuing experiments to detect new mutations at the hemoglobin loci[1] have the following objectives: (*a*) to determine mutation rates for clearly defined biochemical variants, (*b*) to analyze the nature of the mutations in order to determine relative frequencies of point mutations and chromosome aberrations, (*c*) to recover new variants at the hemoglobin loci and possibly at other loci affecting blood characters.

Blood samples of offspring of 101♀ × SEC♂ or SEC♀ × 101♂ crosses, in which one or the other parent was irradiated with acute X rays, are screened for electrophoretic pattern,[2] phosphate solubility, crystal pattern, and red blood cell lysis. The parent strains differ for alleles at the *Hba* and *Hbb* loci. Any exceptional animals are mated in appropriate crosses and their progeny tested for transmission of the abnormal phenotype.

To date, we have analyzed 8621 blood samples from F_1 of irradiated mice. Of these, 7793 and 828 are derived from irradiated males and females, respectively, approximately one-half of each number from each of the two reciprocal crosses. Although tests are still in progress, present indications are that recovered exceptionals are of the following types:

Mutants involving the hemoglobin loci. – Probably five such mutants have been found. Three of them appear phenotypically to be α-chain deletions or inactivations. Transmitting stocks have been set up from two of these, the third being a sterile male (with spermatogenic block in pachytene). One mutation, only recently discovered, may produce β^{SEC} inactivation or deletion. The fifth has turned out to be a tandem duplication involving at least the *Hbb, Mod-2,* and *c* loci. It is described elsewhere in this report.[3]

Anemic variants. – Four of these have been found. In two, there was also a questionable effect on the electrophoretic pattern, but this could merely have resulted from the extreme anemia. Two of the anemics, neither of which showed changes in hemoglobin chemistry, had abnormal red-blood-cell lysis; but neither this effect nor the anemia was transmitted to F_1 in one case, or F_2 in the other. A third anemic died without producing progeny. The fourth is under study.

Miscellaneous variants. – Six exceptional young, recognizable by pigment or hair-quality characteristics, are being tested. At least four of these are transmitting the effect.

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AN EXAMPLE OF CONDITIONS THAT MAKE THE MOUSE SPECIFIC-LOCUS TEST HIGHLY EFFICIENT AT LOW EXPENSE

W. L. Russell and R. B. Cumming

The data obtained in our laboratory by Cumming *et al.*[1] in tests on the effect of 5-chlorouracil in mice provide a powerful example of a point that is not generally appreciated in the methodology of mutagenicity testing in mice. The specific-locus test that we have developed and used for many years both in radiation and chemical mutagenesis is generally recognized as the only reliable test so far available for scoring transmitted gene mutations and small deficiencies in mammals. However, the test is almost always regarded as so expensive, in terms of number of animals that must be scored, that it is recommended only for occasional special use, at the top of a tier of less expensive tests.

The point that is missed is that when a chemical can be administered to mice at a dose level much greater than the human exposure, the specific-locus test can be relatively inexpensive: the larger the difference in dose levels, the smaller the cost for the same degree of mutagenesis detection.

The reason the Cumming *et al.* data on 5-chlorouracil furnish such a good example of this point is that it was possible to administer this chemical at levels much higher than are found in human drinking water.[1] If we assume that 5-chlorouracil in human drinking water does not exceed 1 ppb, then the mice were receiving water with a concentration at least 1 million times higher than that in human drinking water. Their offspring were conceived, on the average, 3 months after a steady-state value for incorporation of 5-chlorouracil into DNA had been reached. Although this was only 1/120 of the 30-year generation time exposure in man, the mouse dose (concentration × time) was still more than 8000 times the human dose ($10^6/120$).

With this, and the observation of zero mutations in 314 offspring in the specific-locus test,[1] we can

compute an estimate of the upper limit of genetic risk that might occur at the human exposure level. Taking 3.3 as the upper 95% confidence limit of the observed zero mutation frequency in the 314 offspring, subtracting the known spontaneous mutation frequency of 28 in 531,500 (which is so small it has almost no effect on this step in the calculation), and dividing by $10^6/120$, we come out with an estimated induced mutation rate, at the human exposure level, that is only 2% of the spontaneous rate:

$$\frac{3{\cdot}3/314 - 28/531{,}500}{10^6/120} \Big/ \frac{28}{531{,}500} = 0.02 .$$

Thus, if our assumptions are correct, we have here a demonstration of the remarkable efficiency and extremely low cost of the specific-locus method as applied to this case. With a total of 313 offspring, which were obtained from only 11 treated males, we have shown, with 95% confidence, that the genetic risk from a presumed maximum level of 5-chlorouracil in human drinking water cannot be greater than 2% of the spontaneous mutation rate.

Are the assumptions valid? There is, of course, the question of whether the experimental result in mice (ratio of induced to spontaneous mutation rate) is applicable to man, but that is a general problem for all mouse tests, and not much can be done about it. Apart from that, there are two other questions that might be raised about the assumptions used in the calculation. The first is whether it is correct to compare mouse and human doses on the basis of concentration multiplied by time of exposure. It might be more appropriate in this case to use number of cell generations during exposure instead of elapsed time. However, if this is done, and if it is assumed that the human cell generation time is the longer of the two, then the difference in number of cell divisions during exposure in the two species will be less than a factor of 120, and the upper limit of the genetic risk will accordingly be lower. Thus, if our assumption is incorrect in this respect, we are still safe in taking our calculation as a maximum risk. The second question is whether it is correct to assume linearity of response over the wide range of dose levels from high in the mouse to low in the human. This is quite likely not valid. From our experience with mammalian chemical mutagenesis, it seems probable that the effect at the human dose level will be less than that estimated on a linear interpolation. In that case, we will, again, have overestimated the risk and still be safe in taking our calculation as a maximum. It is theoretically conceivable that the effect at the human dose level could be greater than expected on a linear basis. In other words, the dose-response curve might be humped. However, we have seen such curves only when there was extensive spermatogonial killing, and that is not occurring with 5-chlorouracil. Furthermore, Cumming *et al.*[1] have data from a dose level only one-tenth of that used for the results cited here, and that also gave no mutations.

In summary, whenever a chemical can be tested in the mouse, without serious toxicity, at dose levels much higher than are involved in human exposure, then the specific-locus test can be used with high efficiency at low expense. This condition, making an efficient test feasible, may well apply to many pollutants.

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DISCOVERY OF A TANDEM DUPLICATION IN THE MOUSE

Liane B. Russell, W. L. Russell, N. L. A. Cacheiro, Carolyn M. Vaughan, and R. A. Popp

In the course of a search for induced mutations involving the mouse hemoglobin loci,[1] we discovered a chromosome aberration not heretofore found in the mouse. The mutant animal, daughter of an irradiated SEC/RΙ ♀ (*Hba*^{b}/*Hba*^{b}; *Hbb*^{s} *c*^{ch}/*Hbb*^{s} *c*^{ch}) and an unirradiated 101/RΙ ♂ (*Hba*^{a}/*Hba*^{a}; *Hbb*^{d} *C*/*Hbb*^{d} *C*), had hemoglobin that was not of the usual F_1 type by the criteria of electrophoretic pattern (fast-moving band relatively fainter), solubility (low), and crystal pattern. The presumed mutant was also of small stature. The abnormal hemoglobin phenotype was not transmitted in backcrossing to SEC/RΙ (although the small size was) but was transmitted, together with small size, in backcrosses to 101/RΙ.

The first evidence for the presence of a duplication came from crosses of the presumed mutant to an albino stock (*Hbb*^{d} *c*/*Hbb*^{d} *c*). These yielded – instead of the expected *c*^{ch}/*c* – offspring which were *c*^{ch}/*c*^{ch} in coat color, possessed the abnormal hemoglobin phenotype, and were of small size. Subsequent cytological analysis by the use of quinacrine banding clearly shows a chromosome 7 that is approximately 20% longer than normal, and in which there is a repetition of the bright E band and adjacent subbands (D and F).

The combined genetic and cytological findings indicate a tandem duplication within chromosome 7, which involves a segment including at least the *Hbb* and *c* loci. The abnormal hemoglobin phenotype is consistent with overproduction of β^{SEC}, and the coat color is con-

sistent with the presence of two doses of c^{ch} and one of *c*. A stock has been set up, and it has been found that crossing over apparently occurs readily.

This tandem duplication, the first recorded in mammals, provides a valuable new tool in mouse genetics, for example, in the study of gene–dosage effects. The duplication is expected to be particularly valuable in view of the fact that it involves the *c*-locus region which is (*a*) well characterized as a result of complementation analysis[2] and (*b*) involved in a number of X-autosome translocations.[3] We plan to combine these various genetic tools in future studies.

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SOMATIC-MUTATION METHOD IN CHEMICAL MUTAGENESIS STUDIES IN THE MOUSE

Liane B. Russell, Clyde S. Montgomery, and Sandra W. Huff

About 18 years ago, we developed a method for the detection of somatic mutations in the mouse and determined that the rate of radiation induction at four specific loci was of the same order of magnitude as that in spermatogonia.[1] We also showed that dominant or nongenetic coat-color effects would not unduly interfere with the determination of somatic-mutation rates, that a radiation-induced increase in ventral white spots occurs in a genetic background that is on the threshold for this character, and that the modal number of prospective pigment cells in $10\frac{1}{4}$-day embryos is between 150 and 200. We are now exploring the usefulness of the somatic-mutation method for chemical mutagenesis studies.

Embryos heterozygous at a number of coat-color loci are exposed to the putative mutagen *in utero* on day $10\frac{1}{4}$ postconception. The animals are subsequently checked at birth, and their coats are carefully examined on about days 12 and 30. If spots other than black are found, animals are kept for further study, such as identification of pigment (to determine the locus involved) and estimation of the proportion of fur involved (to calculate number of cells present at time of mutation).

A preliminary to this experiment became necessary as a result of the fact that one of the mouse strains (NB) used in the original X-ray somatic-mutation study had become extinct. As a source of the coat-color loci to be studied, we now have to use the T stock, which also carries piebald spotting, *s/s*. Different sublines of C57BL were therefore crossed to T to determine the extent of "noise" from small white spots due to the +/*s* condition.

Chemical mutagens used to date are TEM, mitomycin C, EMS, and MMS. These are injected intraperitoneally into the pregnant female on the morning of the tenth day following discovery of a vaginal plug. Control females are injected with Hanks' saline solution. To date, 297 animals have been classified in these studies.

Preliminary experiments, in which three different sublines of C57BL were crossed to T and over 400 offspring checked, showed that the frequencies of small white belly spots varied from 1.7 to 12.1% in the different crosses. We chose the subline giving the lowest frequency.

With TEM we have used doses of 0.8 mg/kg and 0.5 mg/kg. The former produced a 91% incidence of appendicular and tail anomalies (the bulk being posterior preaxial polydactylies) and a high frequency of neonatal and postnatal death. Of 126 animals born, only 41 survived to an age when the coat could be examined. One had a "gray" spot but died before analysis. The lower dose, while still yielding 87% with posterior preaxial polydactylies, allowed 79% of the newborns to survive at least 12 days. (Control survival was 81%.) Of 45 examined, 13 had white midventral spots and three had spots that were neither white nor midventral. After treatment with 2.0 mg/kg of mitomycin C, the respective incidences were 2 and 10 in 66 offspring scored. About 8% of the total also had posterior preaxial polydactyly. Neither 50 mg/kg EMS nor 50 mg/kg MMS has produced nonwhite spots in preliminary groups of 33 and 60 classified offspring respectively. There were 2 white spots in the EMS group.

It will be of interest to compare somatic-mutation results with specific-locus data (for the same loci) in spermatogonia, and our future work in this area will be so directed. If a close correspondence is found, the method will be concluded to be of predictive value. In past spermatogonial experiments, EMS and MMS gave negative results,[2] while TEM[3] and mitomycin C[4] produced increases in mutation rate.

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EFFECT OF AGE ON INDUCED AND SPONTANEOUS NONDISJUNCTION AND CHROMOSOME LOSS

Liane B. Russell and Clyde S. Montgomery

The well-known association between maternal age and increased incidence of Down's syndrome and of certain X-chromosome anomalies in man[1] has long led to the supposition that oocyte aging predisposes toward spontaneous nondisjunction. In the mouse, increased age has been found associated with decreased frequency of chiasmata and increased frequency of univalents in oocytes at meiosis-I,[2] and with an increase in aneuploidy in fetuses conceived.[3] A measurable effect of only 5 R of irradiation on aged females has also been claimed on the basis of fetal chromosome analysis,[4] but the results are somewhat open to doubt.

We have undertaken a cytogenetic study on the effect of maternal age on spontaneous and induced sex-chromosome nondisjunction and loss in the mouse. Maternal X-chromosome nondisjunction has not been reported in the past, and the upper 95% confidence limit of the zero spontaneous frequency is 0.04%.[5] The spontaneous frequency of X^M loss averages only 0.05%, and the effects of acute irradiation are readily measurable.[5]

In an experiment still in progress, genetically uniform X^{Ta}/X^+ females (F_1 between the inbred TABR and C3Hf strains) are mated at either *ca.* 3 or *ca.* 9 months of age to X^{Ie}/Y males. Part of each group of females is irradiated with a single dose of 200 R X rays 1 week prior to mating (1 day prior to mating in the early part of the experiment). All four groups are handled concurrently. This procedure is replicated every 2 weeks as animals become available. The mating scheme permits the phenotypic detection of both maternal and paternal sex-chromosome losses, and of both maternal and paternal first-division nondisjunction (or second-division nondisjunction of crossover chromosomes). Certain mosaics and trisomics may also be occasionally detectable. Exceptional animals are tested genetically and analyzed cytologically by means of tissue cultures derived from the pinna. The phenotype by which maternal nondisjunction would be detected in sons, $X^{Ta}/X^+/Y$, is unequivocal. However, X^M loss (from any cause) would yield $0/X^{Ie}$, the phenotype of which is mimicked by one extreme of the range of expression of the normally segregating X^+/X^{Ie}. Therefore, most of the "possible $0/X^{Ie}$" animals that are saved turn out, on testing, to be in fact X^+/X^{Ie}.

No irradiated female had more than two litters or produced a litter more than 3 months after treatment, while controls have yielded up to 8 litters (even in the old group) and are still producing litters 10 months after initiation of the experiment. For more exact comparison, we have listed separately the first two litters of controls, provided these are born within 3 months after mating (Table 23). It may be seen that old

Table 23. Incidence of offspring with X-chromosome anomalies from irradiated and nonirradiated females of different ages

Age of females (months)	Dose (R)	Number of offspring classified	Number of litters per female	Offspring per litter	Progeny						
					Normal		X^MX^MY	$0X^P$	X^MX^PY	X^M0	Other
					Male	Female					
Irradiated females											
9	200	806	0.93	6.6	402	389	0	0 + 7?[a]	0	2 + 2?	4?
3	200	541	1.00	4.9	271	259	0	1 + 5?	1	1 (mosaic)	3?
Contemporary controls											
9	0	553[b]	1.91	6.5	258	290	0	0 + 3?	0	1	1?
3	0	300[b]	1.60	5.6	135	164	0	0	0	0	1?
Total controls											
9	0	1035	*c*	*c*	501	523	0	0 + 6?	0	2 + 1?	2?
3	0	833	*c*	*c*	403	424	0	0 + 3?	0	0	3?

[a] A ? symbol following a number indicates that no genetic or cytological test has so far been carried out. Classification by phenotype only is uncertain (see text).

[b] Includes only first two litters, provided that these are born within 3 months after mating.

[c] Since some of the control females are near the end of their reproductive life-span, while others have only recently started breeding, these ratios would be meaningless at this time.

females produced better than young, both in the irradiated and control groups, and that, while there was an obvious effect of radiation on number of litters, there was little or no effect on average litter size.

The results with respect to nondisjunction and loss are as yet quite preliminary. Because of phenotypic uncertainties (see above), most of the possible exceptional animals listed in Table 23 must be viewed as very tentative. No unequivocal instances of maternal nondisjunction have so far been observed, although there has been one case of paternal (spontaneous) nondisjunction. We are continuing with the present groups and adding others at older ages and higher doses.

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A CASE OF NONRANDOM X-CHROMOSOME INACTIVATION EXPLAINED BY SELECTION

Liane B. Russell, N. L. A. Cacheiro, and Margaret S. Swartout

We have earlier reported[1] the result of autoradiographic studies on seven X-autosome translocations [T(X;A)'s] that involve either chromosome 7 or 4. Using an early-labeling method in cell cultures derived from adult kidney tissue, we found that either the intact X chromosome (X^n) or the long translocation product (X^t) was differentially unlabeled (presumed inactive) while the short translocation product in each case was labeled indistinguishably from autosomes. However, nonrandom inactivation was found in three of the four T(X;7)s; namely, in considerably more than 50% of the cells, the differentially unlabeled chromosome was the X^n. In the case of the T(X;4)s, approximately equal numbers of cells had X^n or X^t unlabeled. This was true in spite of the fact that, both within the T(X;7)s and the T(X;4)s, there are some translocations in which X^t has an autosomal centromere and proximal portion, and others in which it has an X centromere and proximal portion. Since we had reasons (discussed below) to suspect that nonrandomness might be due to selection resulting from the use of adult kidney tissue, we decided to carry out autoradiographic studies in fetal nonkidney and kidney tissue.

One T(X;4) and one T(X;7) were chosen for this study, namely T(X;4)1Rℓ[2] and T(X;7)6Rℓ.[3,4] These will be referred to as R1 and R6 respectively. Fetuses were removed from the uterus 18 days after discovery of a vaginal plug. At that age, sex can be recognized; and, when appropriate markers have been used in the matings, translocation fetuses can be recognized by eye color (except for rare cases of crossing-over). Tissue cultures were initiated either from the kidneys of these fetuses or from their remaining tissues. Chromosome preparations were made by a technique combining fluorescence banding (for identification) and early-labeling autoradiography.[5]

Table 24 compares the earlier results for R1 and R6 (first 2 lines) with the present ones from 18-day fetuses. It may be noted that R1 shows random inactivation under both circumstances. R6, which gave extreme nonrandomness in adult kidney cultures, behaves randomly in fetal nonkidney cultures and gives intermediate results in fetal kidney cultures.

Since considerable portions of the autosome are subject to inactivation in T(X;A)s,[4] functional hemizygosity for a large number of autosomal loci exists in about 50% of all cells in cases of random inactivation.[6] It is conceivable that such a situation may not be tolerable in tissues where the loci involved play a critical role, and an extension of this explanation has, in fact, been invoked to account for the fact that T(X;A)s are recovered with a frequency considerably below that which would be expected from the incidence of T(A;A)s.[3]

The present results suggest that in R6 [and possibly in some of the other T(X;7)s also] one or more autosomal genes are inactivated that are critical for survival of adult kidney tissue. Selection therefore occurs for those cells in which X^n is inactive, *i.e.*, in which the critical gene(s) on X^t is (are) active and thus functionally diploid. The intermediate result obtained for fetal kidney indicates that the critical period of activity for the gene(s) in question starts before birth. Nonkidney tissue from the same fetuses reveals no need for functional diploidy. Similarly, Nesbitt, in an earlier autoradiographic study[5] involving the *fd* insertion, showed randomness in cells derived from total embryos. This insertion inactivates a region roughly similar to that affected by R6.

The present results have provided a good demonstration that nonrandomness can result from selection. Further studies to determine randomness vs nonrandomness in various tissues of different T(X;A)s may

Table 24. Distribution of cells with regard to X-chromosome labeling in tissues of different origins

Age of female	Tissue	Translocation	Number of females	Number of cells	Percent of differentially labeled cells with –	
					$X^n = 0^a$	$X^t = 0^a$
Adult	Kidney	R1	2	172	45.4	54.6
Adult	Kidney	R6	2	56	98.2	1.8
18-day fetus	Kidney	R6	2	77	70.1	29.9
18-day fetus	Total[b]	R1	2	67	55.5	44.5
18-day fetus	Total	R6	3	137	55.5	44.5

[a] $X^n = 0$: fewer than 3 grains over the intact X; $X^t = 0$: no grains over X^t or grains only over autosomal region of X^t.
[b] Homogenized tissue from the entire fetus, after removal of the kidneys.

localize critical genes for such tissues in specific chromosomal regions.

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A NEW X-AUTOSOME TRANSLOCATION THAT MAY GIVE NONRANDOM INACTIVATION

Liane B. Russell, N. L. A. Cacheiro, Jean W. Bangham, and Margaret S. Swartout

Information derived from the study of X-autosome translocations [T(X;A)s] has contributed heavily to our existing knowledge of gene action, gene inactivation, and gene-dosage effects in mammals. Of ten mouse T(X;A)s known until recently, nine have involved either chromosome 7 or 4, and have shown random inactivation, except in special circumstances.[1] We have this year obtained information on a new T(X;A) which involves neither of these autosomes and may produce nonrandom inactivation of a different sort from that observed in the past. This translocation, R13, was briefly reported last year[2] (when it was erroneously named R9).

R13 was found in a stock set up from a small daughter of an irradiated male (300 R of X ray to postspermatogonial stages). In propagating the stock from small females outcrossed to normal males of other strains, an average litter size of 2.7 is obtained. Segregation is shown in the table. About a third of the females, but only 2% of the males, are small. A number of normal-sized males were tested for fertility: 8 were found to be fertile and 15 sterile. Three small males currently being tested are apparently sterile. We shall analyze these cytologically to check the assumption that they may be R13 males with an extra X.

Sex	Small	Normal	Not classified	Total
Male	22	1019	18	1059
Female	273	585	24	882

Cytological analysis (by quinacrine-mustard banding) of kidney cultures from small females indicates a reciprocal translocation, with one break near the centromere of chromosome 12, the other in the X chromosome, at about one-third of its length from the centromere. The two translocation products designated 12^X and X^{12} (with centromeres of chromosomes 12 and X respectively), are of medium size. Early pulse labeling with [^{3}H]thymidine was carried out in kidney cultures from two adult R13 females. In this procedure, an asynchronously late-replicating (presumed inactive) chromosome will be selectively unlabeled. Among 25 cells in which asynchrony was observed, there were 18 in which the 12^X chromosome was the unlabeled one. In four cells the single unlabeled chromosome was probably the normal X (although diffuseness of X-chromosome fluorescence presents occasional difficulties of identification). In the remaining three cells, two chromosomes – the normal X and one or the other of the translocated chromosomes – were lightly labeled, while the rest of the complement was heavily labeled.

If the preliminary indications from the labeling studies prove to be correct, they would indicate nonrandomness in an opposite direction from that

observed in T(X;16)16H, where both translocated chromosomes are almost always active while the normal X is inactive in all cells. Crosses that introduced X-chromosome markers (*spf*, or *Ie*) and chromosome-12 markers (*ma*, *ft*) have so far not yielded results indicating nonrandomness. However, these loci may not be situated in 12^X.

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A PRESUMED PERICENTRIC INVERSION IN THE MOUSE

Liane B. Russell, N. L. A. Cacheiro, Clyde S. Montgomery, and Georgia M. Guinn

In the course of an experiment designed to measure sex-chromosome loss after irradiation of spermatogonia,[1] we found, within the presumed X^M0 (*i.e.*, paternal-loss) class, a female that had a submetacentric chromosome in a complement of 39. Further genetic and cytological work showed that the submetacentric segregated independently from the 39-chromosome (X0) condition, indicating that the abnormal chromosome was wholly autosomal. Meiotic preparations from animals heterozygous for the submetacentric failed to yield multivalent configurations and had only 20 bivalents. Since the shorter arm of the submetacentric is of considerable length, multivalent configurations would be expected if the abnormal chromosome were the result of a reciprocal translocation. A pericentric inversion was, therefore, suggested by this finding. The suggestion has been tentatively confirmed by the results of cytological banding studies, which indicate that the affected chromosome is 8. A breeding stock has now been successfully established, and homozygotes have recently proved to be viable and fertile. We shall attempt to introduce chromosome-8 markers in order to determine the effects of the presumed pericentric inversion on recombination. The submetacentric may also prove to be a useful cytological marker chromosome.

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CYTOLOGICAL STUDIES OF STERILITY IN SONS OF MICE TREATED AT SPERMATOGONIAL OR EARLY SPERMATOCYTE STAGES WITH MUTAGENIC CHEMICALS

N. L. A. Cacheiro, W. M. Generoso, and Margaret S. Swartout

We have studied cytologically and histologically 14 sterile sons derived from spermatogonia treated with cyclophosphamide, TEM, or TEPA. The 14 sterile mice were found among 3812 F_1 males tested for fertility. As was the case after X-ray treatment of spermatogonia,[1] only a small proportion (3 of 14) have chromosome anomalies. One male each was XYY; XX/XXY mosaic; and 40 chromosomes XX, T(Y;17). All three had small testes and spermatogenic arrest.

In addition, we have studied four sterile sons (out of 615 F_1 tested for fertility) derived from differentiating spermatogonia and very early spermatocytes treated with 6-mercaptopurine. Chromosome abnormalities were observed in three males, all of which were XYY and had small testes and spermatogenic arrest. Although this number is still small, the frequent XYY condition in sterile males from the 6-MP study may be attributable to induced nondisjunction. This problem is now under study.

None of these 18 sterile males derived from chemically treated spermatogonia or very early spermatocytes had the type of aberration most frequently found in sterile sons derived from irradiated postspermatogonial stages — namely, reciprocal translocations in which at least one break is near the centromere or telomere.[2]

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CYTOLOGICAL STUDIES IN STERILE SONS OF IMS-TREATED FEMALES

N. L. A. Cacheiro and Margaret S. Swartout

In earlier work we studied sterile sons of males treated in postspermatogonial[1] or spermatogonial[2] stages. We have now cytologically examined seven sterile sons of females treated with IMS in experiments of W. M. Generoso.

Chromosome abnormalities were observed in five of the males. Findings of the seven sterile sons may be summarized as follows.

1. 39 chromosomes. Robertsonian translocation of chromosomes 2 and 3.
2. 39 chromosomes. Robertsonian translocation of two chromosomes 3.
3. 41 chromosomes, XYY.
4. Mosaic XY/X0.
5. Insertion of small piece of chromatin of unknown origin into the short arm of chromosome 18.
6. Failure of X-Y pairing at diakinesis, but no obvious abnormality in karyotype.
7. No anomalies detectable.

It should be noted that the types of anomalies found are on the whole different from those in sterile sons of irradiated or EMS-treated postspermatogonial stages, most of which carry reciprocal translocations having one or both breaks near the end(s) of chromosome(s).[1]

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ATTEMPTS TO FIND PROTEIN DIFFERENCES RELATED TO GENETIC DIFFERENCES AT SINGLE LOCI

E. G. Bernstine and Liane B. Russell

The availability of a detailed complementation map of the dilute (*d*)–short ear (*se*) region of chromosome 9 of the mouse[1] makes the biochemical identification of the product of the dilute gene of interest. The mutation d^{op} affects pigmentation by causing clumping of melanosomes and opisthotonic seizures believed to be due to myelin degeneration.[2] Since both of these defects may result from an altered membrane component, we are comparing myelin proteins from mutant and wild-type animals by analytical SDS-polyacrylamide gel electrophoresis.

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ECO R1 DIGESTS OBTAINED FROM INTACT MOUSE LIVER NUCLEI

E. G. Bernstine

The current goal of this program is the isolation of defined regions of the mouse genome as DNA-protein complexes. When intact nuclei from the livers of C3H mice were incubated in the presence of the Eco R1 restriction endonuclease, 15–30% of the total nuclear DNA was recovered in soluble form as high-molecular-weight material. The digest contains DNA, RNA, and protein. Over 90% of the digest is excluded from large-pore columns of agarose gel. DNA purified from the digest is of high molecular weight as determined by agarose gel electrophoresis. Proteins from the agarose-column eluate extractable with SDS have been analyzed by electrophoresis in SDS-polyacrylamide gels. The results show that most of the protein is histone, the rest being a complex array of other proteins.

A variety of fractionation procedures will be used in attempts to obtain homogeneous preparations of various portions of the mouse genome. Such material should provide a basis for the detection of proteins that are not uniformly distributed over the genome as well as for the study of a wide variety of fundamental processes in the expression of genetic information.

ENERGY TRANSFER FROM ACETOPHENONE TO DNA–POLYLYSINE COMPLEXES

E. G. Bernstine and R. O. Rahn

The biochemical assessment of damage to the genome by a variety of agents is a logical extension of work in mammalian genetics in progress at ORNL. Preliminary experiments have shown that the number of thymine dimers introduced into DNA by energy transfer from acetophenone molecules excited by irradiation at 310 nm decreases linearly, after a brief lag, as the DNA is titrated with polylysine. The project was begun in order to determine whether an estimate of exposed and covered regions of DNA in chromatin and nuclei might be made by comparing the extent of acetophenone-mediated dimer formation in these samples to that in pure DNA. The sensitivity of the method is not sufficient. Dimers were quantitated in unlabeled material by hydrolysis of irradiated DNA, chromatography on ion exchange paper, elution with HCl, photoreversal at pH > 11, and analysis by difference spectroscopy at 275 nm.

A NEW EXPERIMENT ON THE INDUCTION OF SPECIFIC-LOCUS MUTATIONS IN MOUSE SPERMATOGONIA BY TRITIATED WATER

R. B. Cumming and W. L. Russell

In experiments previously completed[1] in this laboratory on tritium-induced specific-locus mutations in the

mouse, questions were raised which made it important to obtain more data from germ cells irradiated by tritium primarily as spermatogonia. Two points are in need of clarification. We previously found that the RBE for spermatogonia appeared to be higher than 1 with a point estimate that is slightly above 2. More data are needed to determine if tritium does indeed produce more genetic damage in spermatogonia per unit of absorbed dose than X or gamma rays. We also got an indication in our first series of experiments that the distribution of mutations among the seven loci scored might be different than for gamma rays. These two points need to be clarified by additional data.

We have started a new experiment designed to approximately double the number of data from cells treated as spermatogonia. The logistics of this experiment are greatly reduced by not collecting any data from cells irradiated in postspermatogonial stages. Thus the males are not mated in the isolators, and more males can be treated at one time. As before, the males are given a single ip injection of tritiated water at 0.5 mCi per gram of body weight. They are placed in isolators with five treated males per cage or 72 treated animals per isolator. This compares with 18 treated animals per isolator in our previous experiments. The animals are held in isolators for 4 weeks and then removed and placed in a special room. At 10 weeks postinjection they are individually mated to catch the first poststerile offspring, and they will remain mated for life.

To date, two groups have been mated and one has produced offspring. There are presently 492 offspring and one mutation at the *b* locus. The data from this experiment should be much more extensive by next year.

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STUDIES ON POTENTIAL GENETIC EFFECTS OF 5-CHLOROURACIL IN MAMMALS

R. B. Cumming, B. C. Pal, Marva F. Walton, and W. L. Russell

Chlorination is the most common method of disinfecting water used by humans in this country. Most municipal water supplies are chlorinated heavily enough to provide a specified residual chlorine content during distribution. Most sewage effluents are required to be chlorinated, and more than 100,000 tons of chlorine are used annually for this purpose. Large amounts of chlorine are also used in industrial processes involving water, including cooling towers.

Chlorine in water may react with any organic materials it encounters to form stable chlorine-containing organics,[1] many of which may have potent biological effects.[2] It is estimated that, from sewage chlorination alone, more than 1000 tons of such stable chlorine-containing organic materials are produced and dumped into surface waters each year.[1] Jolley[1] has demonstrated the presence of more than 60 chlorine compounds by chromatography of chlorinated Oak Ridge sewage effluents, and 17 of these compounds have been identified. The most abundant of the compounds identified to date is 5-chlorouracil (5-ClU), a thymine analogue which may be incorporated into DNA and which is mutagenic in some bacterial systems.[3] This compound, therefore, may serve as a good model to test whether a known mutagen dumped into natural waters may pose a genetic hazard to man. It is of great interest to know whether mammals which ingest 5-ClU in their drinking water actually incorporate this base analogue into DNA of their germ cells. It is also important to know whether it is logistically possible to set up a meaningful genetic test in mammals exposed to 5-ClU in their drinking water.

Male (C3H × 101)F_1 mice were given water containing either 100 mg/liter or 1 g/liter of 5-ClU for eight weeks. Water consumption was normal, and there were no easily observable ill effects. Weight remained normal. The higher of these two exposure levels is at least 1,000,000-fold as high as man can be expected to receive in his water supply. At the end of 8 weeks these males were individually mated to T-stock females, and the pairs were maintained on the same levels of 5-ClU as previously given to the respective males. Offspring were collected from this small number of matings (12 and 11, respectively, for the lower and higher doses) and were scored for specific-locus mutations. Fertility and litter size were normal in both groups, but there appears to be a small reduction in the percentage of offspring raised to weaning at the higher dose level which, if it is real, is probably due to a slight toxic effect to young exposed at this high dose. To date, 383 offspring have been scored in the lower-dose group and 314 in the higher-dose group. No specific-locus mutations have been seen in these 697 offspring. The use of this very small sample for risk estimation is dealt with separately.[4]

Male offspring from this specific-locus test were saved, when weaned, and maintained on 5-ClU water at the same concentration as their parents had received. As young adults these males were killed, their testes and

livers were removed, and DNA was extracted from these organs following procedures previously used in our laboratory.[5] The DNA was hydrolyzed by successive incubation with DNase-I, snake-venom phosphodiesterase, and alkaline phosphatase to produce a mixture of deoxynucleosides. 5-ClU was identified and quantified by chromatography of the DNA hydrolysate on Aminex A-6 (cation-exchange resin). It was absent in DNA from animals which had not been exposed to 5-ClU. About 1.4% of the thymine residues were replaced with 5-ClU in those animals exposed at the higher dose level. This amounts to about 1 5-ClU for every 250 nucleotides (averaged throughout the genome), or about 2.2×10^7 5-ClU's per genome, or about four 5-ClU's per structural gene.

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A TEST FOR 5-CHLOROURACIL-INDUCED DOMINANT-LETHAL MUTATIONS IN THE MOUSE

R. B. Cumming and Barbara J. Elmhorst*

5-ClU is a compound which is produced by chlorination of sewage effluent,[1] is incorporated into the DNA of mammals when it is consumed in the drinking water,[2] and is mutagenic in bacteria.[3] While no specific-locus mutations have yet been detected in the mouse,[2] it is of interest to know whether this compound is capable of producing other classes of genetic damage in mammals. A dominant-lethal protocol has been devised which allows mating of males under continuous chronic exposure to an agent with females which are not exposed. Using this protocol a dominant-lethal mutation experiment to test potential mutagenicity of 5-ClU is now in progress, and about half of the data we anticipate are already available.

Male offspring from the specific-locus mutation experiment[2] had been exposed to 1 g/liter 5-ClU in drinking water since before their conception. Under these conditions, 5-ClU is incorporated into their DNA.[2] Twelve of these animals were caged individually and supplied with 5-ClU-containing drinking water during the work day. Twelve matching pens, containing three unmated females each, were supplied with regular drinking water during the day. At 4:00 p.m. the 5-ClU males were transferred to the female cages, and the water was removed. At 8:00 a.m. the following day, the females were checked for plugs and the males returned to their separate cages. Each cage was supplied with the appropriate type of water. Plugged females were removed and replaced with virgins so that males would again be placed with three unplugged females that afternoon. Plugged females were collected until a sufficient number were obtained for the experiment. Controls were treated in exactly the same way except that the males had no history of 5-ClU exposure and there was regular drinking water in both male and female cages. Females were killed as usual on the 15th or 16th day of pregnancy and the uterine contents analyzed. Results to date are as follows.

	No. of females	Implants per female	Live embryos per female	Dead implants per female (%)	Dominant lethals (%)
Control	24	7.42	6.71	9.55	
5-ClU	36	6.75	6.25	7.41	6.83

There is a slight and probably not significant reduction in total implants in the 5-ClU-treated group. There is no indication of postimplantation loss. The small calculated dominant lethal is not significant and is limited to apparent implantation loss. Thus there is no evidence for a genetic effect. When the present experiment is complete, approximately twice as many females will have been scored. At that time these results can be reevaluated.

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MUTATIONS PRODUCED BY 5-CHLOROURACIL AND 5-BROMOURACIL IN *ESCHERICHIA COLI*

R. B. Cumming, Donna L. George,*
Marva F. Walton, and Barbara J. Elmhorst†

5-ClU is among the most abundant chlorine-containing organic compounds resulting from the chlorination of sewage effluents; consequently, it is an environmental contaminant for which there is some human exposure.[1] Its analogue 5-bromouracil (5-BrU) has been shown to be mutagenic in bacteria.[2] Both compounds are incorporated into DNA and replace thymine, and we have shown[3] that 5-ClU is incorporated into the

testicular DNA of mice when the animals drink water containing this base analogue. Thus it is of importance to know if 5-ClU is mutagenic in the same bacterial systems which are mutated by 5-BrU and more about the relationship between the mutagenicity of the two base analogues.

In a series of experiments, *E. coli* strain WP1-1 (*trp, thy*) was exposed to various concentrations of either thymine or the halogenated base analogue during logarithmic growth in liquid medium. The bacteria were then plated for survival and mutation frequency. Significant mutation induction was observed after 30 min of exposure to either 5-ClU or 5-BrU. There was an increase in mutation frequency up to a 60–90 min exposure time; at longer times, up to 3 hr, the mutation frequency per survivor decreased. 5-ClU is clearly mutagenic in this system for 25 to 100 μg/ml. At concentrations of 100 μg/ml or more, it is toxic to this thymine-requiring strain. Mixtures of 5-ClU and thymine also are mutagenic, and based on the present data there does not seem to be any great selectivity between these two compounds in this strain. A number of problems remain to be solved in this system. Among these is the significance of the 60–90 min peak in mutation frequency and the apparent disappearance of the mutants after these time periods.

We will measure the levels of incorporation of 5-ClU in DNA of this strain and attempt to relate the mutant frequency to the levels of this material in the DNA. The remaining genetic problems must be solved, however, before these types of measurements can be meaningful.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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THE REDUCTION OF MUTAGEN-INDUCED UNSCHEDULED DNA SYNTHESIS IN MICE PRETREATED WITH ENZYME INDUCERS

R. B. Cumming and Juarine Stewart*

We have previously shown[1] that when mice are pretreated with butylated hydroxytoluene (BHT) or phenobarbital, EMS becomes less toxic for them. BHT protects against MMS toxicity only in females.[1] We have also shown that pretreatment of males with BHT or phenobarbital reduces the amount of genetic damage caused by EMS.[2] Sega has demonstrated that EMS will stimulate unscheduled DNA synthesis in spermatids of treated animals,[3] and Sega *et al.* have shown that other methanesulfonic esters including MMS are also effective.[4] This effect has been interpreted as repair of DNA damaged by the mutagen in the cells involved.[3,4] A positive result with this test can then be interpreted as meaning that the mutagen reached the germ cells and damaged DNA. It is thus of interest to know whether

Table 25. Radioactivity incorporated into sperm cells from [^{3}H] thymidine following exposure to mutagens and enzyme-inducers

	Experiment 1		Experiment 2	
	dpm/10^6 sperm	% reduction	dpm/10^6 sperm	% reduction
Saline				
NP[a]	9.86 ± 0.99	–	13.53 ± 0.97	–
BHT	8.95 ± 0.81	–	13.73 ± 1.34	–
Phenobarbital	12.35 ± 1.24	–	16.88 ± 1.17	–
EMS				
NP[a]	485.56 ± 55	–	604.5 ± 82	–
BHT	384.6 ± 22	21	411.9 ± 29	35
Phenobarbital	341.8 ± 27	29	407.5 ± 30	36
MMS				
NP[a]	962.1 ± 12	–	2065.9 ± 254	–
BHT	659.6 ± 45	32	683.2 ± 42	65
Phenobarbital	935.5 ± 75	3	1540.6 ± 172	25

[a]No pretreatment.

the enzyme inducers which reduce EMS-induced genetic damage will also reduce the level of unscheduled DNA synthesis in germ cells.

Male (C3H × 101)F_1 mice 10–12 weeks old at the start of treatment were given BHT (0.75% in diet) for 2 weeks or phenobarbital sodium 1 mg/ml in the drinking water for 1 week. Matched untreated animals served as controls. At the conclusion of these pretreatments the mice were given 200 mg/kg EMS, 100 mg/kg MMS, or saline as a control and intratesticular injections of tritiated thymidine using the technique of Sega[3] as modified by more recent work in our laboratory. At 16 days following mutagen treatment the animals were killed, and sperm from the caudal epididymides were isolated and counted for radioactivity (Table 25).

In both experiments, EMS gave about equal reduction in incorporation for either of the two inducers. For MMS the reduction was only clear for BHT. It will be remembered that BHT does not protect male mice from MMS toxicity. In view of these results, it is clear that a genetic test should be performed.

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UNSCHEDULED DNA SYNTHESIS IN THE GERM CELLS OF MALE MICE AFTER *IN VIVO* EXPOSURE TO X RAYS

G. A. Sega

Unscheduled DNA synthesis in postmeiotic germ-cell stages of male mice was first demonstrated using the chemical mutagen EMS.[1] It was also of interest to determine if X rays could induce unscheduled DNA synthesis in male germ cells. Therefore, two groups of (C3Hf × 101)F_1 males were both given testicular injections of [^{3}H]thymidine ([^{3}H]dThd), with one group of males also receiving a 600-R dose of X rays over a 10-min time period.

Three animals were killed from both the control ([^{3}H]dThd only) and the X-ray-treated ([^{3}H]dThd + 600 R of X rays) groups at 2- to 3-day intervals 3–27 days after treatment. The [^{3}H]dThd activity contained in several million purified sperm heads taken from the vasa deferentia of both the control and X-ray-treated groups of animals was measured in a liquid scintillation counter for all of the time points. The control group showed no significant [^{3}H]dThd activity in the vas sperm through 27 days posttreatment while the X-ray-treated animals showed the presence of [^{3}H]dThd-labeled sperm in the vas 9–27 days posttreatment. The level of [^{3}H]dThd uptake into the vas sperm of the X-ray-treated animals remained at a relatively constant value during this 18-day time period.

The germ-cell stages showing repair of X-ray damage are the same as those found undergoing repair of EMS-induced damage except that the level of unscheduled incorporation of [^{3}H]dThd after X-ray treatment is much less. This is in spite of the fact that the EMS dose used (250 mg/kg) gives roughly comparable numbers of dominant lethals to those given by 600 R of X rays.[2,3] The 600-R X-ray dose is not inhibiting the repair process because we find an elevated level of repair when 1200 R is used.

Painter[4] has estimated that for every single-strand break induced in DNA by X rays between two and three bases are inserted into the repaired site, while Roberts[5] estimates that for every alkylation-induced lesion in DNA, approximately 100 bases are inserted in the repaired site. It is, therefore, possible that the much lower level of DNA repair measured in male germ cells using 600 R of X rays compared with 250 mg/kg of EMS is a manifestation of the number of bases inserted into the repaired lesions.

Work has also been started relating the level of DNA repair in the germ cells of male mice to the dose of X rays received. For example, the level of DNA repair occurring in early spermatids has been found to increase linearly with X-ray dose from 50 R to 600 R. Higher doses are currently being studied. It should also be noted that the detection of unscheduled DNA synthesis in the germ cells of male mice at X-ray doses as low as 50 R attests to the sensitivity of these experiments since Painter,[4] for example, has indicated that a dose of at least 1000 R was needed to detect DNA repair in the mammalian cells he was using.

Another aspect of DNA repair in mouse germ cells is the determination of how the rate of repair in a given germ-cell stage changes with time after treatment. This can be investigated by giving the testicular injections of [^{3}H]dThd at various times after the end of the X-ray treatment. Since we have found that the labeled thymidine is only available for incorporation into germ-cell DNA for approximately 1 hr after it is injected,[6] it is possible to measure the levels of DNA repair occurring at different times after the end of the X-ray exposure. The results for early spermatids exposed to 600 R of X rays so far indicate that the repair

is greatest immediately after treatment and continually decreases up to 4 hr later, although some repair is still measurable at this time.

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STUDIES ON THE RELATIVE EFFECTS OF METHYL, ETHYL, PROPYL, AND ISOPROPYL METHANESULFONATE IN CAUSING *IN VIVO* UNSCHEDULED DNA SYNTHESIS IN THE GERM CELLS OF MALE MICE*

G. A. Sega, J. G. Owens, and R. B. Cumming

Having established the meiotic and postmeiotic germ-cell stages of male mice that undergo DNA repair when exposed to EMS,[1] we are now looking at four chemical homologs – MMS, EMS, PMS, and IMS – to determine their abilities to induce DNA repair in mouse germ cells. In particular, sperm from caudal epididymides are being studied 16 days after treatment. (Sperm from the caudal epididymides are used because there are about twice as many of them per animal as there are of sperm in the caput epididymis or in the vas.) This means that at the time of treatment these caudal sperm were early spermatids.[2,3]

For each of these chemicals a dose-response curve has been obtained which measures the relative unscheduled uptake of [^{3}H] dThd into early spermatids as a function of administered concentration of the chemical mutagen. Also, the relative amount of repair occurring over a 3-day time period following administration of each chemical has been studied. Before the duration of repair could be investigated it was necessary to establish how long labeled thymidine remained available for incorporation into the germ-cell DNA in the testes after injection. We have found that after testicular injections of the [^{3}H] dThd, its removal from the thymidine pool of the testes is quite rapid, with most of the free [^{3}H] dThd disappearing from the testes within about an hour after injection.[1] This result is in agreement with the work of others using mammalian systems to measure the kinetics of incorporation of [^{3}H] dThd.[4]

The study on the duration of DNA repair in early spermatids of the mouse after treatment with MMS, EMS, PMS, and IMS showed that as long as 3 days after exposure to these chemicals the early spermatid stages are still undergoing measurable DNA repair. The extended repair is not due to continuing alkylation of the DNA during this time period, because these four chemical homologs are quite reactive in aqueous solutions[5] and even more reactive in biological systems,[6] probably disappearing within several hours (at most) after injection.

All four of the chemicals also induced an unscheduled uptake of [^{3}H] dThd into early spermatids when injected at the same time the [^{3}H] dThd was administered. This means that all of these mutagens must reach the DNA of early spermatids and begin producing repairable lesions within 1 hr after treatment. The dose-response effects of all four chemicals in the induction of germ-cell DNA repair appear to be linear over the dose ranges studied. The greatest DNA repair response was seen using MMS, followed by EMS, IMS, and finally PMS. The same relative order for MMS, EMS, and PMS applies in their effectiveness in the induction of dominant-lethal mutations in spermatozoa and late spermatids.[7–10] However, IMS falls between MMS and EMS in this case.[9] Comparing MMS and EMS, for example, it was found that at equimolar administered concentrations of the two chemicals, MMS was about 5 times as effective as EMS in inducing DNA repair in early spermatids. A similar relationship has been found between these two chemicals in the induction of DNA repair in cultured mammalian cells,[11] although in that case the difference was tenfold.

Roberts *et al.*[11] have found that in their cultured mammalian cells repair synthesis is directly related to the overall level of DNA alkylation in the cells. If the same result holds true for the mouse germ cells treated *in vivo*, then measurement of DNA repair in these germ cells could become a fast and economical way of measuring chemical dose in the genetic material. The technique would be economical, since expensive isotopically labeled chemical mutagens could be replaced by much less expensive labeled thymidine and unlabeled mutagens to assay for the molecular dose received by the DNA.

The measurement of DNA repair in the mouse germ cells also appears to be a considerably more sensitive biological end point than measurement of dominant lethals or translocations in the germ cells. The measure-

Table 26. Comparisons of the lowest chemical doses of MMS, EMS, PMS, and IMS needed to detect dominant lethal mutations or translocations with doses that still induce DNA repair

Chemical	Minimum dose for detection (mg/kg)		DNA repair in germ cells still readily measured at a dose (mg/kg) of:
	Dominant lethal	Translocation	
MMS	~50 (ref. 8)		5
EMS	~150 (ref. 12)	~50 (ref. 12)	10
PMS	~400 (ref. 9)		50
IMS	~50 (ref. 9)		10

ment of DNA repair is at least 5 to 10 times more sensitive than the dominant-lethal tests or translocation studies using these four chemical mutagens (Table 26).

*Research sponsored jointly by the National Center for Toxicological Research and the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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A STUDY ON THE INDUCTION OF DOMINANT-LETHAL MUTATIONS IN THE GERM CELLS OF MALE MICE TREATED WITH IODOACETAMIDE*

G. A. Sega, R. E. Sotomayor, and Marva F. Walton

In previous studies[1] we found that when male mice were treated with [2-^{3}H]ethyl methanesulfonate ([^{3}H]EMS) the pattern of sperm ethylation in the vas over a 16-day time period following treatment closely paralleled the dominant-lethal pattern measured for the offspring of treated males. Thus, when the dominant-lethal frequency was low, the number of ethylations per sperm cell in the vas was low, and when the dominant-lethal frequency was high, the number of ethylations per sperm cell in the vas was also high. However, when the ethylations per nucleotide were determined for the vas sperm, it was found that the number of DNA ethylations stayed at a relatively constant value of 2–3 $\times 10^{-5}$ ethylation per nucleotide from 4 hr to 10 days after injection. Since the increased number of ethylations found in the vas sperm by 9 to 10 days after treatment were apparently not due to increased DNA ethylation, it was suggested that another chromosome component, such as protamine, might account for the ethylation peak in the sperm contained in the vas at this time and might also contribute to the production of dominant-lethal mutations.

In order to further investigate the role of non-DNA components of male germ cells in the production of dominant-lethal mutations following treatment with alkylating agents, iodoacetamide has been used in a dominant-lethal study. This alkylating agent has been reported to interact very little with DNA; but it does react with proteins, almost exclusively with thiol groups.[2] In previous work[3] we had obtained indirect evidence that mouse sperm protamine contains cysteine, which should be a target for alkylation by iodoacetamide. It was, therefore, interesting to see if an alkylating agent known to attack the sulfhydryl groups of proteins almost exclusively could induce dominant-lethal mutations in male germ cells of the mouse.

(C3Hf $\times$ 101)F_1 males were given 30 mg/kg ip injections of iodoacetamide, which was just below the lethal dose.[4] Each male was placed with three females (101 $\times$ C3Hf), and vaginal plugs were checked daily. Matings were continued for 3 weeks following treatment of the males. Controls were run concurrently with the treated animals. The females were opened between 14 and 16 days of gestation, and the total number of implants as well as the number of dead implants per female were determined.

There was no significant decrease in the average number of implants per female derived from matings with the treated males compared to the controls, nor was there a significant increase in the average number of dead implants per female derived from matings with the treated males compared to the controls over the 3-week time period studied. The results of this experiment do not indicate any dominant-lethal effect caused by the interaction of sperm-cell proteins and iodoacetamide. Although we have not yet established that the iodoacetamide reaches the proteins of the germ cells, we do have preliminary evidence that this alkylating agent induces a small DNA repair response in the germ cells. This means that at least a small amount of interaction between iodoacetamide and DNA in the mouse germ cells is occurring and also shows that the chemical is, in fact, reaching the germ cells. Further studies are being conducted on the induction of germ-cell DNA repair by this chemical.

*Research sponsored jointly by the National Center for Toxicological Research and the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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DEVELOPMENT OF MORE SENSITIVE PROCEDURES FOR USING UNSCHEDULED DNA SYNTHESIS AS A MEASURE OF GERM-CELL DAMAGE*

G. A. Sega and J. G. Owens

Original procedures used to detect unscheduled DNA synthesis in the germ cells of male mice involved the injection of 50 μl of [^{3}H]dThd solution (50 μCi) into each testis prior to treatment with the chemical mutagen.[1] The [^{3}H]dThd was injected through the scrotum and presumably into the testes. These procedures gave, for example, an unscheduled uptake of [^{3}H]dThd into early spermatid stages amounting to ~50 dpm/million sperm with a 200 mg/kg dose of EMS.

To enhance this unscheduled uptake of [^{3}H]dThd into the germ cells (so that DNA repair might be detected at the lowest possible chemical dose), experiments have been performed to determine the best way to administer the [^{3}H]dThd. When dye solutions were injected into mouse testes using the procedure described above, it was found that about half the testes showed no traces of dye. Therefore, to ensure that the [^{3}H]dThd solution was always injected into the testes, we made small incisions in the scrotums of the animals but without cutting into the peritoneum. The testes were readily visualized this way, and it was quite easy to insert the syringe needle into the approximate center of the testis and inject the [^{3}H]dThd. After the testicular injections no closing operation is necessary: The scrotal incisions heal in a few days without the need of sutures or clamps, and no infection has developed in the several hundred mice we have injected in this manner.

Making the scrotal incisions before injecting the [^{3}H]dThd has greatly improved the sensitivity of our unscheduled DNA synthesis assay in male germ cells. For example, with a dose of 200 mg/kg of EMS, the unscheduled uptake of [^{3}H]dThd into early spermatids is now more than 500 dpm/million sperm, a tenfold increase in activity over what was obtained previously.

It was also noted that when 50 μl of [^{3}H]dThd solution was injected into the testes, the tunica encapsulating the tubules would frequently rupture and some of the [^{3}H]dThd solution would leak out. Therefore, a smaller, 35-μl volume of [^{3}H]dThd solution was used to try to prevent this rupturing. There was no difference in the level of unscheduled incorporation of [^{3}H]dThd in the sperm cells whether 50 μl (50 μCi) or 35 μl (35 μCi) of [^{3}H]dThd was injected per testis, although the variability in uptake of the label among the mice was almost twice as great in the former case as in the latter. With 50 μl (50 μCi) of [^{3}H]dThd injected per testis the coefficient of variation among the treated mice was 29%, while with 35 μl (35 μCi) of [^{3}H]dT injected per testis the coefficient of variation was down to 17%. The reduced variability using 35 μl of the [^{3}H]dThd solution per testis reflects the fact that the tunica was not rupturing with this volume of injected solution.

The [^{3}H]dThd was also administered intraperitoneally in an attempt to further reduce the mouse-to-mouse variability in unscheduled incorporation of labeled thymidine into the germ-cell DNA. The variability in this case turned out to be no less than with the testicular injections, and the level of unscheduled incorporation of [^{3}H]dThd into the germ cells was only about 1/50 of what it was with the testicular injections. Currently, then, the best procedure for administering the [^{3}H]dThd to maximize the sensitivity of our unscheduled DNA synthesis assay in the germ cells of male mice and to minimize the variability

from animal to animal appears to be the injection of 35 μl (35 μCi) of the labeled thymidine directly into each testis, which has first been made visible by a scrotal incision.

*Research sponsored jointly by the National Center for Toxicological Research and the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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UNSCHEDULED DNA SYNTHESIS IN GERM CELLS OF MALE MICE AFTER *IN VIVO* TREATMENT WITH MITOMEN AND CYCLOPHOSPHAMIDE*

R. E. Sotomayor, G. A. Sega, and R. B. Cumming

In our laboratory, Sega[1] has been able to demonstrate, for the first time, that unscheduled DNA synthesis occurs in meiotic and postmeiotic germ-cell stages of male mice after *in vivo* treatment with EMS. Previous findings by other investigators have shown that in mouse spermatogenesis the last scheduled DNA synthesis occurs in the spermatocyte just before entering meiosis, and that no unscheduled DNA synthesis is detected thereafter under normal conditions.

A logical explanation for Sega's results is that the occurrence of unscheduled DNA synthesis in the cells exposed to EMS is related to DNA repair. An important observation was the finding that late spermatids and spermatozoa do not exhibit the capacity for DNA repair, even though their DNA is ethylated. Furthermore, it is known that EMS induces dominant lethals, translocations, and specific-locus mutations in those most mature germ-cell stages, where no unscheduled DNA synthesis was detected in Sega's experiments.

It is interesting, therefore, to determine whether other chemical mutagens show a similar pattern of DNA repair, since it would be very attractive to postulate that the genetic damage induced by EMS and other alkylating agents may result from failure of DNA repair to occur.

To investigate this possibility a number of chemical mutagens are at present being studied in our laboratory. Preliminary results on cyclophosphamide and mitomen will be shown here as examples of two different mutagens that produce different patterns of germ-cell stage sensitivity to the induction of genetic damage.

Cyclophosphamide induces chromosome breakage in all postmeiotic germ-cell stages of male mice, the most sensitive being that of early spermatids.[2] Mitomen, on the contrary, induces chromosome breakage mutations only in mature spermatozoa.[3] Both agents require activation by the liver microsome enzymes.

Our scheme of treatment was as follows: (C3H × 101)F_1 male mice were injected intraperitoneally with either mitomen or cyclophosphamide. [^{3}H] thymidine was injected intratesticularly in doses varying from 72 to 103 μCi, depending on the experiment. Control animals received only saline and [^{3}H] thymidine. One group of animals was used to detect unscheduled DNA synthesis under conditions where no or very little activation of the mutagen could be achieved at the time [^{3}H] dThd was available for incorporation. This was done by injecting the mutagen and [^{3}H] thymidine simultaneously. A second group of mice was used to study unscheduled DNA synthesis after activation of the mutagens. This was achieved by injecting [^{3}H]-thymidine at different intervals of time after treatment.

Sperm samples from caput, caudal, and vasa deferentia were obtained from each group of mice. Purified sperm heads derived from different treated germ-cell stages were recovered, and the [^{3}H] dThd activity from several million sperm heads was measured by liquid scintillation counting.

Results can be summarized as follows: (*a*) Cyclophosphamide and mitomen induced unscheduled DNA synthesis (measured as unscheduled incorporation of [^{3}H] thymidine) in early spermatids. In untreated mice no unscheduled DNA synthesis was observed. (*b*) The amount of unscheduled DNA synthesis in treated germ cells varied according to the time probably required for activating the mutagens. When very little or no activation could be achieved at the time of incorporation of [^{3}H] dThd, unscheduled DNA synthesis was considerably lower than at later times when activation had presumably occurred. Unscheduled DNA synthesis increased with time in both cyclophosphamide- and mitomen-treated germ cells, reached a maximum, and then declined. Mitomen induced a maximum response about 0.5 hr after being injected, while cyclophosphamide did the same about 1 hr after treatment. Unscheduled DNA synthesis remained at significant levels several hours after treatment with both chemicals.

These results are interesting for a number of reasons. It is apparent that cyclophosphamide or its active metabolite triggers a DNA repair response in those germ-cell stages which are the most sensitive to the induction of genetic damage by the compound. In other words, a maximum mutagenicity coexists in time and germ-cell stage, with an active repair process. The possibility, mentioned before, that EMS-induced genetic damage may originate from failure of DNA

repair to occur seems not to be true for cyclophosphamide. Although we have not sampled the entire spermatogenic cycle, using mitomen, our preliminary results indicate that DNA repair occurs during early spermatid stages as in the case of cyclophosphamide, EMS, and other mutagens so far tested. Mitomen is interesting because it induces chromosome breakage only in mature spermatozoa, whereas other mature germ-cell stages, which presumably also lack the capacity for DNA repair, are insensitive to the induction of genetic damage. For the first time we have found two chemicals that show a time delay in the maximum level of DNA repair after treatment, unlike chemicals such as EMS which show a maximum level of repair immediately after treatment.

*Research jointly sponsored by the National Center for Toxicological Research and by the US Energy Research and Development Administration under contract with the Union Carbide Corporation.

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A SEARCH FOR MUTAGEN-INDUCED HERITABLE PREMUTATIONAL LESIONS IN THE MOUSE

R. B. Cumming and R. E. Sotomayor

A project has been initiated under an interagency agreement between the Food and Drug Administration and ERDA to investigate the possibility that some chemical mutagens may induce premutational lesions in the DNA of mammalian germ cells. These premutational lesions could then be transmitted to subsequent generations and increase the apparent spontaneous mutation frequency, or they might be developed into expressed mutations by exposure to a second agent which is not normally mutagenic. In 1971 Lavappa and Yerganian[1] reported such a situation in the Armenian hamster, in which urethane caused chromosome aberrations in certain male germ-cell stages in animals whose grandfathers had been exposed to EMS, while no effect was seen in urethane-treated animals whose ancestors had received no treatment. If this is a real effect, it constitutes a very important class of genetic damage because it provides a mechanism for interaction between agents which are not by themselves mutagenic – a class of genetic damage which would not be detected in any mutational test system currently in use. Our project is an attempt to repeat the Lavappa and Yerganian experiment in mice, using a much more extensive and rigorous experimental protocol. It is hoped that we will be able to define this problem with much greater precision than previously and either design tests for such damage, which can be added to current mutational screens, or show within defined limits that this phenomenon is not a problem and does not constitute an important class of genetic damage missed by present mammalian mutational screening systems.

The protocol for our experiment calls for the collection of F_1 male progeny from matings in which the male parent was treated with EMS at 200 mg/kg, cyclophosphamide at 300 mg/kg, or saline (control). Offspring from the EMS-treated males are conceived 5.5 to 8.5 days after treatment, and those from cyclophosphamide-treated males are conceived 17.5 to 20.5 days posttreatment. Control F_1's are conceived 5.5 to 8.5 or 17.5 to 20.5 days posttreatment. Thus the F_1's are produced from germ cells which are most sensitive to chromosome aberrations induced by the particular agent involved. EMS was selected because it is the agent involved in Lavappa and Yerganian's original report, and cyclophosphamide was selected because there is an indication in previous work[2] that there is a higher "spontaneous" translocation frequency in animals whose ancestors were treated with this agent. F_1 males are unilaterally orchidectomized at approximately seven weeks of age, and cytological preparations (20 slides each) are made using a modification of the air-dry technique of Evans *et al.*,[3] which has been used in our laboratory.[2] Following a recovery period, the F_1 males are mated to produce F_2 males (at least 12 F_2 males for each F_1). The F_1 males are then treated with urethane, and at various times later (from 5 to 15 days) they are killed and cytological preparations are made from the remaining testis. In the event that chromosome abnormalities occur in the spermatocytes of the second testis, preparations from the first testis will be examined in detail to see if the same abnormalities were present prior to urethane treatment. If such an abnormality is observed in F_1 males, F_2 animals from the same F_1 will be examined.

To date we have produced, from EMS-treated fathers, 141 nonsterile F_1 males which have had one testis removed and from which good cytological preparations have been made. Of these, 88 have been mated to produce F_2's, and 29 have produced 12 or more F_1 male offspring. From cyclophosphamide-treated males we have, to date, 145 nonsterile F_1's which have given good cytological preparations.

A preliminary examination of slides from the EMS-treated F_1's showed 9 clear translocation heterozygotes

out of the 141 examined, and there were, in addition, 4 sterile animals in this group. Thus the frequency of presumed heterozygotes (13/145) is lower than would be expected for this dose of EMS. We think, however, that the number will increase as the slides are examined in greater detail, since thus far only a few cells have been analyzed for each F_1. No cytological data are yet available for F_1's from the cyclophosphamide-treated groups; however, there were 5 sterile F_1's out of 150 produced.

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SPERMATOGONIAL STEM-CELL SURVIVAL AFTER IRRADIATION AT LOW DOSE RATES

E. F. Oakberg and Deborah T. Palatinus

These data were obtained to evaluate the possible role of cell survival on mutation frequencies at different dose rates. 101 × C3H hybrid males were given 300 R of cesium gamma rays at 0.0056 and 0.00066 R/min (56 and 6.7 R/wk) continuous exposure and compared with males given 300 R X rays at 94 R/min.

One group of mice was killed immediately after removal from the gamma-ray field; a second group was killed 24 hr later in order to observe initiation of cell repopulation. Cell counts for these intervals were the same for gamma-irradiated mice, and the data were pooled. Mice of comparable age exposed to 300 R X rays at 94 R/min were killed 120 hr after irradiation, when cell survival is minimal.

Since the exposure to low dose rates involved all stages up to the time of killing, data were presented as cell type scored. The most drastic effect was observed after 300 R acute X rays, where only 16% of the stem cells and only a few differentiating spermatogonia survived. At 0.0056 R/min, stem-cell survival was 37% for A_s and 24% for A_1 cells. The remaining spermatogonial types showed depletion to about 5% of control, indicating a marked increase in sensitivity for these stages. At 0.00066 R/min, the number of stem cells was unaffected, with survival values for A_1-A_4 spermatogonia ranging from 81 to 97%. Thus, the equilibrium at lower cell numbers which occurs at this dose rate results from sensitivity of A_1-A_4 spermatogonia. The possibility that stem-cell kinetics is affected is being investigated by [^{3}H] thymidine labeling.

The mutation frequency at 0.00066 R/min therefore is measured on a cell population which is maintaining itself at normal levels. The possibility of selection cannot be ruled out, but it appears unlikely. The rate of 0.0056 R/min, which reduces the A_s spermatogonia to 37% of control, gives about the same frequency of mutation, and 300 R, where cell survival is only 16%, gives the highest mutation rates of all. This result is the reverse of that expected if there is a positive correlation between sensitivity to cell killing and sensitivity to genetic damage. Other mechanisms, such as repair, are indicated as the primary factors in the reduced frequency of mutations observed at low dose rates.

EFFECTS OF 6-MERCAPTOPURINE ON SPERMATOGONIAL SURVIVAL AND DURATION OF SPERMATOGENESIS IN THE MOUSE

E. F. Oakberg and Deborah T. Palatinus

This experiment was prompted by the observation of Generoso *et al.*[1] that a high frequency of dominant lethals was observed 32.5–35.5 days after injection of male mice with 6-MP.[1,2] Chromosome breakage was elevated at days 14 and 15.[1] It was important to determine if rate of spermatogenesis had been affected, or if chromosome breaks were being recovered from spermatogonia.

Five groups of 12-week-old 101 × C3H hybrid male mice were used. Group 1 was used as control for cell counts. Group 2 was given 15 μCi [^{3}H] thymidine, followed 30 min later by an injection of 0.25 ml 0.03 *N* NaOH. Group 3 was given 15 μCi [^{3}H] thymidine. Group 4 was given 15 μCi of [^{3}H] thymidine, followed 30 min later by 150 mg/kg of 6-MP in 0.03 *N* NaOH. Group 5 was injected with 150 mg/kg of 6-MP only. Animals were killed at intervals ranging from 72 hr to 17 days after treatment.

Testes were fixed in Zenker-formol, embedded in paraffin, sectioned at 5 μm, and stained by the periodic acid–Schiff technique with Ehrlich's hematoxylin as a counterstain. Autoradiographs were prepared of sections of testes receiving [^{3}H] thymidine. Surviving spermatogonia were scored at 3 and 10 days. Progression of cells labeled as preleptotene spermatocytes was followed to 17 days, or for two cycles of the seminiferous epithelium.

Number of A_s (stem) spermatogonia was not affected. At 3 days, frequency of A_2-A_4, In, and B spermatogonia was reduced, respectively, to 80, 54, and 50% of control. Number of preleptotene spermatocytes (treated as late A_4–early In spermatogonia) was at the control level. Recovery was slower than expected, with

numbers of A_1-A_4 gonia and preleptotene spermatocytes depressed to 84–93% of control at 10 days. Progression of labeled cells through spermatogenesis was identical for groups receiving [^{3}H] thymidine and [^{3}H] thymidine plus 6-MP.

The results on spermatogonial killing are interesting in that no depletion of stem cells occurred, and that late A_4–In spermatogonia, which normally are the most sensitive cell type, showed no effect as measured by number of preleptotene spermatocytes at stage VII 72 hr after treatment. Also, recovery was slower than we have observed for other chemicals, with a significant depression in differentiating spermatogonia and preleptotene spermatocytes 10 days after irradiation. 6-MP did not affect the rate of spermatocyte and spermatid development. Therefore, it can be concluded that the dominant lethality and chromosome breakage observed by Generoso *et al.*[1,2] can be attributed to effects on A_4-In spermatogonia. Finally, timing of spermatogenesis remains a reliable indicator of germ-cell stages sampled at different times after treatment.

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EFFECT OF URETHANE ON MOUSE SPERMATOGONIA

E. F. Oakberg and Patricia D. Tyrrell

The study of the effect of urethane on mouse spermatogonia was initiated both to evaluate cytotoxicity of a teratogen and because of the appearance of lung tumors in the second (F_2) generation of treated animals.[1]

Male 101 × C3H hybrid mice, 12 weeks old, were given a single ip injection of 20 mg of urethane per mouse (22–23 g weight). Animals were killed over a 12-hr to 12-day interval, and 5-μm paraffin sections of the testis were stained by the periodic acid–Schiff technique with Ehrlich's hematoxylin as a counterstain. Spermatogonia were enumerated in 99 tubular cross sections distributed on the basis of frequency of the 12 stages of the cycle of the seminiferous epithelium.

The number of A_s (stem) spermatogonia was depressed only at 72 hr. A_1-In cells, however, showed a decline by 12 hr, and preleptotene spermatocytes were not reduced until 72 hr (*i.e.*, until cells treated as A_4-In spermatogonia were sampled). Thereafter, all cell types from A_1 spermatogonia to preleptotene spermatocytes were below the control level for the 72-hr to 12-day period. The 72-hr value for A_s spermatogonia at 48 hr was 74%, and 73% survival was observed for A_1 cells at 5 and 12 days. Otherwise, the effect was not marked, and usually ranged from a 10 to a 15% reduction. An increased frequency of degenerating cells was observed at both 12 and 24 hr after injection.

The results bear some resemblance to the effects of 6-MP,[2] and both differ from radiation and hycanthone. The effect on stem cells was slight, being observed only at 48 hr; differentiating spermatogonia showed a greater response. However, the effect persisted throughout the 12-day sampling period. Return to control numbers of cells would have occurred by this time after doses of radiation or hycanthone giving comparable (10–15%) cell loss. The basis for this prolonged effect is not known.

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SECTION V

PATHOLOGY AND IMMUNOLOGY

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Radiation Recovery of Hemopoietic Cells

L. H. Smith

Experimental Hematology

Joan Wright Goodman

Cellular Immunology

E. H. Perkins
C. F. Gottlieb
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Effects of High-LET Radiations

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R. L. Ullrich

Hematology and Radiation Physiology

T. T. Odell

Experimental Radiation Pathology

J. M. Holland

Animal Virus Research

R. W. Tennant
G. C. Lavelle
J. A. Otten
J. M. Quarles, Jr.[a]
R. J. Rascati[a]

Histology and Autoradiography

W. D. Gude

Mammalian Radiobiology

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RADIOPROTECTION OF MICE BY PHENYLHYDRAZINE-DAMAGED ERYTHROCYTES

L. H. Smith and T. W. McKinley, Jr.

Phenylhydrazine ($PhNHNH_2$) is radioprotective if given to mice about 1 week before exposure to X rays.[1] In experiments designed to characterize radioprotection by this drug, preliminary results showed that erythrocytes (RBC) damaged by $PhNHNH_2$ (PhRBC) were also radioprotective.[1] Specifically, the injection of PhRBC 1 day before irradiation resulted in enhanced 30-day survival and splenic hemopoiesis. Apparently, RBC that are altered by $PhNHNH_2$ initiate a chain of events that establishes a preirradiation condition which renders the animal more radioresistant. In a previous progress report,[2] we presented evidence showing that this enhanced radioresistance of mice was not the result of an increase either in the D_0 (*i.e.* radioresistance) of hemopoietic stem cells or in the number of stem cells at risk at the time of radiation.

Since there is histologic evidence that $PhNHNH_2$ increases reticuloendothelial system (RES) activity,[3] injection of PhRBC could also possibly increase RES activity, which in turn might provide the irradiated animal with a better defense and consequently improve survival. Therefore, we have continued our studies of radioprotection by PhRBC by examining the functional state of the RES in the treated mice. As a reflection of RES activity, we measured the $T_{1/2}$ for circulation and the liver accumulation of injected ^{51}Cr-labeled sheep RBC. Enhanced RES activity would be expected to reduce the $T_{1/2}$ and increase accumulation of sheep RBC in the liver. The results show that, compared with controls, neither expectation was observed for any of the groups on days 1, 3, or 6 after treatment. Liver accumulation was slightly, but not significantly, increased above normal by each of the three treatments, and PhRBC alone or in combination with irradiation did not significantly affect accumulation compared with X-ray control values. Accumulation data for lung and spleen were also obtained. Lung values were consistently low in all groups (<2%), and the spleen values were no higher in the PhRBC-treated irradiated mice than in X-ray control mice. From these results we conclude that enhanced activity of the RES is not a major factor in radioprotection by PhRBC.

In another series of experiments, we tested the possibility that PhRBC could create an environment in hemopoietic tissue that was conducive to recovery of radiation-damaged stem cells. To explore this possibility, we measured the spleen-colony-forming capacity of bone marrow in several types of recipients. Injection of unirradiated marrow cells produced about the same number of colonies in mice that received PhRBC as in mice that received saline ($P > 0.2$). This observation appears to exclude enhanced trapping or growth of marrow stem cells in spleens of PhRBC-treated mice. Of greater interest, however, is the fact that the number of spleen colonies arising from irradiated marrow cells was greater ($P = 0.05$) in PhRBC-treated mice than in saline-treated controls. We tentatively conclude, therefore, that PhRBC may create a splenic environment that promotes repair of radiation-damaged hemopoietic stem cells.

The results to date show that PhRBC provide a soluble or particulate factor that acts directly or indirectly on hemopoietic tissue of mice. As a result of this action, hemopoietic tissue has the capacity to recover following lethal whole-body irradiation. The fact that PhRBC are radioprotective when given before but not after irradiation indicates that the factor is not effective after injury has occurred. Our data also appear to rule out both of the following stem-cell factors that would enhance survival: increased radioresistance of stem cells and increased number of stem cells at risk. The only observation which suggests a basis for radioprotection by PhRBC is that irradiated marrow grows better in mice treated with PhRBC than in mice treated with saline. Enhanced marrow growth could indicate the presence of a hemopoietic microenvironment more conducive to repair of sublethal radiation injury to hemopoietic stem cells. Accordingly, the factor provided by PhRBC would be involved in creating this microenvironment in hemopoietic tissue rather than acting directly on stem cells.

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ABILITY OF THYMOCYTES TO INCREASE PROLIFERATIVE RATE OF TRANSPLANTED MARROW CELLS

Joan Wright Goodman, Sarah G. Shinpock, and Nancy L. Basford

Our past work using a P-F_1 poor-growth combination has indicated that thymocytes help control division and differentiation of transplanted nonisogenic bone marrow stem cells. Data from Zipori and Trainin,[1] who studied nude and thymectomized mice, and from Lord and Schofield,[2] who transplanted isogenic marrow damaged by a small exposure to X rays, suggested that the thymus plays a controlling role in hemopoiesis in intact animals and in transplantation situations more nearly analogous to the normal physiological state than is our P-F_1 experiment. Specifically, we found that thymocytes augmented the growth of parental bone marrow in irradiated hybrid recipients. The increased growth in chimeras was deduced from several kinds of measurement including (*a*) 24-hr ^{59}Fe uptake by spleen and erythrocytes, (*b*) spleen weight, and (*c*) splenic nodule number. In addition, histologic examination of spleens removed early (4–11 days) after transplantation allowed us not only to enumerate hemopoietic colonies but also to evaluate their differentiation patterns and to assess lymphoid activity. In presenting our histologic findings,[3] we qualitatively described the "augmented" colonies as larger than comparable ones resulting from the injection of marrow cells, and we interpreted this to mean that thymocytes were promoting more rapid proliferation of differentiating marrow cells. We have recently been challenged on this point and thought it sufficiently important to obtain the following quantitative substantiating evidence.

B6D2F_1 mice were heavily irradiated and given parental marrow or marrow plus thymocytes. After 8 days, spleens were removed, fixed in Tellyesniczky's solution, sectioned, and stained for histologic examination. Under 300X magnification, hemopoietic colonies were counted, classified as to differentiation pathway, and measured by use of an ocular micrometer. Colonies were assumed to be ellipsoids, and for this study the largest and smallest diameters were measured. Data are expressed as the mean colony area for each group. One experiment involving 20 mice per group gave the following significantly different sizes for erythropoietic colonies: marrow only = 56 μm, marrow plus thymus = 110 μm. For granulocytic colonies, the values were: marrow only = 26 μm, marrow plus thymus = 50 μm. A second experiment, the splenic colony measurements of which are only partly completed at present, is producing the same kind of data which permit a similar conclusion.

Another approach was to measure "burst size" of erythropoietic colonies. This experiment had been attempted on earlier occasions with little success because of the difficulty of finding a dose of marrow cells whose progeny would produce enough erythropoieses within a reasonable time to be measurable by the ^{59}Fe-uptake method without, at the same time, producing an excessive (and therefore unscorable) number of splenic colonies. We succeeded in the measurements by (*a*) giving a small number of marrow cells (2.5×10^5), (*b*) giving 80 times as many thymocytes, (*c*) waiting 11 days before testing, (*d*) administering a relatively high dose of ^{59}Fe (2 μCi/mouse), and (*e*) using a 6-hr ^{59}Fe incorporation time.

The ^{59}Fe content and erythropoietic colony numbers of the spleens were counted; the ^{59}Fe incorporation per erythropoietic colony was 614 cpm for recipients of marrow only and 1476 cpm for those receiving marrow plus thymus. Surface nodules were obviously much larger on spleens from mice that had received thymus in addition to marrow. These colonies will be accurately measured when the slides have been processed. Thus we have established experimentally that our qualitative earlier evaluation was correct: thymocytes do increase the proliferative rate of transplanted marrow.

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STUDIES ON CONTROL OF THE HEMOPOIETIC STEM CELL

Joan Wright Goodman and Sarah G. Shinpock

Several laboratories in recent years have suggested that control of hemopoietic stem cells – with respect to initiation of division and to subsequent proliferation rate and differentiation pattern – depends largely on cellular interactions and not just on poietins or other modifiers of the marrow microenvironment. In several experimental situations, thymic and lymph node lymphocytes have been shown to augment growth of transplanted marrow stem cells. We are interested in the type(s) of cell responsible for the effect, and, borrowing

from the immunologists' knowledge of various subsets of thymocytes, we are investigating specific characteristics of different cell populations. Cells obtained from the unstimulated mouse peritoneal cavity are much more effective in augmenting growth of marrow cells transplanted into irradiated recipients than equivalent doses of thymic lymphocytes. We are exploring the origin of the augmenting cell(s) from the peritoneum. Results to date show that they are unaffected by anti-theta serum plus complement. We have found that in antigen-absorption studies the unstimulated peritoneal cavity of the mouse yields relatively few theta-bearing cells.

In a less-direct approach to this identification problem, we are asking experimentally whether there might also be a suppressor T cell involved in controlling either the stem cell itself or the lymphocyte–stem-cell interaction. Despite the negative answer that can be inferred from studies using thymectomized donors and/or recipients in the parent into F_1 hybrid ($P \rightarrow F_1$) poor growth combination, we have transplanted a small amount of isogenic marrow (*i.e.*, $P \rightarrow$ irradiated P) with or without P or F_1 thymocytes. After 8 days we found there was no significant difference in splenic colony number as a result of transplanted thymocytes. Another series of experiments used doubly thymus-deprived F_1's (thymectomized mice that some weeks earlier had been irradiated and given marrow from donors that had earlier also been thymectomized, irradiated, and given isogenic marrow transplants). Had a host suppressor cell been an important factor in the growth of P marrow transplanted to the "normal" irradiated F_1 hybrid, doubly thymus-deprived hosts should have permitted development of many more splenic colonies. This was not the case, and we must conclude with the evidence so far obtained that there is no suppressor T cell operating in hemopoiesis.

DISSOCIATION OF AGE-RELATED REFRACTORINESS OF PHA-INDUCED LYMPHOCYTE TRANSFORMATION AND TUMORIGENESIS

E. H. Perkins, Ching-Yuan Hung,*
J. M. Holland, and Wen-Kuang Yang

The concept that lymphocytes with specific suppressor activity function as an essential part of the regulatory mechanisms of the immune response has attracted much attention. Our recent studies demonstrate that splenic lymphocytes from pathology-free aged mice exhibit dramatically depressed responses of DNA synthesis ($[^3H]$thymidine incorporation) when stimulated with the T-cell-specific plant lectin, phytohemagglutinin (PHA). This loss of activity is thought to be related to the decrease in *in vivo* cell-mediated immune response seen with advancing age. Preliminary efforts have demonstrated that the PHA response can be restored in older adult mice, and to some extent in aged mice, by removal of a plastic-adherent cell population before culturing spleen cells with PHA. It has also been reported that separation of spleen cells unresponsive to PHA produced a population that suppressed the PHA response of normal spleen cells.

Spleen cells from virus-infected mice and animals with a variety of progressively growing tumors respond poorly to PHA. Furthermore, suppression of *in vitro* mitogen-induced lymphocyte stimulation by tumor-cell supernatants has been noted. Therefore, it was important to see if related mechanisms might account for the diminished age-related spleen cell response of $BC3F_1$ mice, where tumorigenesis is marked during senescence (neoplasia is present in 80% of our $BC3F_1$ mice at the time of death) but restricted primarily to Type A reticulum cell sarcoma.

Accordingly, we have determined if the decreased PHA activity we see in spleens of normal size from pathology-free aged mice could be related to suppressor cells arising in response to beginning tumorigenesis, but before histopathology is present. Hence, cells from both normal and pathologic old spleens have been tested for suppressor activity. We first measured the decline in PHA responsiveness of spleen cells from old animals free of pathology and from old tumor-bearing mice. It was found that activity of spleen cells from old animals free of pathology was sevenfold less when compared to young adult animals, whereas the PHA responsiveness of spleen cells from old animals with enlarged spleens due to reticulum cell sarcoma was ~100-fold less, dramatizing the marked additional depression occurring with pathology.

If the decreased PHA responsiveness is due primarily to an active suppressor cell population present in pathologic spleens then these spleen cells should exhibit regulatory activity when added to young-adult spleen cells and dramatically suppress the response of the latter to levels approaching that seen with pathologic old spleen cells. It was found that cells from normal old spleens exhibit some suppressor activity, on the average 33%. This suppressor activity (<twofold) can be contrasted to the ~ sevenfold decrease observed when the activities of spleen cells from normal young and old

animals are cultured separately and compared. Similarly, only slightly more suppressor activity (<twofold) was seen when cells from enlarged pathologic spleens from individual old animals were cultured with young adult spleen cells. This finding is in contrast to the markedly reduced activity of individual old pathologic spleens when cultured separately (~100-fold). The observed decreased activity in these mixture experiments is T-cell specific, because B-cell activity, as measured by LPS stimulation, is not suppressed.

The decreased PHA responsiveness of T cells in the spleens of aged animals can be accounted for by at least four mechanisms: (*a*) dilution of responding lymphocytes by proliferation of PHA nonresponsive cells; (*b*) absolute decrease in the numbers of responsive cells; (*c*) decreased reactivity of individual T lymphocytes resulting from an age-dependent alteration in a pathway essential to PHA responsiveness, DNA replication, or cellular proliferation; and (*d*) decreased reactivity due to increased suppressor cell activity or numbers.

While dilution of PHA-responsive cells by PHA-nonresponsive neoplastic reticulum cells offers the most acceptable explanation for the minimal activity of aged mice bearing enlarged (1–3 g) pathologic spleens when cultured separately, this fails to account for the decrease seen in spleens of normal size and free of pathology. Our earlier studies have demonstrated comparable numbers of T cells in the spleens of young and aged animals, similar sensitivity to *in vitro* cultural conditions, and equal abilities to bind PHA. The present results clearly demonstrate that neoplastic processes that lead to splenic pathology in senescing $BC3F_1$ mice do not give rise to specific suppressor cells that exert regulatory control over T-cell responsiveness. Therefore, the decrease in PHA responsiveness observed in old mice appears to be related to yet unidentified defects, perhaps at the intracellular level, involving processes essential for DNA replication and cellular proliferation. Collectively our finding can be interpreted as support for Gelfant's model of cellular aging,[1,2] described as a progressive conversion of cycling to noncycling cells with increasing age. In other words, immunocompetent T cells become blocked in either the G_1 (prior to DNA synthesis) or G_2 (following DNA synthesis) period of the cell cycle, and the capability of reversible transition to the cycling stage (cellular proliferation in response to stimuli, *i.e.*, release from G_1 or G_2 block) is lost or diminishes with chronological age.

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A LOWER INCIDENCE OF PULMONARY TUMORS IN NUDE MICE FOLLOWING INTRAVENOUS INJECTION OF VARIOUS SYNGENIC TUMORS

C. B. Skov,* J. M. Holland, and E. H. Perkins

Developing tumors, whose antigenic character makes them susceptible to immunological recognition and response, may be eliminated or their growth may be temporarily impaired by immune surveillance mechanisms. On the other hand, initiation and growth of weakly antigenic or nonantigenic tumors may be little affected or actually enhanced in their initial development by means of immunostimulation. There is evidence which suggests that the thymus-dependent branch of the immune system may be primarily responsible for both of the above aspects of the tumor-host relationship. We therefore are investigating the growth of tumors in athymic nude mice to further elucidate the role played by the thymus-dependent immune system in tumor development.

The growth of several transplantable syngenic tumors was compared in athymic nude and normal heterozygous littermates that had been back-crossed to three inbred strains, BALB/c, C57BL/6, and C3H. Five different spontaneous or induced tumors have been used. The tumors, including two sarcomas, two carcinomas, and a melanoma, were passaged subcutaneously or in culture. A constant number of individual tumor cells were injected into age-, sex-, and weight-matched syngenic nude and littermate mice. The cell dose was established in preliminary experiments to produce discrete lung colonies without confluence. The lung colony assay was used to assess tumor growth after permitting tumors to grow for 2 to 3 weeks.

Five experiments have been carried out. In each experiment, the mean number of lung colonies was lower in nude mice than in littermates or inbred controls. Littermate and inbred mice developed 1.3 to 4.4 times more colonies than did nude mice. The development of fewer tumors in the lungs of nude mice was consistent with each of the five tumors tested. However, once the tumor became established, or following subcutaneous injection, the rate of tumor growth apparently did not differ in athymic nude and normal animals. Experiments with radiolabeled tumor

cells demonstrated that the lungs of nude and littermate mice trap tumor cells equally well. A trivial health-related cause cannot account for the observed phenomenon, since all nudes were used when healthy and free from gross and microscopic evidence of wasting.

Since it appears likely that differences in initial trapping of tumor cells and cell growth rate do not account for the observed difference in colony numbers, other possible explanations must be considered. Among these, there are several which deserve attention. The resistance of nudes to systemic spread may be due to decreased cell survival as a result of diminished T-cell-dependent immunostimulation, an exaggerated B-cell response because inhibitor T cells are lacking, or both. The importance of a functional thymus in the above phenomenon is being investigated by repetition of the tumor lung colony experiments in nude mice that have been thymus reconstituted.

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AGE-RELATED CHANGES IN HELPER-SUPPRESSOR FUNCTION OF MOUSE THYMUS-DERIVED SPLENIC LYMPHOCYTES

R. L. Krogsrud* and E. H. Perkins

The immune response to many antigens has been shown to decrease dramatically with age. The deficiency cannot be completely explained by differences in the frequency of thymus-derived (T) lymphocytes or bone-marrow-derived (B) lymphocytes or macrophages, the three cell types which are known to cooperate in these reponses.[1] We have measured the ability of the T cells to participate in the immune response by making use of their "carrier-specificity" in a hapten-carrier system where dinitrophenyl (DNP) groups are the haptenic determinants while bovine gamma globulin (BGG) or human gamma globulin (HGG) serves as "carrier."[2] A hapten-specific B-cell population was generated in young mice by injecting DNP-HGG and, 2 weeks later, treating the isolated spleen cells with anti-theta serum to destroy T cells. Similarly, carrier-specific T cells were generated in both young and old mice by priming with BGG and treating the spleen cells with anti-immunoglobulin serum to kill B cells. When the resultant populations were combined, injected into lethally irradiated recipients, and challenged with DNP-BGG, the carrier-primed young T cells produced a 50-fold increase in the DNP-specific progeny of the B cells. On the other hand, the carrier-primed old T cells gave 20-fold fewer plaque-forming cells than the control level of B cells alone. As the number of T cells was reduced, they became limiting, and the point where the young response began to decrease occurred at approximately 3×10^6 spleen cells. A similar "break" point occurred at about 1×10^6 old spleen cells (however, the number of plaque-forming cells *increased* at that point). We concluded that BGG-priming induced cellular differentiation and proliferation of the T-helper population of young animals in a predictable manner. However, T cells from old animals were not only unable to enhance the activity of the hapten-primed B-cell population, but showed significant suppressor activity. One or more of several alternatives could explain this observation: (*a*) the injection of the carrier expanded both a helper and a suppressor compartment of T cells, (*b*) T-helpers were proliferating but their activity was negated by naturally occurring suppressor cells that have been reported to be present in the spleens of old animals,[3] (*c*) the suppressor compartment, rather than the helper compartment, was expanded by the injection of BGG, or (*d*) neither helper nor suppressor cells proliferated. The existence of suppressor activity demonstrated in this study must be considered when interpreting the decline in immune responses of aging mice.

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AGE-RELATED CHANGES IN SPLEEN CELL RESPONSES TO LIPOPOLYSACCHARIDE

R. L. Krogsrud*

The age-related decrease in humoral immune activity, as suggested by investigations using T-cell-dependent antigens, may be due to a decreased ability of bone-marrow-derived (B) lymphocytes to proliferate. In order to test this possibility directly, we have made use of a B-cell-specific mitogen LPS (*E. coli* lipopolysaccharide) to "artificially" stimulate the B cells. Results showed that the optimum concentration of LPS was the same for both young and old spleen cells, but that there was a twofold difference between the levels of DNA

synthesis as measured by [^{3}H]Tdr incorporation. In contrast, cells from old bone marrow showed no difference from young cells. Our previous investigations using an anti-mouse immunoglobulin reagent revealed that the proportion of B cells in the spleen does not change significantly with age. Therefore, the decreased response seen in the old spleens could be due to either decreased numbers of responsive B cells or fewer divisions per cell. Indeed, Price and Makinodan[1] found a 3.5-fold decrease in the frequency of antigen-stimulable B cells in old mice, but calculations from the same studies[2] show a tenfold decrease in the number of Ab-producing progeny generated from one immunocompetent unit (*i.e.*, the minimum configuration of T, B, or accessory cells which, upon antigen stimulation, generates Ab-producing cells). Although both possibilities may be correct, the present investigation suggests that B cells are not the primary or limiting cell type responsible for the immunological defect seen in old mice. A third explanation, under investigation, involves the existence of regulatory T cells which may exert greater suppressive effects with age.

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FAILURE TO IMPLICATE IMMUNOSUPPRESSION AS A SIGNIFICANT INDUCTIVE MECHANISM OF RADIATION LEUKEMOGENESIS IN THE RFM MOUSE

E. H. Perkins and Lucia H. Cacheiro

If the risk of developing neoplastic disease following irradiation is to be successfully predicted, the relationship of immunosuppression to the early inductive events must be clearly identified, *i.e.*, is immunosuppression a critical obligatory step in the pathogenesis of radiation-induced cancer? Induction of thymic lymphomas in the RFM mouse provides a realistic, readily testable model; when female RFM mice receive a single acute exposure of 300 R at 6 weeks of age, over 70% of the animals die within the first year of thymic lymphoma. If depressed immunocompetence is a significant parameter in the induction processes, immunological reconstitution could reduce both the incidence of thymic lymphoma and the early life-shortening seen in irradiated animals. It was thought that different methods of restoring immunocompetence could yield insight as to the importance and time relationship of this procedure. Three methods for restoring immunocompetence have been used: (*a*) injection of 50×10^6 syngenic spleen cells, (*b*) injection of 50×10^6 syngenic bone marrow cells, and (*c*) shielding of the lower $^1/_3$ of the body. It was thought that spleen cells might be more effective in restoring immunocompetence and reducing the incidence of thymic lymphomas in RFM mice if immediate immunological restoration is of significance in interrupting the inductive processes, for the unequal effects seen following injection of allogenic spleen or bone marrow cells in induction of GVH reaction clearly delineate an immediate acute effect (<30 days) for spleen cells in contrast to bone marrow cells which produce late effects (>90 days). Shielding the lower third of the body of the RFM mouse should permit immediate repopulation of the important lymphoid organs by immunocompetent stem cells from bone marrow and might reverse lymphoma induction.

Significant differences in mortality have been observed using the three methods of immunological reconstitution. No difference (time of onset, rate of increase) was seen in the mortality curves or the final level of mortality between RFM mice that received 300 R only and animals that were irradiated and reconstituted with 50×10^6 syngenic spleen cells (67 vs 60%). In contrast, mortality was only 7% when irradiated mice were reconstituted with 50×10^6 syngenic bone marrow cells and only 23% when the lower $^1/_3$ of the body was shielded. Immunocompetence measured by the direct plaque-forming cell response following sheep RBC injection, which reflects a cooperation of both T and B lymphocytes at 3 days, 18 days, 1 month, and 7 weeks after irradiation and reconstitution, yielded negative rather than positive correlations. Whereas reconstitution with bone marrow almost completely reversed the irradiation induction of thymic lymphoma, there was no significant difference in the immunologic recovery of the bone-marrow-reconstituted animals and animals that received only irradiation at the time periods tested (0.3, 4.0, 41, and 76% of nonirradiated control values vs 0.3, 4.0, 27, and 78%). Although spleen cells fail to protect against the irradiation induction of thymic lymphomas, immunologic recovery was more rapid among these animals (2.3, 7.3, 86, and 97%) than in bone-marrow-reconstituted animals, whereas shielded animals yielded the most rapid recovery of immunocompetence (4.5, 25.4, 100, and 135%). The same pattern of immune recovery was seen among the experimental groups when immunocompetence was assessed by resistance (30-day cumulative

mortality) to intraperitoneal P815 mastocytoma challenge. These findings suggest that immunosuppression is not a critical obligatory requirement in the pathogenesis of radiation-induced thymic lymphomas of RFM mice.

RECOVERY OF IMMUNE COMPETENCE FOLLOWING SUBLETHAL IRRADIATION: THE ROLE OF THYMIC FUNCTION AND AGING

W. J. Peterson

Synergism between lymphocytes is a basic requirement for full immunologic expression against most antigens. Thus thymus-derived T cells interact with bone-marrow-derived B cells in the production of the immune response. Recent studies have shown that lymphocytes can be further divided into additional subsets depending on their origin and function. In general, it has been shown that lymphocytes are highly radiosensitive; however, other cell types have shown a disparity in their sensitivity to radiation. In view of these observations, studies were undertaken to determine whether B cells and T cells are equally radiosensitive and, more importantly, which one is the rate-limiting cell type in the recovery of the immune response following X irradiation.

Young adult (4-month-old) C57BL/6J male mice received a single sublethal dose of X rays, and at various times thereafter, their spleen cells were tested for recovery of their capacity to synthesize antibody and to respond to the T- and B-cell-specific mitogens, phytohemagglutinin (PHA), and bacterial LPS.

The results of these studies show that the recovery of young adult mice from 250 R and 500 R follows an orderly sequence of events, *i.e.*, recovery of the B-cell compartment precedes that of the T-cell compartment, and recovery of both cell types precedes the recovery of the antibody response. Full recovery of B cells was seen at 1 week after 250 R and at 2 weeks after 500 R. On the other hand, full recovery of T cells was seen at 3 and 4 weeks after 250 R and 500 R respectively. In regard to antibody production, full recovery was noted at 8 weeks after 250 R and 14 weeks after 500 R. Thus, recovery of T cells from radiation appears to be a rate-limiting step in the full recovery of the immune response.

The essential role of the thymus (the T-cell-producing organ) in immune competence has been demonstrated in studies where its removal in early life reduces immunologic activity. Involution of this organ is a natural consequence of aging. Therefore, the ability of mice to recover from radiation would seem to be age-related. Studies now in progress show that when 27-month-old C57BL/6J mice are irradiated, recovery of the T-cell compartment to the preirradiation level is seen following 250 R, but only minimal recovery is seen after 500 R. These results are similar to those obtained using thymectomized young mice and suggest that the lack of recovery of immune competence is due to a failure in thymic function. On this basis it would seem that any insult that reduces the number of immunocompetent cells in animals living beyond the period of thymic involution could subject a large number of the population to an increased incidence of malignant, infectious, and autoimmune diseases due to the limited ability of these individuals to recover their full immune potential.

A MICROTECHNIQUE FOR MITOGENIC STUDIES OF MOUSE LYMPHOID TISSUES

C. F. Gottlieb, G. M. Peterman, and W. J. Peterson

We have developed a microsystem for evaluating mitogenic indices in mice. A survey of the literature provided descriptions of numerous techniques, all of which had one or more shortcomings, such as a large lymphoid cell requirement, only one mitogen tested, no evaluation of the peak time of harvest, or no evaluation of the length or amount of pulse with labeled nucleoside. We undertook adaptation of a microsystem which can be used with mouse lymphoid tissue (spleen, lymph node, or peripheral blood leukocytes) and which can be successfully performed on a small number of cells, allowing nondestructive evaluation of the responses of individual mice if peripheral blood is used.

From 10^5 to 10^6 mouse lymphoid cells are cultured in 0.2 ml in Falcon Microtest II plates (flat-bottom polystyrene tissue-culture plates containing 96 wells, each well 6.5 mm in diameter). Outside this range in cells per culture, the dose-response curve is nonlinear — the culturing efficiency declining rapidly. A slight response is usually observed with as few as 10^4 cells, and numbers in excess of 10^6 cells per culture seem to saturate the system. Linbro V-bottom and flat-bottom culture plates were also tested. The V-bottom plates gave significant but inconsistent responses with 10^4 or fewer cells and generally no response at 10^5 or greater cells per culture. Linbro flat-bottom plates gave responses basically like the Falcon plates, except the magnitude was always about 80% of that of the same cells cultured in Falcon plates. Culturing conditions are HEPES-buffered RPMI 1640 medium with glutamine,

supplemented with 8% approved fetal calf serum and antibiotics (50 USP units penicillin and 50 μg streptomycin per ml), with incubation at 37°C in a humidified 5% CO_2 atmosphere. The pH was varied from 6.7 to 7.9 by varying the concentration of CO_2 in the atmosphere, and pH 7.3 (5% CO_2) was found to be the center of a broad plateau. The mitogen is added to each culture in 0.01 ml; optimum amounts are: phytohemagglutinin (PHA), 0.25 and 1.25 μg per culture for spleen and peripheral blood respectively; concanavalin A (Con A), 0.60 μg per culture; and *E. coli* 055:B5 lipopolysaccharide, 1.25 μg and about 5 μg per culture for spleen and peripheral blood leukocytes. The higher optimum concentrations for peripheral blood leukocytes may be accounted for by the presence of residual erythrocytes, surviving a brief lypotonic shock treatment of whole-blood samples. To measure the DNA synthetic response, a pulse of tritiated thymidine (initial concentration 10^{-6} *M*, specific activity 5 Ci/mmole) is added the last 24 hr of culture. Tritiated thymidine incorporation was highly dependent on the concentration of thymidine for concentrations less than 10^{-6} *M*, while increasing the concentration above 10^{-6} *M* made little difference in the measured response. The response was directly proportional to the length of the pulse, with 30 hr the longest pulse tested. The 24-hr pulse was chosen for convenience and to maximize the measured response of a small number of cells. The optimum time of harvest with all mitogens is after 48 hr of culture, although because the responses to PHA and Con A are sustained, the magnitude of those responses at 72 hr is similar. The response to LPS declines after 48 hr, as do the PHA and Con A responses after 72 hr. Several mouse strains have been tested, including BALB/c, C57BL/6, and C3H. Variation among these strains approaches tenfold in some cases, with the BALB/c tending to be the best responder.

DIRECT AND INDIRECT EFFECTS OF FAST NEUTRONS OR X RAYS ON MOUSE EMBRYOS

Wallace Friedberg,* G. D. Hanneman,* E. B. Darden, Jr.,† D. N. Faulkner,* and W. R. Kirkham*

A major goal of this study is to obtain risk estimates of lethal injury to preimplantation embryos for radiation levels below those at which an increase in mortality can be detected experimentally. In initial experiments on two-cell embryos irradiated *in utero* with fast neutrons, we examined the conceptuses grossly on gestation day 16. Our working hypothesis was that lethal injury and subsequent prenatal death resulted from passage of a proton through a sensitive region in each of the two blastomeres. The first data obtained supported this two-hit killing mechanism; however, subsequent experiments at higher radiation doses revealed inconsistencies. More embryos died than had been predicted on the basis of results at the lower doses, and also a greater proportion of the deaths occurred at later stages of gestation. The latter finding suggested radiation injury of the mother as the cause of the unpredicted increase in mortality, since it seemed unlikely that an increase in radiation dose would result in a greater proportion of later deaths as a direct effect of radiation.

To test the indirect lethal-injury concept, we irradiated two-cell embryos *in utero* with an absorbed dose of 500 rads of X rays and examined stained tissue sections of implantation sites on gestation days 7.5 and 11.5. In a previous experiment we found that 95% of the embryos given this dose at the two-cell stage were dead when examined on gestation day 16. The 7.5-day implantation sites were mainly of two general types: one type contained a normal-appearing embryo, and the other had no identifiable embryonic tissue. Both showed a decidual reaction. On day 11.5, the implantation sites were also mainly of two types. In one type a decidual reaction but no embryonic tissue was seen. We are tentatively assuming that the absent embryos of both these and the 7.5-day implantation sites died at implantation, or within a few days thereafter, from direct effects of radiation. The second type of 11.5-day implantation sites had embryos and placental tissues that showed varying degrees of degenerative changes. All the embryos had developed at least a day or more beyond gestation day 7.5. A characteristic finding was hemorrhage of maternal blood in the uterine lumen.

The results seem to be in accord with the view that at the higher doses of radiation, some embryos that would otherwise have survived, died some time after gestation day 7.5 because of radiation injury of the mother. To avoid such indirect effects, we are routinely examining implantation sites on gestation day 7.5. Results to date are consistent with the two-event, direct-effect model of our working hypotheses.

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ALTERED SERUM ESTERASE AND PROTEIN PROFILES IN CYCLIC NEUTROPENIC DOGS

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Esterase analysis of dog serum was performed by gradient acrylamide gel electrophoresis, and protein profiles were determined by cellulose acetate electrophoresis. Cyclic esterase activity was detected in sera of adult and prepubertal dogs suffering from cyclic neutropenia. Activity of two serum esterase bands was correlated with the level of peripheral neutrophils. One band of esterase activity was maximal prior to and during the increase in number of peripheral neutrophils, whereas activity of a second esterase band was maximal when numbers of peripheral neutrophils were maximal. Activity of both these esterase bands was higher in normal dogs than in dogs with cyclic neutropenia and did not appreciably vary with time. Serum from dogs with cyclic neutropenia had increased levels of alpha 2 protein which varied appreciably with time but apparently did not correlate with the peripheral neutrophil count. Since esterase changes occur prior to the precipitous diminution of circulating neutrophils, analysis of mammalian plasma esterases after total-body irradiation might be a useful physiologic indicator of radiation dose or impending marrow dysfunction.

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EFFECTS OF BUTYLATED HYDROXYTOLUENE, DIETHYLNITROSAMINE, AND X RAYS DURING TUMORIGENESIS IN MICE

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Kowetha A. Davidson,† C. F. Gottlieb, and
R. W. Tennant

A major problem in environmental carcinogenesis is whether various environmental agents act additively, synergistically, or possibly antagonistically. Cumming and Walton[1] reported that butylated hydroxytoluene (BHT), an antioxidant widely used as a food preservative, decreased the acute toxicity of the potent carcinogen diethylnitrosamine (DEN), but potentiated the acute lethal effects of ionizing radiation. In an earlier experiment,[2] we attempted to determine whether BHT would also protect against the carcinogenic action of DEN. We found a suggestion of potentiation by BHT rather than a protective effect.

We have now expanded this preliminary investigation to determine whether BHT and DEN show an interaction; whether BHT interacts with ionizing radiation; whether the two known carcinogens, DEN and radiation, interact; and what the effects of each agent are individually. Male and female BALB/c mice are being used. Doses of the agents are (*a*) 0.75% BHT in the feed throughout life, (*b*) DEN in the drinking water for 7 weeks for an average cumulative dose of 300 mg/kg, and (*c*) 250 rads of X rays. Suitable control groups are also maintained. The mice will be killed at either 12 or 18 months for pathological examination. In addition to obtaining pathology data (Clapp), we are making serial observations in individual mice on plasma esterase changes (Tyndall), metabolic fate of the chemicals (Daugherty), hormonal dependence and source of esterases (Davidson), immune status (Gottlieb), and viral activation (Tennant).

Some of our preliminary results are reported in companion papers. We have observed: (*a*) interactions of BHT upon DEN carcinogenesis, most of which appear beneficial and are sex dependent; (*b*) esterase changes that are detectable after 1 week of treatment and that vary with different compounds (these alterations are most pronounced in females); (*c*) tumor-susceptible organs that have different metabolic patterns within the same mouse as do the various subcellular components; (*d*) virus 6–8 months after ionizing radiation which may be associated with a reduced viral antibody titer during the leukemogenic period; (*e*) no dramatic changes in immune status during the later stages (12 and 18 months) when mice are developing tumors. Some of the early pathological data have been completed and are now being analyzed for further correlations.

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†Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

1. R. B. Cumming and M. F. Walton, *Food Cosmet. Toxicol.* **11**, 547–53 (1973).
2. N. K. Clapp, R. L. Tyndall, R. B. Cumming, and J. A. Otten, *Food Cosmet. Toxicol.* **12**, 367–71 (1974).

EFFECT OF BUTYLATED HYDROXYTOLUENE ON DIETHYLNITROSAMINE CARCINOGENESIS IN BALB/C MICE

N. K. Clapp,* R. L. Tyndall,*
Lou C. Satterfield, and W. C. Klima

A preliminary experiment had suggested a potentiation of the carcinogenic effect of DEN by a concurrent

Table 27. Sex-related carcinogenic differences in stomach tumors after DEN administration

	Male	Female
BHT protection against DEN effects	0	+
Reduces number of tumor-positive mice		
Lower degree of malignancy in squamous cell carcinomas	+	0
DEN tumorigenesis (incidence)	++++	+++
BHT potentiation of DEN effects		
Greater degree of tumor malignancy	+	++

treatment with BHT.[1] We expanded the investigation to include larger numbers of mice of both sexes, with the same killing times of 12 and 18 months of age; these times correlated with the first deaths and with a time when a majority of the mice were sick or moribund respectively. Mice received either (*a*) 0.75% BHT in the feed throughout life, (*b*) DEN in the drinking water for 7 weeks (mean cumulative dose of ~330 mg per kilogram of body weight), (*c*) DEN plus BHT, or (*d*) no carcinogen (controls). Other studies are being conducted to determine enzyme changes and alterations in immune competency in these mice.

The pathology data from mice killed at 12 months are completed and are being analyzed. Table 27 demonstrates the complexity of BHT-DEN interactions as they are affected by sex. The mechanism whereby BHT modifies the DEN effect on the forestomach is not clear at this time; however, DEN carcinogenesis is significantly affected by hormonal environment, as is the BHT modification of the DEN process. The 18-month mice have been killed, and the histological evaluation is now being completed. The pathology data and the results from the other investigations will then be correlated for inferences regarding mechanism of action.

*Consultant.

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EFFECT OF WHOLE-BODY X RADIATION UPON BUTYLATED HYDROXYTOLUENE OR DIETHYLNITROSAMINE TREATMENT IN MICE

N. K. Clapp,* Lou C. Satterfield, and W. C. Klima

The effect of BHT upon radiation lethality has been reported to vary with strain. Cumming and Walton[1] reported a potentiation in C3HF_1 male mice, and we have observed a slight protection in BALB/c males and females.[2] The interactions of BHT upon DEN carcinogenesis[3] led us to investigate a further concern – the effect that a leukemogenic dose of X rays might have on treatment by BHT (a free-radical scavenger and an enzyme inducer) or DEN (a known carcinogen which presumably requires intracellular enzyme activation before it is effective within the organism).

Table 28. Incidences of tumors in BALB/c mice after DEN, BHT, and 250 rads of X rays, alone or in combination[a]

Treatment group	Number of mice	Tumor incidence (%)		
		Liver	Stomach	Lung
Females				
Control	37	0	0	3
BHT	50	0	0	4
BHT + X rays	48	0	0	25
X rays	47	0	0	25
DEN	49	0	31	77
X rays + DEN	43	0	53	72
BHT + DEN	46	0	26	54
Males				
Control	50	0	0	20
BHT	50	0	0	6
BHT + X rays	49	0	0	16
X rays	48	0	0	19
DEN	47	0	34	36
X rays + DEN	43	0	60	70
BHT + DEN	45	0	49	67

[a]Killed at 12 months; gross necropsy only.

Male and female BALB/c mice were given either (*a*) 0.75% BHT throughout life, (*b*) DEN (330 mg per kilogram of body weight) for 7 weeks in drinking water, (*c*) 250 R of X rays at 11 weeks of age, or (*d*) X rays plus DEN or BHT. Appropriate controls were maintained. Mice were killed at 12 or 18 months for comparison with previous BHT-DEN studies. Histological examination is not complete, but the preliminary information based on gross necropsy suggests modifications in both sexes (Table 28). Radiation plus DEN appears to cause higher stomach tumor incidences than DEN alone or BHT plus DEN. For lung tumors, radiation alone increases the incidence in females but not in males. Radiation plus DEN increases the incidences in males but not in females. There was no difference in mortality between the treatment groups at 12 months. Information regarding changes in esterases is now being evaluated, as are the pathology data at 18 months.

*Consultant.

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2. N. K. Clapp and L.C. Satterfield, *Biol. Div. Annu. Prog. Rep. June 30, 1974*, ORNL-4993, pp. 151–52.
3. N. K. Clapp, R. L. Tyndall, L. C. Satterfield, and W. C. Klima, this report.

ENZYME ANALYSIS OF PLASMA FROM CARCINOGEN- AND NONCARCINOGEN-TREATED MICE

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Esterase profiles of plasma from BALB/c mice treated with a variety of carcinogenic and noncarcinogenic chemicals were analyzed. Plasma levels of gamma glutamyl transpeptidase and glutamic-oxalacetic transaminase were also determined. Esterase analysis was carried out by gradient acrylamide gel electrophoresis, while levels of the other plasma enzymes were determined with the mini-GeMSAEC system. Mice exposed to the carcinogens diethylnitrosamine, dipropylnitrosamine, dinitrosopiperazine, dimethylhydrazine, dimethyldinitrosopiperazine, and urethane had identically altered plasma esterase profiles after 7 days' exposure to the chemicals. Exposure to the noncarcinogens nitrosohydroxyproline, nitroso-2,6-dimethylpiperidine, nitrosomethoxymethylamine, 1-nitroso-4-methylpiperazine, and ethyl methanesulfonate caused no obvious plasma esterase alterations. Ingestion of carbon tetrachloride resulted in a plasma esterase alteration different from that seen in carcinogen-treated mice. Levels of the plasma enzymes gamma glutamyl transpeptidase and glutamic oxalacetic transaminase did not distinguish the carcinogen-treated from the carbon-tetrachloride-treated mice. Thus, within this group of 12 chemicals, esterase analysis of mouse plasma distinguished the carcinogens from the noncarcinogens 1 week after beginning treatment. We are currently testing a variety of additional carcinogenic and noncarcinogenic chemicals to determine the efficacy of plasma esterase analysis as a potential screen for environmental carcinogens.

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PLASMA ESTERASE ALTERATIONS IN MICE FED CARCINOGENS AND/OR THE FOOD ADDITIVE BUTYLATED HYDROXYTOLUENE

R. L. Tyndall* and N. K. Clapp*

We undertook electrophoretic analysis of plasma esterases in mice fed DEN and BHT, singly or in combination, to detect their early physiologic effects. One group of BALB/c mice was continuously fed chow containing 0.75% BHT. A second was fed BHT and given DEN (total dose ~330 mg per kilogram of body weight) in drinking water for 7 weeks. A third group was given DEN alone, and a fourth was untreated. Mice were necropsied after 10, 20, and 48 weeks.

Plasma esterase changes were apparent in BALB/c mice after feeding them laboratory chow with 0.75% of the antioxidant BHT added. Other esterase changes, different from those in BHT-treated animals, were also apparent in plasma of mice during exposure to the carcinogen DEN. Interference with these DEN esterase alterations was apparent in plasma of mice treated concomitantly with both DEN and BHT. Esterase changes resulting from either the carcinogen or antioxidant exposure precede the overt histologically detected changes induced by these compounds. The early esterase changes and subsequent tumorigenesis resulting from DEN exposure were more severe in female mice. Some of the esterases altered by DEN or BHT exposure were testosterone-related.

The interference of BHT feeding on DEN-induced esterase changes was reflected in the subsequent alteration in type and severity of tumors in mice fed both DEN and BHT as compared with mice fed only DEN. We are currently analyzing esterase alterations in mice exposed to total-body irradiation with or without concomitant BHT treatment to determine whether esterase changes are predictive of pathologic sequelae in such mice.

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ALTERATIONS IN ISOENZYME PATTERNS FOLLOWING TREATMENT WITH CARCINOGENIC AGENTS

J. P. Daugherty,* N. K. Clapp,† and R. L. Tyndall†

Numerous studies have shown that many classes of enzymes in plasma and tissues are changed during

different pathological processes. Plasma esterases were altered when BALB/c mice were given DEN, BHT, or a combination of both.[1] To determine if such alterations are common among different groups of enzymes, we are investigating the electrophoretic mobility of several enzymes under different conditions. Tumorous and neighboring nontumorous tissues (liver, lung, stomach, ovary) and plasma have been obtained from mice treated with BHT, DEN, and X rays, alone or in combination, and will be analyzed for changes in different enzymes by polyacrylamide gel electrophoresis. We are screening several enzymes, including acid phosphatase, alkaline phosphatase, esterases, glucose 6-phosphate dehydrogenase, lactic dehydrogenase, phosphoglucomutase, aldolase, and β-glucuronidase, for alterations and correlations with known carcinogenic activity of treatment.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee.

†Consultant.

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CHARACTERIZATION OF NONSPECIFIC ESTERASES IN MOUSE SERUM

Kowetha A. Davidson,* R. L. Tyndall,*
and N. K. Clapp†

Mouse serum and tissues contain numerous nonspecific esterase enzymes, many of which are altered under different physiological and pathological conditions of the animal. At least 18 plasma esterases have been demonstrated by polyacrylamide gel electrophoresis, eight of which are quantitatively altered in mice fed a chemical carcinogen, DEN.[1] The tissue sources of these esterases are yet unknown, and their identification will depend in part on characterization of serum esterases. We have established parameters by which these enzymes may be characterized by polyacrylamide gel electrophoresis so that the tissue source(s), as well as any qualitative differences in plasma esterases induced by carcinogens, may be identified. These parameters include enhancement or inhibition of esterase activity by cations, sensitivity to enzyme inhibitors, substrate preference, and heat stability.

Divalent cations such as Ca^{2+}, Mg^{2+}, and Mn^{2+} enhanced the activity in five esterases, whereas Hg^{2+}, Cd^{2+}, Cu^{2+}, and Zn^{2+} had essentially no effect on esterase activity. The trivalent cation La^{3+} enhanced activity in one enzyme and inhibited activity in the remainder. An aliesterase esterase inhibitor, eserine, showed some degree of inhibition in all but three esterase bands; only one band was completely inhibited. Manganese in the presence of eserine abolished the inhibitory effect of eserine on three esterases and stimulated or enhanced effect on two enzymes.

Studies to analyze substrate preference revealed that α-naphthyl butyrate showed a lesser preference in nine enzymes and a greater preference in 2. Napthol AS-D acetate and AS-D chloroacetate revealed a very low preference, and only 11 of the 18 enzymes were demonstrated with the former and 6 with the latter.

Thirteen esterases appeared to contain very small amounts of lipase activity when assayed with α-naphthyl caprylate in the presence of sodium cholate; α-naphthyl laurate and myristate were not hydrolyzed. Heat-stability tests showed marked variations in the sensitivity of various bands depending in part on whether the serum was heated prior to or after electrophoresis.

Plasma from mice fed DEN was analyzed using the parameters described above. All esterases had characteristics identical to those in control plasma with the exception of one, band 10. This esterase activity in normal (control) mouse plasma was enhanced by Mn^{2+} and resistant to eserine; however, in plasma from DEN-treated mice it was not affected by Mn^{2+} and was sensitive to inhibition by eserine. Band 10 is also one of the enzymes which shows a quantitative increase during DEN treatment. These results reveal that it is also qualitatively different.

Experiments are now planned to: (*a*) examine plasma from mice treated with other carcinogens to determine if band 10 is qualitatively different, (*b*) measure the level of circulating testosterone to determine if the quantitative alteration in testosterone-dependent esterases may be mediated by an effect of the carcinogen on testosterone production, and (*c*) identify the tissue source(s) of these esterases.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

†Consultant.

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STUDY OF IMMUNE COMPETENCE IN MICE TREATED WITH THE POTENT CARCINOGEN, DIETHYLNITROSAMINE, AND/OR BUTYLATED HYDROXYTOLUENE

C. F. Gottlieb, G. M. Peterman, and N. K. Clapp*

The involvement of immunosuppression in carcinogenesis has been suggested as a partial explanation for the carcinogenicity of ionizing radiation. If immune changes are consistently associated with oncogenesis, the immune depression would be expected to occur with other carcinogenic agents besides radiation. Within this context, we have been evaluating immunocompetence of BALB/c mice which have been treated with DEN, either alone or in conjunction with BHT, a food additive suspected of potentiating the action of DEN. A concentration of 0.75% BHT was administered in the food for life starting at 8 weeks of age; a 7-week DEN treatment was given orally in the drinking water, beginning 3 weeks after initiation of BHT, with cumulative doses of approximately 330 mg per kilogram of body weight. At sacrifice, part of the spleen was taken for two *in vitro* assays – (*a*) mitogenesis by PHA and *Escherichia coli* LPS and (*b*) humoral antibody response to sheep erythrocytes.[1] PHA stimulation provides an estimate of the activity level of one subpopulation of thymus-derived or T cells, and LPS stimulation reflects one functional measure of bone-marrow-derived or B cells; the Mishell and Dutton assay requires interaction of both T and B cells to produce an antibody response, thus giving a functional measure of the immune capability of both cell compartments, individually and in combination. Although the results are not yet complete, no dramatic changes were observed in the immune competence of DEN-treated mice compared with BHT-treated mice or untreated controls, at either 12 or 18 months after treatment. Preliminary analysis suggests a slight increase in response with the Mishell and Dutton assay in DEN-treated mice, as well as a slightly reduced PHA response in those mice with lung tumors. Further analysis of the data is being made, and correlations are being attempted with tumor types and organ sites.

*Consultant.

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MERITS OF HISTOLOGICAL CONFIRMATION OF GROSS NECROPSY DATA

N. K. Clapp,* Lou C. Satterfield, and W. C. Klima

Confirmation of pathological necropsy material by histological examination has obvious merit but has often been restricted by lack of qualified pathology personnel. Consequently, a routine and complete histology may not be performed in some experiments that have pathological end points. In an experiment involving several hundred mice, we analyzed the results both with and without histological examinations.

In Table 29 incidences of forestomach and lung tumors after DEN and/or BHT in BALB/c mice of both sexes are compared. Gross interpretion of early changes

Table 29. Comparison of results by gross necropsy only with results after histological evaluation as affecting tumor incidences in BALB/c mice after BHT and/or DEN

Treatment group[a]	Tumor incidence (%)			
	Stomach		Lung	
	Gross	Histological	Gross	Histological
Males				
Control	0	0	20	20.0
BHT	0	0	6	14.0
BHT + DEN	49	91	67	68.9
DEN	34	93	36	61.7
Females				
Control	0	0	3	10.8
BHT	0	0	4	6
BHT + DEN	26	70	54	84.8
DEN	31	83	77	91.8

[a]Mice killed at 12 months of age.

in the squamous lining of the forestomach is difficult; thus the anticipated increase in tumor incidence with histological confirmation was not surprising. Pulmonary tumors were recorded grossly after inflation of the lungs and were then cleared and subsequently examined under a dissecting microscope. Histological sections were made frequently to differentiate between tumorous and nontumorous nodules. With gross observation, tumors ≥0.5 mm can be detected, while with the dissecting scope we can detect nodules ≥0.1 mm. Our observations show that the data from gross necropsy alone are valuable, but some conclusions would have been different without histological examinations. These results reinforce the desirability of confirming pathological changes by histological observation, and guarding interpretations which do not include histology.

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KINETICS OF APPEARANCE OF COVALENTLY BOUND METHYL LABEL (^{14}C) IN DNA AND RNA OF MOUSE LIVER AND LUNG FOLLOWING A SINGLE INJECTION OF [^{14}C]DIMETHYLNITROSAMINE

J. P. Daugherty* and N. K. Clapp†

The reactions of dimethylnitrosamine (DMN) with cellular nucleic acids are of interest in determining the possible cellular targets that are responsible for the observed organotropism of DMN and DEN.[1] The differences in the reactions with DNA and RNA may help define the cellular reactions which are significant in the carcinogenic process. The experimental protocol is described in an accompanying report.[2]

The greatest amount of methyl label, in both lung and liver, was associated with the RNA components of the cytosol fraction, the tRNA. In the case of the liver cytosol fraction, the radioactivity bound to RNA increased rapidly during the first 2 hr, reached a maximum at 8 hr, and declined thereafter. The distribution of radioactivity in the cytosol fraction of the lung increased rapidly through 4 hr and then declined, the level at 16 hr being equal to the level at 15 min. The radioactivity in the nuclear, mitochondrial-lysosomal, ribosomal, and membrane-microsomal subcellular fractions reached maxima at 16, 4, 4, and 8 hr, respectively, for the liver, and at 8, 4, 0 (no accumulation), and 4 hr, respectively, for the lung. The DNA components of the various subcellular fractions of both liver and lung did not incorporate appreciable radioactivity.

DMN is rapidly metabolized within the body and shows remarkable affinity for tRNA in two organs susceptible to DMN's carcinogenic activity, but has little affinity for DNA or for non-tumor-susceptible tissues. The significance of these observations to the carcinogenic process is uncertain at this time, but is being explored experimentally. In addition, each of the subcellular fractions is being analyzed for radioactivity associated with lipid and protein components, and analysis of the methylated bases of tRNA fractions is in progress.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

†Consultant.

1. N. K. Clapp, R. L. Tyndall, and J. A. Otten, *Cancer Res.* 31, 196–98 (1971).

2. J. P. Daugherty and N. K. Clapp, this report.

SUBCELLULAR DISTRIBUTION OF RADIOACTIVITY IN THE ACID-SOLUBLE AND -INSOLUBLE COMPONENTS OF MOUSE LIVER AND LUNG FOLLOWING A SINGLE INJECTION OF [^{14}C]DIMETHYLNITROSAMINE

J. P. Daugherty* and N. K. Clapp†

A large amount of literature has accumulated on the diversity of the toxic, mutagenic, and carcinogenic activity of numerous nitrosamine compounds. The incidence of primary tumors following DMN and DEN administration in two strains of mice (BALB/c and RF) has been described by Clapp *et al.*[1] Differences were noted between DMN and DEN tumor induction within strains and between strains for each carcinogen. The differences in tumor types suggest that differences may exist at the cellular level in the affinity, transport, and/or metabolism of these compounds.

The present study was undertaken to investigate the distribution of DMN in various tissues of RF mice. It is hoped that these data will provide information to elucidate the observed differences in cellular and organ susceptibility between BALB/c and RF mice and ultimately between DMN and DEN.

Radioactive DMN was administered (10 mg per kilogram of body weight) by gastric intubation to three replicates of 8-week-old male RF mice. The animals were killed at timed intervals (15 min–24 hr), and tissues were removed. The tissues were homogenized

and fractionated by differential centrifugation into five subcellular fractions: nuclear, mitochondrial-lysosomal, ribosomal, membrane-microsomal, and cytosol. Each subcellular fraction was incubated in trichloroacetic acid and filtered to separate the acid-soluble and -insoluble components; the radioactivity of each component was determined and expressed as cpm/mg of DNA in the homogenate.

Radioactivity was detected in the tumor-susceptible tissues (liver and lung) following the administration of DMN, while radioactivity was near background in non-tumor-susceptible tissues (heart). The acid-soluble components of the lung incorporated about six times as much radioactivity as the liver at 15 min after administration; at 16 hr the radioactivity in both tissues had decreased and was approximately equal. The acid-insoluble components of both tissues incorporated much less radioactivity than the acid-soluble components, indicating that only a small amount of methyl label was covalently bound to cellular macromolecules. The results suggest that differences do exist in the metabolic fate of DMN in liver and lung tissues. A similar study is under way using ^{14}C-DEN and including BALB/c mice.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

†Consultant.

1. N. K. Clapp, R. L. Tyndall, and J. A. Otten, *Cancer Res.* **31**, 196–98 (1971).

EFFECT OF NEUTRON IRRADIATION ON THE INCIDENCE OF VARIOUS NEOPLASTIC DISEASES

R. L. Ullrich, J. B. Storer, and M. C. Jernigan

Our studies on the late effects of neutrons in female RFM and BALB/c mice are sufficiently complete to allow analysis of tumor incidences in the RFM groups which received 0–50 rads of fission neutrons. Details of the experimental protocol, as well as the effect of these doses on life-span, have been reported previously.[1]

Table 30 indicates the age-adjusted incidences of leukemias and solid tumors in the various treatment groups. An increased incidence of thymic lymphoma over controls was observed at all doses except 5 rads. When these dose-incidence data for the induction of thymic lymphoma are compared with those of similarly maintained RFM females exposed to X irradiation (J.B.S., unpublished data), an RBE of approximately 3 is obtained. Although there was some fluctuation in incidences of reticulum cell sarcoma and nonthymic lymphoma among groups, no radiation-induced increase in incidence was apparent. This is similar to results obtained after X-ray exposure.

Solid tumors were pooled into three categories: (*a*) endocrine tumors, (*b*) lung adenomas, and (*c*) other tumors, because of sample sizes. The increased incidence of endocrine tumors is primarily a reflection of ovarian tumors, although slight increases in pituitary and mammary tumors were observed. The most interesting observation was the apparent increased incidence of lung adenomas at 50 rads. To our knowledge, such an increase has not been reported previously using either neutrons or X rays except after relatively high doses localized to the thorax.

As a result of these data indicating that neutrons may be significantly more effective in the induction of lung adenomas than low-LET radiation, we have initiated a more detailed, mechanistic examination of the response of the lung to neutrons and X rays. Results of these studies are too preliminary to report at this time.

1. J. B. Storer, G. E. Cosgrove, E. B. Darden, Jr., L. J. Serrano, M. C. Jernigan, W. C. Klima, L. C. Satterfield, H. C. Swann, and F. S. Martin, *Biol. Div. Annu. Prog. Rep. June 30, 1974*, ORNL-4993, pp. 149–50.

Table 30. Age-adjusted incidences of neoplastic diseases in RFM female mice after neutron irradiation

	Incidence (% ± S.E.) for dose of –				
	0 (control)	5 rads	10 rads	20 rads	50 rads
Thymic lymphoma	10.0 ± 3.8	11.7 ± 5.9	24.5 ± 8.5	31.6 ± 8.8	44.6 ± 8.3
Reticulum cell sarcoma	42.3 ± 5.3	43.6 ± 6.5	53.5 ± 7.7	38.3 ± 7.3	47.8 ± 4.1
Other leukemia	11.3 ± 3.7	23.7 ± 8.9	9.3 ± 3.4	12.4 ± 6.3	10.1 ± 4.1
Endocrine tumors	7.0 ± 2.9	5.2 ± 3.1	10.7 ± 5.9	50.4 ± 6.9	54.2 ± 4.2
Lung adenomas	23.9 ± 4.7	10.1 ± 3.7	23.8 ± 8.8	30.1 ± 8.8	45.8 ± 5.3
Other tumors	4.2 ± 2.2	9.5 ± 5.0	4.6 ± 2.5	25.6 ± 7.0	47.2 ± 4.4

EFFECT OF CHLOROQUINE ON RECOVERY FROM CARCINOGENIC INJURY TO MOUSE LUNG INDUCED BY SPLIT-DOSE RADIATION

R. L. Ullrich, N. H. Pazmiño,* and J. M. Yuhas

Numerous studies have shown that fractionation of a radiation exposure is less effective in producing a particular biological end point than a similar single exposure. This decreased effectiveness with fractionation is apparently due to recovery between fractions. Recovery may be a function of intracellular repair and/or intercellular recovery of target and interacting cell populations. Intracellular repair is relatively fast and is essentially complete in a matter of hours. Intercellular recovery, on the other hand, is a slower process. Some intercellular recovery may occur within 24 hr, but most occurs over a period of days and in some cases perhaps weeks.

Although the reduced effectiveness of low-dose-rate or extended-fractionation regimens in tumor induction suggests that recovery occurs, the role of this phenomenon in carcinogenesis is still unclear. An understanding of the importance of this phenomenon in carcinogenesis as well as an understanding of the relative importance of intracellular and intercellular recovery in carcinogenesis is essential for the understanding of the mechanisms involved in the carcinogenic process. Intracellular repair mechanisms may act on two types of injury: injury which would ultimately lead to either neoplastic transformation or potentially lethal damage. In both instances recovery could ultimately influence the carcinogenic effect either by affecting the number of transformed cells (initiating event) or by influencing the activities of the target- and interacting-cell populations (promoting action).

A relatively simple approach to the study of recovery is the split-dose technique. By simply splitting the dose into two fractions, the influence of recovery may be assessed with as few complicating factors as possible. The relative importance of intracellular repair could be approached in two manners, using this technique: first, by a split dose separated by an interval of 24 hr or less, or second, by administering a DNA-repair inhibitor between split doses.

The present study was designed to examine the influence of intracellular recovery on the induction of lung carcinomas in the BALB/c mouse using both methods. Experimental groups included: (*a*) animals receiving single exposures, (*b*) animals receiving split exposures plus an injection of saline immediately after the first exposure, (*c*) animals receiving split exposures with an injection of 30 mg of chloroquine per kilogram of body weight immediately after the first exposure. Animals given split exposures received the same total dose as the single-exposure group. Appropriate non-treated, saline-treated, and chloroquine-treated controls were included. Since there were no differences in the tumor incidences among the control groups, they were pooled for analysis. The 5-hr fraction interval was selected to ensure that only intracellular recovery could occur. Previous studies using this same split-dose scheme had shown that the effects of chloroquine are on the first of the paired exposures using a variety of end points.[1]

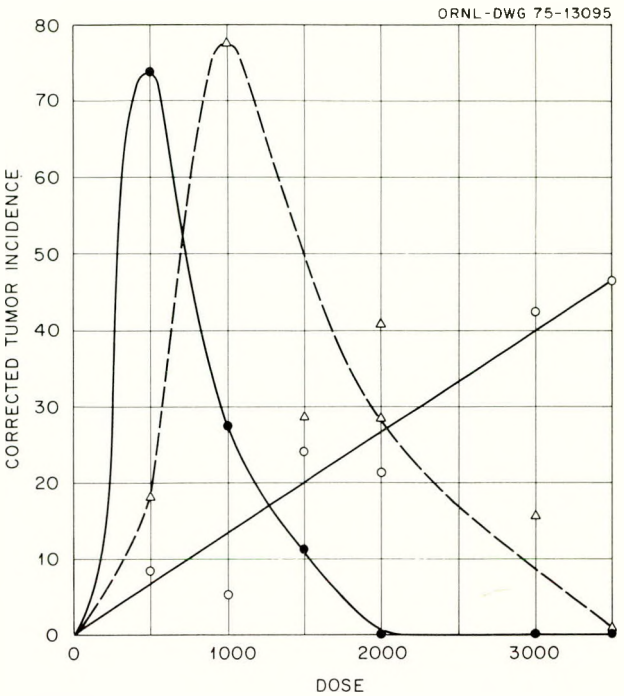

Fig. 15. Incidence of murine lung carcinomas after irradiation in the various treatment groups. ●—● single dose, ○—○ split + saline, △---△ split + chloroguine.

Figure 15 is a plot of tumor incidence vs dose for the three treatment groups. Tumor incidences have been corrected both for control incidence and for any differences in incidence due to differential survival times among groups. This correction was particularly essential in the high-dose groups. Two points are apparent: significant recovery takes place during the 5-hr fractionation interval, and chloroquine inhibits a significant portion of this recovery. These data suggest that early intracellular repair may play an important role in recovery from carcinogenic injury during fractionated or protracted exposures. This influence may be exerted on initiating events or promoting events or both.

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1. N. H. Pazmiño and J. M. Yuhas, *Radiat. Res.* **60**, 54–61 (1974).

INFLUENCE OF LOCAL RADIOTHERAPY ON HOST-TUMOR INTERACTIONS

R. L. Ullrich, J. M. Yuhas, and M. C. Jernigan

The Line 1 alveolar cell carcinoma is a transplantable murine tumor, which, unlike most others, kills the host by means of metastatic spread. Attempts to cure this tumor with localized radiation therapy often fail, in spite of local tumor control, because the metastases evade the treatment. In fact, it appears in this system that localized radiation therapy can actually accelerate metastatic spread, presumably through injury to the weak immunologic control mechanisms which help to inhibit tumor dissemination from the transplantation site. As a possible means of circumventing this problem (local control only at the expense of accelerated metastasis), we have given the mice a radioprotective drug, WR-2721, just prior to their localized radiation exposures. Previous experience indicated that this drug did not protect solid tumors, but did protect the normal tissues included in the radiation field. In drug-treated animals, the same radiation dose was required to achieve local control of the tumor, but many more of these "locally cured" animals survived without showing evidence of accelerated metastasis. Studies are presently in progress to determine the nature of this regional inhibition of metastases, and its response to irradiation in the presence and absence of WR-2721.

A related series of experiments have begun involving the combination of local radiotherapy with *C. parvum* in the treatment of the Line 1 alveolar cell carcinoma. The objective of the study is to identify the radiobiological and immunological factors of importance in maximizing the efficacy of this mode of cancer therapy. This type of combined therapy would allow us to more effectively control distant metastases while achieving local tumor control. Although these experiments are still in progress, it is apparent that fractionation of the total radiation dose in combination with *C. parvum* treatment is significantly more effective than a similar single acute dose plus *C. parvum* in increasing the survival times of tumor-bearing mice.

DNA SYNTHESIS IN MEGAKARYOCYTES OF MICE AFTER STIMULATION OF MEGAKARYOCYTOPOIESIS

T. T. Odell, T. P. McDonald,* and Deborah A. Boran

The labeling index of megakaryocytes 30 min after injection of tritiated thymidine ([^{3}H]TdR) indicates the proportion of the megakaryocyte population that is in DNA synthesis at the time of injection, because [^{3}H]TdR labels DNA and has a very short half-time in the body. Other work had shown that the average ploidy of megakaryocytes increases in animals that have been stimulated to increase their platelet production. It was therefore predicted that an increase in DNA synthesis resulting from a ploidy increase in stimulated mice could be seen as an increase in the labeling index of the megakaryocytes after [^{3}H]TdR injection. This measure might serve as a method to assay the stimulation of megakaryocytopoiesis.

C3H mice were injected with rabbit anti-mouse-platelet serum and then injected at intervals up to 96 hr later with [^{3}H]TdR. Mice were killed 30 min after [^{3}H]TdR administration, and bone marrow smears were prepared. Autoradiograms were made, and labeling index was determined. The antiserum reduced the platelet counts to about 4% of the normal level. The normal level was regained at approximately 96 hr. The labeling index was greater than that of controls ($p < 0.05$) at 24 hr and less ($p < 0.025$) at 72 hr after injection of antiplatelet serum. These results indicate a stimulation of megakaryocytopoiesis followed by a depression. It may be possible to use either of these changes from normal as an end point for studying stimulation of megakaryocytopoiesis. Additional experiments will be needed to determine whether a more moderate stimulation of megakaryocytopoiesis will produce a measurable effect by this method.

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CHANGES IN PLATELET SIZE AFTER INDUCED ACUTE THROMBOCYTOPENIA

T. T. Odell

Earlier studies in several laboratories have indicated that there is an increase in the size of some of the circulating blood platelets in thrombocytopenic states.

It has been suggested by many authors that young platelets are larger than average, which would account for increased average platelet size in individuals recovering from thrombocytopenia, and that platelet size decreases with age. On the other hand, it has also been suggested that large platelets are produced as a consequence of the stress of acute thrombocytopenia and that these large "stress" platelets may not be the same as the large young platelets in normal individuals. In studies of megakaryocytopoiesis and platelet production after induced acute thrombocytopenia, we had noted the presence of large platelets in platelet-counting hemacytometer chambers at about 18 hr after induction of thrombocytopenia with antiplatelet serum. We wondered whether these large platelets were normally produced young platelets present in high proportion soon after thrombocytopenia induction or whether they were produced specifically in response to the stress of acute thrombocytopenia. To learn more about these platelets, we made measurements of the size of individual platelets at intervals during 60 hr after injection of antiplatelet serum into rats. The dose of antiserum used was adjusted to reduce the peripheral platelet count to about 2% of the pretreatment count. In such rats there is a slow increase in the number of circulating platelets for about $1\frac{1}{2}$ to 2 days and then a very rapid increase between 48 and 96 hr. Platelet size measurements were made on platelets of a control rat and of rats killed at 12, 18, 24, 30, 42, and 60 hr after injection of APS. Relative sizes were obtained by taking 35-mm photomicrographs of platelets in hemacytometer counting chambers, placing the photomicrographs in an enlarger and tracing the platelet perimeter on paper, cutting out the tracing, and weighing it.

Average platelet size was larger statistically ($p < 0.001$) than in the control at 12, 18, 24, 30, and 42 hours. By 60 hr, average platelet size and size distribution had returned to a pattern very similar to that of the control. In the 18-hr sample the frequency distribution appeared to be bimodal, and nearly half of the platelets were larger than any observed in controls. Because the new large platelets at 18 hr were bigger than any present in controls, they apparently do not correspond to normally occurring large young platelets, but rather represent a specific response to the acute thrombocytopenic condition. The early appearance of these extra-large platelets suggests that they were derived from megakaryocytes that were already undergoing the later stages of their maturation process at the time of antiserum injection. If so, the message to produce these large platelets was received by postreplication megakaryocytes, and the stimulus was platelet deprivation. By 24 hr the frequency distribution of platelet size had begun to shift back toward the control condition, suggesting that the period of delivery of these large platelets to the circulation is quite short. Moreover, these large platelets may possibly have a shorter than normal survival time, because they seem to be essentially gone by 60 hr, whereas it has been reported that the average lifetime of rat platelets is 4 to 5 days. Alternately, these large platelets may decrease in size with age, as has been suggested for normally produced platelets.

In addition, the data collected in these experiments are consistent with a shift in the proportion of platelets in the circulating population toward the upper (larger) end of the normal size distribution about the time when the most rapid platelet production begins (sample taken at 42 hr).

These results suggest that "stress" platelets that are larger than any found in normal individuals can be produced in response to acute, severe thrombocytopenia. The results do not rule out the possibility that newly produced platelets in normal or treated individuals are greater in size than the average of the circulating population.

PERSISTENCE OF INJECTED ANTIPLATELET SERUM IN THE CIRCULATION OF RATS AND MICE

Lucia L. Hodges,* T. T. Odell,
Diane K. Beeman, and Deborah A. Boran

Antiplatelet serum (APS) has been used to induce thrombocytopenia in experimental animals in order to study platelet production and megakaryocytopoiesis. It has been shown that the degree of thrombocytopenia is related to the dose of APS injected. Results have also suggested that the duration of thrombocytopenia may be related to the dose of APS. The latter observation raises the question whether larger doses of APS persist in the circulation and produce a continuing effect of remaining APS on newly produced platelets or whether a longer-lasting depression of platelet production after larger doses of APS is due to an effect of the APS on megakaryocytes, the precursors of platelets. The present investigation was begun to examine the length of time APS remains in the circulation after intravenous injection.

Rats or mice were injected intravenously with APS that was made in rabbits against rat platelets. The serum was absorbed with rat red blood cells. The doses of antiserum were sufficient to reduce the circulating

platelet count to about zero. At intervals during the 24 hr after APS injection, blood samples were taken and allowed to clot. The serum was then collected. Rat serum was tested for its ability to agglutinate rat platelets *in vitro* or to take part in formation of a precipitation band on an Ouchterlony immunodiffusion plate with sheep anti-rabbit gamma globulin; mouse serum was tested only with the Ouchterlony method.

The results established that antiserum specific to rat platelets can be detected in serum of APS-injected rats by an *in vitro* platelet agglutination technique. The APS in the serum of recipients can also be detected indirectly using sheep anti-rabbit gamma globulin to detect the rabbit gamma globulin of the APS. APS was present in the serum of recipients for 3 hr after injection in rats and for 24 hr in mice. Additional experiments will further delineate the relationships between dose of APS injected and its persistence in the circulation.

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MORTALITY INTERACTION BETWEEN SINGLE-DOSE X RAY AND CONTINUOUS LOW-LEVEL DIETHYLNITROSAMINE IN MALE C3H MICE

J. M. Holland and T. J. Mitchell*

The possibility that a single exposure to a sublethal dose of radiation given at an early age could alter the sensitivity of an individual to subsequent continuous ingestion of a chemical carcinogen is the subject of the present preliminary report. Treatment groups, number per group, dosage, and mean survival time are given in Table 31. As a measure of the overall effect of a given individual or combined treatment, the survival data were analyzed using the odds-ratio approach.

The concept of odds ratio, as we have applied it to survival data, may be understood as follows. For each of two populations of size N, the life-span is divided into successive intervals, I_1, I_2, I_3, ..., I_k. Interval length is arbitrary but is usually chosen to reflect the pattern of mortality peculiar to the species under consideration. For mice a 50-day interval is convenient. For each group the odds for dying (R^*) in a given interval are given by the probability of dying in the interval divided by the probability of surviving the interval. For each interval, then, R^* can be estimated by the ratio:

$$R = D/A , \quad (1)$$

where D is the number which die in the interval and A is the number which survive to the end of the interval. If this procedure is applied to all intervals, an array is obtained that represents the changing force of mortality in each group. As a measure of the degree of difference between two groups, the odds ratio γ is defined in each interval to be:

$$\gamma = R_1^*/R_2^* , \quad (2)$$

where R_1^* and R_2^* are the odds for dying in that interval corresponding to groups 1 and 2 respectively. In the present context, where group 1 is a treated group and group 2 is an untreated control group, we shall refer to γ as the mortality factor (MF) associated with the treatment. An MF of 2 is interpreted to mean that the odds of dying in the treated group are twice the odds for dying in the control. In principle, the mortality factor can change from interval to interval, but if the differences in mortality between the groups are reasonably consistent, a single measure of the difference is obtained by assuming that γ is constant for

Table 31. Survival parameters in C3H mice exposed to X rays, DEN, or both

Group	Treatment	Dose		Number	Mean survival time (days)	S.E.
		X ray[a] (R)	DEN (μg/ml)[b]			
1	Aging	0	0	139	808	16
2	X ray	300	0	147	760	16
3	DEN	0	1.2	156	632	14
4	DEN	0	6.0	225	406	7
5	DEN + X ray	300	1.2	158	532	15
6	DEN + X ray	300	6.0	226	386	7

[a]X ray: 300 kVp, 20 mA, HVL 0.5 mm Cu, dose rate ~130 R/min, 5–6 weeks of age.
[b]DEN: 1.2 or 6.0 μg/ml in drinking water for life commencing at 12–13 weeks of age.

all intervals. An iterative procedure has been implemented in a computer program to determine the maximum likelihood estimate of γ, together with approximate 95% confidence limits.[1] The program also provides an exact test of the hypothesis that $\gamma = 1$, *i.e.*, that there is no difference between the treated and control group.

The results of these calculations are summarized in Table 32. It is of interest to examine the results for evidence of interaction between the radiation and DEN treatments. In the present context we shall say there is no interaction if the effects of these treatments (as measured by the MF) are multiplicative, *i.e.*, the MF for the combined treatment is approximately equal to the product of the MFs for individual treatments.

Table 32. Mortality factor for treatment groups relative to aging control

Group	MF	95% confidence interval	*P*
2	1.44	1.10, 1.91	0.009
3	2.16	1.63, 2.86	<0.001
4	66.42	35.41, 136.73	<0.001
5	8.40	5.74, 12.59	<0.001
6	93.50	44.39, 233.22	<0.001

The MF for DEN at 1.2 μg/ml was 2.16, while the MF for 300 R of X rays was 1.44. Under the assumption of no interaction, we would expect the combined MF to be 2.16 × 1.44 = 3.11. However, the observed MF in group 5 was 8.40, indicating a synergistic interaction between the chemical and radiation treatments.

A similar analysis of the interaction between DEN at 6.0 μg/ml and X rays showed that the combined effect of these treatments (MF = 93.5) was very nearly equal to the product of their individual effects (1.44 × 66.42 = 95.64). Thus, by our previous criteria, DEN at 6.0 μg/ml does not exhibit interaction under the same set of experimental conditions which revealed clear-cut evidence of interaction at a lower DEN dose rate. The most obvious interpretation of the demonstrated dependence of interaction on DEN dose rate is the rather sizable MF associated with DEN alone at the higher dose (66.42). This effect is so large relative to the X-ray component (1.44) that the potentiating effect of X rays may have been masked. This observation illustrates why it is important to choose carefully the dosage in any interaction experiment. With too high a dose of either agent, animals will be killed before enough time has passed for interaction to become manifest.

Having demonstrated interaction between radiation and DEN, our next objective will be to establish the basis for this interaction by an analysis of tissues obtained from necropsy examination of all animals dying in these experiments.

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1. *Math. Stat. Res. Dept. Annu. Prog. Rep. June 30, 1975*, UCC-ND-CSD 18.

SEX- AND STRAIN-DEPENDENT DIFFERENCES IN THE MAGNITUDE OF THE CHRONIC RESPONSE TO RADIATION

J. M. Holland, Mary S. Whitaker, L. C. Gipson, and T. J. Mitchell*

As part of a continuing program to identify, characterize, and assign relative importance to those genetic factors which influence effects of radiation, a series of multiple strain and hybrid survival experiments were initiated. Four inbred strains (C3H, BALB/c, RFM, C57BL/6), two F_1 hybrids ($C3CF_1$, $B6RFMF_1$), and a four-way cross (C3CB6RFM) were exposed to 300 R of 300-kVp, 20-mA X rays. Both sexes were exposed at 5–6 weeks of age and allowed to survive under SPF conditions which minimized infectious-disease mortality. Unirradiated controls were maintained under the same conditions concurrently. This report is a preliminary analysis of life-shortening in these experiments.

The odds-ratio parameter, described in detail elsewhere in this report,[1] was used to compare overall risk of mortality between irradiated and control groups. MFs were calculated for females and males separately by the method indicated.[1] The magnitude of the sex effect is measured by the ratio of MFs. These data are presented in Table 33 along with results of a test of the hypothesis that female and male MFs were the same.

In Table 33, strains are in the order from the greatest to the least significant sex difference. In all groups except the four-way cross and the C57BL/6, the female was significantly more sensitive than the male. In the latter two comparisons, the trend was also in this direction, but the results were not significant at the 5% level. Thus it was not possible to conclude definitely that females were consistently more sensitive than males, although the trend was in this direction.

Tests of the resemblance of hybrid and parental-strain MFs reveal that female $C3CF_1$ mice have a response not significantly different ($P > 0.05$) from females of either parental strain. The response of $C3CF_1$ males, however, tends to be more like the male parent (BALB/c), although we cannot reject equal contribution from both parents. If both sexes are pooled, the general conclusion

Table 33. The relation of sex and mortality following radiation

Rank	Strain	Mortality factor		Ratio (female/male)	P
		Female	Male		
1	C3H	4.31	1.44	2.98	<0.001
2	$C3CF_1$	4.23	2.19	1.93	<0.01
3	$B6RFMF_1$	3.44	1.84	1.86	<0.01
4	RFM	6.83	3.70	1.85	<0.05
5	BALB/c	4.02	2.40	1.68	<0.05
6	4-way cross	2.78	2.28	1.22	NS[a]
7	C57BL/6	2.30	2.03	1.14	NS

[a]NS = not significant.

is that the response of $C3CF_1$ hybrids is so similar to each parental strain that we cannot make genetic inferences. Female $B6RFMF_1$ mice have a response intermediate between the two parents, although there is a tendency for the hybrids to more closely resemble C57BL/6. Male $B6RFMF_1$ respond as the more resistant C57BL/6. Both sexes pooled follow this same trend, that the F_1 of this cross between resistant C57BL/6 and sensitive RFM resembles the resistant parental strain. Female four-way-cross mice also resemble the resistant $B6RFMF_1$ male parent. Male four-way-cross mice have a response that cannot be distinguished from either parental response. To summarize the results of the hybrid analysis, if we exclude the $C3CF_1$, which is a cross between two related strains that show very similar responses to radiation, inheritance of resistance appears dominant over sensitivity in F_1 males of a cross between the divergent C57BL/6 and RFM strains. Female hybrids show a slightly different pattern in that their response is clearly intermediate. An intermediate response is consistent with the hypothesis that factors which control the somatic response to radiation are multiple and that these factors manifest additive dominant interactions.

Genetic factors are obviously important in determining the magnitude and direction of the radiation response. Are these differences due solely to the differential induction of leukemia and ovarian tumors in females as has been suggested, or are there additional factors involved in the male–female difference? Are the same mechanisms responsible for between-strain differences as between sexes, or must we assign certain effects to sex and others to strain? How representative of the "average mouse" is the range of responses observed in this series of inbred mouse experiments? Are observations obtained from genetically divergent but homologous strains representative of the range of responses likely to be observed in a random breeding population? We only raise these questions now. Eventually we hope to have some of the answers.

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1. J. M. Holland and T. J. Mitchell, this report.

PERCUTANEOUS TOXICITY OF EPOXY RESINS AND AMINE HARDENERS IN INBRED C3H AND C57BL/6 MICE

J. M. Holland, L. C. Gipson,
and Mary S. Whitaker

The epoxy resins bis[2,3-epoxy-cyclopentyl] ether (Chem. Abstr. Serv. Reg. No. 2386-90-5) and diglycidal ether of bisphenol A (1675-54-3) are representative of a large class of materials that are used extensively throughout industry. An amine hardener, *m*-phenylenediamine (108-45-2), is used with various resins and resin mixtures as a polymerizing agent. Given the extensive use, economic importance, and opportunity for skin contact associated with epoxy resin components, a long-term study was initiated to evaluate the cumulative effects of these materials applied to the skin of mice. Preliminary toxicity-range-finding experiments were performed in order to establish the maximum dose that would be tolerated without inducing acute toxicity.

Fifty microliters of a 50% v/v acetone solution of each resin and a 1:1 mixture of both resins were applied to the shaved interscapular skin of male and female inbred C3H and C57BL/6 mice. The amine hardener was also diluted in acetone, but to a concentration of 10% v/v. Treatments were given daily 5 times/week for 2 weeks or until death. Survivors at the end of the

2-week treatment period were killed and examined for gross or microscopic evidence of toxicity.

C3H mice tolerated the resin exposures without mortality and with no clinical evidence of overt toxicity. The amine hardener did, however, induce 100% mortality in females and 60% mortality in males over the period of exposure. The observed difference between sexes is of questionable significance, because survivors had gross and microscopic lesions indicative of systemic toxicity.

C57BL/6 mice exhibited 50% mortality after exposure to resin 2386. No mortality or signs of toxicity were observed with either of the other resin treatments. As was the case with C3H mice, C57BL/6 mice exhibited 100% mortality after exposure to the amine hardener.

In both strains the clinical response to the amine hardener was the same. After one or two treatments, signs of toxicity were observed as dehydration, lethargy, and brown, discolored urine. With continued treatment the animals became depressed and comatose before death. It was originally assumed that the discolored urine was the result of massive intravascular erythrolysis and excretion of hemoglobin. That this was not the case was concluded by failure to detect hemoglobin in urine using the benzidine test. Acetone solutions of commercial-grade *m*-phenylenediamine are dark brown. If the compound was absorbed through the intact skin in sufficient quantity, it or its catabolites could have caused urine discoloration. A UV chromatographic profile of individual urine samples revealed that the material was absorbed. Some of the material was excreted apparently unchanged, but several new peaks were observed, suggesting that metabolism of the material had taken place.

Toxicity of resin 2386 in C57BL/6 mice was unexpected, since the same material had no clinical effect in C3H mice. Death in C57BL/6 mice was the result of a Schwartzmann-like phenomenon with massive renal cortical and hepatic necrosis, suggesting an angiovascular or allergic pathogenesis. This conjecture is supported by the observation that death was very rapid and occurred after only one or two applications. If the animal survived the initial allergic syndrome, gradual recovery ensued even though treatment continued. This tolerance or resistant state persisted throughout the remainder of the treatment period.

On the basis of these preliminary experiments, 50% acetone solutions of resin 2386, the resin mixture, and resin 1675 have been applied three times weekly in 50-μl amounts to the skin of C3H mice for several months without mortality or evidence of toxicity. C57BL/6 mice are being exposed to the same spectrum of materials but at a lower (25%) concentration of resin 2386. Under these conditions no toxic deaths have occurred in C57BL/6 mice. The amine is being applied to both strains at a 2% concentration without signs of toxicity. It is concluded, based on the experience accumulated to date, that maximum concentrations of each material are being applied which will be tolerated without evidence of cumulative toxicity and shortened survival time. It is under these conditions that those adverse biological responses which are likely only after prolonged exposure, such as tumor induction or autoallergic phenomena, are likely to become manifest. While the long-term study has only just begun, after over 5 months of continuous exposure, no evidence of any deleterious effect has been observed in either sex or strain.

TRANSPLANTABILITY OF SPONTANEOUS HEPATOMAS IN C3H MALE MICE

J. M. Holland and Mary S. Whitaker

Spontaneous or induced hepatocellular tumors are difficult to classify as benign or malignant on a purely morphologic basis. The liver, due to its practically unlimited growth potential, typically responds to chronic injury, and in some mouse strains to senescence, by irregular compensatory hyperplasia. These compensatory hyperplastic changes grade continually into frank autonomous neoplasia. The C3H male mouse very commonly develops hepatoma with an average latency of 14 months and an incidence between 40 and 50%. These tumors vary greatly in gross form and location. Most are well circumscribed, pedunculated, and resemble normal liver in color and texture. They rarely exhibit malignant behavior such as local invasion or systemic metastasis. Usually they kill the host by rupture and hemorrhage into the abdominal cavity, by infarction, or by obstruction of portal and post caval blood flow which leads to circulatory collapse.

In an effort to better characterize the biological behavior of this common tumor, we have initiated a transplantation experiment to determine (*a*) whether there is any correlation between gross and microscopic characteristics of the primary and growth of the tumor following transplantation, (*b*) the average latency and growth rate, (*c*) how the morphology of the transplant resembles or differs from the primary, and (*d*) what host factors are involved in maintenance, latency, and growth rate of transplanted hepatomas.

The procedure we have adopted involves determining the characteristics of the primary which include gross form, location, color, texture, size, and evidence of metastasis. Sections of the primary, residual normal liver, and representative other tissues are taken. The tumor is minced in Hanks' buffered salt solution, and small fragments of tumor tissue are grafted subcutaneously into five or ten 10- to 12-week-old C3H recipients of the same sex as the donor. Transplant recipients are examined at 2-week intervals for evidence of tumor growth. Latency is established as the elapsed time from tumor implantation to the first palpable evidence of tumor growth. Tumors are allowed to progress for 30 days after first observation. The hosts are then killed, and the tumor is dissected and weighed to obtain an estimate of growth rate. Gross and microscopic appearances of transplants are compared with the original primary to determine if one or more cell components have become predominant. While the experiment has only just begun, preliminary observations are interesting. Of 19 hepatocellular tumors which have been transplanted, 13 grew successfully to resemble the original primary closely. All of these tumors, both primary and transplant, were classified as minimal deviation hepatoma. A variable small cell component, resembling the "fetal hepatocyte," was observed, but the presence of this cell did not correlate with transplantability of the tumors. Only 1 of 19 primaries had undergone spontaneous metastasis. In this case, multiple pulmonary nodules resembling normal liver tissue with sinusoids and hepatic plate architecture were observed.

Latency varied from 27 to 267 days, with an average around 180 days. For this entire period, tumor cells remain viable but presumably do not divide. Eventually, however, the tumor cells begin to divide and give rise to large subcutaneous tumor masses. Data on differential growth rate and correlation of growth rate with latency are incomplete. These observations are consistent with early reports[1] which also encountered long latency in the first-generation transplant. Our current working hypothesis to explain prolonged latency is that host factors are involved in maintaining the dormant state in transplanted syngenic hepatoma. These same host factors may be responsible for controlling the time of appearance and rate of growth of the primary. For this reason, the identification and characterization of these putative host factors and the extension of our data on the biology of the primary are in progress.

1. H. B. Andervont and T. B. Dunn, *J. Natl. Cancer Inst.* **13**, 455 (1952).

ACTIVATION OF MURINE LEUKEMIA VIRUS BY GAMMA RADIATION

J. A. Otten, J. M. Quarles,* and R. W. Tennant

Oncornavirus genetic information is present in all mouse cells in an unexpressed state. These endogenous viruses may be activated by chemicals such as halogenated pyrimidines or protein synthesis inhibitors. Large acute doses of X irradiation also induce the expression of endogenous virus, although X irradiation is relatively inefficient compared with halogenated pyrimidines. Since the activation of virus by such chemicals has been shown to be dependent on cell division, we devised an experimental procedure for low-dose-rate, long-term irradiation of actively dividing cells. Non-virus-producing AKR cells were grown in flasks filled with medium and incubated at 37°C with the cell monolayer perpendicular to a ^{137}Cs source providing 17.5 rads/hr of gamma rays. Total doses of 350 rads (20 hr) and 700 rads (40 hr) were applied. Activation and expression of virus was determined by conducting XC-plaque assays on the subcultured irradiated cells. The minimum total dose of 350 rads resulted in virus activation, and 700 rads induced a 40-fold increase in plaque-forming units. Serum deprivation for 18 hr prior to and during irradiation inhibited cell division and prevented virus activation. These results show that low-dose-rate gamma radiation activates endogenous leukemia virus from AKR mouse cells *in vitro* and that cell division during irradiation is required for virus activation.

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STUDIES ON THE PATHOGENESIS OF RADIATION-INDUCED LEUKEMIA IN RF MICE

J. A. Otten, Susan Custead Jones,* N. K. Clapp,† and R. W. Tennant

The RF-strain mouse is sensitive to radiation-induced neoplasms, and an RNA tumor virus has been associated with radiation-induced lymphomas and myeloid leukemia in the RF-strain mouse. However, the relationship of irradiation to activation of endogenous RNA tumor viruses and effects on the autogenous immune response of mice to these viruses is not understood.

We have examined RF mice for endogenous type C viruses and naturally occurring antibody to these viruses following irradiation. The study is designed to follow

individual animals throughout their life to determine the relationship between expression of endogenous levels of virus-specific antibody to the virus. These *in vivo* studies are paralleled by *in vitro* activation studies described in this report. RF mice were irradiated at 2 months of age with 250 rads of whole-body X radiation; at 2-month intervals following irradiation, serum samples for antibody titration and a small portion of tail for isolation of virus were taken from individual mice. The individual mice were then held and examined at further 2-month intervals. The radioimmune assay was used for determining viral antibody levels, and the XC-plaque assay was used for detection of infectious virus. Preliminary results suggest that virus appears by 6–8 months following irradiation and that with the appearance of virus there is a concomitant drop in antibody titer to the endogenous leukemia virus. These studies are being continued in order to further understand host regulation of endogenous leukemia viruses and the role of irradiation in the loss of this regulation.

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REPLICATION OF THE DNA OF KILHAM RAT VIRUS

G. C. Lavelle and Anna Tai Li*

Kilham rat virus (KRV) is a member of the parvovirus group of small (18–26 nm) isometric viruses containing linear, single-stranded DNA. KRV has the important capacity to induce developmental defects in the rat and hamster. Our interest in parvovirus multiplication has been further stimulated by recent evidence that a parvovirus-like agent is the cause of acute gastroenteritis in humans.

As shown earlier by R. W. Tennant in this laboratory, KRV production requires cellular functions found only in actively dividing cells and associated with DNA synthesis. Specific events in the replication cycle of the virus which are dependent upon cellular function, however, have not been defined.

The purpose of present research is to examine the effect of the cell physiological state, particularly DNA synthesis, on the replication of viral DNA. DNA of parvoviruses is believed to replicate by means of a double-stranded intermediate as is the case of bacteriophages having single-stranded genomes. In one series of experiments, designed to identify formation of a double-stranded intermediate, intracellular forms of input KRV DNA were examined after infection of cells with virus containing radioactively labeled DNA. Between 6 and 10 hr after infection of actively growing NRK cells, viral DNA showed a shift in density by CsCl isopycnic centrifugation. DNA banding at the new density was primarily of viral unit length in alkaline sucrose velocity gradients, which suggested conservation, rather than degradation and reutilization, of viral DNA. In agreement with the appearance of viral DNA in new density species, hydroxyapatite chromatography showed an increase in the double-strandedness of viral DNA between 6 and 10 hr after infection. New species of input DNA were also prominent in neutral sucrose gradients by 10 hr after infection. In alkaline gradients, DNA from the broad 14–20S (neutral sucrose) fraction was homogeneous in size, but appeared to be slightly less than unit length. Analysis of resistance to single-strand-specific nuclease, S1, before and after denaturation/reannealing showed that 14–18% of 14–20S DNA was double-stranded. Addition of excess virion DNA to annealing reactions completely inhibited reassociation of 14–20S viral DNA, confirming the presence of complementary viral sequences in 14–20S molecules. All of these results support the conclusion that parental viral DNA is present in cells in virus-specific molecules which are at least partly double-stranded.

The replication of the RF DNA was also studied by infecting synchronized NRK cells with virus and pulse-labeling with [^{3}H]thymidine. DNA obtained at different times contained labeled complementary strands which could be identified by hybridization to unlabeled virion DNA fixed to nitrocellulose filters. Preliminary results revealed that maximum RF replication occurred from 15 to 20 hr postinfection and always after cellular DNA synthesis was initiated. In cells which were blocked continuously in G1, little RF could be detected by hybridization. Thus the replication of viral RF appears to be cell-dependent. The time required before the detection of progeny RF agrees with the time of formation of parental RF. Whether the formation of parental RF is also cell-cycle dependent is not yet known.

Finally, hydroxyapatite chromatography of virion DNA revealed self-complementary sequences in 50% of unit-length molecules which, when denatured, reassociate in a monomolecular fashion indicative of hairpinlike structure. The biological role for such a self-complementary region is not clear, but the feature is common to the DNA of at least two other parvoviruses and the human adenoviruses.

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DETECTION OF MURINE TUMOR VIRUS ANTIGEN IN CULTURED CELLS BY FLOW MICROFLUOROMETRY

R. E. Hand, J. M. Quarles,* and R. W. Tennant

Immunofluorescent tests are widely used to detect the presence of viral antigens in infected cells. The standard procedure involves infecting cells growing on glass coverslips and fixing and staining the cells for immunofluorescent microscopic examination. The technical limitations of this method allow examination of only relatively low numbers of cells (on the order of 500–1000 per experimental sample for a typical assay), with a resulting wide statistical variation. Recently flow microfluorometric techniques for measuring selected parameters of single cells have become available. These techniques provide rapid examination of large numbers of cells (10,000 cells/sample for a typical assay) and thus yield very precise statistical results.

We have developed a method for preparing cells for flow microfluorometric measurement of specific staining by immunofluorescent reagents reactive against murine RNA tumor virus antigen and are defining quantitative parameters of the technique. Cells, either uninfected or infected with Moloney leukemia virus, are fixed in acetone, washed in buffered saline, incubated with fluorescein isothiocyanate-labeled antibody, re-

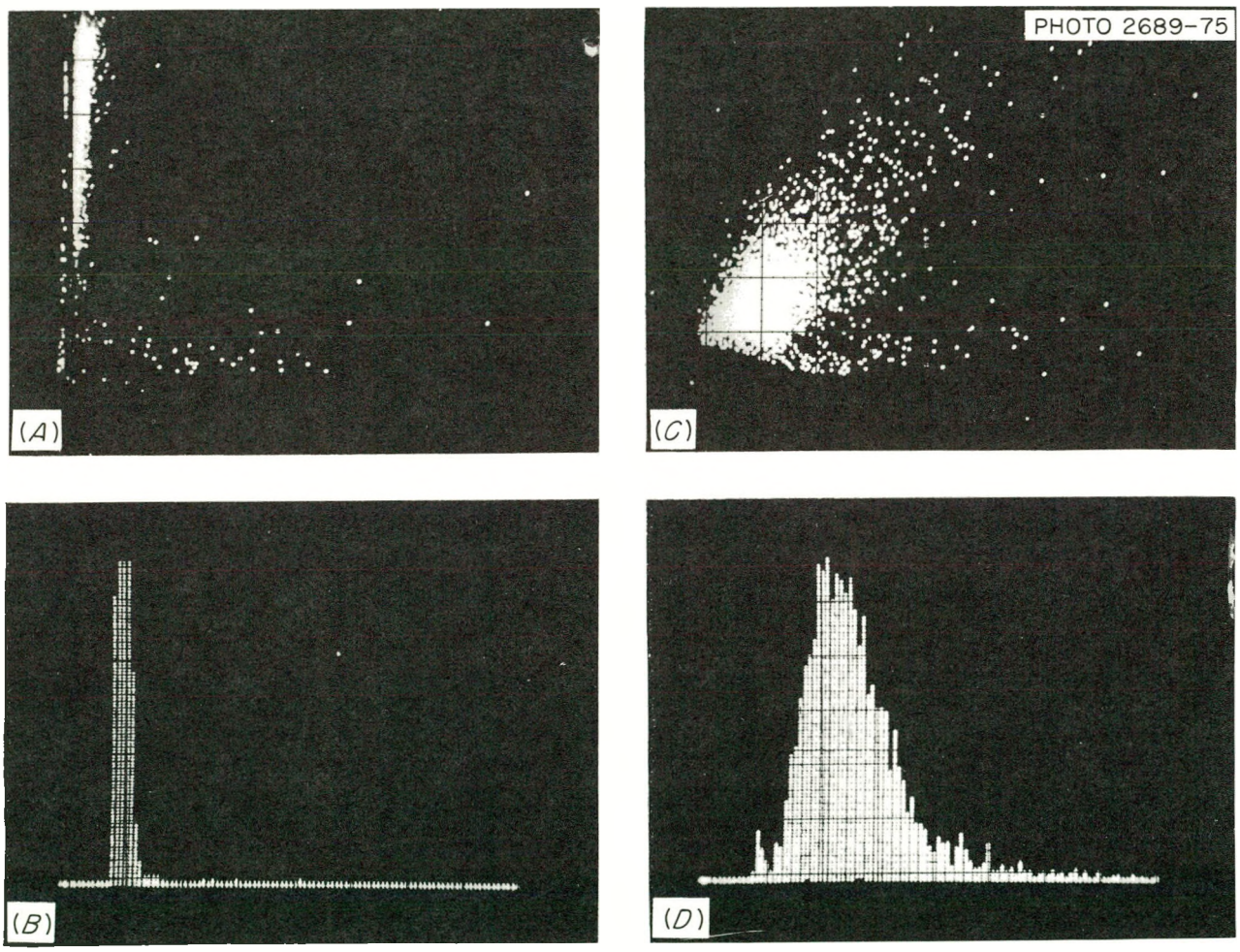

Fig. 16. Scatter pattern and histogram of a population of negative (uninfected) cells (A and B) and positive (murine-leukemia-virus-infected) cells (C and D). For the scatter patterns, the Y axis represents light scatter and the X axis fluorescence. For the histograms, the Y axis represents number of cells and the X axis fluorescence.

washed, and examined by flow microfluorometry. The number of fluorescent cells as a percentage of the total cell population is determined for each sample using a Cytofluorograf, Model 4801 (Bio/Physics Systems, Inc.). This instrument, which has the capacity of simultaneously measuring two optical properties of cells at a maximum rate of 5000 cells/sec, is used to determine light scatter (representing cell size) vs fluorescence (representing the specific antigen–antibody reaction); these properties are displayed on an oscilloscope with a grid overlay. Typical displays of negative and positive cells are shown in Fig. 16. A scatter pattern (*A*) and histogram (*B*) of a negative test are shown in the left panels and the corresponding displays for a positive test in the right panels (*C* and *D*). This technique, which is being standardized and adapted to other virus–cell systems, offers the advantages of much more rapid detection of specific viral antigen on a per-cell basis and a larger number of cells than can be obtained with microscopic examination and thus yields a better statistical analysis of the results.

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USE OF FLOW MICROFLUOROMETRY FOR DETERMINATION OF DNA SYNTHESIS RATES

J. W. Heidel,* J. M. Quarles,† and R. W. Tennant

Flow microfluorometric techniques provide rapid measurements of selected parameters of single cells in suspension. The use of large numbers of cells, approximately 100,000/min, provides high statistical precision. Fluorescent dyes specific for DNA are available and have been used to stain cellular DNA for flow microfluorometric determinations of the DNA content of single cells. The life cycle of mammalian cells in culture can be divided into four main periods: G_1 (gap), S (synthesis), G_2, and M (mitosis). Ideally, cells in G_1 (*i.e.*, prior to synthesis) have one unit of DNA, cells in G_2 and M have two units, and cells in S have between one and two units. Histograms of the number of cells vs fluorescence (*i.e.*, the number of cells vs the amount or units of DNA) yield peaks at G_1 and at G_2 + M, with cells in the synthetic phase between the two peaks.

Working on the assumption that the rate of DNA replication during the synthetic phase is an important and distinguishing characteristic of cells and that the shape of the histogram described above is related to the rate of DNA synthesis if the generation time is known for defined conditions, we conducted a mathematical analysis of the synthetic phase of several types of normal cells using data obtained from the Los Alamos Scientific Laboratory. A formula was derived giving rate of DNA increase as a function of the distribution of flow microfluorometric data. The resulting differential equation was integrated and graphed to yield the amount of DNA and synthesis rate as a function of time. This provides a theoretical model which we are currently testing to determine how these curves vary as a function of altered growth properties, and whether external perturbations of the cells (*e.g.*, infection with viruses or treatment with carcinogenic chemicals) can be detected as alterations in DNA synthesis. The usefulness and applicability of the mathematical model will be evaluated experimentally.

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SIMULATION OF CLINICAL RADIOTHERAPY WITH WR-2721 IN THE SPONTANEOUS DOG-TUMOR SYSTEM

J. M. Yuhas and Darryl Biery*

All of the information which suggests that *S*-2-[3-aminopropylamino]ethylphosphorothioic acid (WR-2721) offers differential protection to normal and malignant tissues has been obtained in rodent systems. While such studies are of value for preliminary work, they do not take into account the gross differences between the mouse and man, and the tumors each bears. Since the dog represents a closer approximation of man, both physiologically and anatomically, and certain canine tumors are essentially the same as their human counterparts, studies on the effectiveness of WR-2721 in increasing the efficiency of radiotherapy of spontaneous canine tumors were originated.

The Department of Radiology, University of Pennsylvania, is a referral center for the East Coast for the radiation treatment of spontaneous tumors in canine pets. The two tumors selected for this study were squamous cell carcinomas and fibrosarcomas of the head and neck. The overall plan calls for the administration of 0, 50, or 75 mg of WR-2721 per kilogram of body weight before each of four to eight localized exposures totaling up to 4000 rads. Full clinical work-up and follow-up are made on each dog.

The first study, which is now nearing completion, involved the exposure of non-tumor-bearing dogs to

four localized doses of 1000 rads on alternate days. A 3 × 3 cm field on the side of the face was employed. In accord with past experience, dogs which did not receive WR-2721 suffered from severe necrosis both at the skin (entrance port) and the oral mucosa (exit port). Dogs given WR-2721 prior to each of their 1000-rad exposures ($N = 4$) did not suffer detectable injury to their oral mucosa, and only one suffered a mild epilation of the skin.

A single fibrosarcoma-bearing dog has been treated thus far. Again using a 4 × 1000-rad protocol, we gave the dog 75 mg/kg of WR-2721 before each exposure. At the time of writing (60 days posttreatment) the dog showed no signs of radiation injury to the normal tissues, and the tumor had regressed with no evidence of recurrence. Additional tumor-bearing dogs are presently being studied under a grant provided by the East Tennessee Cancer Research Center.

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ANTITUMOR PROPERTIES OF *CORYNEBACTERIUM PARVUM* IN SENESCENT IMMUNOINCOMPETENT MICE

J. M. Yuhas and R. L. Ullrich

Most experimental studies on specific and nonspecific immunotherapy are conducted in young adult mice bearing strongly immunogenic transplanted tumors. In this respect, they bear little similarity to the clinical situation in that the autochthonous tumors are weakly immunogenic and the immune responsiveness of the host is reduced due to age, tumor growth, side effects of therapy, or their combination. We have therefore initiated studies which attempt to circumvent these problems: both young and senescent immunologically incompetent mice are employed, strongly and weakly immunogenic tumors are studied, and, in certain circumstances, spontaneous tumors growing in old animals are studied.

The nonspecific immunostimulant being studied is *Corynebacterium parvum,* which has been shown by others to be a potent inhibitor of tumor growth in the aforementioned systems. In our laboratory, the use of *C. parvum* does inhibit tumor growth, but no cures have yet been obtained, even in young mice. Further, the overall effectiveness is far lower in weakly immunogenic tumor systems, such as the Line 1 lung carcinoma. Senescent mice are less responsive to the antitumor properties of *C. parvum* both because of their poor immunologic reserves and because at least part of their increased sensitivity to tumor transplant is nonimmunologic and therefore nonresponsive to *C. parvum.* In spite of the reduced effectiveness of *C. parvum* in these studies, repeated injections of the vaccine were able to induce temporary regression of spontaneous mammary tumors growing in old mice (26 to 33 months) and thereby increase their survival time. We conclude therefore that *C. parvum* remains an effective antitumor immunostimulant, but that its effectiveness is less than originally predicted.

PREDICTION OF THE EFFECTIVE DOSE OF WR-2721 FOR HUMANS FROM AN INTERSPECIES DISTRIBUTION STUDY

L. C. Washburn,* J. J. Rafter,* R. L. Hayes,* and J. M. Yuhas

Preirradiation injection of the radioprotective drug *S*-2-[3-aminopropylamino]ethylphosphorothioic acid (WR-2721) has potential value in the radiotherapy of solid tumors, since the drug increases the radiation resistance of normal tissues markedly while affording barely detectable protection to the tumor, at least in experimental systems. Deficient absorption of the drug by a variety of solid tumors has been demonstrated; this deficiency appears to be the major factor responsible for the observed differential protection of normal and malignant tissues.

The question we are presently addressing is composed of two parts: How much WR-2721 must be administered to a human in order to obtain the same tissue concentrations which offer excellent protection to lower species, and does the same tissue concentration offer similar protection to all species? The first portion of the question is covered in this report.

The tissue concentration of an injected drug can range from being a simple function of the dose per unit body weight through the more complex relation of dose per unit surface area. This latter relationship is a reflection of the fact that the surface area is a good correlate of the metabolic rate.

In the hope of being able to predict the drug dose required to achieve excellent protection of human normal tissues, we have studied the distribution of ^{35}S-labeled WR-2721 in mice, rats, rabbits, and dogs. These studies have demonstrated that the tissue distribution in these species is not a strict function of either body weight or surface area. Extrapolating this inter-

mediate relationship to man, we would predict that a dose of 10 to 20 mg/kg of WR-2721 would offer as much protection to the normal tissues of man as 100 mg/kg would for the mouse, *i.e.*, a 50–80% increase in radiation resistance. This total dose of 700 to 1400 mg for the average man places this drug in the realm of practicality for clinical application.

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DOSE-RATE EFFECTS IN MURINE RADIATION CARCINOGENESIS

J. M. Yuhas and Anita E. Walker

This experiment was designed to determine the effects of ^{137}Cs gamma-radiation dose rate (1.75 rads/day through 41 rads/min) on the carcinogenic effectiveness of total doses of 49, 98, 196, and 392 rads in the BALB/c mouse. Of the more than 7000 mice included in this experiment, less than 10 are still alive. We have previously reported some preliminary conclusions.[1]

The histological examination of the autopsy material is still in progress, but even the gross diagnosis information allows certain tentative conclusions. All of the radiation-inducible tumors show at least some dependence on the dose rate for their induction. The classic example is that of the ovarian tumor, in which effectiveness declines with decreasing dose rates, even after correction for age-dependent loss of sensitivity. Other tumors which conform to this pattern include mammary tumors, liver tumors, and possibly reticulum cell sarcoma.

An exception to this rule is the induction of malignant lung carcinomas. Instead of a direct relationship between dose rate and carcinogenic effectiveness, we have observed that intermediate dose rates are optimally effective, *i.e.*, in the range of 7–14 rads/day. Preliminary studies on the target-cell population, the type II alveolar cells of the mouse lung, suggest that this parabolic relationship results from the delicate balance of malignant transformation and cell cytotoxicity.

Also included in these studies were analyses of the role of age at exposure in determining sensitivity to tumor induction. As a general rule, it appears that cell systems whose function declines with age (*e.g.*, the ovary) show a marked decline in sensitivity to tumor induction with advancing age, whereas cell systems whose integrity remains essentially constant throughout life (*e.g.*, alveolar lining cells of the lung) show no decline in sensitivity with advancing age. Although not significant, it appears that sensitivity to the induction of certain tumors (*e.g.*, liver tumors) actually increases with age at exposure.

Lastly, a comparison of fractionated and chronic exposures, delivered over a period of 112 days, has been made. Again, the response varied as a function of the tumor type in question. Fractionated exposures were more effective than chronic ones in inducing ovarian tumors, but the reverse was true for the induction of malignant lung tumors.

These results suggest that the dose-rate effect varies as a function of the tumor type in question, and that each may eventually be predictable from an understanding of the sensitivity of the target cells to the opposing effects of transformation and cytotoxicity.

1. J. M. Yuhas and A. E. Walker, *Biol. Div. Annu. Prog. Rep. June 30, 1974*, ORNL-4993, pp. 158–60.

RECOVERY FROM RADIATION AND CHEMICAL CARCINOGENIC INJURY TO THE MOUSE LUNG

J. M. Yuhas

In choosing alternative energy sources for the future, one of the most critical considerations is the health hazard associated with the by-products of each technology. Through a variety of screening procedures we are presently accumulating information on the hazards of various by-products when they are given to experimental animals as acute exposures. These studies do not take into account, however, the possibility that two equally potent carcinogens might differ markedly in their carcinogenic efficiency when they are given chronically as opposed to acutely. In brief, it is possible that the mammalian system might be able to "recover" from the carcinogenic injury induced by one, but unable to recover from the injury induced by the other.

The possibility that such a pattern existed was suggested by our preliminary studies on the acute dose-response curves for urethane and ionizing radiation. Both carcinogens yielded a two-component dose-response curve for the production of lung tumors in the mouse (Fig. 17). In the low dose range the yield of tumors was linearly proportional to dose (log-log slope = 1.0), while in the higher dose range the yield was proportional to a greater than 1.0 power of the carcinogen dose. These data suggest that in the low dose range a single event is responsible for the production of tumors, while in the higher dose range, not only single

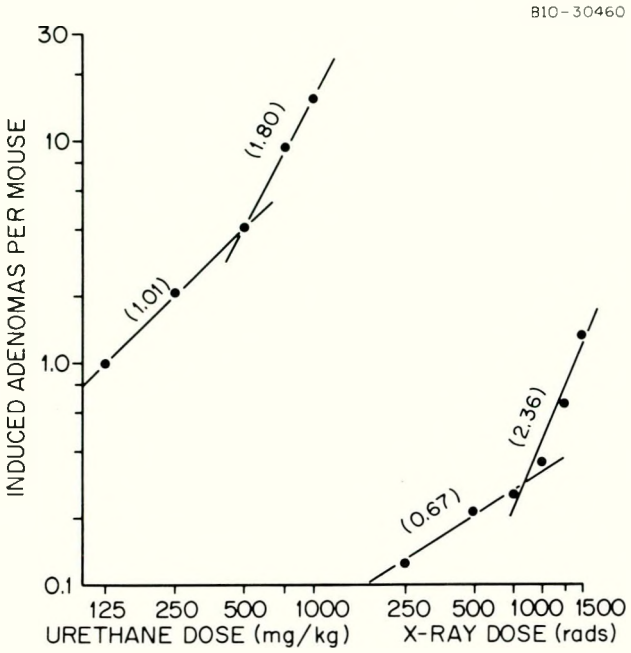

Fig. 17. Mean number of papillary lung adenomas per mouse induced by graded doses of urethane or localized X rays. Induced values corrected for spontaneous control value (0.39 to 0.43 adenoma per mouse). Numbers on each of the curves represent the maximum likelihood estimates of the log-log slope.

events, but event interaction is contributing. Carrying the analysis a step further, one could argue that there should be no recovery from total doses which fell on the linear portion of the curve but that significant recovery should be observed in the event-interaction range, *i.e.*, if more than one event is required for tumor production, the first of a series of events might be repaired before the others were induced under chronic exposure conditions. This point was verified for both urethane and ionizing radiation by giving doses on the linear or greater than linear portions of the curve either acutely or as two fractions separated by 24 hr. Recovery in excess of 50% was observed when the high total doses of urethane and ionizing radiation were fractionated, but no recovery could be detected when the low total dose was fractionated for either carcinogen.

The importance of these observations stems from the interrelation of carcinogenic efficiency and recovery, taking as an example the dose of each carcinogen which induces a mean of one adenoma per mouse (Fig. 17). With urethane, this dose is well within the linear, no-recovery portion of the dose response curve, while for ionizing radiation it is far into the dose range which is recoverable. If we used only acute-dose information, we would argue that 125 mg/kg of urethane is about as hazardous as 1250 rads of ionizing radiation. However, when one takes into account the fact that chronic administration of this dose of urethane would also yield a mean of one adenoma per mouse, while chronic administration of 1250 rads would yield far fewer tumors, it can no longer be argued that these doses of the two carcinogens are equally hazardous. Unless information such as this is obtained for the by-products of energy technologies, cost-benefit analyses will be somewhat tenuous.

SECTION VI

CARCINOGENESIS PROGRAM

F. T. Kenney

Respiratory Carcinogenesis

Paul Nettesheim
D. A. Creasia
W. E. Dalbey
R. A. Griesemer
Roudabeh J. Jamasbi[a]
Ann C. Marchok

Nitrosamine Carcinogenesis

William Lijinsky
A. R. Jones
G. M. Singer
H. W. Taylor

Repair Mechanisms in Carcinogenesis

James D. Regan
J. S. Cook

RNA Tumor Virus – Cell Biology

R. W. Tennant
G. C. Lavelle
J. A. Otten
J. M. Quarles, Jr.[a]

Enzymology of Carcinogenesis

Wen-Kuang Yang
D. W. Fountain[a]
Ih-Chang Hsu[a]
Beth C. Mullin[a]
G. David Novelli
L. C. Waters

Regulation of Gene Expression

F. T. Kenney
J. G. Farrelly
J. N. Ihle
Kai-Lin Lee
Nicholas Pomato[a]

*Research sponsored by the National Cancer Institute.
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A SENSITIVE ASSAY FOR RESPIRATORY CARCINOGENESIS, USING TRANSPLANTED TRACHEAS

R. A. Griesemer, Paul Nettesheim, D. H. Martin, and J. E. Caton, Jr.*

In most experimental studies on respiratory carcinogenesis, large amounts of carcinogens have been required to produce neoplasms. This study was designed to determine the sensitivity of a well-defined respiratory tract tissue, the tracheal transplant, to carcinogens.

Entire tracheas were grafted subcutaneously in isogenic rats or hamsters. When the grafts were established, pellets of 7,12-dimethylbenz[*a*]anthracene (DMBA) or benzo[*a*]pyrene (BP) in beeswax were inserted. The treated grafts were collected at intervals and examined microscopically.

Assay of pellets for the amount of carcinogen remaining when the animals were killed permitted measurement of total dose delivered to the graft and the dose rate. Analysis of the data revealed a rate of release of carcinogen from beeswax that approached first order. The amount of carcinogen released was concentration dependent (1.7%/day for DMBA and 2.2%/day for BP).

In grafted rat tracheas, toxic effects were produced by pellets containing as little as 10 μg of DMBA; 280 μg delivered over 60 days produced 100% carcinomas in 60 days. BP was also toxic in the low-microgram range; the 100% tumor dose was approximately 1 mg delivered over six months.

Hamster tracheal transplants were highly sensitive to the toxic effects of DMBA. Pellets containing 1.0 μg of DMBA destroyed nearly the entire mucosal epithelium. BP was tolerated up to several hundred micrograms, but toxic effects were severe. Carcinoma induction is still under study. The results are compatible with previous studies in which we showed considerable species variations in penetration of airway epithelium by carcinogens and in distribution of experimentally produced tumors in the respiratory tract.

Assay in the tracheal graft appears comparable in sensitivity to skin testing and far more sensitive than intratracheal or inhalation exposure methods.

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COMPARATIVE EFFECTS OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE AND BENZO[*a*]PYRENE ON TRACHEAL GRAFTS

R. A. Griesemer, Paul Nettesheim, D. H. Martin, and J. E. Caton, Jr.*

We have recently reported the feasibility of transplanting entire tracheas to subcutaneous sites on isogenic recipient animals.

When rat tracheal grafts were exposed to beeswax pellets containing 0.5 to 3.0 mg of DMBA, the mucociliary epithelium was replaced by squamous metaplasia in one week. Keratinizing squamous metaplasia became progressively more severe over the next several weeks and developed into invasive squamous carcinoma. All of 104 grafts collected after two months or longer exposure to DMBA were carcinomatous regardless of dose.

The response of rat tracheal grafts to BP was qualitatively different from that to DMBA. When exposed to beeswax pellets containing 0.5 to 3.0 mg of BP, the tracheal mucosa underwent initial mild hyperplasia with subjacent lymphoreticular proliferation and myxoid degeneration of connective tissue. One month later the hyperplastic epithelium was replaced by a single cell layer of dystrophic and atrophic epithelium with considerable cellular atypia. The thin, dystrophic epithelium persisted through six months of BP exposure. Eight months after the start of the experiment, invasive carcinomas were found in all remaining grafts.

The commonly accepted preneoplastic morphogenic sequence of metaplasia, dysplasia, and carcinoma *in situ* was not observed in either experiment.

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DEVELOPMENT OF AN EPITHELIAL-CELL CULTURE SYSTEM FOR DETECTING TRANSFORMATION IN EXPOSED TRACHEA

Ann C. Marchok, Joyce C. Rhoton, and Rhonda F. Irwin

We are exploring the possibility of using the change in growth behavior of cell outgrowth from tracheal explants as a system for detecting transformation in carcinogen-exposed tracheas. The method consists in placing pieces of trachea, epithelial side up, on the bottom of tissue culture dishes and allowing cells to migrate out from the explant. In our system, most of the outgrowth consists of epithelial cells which initially show some differentiated properties such as motile cilia

as well as cell division. The epithelial cells typically increase tremendously in size and become multinucleate before sloughing from the dish. After removal from the original outgrowths, the explants can usually be used to produce several new outgrowths of epithelial cells before fibroblasts become predominant. However, we can selectively remove most of the fibroblasts from the cell cultures by short exposure to low concentrations of trypsin.

At present we are using rat tracheal transplants treated for short periods of time with pellets containing carcinogen as a source of precancerous and cancerous lesions. After exposure to the carcinogens, the outgrowth from cultured pieces of the tracheal transplants are being scored for extent of outgrowth and changes in cellular morphology under selected culture conditions. In this manner the stability of the lesions in different environments can be determined. These cell outgrowths will also be tested for tumorigenicity by implantation in the kidney capsules of isogenic host rats.

EFFECTS OF VITAMIN A ON PROLIFERATION AND DIFFERENTIATION IN TRACHEAL ORGAN CULTURES

Ann C. Marchok, Joyce C. Rhoton, Rhonda F. Irwin, and M. Virginia Cone

Last year we described a tracheal organ-culture system we developed for the purpose of studying normal and abnormal epithelial tissue differentiation *in vitro*. One way we are utilizing this system is to investigate further the effects of vitamin A (all-*trans*-retinol) on cell proliferation, secretory cell activity, and inhibition of squamous metaplasia.

We have reported that 0.2 and 2.0 μg per ml of medium stimulates [^{3}H] thymidine incorporation, cellular hyperplasia, and hypersecretory activity in tracheal explants cultured in Waymouth's MB 752/1 medium supplemented with 10% horse serum.[1] As a continuation of these studies, the following series of experiments are being carried out. (*a*) The effects of 0.2 and 2 μg of retinol per ml are being tested on tracheal explants cultured in Waymouth's medium supplemented with low serum (2%) or in the defined medium alone. In both cases a marked increase in [^{3}H] thymidine-labeled epithelial cells is found on autoradiographs, and cellular hyperplasia and hypersecretory activity are stimulated. (*b*) We are carrying out these kinds of analyses using tracheal explants from 11- to 12-week-old normal rats and rats maintained on vitamin-A-free diet after weaning. In the deficient animals, vitamin A is down one-half in the serum, barely detectable in the liver, and at least 6 times lower in the lungs. Using these animals, vitamin A doses (0.02 to 2.0 μg of retinol per ml) are being tested in several different types of media. One very striking finding in these experiments is the effect of insulin and hydrocortisone media supplements on epithelial tissue differentiation *in vitro*. Keratinization is much more extensive on explants cultured in this medium, and tracheal explants from the vitamin-A-deficient rats exhibit a greater degree of squamous metaplasia and keratinization earlier in culture. Vitamin A supplements inhibited the squamous metaplasia and maintained a mucociliary epithelium with prominent secretory cells in tracheal explants from the normal and deficient animals. (*c*) A vitamin-A-reversal experiment is also in progress. In this study, tracheal explants were cultured in Waymouth's medium plus 0.1 μg of insulin and 0.1 μg of hydrocortisone per ml, or in Waymouth's medium plus low serum (2%) for 57 days. Vitamin A concentrations of 0.02 μg or 2.0 μg/ml were then added. It remains to be determined whether these concentrations of vitamin A can reverse the squamous metaplasia that should be found on explants cultured in serum-free medium and/or whether hypersecretory activity can be induced in explants cultured in the low-serum medium.

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EFFECT OF RETINYLACETATE AND RETINOIC ACID ON DEVELOPMENT OF CARCINOGEN-INDUCED METAPLASTIC LUNG NODULES

Paul Nettesheim and Mary L. Williams

We have previously reported that rats maintained on different levels of retinylacetate (RA) show different sensitivities to lung cancer induction. Animals on low RA intake show an increased sensitivity when compared with rats on high levels of RA. However, it was not clear from the previous study whether this was indicative of a state of increased susceptibility in deficiency and its mere correction by addition of vitamin A, or whether a general anticarcinogenic effect of vitamin A was suggested.

In a new study, three groups of rats were maintained (beginning at 12 weeks of age) on a diet providing 250–300 μg of RA per week. In addition, two of the three groups received daily doses of either 1500 μg of RA or 1000 μg of 13-*cis*-retinoic acid. A fourth group was kept on a vitamin-A-free diet from 12 weeks of age on. All rats received a carcinogenic dose of 3-methylcholanthrene given by intratracheal injection and were

killed 16 weeks later to score number and size of the carcinogen-induced lung nodules which are the precursor lesions for the later-developing squamous-cell carcinomas. We found that the amount of metaplastic tissue in the lungs of rats on vitamin-A-free diet was three to four times larger than that in the rats of the other groups. This study thus strongly supports our earlier finding which suggests that inadequate vitamin A intake creates a state of increased susceptibility to lung cancer induction, which can be corrected by adding moderate amounts of vitamin A to the diet. However, contrary to reports from other laboratories, our studies do *not* show an inhibitory effect on pulmonary carcinogenesis by pharmacological doses of retinoids.

ELUTION OF CARCINOGEN FROM CARRIER PARTICLES IN THE RESPIRATORY TRACT OF MICE

D. A. Creasia

Effect of carrier particle size. — Several studies from other laboratories have reported an increased retention in the lung and/or a greater tumorigenic efficiency when BP is intratracheally administered together with particulate material. However, studies with certain carrier particles (carbon, in particular) have shown that increased carcinogen retention does not necessarily result in increased tumorigenicity. A possible explanation for the discrepancy is that under certain conditions the carcinogen is *not* released from the carrier particle and therefore is not available to interact with tissue. We previously reported on the development of a method to simultaneously measure the pulmonary clearance of the carrier particle and the elimination of the carcinogen (adsorbed to the carrier particle) from the lung. Briefly, the method consists in labeling carbon particles (to which BP has been adsorbed) with ^{103}Ru, a gamma-emitting radioisotope. This methodology was used to show that the *in vivo* release of BP from two different carbon particle sizes (0.5–1.0 μ and 15–30 μ) was vastly different. When BP adsorbed to 0.5- to 1.0-μ carbon particles was administered intratracheally to mice, 50% of the initial lung burden was eliminated at about 36 hr, and 90% was eliminated at about four

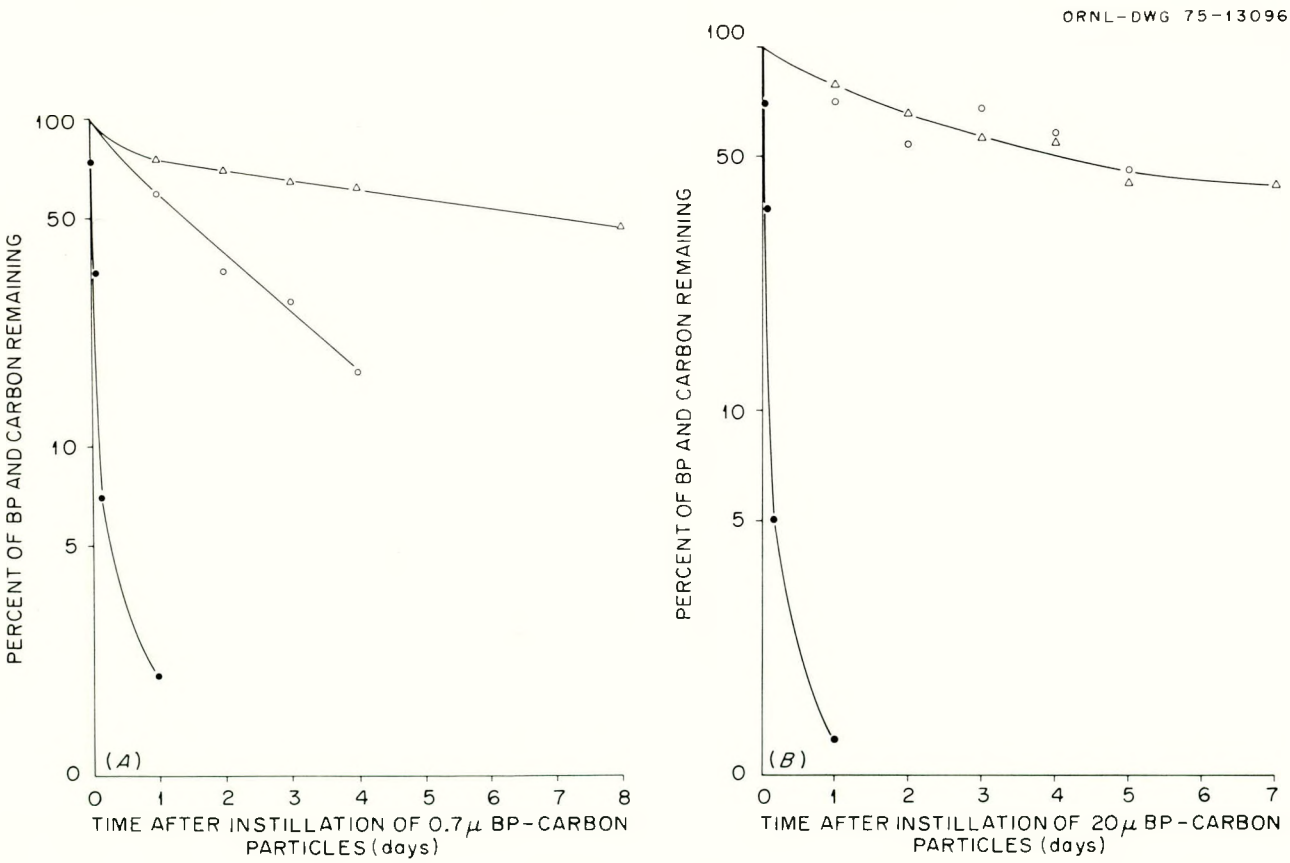

Fig. 18. Elimination of BP from the respiratory tract of mice. Mice were intratracheally injected with BP crystals alone or with BP crystals adsorbed to (*A*) 0.7-μ or (*B*) 20-μ carbon particles. Data is expressed as percent of the initial lung burden. •, BP crystals alone; ○, BP eluted from carbon particles; △, carbon particles.

days. At seven days, 50% of the carbon particles were eliminated (see Fig. 18*A*). This indicates that the BP is being eluted from the carbon while in the lung. The elution rate is approximately 16%/day for at least four days. This means that there is always a steady, relatively constant quantity of free carcinogen that persists in the lung for four days. In contrast, when the same quantity of free BP crystals (also 0.5–1.0 μ) is intratracheally administered, 50% is eliminated within 1.5 hr and >90% eliminated within one day. These facts are important with regard to carcinogen activity, since persistent carcinogen contact is probably more conducive to tumorigenic activity than a rapid acute carcinogenic exposure.

In contrast to the elimination rate of free BP crystals and BP adsorbed to 0.5- to 1.0-μ particles, the elimination from the lung of BP adsorbed to 15- to 30-μ particles is much slower. In this case <50% of the BP is eliminated at four days (see Fig. 18*B*). This is almost the same pulmonary clearance rate as for the carbon particles, indicating that the BP is *not* dissociating from the carbon and therefore relatively little is free to interact with tissue.

It is noteworthy that with tumorigenic studies, both free BP crystals and BP adsorbed to 15- to 30-μ carbon have a low carcinogenic activity, while adsorption of BP to 0.5- to 1.0-μ carbon particles produces an enhanced carcinogenic activity.

Effect of respiratory infection. – In the past we have shown that severe viral pneumonitis causes a lasting defect in the respiratory clearance of insoluble particles. Utilizing our ^{103}Ru-labeling technique described above, we studied the effect of viral infection on the rate of elimination of BP from mouse lungs. When the 0.5- to 1.0-μ BP carrier particle is intratracheally administered two days prior to the peak pneumonitis induced by the infection, there appears to be an enhanced elimination of BP from the lung (see Fig. 19). Within 24 hr, 75% of the initial dose is eliminated. Uninfected controls eliminate 40% of the BP within 24 hr, and 75% is eliminated by three days after instillation. A massive proteinaceous exudate is produced in the lung as a result of the viral infection. It is conceivable that this proteinaceous exudate enhances the dissociation of BP from the carbon carrier particle.

There was no apparent effect from the virus infection on the pulmonary clearance of carbon particles during the seven-day observation period. Based on our previous clearance studies with insoluble particles, seven days for carbon-particle clearance is too short an interval for an effect to be noted.

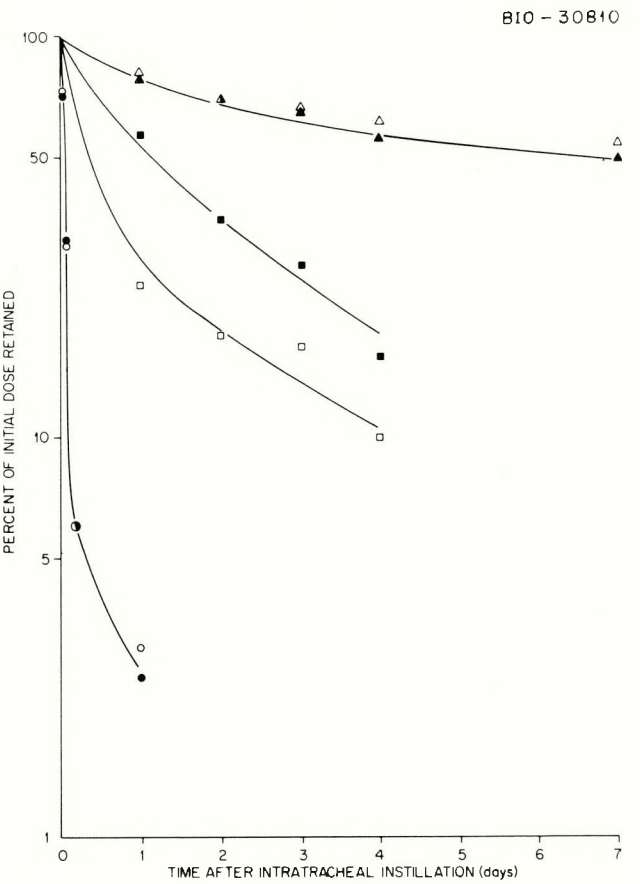

Fig. 19. Elimination of BP from the respiratory tract of PR8-infected and noninfected mice. The mice were intratracheally injected with BP crystals alone or with BP crystals adsorbed to 0.7-μ carbon particles. Data are expressed as percent of initial lung burden. ●, BP crystals alone, noninfected mice; ○, BP crystals alone, infected mice; ▲, carbon particles, noninfected mice; △, carbon particles, infected mice; ■, BP eluted from carbon particles, noninfected mice; □, BP eluted from carbon particles, infected mice.

TOBACCO SMOKE BIOASSAY PROJECT

W. E. Dalbey, R. A. Griesemer, and Paul Nettesheim

There has been a need for a bioassay of the impact of chronic tobacco-smoke exposure directly on respiratory tract tissues. The problem has been approached here in two ways: inhalation exposure of rats and delivering smoke to subcutaneously transplanted tracheas.

Small laboratory animals are to be preferred for such bioassays because of economical and logistical considerations. The only small laboratory animal that had been systematically explored for inhalation exposures was the hamster. The only significant lesions induced in these animals were laryngeal leukoplasias and carci-

nomas and accumulation of pulmonary macrophages in the lung. Chemical carcinogenesis studies suggested that the rat might be significantly more sensitive to cancer induction in the respiratory tract than the hamster. However, the rat has not been used extensively in smoke inhalation studies because of its greater susceptibility to respiratory tract infections and acute cigarette-smoke toxicity.

A series of acute experiments led to a feasibility study for the use of rats as an inhalation bioassay model for chronic cigarette exposures. Female SPF rats were exposed to 10% smoke from standard test cigarettes on an intermittant smoking machine. Deposition of [^{14}C]dotriacontane ([^{14}C]DTC) from labeled cigarettes was measured simultaneously in rats and hamsters for comparison. Total deposition was 1.43 ± 0.09% and 1.29 ± 0.13%, respectively, of the total dose offered the animals; pulmonary deposition was 0.90 ± 0.07% and 0.82 ± 0.11% respectively. And, as in hamsters, respiratory minute volume greatly decreased (to 25% of original) during smoke exposure of rats. With appropriate use of gradually increasing exposure regimens and breaks between individual cigarettes, rats have been exposed to up to ten cigarettes/day for several months. Accumulation of [^{14}C]DTC from ten successive cigarettes in one day was about ten times that after one cigarette, although lung retention was about eightfold. [^{14}C]DTC distribution the following day indicated slow clearance of label from the deep lung to the gut. Blood carboxyhemoglobin remained under 30% during the day's exposure to ten cigarettes and had returned to normal levels the following morning.

The importance of preexposure in survival of acute smoke exposure was demonstrated by deaths of seven of ten naive rats after exposure to five consecutive NCI code-16 cigarettes on the half-hour, compared with deaths of five to ten rats preexposed for three weeks after 19 cigarettes. Blood carboxyhemoglobin did not account for the difference. Therefore gradually increasing exposures were used to bring 160 rats to either seven or ten cigarettes/day as part of a chronic inhalation experiment. Eighty of the rats will be exposed to seven cigarettes/day until death. The remainder are split into those receiving seven and ten cigarettes/day, both to be killed after 12, 18, and 24 months of exposure. After killing, extensive biochemical tests on urine and blood, cardiovascular and pulmonary function tests, and histological examination will be made to assess the overall impact of smoke exposure.

To date, smoke-exposed rats have exhibited low but stable body weights and occasional hindlimb ataxia of unknown origin.

Of rats chronically exposed to seven and ten cigarettes/day, 5 and 20% respectively have died spontaneously after 15 months. At autopsy, lesions have been observed only in the respiratory tract. Histologically, these lesions included occasional mild focal hyperplasias of the upper airways, bronchiolitis of the terminal bronchioles, and alveolitis.

Tracheas transplanted subcutaneously between inbred rats show promise for assaying carcinogenicity of whole smoke without the limiting complication of toxicity seen in inhalation exposures. Smoke dose per unit area of epithelium probably can be greatly increased. Initial acute exposures resulted in hyperplastic transitional epithelium. A new modified Hamberg II machine is being used for a longer-term feasibility study.

MECHANISMS OF CHEMICAL CARCINOGENESIS

G. M. Singer

During the past year we have made appreciable progress in several areas related to nitroso compound carcinogenesis. Development of our procedure for determining naturally occurring nitrosatable secondary amines has been completed, and the method has been applied to a variety of foodstuffs, tobacco, and cigarette smoke condensate ("tar"). We find that, as expected, dimethylamine is generally the most prevalent amine, but that its concentration varied widely depending on the material. In a number of fish samples, for example, the range is more than 1000-fold. Among the other amines found, morpholine was the most common; in fact, it was ubiquitous. This was surprising, since its natural occurrence had not previously been reported. (The artifactual formation of morpholine has been ruled out.)

We are studying the mechanism of nitrosation of tertiary amines, a problem which is still unresolved despite the widespread occurrence of this class of compounds and their known ability to form carcinogenic nitrosamines, both *in vitro* and *in vivo*. The reaction is second order in nitrous acid, in the established second-order dependence in secondary amine nitrosation. Further kinetic and product studies are continuing in an attempt to elucidate completely this mechanism.

With the aid of the Laboratory's recently acquired XL-100 Nuclear Magnetic Resonance Spectrometer and its associated computer data system, conformational analysis studies of alicyclic nitrosamines have been carried out to seek any possible correlation with relative carcinogenicities. These studies are almost complete.

The NMR spectrometer will be used in the near future to study hydrogen-deuterium exchange kinetics of nitroso compounds to investigate the possibility of carbanion participation in the mechanism of carcinogenesis.

High-pressure liquid chromatographic techniques are being developed for the analysis of complex thermally unstable, nonvolatile *N*-nitroso compounds and/or their precursors, which are often important pesticides and herbicides. Rapid, accurate separation techniques have been worked out. More importantly, a new, sensitive, nitroso-compound detector is being developed, and initial results are very encouraging. This flowing wet-chemistry detector will detect microgram quantities of, for example, nitrosocarbamates which cannot be readily analyzed by gas-liquid chromatography. Refinements of the detector are in progress.

RELATION BETWEEN STRUCTURE AND CARCINOGENIC ACTIVITY OF *N*-NITROSO COMPOUNDS

William Lijinsky and H. W. Taylor

It has become obvious that small changes in chemical structure of nitrosamines and other *N*-nitroso compounds can make a large difference in carcinogenic activity, either in potency or in target organ of the carcinogen. By comparing the potencies of several nitroso compounds of similar structure, some indications can be gained of the chemical characteristics related to carcinogenicity and hence inferences about the mechanism of their action. Several nitroso compounds have been given to rats at the same molar concentration for the same time period, and the survival and tumor incidence in the animals have been measured. Dichloronitrosopiperidine is much more potent than nitrosopiperidine, and dibromonitrosopiperidine is a little less potent than the dichloro compound, all three giving rise to esophageal tumors in high incidence. Similarly, dichloronitrosopyrrolidine is much more potent than nitrosopyrrolidine, causing esophageal tumors rather than liver tumors, and nitrosonornicotine, another derivative of nitrosopyrrolidine present in tobacco smoke, causes nasal cavity tumors, again with higher potency than nitrosopyrrolidine. Nitroso-2,5-dimethylpyrrolidine was noncarcinogenic, showing that blockage of the alpha carbon atoms reduced carcinogenic activity. The reduced carcinogenicity of alpha-deuterium-labeled nitrosomorpholine compared with the unlabeled compound, together with the above results and the greatly increased carcinogenicity of 2,6-dimethylnitrosomorpholine (with methyl groups in the beta position), all point to activation of hydrogens alpha to the nitroso group as a rate-limiting step in carcinogenesis by nitrosamines.

The increased carcinogenicity of nitrosotetrahydropyridine compared with nitrosopiperidine, and the liver tumors induced in rats by the former but not the latter, together with the noncarcinogenicity of the closely related nitrosoguvacoline (3-carboxymethylnitrosotetrahydropyridine), are difficult to explain in this way and undoubtedly involve other factors.

STUDIES ON TUMORIGENESIS BY NITROSOMETHYLDODECYLAMINE

H. W. Taylor

In previous studies, nitrosomethyldodecylamine induced transitional cell carcinomas of the urinary bladder in Sprague-Dawley rats. It has also been shown that urinary bladder epithelium in rats can be transplanted to a subcutaneous position and will develop into "pseudo-bladders" lined by a typical transitional type of epithelium. Animals bearing such aberrant bladders are being fed nitrosomethyldodecylamine to determine if these ectopic bladders will be affected similarly to the natural bladder. Systemic effects of the compound can thus be distinguished from the local action of some possible metabolite in the urine of treated animals.

SYSTEMIC EFFECT OF NITROSOHEPTAMETHYLENEIMINE ON TRANSPLANTED AND HOST TRACHEAS OF RATS

H. W. Taylor, R. A. Griesemer, and Paul Nettesheim

Nitrosoheptamethyleneimine (NHMI), which is known to induce neoplasia in the trachea and lung of rats, was given by gavage to F-344 rats bearing subcutaneous transplanted tracheas. The effects of this nitroso compound on the host tracheas and on the transplanted tracheas were compared at five-week intervals up to 30 weeks. The initial effects on host tracheas were hyperplasia and metaplasia to stratified squamous epithelium, accompanied by a lymphocytic infiltrate. Later, hyperplastic mucociliary epithelium returned to most of the tracheal lining, except for isolated foci. Finally, polyps, papillomas, and squamous cell carcinomas developed. The effects on the transplanted tracheas were those of atrophy and dystrophy, with no

neoplastic changes. These studies strongly suggest that the effect of NHMI on the trachea involves more than a simple organotrophic action. Some metabolite may be formed in the lung, or may be formed elsewhere and removed by way of the lung and trachea, and exert its action locally on these organs.

STUDIES ON METABOLISM OF DIMETHYLNITROSAMINE IN RATS

H. W. Taylor, Catherine Snyder, and William Lijinsky

We have initiated metabolic studies with tritium-labeled dimethylnitrosamine (DMN). The distribution of DMN among the five liver lobes at various time intervals after oral feeding of the nitrosamine has been determined, as well as its distribution in four liver tissue fractions: acid-soluble, lipid, protein, and nucleic acid. Distribution in the lobes is approximately equal after recirculation time. Although most of the liver radioactivity remains in the acid-soluble fraction throughout the first 24 hr after oral administration of tritiated DMN, a significant portion of label (>30%) is in the lipid fraction after the 1-hr time point. Work is under way to determine the location of the label within the various lipid classes. Methodology is also being developed for studying the morphologic distribution of the label through autoradiography. Because DMN is soluble both in water and in nonpolar solvents, frozen tissues must be cut in a cryostatic microtome and freeze-dried under vacuum before they can be used to expose photographic film in a meaningful manner. We are, in addition, studying techniques for separating Kupffer cells and hepatocytes and quantitating the labeled DMN in each cell type at periods after carcinogen administration.

HISTOGENESIS AND BIOLOGY OF HEPATIC TUMORS INDUCED BY DIMETHYLNITROSAMINE IN RATS

H. W. Taylor

Several studies were performed in an effort to help clarify the cellular origin of tumors induced in the liver by DMN. In metabolic studies of DMN, it is imperative to know what cell type actually gives rise to tumors, so that relevant cellular metabolism can be elucidated. The influence of concurrent administration of CCl_4 and the influence of partial hepatectomy on DMN tumorigenicity have been studied. It appears that CCl_4 had little influence on the action of DMN, while partial hepatectomy increased the time-to-death with tumors induced by DMN. While these studies are not yet completed, the results suggest that tumorigenicity is not enhanced by a population of dividing hepatocytes. Histogenesis studies, in which animals were killed at four-week intervals after initiation of treatment with DMN, show that this compound is toxic to hepatocytes and that tumors probably arise from small foci of undifferentiated cells that arise in the periportal regions. These cells seem to have a pleuripotent potential; some foci later resemble small oval hepatocytes, some areas proliferate as endothelial cells that are capable of phagocytizing carbon particles, and cells in some foci acquire cilia and resemble primitive cholangiolar cells. Transplantation studies with these tumors are under way, as are histoenzyme studies.

FURTHER CLASSIFICATION OF CHEMICAL CARCINOGENS ACCORDING TO THE MODE OF DNA REPAIR THEY INDUCE IN HUMAN CELLS

James D. Regan and A. A. Francis

Using the bromodeoxyuridine photolysis assay, we have previously described in detail the two modes of DNA repair which occur in human cells.[1] They are short (ionizing-type) repair and long (UV-type) repair. These two modes are observed in human cells after treatment with a variety of chemical carcinogens and mutagens. We have extended these studies to include a number of other chemical carcinogens representative of certain classes of chemicals having similar structures but varying in their ability to induce cancer. Results of these studies (along with our previous results for comparison) are shown in Table 34. 7-Bromomethylbenzanthracene at 10^{-5} *M* induces about four breaks per 10^8 daltons after BrdUrd incubation and 10^6 ergs/mm^2 of 313-nm radiation. The activity of this compound in regard to repair in xeroderma cells has not yet been tested. It is conceivably a complex agent similar to 4-nitroquinoline-1-oxide (4NQO) which produces more than one type of damage. The K-region epoxide, BP 3,4 epoxide appears to be a classical short-type agent similar to previous short-type agents we have described. The non-K-region epoxide of BP, the 7,8 epoxide, has a complex interaction with DNA in the presence of the 313-nm light and appears to induce DNA cross-links. The dimethylbenzanthracene 5,6 epoxide appears to be a long-type agent, and its repair in xeroderma cells is defective. Dr. James A. Miller supplied 1,1-bis(phenyl)-2-propynyl-*N*-cyclohexylcarbamate, which we find to be a classical long-repair-inducing agent with defective repair in xeroderma cells.

Table 34. Classification of chemical carcinogens according to the mode of repair they induce in human cells

Agent	Dose	Duration (min)	Repair period (hr)	No. excess strand breaks per 10^8 daltons after BrdUrd incubation and 10^6 ergs/mm^2 313-nm radiation[a]	Type of repair
Ultraviolet light (254 nm)	200 ergs/mm^2	0.4	20	10	Long
γ rays (^{60}Co)	10 krads	4	0.5–1	0.6	Short
EMS	10^{-2} *M*	120	2	1.5	Short
			20	1.5	Long
MMS	5×10^{-5} *M*	5	1	0.4	Short
N-acetoxyacetoaminofluorene	7×10^{-6} *M*	60	20	4	Long
Propane sulfone	2×10^{-4} *M*	120	2	0.4	Short
ICR-170	10^{-6} *M*	60	20	1	Long
4NQO	5×10^{-7} *M*	90	1.5	?	Short
			18	~2	Long
7-Bromomethylbenzanthracene	10^{-5} *M*	90	18	~4	?
BP 3,4 epoxide (K region)	2×10^{-5} *M*	90	18	0.6	Short
BP 7,8 epoxide (non-K region)	5×10^{-5} *M*	90	18	Cross-links DNA	?
Dimethylbenzanthracene 5,6 epoxide	10^{-5} *M*	90	18	1.4	Long
1,1-Bis(phenyl)-2-propynyl-*N*-cyclohexylcarbamate	5×10^{-5} *M*	90	18	0.5	Long
1′-Acetoxysafrole	5×10^{-5} *M*	90	18	0.4	Short

[a] $2 \times \Delta\ 1/M_w$; $\Delta\ 1/M_w = (1/M_w)_B - (1/M_w)_T$.

A similar compound, but having one less benzene ring, is 1-acetoxysafrole. This agent, by contrast, is a short-repair-type agent which produces repair patterns identical in normal and xeroderma cells, even at long times, *i.e.*, 18 hr, after damage. We are continuing our studies with these agents and will further characterize them, particularly with regard to the number of nucleotides inserted in the average repaired region and the level of repair in the xeroderma pigmentosum cells.

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DNA DAMAGE AND REPAIR BY 4-NITROQUINOLINE-1-OXIDE IN NORMAL AND RAUSCHER LEUKEMIA-VIRUS-INFECTED RAT CELL LINES

Raymond Waters,* F. M. Faulcon, Giampero DiMayorca,† and James D. Regan

4-Nitroquinoline-1-oxide is a potent carcinogen[1] and produces damage to DNA either directly or via a metabolite. Primarily purines, especially guanine, are attacked.[2] A tautomeric form of deoxyguanosine that will pair with deoxythymidine is produced. Hence the G-T gives rise to a GC and an erroneous A-T at the next replication.[3] In both bacterial[4–6] and human[7,8] cells, a considerable amount of this damage is removed via the excision-resynthesis mechanism.

Rodent cells, unlike those of humans, are unable to excise more than a small percentage of UV-induced pyr()pyr. However, they are able to tolerate a certain number of pyr()pyr because they possess a postreplication repair mechanism similar to that already described in human cells.[9,10] Colony-forming ability in the R111 rat cell line is three times more sensitive to 4NQO treatment than is the F111 cell line. The sole difference between the two cell lines is that R111 has a Rauscher leukemia virus integrated into its genome. Hence the presence of this virus has conferred a sensitivity to 4NQO. Some differences in prereplication repair after 4NQO treatment are observed. The virus-infected cell appears (by bromodeoxyuridine photolysis assay) to be somewhat defective in this repair mode, although the magnitude of these differences, while consistent, is not great. We are now in the process of

studying whether the postreplication repair of 4NQO damage in the R111 and F111 cell lines is influenced by the presence of this cancer virus and, if so, to what extent.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

†University of Illinois School of Medicine, Chicago, 60612.

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EFFECTS OF ISOMERIC METHYLCHRYSENES ON DNA OF HUMAN CELLS

A. A. Francis and James D. Regan

Methylchrysenes are present in tobacco smoke and in lower concentrations in other pollutants, including gasoline engine exhaust. Various isomeric forms of methylchrysenes have been tested for carcinogenicity in mice; among these the 5-methyl isomer was found to be a strong carcinogen, whereas the 4-methyl isomer was inactive.[1] While investigating the effects of these two methylchrysene isomers on the repair of DNA in human cells using the 5-bromodeoxyuridine photolysis technique,[2] we found that these chemicals cross-link the DNA photodynamically.

Cells are labeled with [^{3}H]thymidine and [^{14}C]thymidine and are exposed to 200 ergs/mm^2 of 254-nm radiation. The ^{3}H-labeled cells are then allowed to undergo repair replication in the presence of BrdUrd, the ^{14}C-labeled cells with thymidine. After a repair period of 18 hr, the cells are mixed and irradiated with 313-nm light, which photolyzes the BrdUrd-containing DNA. The cells are lysed on alkaline sucrose density gradients and the difference in the molecular weights used to follow the repair process. Inhibition of repair can be tested by adding various concentrations of methylchrysenes to the cells during the period of repair replication.

The 5-methylchrysene appeared to be a strong inhibitor of repair of 254-nm radiation-induced damage at a dose of 10^{-5} *M*. The 4-methyl isomer was less active but still inhibitory. Further investigation, however, has revealed an increase in molecular weight of the control DNA from mol wt = 230×10^6 to mol wt $\cong 439 \times 10^6$ in the samples receiving both methylchrysene and 6×10^5 ergs/mm^2 of 313-nm radiation, indicating photodynamic cross-linking of this DNA. This shift was seen with both isomers, but, since molecular weights this large or larger are not resolved on these gradients, any differences in cross-linking between the methylchrysenes were not measured. The apparent differences in repair replication inhibition shown by these two compounds could be artifactual in an assay and actually due to differences in cross-linking characteristics of the different isomers. These photodynamic cross-linking characteristics are now being investigated.

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COUPLING OF TRANSPORT IN CULTURED HUMAN CELLS

Emily Tate Brake and J. S. Cook

Changes in the Na^+-K^+ transport system may be indirectly linked to uptake of other metabolites; specifically, amino acid uptake may be linked to the membrane potential or to the Na^+ gradient.

With the nontransformed human strain HSWP we have been measuring the transport response to serum stimulation of quiescent cells. Our preliminary results are that the K^+ turnover doubles in both the transport and leak components within minutes of stimulation, and, as reported last year, these remain at the elevated level and proportional to cell size throughout the subsequent cell cycle.

The quiescent cells, as observed by scanning electron microscopy, are quite flat and smooth. The early transport changes are *not* accompanied by an increase in microvilli that might indicate an increase in surface area; the microvilli arise later in the cell cycle. Since this stimulated transport yields no net change in cell electrolytes, it has no immediately obvious physiological significance. However, the enhanced passive K flux suggests an enhanced membrane potential, which in

turn would increase the electrochemical force on Na^+ and on any Na^+-gradient-coupled transport such as that of α-aminoisobutyric acid (AIB). An enhanced uptake of AIB in stimulated cells is in fact observed. The serum-stimulation effect can be mimicked by treating the cells with valinomycin, a drug which enhances the passive K flux; as expected, valinomycin also enhances AIB uptake in HSWP cells. This argument gives meaning to the repeatedly observed enhanced electrolyte fluxes in other stimulated systems such as PHA-stimulated lymphocytes and SV40-transformed 3T3 cells.[1-3]

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TURNOVER OF OUABAIN-BINDING SITES IN HeLa CELLS

J. S. Cook, Emily Tate Brake, and P. C. Will*

Using [^{3}H] ouabain both as a ligand to find and count Na^+-K^+ transport enzyme and as a specific inactivator of that enzyme, we have continued the study of the turnover of ouabain-binding sites on the HeLa cell surface. We make extensive use of the Atkinson-Summers sucrose-gradient technique[1] for isolation of the membranes, which may be prepared in less than 1 hr. Since the isolation is carried out in the cold, virtually no bound ouabain is lost during this time. The preparation contains 1–4% cell protein, no detectable glucose-6-phosphatase, less than 2% of the total acid-phosphatase, and a very small amount of DNA; 5′-nucleotidase is enhanced 30- to 80-fold in specific activity. We use the latter as our quantitative marker.

As an explanation of our earlier data, we had suggested[2] that the inactivating effects of ouabain may specifically induce the enhanced synthesis of ouabain-binding sites; Boardman *et al.*[3,4] have concluded that such induction in fact occurs. Their argument is based (in part) on the fact that cells incubated for long periods in low ($<K_I$) concentrations of [^{3}H] ouabain for several days and then challenged with high (2×10^{-7} *M*) [^{3}H] ouabain for a few minutes show more cell-associated radioactivity than controls. We agree with the data, but find the excess ouabain to be inside the cell (internalization; see below). We have examined the induction question by treating cells as follows: (*a*) short challenge with 2×10^{-7} *M* ouabain (control for total number of binding sites); (*b*) growth of cells 48 hr in 10^{-8} *M* [^{3}H] ouabain, K_I being 3×10^{-8} *M*; (*c*) growth of cells as in *b* followed by challenge as in *a*. In each case, ^{3}H per cell was determined as well as ^{3}H per 5′-nucleotidase in the isolated membranes (Table 35). We conclude from this and similar experiments that the enhanced ouabain per cell does not reflect increased surface binding sites; in long-term experiments most of the [^{3}H] ouabain is not found with the membranes. Similarly, growth of the cells in low K^+ (0.5 m*M*) slows growth but does not increase ouabain-binding sites per nucleotidase on the membranes. We thus find no evidence for specific induction on the membranes of K^+-depleted cells. This is an important conclusion for what follows.

Cells pulse-labeled with [^{3}H] ouabain at low enough concentrations so that growth is not noticeably inhibited, slowly (hours) lose a fraction of the label into the medium by simple dissociation. This dissociation rate as measured by ouabain bound to isolated membranes is the same, within experimental error, as the rate measured in whole cells whose protein turnover is stopped by treatment with NaN_3 and 2-deoxyglucose. The latter treatment rapidly and effectively depletes cells of ATP, since 2-deoxyglucose is readily phosphorylated but yields no new ATP via metabolism. Allowing for this dissociation, we find that ouabain binding sites are taken into the cells at a rate of ~15%/hr. The [^{3}H] ouabain so internalized is found in the non-membrane fractions and is bound to a site of molecular weight >25,000; *i.e.*, it is too large to be free ouabain

Table 35. Short- and long-term [^{3}H] ouabain binding to HeLa cells

Treatment	^{3}H per cell (percent of control)	^{3}H per 5′-nucleotidase on isolated membranes (percent of control)
(*a*) 2×10^{-7} *M* [^{3}H] ouabain, 90 min (control)	100	100
(*b*) 2-day growth in 10^{-8} *M* [^{3}H] ouabain	60	18
(*c*) *b*, then *a*	146	99

but with sedimentation properties of a lysosome. At a rate about equal to the internalization rate, the ouabain is released within the cytoplasm, presumably by the degradation of the binding site. Eventually the drug escapes in its original form into the medium.

From the above, a three-compartment kinetic model has been constructed for flow of the drug after its binding to the cell surface, the compartments being membrane, cytoplasm, and medium. Given data only for the total cell-associated drug as a function of time, a computer program, CRICF,[5] has computed the optimal fit for all the kinetic and compartment parameters, yielding rate constants for dissociation, internalization, and escape which agree well with those independently derived from fractionation and ATP-depletion studies.

We conclude that the Na^+-K^+ transport enzyme on the surface of HeLa cells is turning over with a rate constant of ~0.07 hr^{-1}, or about twice per cell cycle, and that its synthesis is not enhanced or derepressed by depleting the cell of its alkali-cation substrate.

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GLUCOSE TRANSPORT IN CULTURED HUMAN CELLS

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We have used radioactive compounds to investigate binding of known inhibitors of glucose transport in the diploid human fibroblast HSWP. (This work was greatly impeded by the finding, since confirmed by the manufacturer, that commercially available [^{3}H]phloridzin and [^{3}H]phloretin are highly impure products. The phloridzin was initially 4% pure; further purification by the manufacturer gave a 100% pure contaminant with no detectable phloridzin. The phloretin is 90% exchangeable ^{3}H but can be readily purified by lyophilization.) Phloretin binds to HSWP cells with a two-component curve, the saturable component having a dissociation constant equal to the K_I for transport (10–15 μM). The amount bound at saturation is independent of whether transport is quiescent, as in glucose-fed confluent cells, or stimulated by serum or starvation. Binding is not inhibited by phloridzin, hexoses, or cytochalasin B, although diethylstilbesterol is as effective as phloretin itself. The number of phloretins per cell at saturation is 3×10^9, which far exceeds the number of all membrane proteins. Our working hypothesis is that phloretin (and diethylstilbesterol) do not interact with the transport molecules at their active site but specifically interact with the lipid matrix in which the transport proteins are embedded.

Cytochalasin B has a K_I for glucose transport of 0.1 μM in HSWP cells. Its ^{3}H derivative binds rapidly at all temperatures. At the K_I there are *ca.* 1.5×10^6 molecules bound per cell. The diacetate derivative of cytochalasin B binds very extensively but is inactive against glucose transport and does not affect cell morphology, even at high doses.

In glucose-starved cells, where the glucose transport system is stimulated (see last year's report), the morphological effects of cytochalasin B at 1 μM are readily demonstrated. Once the morphology is altered, removal of the cytochalasin B does not lead to reversal to normal morphology until glucose is restored to the medium. Presumably the restoration of normal microfilament structure requires glucose, whereas the other metabolites in Eagle's basal medium are not utilizable for this reversal; this observation is still under investigation.

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PROPERTIES OF AN INHIBITOR OF MOUSE LEUKEMIA VIRUS INFECTION ASSOCIATED WITH THE *Fv-1* GENE

R. W. Tennant, Bonnie Schluter,* F. E. Myer, Wen-Kuang Yang, and Arthur Brown*

The *Fv-1* locus of mice is the major determinant of resistance to most naturally occurring leukemia viruses. The two alleles of the gene, $Fv\text{-}1^n$ and $Fv\text{-}1^b$, reciprocally restrict N- and B-tropic leukemia virus infection *in vivo* and in cell culture. We have reported evidence for a cellular product, apparently specified by *Fv-1* gene alleles, which specifically and reciprocally transfers resistance to mouse leukemia viruses.[1] Inhibitors have also been obtained from soluble extracts of embryo cell cultures of several mouse strains, and the inhibition was specific for the mouse leukemia virus host range type restricted by the appropriate *Fv-1* allele. Inhibitory

activity could also be extracted from tissues of adult mice (liver, muscle, kidney) which reciprocally inhibited N- or B-tropic but not NB-tropic virus, which is not naturally sensitive to this gene.

Direct association of the inhibitor with the *Fv-1* locus was established by independently determining the segration of the alleles in the F_2 generation embryos of an $Fv\text{-}1^{nn} \times Fv\text{-}1^{bb}$ mating and the inhibitory activity of extracts obtained from each embryo. The products of five separate matings, comprising a total of 47 embryos, were retested for direct resistance to N- or B-tropic virus and the ability of extracts of cultured embryo cells to transfer resistance to either virus. Resistance to either virus designated the homozygous individuals, and resistance to both viruses designated the heterozygotes. The segregation of the alleles closely approximated the expected 1:2:1 ratio, and the inhibitory activity extracted from the cells segregated according to the *Fv-1* genotype.

Studies directed toward the mechanism of the *Fv-1* restriction and the nature of the inhibitor are in progress.

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EFFECT OF *Fv-1* GENE ON MURINE LEUKEMIA VIRUS PROVIRAL DNA SYNTHESIS

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We are currently investigating the steps in the replication cycle of murine leukemia viruses which may be sensitive to the *Fv-1* gene restriction. Earlier work from this laboratory has indicated that an early event after infection is probably involved.[1] Others have reported,[2–4] and we also have found, that the adsorption process is not involved in this host range restriction. Our aim now is to investigate the transcription of the infecting viral RNA into proviral DNA in cells which are permissive or restrictive. The process of reverse transcription seems a likely step for regulation of virus infection by the *Fv-1* gene. Our approach is to isolate low-molecular-weight DNA at early time points after infection and assay by hybridization for unintegrated proviral DNA. Our recent efforts are concerned with synthesizing and characterizing a ^{3}H-labeled complementary DNA (cDNA) to use as a molecular probe for the detection of viral DNA. The cDNA was synthesized by detergent-disrupted, purified AKR virus using a ^{3}H-labeled thymidine triphosphate and the other three nucleoside triphosphates unlabeled. Actinomycin D was used to decrease the transcription of a double-stranded product.

The cDNA product is 95% single stranded as assayed by hydroxyapatite chromatography, is approximately 3S in size, and has a specific activity of 4.8×10^7 cpm/μg. The cDNA has been hybridized to excess purified 70S AKR virus RNA, fractionated by hydroxyapatite, and alkaline hydrolyzed to remove the viral RNA. This virus-specific cDNA is the probe for detecting proviral DNA and will be used to assay virus expression in *Fv-1* permissive and nonpermissive cells.

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EARLY EVENTS IN MURINE LEUKEMIA VIRUS INFECTION OF CULTURED CELLS*

J. M. Quarles,† L. R. Boone,‡ D. P. Allison, and R. W. Tennant

Murine leukemia viruses show host-range restrictions which are determined at least in part by an active cell function which occurs after attachment and penetration of the virus into the cell. These mouse viruses may be classified as "ecotropic" if they will infect other mouse cells or as "xenotropic" if they infect only cells of species other than mice. Within the ecotropic class, the viruses are further subdivided into "N-tropic," those which infect cells of mice like the NIH/Swiss, and "B-tropic," those which infect cells like the BALB/c line.

We are attempting to determine whether these restrictions involve differences in the kinetics of attachment and penetration, in uncoating the virus after penetration, or in differential virus degradation in permissive and restrictive cells. Our approach has been to determine the fate of labeled virus during the early periods of infection of various cell types. Partially purified [^{3}H]uridine-labeled viruses of N type (AKR) and B type (BALB/3T3) were used. Preliminary work using labeled xenotropic virus from BALB cells has been inconclusive due to lack of a satisfactory source of

virus. We have verified the work of other laboratories which showed the restriction of N and B cells is not a function of the cell membrane. Attachment rates were essentially identical on these and several other cell types. There is some indication, however, that the virus may be less tightly bound by one human cell line (WI38) and may elute more easily from these cells than from mouse cells. This is being further investigated. We have prepared labeled virus preparations of several different known particle numbers, infectivity titers, radioactivity, and particle-to-infective-virus ratios. These reagents are currently being used to obtain kinetic data on the rate of attachment and degradation of virus and the number and specificity of the attachment sites of permissive and restrictive cells.

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EFFECTS OF SELECTED PESTICIDES AND THEIR NITROSATED DERIVATIVES ON CELL TRANSFORMATION AND RNA TUMOR VIRUS EXPRESSION

J. M. Quarles* and R. W. Tennant

Nitroso compounds are known to have carcinogenic activity in a wide variety of animal species, and several have been shown to have mutagenic and transforming activity in cell-culture systems. Certain nitroso compounds, particularly nitrosamines, may be important environmental carcinogens, due in part to their ready formation from nitrite and secondary amines under conditions similar to those found in the mammalian stomach and to their common use throughout the environment. For example, Elespuru *et al.*[1] reported the formation of *N*-nitrosocarbaryl from a common food additive, sodium nitrite, and the widely used pesticide carbaryl. Many other commonly used pesticides contain nitrogen atoms which can be nitrosated to form *N*-nitroso compounds which are similar in structure to known carcinogens. Therefore these compounds form a class of chemicals which are potentially hazardous to humans. Our preliminary investigations indicated that nitrosocarbaryl could induce morphological and biological transformation of cultured BALB/3T3 cells. We then initiated the testing of selected widely used pesticides and their nitrosated derivatives for both RNA tumor virus activation and transforming activity in the BALB/3T3 cell system. This test system was chosen because the cells are sensitive to potential carcinogens and also contain endogenous leukemia viruses which can be activated by chemicals such as halogenated pyrimidines and protein inhibitors.

In work completed to date we have found that nitrosation can cause profound changes in the biologic activity of a parent pesticide. Nitrosocarbaryl, but not carbaryl, caused morphological transformation of the BALB fibroblasts. The transformed cells differed from parental control cells by growth to higher saturation densities, loss of contact inhibition, change in morphology, and growth in soft agar (Fig. 20). The transformed cells formed tumors at the site of injection in normal newborn and irradiated weanling mice and athymic nude mice, but untransformed control cells did not form tumors. Neither carbaryl, nitrosocarbaryl, nor the

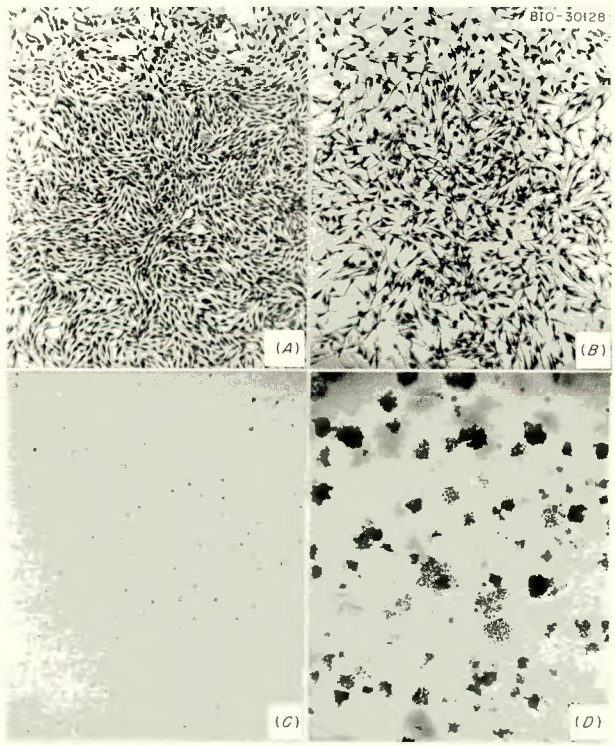

Fig. 20. (*A*) Control, untransformed BALB/3T3 cells. The cells are contact-inhibited, epithelioid in morphology, and show no crisscrossing. **(*B*) Nitrosocarbaryl-transformed BALB/3T3 cells.** The cells have lost contact inhibition, are more spindle-shaped than control cells, and are crisscrossed. **(*C*) Untransformed BALB/3T3 cells after 10-day incubation in soft agar.** These control cells show no growth, and only single cells or pairs of cells are visible. **(*D*) Nitrosocarbaryl-transformed BALB/3T3 cells after 10-day incubation in soft agar.** Cells have divided several times to form large visible colonies. All photomicrographs X100

nitrosated derivatives of the pesticides methomyl, bux-ten, and aldicarb induced activation of ecotropic or xenotropic murine leukemia viruses when tested for infectious virus and viral-antigen expression. It is possible, however, that only partial transcription of the viral genome was induced and the assays were not sufficiently sensitive to detect this activation. Assays for the transforming activity of these chemicals are in progress. It was shown that nitrosocarbaryl can transform BALB/3T3 cells to tumorigenic cells with altered biologic properties but without complete activation of murine tumor viruses in the transformed cells. Expression of viral antigen in the transformed cells was inducible by a halogenated pyrimidine, iododeoxyuridine, indicating that the endogenous viral genome was retained in an unexpressed state. Based on these studies, and its known mutagenic activity and structural similarity to the active carcinogen nitrosomethylurethane, we suggest that nitrosocarbaryl may also be carcinogenic *in vivo*.

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HOST-MEDIATED *IN VIVO–IN VITRO* ASSAY FOR CHEMICAL CARCINOGENS

J. M. Quarles,* Cynthia K. Schenley, and R. W. Tennant

The identification of potential chemical carcinogens requires rapid and effective screening tests. *In vitro* cell-culture systems, using morphologic changes in the cells as an indication of carcinogenicity, may not detect potential carcinogens which require metabolic activation. Dr. J. A. DiPaolo of the National Cancer Institute developed an assay system which provides the necessary metabolic activation and retains the advantages of *in vitro* culture systems. In this assay, pregnant hamsters are injected with the test chemical; fetuses, which have thus been exposed transplacentally to the chemical and its metabolic products, are removed and prepared as cell cultures. During subsequent passage of the fetal cells, transformed cells are detected and quantitated by examining colonies and scoring colony morphology.

We have conducted approximately 50 assays with 25 different chemicals and solvents using this system and have correlated morphological changes with growth in soft agar. A series of known positive chemicals [diethylnitrosamine, urethan, benzo(*a*)pyrene, nitrosomethylurethane] and negative controls (untreated and solvent treated) were used to standardize and evaluate the assay. The positive control chemicals yielded transformation rates as high as 5–6%, and the negative controls showed no transformation in more than 20,000 colonies examined. Clones of transformed cells are being tested for tumorigenicity in athymic nude mice. We are currently testing a series of unknown chemicals supplied by the NCI and a series of pesticides and their nitrosated derivatives provided by W. Lijinsky, ORNL. Preliminary results indicate that several nitrosated pesticides, including nitrosomethomyl, nitrosobaygon, nitrosobux-ten, and nitrosoaldicarb, cause morphological transformation and induce growth in soft agar.

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BIOCHEMICAL CHARACTERIZATION OF *Fv-1*-ALLELE CELL EXTRACTS WHICH INHIBIT MOUSE LEUKEMIA VIRUS INFECTION*

Wen-Kuang Yang, R. W. Tennant, Bonnie Schluter,† J. O. Kiggans,† F. E. Myer, and Arthur Brown†

We have previously demonstrated that mouse embryo culture cells which belong to a given *Fv-1* genotype and are susceptible to an ecotropic MuLV can be rendered partially resistant to the infection of this virus by treatment with cell-free extracts made from cells of a different *Fv-1* genotype which is resistant to the infection.[1] We describe our recent results concerning biochemical properties of the active components in the cell extracts.

Our initial studies of the inhibitory activity were all performed with embryo culture cells homogenized by sonication. Disruption of the cells by hypotonic treatment and Dounce homogenization was also found to be effective for the extraction of the inhibitor. Subcellular fractions were obtained by differential centrifugation of the cell homogenate. Subsequent tests showed that the inhibitor activity was found predominantly in the microsomal fraction and also in the postmicrosomal supernatant. The inhibition of the virus infection by these subcellular fractions also followed the *Fv-1* gene properties of the cells. The presence of inhibitor activity in the postmicrosomal supernatant provided the possibility of further separation. Gel filtration on Sephadex G-25 indicated that the inhibitor is of high molecular weight. Chromatography on DEAE-cellulose and phosphocellulose showed that the inhibitor activity spread in broad diffused patterns of elution. The activity of the two subcellular fractions also showed relative instability on storage.

Tests of the cell extracts with various hydrolytic enzymes demonstrated that the inhibitor activity was sensitive to inactivation by ribonucleases (but not by deoxyribonuclease or by proteases). Together with the subcellular distribution, this observation suggested that the inhibitor molecules were presumably ribonucleic acid and might be isolated by biochemical procedures. RNAs of $Fv\text{-}1^{nn}$ and $Fv\text{-}1^{bb}$ embryo culture cells were isolated by phenol deproteinization at pH 7.6 and pH 9.0 and subsequent ethanol precipitation. These RNA preparations were able to transfer the specific virus resistance according to the *Fv-1* gene determinants of their original cell materials, namely, RNA preparations from $Fv\text{-}1^{bb}$ cells (or $Fv\text{-}1^{nn}$ cells) can induce increased resistance to N-tropic (or B-tropic) MuLV infection in susceptible mouse-cell cultures. The inhibitory activity of the RNA preparation becomes more stable after phenol extraction, presumably due to removal of endogenous ribonucleases from the extract. It is resistant to heat inactivation at 37°C for 30 min, 56°C for 15 min, and 80°C for 3 min, and can be stored for some time in a liquid-nitrogen freezer without change in activity. RNA preparations with high activity can also be isolated from livers of young adult mice, thus providing sources for obtaining a large pool of the inhibitor(s). The inhibitor activity can be demonstrated both in the pH 7.6 and pH 9.0 fractions of RNA prepared by differential pH extraction with phenol; quantitative titration suggested that more activity was present in the pH 9.0 fraction. Preliminary results showed that the active component sedimented at the 20–25S region of the sucrose gradient centrifugation. Also, preliminary results suggest that the participation of a certain vehicle is important for efficient uptake and utilization of the RNA in the medium by the culture cells.

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EFFECTS OF CELLULAR RNA ON REVERSE TRANSCRIPTASE ACTIVITY: PRELIMINARY STUDIES*

Ih-Chang Hsu,† Wen-Kuang Yang,
R. W. Tennant, and Arthur Brown‡

Previous studies have indicated that the active ingredient in *Fv-1*-allele cell extract responsible for specific resistance to murine leukemia virus infection is RNA in nature. To study the mechanism of action of the *Fv-1* gene at the molecular level, we have initially focused on (*a*) reverse transcription and (*b*) integration of provirus DNA. These two biochemical functions occur at the early stage of the virus infection, in which *Fv-1* gene products appear to exert their effect. We describe some experimental results related to these studies.

AKR murine leukemia virus, an N-tropic virus, was isolated from culture medium of a high-passage line of AKR mouse embryo culture actively producing the virus. RNA materials used for the study were prepared by phenol extraction procedures from embryo primary culture cells of NIH Swiss mice (carrying $Fv\text{-}1^{n}$ allele) and BALB/c mice (carrying $Fv\text{-}1^{b}$ allele); the RNA preparations were previously shown to contain *Fv-1*-gene-specific inhibitory activity in the cell-culture infection system. Endogenous reverse transcription activity of the AKR viruses was not inhibited when the total cellular RNA preparations were included in the reaction mixture up to 0.3 mg/ml. Utilization of synthetic template-primer, $(rA)_n \cdot (dT)_9$, by the viral reverse transcriptase was markedly inhibited by the cellular RNA; however, both $Fv\text{-}1^{n}$ and $Fv\text{-}1^{b}$ cell RNA showed the same marked inhibitory activity on AKR viruses. The cellular RNA preparations were fractionated by sucrose-gradient sedimentation, and all RNA fractions were found to have similar inhibitory effect on the reverse transcription of $(rA)_n \cdot (dT)_9$. The fractions containing 20–28S RNA were pooled for further study. Enzyme kinetic analyses demonstrated that the inhibition is competitive with regard to $(rA)_n \cdot (dT)_9$. Utilization of $(rA)_n \cdot (rU)_n$ and $(rA)_n \cdot (dT)_n$ as template-primer by the AKR-virus reverse transcriptase was also affected by the 20–28S cellular RNA, but again the inhibition was nonspecific as far as the *Fv-1* gene is concerned.

A direct examination of the possible effect of *Fv-1* gene on insertion and integration of provirus DNA into host cell genome depends on the feasibility of isolating biologically infectious DNA from cells infected with N-tropic and B-tropic murine leukemia viruses. Although infectious DNA of Rous sarcoma virus for chick cells has been isolated from rat cells transformed by this virus, similar results have yet to be reported for the MuLV system. Recently, this infection system ("transfection") has been demonstrated in human cells with DNA isolated from RD-114-virus-infected cells, and we have confirmed the results. Attempts to develop procedures of transfection in the MuLV system are in progress.

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COMBINED USE OF HYDROCORTISONE AND INSULIN FOR PRODUCTION OF AKR MURINE LEUKEMIA VIRUS*

Wen-Kuang Yang, Ih-Chang Hsu,† L. C. Waters, Beth C. Mullin,‡ D. W. Fountain,‡ L. G. Hardin, and Den-Mei Yang§

For biochemical studies of RNA tumor viruses, a large quantity of virus material is generally required. Also, virus preparation should be of considerable purity and minimal degradation for analyses since intact nucleic-acid structure is critical. This is often difficult in the case of RNA tumor viruses produced by the culture cells, which are generally of low yield and known to be degraded rapidly in the culture medium. In our laboratory, we have devised a procedure to obtain from cultured AKR mouse embryo high-passage cell lines sufficient quantity of the virus for the purpose of our biochemical studies. The procedure is briefly described as follows: AKR cells are transferred from culture dishes into roller tubes and grown to confluency in the roller drum. Hydrocortisone, at the concentration of 10^{-6} *M*, is then included in the culture medium. Harvest of viruses is also started using 3- to 4-hr daytime media and 15- to 18-hr overnight medium by a discontinuous and a continuous sucrose density gradient centrifugation. At 5–7 days later, 100 mU/ml of insulin (Iletin, Eli Lilly) is also included in the medium in addition to hydrocortisone, and the virus harvest continues for the next 2–3 weeks. Addition of hydrocortisone in the medium has two effects: (*a*) increasing the virus yield two- to threefold and (*b*) preventing detachment of the confluent cell sheet from the tube wall. Addition of insulin 1 week later evidently stimulates growth of the cells to overconfluency, which apparently enhances the virus-producing function, and a further increase of two- to fivefold yield of viruses is often obtained. With the use of hydrocortisone, medium volume per roller tube can be decreased to 15 ml at 3- to 4-hr intervals and to 30 ml overnight, thus saving medium and also the process of centrifugation. In a typical operation of 22 roller tubes and 3-week harvest, we obtained a lot of AKR viruses containing about 10 mg protein. Effect of insulin is very much dependent on time of addition and state of culture cells. Our results showed that AKR viruses produced by this procedure were of considerable specific infectious titer, of the same host-cell tropism (N-tropic), and gave improved efficiency of endogenous reverse transcription.

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†Postdoctoral investigator, Carcinogenesis Training Grant No. CA 05296 from the NCI.

‡Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

§Consultant.

TRYPTOPHAN tRNA PRIMES REVERSE TRANSCRIPTION OF AVIAN MYELOBLASTOSIS VIRUS (AMV) 35S RNA *IN VITRO**

L. C. Waters, Beth C. Mullin,† Ti Ho, Wen-Kuang Yang, and L. G. Hardin

Several lines of evidence indicate that 4S RNA molecules initiate (or prime) reverse transcription of tumor virus RNA to DNA *in vitro*. These primer RNAs can be dissociated from the viral 70S or 35S RNA only at relatively high temperatures. This dissociation coincides with the loss of template-primer activity as measured *in vitro*. The 70S-associated 4S RNA has several properties of tRNA.

The properties of the primer molecules indicate a great degree of complementarity between them and the viral 35S RNA. We used this property to show that specific cellular 4S RNA molecules will hybridize with AMV 35S RNA *in vitro*.[1] A major proportion of the 4S RNA which hybridizes is tRNA, and of the tRNA, more than 60% is identified as tryptophan tRNA.[2] The hybrid formed *in vitro* between AMV 35S RNA and tryptophan tRNA, but not lysine, methionine, and other unidentified tRNAs, was as efficient a template-primer for DNA synthesis *in vitro* as was the native AMV 70S RNA.[2] Based on these results and those obtained by Dr. J. E. Dahlberg and co-workers at the University of Wisconsin with the Rous sarcoma virus, indications are that tryptophan tRNA is important in the replication of avian RNA tumor viruses.

We are currently using the purified components, *i.e.*, viral 35S RNA, reverse transcriptase, and tryptophan tRNA, to study the mechanism of replication of these viruses.

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†Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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PURIFICATION OF CHICK-CELL TRYPTOPHAN tRNA BY RPC-5 COLUMN CHROMATOGRAPHY*

L. C. Waters, Beth C. Mullin,† Wen-Kuang Yang, J. L. Nichol,‡ and L. G. Hardin

Nonacylated tryptophan tRNA elutes early from RPC-5. However, tryptophyl tRNA is one of the last tRNAs eluted from the column. We exploited this unique behavior to identify tryptophan tRNA as a major tRNA component which hybridizes to AMV 35S RNA *in vitro.*[1] By first chromatographing nonacylated chicken-liver tRNA followed by rechromatography of the tryptophan tRNA region of the first column after tryptophylation, we have purified chick-liver tryptophan tRNA to homogeneity.[2] Oligonucleotide fingerprints of ^{32}P-labeled tryptophan tRNA purified by the method described are identical to those reported for "spot 1" RNA isolated from, and shown to prime DNA synthesis in, Rous sarcoma virus.[3] By this method, pure tryptophan tRNA can easily be prepared for use in studying the biochemical mechanisms by which RNA tumor viruses replicate.

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SELECTIVE ASSOCIATION OF TRYPTOPHAN tRNA WITH THE 35S RNA OF AVIAN MYELOBLASTOSIS VIRUS *IN VIVO**

L. C. Waters, Beth C. Mullin,† E. G. Bailiff, R. A. Popp, and L. G. Hardin

Tryptophan tRNA has been shown by Dr. J. E. Dahlberg and co-workers at the University of Wisconsin to be associated with viral 35S RNA within the Rous sarcoma virions and also to serve as a primer for DNA synthesis *in vitro.* We have shown that tryptophan tRNA isolated from chick cells will hybridize with AMV 35S RNA *in vitro.* The hybrid, but neither tryptophan tRNA nor 35S RNA alone, is a very efficient template-primer for DNA synthesis *in vitro.*[1] To show a unique association between tryptophan tRNA and AMV 35S RNA in the isolated virus particles would lend strong support to the notion that this tRNA is biologically important as a primer of DNA synthesis in AMV.

In the present study we have made two assumptions: (*a*) the potential primer is a biologically active tRNA, and (*b*) it is thermally dissociated from viral 70S or 35S RNA at a temperature which is consistent with the heat inactivation pattern for the native template-primer (70S RNA). The 4S RNA within the virion was isolated as three fractions: (*a*) "Free 4S RNA" – which is not associated with the viral 70S RNA; (*b*) "60°C 70S-associated 4S RNA" – that which is dissociated from the 70S RNA by heating to 60°C; (*c*) "60–80°C 70S-associated 4S RNA" – that which is obtained by heating the 70S or 35S RNA obtained at 60 to 80°C. The RNA samples are aminoacylated with a mixture of tritium-labeled amino acids, the aminoacyl-tRNAs isolated, and the amino acids discharged and analyzed with an amino acid analyzer. In this manner a qualitative and semiquantitative estimate of the tRNAs within a sample can be made. Relative to that in myeloblasts, AMV "free" 4S RNA was characterized by very low quantities of glutamate, valine, and tyrosine tRNAs. Transfer RNAs accepting all 17 amino acids with the exception of tyrosine were shown to be present in the 70S-associated 4S RNA which dissociates at 60°C. The bulk of the 70S-associated 4S RNA was dissociated at 60°C at low ionic strength with a concomitant conversion of 70S RNA to 35S RNA. However, under these conditions, more than 75% of the original template-primer activity remains. Further heating to 80°C abolishes template-primer activity, and in the 4S RNA fraction released (60–80°C), more than 90% of the total amino acid tRNA was tryptophan tRNA. These results support our other studies in suggesting that tryptophan tRNA is biologically important as a primer for DNA synthesis in AMV.

We anticipate that this method, using the biological specificity of the aminoacylation reaction and the resolving power of the amino acid analyzer, will find expanded use in the study of tRNAs and amino acyl-tRNA synthetases.

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SELECTIVE ASSOCIATION OF PROLINE tRNA WITH THE 35S RNA OF THE AKR MURINE LEUKEMIA VIRUS *IN VIVO**

L. C. Waters, Beth C. Mullin,† E. G. Bailiff, R. A. Popp, and L. G. Hardin

We showed that specific mouse-cell tRNAs would hybridize with AKR viral 35S RNA *in vitro*;[1] this is similar to our findings in the chick-cell–AMV 35S RNA system. However, the same technique used to show tryptophan tRNA involvement in the avian system indicated that tryptophan tRNA was not a major component of the hybridized tRNA in the mouse-cell–AKR viral 35S RNA system. To find which if any tRNA has the affinity with AKR viral 35S RNA expected of a potential primer molecule, the 4S RNA fractions obtained from this murine tumor virus were analyzed for their tRNA content as was described for AMV.[2] In the 70S-associated 4S RNA, which dissociates between 60 and 80°C, tryptophan, lysine, arginine, threonine and/or serine, glutamate, proline, glycine, alanine, isoleucine, and leucine tRNAs were repeatedly demonstrated. However, with the exception of proline tRNA, none are present at a level greater than 12%. Proline tRNA comprises 50–60% of the identified tRNAs in this fraction.[3] By analogy with the avian tumor viruses and since our preliminary results with Rauscher murine leukemia virus also indicate proline tRNA, it is possible that in murine RNA tumor viruses, proline tRNA might be the primer molecule. Currently we are attempting to demonstrate a primer function for proline tRNA *in vitro*.

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†Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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AN *IN VITRO* LYMPHOCYTE-MEDIATED CYTOTOXICITY ASSAY USING LABELED ADENOSINE*

Den-Mei Yang† and Wen-Kuang Yang

Our study on *in vitro* bioassay for transfer factor activity has been initiated as a collaborative effort with G. David Novelli's group, who have been concentrating on separation and purification of transfer factor(s) for cell-mediated immunity against various human cancers, including osteogenic sarcomas (a major plutonium hazard) and breast cancers. Our specific aim is to develop a dependable and reproducible method for assaying the transfer factor activity in the separation and purification operation. We have approached the problem from two aspects, namely, quantitation of tumor-cell killing by immune lymphocytes and induction of immune property in normal lymphocytes. This report describes the results of our efforts for the first aspect of the study.

Groups of young adult female rats were immunized with three tissue-cultured tumor-cell lines (PR mouse mammary tumor, ALAB human breast cancer, and HT-29 human cancer). The cells, 5×10^7 in 1.0 ml phosphate-buffered saline, were injected intraperitoneally at 3-week intervals for 3–4 times. Ten days after the last injection, lymphocytes were obtained from peritoneal washings, peripheral blood, or the spleen of the rats. The lymphocytes, with appropriate controls, were used to examine the applicability of various *in vitro* cytotoxicity methods, such as microplate test (to count the reduction of target cell number), iododeoxyuridine labeling and release method (measuring release of radioiodine radioactivity from target tumor cells), thymidine labeling assays (DNA synthesis and breakdown), and ^{51}Cr labeling and release assay (detecting release of cytoplasmic molecules with ^{51}Cr from target cells). Three criteria used to assess the applicability were simplicity (for handling large numbers of sample), sensitivity (for working with small cell quantity of lymphocytes), and reproducibility (for dependable quantitative measurement). Most of the methods fulfilled one or two but not all three criteria. In the case of the human breast cancer line ALAB, the ^{51}Cr method was found to be simplest and generally dependable, but this method has the disadvantages of not being physiological and of giving a high background (spontaneous release of radioactivity) upon prolonged incubation of target-cell cultures. Thus, searching for an

alternate method other than the conventional cytotoxicity methods seemed desirable. We have found that improvement of the labeling and release method can be achieved by using adenosine, which can be incorporated into a large nucleotide pool and which also serves as a precursor for RNA and DNA synthesis within the cell.

General procedures of the cytotoxicity test employing labeled adenosine are as follows: Labeled target cells are prepared by incubation of trypsinized logarithmically growing cells in suspension with ^{3}H- or ^{14}C-labeled adenosine of high specific activity. The labeled cells are washed by centrifugation through a layer of fetal calf serum. Appropriate numbers of the labeled cells are introduced into wells of cluster dishes for cell attachment, which generally requires 3 hr. Lymphocytes are then added. Release of radioactivity can be studied for the next 24 hr in the medium containing excess nonlabeled adenosine. This method has the advantage that the labeling is physiological, spontaneous release is generally low, considerably high specific radioactivity of the cells can be achieved, and it applies to various cell lines. With this method, effect of lymphocyte cytotoxicity on the release of free nucleotides (rapid) and nucleic acids (slow) from the target cells can be simultaneously studied.

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ATTEMPTS TO SELECT TUMOR-SPECIFIC LECTINS FROM LEGUMES*

Wen-Kuang Yang, M. Margaret Williams,†
D. E. Foard, and D. W. Fountain‡

This research project was initiated because almost all conventionally known lectins used for cancer research have been isolated from natural sources by investigators using red blood cells as selecting agents. To be useful for studying cancer cell membranes, a lectin should be minimally, rather than highly, reactive to red blood cells, which are normal differentiated cells. Also, tumor-specific antigens are generally known to be glycoproteins of the membrane. Thus, using tumor cells as selecting agents, one may be able to isolate specific lectins from whole multitudes of such molecules existing in nature. We describe here the results of an initial attempt of such an investigation.

Fifteen legumes were selected for the present study: three varieties of *Phaseolus vulgaris* (red kidney, cranberry, and navy beans), *Phaseolus mungo, Glycine max, Gleditsia triacanthos, Vigna sinesis, Ulex europaeus, Lupinus succulentus, Pachyrhizus* (jicama), *Acacia nilotica, Albizzia distachya, Cercis siliquastrum, Kennedia prostrata,* and *Prosopis glandulosa.* Seeds from these plants were pulverized and extracted with a solution containing 0.1 *M* Na acetate and 1.0 *M* NaCl at pH 4.5. The resultant extracts were used for immunization of rabbits to obtain specific antisera and also for membrane absorption tests using tumor cells and red blood cells. Tumor cells used for the tests included a mouse plasma cell tumor (B-lymphocyte neoplasm), AKR mouse thymoma (T-lymphocyte neoplasm), human chronic lymphocytic leukemic cells (B-lymphocyte neoplasm), and human acute lymphocytic leukemic cells (T-lymphocyte neoplasm). Control cells included appropriate normal lymphocytes and red blood cells from humans, sheep, rabbits, rats, and mice. Examination of antisera by immunodiffusion and immunoelectrophoresis indicated generally high potency. Agglutination tests showed considerable titer differences among various cells reacting with various legume seed extracts. After reaction with legume seed extract, the cells were extensively washed and subsequently eluted with the extracting buffers. The eluted proteins were analyzed by immunoelectrophoresis using rabbit antisera. Preliminary results indicated that lectins bound to lymphocyte neoplasms can be different from those bound to red blood cells. To investigate this problem with more precision, legume seed extracts were first labeled with radioiodine before absorption tests with tumor cells. Tumor cells were also formalinized to increase cell rigidity for the drastic elution procedures. It was found that proteins bound to tumor cells could be very heterogeneous in terms of binding affinity and binding specificity. Lectins which tend to cause cell agglutination usually bind strongly and irreversibly to both normal and tumor cell membranes. Some lectins were found to bind weakly to cell membrane and could be recovered by subsequent elution at low pH. We have not been able to show competition of binding of these weakly binding lectins by simple sugars, which usually compete rather well with the strongly binding lectins. Work is in progress to identify individual lectins bound to T- and B-lymphocyte neoplasms and also to employ the weakly and reversibly binding lectins in the affinity gel chromatographic systems.

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PRELIMINARY STUDIES OF MURINE LEUKEMIA VIRUS SURFACE PROTEINS BY LECTIN AFFINITY CHROMATOGRAPHY*

D. W. Fountain† and Wen-Kuang Yang

Viral envelope proteins, particularly glycoproteins, play an important role in the initiation of infection of susceptible cells by RNA tumor viruses. In addition to providing sites for the initial adsorption of virus particles to the cell membrane, these envelope components (morphologically identifiable as spikes or knobs on the virion membrane) are also associated with the type species and group specificity of virus antigenicity. Although immunological approaches have provided much information on viral proteins — their relative abundance, location in the virion, and relative antigenic properties and cross reactivities — they remain difficult to obtain in any large quantity of purified materials by conventional biochemical separation techniques and are thus poorly defined biochemically.

The ability of plant lectins to bind to and agglutinate RNA tumor viruses[1,2] suggests specific interactions of externally located glycoproteins with lectins of different oligosaccharide specificity. We are exploring the potential use of these reversible binding interactions to purify and examine surface glycoproteins of MuLV.

Affinity chromatographic gels have been prepared by covalently coupling a lectin isolated from soybeans (cultivar D68-127) to CNBr-activated Sepharose 4B (SBH-Sepharose) or to glutaraldehyde-activated polyacrylamide (SBH-PA) supports. A commercial preparation of Con A-Sepharose has also been tested. Soybean lectins combine specifically with D-galactose and *N*-acetyl-D-galactosamine-like residues, while Con A is specific for D-glucose and D-mannose-like residues. Two viral protein preparations have been tested to date: (*a*) AKR MuLV labeled by the lactoperoxidase (surface labeling) technique and its lithium diiodosalicylate-solubilized extract (glycoprotein), and (*b*) ^{125}I-labeled viral proteins containing gp69/71, kindly supplied by Dr. J. N. Ihle.

Affinity binding experiments using the lectin gel preparations and the ^{125}I-labeled virus protein preparations obtained the following preliminary results: (*a*) Reaction of virus proteins and lectin gel usually resulted in binding of certain percentages of the radioactivity to the lectin gel (25–27% for SBH and about 50% for Con A). (*b*) The radioactive virus proteins which showed no binding to the lectin gel, showed no binding upon rechromatography. (*c*) The binding, however, was almost totally irreversible, even with the elution of solution containing α-methyl-D-mannoside (specific to Con A) and *N*-acetyl-D-galactosamine (specific for SBH).

Thus, the lectin gels prepared by using plant lectins of strong binding affinity were shown to be not suitable for the purpose of isolating the glycoproteins of RNA tumor viruses. Research is in progress to use plant lectins which show weak and reversible binding properties. These include lectins from *Lens culinaris* seeds and *Phaseolus vulgaris* varieties.

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LECTIN CONTENT OF FOURTEEN VARIETIES OF SOYBEAN*

D. W. Fountain,† M. Margaret Williams,‡ Wen-Kuang Yang, and D. E. Foard

Lectins native to legume seeds have been suggested to play a major role in the establishment of the *Rhizobium*-nodule symbiosis required for fixation of atmospheric nitrogen. Specific membrane binding properties of the lectins[1,2] may serve to positively recognize and actively bind the symbiont *Rhizobium* species onto the growing roots in the soil. Thus, studies of lectins in the legume can be very important in view of energy conservation, *i.e.,* decreasing the need of industrial energy for nitrogen fertilizer production. In the present study, seeds of 14 varieties of soybean were analyzed quantitatively and qualitatively for their lectin content in the hope of establishing a relationship between this and the nitrogen-fixing capacity and productivity of individual soybean strains. This study is necessary before a full-scale project of genetics of soybean lectins is instituted.

The seeds were extracted at low pH, and the 47–85% ammonium sulfate precipitable protein was recovered for quantitative and qualitative analysis of lectin content and activity. A radial immunodiffusion assay using an antiserum produced against the lectin purified from the D68-127 cultivar of soybean indicated that although all of the extracts tested contained some lectin activity, the seeds of certain varieties were depleted in

lectin content compared with others (relative to radial data produced in this assay in response to standard dilutions of purified D68-127 soybean lectin). The lectin contents ranged from 41 μg per milligram of protein (*cultivar* "Chippewa") up to 530 μg per milligram of protein (*cultivar* "Select Bragg"). Lectin activity of the crude extracts assayed by a standardized hemagglutination test gave a ranked list of soybean varieties consistent with the ranking obtained from the immunological assay.

Immunoelectrophoresis of crude lectin extracts using D68-127 soybean-lectin antiserum indicated that an antigenically similar lectin was present in seeds of each of the 14 varieties, and the relative intensity of precipitin formation suggested that this lectin (or isolectin complex) was responsible for the differences in lectin quantity observed in the quantitative assays.

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‡Southern College and University Union–ORNL Science Semester student participant, spring 1975, and also ORAU undergraduate research participant, summer 1975, from Centre College of Kentucky, Danville 40422.

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ISOLECTINS IN *GLYCINE MAX**

D. W. Fountain† and Wen-Kuang Yang

Although lectin from a single seed species may differ substantially in saccharide specificity (*e.g., Ulex* sp.) or cell-type specificity (*Phaseolus vulgaris*), many seed types contain multiple similar lectin species. Such isolectins can be distinguished on the basis of differences in electrophoretic or chromatographic behavior and appear to be a consequence of small differences in primary structure of polypeptide portions of the molecule.

In the course of extraction and purification of a soybean lectin for use as a probe for RNA tumor virus membrane components, a brief study was made of the major isolectin of seeds of D68-127, an economically important soybean cultivar. This lectin appears to differ in several respects from previously reported characteristics of soybean lectin.[1] Gel filtration on Sephadex G-100 of the electrophoretically homogeneous D68-127 lectin indicates a glycoprotein of mol wt 95,000, somewhat lower than the mol wt of 110,000–120,000 daltons reported earlier. SDS-gel filtration and SDS-polyacrylamide gel electrophoresis data suggest that the native lectin is a tetramer with a subunit molecular weight of approximately 23,000. This lectin is the major isolectin component of D68-127 soybean seeds and is readily distinguishable from two minor isolectins by its unretarded behavior on DEAE-cellulose at near neutral pH. The two minor isolectins, although antigenically and electrophoretically identical to the DEAE breakthrough lectin, are released from DEAE-cellulose by an elevated salt concentration and in this respect are similar to the "major" isolectin previously reported by Lis *et al.*[2] It thus seems likely that seeds of different soybean varieties may contain quite different isolectins as their major lectin species.

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†Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

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VIRAL RNA SUBUNITS*

J. N. Ihle,† J. G. Farrelly, and F. T. Kenney

We have reported previously that the 34S RNA subunits of several C-type viruses can be fractionated on poly(U)-Sepharose columns to yield two subunit populations, one containing poly(A) segments of 150 to 200 nucleotides and the other not hybridized to the column and not containing poly(A) [*i.e.*, no poly(A) segment longer than 20 nucleotides]. Essentially all of the 70S viral RNA hybridizes to the column and is only eluted when the temperature is increased above 40°C; thus, each 70S molecule contains poly(A). However, only two-thirds of the isolated 34S subunits behave similarly, and one-third of them fail to hybridize and elute at the loading temperature of 20°C. Analysis of the eluted subunits on PAGE confirms that they retain the 34S configuration; thus this result is not due to degradation.

A cDNA complement of 70S RNA was synthesized in an endogenous reverse transcriptase reaction under conditions yielding a complete transcript. When the cDNA was hybridized to total 34S viral RNA, more than 95% of the DNA hybridized to the RNA and banded in the RNA region of density gradients. In contrast, when cDNA was hybridized to excess amounts of either the poly(A)-containing or nonpoly(A)-containing 34S subunits, only about 50% of the DNA formed

hybrids with each fraction. Similar results were obtained when hybridization was examined by single-strand specific nuclease (S-1) assays.

To establish further that each fraction of subunit RNA contains unique sequences, we tested the ability of the cDNA, isolated from each of the hybridized fractions, to rehybridize with its original RNA complement as well as with the other fraction. The specific hybrids formed (as described in the paragraph above) were subjected to alkaline hydrolysis, and each of the cDNAs was reisolated. The cDNA isolated by hybridization to nonpoly(A)-containing RNA rehybridized completely to this RNA, but not to poly(A)-containing RNA, and vice versa. Thus the two subunit populations from AKR leukemia virus contain different sequences and are genetically distinct. Under our experimental conditions (RNA in great excess) the sequence differences appear to be complete, but futher experimentation is needed to determine the absolute degree of similarity in the two subunit species.

This is a controversial finding, in that several reports have appeared in which indirect approaches (RNA fingerprint analyses, estimates of genome complexity) have led to the conclusion that the viral genome is polyploid. However, we feel that our direct approach has yielded results which are incontrovertible and argue that the genome must be at least partially haploid. Our data suggest a genome composed of three subunits, two containing poly(A) and one without poly(A), and show that the latter is genetically distinct from the former. Whether the putative two subunits which contain poly(A) also differ in nucleotide sequence cannot be determined until a method of separating them is developed.

The capacity to prepare cDNAs specific for fractions of the viral genome is expected to be of importance in definitively examining independent expression of discrete viral components during the process of activation.

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VIRAL ANTIGENS*

J. N. Ihle† and F. T. Kenney

During the past year we have attempted to develop purification techniques applicable to the isolation of a number of viral proteins in the undenatured state; special emphasis was placed on those antigens involved in the autogenous immune response, especially gp71. The development of these techniques is particularly significant to further analysis of the autogenous immune response in that purification of these proteins by conventional techniques results in the loss of their natural antigenic determinants.

The techniques we have tried have been numerous and included the use of dilute solutions of a number of nonionic detergents (such as triton X-100 and NP-40) and ionic detergents (DOC). These approaches have generally yielded unsatisfactory results, primarily because the detergents could not be removed without denaturing the viral proteins. More recently we have found that lithium diiodosalicylate can be used to disrupt the virion and solubilize viral proteins with much better results. Briefly, we used 0.3 *M* lithium diiodosalicylate solutions to disrupt the virus, diluted the resulting solution tenfold with water, and centrifuged at 40,000 rpm for 1 hr to remove undisrupted virus or aggregate material. These techniques generally result in the solubilization of more than 90% of the viral protein. The solution is then dialyzed against 0.01 *M* sodium phosphate buffer, pH 7.5, to remove the lithium diiodosalicylate. The dialysate is then made 0.1 *M* in sodium acetate, pH 5.0, and allowed to stand at 4°C for 30 min. The resulting precipitate is removed by centrifugation at 2000 rpm for 30 min, and the supernatant is dialyzed against 0.01 *M* Tris buffer (pH 7.5). This precipitate contains a number of the low-molecular-weight viral proteins and some p30. The supernatant, which contains most of the p30, p15, and gp71, is subsequently chromatographed on a G-150 column, which results in the production of two fractions. One fraction elutes at the void volume of the column and is approximately 50% gp71. We are currently using this fraction in attempts to further purify gp71. At this point the gp71 is still reactive with natural immune sera. The second fraction from the column elutes slightly behind the void volume, contains predominantly p30, but is slightly contaminated with p15. The p30 can be purified to homogeneity from this fraction by chromatography on DEAE-Sepharose columns from which it elutes as a homogeneous peak at approximately 0.06 *M* NaCl. The identity of this fraction has been verified by gel electrophoresis and immunologically with anti-p30 sera (obtained from NCI) and with antisera produced to p30 purified by conventional techniques. Furthermore, p30 purified by these techniques has been used to establish a competition radioimmunoassay for p30 to be used in activation experiments and in studies of natural expression of p30 *in vivo*.

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VIRUS ACTIVATION*

J. N. Ihle† and F. T. Kenney

The consequences of IdUrd incorporation into DNA that result in expression of the latent virus genome are not known, but presumably can be related either to altered binding of DNA to regulatory proteins or to the breakage which occurs in substituted DNA. We have attempted to explore this question by testing the effect on activation of inhibitors of DNA repair, so far with quite inconclusive results. Concentrations of caffeine known to inhibit repair did not appear to influence activation. Chloroquine, a more specific repair inhibitor, yielded frustratingly variable results, ranging from no effect to a pronounced degree of inhibition restricted to the early events prior to establishment of the stable activation intermediate, as would be expected. The basis for the extensive variability in these experiments is being sought.

A second approach to defining the role of IdUrd incorporation into DNA is to examine the "hitness" of the process. Preliminary results suggest a single-hit process, *i.e.*, a plot of the logarithm of IdUrd concentration vs percent activation is linear. This implies that a single IdUrd substitution (or breakage point) in the DNA is sufficient for activation of the viral genome, *i.e.*, coordinate substitution at more than one site is not required. The data do not allow determination of the number of sites which individually could give rise to activation. This question is of particular significance in that the multiple components of the viral genome may be regulated independently; *i.e.*, there may be multiple regulatory sites.

We have examined the effect of cordecypin (3′-deoxyadenosine), an inhibitor of mRNA processing and of nuclear-cytoplasmic transport of mRNA in other systems, on the activation of AKR virus by IdUrd. These experiments have involved dissociating the activation process into two phases, using serum starvation techniques as previously described. Results have been consistent in demonstrating that an interval of cordecypin sensitivity does exist, beginning shortly after the period of IdUrd incorporation and lasting approximately 6 to 8 hr. This sensitive interval is restricted to the first phase of activation, implying a requirement for synthesis of a cellular mRNA in order to establish the activation intermediate. Since no synthesis of virion protein appears to occur during this period, the putative mRNA (or its translational product) may be regulatory in function. Also, this RNA may be the first and critical product of perturbation of the cellular genome by incorporation of IdUrd.

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ATTEMPTS TO IDENTIFY SPECIFIC TYROSINE AMINOTRANSFERASE POLYRIBOSOMES*

Kai-Lin Lee and F. T. Kenney

Specific polyribosomes of ovalbumin, albumin, collagen, and immunoglobulin have been identified and isolated with their specific antibodies. Such an approach has proved successful in isolation of specific mRNA. Attempts were made to bind tyrosine aminotransferase (TAT) antibody to liver polysomes. Intact rat liver polysomes were investigated for the presence of TAT antigenic groups with the use of antibody to TAT subunits or total CNBr fragments of TAT. Monospecific ^{125}I-labeled antibodies were incubated with rat liver polysomes, and the polysomes were distributed on a sucrose density gradient to see whether the antibodies associated with particular sizes of ribosomal aggregates. The radioactivity was not evenly distributed among the ribosomes, but no discrete binding peak could be detected. When results were expressed as the binding of antibodies per unit of ribosomes (cpm/A_{260}), a small binding peak in the large polysome region could be detected. These results indicate that some specific binding of antibody to the polysome was achieved, although considerable nonspecific absorption of antibodies onto the ribosomes masked the specific binding. These conclusions were substantiated by the results that (*a*) the amount of ^{125}I-labeled antibody binding to polysomes was not reduced by rabbit γ-globulin but was reduced by nonlabeled monospecific TAT antibody to its subunits, (*b*) the amount of monospecific ^{125}I-labeled antibody binding to EDTA-pretreated polysomes was reduced, (*c*) we constantly observed considerable amounts of radioactivity associated with pelleted polysomes during sucrose gradient centrifugation. The radioactivity in the pellet did not change when the amount of ^{125}I-labeled antibody was increased, but did increase when more polysomes were used. Since the nonspecific adsorption of antibodies to ribosomes has been attributed to the interaction of Fc fragments of γ-globulin to ribosome, this nonspecific

adsorption should be eliminated or reduced by using antibody-binding fragments [$F(ab')_2$ or F(ab)] instead of intact γ-globulin. We are currently trying to repeat the binding experiments using ^{125}I-$F(ab')_2$ antibody fragments.

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PREPARATION OF ANTIBODIES AGAINST SEQUENTIAL ANTIGEN DETERMINANTS OF HEPATIC TYROSINE AMINOTRANSFERASE*

Kai-Lin Lee and P. L. Darke

Rabbit antibodies prepared against enzymatic active TAT were found to react with biologically active TAT antigen, but did not react at all with either TAT subunits or the peptide fragments produced by CNBr cleavage of the transferase. These results indicate that antibodies elicited by native TAT primarily recognize the conformational antigen determinants rather than sequential antigen determinants. An attempt was made to prepare anti-TAT antibodies which primarily recognize the sequential antigen determinants. Such antibodies should be useful in isolation of TAT-specific polysomes and the intermediates of TAT degradation. Homogeneous TAT, isolated from rat liver, was used to prepare carboxymethylated subunits of the enzyme. The carboxymethylated subunit was further cleaved by CNBr. Analysis of the fragments shows that there are five major fragments by SDS-gel electrophoresis (molecular weight ranges from 25,000 to 10,000) and ten fragments by urea-gel electrophoresis. Antibodies prepared against carboxymethylated subunits react with either native TAT or the subunits of the enzyme. However, antibodies prepared against total CNBr-cleaved fragments of the enzyme react only with subunits of the transferase. These results demonstrate that antibodies elicited by peptide fragments or subunits of tyrosine aminotransferase can recognize some sequential antigen determinants of the enzyme. Preliminary results indicate that specific TAT polyribosomes can be identified by such antibodies.

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ATTEMPTS TO ASSAY TYROSINE AMINOTRANSFERASE MESSENGER RNA IN CELL-FREE TRANSLATION SYSTEMS*

Joanne M. Nickol,† Kai-Lin Lee, and F. T. Kenney

The liver-specific enzyme TAT can be induced in rat liver by administration of the glucocorticoid hydrocortisone. The induction is due to an increase in the rate of synthesis of the enzyme, but the exact biochemical mechanism of action of the hormone is not clearly understood. Indirect evidence indicates that the hormone exerts a stimulatory effect at the level of transcription, thus providing more TAT messenger RNA to be translated into a correspondingly greater number of TAT enzyme molecules. To ascertain whether this is the mode of action involved in hydrocortisone induction, we are attempting to directly assay the amount of functional TAT mRNA present in rat liver before and after hydrocortisone administration. This can be accomplished by adding into heterologous cell-free translation systems certain aliquots of rat-liver RNA fractionated in two ways: by sucrose gradient sedimentation to separate primarily according to size, and by poly(U)-Sepharose column chromatography to enrich for poly(A)-RNA. The amount of TAT synthesized in response to the exogenously added RNA can be quantitated by specific precipitation using antibody directed against the TAT enzyme. We have isolated both total polysomal RNA and poly(A) containing RNA from rat liver, fractionated according to size on sucrose gradients, and are currently trying to assay TAT mRNA in both the reticulocyte system and the wheat germ system. Both of these translation systems have been used successfully to translate a variety of different eukaryotic messenger RNAs.

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†Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

EFFECT OF VITAMIN B_6 DEFICIENCY ON THE SYNTHESIS AND DEGRADATION OF HEPATIC TYROSINE AMINOTRANSFERASE*

Kai-Lin Lee, P. L. Darke, and F. T. Kenney

It has been suggested that the rate of dissociation of cofactor of vitamin B_6-dependent enzymes is the

rate-limiting step to control the degradation rate of these enzymes *in vivo*. In agreement with this hypothesis, the half-life of hepatic serine dehydrase is prolonged by administration of pyridoxine *in vivo* to B_6-deficient rats. In view of these observations and to substantiate the role of cofactor in regulation of the enzyme level, we have studied the turnover rates of hepatic tyrosine aminotransferase in B_6-deficient rats. Weanling rats were placed on B_6-deficient diets, and control rats were placed on regular Purina chow diets. During depletion of coenzyme (vitamin B_6 deficiency) the total hepatic TAT was less than that of normal rat liver; particularly, the amount of holoenzyme was decreased in B_6-deficient animals. These findings are in accordance with the idea that the coenzyme is involved in the regulation of the transaminase level *in vivo*. Direct analysis of the transferase synthesis and degradation by isotopic immunochemical technique has revealed that the changes in TAT level in B_6 deficiency are primarily due to the decreased rate of enzyme synthesis. The rates of enzyme degradation are the same in B_6-deficient rats and control rats. These results clearly demonstrate that the cofactor, pyridoxal phosphate, does not protect TAT against degradation *in vivo*. It can be envisioned that, *in vivo,* multiple steps are involved in the process of protein degradation. The limiting step for such a process may be different from one protein to another.

*Research supported by the Virus Cancer Program of the NCI.

STUDIES ON THE DEGRADATION OF HEPATIC TYROSINE AMINOTRANSFERASE DURING INDUCTION BY HYDROCORTISONE*

Nicholas Pomato,† Kai-Lin Lee, and F. T. Kenney

We have extended our study on the degradation of TAT in the H-35 hepatoma cell line during its induction by hydrocortisone. A detailed study of the rate of degradation of the enzyme by immunochemical techniques has revealed that no TAT degradation is observed between 5 and 8 hr after the addition of hydrocortisone. After 8 hr, degradation of the enzyme proceeds at a normal rate.

We suggest that the phenomenon observed is caused by the inability of some component of the cellular degradative system to respond quickly enough to the tremendous increase in synthesis of TAT in the presence of hydrocortisone. To determine whether or not this cessation of degradation is coupled to the synthesis of the enzyme, we examined the enzyme degradation during hydrocortisone induction in the presence of actinomycin D. It had been previously demonstrated in this laboratory that in cells grown in the presence of low levels of actinomycin D (0.2 μg/ml), TAT synthesis is prevented but general protein synthesis is not affected. We have found that the cessation of degradation was not observed in cells grown in the presence of hydrocortisone and low levels of actinomycin D, thus indicating that the synthesis of TAT and the transient prevention of degradation during its induction are coupled processes.

Experiments were also performed to find out if there was any relationship between the amount of time needed to attain full induction of the enzyme and the time at which degradation of the enzyme was prevented. In order to do this, cells were fully induced with hydrocortisone, the hormone was then removed for 3 hr, and finally the hormone was added back to the cells and the rate of enzyme degradation was measured. If there was a fixed relationship between the time at which the hormone was added and the time at which degradation was stopped, the degradation should have been shut off from 5 to 8 hr after the last addition of hormone. If there is a relationship between the time at which full enzyme induction is observed and the time at which degradation is prevented, then the cessation of degradation should occur within 3 hr after final addition of the hormone, since the steady state is reached in this time. Normally, steady-state conditions are reached at about 9–10 hr after addition of the hormone. Since no change in the rate of degradation was observed at any time after the final addition of hydrocortisone, we have concluded that there is no fixed relationship between the time of hormone addition and the cessation of degradation and, also, there is no relationship between the amount of time necessary to reach steady-state conditions and the time at which degradation of the enzyme is prevented.

Finally, an experiment was designed to determine how long the hormone had to be present after the addition of hydrocortisone to the cells in order to see the prevention of degradation. It has been shown in this laboratory that the addition of progesterone to cells being grown on medium containing hydrocortisone will prevent further induction of TAT by the hydrocortisone. In this experiment, progesterone was added to cells at various times after the addition of hydrocortisone, and enzyme degradation was measured between 5 and 7 hr after the initial addition of the hydrocortisone. The results demonstrated that hydrocortisone must be present for at least 3 hr in the cell medium in order to observe a cessation of degradation between 5 and 7 hr after addition of the hormone.

Thus it is evident that the synthesis of TAT and the prevention of its degradation are coupled processes. The hormone must be present for at least 3 hr in order to observe this effect, and there is no fixed relationship between the time at which the hormone is added and the time at which the effect is observed. Finally, there is no relationship between the time necessary to reach full hormonal induction and the time at which the effect is observed.

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