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ANNUAL PROGRESS REPORT
For Period Ending June 30, 1974

H. I. Adler, Director
S. F. Carson, Deputy Director

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INTRODUCTION

AS INDICATED in introductions to earlier reports in this series, we are eager to have these volumes be as useful as possible to the reader. Our primary purpose is to provide short statements of research progress in the past year. In order to do this in a minimum of space, we omit many of the graphs, tables, and photographs that are very important to the individual investigators. This is admittedly a less than satisfactory procedure, but I am reassured by the fact that most of the information contained in this report has appeared or shortly will appear in the open literature in one of the more than 200 research publications produced by the Biology Division each year. Also in the interest of brevity, we have omitted any discussion of the many seminars, meetings, and other activities sponsored by the Division during the year. Likewise, little has been included about the progress of the University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences.

We have for the first time this year attempted to provide capsule statements of the work of each section, written by the Section Leaders. You will notice that these have taken somewhat different forms. This is as it should be since the work of each of our sections has characteristics different from the others. I hope that these introductions are useful. If they are, or if you have suggestions for their improvement, please contact me so that we can make future reports more helpful.

Howard I. Adler
Director, Biology Division

BIOCHEMISTRY

Elliot Volkin

Chemical Protection and Enzyme Catalysis

D. G. Doherty
J. R. Totter

Biochemistry of Cell Differentiation

John Papaconstantinou
Jean Delcour[a]
B. E. Ledford[b]
C. L. Parker[b]

Protein Synthesis

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

Enzymatic Mechanisms in Protein Synthesis

M. P. Stulberg
Vivian S. Hiatt[b]
L. R. Shugart
B. L. Whitfield[b]

Enzyme Regulation

K. Bruce Jacobson
J. B. Bell[b]
J. E. Geltosky[b]

Nucleic Acid Chemistry

W. E. Cohn
J. P. Brown[b]
B. C. Pal
Diane G. Schmidt
Mayo Uziel

Nucleic Acid Enzymology

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

Nucleotide Metabolism

F. D. Hamilton
S. Munavalli[d]

Developmental Biochemistry

F. J. Finamore
Peter Pfuderer
W. F. Zapisek[d]

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[b]Postdoctoral investigator.
[c]Consultant.
[d]Visiting investigator.

INTRODUCTION

THE BIOCHEMISTRY SECTION is made up of many investigators working on a variety of highly detailed basic projects. Therefore, the summary of the work of the Section consists, of necessity, of thumbnail descriptions of only the major efforts of each senior scientist.

Barnett is attempting to establish the evolutionary origin of organelles, particularly chloroplasts, by homology comparisons of purified transfer RNAs from chloroplasts and cytoplasm and the specific tRNA synthetases from the two cellular sources. Niyogi uses specific nucleoside diphosphates (with ATP and GTP) as chain initiators for *in vitro* transcription with *Escherichia coli* RNA polymerase and has derived a tentative model for "early" phage T4 mRNA synthesis. By using the sigma subunit of the enzyme and selective enzymic degradation to advantage, he has been able to determine nucleotide lengths corresponding to the specific binding sites for *E. coli* RNA polymerase on phage T4 and T7 DNAs. Stevens has described many of the unique properties of the sigma subunit of RNA polymerase which determine its function. Fujimura shows that a single-step mutation in phage T5 can cause two enzymic changes: temperature sensitivity of a DNA polymerase activity and an enhanced exonuclease activity. Volkin and Bynum show that in myeloma cells nucleic acid processing is slow, the precursors to ribosomal RNA existing for many hours after a pulse-chase. Mead is separating the proteins of chromosomes of Chinese hamster ovarian cells and studying their enzymic activities. Using biochemical and electron microscopic approaches, Mitra has concluded that, in the stages of phage M13 DNA synthesis, single-strand synthesis takes place by a "rolling circle" mechanism, but replication of the double-stranded replicative form occurs by the "Cairns" model.

McClintock in Papaconstantinou's group, using a variety of techniques to synchronize cells and increase and decrease the generation time, has shown that exactly two generations of growth (after proper stimulation) are required before hemoglobin synthesis can start in Friend leukemia cells. Ledford and Papaconstantinou have shown that in mouse hepatoma cells the rate of albumin synthesis is inversely proportional to the rate of cellular replication; these and other data indicate that, as cells enter the stationary phase, processes of biochemical specialization are enhanced. They have further shown that albumin and α-fetoprotein are synthesized in a linear way throughout the cell cycle, and that both show a critical point when this synthesis increases sharply: early mitosis for albumin, mid-G_2 for α-fetoprotein. But Parker, of the same group, has shown that dibutyryl cyclic AMP can shift the critical point of albumin synthesis into the G_2 phase. All of these studies are designed to show the temporal relationship from the time of gene doubling to transcription to translation. Other work of this group with *Rhynchosciara* spermatocytes shows that this tissue's mitochondrial DNA synthesis occurs specifically during mitochondrial differentiation. Furthermore, this DNA has a contour length of 9 μm rather than 5 μm as has been found with other eukaryotic mitochondrial DNAs.

Jacobson's laboratory has been studying the tRNAs and synthetases of a variety of *Drosophila* mutants, attempting to establish the biochemical mechanism of suppression. Thus, suppression of the "vermillion" mutation by the *su(s)*[2] locus is apparently the result of an alteration in the isoacceptor forms of $tRNA^{Tyr}$ Their work with the ribosomal 5S RNA, using ^{125}I labeling and specific hybridization, indicates that the gene sequences for the 5S RNA have been faithfully conserved during the evolution of *Drosophila* species. Other aspects of tRNA biochemistry are undergoing continued investigation in Stulberg's group. In particular they are attempting to describe the details of tRNA-specific synthetase interaction and the effect of methylation (or its deficiency) on tRNA activity in protein synthesis. An *in vitro* protein-synthesis system from ascites tumor cells has been optimized and is being used in studying the components of the reaction obtained from aging tumors and from irradiated animals.

Novelli's group, while still doing considerable work on tRNAs (purification of aminoacyl-tRNAs with immobilized elongation factor Tu, a partial description of precursor tRNAs in *E. coli*, the induction of certain tRNA synthesis during lymphocyte blastogenesis), have diversified their efforts considerably. In connection with energy-related programs, they are attempting to immobilize methane-producing bacteria and the enzyme cellulase (for glucose production) and to purify a spinach enzyme vital to the photosynthetic process (as a potential source of solar energy). In addition to these efforts, Novelli's group have initiated a strong program on the isolation of "transfer factor." They are studying this material's established ability to confer the *in vivo*

tuberculin sensitivity in humans and also its apparent efficacy in the treatment of osteogenic sarcoma.

In attempting to determine the biological function of ascorbic acid sulfate, Finamore has isolated an ascorbic sulfohydrolase from *Helix pomatia* which converts the compound to vitamin C. Pfuderer continues his work on a variety of fish pheromones; the pollutant dibutylphthalate has been shown to depress heart rate, while natural lipid factors which affect heart rate and growth have been isolated from aquatic animals and partially identified. In Cohn's group, Pal has successfully synthesized the minor nucleoside 5-methylaminoethyl-2-thiouridine and has shown that chemical labeling of tRNA results in 83% of the ^{35}S being found in the 4-thiouridine. Uziel has tested an automatic RNA sequenator (fabricated in a joint effort with the Instrumentation and Controls Division) and has determined the sequence of 21 residues in two purified tRNAs. The process, for unknown reasons, stops at pseudouridine residue 56 and thus leaves "¾ molecules," which have a unique value in RNA analysis. The investigators hope to describe chemical reactivity of tRNA as a function of nucleotide sequence. Hamilton has shown that three protein components in *Euglena* extracts are necessary for ribonucleotide reductase activity; two components are probably thioredoxin reductase and thioredoxin, while the function of the third protein has not been assigned as yet. Doherty and collaborators from the Chemistry Division are using electron spin resonance to study the photolysis of peptides. By employing a well-defined series of peptides they show that •OH attack yields a single radical of the *trans* configuration and that the hydrogen abstracted comes from the carbon atom adjacent to the nitrogen of the peptide.

It is evident from these descriptions that there is increasing emphasis in the Section toward work with eukaryotic systems, particularly with cells grown in culture. A consequence of this has been an increasing collaboration of biochemists with members of other sections. Particular mention should be made of the collaborative biochemical research within the Division with James D. Regan, D. P. Allison, F. C. Hartman, W. L. and Liane B. Russell, E. A. Hiss, E. H. Grell, and L. H. Smith. In addition to these, Doherty is actively working with R. Livingston and H. Zeldes of the Chemistry Division. Purified tRNAs prepared by the Macromolecular Separations Group of ORNL are still being studied. The investigations by the Novelli, Barnett, and Hamilton groups are examples of collaborative work with members of other institutions. The trend toward studies with higher animals is exemplified by the efforts of some biochemists to receive advanced training in these directions. Mitra has spent three months with Al Kaplan at Vanderbilt University learning techniques of herpes virology, S. Niyogi has received training at Cold Spring Harbor from Sambrook in adenovirus work, while Uziel has spent the summer at Woods Hole as a recipient of a grant to participate in a course on eukaryotic RNAs. We look forward to these changes in direction and, furthermore, predict a transition from our traditionally strong biochemical orientation toward genetics and related work, to an increased emphasis on other, equally important areas in biology. These include biochemical studies on cell membranes, hormonal action, tissue and humoral immunology, and cellular differentiation.

ESR STUDIES OF PHOTOLYZED PEPTIDE SOLUTIONS

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

Livingston and Zeldes examined by microwave spectroscopy the radicals produced by photolysis of two Bence Jones proteins obtained from Alan Solomon of the UT Memorial Research Center. Broad electron spin resonance (ESR) signals were obtained with intensities characteristic of the individual proteins; however, little could be said about the nature of the radicals from a direct analysis of the spectra. Thus a cooperative study of simple peptides was begun with the expectation that detailed analysis of the spectra would reveal systematic trends that would be applicable to more complex systems.

Glycylglycine was initially studied, but, unlike with the proteins, signals were not obtained upon photolysis in aqueous solution. The addition of a small amount of hydrogen peroxide (about 1%), however, did give an excellent, well-resolved spectrum for a single radical. Under these conditions, photolysis of the peroxide gives •OH, and the observed radical is the product of •OH attack on the peptide, a process of great importance in radiation biology. The glycylglycine was present as the zwitterion. Upon adding acid, the spectrum broadened and became weaker. No spectrum was obtained from the hydrochloride. In all of the work described here, the peptides were in nearly neutral aqueous solutions containing about 1% hydrogen peroxide.

The finding of only a single radical from glycylglycine was of great interest. The peptide and also the resulting radical may exist in *cis* and *trans* forms. It is well known that there is a lack of free rotation about the peptide bond, and the *trans* isomer is presumably the

more stable form. Each of the radicals described here is a single isomer, presumably the *trans* configuration. There are two likely positions in glycylglycine – either CH_2 group – from which •OH might abstract hydrogen, but it was clear from the spectrum that attack is selective for only one of these positions. To resolve this ambiguity and gain further insight into the products of radical attack, a series of peptides were prepared in the necessary 5- to 10-g quantities, beginning with the pairs β-alanylglycine and glycyl-β-alanine, and glycyl-L-alanine and L-alanylglycine. There is no ambiguity for assignments of the radicals from β-alanylglycine, L-alanylglycine, and glycyl-L-alanine (Fig. 1), where again only a single radical is formed. The assignment for glycylglycine was taken by comparison with these peptides. In each case the abstracted hydrogen comes from the carbon adjacent to the nitrogen of the peptide link. There is also ambiguity in the assignment of the radical from glycyl-β-alanine (Fig. 1), the two possible choices being abstraction from either of the adjacent CH_2 groups. The choice illustrated was made by comparison with the other peptides.

This study is in its initial stages but already illustrates systematics in the nature of •OH attack and in the values of hyperfine couplings and *g* values. It is planned to extend the work to other dipeptides such as β-alanyl-β-alanine, β-alanyl-L-alanine, glycyl-L-valine, β-alanyl-L-valine, glycyl-L-proline, and β-alanyl-L-proline, and then to other dipeptides with functional groups and simple tripeptides.

ORNL-DWG. 74-4550

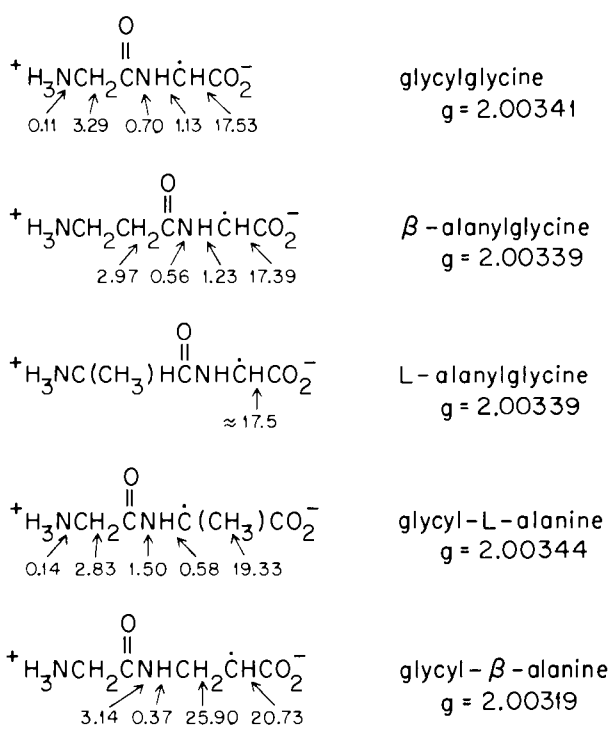

Fig. 1. Formulas showing the radicals formed by •OH attack in aqueous solutions of the indicated dipeptides.

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*Chemistry Division.

REGULATION OF HEMOGLOBIN SYNTHESIS IN A MURINE ERYTHROBLASTIC LEUKEMIA CELL: THE REQUIREMENT FOR REPLICATION TO INDUCE HEMOGLOBIN SYNTHESIS

P. R. McClintock* and John Papaconstantinou

Friend leukemia cells (FLC) grown in tissue culture can be induced to differentiate along erythroid lines. The induction can be stimulated by the addition of 2% (v/v) dimethylsulfoxide (Me_2SO) or 1% (v/v) dimethylformamide to the culture medium.[1,2] The most striking manifestation of this differentiation is the appearance of mouse hemoglobin in the cytoplasm. After about three days of exposure to Me_2SO, the cells have a discernible pink tinge, and after five days they are quite red. RNA sequences complementary to transcripts of mouse globin mRNA can be detected in FLC after two days of exposure to Me_2SO, but little or no hybridization is observed in uninduced cells.[3] The hemoglobin produced is reportedly the normal adult form for DBA/2 mice.[4]

The length of time between addition of Me_2SO and the appearance of hemoglobin suggested the possibility of a requirement for a precise number of mitoses before the expression of differentiated functions. To demonstrate a relationship between the induction of hemoglobin synthesis and cellular replication, cultures were treated with Me_2SO under conditions that were either conducive or nonconducive to growth. Nonconducive conditions included seeding at high cell density, seeding in medium containing low serum, and adding $N^6,O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphate [$(But)_2$cAMP] to the culture medium.

The time course of hemoglobin accumulation was also studied in synchronous cultures of FLC. The results of these experiments indicate that two generations after stimulation with Me_2SO are required before hemoglobin synthesis can be initiated.

Requirement of two generation times for the induction of hemoglobin synthesis by Me_2SO. Time courses of cell growth and the appearance and accumulation of hemoglobin in FLC cultures seeded at various densities were studied. In the absence of Me_2SO, negligible

amounts of hemoglobin are made. Addition of Me_2SO (2% v/v) to the medium results in a tenfold or greater stimulation of hemoglobin synthesis without any significant effect on cell growth. When cultures are seeded at a low density, the period of cell division prior to hemoglobin synthesis is approximately 48 hr. At this time the cells are in log phase, and the cell number increases from 1.5×10^5 to 5.6×10^5 cells per cm^3. This is equivalent to two generation times, and on the basis of these data we assign a mean generation time of 24 hr to these cells. These studies clearly indicate that most cells pass through two generations after the addition of the inductive stimulus (Me_2SO) before hemoglobin synthesis is initiated.

When cultures are seeded at a moderately high cell density (4.2×10^5 cells per cm^3), hemoglobin synthesis begins at about 48 hr. In these experiments also, cells pass through two generations in the presence of Me_2SO before hemoglobin synthesis occurs. (The cell number increased from 4.2×10^5 to 16×10^5 cells per cm^3 in 48 hr.) In contrast to the above observations, when cultures are seeded at a high cell density (36×10^5 cells per cm^3), so that cellular replication does not occur, hemoglobin synthesis is not detected. At this high cell density, a stationary population of cells is maintained for a period of 72 hr, and no hemoglobin synthesis is induced during this time.

We conclude from these studies that the FLC require approximately two generations after the addition of the inductive stimulus (Me_2SO) for the initiation of hemoglobin synthesis.

The effect of reduced serum content on the generation time of FLC and the induction of hemoglobin synthesis by Me_2SO. When FLC are grown in medium supplemented with less than 15% fetal calf serum, the generation time is extended. The generation time of cells grown in Eagle's minimal essential medium plus 15% fetal calf serum is approximately 24 hr. When the serum content is reduced to 7.5% and 2.5%, the generation time is increased to 34 hr and 48 hr, respectively. In each case it was observed that the cells must pass through two generation times after the addition of Me_2SO for the induction of hemoglobin synthesis.

The effect of (But_2)cAMP on the generation time of FLC and the induction of hemoglobin synthesis by Me_2SO. Treatment of FLC cells with $(But)_2$cAMP results in an extension of the generation time of these cells. Studies were carried out to determine whether this would affect the induction of hemoglobin synthesis. Addition of 1 m*M* $(But)_2$cAMP to cultures 24 hr after the addition of Me_2SO extended the generation time to 38 hr and delayed the appearance of hemoglobin to 78 hr. Thus, a time period equivalent to two generation times elapsed before hemoglobin synthesis was initiated. On the other hand, when $(But)_2$cAMP was added to cultures after 48 hr, there was no effect on the time of appearance of hemoglobin, even though cellular replication was inhibited. In this case, the cells replicated two times, and, although the $(But)_2$cAMP inhibited further growth, hemoglobin synthesis was initiated, since the required two replications were completed.

Induction of hemoglobin synthesis in synchronized FLC. Synchronization of the FLC was achieved by the isoleucine-deficiency method of Ley and Tobey.[5] The cells were suspended in isoleucine-deficient medium for 40 hr to bring about cellular arrest at the G_1 phase. The cells were released from the block by addition of isoleucine-containing medium, and Me_2SO was added to induce hemoglobin synthesis. Hemoglobin appeared at 47 hr after the release. During this time the cells went through two mitoses prior to the appearance of hemoglobin, and we tentatively assign the appearance of hemoglobin to the G_1 phase of the cell cycle.

The phenomena of obligatory DNA synthesis and critical mitosis have been described for the erythropoietin stimulation of hemoglobin synthesis in fetal mouse liver[5] and in chick-embryo erythroblasts.[6] In these studies we have been able to show that in the presence of Me_2SO, which is not a physiological inducer, these cells must go through two rounds of DNA synthesis and/or mitosis before hemoglobin synthesis is initiated. Thus we conclude that this model system can mimic erythropoietin with respect to the role of critical mitosis in the induction of hemoglobin synthesis. We are presently attempting to determine the mechanism of Me_2SO-mediated induction.

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KINETICS OF ALBUMIN SYNTHESIS BY CULTURED MOUSE HEPATOMA CELLS

B. E. Ledford* and John Papaconstantinou

Differentiation of eukaryotic cells is usually accompanied by the acquisition of morphological, functional, and biochemical specificity. In most cases the highly differentiated cell synthesizes large quantities of tissue-specific structural proteins. This is clearly seen in the synthesis of crystallins in the lens,[1] globin in the reticulocyte,[2] albumin in the liver,[3] and other highly differentiated tissues. In recent years a number of tumor-derived cell lines have been established in continuous tissue culture and have been shown to synthesize tissue-specific proteins over a long period of time. One of these cell lines (Hepa) derived from the mouse hepatoma BW7756, has been shown to maintain a high level of albumin synthesis in long-term culture.[4,5] Cultures such as these, which retain specific genotypic properties of the tissue of origin, are of particular value in studying the role of a variety of factors in the regulation of the synthesis of these specific proteins. We have used these hepatoma cells to study the regulation of synthesis of liver-specific secretory proteins. In these studies we have shown that albumin is synthesized and secreted into the culture medium, that synthesis occurs at levels reported to occur *in vivo*, and that the rate of albumin synthesis is inversely proportional to the rate of cellular replication.[5]

Identification of albumin as a component of the proteins secreted by Hepa. One of the characteristics of this cell line is that it secretes large amounts of protein into a serum-free medium. By polyacrylamide gel electrophoresis, the complex protein solution was resolved into three distinct major bands and several minor bands. Albumin was identified by the use of specific precipitation with mouse albumin antiserum. Further evidence supporting the identification of albumin is based on (1) coelectrophoresis with pure mouse albumin and (2) solubility properties and molecular weight.

Rate of intracellular albumin synthesis and secretion. The rates of total protein synthesis, albumin synthesis, and albumin secretion were determined. [^{3}H] Leucine was incorporated linearly into intracellular albumin for a period of 60 min, after which the labeling leveled off, indicating that the small intracellular pool had been completely labeled. The slope of the secretion was approximately equal to the slope of intracellular incorporation, indicating that the rates of secretion and synthesis are comparable. The slope of the rate of secretion was approximately 10% that of the total incorporation, which was comparable to the ratio of albumin synthesis to total protein synthesis.

The effect of cellular growth rate on albumin synthesis. We have recently completed a series of studies that indicate that the rate of albumin synthesis is inversely proportional to the cellular growth rate. Our data show that during logarithmic growth the cellular rate of albumin synthesis in Hepa cells falls to approximately 20,000 molecules per cell per min. When the cells approach stationary phase, there is an increase in the cellular rate to 60,000 molecules per cell per min. There is a further increase in the rate of albumin synthesis when the cells are in stationary phase, where a level of 100,000 molecules per cell per min is reached.

The experiments described above indicate that albumin synthesis can be increased by extending the generation time of Hepa cells. In Dulbecco's medium the generation time of Hepa cells is 24 hr. We were able to alter this by growing the cells in Waymouth's medium supplemented with insulin and 10% fetal calf serum (generation time of 15 hr) and in the same medium without fetal calf serum (generation time 24 hr). The data from these experiments show that in rapidly growing cultures the rate of albumin synthesis follows the same pattern as in Dulbecco's medium; that is, the rate of synthesis decreases rapidly in log-phase cells to a level of 11,000 molecules per cell per min and then increases to a maximum level of 137,000 molecules per cell per min in stationary-phase cells. When fetal calf serum is removed from the medium, the generation time increases and the rate of albumin synthesis remains at a relatively high level (50,000–60,000 molecules per cell per min) throughout the log phase; when the culture enters stationary phase, a twofold increase in the rate of albumin synthesis occurs. These data further indicate that the rate of albumin synthesis is inversely proportional to the generation time of the cell.

Extension of the generation time of cells in culture can also be achieved by adding $(But)_2$cAMP to the medium. We used this technique to extend the generation time of Hepa cells. When $(But)_2$cAMP was added to log-phase cultures, cellular replication was arrested, and within 24 hr the rate of albumin synthesis was stimulated to its maximal level. From these studies we conclude that the rate of albumin synthesis in asynchronous cultures is inversely proportional to the rate of cellular replication.

In a recent paper, Bernhard et al.[4] described a clonal line, Hepa-1, which has a generation time of 28 hr when grown in Waymouth's MAB/87/3 medium supplemented with 10% fetal calf serum. These are the same

growth conditions that support a generation time of 15 hr for the parent culture, Hepa. Because of this significant difference in generation time, we determined the rates of albumin synthesis in this slow-growing cell line during log phase and stationary phase. In Hepa-1 cells, the rate of albumin synthesis is relatively constant at 50,000 molecules per cell per min throughout the cell cycle. Although a decline to 32,000 molecules per cell per min is seen in log-phase cells, indicating that there is some degree of control of albumin synthesis similar to that seen in Hepa cells, this is not comparable to the tenfold difference seen in log-phase vs stationary-phase Hepa cells.

A coupling of the regulation of tissue-specific biochemical functions to the inverse rate of cellular replication has been described for both structural protein synthesis[6] and enzyme activity.[7,8] In cultures of chick muscle cells, the concentration of myosin per cell and the creatine kinase activity increase fivefold when myoblasts fuse to form myotubes. Similar observations have been made for the regulation of acetylcholinesterase activity in neuroblastoma cells[8] when they differentiate to form dendrites, and for procollagen peptidase activity in human fibroblasts.[7] In all of these cases, the structural protein concentration or specific activity of an enzyme is low in the rapid cellular proliferation stage and increases when the cells reach the postmitotic stage (muscle cells) or saturation density. Thus, in all of these cell lines, Hepa included, the shift to stationary phase enhances the expression of biochemical specialization. Although the mechanism for this is not clearly known, there are studies which suggest that, in addition to transcriptional regulation, the level of a protein may be determined by a modulation of synthesis and degradation. In the Hepa cells there is a change in the rate of protein synthesis, and since all of the albumin is secreted into the medium, changes in the rate of degradation would not be involved in this mechanism. We propose therefore that this stimulation in synthesis can be attributed either to an increase in the amount of mRNA available for translation or to a specific translational regulatory event. We are presently attempting to determine which of these mechanisms is involved in the regulation of levels of albumin synthesis.

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ALBUMIN AND α-FETOPROTEIN SYNTHESIS BY SYNCHRONOUS MOUSE HEPATOMA CELLS

John Papaconstantinou and B. E. Ledford*

In recent years, studies with synchronized eukaryotic cells have shown that patterns of enzyme activity (and presumably specific enzyme and protein synthesis) can be classified into two broad groups – discontinuous synthesis and continuous synthesis throughout the cell cycle.[1,2] A survey of the literature indicates that patterns of discontinuous protein synthesis are more common than patterns of continuous synthesis. Continuous protein synthesis throughout the cell cycle is either exponential or linear, and in the latter case a constant rate of synthesis is maintained until a characteristic point in the cell cycle is reached where the rate doubles (critical point).

The activities (synthesis) of many enzymes have been studied in the yeast *Schizosaccharomyces pombe,* of which only three (acid phosphatase, sucrase, and alkaline phosphatase) have a continuous linear pattern. All of these enzymes show a doubling of activity at a point in the cell cycle called a critical point, and in each case the critical point occurs in G_2. If the pattern of enzyme activity can be extrapolated to enzyme (or protein) synthesis, then some of the questions that arise as a result of this type of synthesis pattern are: (1) How does the eukaryotic cell accomplish a significant delay from the gene-doubling point in S phase to the period of transcription and then translation (which probably occur in G_2)? (2) Is the time for gene doubling to transcription to translation constant and reproducible from one cell cycle to the next? (3) Is this temporal relationship from gene doubling to transcription to translation a mechanism by which the cell can achieve coordination of macromolecular synthesis and assembly and the formation of complex functional units? (4) Can the time in S phase when a gene is doubled be altered, and does this affect the subsequent coordination of

macromolecular synthesis? Furthermore, can this be the basis for metabolic changes seen in cellular transformation?[3]

Recently we demonstrated that the cell line Hepa, derived from the mouse hepatoma (BW7756), retains the capacity to secrete albumin and α-fetoprotein. These cells have been synchronized, and studies on the synthesis of albumin and α-fetoprotein during the cell cycle have been carried out. The results indicate that albumin and α-fetoprotein syntheses are linear and continuous throughout the cell cycle, and that both show a specific critical point where the rate of synthesis increases sharply. The critical point for albumin is in early mitosis and for α-fetoprotein is in mid-G_2. To carry out the synchrony studies, Hepa cells were arrested at G_1/S by the isoleucine-deficiency method.[4] The blocked cells were released by the addition of complete medium containing [^{3}H]leucine. Secretion of albumin and α-fetoprotein was then followed throughout the cell cycle. Six hours after the addition of complete medium, radioactive albumin was detected in the medium. Incorporation was linear until midmitosis, where a threefold increase in the incorporation rate occurred. After the first critical point, the rate of incorporation remained constant until the second mitosis was reached. at which time a 60% increase occurred. (At this point the cells are beginning to phase out of synchrony.)

Incorporation of [^{3}H]leucine into secreted α-fetoprotein was first detected 20 hr after release from the isoleucine-deficiency block. Synthesis of α-fetoprotein began in late G_2 but significantly prior to mitosis. The incorporation rate remained linear and increased twofold prior to the second mitosis.

Our studies thus far indicate that the syntheses of albumin and α-fetoprotein are continuous and linear throughout the cell cycle, with characteristic critical points in the G_2 phase.

The existence of a defined period of transcription for a specific gene during the cell cycle has been indicated by studies with a number of eukaryotic cells. The majority of the evidence is based on enzymatic activity in synchronous cells (although synthesis data are rapidly accumulating). Thus it appears that transcription can be removed, in time, for the gene-doubling time in eukaryotic cells. This is also true for the time from transcription to translation. We are presently studying the temporal relationship of gene doubling to transcription to translation, using albumin and α-fetoprotein as marker proteins.

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EFFECT OF DIBUTYRYL CYCLIC AMP ON THE SYNTHESIS OF ALBUMIN IN SYNCHRONIZED MOUSE HEPATOMA CELLS

C. L. Parker and John Papaconstantinou

Our previous studies indicated that the syntheses of albumin and α-fetoprotein in synchronized mouse hepatoma cells are linear and continuous throughout the cell cycle. Both proteins were found to have a critical point where their rate of synthesis increases sharply. The critical point in albumin synthesis occurs during early mitosis (or very late G_2), whereas the critical point in α-fetoprotein synthesis occurs in late G_2 but prior to that for albumin.

Since the Hepa cells used in our experiments are transformed cells, we have considered the possibility that the critical point for albumin synthesis (or any other protein) may be shifted from its normal position within the cell cycle. This shift might occur, for example, if the temporal relationship of gene doubling to transcription to translation is altered in the transformed cell. To test this hypothesis, experiments were done to determine whether the critical point for albumin synthesis could be shifted. These experiments involved the treatment of synchronized Hepa cells with $(But)_2$cAMP. The use of this compound is based on its known ability to decrease the rate of replication of transformed cells and its ability to bring about changes in the membrane characteristics of the transformed cell so that they are similar to those of normal cells. The results of our studies indicate that the critical point for albumin synthesis can be shifted into the G_2 phase of the cell cycle approximately 2 hr prior to mitosis. We interpret these data to indicate that the critical point in the synthesis of albumin is reproducible in successive cell cycles, and that this critical point can be shifted by treatment with $(But)_2$cAMP. The occurrence of such a shift may indeed indicate that the transformed cell undergoes changes that affect the coordinated synthesis and assembly of macromolecules. We are presently attempting to locate the period of transcription of albumin genes in synchronized cells, so that we can determine whether treatment of these transformed cells with $(But)_2$cAMP will alter this sequence of events.

ELECTRON MICROSCOPIC ANALYSIS OF MITOCHONDRIAL DNA FROM *RHYNCHOSCIARA* SPERMATOCYTES

John Papaconstantinou, D. P. Allison, and Emilia M. Julku

The morphological changes seen in the mitochondria of both invertebrate and vertebrate spermatocytes are specific for these cells and occur at specific stages during spermatogenesis. In a variety of insects, where these changes have been studied in detail, the mitochondria first appear as aggregates of small granular particles located in specific regions of the cell. As development of the spermatocyte proceeds, mitochondria acquire different shapes, such as spheres, rods, or threads, and are scattered throughout the cytoplasm.[1] Such specific mitochondrial differentiation is observed both in *Rhynchosciara hollaenderi*[2] and in the closely related dipteran, *Sciara coprophila*.[3] In the initial stages of spermatogenesis, the mitochondria appear as discrete granules closely associated with the nucleus; they enlarge, occupying practically the entire cytoplasmic area, and then are arranged along the spermatid flagellum, where they ultimately fuse to form a single mitochondrial derivative extending from the nucleus to the tip of the tail of the mature spermatid.

In a previous publication,[4] we presented evidence for a specific pattern of DNA synthesis concurrent with the morphological changes in the spermatocyte mitochondria. The synthesis of a circular DNA ($\rho = 1.681$ g/cm^3) is first detectable when the mitochondria become visible as granules aggregated around the nuclear envelope. As the mitochondria increase in size, the synthesis of this DNA becomes a major part of the total DNA synthesized, and when meiosis is initiated, 80% of the total DNA synthesized is this circular DNA.

Studies have also been done to show that this circular DNA is associated with mitochondria.[5] Ethidium bromide analysis of DNA extracted from a mitochondrial pellet revealed a circular DNA. One of the interesting observations made in these studies is that the circular DNA synthesized in *Rhynchosciara* spermatocytes has a contour length of 9 μm, which is equivalent to a molecular weight of 18×10^6. To date, mitochondrial DNA of all metazoan species examined is in the form of circular molecules, with a contour length of approximately 5 μm (ref. 6). This is also true with respect to insects, where mitochondrial DNA isolated from *Musca* has a length of 4.6 to 5.6 μm (ref. 7) and that from thoracic muscles of *Schistocerca gregaria* has a contour length of 4.74 μm (ref. 8). In *Rhynchosciara* spermatocytes, however, the only circular DNA we detect is 9 μm in contour length. The possibilities exist therefore that (1) all mitochondrial DNA of *Rhynchosciara* has a contour length of 9 μm (2) only the mitochondria of the spermatocytes have DNA of 9 μm contour length, or (3) the mitochondrial DNA of the spermatocytes represents circular dimers of a 4.5-μm form.

In the past year we have succeeded in extracting circular DNA from somatic tissues (salivary gland, fat body, and gastric caecum). In each case the circular DNA isolated has a contour length of 9 μm. Our data now strongly indicate that mitochondrial DNA from both somatic and germ cells is uniquely large. To determine whether this 9-μm circular DNA is a monomer or dimer, we carried out a denaturation-map analysis, using the Inman electron microscope technique. The denaturation maps indicate that the circular DNA is a monomer; that is, there are no repeating patterns of denaturation in the 9-μm circle.

Our cytological observations confirm the highly specific morphological changes in the mitochondria of primary spermatocytes that have been described for many species of insects. The mitochondria undergo a striking enlargement and accumulate proteinaceous material during spermatogenesis. The process of mitochondrial differentiation culminates in the fusion of the mitochondria into a single body extending the length of the mature sperm tail. We have demonstrated that the specific synthesis of mitochondrial DNA in spermatocytes occurs during the period of mitochondrial differentiation. The high level of synthesis of circular DNA molecules is observed only in primary spermatocytes and only at the time when mitochondrial differentiation is occurring. We are thus observing a stage-specific, tissue-specific, and organelle-specific developmental response, which we feel can be attributed to a highly specific regulatory mechanism.

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STUDIES ON THE TRANSFER RNAs OF METHANE-PRODUCING BACTERIA AND THE IMMOBILIZATION OF METHANE-PRODUCING BACTERIA

Audrey N. Best

The role of bacteria in the production of methane from various carbon sources has been of interest to this laboratory. We have studied two pure cultures with the object of immobilizing them to a solid support in order to carry out the production of methane *in situ* without growth of the cells. The results have not been satisfactory. These bacteria are strict anaerobes, and this condition is a possible complication, along with damage to the cell wall during treatment for immobilization. We had elected to immobilize the whole cells rather than the enzymes involved in methane production due to the multiplicity of the latter.

We are interested in isolating and identifying altered species of transfer RNAs. At the present time there is no satisfactory procedure for the separation of an individual transfer RNA other than by laborious, large-scale separation. Investigators in our laboratory (Jan Dijk and R. P. Singhal) are working on a procedure for the isolation of small amounts of purified transfer RNA by means of the formation of an aminoacyl-tRNA–GTP–protein factor Tu complex. We know that the Tu factor from *Escherichia coli* can form a complex with human placental aminoacyl-tRNA (A. N. Best, unpublished results), so that the procedure could be applied to transfer RNAs from various organisms. We have begun initial studies on the nucleoside composition of the purified bulk transfer RNA from one of these methane-producing bacteria, using ion-exclusion chromatography. The composition appears to differ from that of bulk transfer RNA from *E. coli.* Further experiments are planned with the object of purifying one or several transfer RNA species from this bacterium and studying the nucleoside composition.

PURIFICATION OF AMINOACYL-tRNAs WITH IMMOBILIZED ELONGATION FACTOR Tu

Jan Dijk, R. P. Singhal, Shigemi I. Simms, and G. David Novelli

Elongation factor Tu (*E. coli*) binds only aminoacylated tRNAs in the presence of GTP, forming a ternary complex Tu–GTP–aminoacyl-tRNA. It is strictly specific for *aminoacyl*-tRNA (except for the initiator $tRNA^{fMet}$) and does not bind any *free* tRNA or aminoacyl-tRNAs in which the NH_2 group of the amino acid has been modified. In immobilized form it can be used in a one-step procedure to obtain one aminoacyl-tRNA from a mixture of tRNAs (from both prokaryotes and eukaryotes). The mixed tRNA is aminoacylated with one amino acid and passed through a Tu column in the presence of GTP. The aminoacyl-tRNA will bind to the column, while all others are eluted and can be recovered. The aminoacyl-tRNA is obtained by disrupting the ternary complex; isoaccepting species can then be separated by established procedures, such as reversed-phase chromatography.

The realization of the procedure described above has been delayed by several technical difficulties, the two major ones being lack of pure Tu and the problem of establishing proper conditions for its immobilization.

Tu isolated from *E. coli* B or Q-13 is contaminated with a GTP-hydrolyzing activity, which prevents the formation of the ternary complex with aminoacyl-tRNA. It persistently cochromatographs with Tu on DEAE-Sephadex and Sephadex G-100, the last two purification steps. So far, only a small amount of pure Tu has been obtained by first isolating the Tu-Ts complex, which is subsequently split up into Ts and Tu-GDP.

Recently, experiments on the use of immobilized GDP for the isolation of Tu have been started. GDP has been immobilized on Affi-Gel 10 (BioRad), using the NH_2 group for coupling. The coupling efficiency of this reaction is very low (less than 35 nmoles of GDP per ml of gel). Consequently, only a maximum of 360 pmoles of Tu (= 14 μg) can be bound (an excess of GDP on the gel is needed to displace the Tu-bound GDP). Coupling of periodate-oxidized GDP to NH_2 groups on Sepharose or glass beads is much more efficient (5.5 μmoles GDP per ml for glass, 150 nmoles GDP per ml for Sepharose). However, Tu does not bind to this immobilized GDP, even after reduction of the residual aldehyde group. Other possibilities for the immobilization of GDP will be explored.

As an alternative, Tu will be isolated from *Bacillus stearothermophilus.* It is reported to have less GTPase activity, and as an additional advantage it is more temperature-stable than Tu from *E. coli* (Tu is the abbreviation for temperature-unstable).

The conditions for immobilization of Tu have been worked out. CNBr-activated Sepharose cannot be used without an additional step; the activation with CNBr introduces a positively charged imido group which will bind all tRNAs without discrimination. However, it can be shielded, after coupling of Tu, by the introduction of a negatively charged molecule. As an alternative, Affi-Gel 10 (BioRad) can be used, which has an arm

10 Å long that is linked to the agarose backbone by an ether linkage, ending in an activated COO^- residue, and reacts with the NH_2 group of proteins.

Immobilization of Tu-GDP by either method results in bound Tu, which will still bind GDP or GTP but no longer aminoacyl-tRNA. Therefore, it will have to be immobilized as the ternary complex. Aminoacyl-tRNA can be recovered from the immobilized complex by hydrolyzing the aminoacyl-tRNA linkage; a 10-min treatment with 1.5 *M* Tris acetate, pH 8.0, at 30°C hydrolyzes more than 99% of the aminoacyl-tRNA, without detectable damage to Tu.

PRECURSOR tRNAs IN *ESCHERICHIA COLI*

Jan Dijk and R. P. Singhal

RNAs from *E. coli* χ402 (Met^-, RC^{rel}) pulse-labeled with [^{32}P] phosphate after chloramphenicol treatment are separated into four major peaks on Sephadex G-100: high-molecular-weight RNA (rRNA), α, β, and tRNA. Peaks α and β have been fractionated further by RPC-5 and polyacrylamide gel electrophoresis on 40-cm-long slabs. Peak α, in addition to 6S RNA, contains a large number of unknown bands with an estimated size of 140–250 nucleotides. Peak β also has a large number of bands, in addition to 4.5S and 5S RNA and its precursors, size range 100–150 nucleotides.

We have demonstrated by chase experiments, *in vitro* maturation, and competitive hybridization, that more than 60% of the α and 70% of the β fraction is structurally related to mature tRNA and represents precursor molecules for tRNAs. The α species are sufficiently large to accommodate the two or three tRNA sequences that have been demonstrated by other investigators. The β precursors most probably have only one tRNA sequence, similar to the $tRNA^{Tyr}$ precursor in phage-infected *E. coli*. The α species do not contain modified nucleosides, while some of the β fractions show considerable modification levels, approaching the levels of tRNA. It is therefore possible that the α fraction represents an earlier stage in the maturation process than β; however, at this point in the investigation this cannot be proven.

A modest approach to the identification of one of the β precursors has been made. RNase-T_1 and RNase-A digests of β_4 have been separated by RPC-5 and Aminex A-28 chromatography. The nucleotide compositions of the peaks and the sequences of some of them have been determined. The results allow the following conclusions: (1) The size of β_4 is between 125 and 135 nucleotides. (2) the 5′ end is a mixture of 80% pGp--- and 20% pCpGp---. (3) A tetranucleotide is found, consisting of a mixture of U-U-C-G and U-T-C-G, indicative of a not fully mature Ψ-T-C-G sequence, characteristic for most tRNAs. (4) The 3′ end is heterogeneous, and no sequence has yet been established.

tRNA ALTERATIONS OCCURRING DURING LYMPHOCYTE BLASTOGENESIS*

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

When certain plant extracts (called mitogens) or bacterial products are added to cultures of human peripheral blood lymphocytes, a process called blastogenesis ensues, characterized by dramatic increases in cell size and, on a biochemical level, large increases in RNA, protein, and DNA synthesis. RNA synthesis begins to increase within 30 min of the initial stimulation, and much of this early synthesis is apparently 4S material.[1] Using the high resolving power of reversed-phase chromatography (RPC-5), we have attempted to see whether alterations in tRNA are associated with the process of blastogenesis.

Lymphocytes were isolated from peripheral blood and placed in culture, and some cultures were stimulated with mitogens such as phytohemagglutinin (PHA), conconavalin A, pokeweed mitogen, or *E. coli* lipopolysaccharide. tRNA was either labeled by incorporation of radioactive uridine or $^{32}PO_4$ *in vitro* or charged, after appropriate isolation procedures, with radioactive amino acids, using a synthetase preparation from human placenta.

Cochromatography of uridine-labeled tRNAs from PHA-stimulated lymphocytes on RPC-5 columns showed that the greatest alteration (in terms of changes seen in chromatograms) in tRNAs, compared to non-stimulated lymphocytes, occurred within 4 hr after addition of PHA. This distinctive early profile very rapidly underwent further alteration, so that by 8 hr after PHA, the total cellular tRNA distribution assumed a characteristic appearance different from profiles of control cultures but consistently the same as profiles obtained with tRNAs labeled later in the course of PHA stimulation (up to 68 hr after PHA stimulation).

Comparison of uridine-labeled tRNA profiles from lymphocytes stimulated with different mitogens indicated that, regardless of the specific blastogenic trigger, the tRNA species synthesized in response to this stimulus were generally the same. Exceptions to this were seen upon stimulation with pokeweed mitogen and anti-human immunoglobulin, in which cases rather

specific differences in certain parts of the chromatogram were seen, as compared to profiles from other mitogen-stimulated cultures. One might expect that mitogens which activate preferentially T and B lymphocytes would induce synthesis of somewhat different complements of tRNA, but it rather appears that the blastogenic signal induces synthesis of unique tRNAs, these tRNAs being the same in T and B cells.

We have also studied specific isoaccepting tRNA species in control and PHA-stimulated lymphocytes and have found quantitative and qualitative (appearance of a new, very minor isoaccepting species) changes in tyrosine tRNA from stimulated cells compared to control cells. The same quantitative change (a decrease in amino acid acceptor activity) is seen in phenylalanine tRNA from PHA-stimulated cells, while aspartyl-tRNA shows no difference. These changes in specific tRNAs underscore the idea of the more or less drastic differences in tRNA species required by this mammalian cell as it undergoes significant changes in metabolic activity leading to a dedifferentiated state, and they suggest possible regulatory roles of tRNA in this total process.

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†American Cancer Society Postdoctoral Fellow.

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CELLULASE

Helen G. Sellin

Cellulose is the major constituent of all vegetation, and after a variety of manufacturing processes much of it remains as sawdust, sugar cane bagasse, corn cobs, etc. If waste cellulose could be effectively reutilized as an industrial raw material which is annually replenished, a major pollution problem would be curtailed and an inexpensive starting material would be used. One potential use of cellulose is as an energy source. Cellulose can be degraded microbiologically by cellulase, a complicated enzyme complex, to glucose. Using enzyme systems from other organisms, glucose can be converted to methane, an energy source, or ethanol, in demand industrially.

We have examined, briefly, the cellulose-to-glucose conversion. There are many problems in this system. The conversion occurs stepwise, but many of the steps are unknown or there is no known assay available for some of them. The cellulase system is composed of at least three components. The first, C_1, acts on native cellulose to "loosen" its structure into a reactive cellulose which C_x can attack and convert to oligosaccharides. The oligosaccharides are hydrolyzed to cellobiose by glucanases and finally to glucose by glucosidase. At the present time, however, no assay exists for the "loosening" action of C_1. The overall activity can be measured by determining reducing sugar or glucose. Further problems arise from the insoluble nature of the cellulose substrate and the lengthy incubation time required for production of reducing sugar. Ideally one would like to look at an earlier stage of the reaction.

Using *Trichoderma viride* as the source of cellulase, we examined growth and cellulase production on a variety of carbon sources ranging from glycerol and some sugars through substituted celluloses to avicel, a microcrystalline cellulose. As might be expected, since cellulase is an inducible enzyme, the level of enzymatic activity varied widely with the carbon source. Cultures grown on cotton linter pulp or cellulose flock (wood α-cellulose) gave the highest cellulase specific activity when avicel was used as the assay substrate. When grown in shaker flasks, the enzyme activity of the culture on cotton linter pulp peaked the eighth day after inoculation. The cellulose flock culture had a peak five to eight days after inoculation. When the cultures were grown in a New Brunswick fermentor under pH control, the cellulase activity of the cotton linter pulp culture did not begin to increase until the twelfth day. The cellulose flock culture under these conditions had good activity after the fourth day.

Much work remains to be done on purification and characterization of cellulase.

NONREVERSIBLE D-GLYCERALDEHYDE-3-P DEHYDROGENASE

Helen G. Sellin

The availability of sunlight makes it attractive as a potential energy source if its energy could be harnessed. Activated by sunlight, photosynthesis occurs in chloroplasts, yielding NADPH and ATP. However, the NADPH is not readily available for use outside the chloroplasts because it cannot penetrate the chloroplast envelope. Within the chloroplast, NADPH can be utilized for the conversion of 3-P-glycerate to D-glyceraldehyde-3-P, which can penetrate the chloroplast envelope and thus be an indirect transfer of reducing power across the chloroplast. Outside the chloroplast, using the nonreversible D-glyceraldehyde-3-P dehydrogenase, any triose-P would be rapidly reoxidized to 3-P-glycerate using NADP. The resulting NADPH could

be linked through ferredoxin to other enzyme systems (e.g., hydrogenase) to yield H_2 or CH_4, both energy sources.

In preliminary experiments, several possible sources of nonreversible D-glyceraldehyde-3-P dehydrogenase were screened. Spinach was the most readily available material with high activity. A few initial steps of purification, including high-speed centrifugation, G-100 chromatography, and DEAE-cellulose chromatography, were performed in preparation for further purification by affinity chromatography.

PROGRESS IN PURIFICATION OF OSTEOGENIC-SARCOMA-SPECIFIC TRANSFER FACTOR*

G. D. Griffin,† G. David Novelli, and R. P. Singhal

The capacity to defend the integrity of the mammalian organism against attack by intracellular parasites (such as viruses) or invasion by "foreign" cells (such as tumor cells) seems to depend mainly upon the cellular immune system, the cells involved being apparently the T lymphocytes. This cellular immune competence can be transferred from one individual to another by living lymphoid cells or (at least in the human) a low-molecular-weight dialysate of ruptured white blood cells called transfer factor. Levin and co-workers[1] have obtained evidence that transfer factor appears to be of therapeutic use in the treatment of the human bone malignancy, osteogenic sarcoma. We have been attempting to isolate the factor involved in transferring cellular immunity against the tumor from crude transfer-factor preparations.

White blood cells were isolated from individuals whose lymphocytes were shown to be cytolytic to osteogenic sarcoma cells *in vitro*, and transfer factor was prepared by cell lysis and subsequent dialysis against water, the lyophilized dialysate being termed transfer factor.

This transfer factor was separated into 15–18 uv-absorbing (260 and 280 nm) components, using an Aminex A-28 column. The components were assayed for their ability to convert *in vitro* nonsensitized lymphocytes to so-called "killer cells," which will attack and lyse osteogenic sarcoma cells in culture. It was found that activity resided in three components, two of which formed an incompletely resolved double peak, while the other component was found in a different region of the chromatogram. All action components had A_{260}/A_{280} ratios close to 1, and the double-peak components showed different biological activity from the other component in that they inhibited PHA transformation of lymphocytes, while the single-peak component did not. Fractionation of another sample of transfer factor with the same system resolved 11 components, with two rather widely separated uv-absorbing components containing the cytolytic transferring capacity. These components again had an A_{260}/A_{280} ratio of nearly 1 and were apparently specific for osteogenic sarcoma tumor, since they did not enhance nonspecific cytolytic activity against a nephroma cell line.

If this purification procedure can be confirmed and extended, it may be possible to isolate in a pure form the molecular species responsible for this antitumor activity and to characterize its molecular structure preparatory to undertaking its synthesis.

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*In collaboration with A. Levin, V. Baers, L. Spitler, and H. Fudenberg of the University of California Medical Center, San Francisco.

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BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF TUBERCULIN-SPECIFIC TRANSFER FACTOR

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

The biochemical characterization of the molecular species responsible for the *in vivo* transfer of tuberculin sensitivity from one human to another using transfer-factor preparations remains a vexing problem, due to the potentially widely variable conditions used in its preparation. The normal procedure[1] involves obtaining white blood cells (WBCs) from a strongly tuberculin-reacting person, lysing these cells (by freezing and thawing), and carrying out a DNase digestion followed by dialysis, the dialysate being referred to as transfer factor. This material will then transfer tuberculin reactivity to tuberculin-negative individuals by injection *in vivo*, but conversion of nonsensitive lymphocytes to an antigen-reactive state *in vitro* has not been achieved with any consistent success. The purification of an active tuberculin-specific transfer factor would be of more than academic interest, since an adequate biochemical understanding of this transfer factor would enable us to study the mechanism by which transfer factor acts in inducing cell-mediated immunity. Since cellular immunity is involved in defense against viral agents and foreign tissue, such as tumor cells or transplanted organs, the potential use of the knowledge gained is obvious.

Transfer factor from tuberculin-positive individuals has been prepared both from total WBCs and from isolated peripheral blood lymphocytes. Separation of these crude preparations into constituent fractions of uv-absorbing (260 and 280 nm) material has been achieved by Aminex A-28 chromatography, using a volatile-salt gradient. This single chromatographic step has been consistently successful in resolving 14–18 components from crude transfer-factor preparations. A rather fundamental change has also been introduced in the original procedure, in that, after initial homogenization, large- and small-molecular-weight materials are separated on Sephadex G-25, and the retained material from Sephadex (small molecular weight) is then introduced to the Aminex column.

Efforts are currently under way to assess the biological activity of these various transfer-factor components separated by different procedures, but so far the *in vitro* assays have proven to be unreliable indicators of transfer of cellular immunity. Indeed, there is some question whether such standard assays of cell-mediated immunity as blastogenesis and/or production of various lymphokines (soluble factors which affect cellular functions) have any fundamental relationship to cell-mediated immunity, as expressed by delayed-hypersensitivity reactions or cytotoxicity. We have completed a study of the blastogenesis assay and found it rather deficient as a reliable indicator of transfer of antigen sensitivity. Current efforts are geared toward developing a reliable assay procedure involving other parameters of cell-mediated immunity.

*American Cancer Society Postdoctoral Fellow.

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PROPERTIES OF THE SIGMA FRACTION OF DNA-DEPENDENT RNA POLYMERASE FROM A T4-PHAGE-INFECTED SYSTEM

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 unique 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 purified 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, while uninfected polymerase contains about 0.6 equivalent. The sigma fraction derived from the infected holoenzyme by phosphocellulose column chromatography has the following properties: (1) It poorly stimulates (1.5- to 2-fold) the activity of core enzyme fractions from either uninfected or infected *E. coli* using T4 DNA as a template. The stimulation by the infected sigma fraction is salt sensitive; however, the stimulation is enhanced severalfold by the addition of certain nonionic detergents, leading to asymmetric RNA synthesis. (2) It inhibits the comparatively high stimulatory activity of sigma fractions from uninfected *E. coli* under certain assay conditions, particularly in the presence of high salt. All the properties can be demonstrated at the level of rifampicin-resistant preinitiation complex formation and with sigma fractions further purified by gradient centrifugation. Labeling studies using ^{14}C-labeled amino acids with infected cells show that the infected sigma subunit is not newly formed after infection. It does bind the polymerase-T4-specific binding protein of mol wt 10,000 (ref. 1). ^{32}P-labeling studies with infected cells show that the sigma fraction does contain label, predominantly in phospholipid. The possibilities that the binding protein of mol wt 10,000 or the lipid content affects the activity of the sigma subunit are under investigation.

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INTERACTIONS OF $tRNA^{Phe}$ AND PHENYLALANYL-tRNA SYNTHETASE

M. P. Stulberg, K. R. Isham, A. J. Weinberger, and Mayo Uziel

This project is a continuation of our past studies on the specific interaction of $tRNA^{Phe}$ with its cognate enzyme, Phe-tRNA synthetase. Both components are highly purified. We have previously published data on the location of the enzyme-recognition site in the tRNA.[1] This work was accomplished by enzymic degradation of the $tRNA^{Phe}$ to limit-digest oligonucleotides, which were then utilized in kinetic studies of competitive inhibition to detect the number and location of specific binding sites.

This study will make use of the products of stepwise base elimination produced by the automated ribonucleic acid sequencer developed by Uziel and associates.[2] The oligonucleotides produced will afford us the opportunity to test the inhibition properties of defined, stepwise-degraded, partial molecules of $tRNA^{Phe}$. Thus we should be able to elucidate the finer details of enzyme-tRNA interaction.

The $tRNA^{Phe}$ has been purified and concentrated and is being degraded by the sequencer for utilization of products in our studies.

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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 $tRNA^{Phe}$ (UU-$tRNA^{Phe}$) from the RC^- Met^- mutant of *E. coli.* We have now completed this analysis and interpreted the results in relation to the function of $tRNA^{Phe}$ (ref. 2).

We have confirmed previous indications that guanine is partially methylated to 7-methylguanosine (7MeG) by *in vitro* methylation of UU-$tRNA^{Phe}$. Thus, the fractional content of 7MeG could be due to differential methylation from a small residual pool of *S*-adenosylmethionine in the methionine-starved growth or to the fact that 60% of the methylatable guanosine cannot accept methyl groups *in vitro.*

The lack of the methylthio group from 6MeS6iPeAdo in the UU-$tRNA^{Phe}$ may be responsible for the decreased activity of the molecule in protein synthesis rather than due to general undermethylation, because this modification is adjacent to the anticodon and is involved in binding to the ribosome. The discrepancy in uridine content in normal vs UU-$tRNA^{Phe}$ is accounted for by the lack of ribosylthymidine and, apparently, 3-(3-amino-3-carboxypropyl)uridine in the UU-$tRNA^{Phe}$. We have no explanation for the final discrepancy of one uridine.

We are continuing these studies by attempting to identify the enzyme(s) responsible for the methylthio modification of 6iPeAdo, using UU-$tRNA^{Phe}$ as substrate. Furthermore, we are determining the capability of a complete mixture of UU-tRNAs to support protein synthesis in an *in vitro* protein-synthesis system from *E. coli.*

*Student trainee, Carnegie Corporation Program.

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PURIFICATION OF tRNA METHYLTRANSFERASES FROM *ESCHERICHIA COLI*

L. R. Shugart and Barbara H. Chastain

Some aspects of the preparation of individual tRNA methyltransferases for the *in vitro* stepwise remethylation of purified methyl-deficient tRNA species (see the following article) have not progressed satisfactorily for several reasons.[1] The difficulties encountered are (1) instability of enzyme preparations during purification, (2) frequent contamination of these preparations with ribonuclease and/or endogenous tRNA, and (3) inability to demonstrate a specific methylase activity.

However, the isolation and partial purification of tRNA ^{5}U-methyltransferase were reasonably successful, consisting-of the following steps: (1) chromatography of the 100,000*g* supernatant solution on a Sephadex G-200 glass-bead column, (2) concentration of the enzyme activity by precipitation with 75% $(NH_4)_2SO_4$ and rechromatography of the material on the same column but at a higher magnesium-ion concentration, and (3) $(NH_4)_2SO_4$ fractionation of the enzyme activity subsequent to the second Sephadex column step. This procedure results in a sevenfold increase in purity with a 40% recovery of total enzyme activity; however, the preparation is apparently free of ribonuclease activity and endogenous tRNA and is stable at 0°C for several months.

1. L. R. Shugart and M. P. Stulberg, *Methods Enzymol.* 29, 492 (1974).

REMETHYLATION OF METHYL-DEFICIENT $tRNA^{Phe}$

L. R. Shugart

Five hundred micrograms of methyl-deficient $tRNA^{Phe}$ was remethylated *in vitro* with a partially purified tRNA ^{5}U-methyltransferase (see the preceding article). The tRNA was recovered from the assay mixture by Sephadex G-200 column chromatography and was found to have incorporated 1450 pmoles of methyl groups per A_{260} unit of tRNA. A nucleoside analysis showed that more than 90% of the incorporated methyl groups were recoverable as ribothymidine. The initial velocity of phenylalanyl-tRNA formation in this remethylated material at saturating concentrations of the tRNA was determined and compared to the initial velocity in the methyl-deficient material.[1] No significant difference was noted. It is concluded that the presence or absence of the methyl

group on uridine at position 54 (ribothymidine) in $tRNA^{Phe}$ is not important for the interaction of the tRNA with its cognate tRNA ligase.

1. L. R. Shugart, G. D. Novelli, and M. P. Stulberg, *Biochim. Biophys. Acta* 157, 83 (1968).

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

K. R. Isham, Vivian S. Hiatt,* and M. P. Stulberg

Previously[1] we described progress in constructing and optimizing an *in vitro* protein-synthesis system from ascites tumor cells. The system is now optimized; it is absolutely dependent on a source of exogenous mRNA and is stimulated up to fourfold by the addition of exogenous tRNA. We are now finalizing a slab, disc gel electrophoresis system to determine product formation.

In collaboration with J. N. Ihle and Kai-Lin Lee we have tested mRNAs from encephalomyocarditis and murine leukemia viruses and the messenger from tyrosine-transaminase-induced cells, with contrasting results. Further studies are under way to elucidate the differences in translational activity of these messengers.

We have isolated tRNA from young and old tissues of the mouse and are testing their activity in the depleted *in vitro* system. Following this, we will test other components from aging tissues such as ribosomes, initiation factors, and mRNA. Equivalent experiments with tissues from irradiated mice are planned.

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1. V. S. Hiatt, K. R. Isham, and M. P. Stulberg, *Biol. Div. Annu. Progr. Rep. June 30, 1973*, ORNL-4915, p. 19.

PROPERTIES OF TYROSYL-tRNA OF *DROSOPHILA MELANOGASTER*

K. Bruce Jacobson and J. B. Murphy

Tyrosyl-tRNA is one of a family of four aminoacyl-tRNAs that are candiates for regulatory roles in the growth and metabolism of *Drosophila*. This family of tRNA has more than one isoacceptor, as do many tRNAs, but it is unique in that the amounts of the isoacceptors change dramatically as the organism passes from one metamorphic stage to another.[1] Furthermore, one of the isoacceptors of tyrosyl-tRNA was implicated in a primary role in genetic suppression of the "vermilion" locus.[2] We are continuing to study tyrosyl-tRNA of wild type and the "suppressor of sable" mutant.

Purification of the two major isoacceptors of tyrosine tRNA from both wild type and suppressor has been accomplished. Uncharged tRNA is chromatographed on BD-cellulose, and the tyrosine tRNA is located, pooled, precipitated, and tyrosylated. The tyrosyl-tRNA is chromatographed on BD-cellulose again, and then on RPC-6 to separate the isoacceptors. The isoacceptors are pooled individually, discharged, and chromatographed on either RPC-5 or Aminex A-28. Each isoacceptor product thus obtained has an acceptance of >1200 pmoles per A_{260} unit. These highly purified tyrosine tRNAs are being used to determine the locus of the tRNA gene by *in situ* hybridization and to measure the size of the tyrosine tRNA gene by hybridization to DNA that is bound to cellulose acetate.

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2. K. Bruce Jacobson, *Nature New Biol.* 231, 17 (1971).

TYROSYL-tRNA LIGASE IN *DROSOPHILA MELANOGASTER*

Cynthia K. Warner,* J. B. Murphy, and K. Bruce Jacobson

During a study of the mechanism of genetic suppression of the "vermilion" locus in *Drosophila melanogaster*, we have found that suppressor flies [$su(s)^2$] have two tyrosine tRNA isoacceptors [$tRNA^{Tyr}_{su(s)A}$ and $tRNA^{Tyr}_{su(s)B}$] which appear to be different from the two tyrosine tRNA isoacceptors ($tRNA^{Tyr}_{SAMI}$ and $tRNA^{Tyr}_{SAMII}$) present in wild-type flies. Under appropriate conditions we can prepare a ligase which can aminoacylate $tRNA^{Tyr}_{SAMI}$, $tRNA^{Tyr}_{SAMII}$, and $tRNA^{Tyr}_{su(s)A}$ but not $tRNA^{Tyr}_{su(s)B}$. We have as yet been unable to determine exactly what defines "appropriate conditions."[1]

In an attempt to characterize the conditions necessary for the specific aminoacylation cited above, we have concluded that there are at least three forms of tyrosyl-tRNA ligase which can be prepared from adult wild-type flies. One form of the ligase elutes from a DEAE-cellulose column in 0.1 *M* NaCl and can achieve the specificity of charging. Potassium ions enhance the activity of this form but are not an absolute requirement. A second form of the enzyme elutes from DEAE-cellulose in 0.3 *M* NaCl and always accompanies the first form. The third form of the ligase elutes from DEAE-cellulose in 0.1 *M* NaCl, charges all four tyrosine isoacceptors, and absolutely requires K^+ for activity.

Postulating that perhaps we could find chemical conditions which would allow the latter form of the ligase to achieve the specificity of this first form, we began a series of kinetic studies on the latter form. A thorough study of all of the substrates and some inhibitors of the ligase has failed to yield a chemical condition under which the third form of the enzyme can charge only $tRNA^{Tyr}_{SAMI}$, $tRNA^{Tyr}_{SAMII}$, and $tRNA^{Tyr}_{su(s)A}$.

From an enzymological point of view, the third form of tyrosyl-tRNA ligase in *Drosophila* has two interesting properties: (1) a plot of v vs μl enzyme is not linear but has a distinct lag at low concentrations, and (2) rechromatography of the enzyme on DEAE-cellulose yields a form which is active at 20°C but not at 37°C. We do not yet understand what, if any, relationship these two properties may have to the other forms of the enzyme.

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1. C. K. Warner, J. B. Murphy, and K. B. Jacobson, *Biol. Div. Annu. Progr. Rep. June 30, 1973,* ORNL-4915, p. 20.

PURIFICATION OF TYROSYL-tRNA LIGASE FROM *DROSOPHILA*

J. E. Geltosky and K. Bruce Jacobson

Suppression of the "vermilion" mutation by the $su(s)^2$ locus in *Drosophila* is apparently the result of an alteration in the isoacceptor forms of $tRNA^{Tyr}$. At least two forms of tyrosyl-tRNA ligase activity can be demonstrated in adult flies: one form which charges both isoacceptors of wild type (termed $tRNA^{Tyr}_{I}$ and $tRNA^{Tyr}_{II}$) and also both isoacceptors derived from flies carrying the $su(s)^2$ locus (termed $tRNA^{Tyr}_{A}$ and $tRNA^{Tyr}_{B}$); and a second form which charges both wild-type moieties but only $tRNA^{Tyr}_{A}$ derived from the suppressed flies. Hence it is inferred that wild-type $tRNA^{Tyr}_{II}$ and suppressor $tRNA^{Tyr}_{B}$ are distinct, non-identical molecules.

An attempt has been made to purify the two tyrosyl-tRNA ligases to the extent that they can be used as reagents to dissect the role of $tRNA^{Tyr}$ in genetic suppression specified by the $su(s)^2$ locus. Additionally, a pure ligase is a useful tool for preparing quantities of charged $tRNA^{Tyr}$ to be used in purification procedures for the $tRNA^{Tyr}$ (i.e., BD-cellulose chromatography).

Use has been made of the following chromatographic procedures: DEAE-cellulose, CM-cellulose, and hydroxylapatite. By following these steps, a relatively pure enzyme preparation can be obtained, and each step results in at least a 70% recovery. It is also planned to use affinity chromatography as an additional purification procedure.

Unfortunately, the purified ligase does not possess the ability to discriminate between $tRNA^{Tyr}_{II}$ and $tRNA^{Tyr}_{B}$.

SUPPRESSION IN *DROSOPHILA MELANOGASTER*

Cynthia K. Warner,* K. Bruce Jacobson, and E. H. Grell

We are interested in the biochemical mechanism of suppression in *Drosophila melanogaster.* Although there are several known enzymatic mutants of *Drosophila,* most of them have no visibly recognizable phenotype and thus are difficult to screen for the effect of possible suppressors. We have found that the mutant "speck" (*sp*) has a recognizable enzymatic phenotype[1] as well as a visible phenotype. We have also found that suppressed "speck" flies [*su(s) sp*] are identical to wild type in both enzymatic and visible phenotype.

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STUDIES ON THE SUPPRESSION OF THE "PURPLE" MUTANT IN *DROSOPHILA*

T. G. Wilson* and K. Bruce Jacobson

"Purple" (*pr*) is a mutation in *Drosophila* whose phenotype is characterized by a reddish-purple eye color instead of the wild-type reddish-brown eye color. *Pr* can be suppressed by either of two suppressor mutations, *su(s)* on the X chromosome or *su(pr)* on the third chromosome.

The biochemical mechanism of suppression of *pr* is being studied by comparing the amounts of pteridines in the heads of *pr* flies, $su(s)^2$; *pr* flies, and $su(pr)^{e3}$; *pr* flies. Compared with wild-type flies, *pr* flies have reduced amounts of drosopterins, sepiapterin, several green fluorescent compounds which appear in the absence of reducing agent and thus may be degradation products of other compounds, and an unknown dull-green fluorescent compound. These pteridines are restored to wild-type levels in $su(s)^2$; *pr* flies. However, in $su(pr)^{e3}$; *pr* flies, (1) the amounts of sepiapterin and the degradation compounds remain low, (2) drosopterins and the unknown dull-green fluorescent compound are restored to wild-type levels, and (3) an

unidentified blue fluorescent compound is enhanced over the level in wild-type flies. These results indicate that the two suppressor mutations act by different mechanisms to suppress *pr* flies, but the end result, the restoration of drosopterins and consequently the reddish-brown eye color to wild-type levels, is the same for both suppressor mutations.

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THIN-LAYER CHROMATOGRAPHY OF *DROSOPHILA* PTERIDINES

T. G. Wilson* and K. Bruce Jacobson

Although several procedures have been published for the separation of pteridines from *Drosophila* by paper chromatography, resolution is often incomplete and development times are long. We have found that excellent resolution is achieved by using the solvent systems described by Descimon and Barial[1] and using Eastman analytical cellulose thin-layer chromatography sheets. Chromatography of extracts of *Drosophila* heads results in the resolution of more than 20 fluorescent compounds in a comparatively short development time of 6 hr. Several of these fluorescent compounds (presumably pteridines) have not been reported before due to poor resolution by previous chromatography methods.

Two fluorescent compounds have been identified as xanthurenic acid and 3-hydroxykynurenine, both of which are tryptophan metabolites. Pteridines that have been identified include five drosopterins, biopterin, neopterin, sepiapterin, isosepiapterin, isoxanthopterin, and 2-amino-4-hydroxypteridine. Identifications were made by oxidative degradation studies, comparison with commercial or synthetic pteridines, and the use of various *Drosophila* mutants which are known to be deficient in or have an excess of known pteridines. Several of the unidentified pteridines appear to be degradation products arising during homogenization, since the presence of a reducing agent, 2-mercaptoethanol, prevents formation of these fluorescent spots. Studies are in progress to determine the structure of at least one of the three unidentified pteridines.

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5S RNA GENES IN THE GENUS *DROSOPHILA*

Maurice Cohen, Jr.,* and Janice Harmon†

Because of their polytene nature, chromosomes from third-instar larvae of *Drosophila* are an excellent substrate for cytological genetic study. When a radioactive complementary RNA or DNA is allowed to anneal to denatured salivary chromosomes on glass slides, the genes for that complementary nucleic acid can be localized by autoradiography. Using such hybridization *in situ*, several authors have been able to locate the genes for ribosomal 5S RNA in the *Drosophila melanogaster* chromosomes.

Ribosomes were isolated from adult *Drosophila melanogaster* Samarkands, and RNA was extracted and fractionated by gel filtration. 5S RNA was labeled *in vitro* with ^{125}I to a specific activity of about 10^8 dpm/μg and hybridized *in situ* to salivary-gland chromosomes of giant mutants of *D. melanogaster*. Autoradiographic localization of the 5S RNA genes was two bands 56E–56F on the right arm of chromosome 2, the localization reported by earlier workers. Hybridization of the same ^{125}I-labeled 5S RNA to salivary chromosomes of *Drosophila virilis* and *Drosophila hydei* allowed us to map the 5S RNA genes in these two species. The sequences in *D. hydei* are located at bands 53a–c of chromosome 3. In *D. virilis*, in contrast, there are two loci containing gene sequences for 5S RNA: bands S(d–f) and W(c–j), both on chromosome 5.

These studies (1) indicate that the gene sequences coding for 5S RNA have been well conserved in the evolution of *Drosophila* species, since RNA transcribed from one species (*D. melanogaster*) is quite capable of efficient hybridization to the DNA of other species in the genus; (2) confirm data of others which indicate that chromosome 2R of *D. melanogaster* is the evolutionary homolog of chromosome 3 of *D. hydei* and chromosome 5 of *D. virilis*.

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MULTIPLE ISOACCEPTORS FOR VARIOUS tRNAs IN A MUTANT STRAIN OF *SACCHAROMYCES CEREVISIAE* AS RESOLVED BY RPC-5 CHROMATOGRAPHY

J. B. Bell* and K. Bruce Jacobson

Several species of tRNA from aerobically grown wild-type (S288C) yeast and aerobically grown mutant

(XB109-5B) yeast have been examined by aminoacylating with isotopically labeled amino acids and observing the profiles obtained after RPC-5 chromatography. Wild-type yeast consistently give only one isoaccepting form of tRNA for each aminoacyl-tRNA examined, which includes those for $tRNA^{Phe}$, $tRNA^{Tyr}$, $tRNA^{Ser}$, $tRNA^{Asp}$, and $tRNA^{Val}$. However, RPC-5 chromatograms of XB109-5B tRNA exhibit multiple isoacceptors for all of the above tRNAs except $tRNA^{Asp}$. Moreover, if XB109-5B is grown under near anaerobic conditions, then tRNAs can be obtained with single isoacceptors that cochromatograph with the single isoacceptor from wild-type tRNAs. It may be that the extra isoaccepting forms of tRNA from aerobically grown cultures of XB109-5B represent tRNAs lacking a complete complement of modified bases as a result of some poorly functional modifying enzyme, for example. When the cultures are grown more slowly, as under near anaerobic conditions, this enzyme may be able to modify the tRNA as fast as it is made, whereas under better growth conditions the production of tRNA is much faster than a faulty modifying enzyme can handle. These speculations are currently being tested.

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TRANSFER RNA AS A NONSPECIFIC INHIBITOR OF AN ENZYMIC ACTIVITY IN *SACCHAROMYCES CEREVISIAE*

J. B. Bell* and K. Bruce Jacobson

For the yeast *Saccharomyces cerevisiae*, we previously reported the indication that $tRNA^{Phe}$ played a specific role in inhibiting the first enzyme in the aromatic amino acid biosynthetic pathway, namely, 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthetase, whereas other purified tRNAs from the organism had no effect on the enzyme.[1,2] However, it became apparent that the effect might not be specific for $tRNA^{Phe}$, since crude uncharged tRNA inhibited the enzyme about as well as pure $tRNA^{Phe}$. Further testing with *E. coli* rRNA (free of any tRNA) and with heparin (as a typical polyanionic reagent) resulted in inhibition of enzymic activity also. In addition, no inhibition occurs when Co^{2+} is added to the assay mix in the presence of sufficient RNA to cause an 80% drop in DAHP synthetase activity. Fully digested tRNA will not inhibit the enzyme, but whole molecules or even oligonucleotides from partial digestions will, thus indicating a requirement for a polymer in order to observe inhibition. It seems apparent that tRNA is acting as a metal scavenger, depleting the Co^{2+} pool that is essential for enzymic activity. This role can be duplicated by other polyanionic polymers.

The finding that inhibition of DAHP synthetase by $tRNA^{Phe}$ is nonspecific negates the value of this once promising system for studying the possible role of tRNA in regulating enzyme activity.

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AUTOMATED SEQUENTIAL DEGRADATION OF RNA

Mayo Uziel, A. J. Weinberger, and A. Jeannine Bandy

Automated sequential degradation of RNA is a useful tool both for providing sequence information and in the preparation of specifically truncated RNA molecules for further chemical, physical, or biological studies.

The apparatus described in previous reports[1,2] has been tested with *E. coli* $tRNA^{fMet}$ and $tRNA^{Glu}_{1,2}$ prepared at ORNL by the Macromolecular Separations Group. Approximately 2 mg of each preparation was used for each experiment. A total of 21 residues have been removed from both tRNAs, with an average yield of 94% per cycle. The remaining 6% can be accounted for by mechanical removal of about 5% from each cycle and less than 1% due to irreversible chemical losses.

$$\left.\begin{array}{l} \overset{1}{p}G\text{-}U\text{-}C_4\text{-}U_2\text{-}C\text{-}G\text{-}U\text{-}C\text{-}\psi\text{-}A\text{-}G\text{-}A\text{-}G_2\text{-}C_3\text{-}A\text{-}G_2\text{-}A\text{-}C\text{-}A\text{-}C_2\text{-}G\text{-}C_3\text{-}\overset{34}{U}\text{-} \\ mam^2\overset{35}{S}\text{-}U\text{-}C\text{-}m^2A\text{-}C\text{-}G_2\text{-}C\text{-}G_2\text{-}U\text{-}A_2\text{-}C\text{-}A\text{-}G_4\text{-}T\text{-}\psi\text{-}C\text{-}G\text{-}A_2\text{-}U\text{-}C_4\text{-}\overset{65}{U}\text{-} \end{array}\right\} \text{In both}$$

$$\text{--}\overset{66}{\underline{A}}\text{-}G_4\text{-}A\text{-}C\text{-}G\text{-}C_2\text{-}A \qquad \text{In major component}$$

$$\text{--}\overset{66}{\underline{G}}\text{-}G_4\text{-}A\text{-}C\text{-}G\text{-}C_2\text{-}A \qquad \text{In minor component}$$

Fig. 2. Sequence of $tRNA^{Glu}$ from *E. coli*.

The sequence data on $tRNA^{Glu}$ indicate that the minor component and the major component differ only in the presence of a G at position 66 instead of an A. Comparison of the oligonucleotides produced by specific enzymatic cleavage of chromatographically pure $tRNA_2^{Glu}$ and $tRNA_1^{Glu}$ shows essentially no differences in the remaining sequences. Thus we have proven the sequence of the minor $tRNA^{Glu}$ component (Fig. 2).

An unexpected property of the sequential degradation process is that the removal of bases ceases when we reach the pseudouridine residue 56. This has been found true for *E. coli* $tRNA^{fMet}$, *E. coli* $tRNA^{Glu}$, and *E. coli* $tRNA^{Phe}$ (ref. 3). The 4-thiouridine residue often found at position 8 does not play a role here, since $tRNA^{Glu}$ does not have this substitution. This has a unique value in that "¾ molecules" are readily prepared. The basis for the interference in sequential degradation is under study.

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CHEMICAL REACTIVITY AS A FUNCTION OF NUCLEIC ACID SEQUENCE

Mayo Uziel, Nathan Brooks,* and A. Jeannine Bandy

The structure of RNA in solution will be defined by the combined data from (1) high-resolution physical techniques such as x-ray crystallography and electron spin and nuclear magnetic resonance spectral analyses, and (2) quantitative and qualitative chemical studies of sequences and the kinetics of the reaction of the various groups on the RNA. The last category provides insight into the microenvironment of individual reactive atoms. One selective reagent discovered by Cramer's group is the monoperphthalate oxidation of adenine residues. They demonstrated that base-paired adenines are not attacked by the reagent. However, there are a number of adenine residues in tRNA that are not attacked, yet there is no evidence that they are base-paired. We have begun an investigation of the influence of nonadjacent residues on the reactivity of selected residues in a nucleic acid chain. We have observed that the adenine in the "discriminator site" of *E. coli* $tRNA^{Phe}$ does not react with the monoperphthalate reagent. Removal of the terminal adenosine, however, makes this adenine susceptible to oxidation. We are exploring the reactivity of other "nonreactive" adenine residues after sequential removal of a series of bases from the amino-acid-acceptor terminus to probe the basis of "nonreactivity."

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C57BL MOUSE THYMUS RNA

Mayo Uziel, L. H. Smith, A. Jeannine Bandy, and Linda A. Ferguson*

The radiation-induced thymic lymphoma in C57BL mice presents a unique opportunity to study precancerous biochemical changes. Since protein synthesis is altered in the tumor development, we have begun a study of thymus RNAs.

The normal mouse thymus appears to have substantial nuclease activity, so our initial efforts have been directed at procedures for the isolation of undegraded RNA. We have confirmed Weymouth and Kaplan's earlier data on the level of RNA in the normal thymus (about 10 mg per g wet tissue). The nuclease activities were suppressed by the initial acid precipitation of all the nucleic acids. The resulting pellet was extracted with buffered phenol to solubilize the RNA, leaving the DNA in an essentially insoluble form. The RNA fraction was separated into high-molecular-weight and tRNA fractions by gel filtration on Sephadex G-100. We can account for up to 70% of the total RNA by this process.

Analysis of the tRNA preparations shows the minor component to be quite similar to the minor components of tRNA isolated from C57BL mouse liver. One interesting observation is that the thymine content appears to be relatively low. This has also been observed in other eukaryotic systems. Sequential degradation of the RNA may provide some data on the nature of the sequences in this region.

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IN VITRO LABELING OF tRNA WITH ^{35}S

B. C. Pal and Diane G. Schmidt

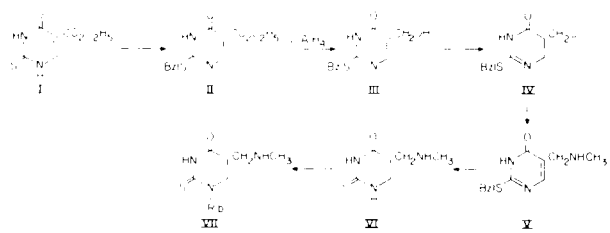
The reduction of ^{35}S-labeled 1-methyl-4-thiocyanatouracil to 1-methyl-4-thiouracil by SH^- has been shown to proceed via scission of the pyrimidine-ring C–S bond and the exocyclic C–S bond to the extent of 70% and 30%, respectively. The principle has been utilized in developing a method for labeling the 4-thiouridine moieties in tRNA *in vitro*. *E. coli* mixed

tRNA was treated with CNBr at pH 8 and then with $NaH^{35}S$. It was freed from excess reagent by chromatography on hydroxylapatite followed by dialysis. The labeled tRNA (9550 cpm per A_{260} unit) was enzymically hydrolyzed to a mixture of nucleosides, and about 83% of the radioactivity was shown to be associated with 4-thiouridine.

SYNTHESIS OF 5-METHYLAMINOMETHYL-2-THIOURIDINE

B. C. Pal and Diane G. Schmidt

The minor nucleoside 5-methylaminomethyl-2-thiouridine has been found in the first position of the anticodon in $tRNA^{Glu}$ from several sources. To study its properties at monomer and polymer levels, we attempted its synthesis. Of the various routes tried, the following scheme looks promising:



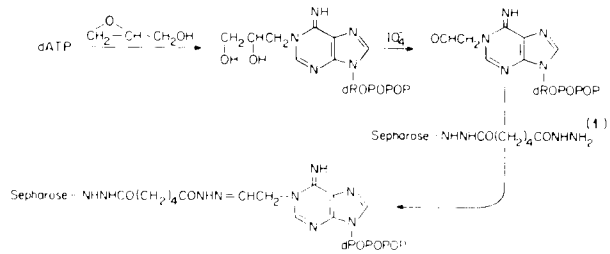
We have successfully prepared compound IV in good overall yield. The three remaining steps are being worked out.

SYNTHESIS OF dATP-SUBSTITUTED SEPHAROSE FOR THE PURIFICATION OF RIBONUCLEOTIDE REDUCTASE

B. C. Pal and F. D. Hamilton

The enzyme ribonucleotide reductase has a high affinity for dATP. Covalent attachment of dATP to Sepharose should result in an affinity resin which could be used to isolate this enzyme selectively and quickly from any crude cell lysate. A simple isolation technique would be valuable for the study of ribonucleotide reductase in mammalian cells. Inasmuch as the levels of reductase activity affect DNA synthesis, knowledge obtained from studying the purified enzyme may lead to the design of new compounds which could be used in cancer chemotherapy.

A dATP-substituted Sepharose has been prepared according to the following scheme:



A column of this dATP-Sepharose is being tested for its ability to bind ribonucleotide reductase from *Euglena gracilis*.

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

J. E. Fritz and W. E. Barnett

The two major theories concerning the origin of cellular organelles are, briefly stated, (1) that organelles evolved from prokaryotic endosymbionts and (2) that organelles simply reflect subcellular compartmentalization of function, for example, energy or ATP production. In support of the former, we have shown that, not unlike the prokaryotic cell, mitochondria and chloroplasts contain their own unique tRNAs and aminoacyl-tRNA synthetases. We have also found, however, that the chloroplastic synthetases are coded by nuclear genes, synthesized within the cytoplasm, and rapidly compartmentalized within the organelle. Thus, if indeed the genetic information for these enzymes originated from an endosymbiotic prokaryote, it must be concluded that the cistrons involved were physically transferred to the host's nuclear genome.

In order to evaluate more critically the evolutionary origin of chloroplasts, we have initiated a program to purify to homogeneity a typical chloroplastic and cytoplasmic synthetase, Phe-tRNA synthetase. Methodology for purification of the chloroplastic enzyme is approaching completion. In brief, the approaches we will use are (1) use of the amino acid sequenator to sequence the first 50–60 amino acids for homology comparisons, and (2) an immunological approach in which the degree of homology between functionally

identical enzymes can be related by virtue of cross-reactivity. These studies should provide basic insight regarding the evolutionary origin of the chloroplastic synthetase specifically and organelles in general.

SEQUENCE HOMOLOGY STUDIES WITH ISOACCEPTING tRNAs FROM *EUGLENA* CHLOROPLASTS AND CYTOPLASM

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

The tRNAs that are found in isolated *Euglena* chloroplasts have been shown to be chromatographically distinguishable from their cytoplasmic counterparts. Thus there appear to be two *sets* of structural genes for tRNAs in *Euglena:* the structural genes for the cytoplasmic tRNAs undoubtedly reside within the nuclear genome, and probably (though not yet experimentally verified) the organellar tRNAs reflect chloroplast transcription.

The two basic issues we have addressed are (1) to establish the sites of transcription for both cytoplasmic and chloroplastic tRNAs and (2) to attempt to determine whether the chloroplastic tRNA structural genes arose by duplication of the cytoplasmic tRNA structural genes. To these ends we have purified to homogeneity, in milligram quantities, four tRNAs: cytoplasmic $tRNA^{Phe}$ and $tRNA^{Asp}$ and chloroplastic $tRNA^{Phe}$ and $tRNA^{Asp}$. Nuclear and chloroplastic DNAs are also being purified.

With regard to the site of transcription, our approach is to iodinate the tRNAs with ^{125}I and perform the appropriate hybridization experiments. To approach the question of whether chloroplastic tRNA cistrons evolved via gene duplication – as opposed to having evolved from a prokaryotic endosymbiont progenitor of the chloroplast – we plan to sequence the four tRNAs mentioned above. A sequence comparison between the two chloroplastic tRNAs and their cytoplasmic counterparts (as well as published sequences from both prokaryotic and eukaryotic sources) should provide considerable insight into the evolutionary origin of the chloroplastic genes.

THE ELUCIDATION OF RNA INITIATION (DNA PROMOTER?) SEQUENCES DURING T4 DNA TRANSCRIPTION WITH *E. COLI* RNA POLYMERASE AND WELL-DEFINED OLIGORIBONUCLEOTIDE INITIATORS

D. J. Hoffman* and S. K. Niyogi

As an important parameter, the effect of ionic strength was examined at the extremely low substrate concentrations employed in these studies. KCl has an inhibitory effect[1] at the level of chain initiation, since both [γ-^{32}P] ATP and [γ-^{32}P] GTP incorporations are greatly reduced by KCl concentrations that inhibit overall RNA synthesis. Also, addition of KCl after the initiation of the reaction has little inhibitory effect.

The stimulatory effects of dinucleoside monophosphates[2] and trinucleoside diphosphates of defined sequences on the transcription of phage T4 DNA by *E. coli* RNA polymerase were examined at various concentrations of the sigma subunit and extremely low concentration (10 μM) of ribonucleoside triphosphate. Labeled specific dinucleoside monophosphates are incorporated as chain initiators and, depending on whether these represent ATP starts or GTP starts, effectively reduce initiation by either ATP or GTP, respectively. Certain dinucleoside monophosphates stimulate selectively depending on the ratio of sigma factor to core RNA polymerase; when the ratio is small, G-containing dinucleoside monophosphates stimulate, whereas A-containing ones are predominant at higher ratios. With holoenzyme, two sets of compounds stimulate the reaction: C-A, A-U, and U-A, representing ATP initiations; and G-U, U-G, and A-G, representing GTP initiations. The RNAs initiated by these dinucleoside monophosphates are of the T4 "early" type, as indicated by competition hybridization studies. Studies involving both combinations of stimulatory dinucleoside monophosphates and competitive effects of these compounds on chain initiation by ATP and GTP suggest that the stimulatory dinucleoside monophosphates act as chain initiators and may recognize part of a continuous sequence in RNA initiation (DNA promoter?) regions in T4 DNA. There may be two such DNA sequences serving as initiation regions, one for ATP and the other for GTP initiation. Studies with specific trinucleoside diphosphates and labeled dinucleoside monophosphates support the above conclusions. On the basis of these studies, a *speculative* model for T4 "early" RNA initiation (DNA promoter?) sequences is proposed:

DNA ---- 3′ ---- G-T-A-T-A-T-$(N)_n$-A-T-C-A-C ---- 5′

RNA ---- 5′ ---- C-A-U-A-U-A-$(N)_n$-U-A-G-U-G ---- 3′.

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ISOLATION AND PROPERTIES OF AN OLIGORIBONUCLEASE FROM *E. COLI*

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

We have isolated a new ribonuclease from *E. coli* Q13,[1,2] a mutant deficient in both RNase I and polynucleotide phosphorylase, and also from *E. coli* AB105, a mutant deficient in both RNase I and RNase III. This enzyme has now been purified free of RNase II, the major contaminating activity. The enzyme prefers Mn^{2+} over Mg^{2+} for its activity. It preferentially degrades oligoribonucleotides, the activity being inversely proportional to the chain length of the oligoribonucleotide. The products are 5′-nucleotides, probably liberated from the 3′ end of the substrate. The enzyme activity is markedly inhibited by secondary structure; oligoribonucleotides combined with complementary polynucleotides are attached poorly below the T_m of the complex and efficiently above the T_m.

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ISOLATION AND PROPERTIES OF THE SPECIFIC BINDING SITES FOR *E. COLI* RNA POLYMERASE ON T4 AND T7 PHAGE DNAs

S. K. Niyogi and Brenda H. Underwood

RNA polymerase of *Escherichia coli* was allowed to bind to labeled T4 or T7 phage 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 hydroxylapatite chromatography. Studies of the DNA recognition regions led to the following conclusions: (1) No binding is observed in the absence of the sigma subunit or at low temperatures. (2) The amount of protection ranges from 0.18 to 0.24% of T4 DNA and 0.25 to 0.28% of T7 DNA. In the absence of poly(I), much higher protections are observed; under these conditions, the protected regions display heterogeneity in size and secondary structure. (3) The protected regions are double-stranded as shown by hydroxylapatite chromatography, base-composition analysis, and thermal chromatography. (4) The length of the protected regions comprises about 50 to 55 nucleotide pairs, as suggested by end-group analysis, sucrose density gradient centrifugation, and polyacrylamide gel electrophoresis. (5) The results suggest the interaction of dimeric polymerase molecules at these sites. There are six to eight such sites on T4 DNA and two on T7 DNA. (6) The protected regions are high in A + T – 68% for T4 and 62% for T7 DNA. (7) Thermal chromatograms reflect these base compositions and suggest the homogeneity of these regions with respect to size and base composition. (8) The recognition regions are unique in that they will anneal only to their homologous DNAs: T4 with T4 and T7 with T7. Thus, RNA polymerase recognizes different sequences in these DNAs. (9) The annealing of the recognition regions to their homologous initial RNAs suggests that at least part of the "early" promoter region is transcribed in T4 and T7 DNAs *in vitro*. (10) The recognition regions can be transcribed, suggesting interesting possibilities for the future.

COMPARISON OF PROPERTIES OF TEMPERATURE-SENSITIVE T5 DNA POLYMERASES WITH TEMPERATURE-STABLE POLYMERASES

R. K. Fujimura and Barbara R. Bussell

Two temperature-sensitive DNA polymerases of T5, a revertant of one, and a wild type were isolated to at least 90% purity. Three enzymatic activities were detectable in the temperature-sensitive polymerases. They are DNA polymerase activity with denatured DNA, which is temperature sensitive; DNA polymerase activity with nicked DNA, which is temperature stable; and an exonuclease activity enhanced at higher temperature, which is detected when assayed with nicked DNA or fragmented denatured DNA. Differences in these activities were studied among the four strains. There were two changes in enzyme activity with one-step mutation: temperature sensitivity of polymerase activity when assayed with intact denatured DNA, and enhanced exonuclease activity at the higher temperature (43°C). The polymerase activity with nicked DNA is temperature stable and remained unchanged by mutations. The polymerase activities are inactivated at the same rate by heating. The presence of nicked DNA during heating protects the enzyme from inactivation. The polymerase activity with nicked DNA may be due to the following: (1) the intrinsic property of the same protein that has temperature-sensitive polymerase activity with intact denatured DNA, (2) contamination with

some other polymerase, which may or may not be phage induced, and (3) contamination with 5′ → 3′ exonuclease or DNA-unwinding protein. These possibilities are still under investigation.

PROPERTIES OF EXONUCLEASE ASSOCIATED WITH T5 DNA POLYMERASE

R. K. Fujimura and Barbara R. Bussell

The exonuclease associated with purified T5 DNA polymerase was characterized. The exonuclease activity requires $MgCl_2$ and is inhibited by salts. The presence of four deoxynucleoside triphosphates under our standard conditions for polymerase assay has no effect. The activity is rapidly inactivated in the absence of dithiothreitol. DNA with dBrUMP is hydrolyzed at about the same rate as one without analog. Most of the products of hydrolysis are mononucleotides. When tested with ϕX RFI, there was no detectable endonuclease activity. When tested with terminally labeled DNA, hydrolysis of 3′ termini occurred faster than that of 5′ termini. Thus, hydrolysis occurs in the 3′ → 5′ direction, but available data do not yet exclude the presence of 5′ → 3′ exonucleolytic activity. The temperature-sensitive mutation of the polymerase enhances the rate of hydrolysis in the 3′ → 5′ direction. There is no RNase activity that hydrolyzes poly(A)-poly(dT) hybrid. The hydrolysis of DNA occurs randomly among the ends, not progressively, but partially degraded molecules are hydrolyzed faster than intact DNA. For example, RFI with a single nick is not hydrolyzed, but one with many breaks is hydrolyzed. Intact denatured T7 DNA is resistant, but fragmented denatured DNA is one of the best substrates. Thus, the exonuclease of T5 DNA polymerase is very similar to that of T4 DNA polymerase.

DNA REPLICATION *IN VITRO* WITH NATIVE T5 DNA

R. K. Fujimura and Barbara R. Bussell

Purified DNA polymerase induced by T5 phage infection prefers denatured DNA as a template, as do other T-phage DNA polymerases. Recent work from other laboratories has shown that synthesis with crude extracts from infected cells prefers native DNA as template. We have been studying the effect of extracts from *su*⁻ cells infected with a T5 amber mutant in the DNA polymerase gene. The results show that with purified T5 polymerase the addition of such crude extract causes more DNA synthesis with native DNA than with denatured DNA. Such synthesis is enhanced at least twofold in the presence of four ribonucleoside triphosphates. Factors involved in these syntheses are being characterized.

ELECTRON MICROSCOPIC STUDIES OF THE MECHANISM OF REPLICATION OF M13 BACTERIOPHAGE DNA SYNTHESIS

D. P. Allison, A. T. Ganesan,* and Sankar Mitra

In continuation of the work reported in last year's annual progress report,[1] we have further investigated the replication of M13 DNA, using wild type and gene-5 mutants. Log-phase *E. coli* cells at different times after infection were quickly poisoned with NaCN-pyridine after the cultures were pulse-labeled with [^{3}H] thymidine. The cells after washing were lysed with Sarkosyl, and host DNA was removed by band sedimentation in sucrose. The phage DNA was then fractionated by equilibrium centrifugation in CsCl in the presence of ethidium bromide, to separate supercoiled double-stranded circular molecules from single-stranded and nicked double-stranded circular DNA. Different fractions were then prepared for electron microscopy.

The following conclusions have been reached:[2] (1) In confirmation of the earlier observation, double-stranded nicked molecules with single-stranded tails are seen both early (8 min) and late (45 min) after infection with wild-type phage. Although single-stranded (SS) progeny DNA synthesis predominates late after infection, the frequency of tailed molecules (15–20%) in the nicked double-stranded circles is not very different early and late after infection. (2) The tails are always single-stranded. Sometimes two tails come out of double-stranded DNA (RF) within a short region of the molecule, presumably arising from branch migration. (3) The tails are always less than unit length (av 1.4 μm, compared to 2 μm for the contour length of RF). (4) The tails can be mapped on the partially denatured molecules and can be ascribed to a unique region in the RF with respect to the denaturation map. (5) When infection is carried out with gene-5 mutants of the phage, when there is no SS DNA synthesis, no tailed molecules are seen. (6) A small number of supercoiled molecules after infection with wild type and gene-5 mutants have structures with two Y forks, and all the segments are double-stranded.

It has therefore been concluded that in M13 phage (1) SS synthesis occurs by a rolling circle with a unique origin of synthesis on RF; (2) the ring closure for SS circles is more efficient than DNA chain elongation; (3) the incipient progeny SS synthesis can occur very early after infection and is presumably regulated in a positive

manner by M13 gene-5 protein; and (4) RF replication does not occur by the rolling-circle mode, rather according to the Cairns model of DNA replication.

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DISCONTINUOUS REPLICATION OF M13 COLIPHAGE REPLICATIVE-FORM DNA

Sankar Mitra

The mechanism of replication of M13 phage replicative-form (RF) DNA has been investigated by analyzing pulse-labeled phage DNA after infection with a gene-5 mutant in the absence of single-stranded (SS) DNA synthesis. Wild-type *E. coli* as well as *E. coli* mutants deficient in DNA ligase and DNA polymerase I have been used as hosts. Band sedimentation of pulse-labeled phage DNA in alkaline sucrose gradients shows the presence of heterogeneous fragments smaller than unit length which are eventually incorporated into covalently closed supercoiled RF molecules. The fragments are first joined to form open-circular unit-length molecules. Self-hybridization and competition hybridization of the fragments in the presence of excess virus DNA show the presence of both virus and complementary-strand sequences in the fragments. Equilibrium centrifugation in alkaline CsCl shows equal amounts of synthesis of both types of strands in the unit-length linear molecules and also in supercoiled RF. The joining of fragments to form unit-length linear molecules and covalently closed RF is poor in the DNA-ligase-mutant and DNA-polymerase-I-deficient hosts. SS DNA molecules longer than unit length are not synthesized after infection with the gene-5 mutant, unlike in the case of wild-type phage.

It is concluded that M13 RF replication occurs by discontinuous synthesis of both strands, mimicking the replication of the host DNA.

RNA PROCESSING IN HUMAN MYELOMA CELLS

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

Human myeloma cell lines are transformed cells of lymphocytic origin. Under normal physiological conditions, the lymphocyte can exist for prolonged periods in an intermitotic state. During this period, there is no net ribosomal RNA synthesis. Using human and mouse myeloma lines, our studies have shown that after a 1-hr pulse with [^{3}H]uridine and a 6-hr chase, the 45S and 32S ribosomal RNA (rRNA) precursors are still present and constitute an unusually high percentage of the labeled cellular RNA. Interim time points revealed the synthesis and processing of the various rRNA precursors.

Ether-ethanol extraction of the residual interphase and phenol treatment after a pH 5.2 acetate-EDTA extraction resulted in two additional RNA fractions. Both fractions were unique in their uridine-to-cytidine ratios and Agarose-polyacrylamide electrophoresis profiles. However, analysis of the poly(A) content of these fractions showed a percentage only slightly higher than that in the bulk of the RNA.

Stepwise gradient elution of poly(A)-containing RNA from a poly(U)-Sepharose column revealed three main peaks of RNA. The first eluted with 0.5 *M* KCl and contained a presumptive short poly(A) region, the second and major peak eluted with 0.25 *M* KCl, and the third with 0.1 *M* KCl. These three species were present in three different RNA fractions and in various isolated cellular organelles. After a pulse with [^{3}H]uridine, the third peak is modified and elutes in a larger volume. The uridine-to-cytidine ratios of all three peaks are different from that of the total RNA, and electrophoresis indicates that they are heterogeneous with a size range from 50S to 5S.

CHARACTERIZATION OF THE ENDS OF SHEARED DNA

Ann C. Olson and Elliot Volkin

DNA is broken by shearing to produce smaller pieces which can be used in reassociation studies. One of the methods of shearing is passing the DNA solution through a syringe needle of small diameter. It is important for the subsequent reassociation studies that the breakpoints be random with respect to the total nucleotide sequence. The ends produced by shearing native phage T7 DNA to $\frac{1}{4}$-length molecules are being studied with respect to the following properties: (1) single-stranded tails, by looking for regions in the sheared DNA that are sensitive to single-strand-specific nuclease (*N. crassa*); (2) nucleotide composition of such regions, if shown to exist, in comparison to that of total T7 DNA; (3) identification of the 5′-dinucleotides at the break, by labeling them with ^{32}P (using polynucleotide kinase), digesting the DNA with exonuclease I, and

analyzing the dinucleotides by two-dimensional thin-layer chromatography; if there is no specificity to shearing, the distribution of dinucleotides should reflect the total nucleotide composition of T7 DNA. Preliminary experiments on the 5′-terminal mononucleotides indicate that shearing is random with respect to the nucleotide on the 5′ side of the break. The two-dimensional thin-layer chromatography system is being developed. The required nucleases have been purified.

CHARACTERIZATION OF CHROMOSOMAL PROTEINS

C. G. Mead and E. A. Hiss

Proteins isolated from purified chromosomes are being examined for enzymatic activities thought to be involved in DNA synthesis and/or genetic recombination. Chinese hamster ovary tissue-culture cells are synchronized with 5-amino uracil and then blocked at metaphase with colcimid. The cells are then harvested and invariably display a mitotic index of 95–99%. Chromosomes are prepared by the method of Wray.[1] This procedure involves treating the cells with hexylene glycol, disrupting the cells by shear, and differential centrifugation of the chromosomes. The chromosomes are then purified by sucrose density gradient centrifugation. Gradient sievorptive chromatography[2] is employed to separate the chromosomal proteins from each other and from the chromosomal nucleic acids.

Preliminary experiments have been done using chromosomes labeled with thymidine and leucine to work out the techniques and examine the distribution of proteins generated by gradient sievorptive chromatography. The chromosomes are disrupted by the high salt when they are applied to the column. The chromosomal DNA, labeled with thymidine, has been shown to be adsorbed to the column and eluted in one sharp peak. To date we have been unable to get the desired degree of labeling of the chromosomal proteins. However, the chromosomal proteins labeled with leucine appear to be distributed throughout the elution pattern, indicating the separation of several classes. The separated chromosomal proteins are being examined for enzymatic activities such as DNA polymerase, RNA polymerase, DNA ligase, nucleases, and DNA-binding proteins.

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RIBONUCLEOTIDE REDUCTASE FROM *EUGLENA GRACILIS*

F. D. Hamilton

A ribonucleotide reductase has been purified to homogeneity from *Euglena gracilis.*[1] A single enzyme reduces the four common ribonucleoside triphosphates when a dithiol and 5′-deoxyadenosylcobalamin, required for activity, are added. Magnesium ions are not required for ribonucleotide reduction but can stimulate the reaction. The reduction of CTP can be stimulated by dATP or ATP and can be inhibited, as can ATP and GTP reduction, by dTTP. The reduction of UTP is unaffected by deoxyribonucleotides. The molecular weight of the *Euglena* enzyme as determined by gel filtration is 440,000. Sodium dodecyl sulfate gels indicate subunits of approximately 100,000.

1. F. D. Hamilton, *J. Biol. Chem.* (in press).

IDENTIFICATION OF THIOREDOXIN AND THIOREDOXIN REDUCTASE IN *EUGLENA GRACILIS*

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

The *in vivo* reduction of ribonucleotides has been shown to involve the oxidation of NADPH, with the participation of two proteins in the hydrogen-transfer process. The ribonucleotide reductase from *Euglena* extracts will also utilize NADPH as a hydrogen donor. Three protein components which are necessary for this reaction have been isolated from *Euglena* extracts. One has been shown to be a flavoprotein and to contain the reactive sulfhydryl group necessary for activity. The second is a low-molecular-weight protein (<15,000). These two components are considered to be thioredoxin reductase and thioredoxin, respectively. A third protein component which has been isolated has a molecular weight of 240,000 and can be separated into two nonidentical subunits. Presently, no function has been assigned to this protein. All three proteins are needed, along with ribonucleotide reductase, to obtain NADPH-supported reduction of ribonucleotides.

IDENTIFICATION OF RIBONUCLEOTIDE REDUCTASE IN *PSEUDOMONAS STUTZERI*

Bam Mehrotra,* Dorothy Sallis,† and F. D. Hamilton

Pseudomonas stutzeri contains a ribonucleotide reductase which requires added deoxyadenosylcobalamin and a dithiol for activity. Preliminary results with crude

extracts indicate that ribonucleoside triphosphates are substrates for the enzyme and the reaction can be stimulated by ATP. Enzyme activity can be obtained in a 0–60% $AmSO_4$ fraction; however, even at high salt concentration the enzyme is unstable when frozen.

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ASCORBIC SULFATE AND ITS RELATION TO VITAMIN C

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, and it may very well prove to be ubiquitous to the animal kingdom. At present, several laboratories are actively engaged in studies to determine the function of this derivative, and at best, the results are confusing.

In an attempt to determine whether ascorbic sulfate is a storage form of vitamin C, we have embarked on a search for an ascorbic 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 ammonium sulfate precipitation, gel filtration, DEAE-cellulose column chromatography, and finally isoelectric focusing. We have observed that the enzyme also exhibits arylsulfatase activity when *p*-nitrocatechol sulfate is used as substrate, and so far we have been unable to separate this activity from the ascorbic sulfohydrolase activity. These results suggest the presence of a single enzyme in our preparations that is active toward both substrates. When ascorbic sulfate is used as substrate, the enzyme shows a pH optimum of 4.0 and a K_m of approximately 16 m*M*; however, when *p*-nitrocatechol sulfate is the substrate, the pH optimum is 6.9 and the K_m is 3.0 m*M*. Inorganic orthophosphate, nucleotides, and sulfate ions inhibit the activity toward ascorbic sulfate but not toward *p*-nitrocatechol sulfate.

We are currently attempting to purify this enzyme further and determine its properties, regulation, and distribution.

ISOLATION OF A GROWTH INHIBITOR FROM CROWDED *XENOPUS LAEVIS*

Peter Pfuderer and J. N. Dumont

We have observed that a diglyceride fraction from tungseed oil is a potent growth inhibitor to *Xenopus* tadpoles. This preparation inhibits growth at concentrations less than 1 ppm and is lethal at 25 ppm. The analogy with the goldfish crowding factor previously isolated would suggest that a similar mechanism is operational in *Xenopus.* We have set up a growth assay for this postulated *Xenopus* growth inhibitor and are doing fractionation experiments to isolate, purify, and identify it.

PHTHALATE ESTERS AS ENVIRONMENTAL POLLUTANTS

Peter Pfuderer

A fish heart-rate-depressing substance isolated from tissues of carp taken from local lakes has been identified as dibutyl phthalate. This industrial plasticizer appears to affect the fish heart rate through the nervous system, since the effect is abolished by low concentrations of atropine. The threshold for the heart-rate depression effect is approximately 1 ppm of an aqueous emulsion of dibutyl phthalate. Concentrations higher than this have a proportionally greater effect on depressing the heart rate of juvenile goldfish. The toxicity of these emulsions to goldfish fry was also investigated. Concentrations of dibutyl phthalate equal to or greater than 3 ppm were lethal within 18 hr, while 1 ppm had no effect.

FACTORS AFFECTING GROWTH AND AGING IN VERTEBRATES

Peter Pfuderer

We are studying natural lipid factors that appear to control growth, heart rate, and reproduction in lower vertebrates, and probably affect higher vertebrates also.

Many aquatic animals have been shown to secrete a growth and reproduction inhibitor into their water when crowded. We have found that a family-specific growth inhibitor could be extracted from the water of crowded goldfish with carbon tetrachloride. This extract also contained a family-specific heart-rate-depressant activity, which may be identical to or similar to the growth inhibitor. We have set up an assay to measure the heart-rate depression of a goldfish in water

and have used this assay to follow the purification of this crowding pheromone in the goldfish.

The heart-rate-depression activity behaves as a diglyceride on column chromatography. It has the unique ultraviolet spectrum characteristic of conjugated trienoic fatty acids.

A diglyceride fraction from tungseed oil, a natural source of conjugated trienoic oils, having approximately the same uv spectrum and chromatographic behavior as the pheromone, was also active in the goldfish heart-rate assay. Tungseed-oil preparations and also fish-oil preparations have been reported in the literature to be effective in treating mammalian hypertension, although the active principle was never isolated. We are doing preliminary experiments with hypertensive rats to see whether an assay for the active principle can be developed and to determine whether it is related to the fraction that is active in goldfish.

BIOPHYSICS AND CELL PHYSIOLOGY

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INTRODUCTION

THE CENTRAL INTEREST of a majority of the Biophysics and Cell Physiology Section is the response of biological macromolecules and cells to electromagnetic radiation. But within this central theme the research ranges from the atomic level to whole organisms. Studies at the former level are concerned with the nature of excitation and the decay and transfer of the excited state in nucleic acids, proteins, and chloroplasts. These processes are important in photosynthesis and in radiation damage to nucleic acids. They also serve as useful probes of the conformation of macromolecules.

The interaction of uv radiation with DNA continues to receive considerable attention. An initial electronic excitation underlies the photochemical changes which uv induces, and the energy of the excited state can be transferred to other regions of the affected molecule. This past year we have proposed a theory to resolve a long-standing conflict between optical activity data that strongly imply wavelike excitons and fluorescence kinetic data that imply slowly hopping excitons.

At the molecular level the focus is on the nature of the uv-induced lesion and the repair mechanisms by which cells eliminate its lethal effects. The chief photochemical product in DNA is the pyrimidine dimer, and one repair path involves its enzymatic removal in the dark (excision-repair). The kinetics of the process and the enzymatic mechanisms involved are being studied in microorganisms (*Micrococcus, E. coli,* and yeast), in human, mouse, and hamster tissue-culture cells (including those of different passage ages), and in tissue-culture cells from higher plants. Some studies are also under way on the repair of bacterial DNA by two other routes: postreplication repair and photoreactivation.

Lesions in DNA are produced by mutagens, carcinogens, and gamma radiation as well as by uv. Knowledge of the physical and chemical nature of the lesions and the relationship between the detailed primary and secondary structure of the nucleic acid and the type of lesion produced is central to an understanding of the lesions and their repair. These questions are being pursued with both synthetic and naturally occurring nucleic acids in which the base sequence is known, with normal DNA, and with DNA perturbed by the binding of heavy metals. The structure of the lesions is being investigated by proton magnetic resonance and fluorescence and phosphorescence measurements. Most lesions behave as though they are locally denatured regions of the polymers, as evidenced for example by their rapid reaction with formaldehyde.

The lesions induced by gamma irradiation and the mechanisms by which they are repaired are less well known than those for uv irradiation. After gamma irradiation the DNA in *Haemophilus* undergoes degradation. Kinetic models of degradation have been developed, consistent with the experimental findings, which consider such factors as the initial state of the DNA (e.g., whether circular or linear), whether degradation proceeds unidirectionally or bidirectionally along the DNA strand, and the probability distribution in the strands of functional or structural devices that interrupt the degradation. Essential to any studies of DNA damage and its repair are methods for accurately assessing the molecular weights of DNA fragments. One of the most important of these methods is sedimentation in sucrose gradients, and progress has been made on a detailed physical and mathematical analysis of the behavior of DNA during centrifugation.

Not all of the lethal effects of radiation and chemicals may be due to the inability of damaged DNA to replicate; some effects reflect adverse consequences of the DNA lesion on the physiology of the cell. For example, uv irradiation adversely affects the growth and respiration of *E. coli* in a way that suggests that the metabolic control systems of the cell are upset, so that there is excess synthesis of a normal protein involved in respiratory control. If these control systems are too seriously perturbed, the cell may not remain viable long enough to permit enzymatic repair of the DNA per se. Damage to DNA by irradiation is also coupled to cell function by its effects on cell division. Low doses of ionizing radiation and uv block cell division in certain blue-green algae and bacteria, for example. In some cases, substances obtained from normally dividing cells can reverse the blockage.

The function (and misfunction) of every biological molecule resides ultimately in its structure and conformation. The most direct and persuasive method of determining such structure is x-ray diffraction. This past year several plant lectins and phytotoxins have been crystallized, and a determination of their structure is under way using x-ray diffraction. These compounds are of considerable importance both as probes of membrane structure and as agents which act differentially on normal and malignant cells.

Although x-ray diffraction is the most direct approach to structure analysis, it is limited to molecules for which suitable crystals and isomorphous heavy-atom derivatives can be obtained. For compounds not meeting these stringent requirements, other methods must be used. One approach is chemical. Catalysis is one of the important properties of most proteins, and although even the smallest enzymes contain more than 100 amino acid residues, only a small number of the residues are actually involved intimately in catalytic action on the substrate. Identifying these residues and the active site and determining their three-dimensional structure bear directly on questions of enzyme specificity and catalytic efficiency. The approach being used is "affinity labeling," a procedure whereby a chemical reagent is endowed with a high degree of specificity for an active site by incorporating into its structure by organic synthesis functional groups of the natural substrate that are required for binding.

Another approach to elucidating molecular structure is photophysical. The kinetics of fluorescence and phosphorescence of proteins and their subunits is chiefly a function of their tryptophan and tyrosine content, the spatial separation of these groups, and the perturbation of the excited states of these residues by the local environment, including the solvent. Hence, measurement of the kinetics under various conditions, combined with knowledge of the spectroscopic behavior of simple molecular systems and chemical knowledge of primary and secondary structure, permits conclusions to be drawn about the conformation of complex macromolecules of biological and medical interest. These techniques are being used to study an important group of proteins, immunoglobulins.

Biological function will ultimately be accounted for in terms of the structure and conformation of the component molecules. Gaining this understanding will require an examination of structure-function relations at the supramolecular level. One such study is seeking to explain the structure of the eukaryotic chromosome in terms of its component nucleic acids and histones. It has recently been discovered by electron microscopy that chromatin fibers from rat and chicken cells exhibit a "beaded" appearance, with beads 60–80 Å in diameter connected by thin (~15 Å) filaments. Hydrodynamic studies suggest that each bead contains ~200 nucleotide pairs of DNA and ~160,000 daltons of protein. This amount of protein could represent two molecules of histone.

We have already noted that there is an interaction between the effects of radiation on DNA and on bacterial respiration and cell division. In bacteria, the location of both of these physiological processes is the plasma membrane, and the DNA itself is physically associated with the plasma membrane. It is significant that large transient changes in the DNA-membrane association in certain strains of *E. coli* have been found to be correlated with the cell division cycle.

Cell division is the basic unit of the more complex biological problem of growth and development. Crustacea provide a useful model because regeneration of new tissues is part of their natural growth cycle, because the regeneration is amenable to detailed experimental control, and because they contain satellite DNAs with high concentrations of highly reiterative molecular sequences. The rapid regeneration of highly differentiated tissues in crustaceans offers a unique opportunity to study the control of replication of the satellite DNAs as well as to determine their role in the differentiation process. Current emphasis is on the turnover and synthesis of muscle proteins, the synthesis of DNA and its hormonal control, and physicochemical characterization of the satellite DNAs.

The Section is devoting increasing attention to cell surfaces and membranes because of their central importance to cell function and to the response of cells to their environment. The rate of synthesis of one enzyme system responsible for the transport of Na^+ and K^+ in HeLa cells has been found to correspond to the rate of increase of the cell surface during growth. This transport system is inactivated by the binding of ouabain to the cell surface. However, evidence has been obtained that in 3–5 hr these inactivated sites are removed from the surface and replaced by freshly synthesized sites. The cells have an interesting feedback control mechanism for maintaining Na/K balance during the repair period. Inactivation of some of the sites leads to increases in internal Na^+, which in turn increases the transport efficiency of the remaining unaffected sites.

Two parameters important to living cells are water and temperature. One approach to studying their role is to study the response of cells when water and temperature are perturbed – as by freezing. As with radiation, the cell surface is being found to play an important role in the cellular response to freezing. Its permeability to water affects osmotic events during freezing, which in turn determine the site of ice-crystal formation and lethality. The cellular functions most sensitive to freezing are membrane associated, and substances which prevent this damage appear to act at the cell surface. Studies on the freezing of cells have other implications. For example, the fact that early mouse embryos can now be frozen and stored at −196°C means that

mutant stocks can be stored in an unchanged state indefinitely.

An important parameter in world energy utilization is the efficiency with which energy (ultimately solar energy) can be converted into foodstuffs. At the molecular level, increased efficiency requires a better understanding of photosynthesis. Therefore we have undertaken an analysis of the primary light-induced electronic events in isolated chloroplasts and an analysis of how energy is transferred from the points of absorption in chlorophyll to the photochemically reactive sites. Emphasis the past year has been on understanding the theoretical aspects of interactions between the transfer of excited-state energy and intramolecular vibrations (phonons), and on the development of necessary experimental tools (a tunable laser, a nanosecond fluorescence polarization chronometer, and a photographic procedure for detecting the photochemical activity of single chloroplasts). At the cell and organism levels, attempts are being made to produce soybeans with higher levels of methionine, an amino acid in which legumes are nutritionally deficient. The approach involves the induction of mutations in seeds by irradiation and chemicals. A procedure for rapidly screening the methionine content of the progeny is being developed. Attempts are also being made to speed the genetic upgrading of cattle by developing techniques for inducing multiple ovulations and for transferring the resulting fertilized ova to foster mothers. An important component of the technique will be the ability to preserve the early embryos by freezing.

Despite steps to increase efficient use of energy, man's energy requirements will increase, and it has become vital to know the tolerance of biota to energy production, whether nuclear or nonnuclear. This includes health tolerances of humans and also the tolerance of the important components of the biosphere to environmental perturbations. If we are not to engage in the prodigious task of studying the effects of hundreds of environmental pollutants on thousands of species, it will be necessary to develop generalizations through fundamental studies at the molecular and cellular level.

SUBUNITS (ν BODIES) IN EUKARYOTIC CHROMATIN*

D. E. Olins, Ada L. Olins, Marilyn B. Senior, R. Douglas Carlson, Gwendolyn B. Howze, Everline B. Wright, and Mayphoon Hsie-Hsu

Electron microscopic studies within out laboratory have indicated that eukaryotic chromatin from a variety of tissues contains arrays of spheroid particles (ν bodies) 60–80 Å in diameter connected by thin strands ~15 Å wide. These particles are best visualized following swelling of isolated nuclei at low ionic strength, fixation with formaldehyde, centrifugation onto microscope grids, and positive or negative staining.

In an effort to determine whether these particles contain both DNA and protein, fractions enriched with monomer ν bodies were isolated from fixed chromatin by sonication and separated on sucrose gradients. Data from analytical ultracentrifugation indicated that monomer ν bodies have a molecular weight of ~300,000, a protein/DNA ratio of 1.2/1, and a DNA fragment molecular weight of ~140,000. Thus, each particle would contain ~200 nucleotide pairs and ~160,000 daltons of protein. We have calculated that each particle could contain two of each of the five classes of histones, possibly related by a dyad axis of symmetry.

Native chromatin exhibits a series of low-angle x-ray reflections, indicating an ordered periodic structure. We have observed that swelling, fixation, sonication, and fractionation of the chromatin do not disrupt the x-ray reflections. We believe, however, that drying of the fixed chromatin leads to a loss of the reflections characteristic of native chromatin, and to a shrinkage of the chromatin particles. We are currently studying the origin of the x-ray reflections to determine whether they arise from particle packing or particle structure.

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DEGENERATION AND REFORMATION OF MUSCLE IN THE LAND CRAB, *GECARCINUS LATERALIS*

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

The molt-stage-specific degradation and reformation of the claw (chela) muscle of the land crab, *Gecarcinus lateralis*,[1] provides a model system for obtaining morphological and biochemical evidence on degenerative[2] and regenerative processes during normal growth. Crustacean muscle turnover is a favorable model because of the magnitude of degeneration (40% by weight) and because a method of inducing precocious molts provides premolt animals throughout the year.[3,4] We have begun to study the specific characterization of myosin, including its synthesis and turnover.

The magnitude of the muscle mass lost implies that the major structural proteins are involved. Whether the observed loss of muscle protein represents accelerated degradation cannot be known without information on

the other half of the question, namely, changes in the rates of synthesis. Estimations of the uptake and loss of isotope from a protein require (1) information on the concentration of free amino acids in hemolymph and, more importantly, cell water and (2) isolation and clear identification of the protein. We have initiated studies on the synthesis of muscle protein and have defined the period of maximal protein synthesis – characterized by the uptake of isotopically labeled amino acids.

Amino acid pools. Crustacean tissues contain relatively high concentrations of free amino acids, whose primary function is believed to be in osmotic regulation. We have been carrying out a molt-stage analysis of the free amino acid levels in *Gecarcinus* hemolymph and muscle, using a Beckman amino acid analyzer. The concentrations of hemolymph amino acids show a rather wide range of variability from animal to animal of the same molt cycle stage, making comparisons difficult. However, in muscle, which contains higher concentrations than hemolymph, there is less variation, and several amino acids show changes correlated with the molt stage. Proline, implicated in energy metabolism in insects, decreases six- to eightfold after ecdysis, while several of the essential amino acids increase during the same time. Whether these changes in the free amino acids reflect dietary influences during captivity or are the consequence of changing metabolic demands of muscle and exoskeleton synthesis at ecdysis awaits further evidence. Observations such as these have led us to utilize *in vitro* methods for labeling muscle protein to overcome low and variable specific activities of isotopes difuted by endogenous amino acids *in vivo*.

Isolation of muscle proteins. Methods have been developed for the isolation of myosin from crustacean muscle. Attempts to isolate myosin without significant contamination by other contractile proteins, using methods for isolating mammalian myosin, were not adequate. In arthropods, myosin is extracted as actomyosin and dissociated therefrom by centrifugation with ATP and Mg^{2+}. Recovery of *Gecarcinus* myosin by this method was less than 5%. Neither actomyosin nor myosin could be eluted from DEAE–Sephadex A-50 columns. The method presently used was developed for insect myosin[4] and, although only a partial purification, shows fewer contaminants than other methods. Since preparations are displayed on acrylamide gels, the specific activity of the myosin and its subunits can be quantitated, despite these contaminants.

For *in vitro* labeling, fragments of chelae muscle were incubated for 24 hr at 24°C in Earl's balanced salt solution made isotonic with *Gecarcinus* hemolymph containing 10% fetal calf serum and a ^{3}H-labeled protein hydrolysate as a source of amino acids. Unlabeled glutamine, tryptophan, glutamic acid, asparagine, and aspartic acid, which were not present in the labeled mixture, were added to the medium. Myosin was isolated and separated by SDS-polyacrylamide gel electrophoresis, and the gels were sliced and counted. Incorporation of isotope into all proteins was elevated during the postmolt period as compared with the intermolt period. Myosin heavy chain and a protein with molecular weight 105,000 showed the greatest increase in radioactivity. Newly regenerated chelae showed a five- to tenfold higher uptake than normal (nonregenerated) chelae during the postmolt period.

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CRABS CAN COUNT: THE INTERDEPENDENCE OF LIMB REGENERATION AND MOLTING IN CRUSTACEA

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

In crustacea, limb regeneration and molting are intrinsically and complexly linked. This inseparable relationship depends upon the complex interplay of multiple hormones by which the molt cycle is regulated. In the land crab, *Gecarcinus*, loss of more than five limbs during the intermolt period initiates precocious ecdysis.[1] Conversely, the loss of one or more of the partially regenerated limbs during the premolt period stabilizes the animal in the stage of the premolt period when regeneration occurs,[2] delaying further preparations for ecdysis (i.e. exoskeleton synthesis). We have studied the effect of the loss of several regenerating limbs on the growth and DNA-synthesis rates of the remaining regenerates. In the early stages of regeneration, the loss of one or more regenerates (1) inhibits growth of the remaining regenerates, (2) retards or halts DNA synthesis (i.e. rates of incorporation of [^{3}H]dThd into autotomized limb buds incubated *in vitro*), and (3) induces a premolt pause lasting eight to ten days. During this pause, the missing limbs are reregenerated and the animal molts with a full complement of eight legs. In very late stages of regeneration, loss of regenerates (1) does not inhibit growth or DNA synthesis in the remaining limbs and (2) does not lengthen the duration of the molt cycle, and (3) the

missing limbs are not reregenerated. Several lines of evidence suggest the involvement of an inhibitory factor: (1) Crustacea undergo basal growth of autotomized limbs in the intermolt period in the presence of the molt inhibitory hormone (MIH), which is synthesized and stored in the eyestalks. (2) The time required for basal growth of a new blastema is identical in duration to the period of suppressed growth.

One hypothesis is that the loss of partial regenerates causes the release of MIH, which does not inhibit formation of new blastema at the autotomy plane of each lost regenerate but does inhibit premolt growth of the partial regenerates remaining *in situ*. To test this, partial regenerates have been removed from eyestalkless animals. Since preliminary evidence indicates that the inhibitory effect is still present, an inhibitory factor other than MIH must be released. The effect of loss of partial regenerates on the rates of growth and DNA synthesis in eyestalkless and parabiosed animals is also being studied.

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PARABIOSIS OF CRUSTACEA

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

For our studies on the control of crustacean molting and regeneration we have required that the circulatory systems of pairs of animals be conjoined. To this end, we have exposed the pericardial chambers of pairs of animals by excising the exoskeleton and removing the underlying epidermis. We have then joined the animals with dental cement. To test the effectiveness of this cross-circulation, we have injected [^{3}H]dThd into one animal and sampled the blood of its partner at intervals thereafter. Within 1 hr, the specific activity of the hemolymph of the noninjected animal equals that of the injected specimen. Experiments with such animals will indicate whether the inhibitory factor that turns off DNA synthesis and growth of partial regenerates on the loss of other partial regenerates is hemolymph borne.

Although parabiotic experiments have long been performed with various insect species, to our knowledge this is the first successful method devised for a crustacean species.

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NUCLEOSIDE ANALYSES OF SATELLITE DNAs

W. G. Beattie and D. M. Skinner

In 1970 we showed that two (A + T)-rich crustacean satellite DNAs of identical density in CsCl dissociated at different temperatures and had different solubilities in phenol, the protein denaturant commonly used in isolating DNA.[1] Phenol was shown to extract selectively the crab satellite "poly[d(A-T)]" – satellite DNA composed of more than 90% alternating adenylate and thymidylate residues.[2] From the thermal dissociation temperature (T_m) of the (A + T)-rich satellite of the edible blue crab, *Callinectes sapidus*, we concluded that the DNA contained 26% G + C residues. Other corroborative data were obtained from our circular dichroism studies of the two (A + T)-rich satellites.[3] Direct confirmation has now been obtained from nucleoside analyses of satellite degraded by a mixture of DNase I, snake venom diesterase, and alkaline phosphatase.

We have also determined the base composition of main-component DNA and satellites of six different crustacea. All of the main-component DNAs contain approximately 40% G + C residues. In cases of satellite DNAs having unequal distribution of one or more bases on individual strands,[4] we have analyzed each strand separately. We find that the two satellites of most simple sequence – poly[d(A-T)] and poly-[d(T-A-G-G)]:poly[d(A-T-C-C)] – have G + C contents differing from those predicted from their densities in CsCl gradients or their T_m's.

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A EUKARYOTIC SATELLITE DNA COMPOSED OF ONLY THREE BASES IN EACH STRAND

D. M. Skinner and W. G. Beattie

We have isolated and characterized a pair of isopycnic twin satellite DNAs from the hermit crab, *Pagurus pollicaris*.[1,2] We have determined the nucleotide sequence of satellite I, the less complex of the two.[3]

Nucleoside analyses of the separated strands of satellite I indicated that the dense (H) strand contained 3% C residues and the light (L) strand 3% G residues. To determine whether the small amount of dC in the H strand and the equally small amount of dG in the L strand were due to contamination by trace amounts of the opposite strands, several types of experiments were performed with either H- or L-strand DNA labeled by iodination with $Na^{125}I$. Prefatory to the experiments with the satellite DNAs, *E. coli* DNA was iodinated, degraded to free bases by incubation with 90% formic acid at 175°C for 45 min, and chromatographed on Whatman #1 paper in two solvent systems, butanol:water (86:14) and water. The ^{125}I, presumably in the form of 5-iodocytosine,[4] migrated as a single peak in both solvent systems. There is thus no indication of bases other than cytosine being labeled by this technique.

It has been reported that the presence of a large number of 5-iodocytosine residues causes an increase in density; 21% 5-iodocytosine residues in calf thymus DNA increases the density by 35 mg/cm^3 (ref. 4). As a control on our own technique we centrifuged iodinated *E. coli* DNA and found its density to be identical with that of cosedimented nonradioactive *E. coli* marker DNA.

We then studied the behavior of the H and L strands of iodinated satellite I in alkaline CsCl gradients. Three possibilities were considered. If the H strand were contaminated by a small amount of iodinated L-strand DNA, then a band should form at ρ = 1.724 g/cm^3. Even if the DNA were completely iodinated, its density would not be expected to shift to more than ρ = 1.794 g/cm^3. If the dC residues formed an integral part of the H strand, a band should appear at ρ = 1.828 g/cm^3. If the dC residues were contained in a species of DNA other than the H and L strands of satellite I, a band should form with a density different from either of the strands of satellite I or II.

Iodinated H-strand DNA preparations formed two bands, one superimposed on the main band of *P. pollicaris* (ρ = 1.760 g/cm^3) and the other at ρ = 1.775 g/cm^3. It is possible that the lighter of the two components was contaminating L-strand DNA, since the increase in density (35 mg/cm^3) was that expected if 10.5% of the dC residues of a DNA containing 50% dC residues were iodinated. There was essentially no radioactivity at the density of H-strand DNA (ρ = 1.828 g/cm^3). From these experiments we conclude that the dC residues are not an integral part of the H-strand DNA.

Iodination of the dC-rich L strand yielded more than 25 times as many counts on the DNA, which banded principally in the expected region of the gradient (i.e., it was less dense than the main-band marker). A small peak (defined by only a single point) was observed on the dense side of the main band. This again may represent a DNA contaminant that is neither H nor L strand.

Experiments with radiolabeled H strand of the guinea pig α satellite should indicate whether the few dC residues (and, by complementarity, the few dG residues in the L strand) are contaminations or are an integral part of the H strand.

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GROWTH AND DIVISION SITES IN BACTERIAL CELL-DIVISION MUTANTS

Karen Borneman,* D. R. Stallions, and W. D. Fisher

Radioautographic studies of cells pulse-labeled with diaminopimelic acid (DAP) indicate that new cell-wall synthesis is localized in the central region of the rod-shaped *E. coli* cell.[1] Treatment of cells with low concentrations of penicillin confirms that there is active wall synthesis at the future division site.[2] Pritchard[3] has proposed that septum formation occurs at the site of wall synthesis as a consequence of osmotic pressure changes (decreased turgor) caused by an increase in the rate of wall synthesis after the replication of distal genes. We are therefore examining wall synthesis in selected cell-division mutants to look for possible alterations in the pattern of synthesis.

Two filamentous mutants which have distinctly different patterns of cell division also exhibit different patterns of growth-site locations after penicillin treatment. A temperature-sensitive filamentous mutant (Bug-6), which divides into cell equivalents when returned to the permissive temperature, shows regularly spaced growth sites along its filament at approximate one-cell-length intervals. The other strain, 1899NM, divides only at the polar ends of radiation-induced filaments, but growth sites appear to be randomly distributed along the filament rather than at unit intervals or at the polar regions. The minicell-producing strain P678-54 has active sites of synthesis both in the

central region and at the polar sites where minicells are produced, but it appears probable that these two sites are mutually exclusive in any one cell. The "mon" mutant, whose phenotype is a highly irregular cell shape, reverts to a rod-shaped cell with "blips" in the central region following treatment with penicillin.

If the results obtained with penicillin treatment are confirmed by pulse-labeling studies, it appears that the altered patterns of cell division in some of these mutants are associated with changes in the pattern of wall synthesis. We have obtained DAP-requiring mutants of P678-54, 1899NM, and Bug-6, and radioautographic studies are in progress with these strains to confirm the observations obtained with penicillin treatment.

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MUREIN HYDROLASE ACTIVITY OF DIVISION-PROMOTING EXTRACTS OF BACTERIAL CELLS

R. D. Deufel and W. D. Fisher

We have described and partially characterized a heat-labile, particulate fraction from extracts of bacterial cells which can initiate cell division in radiation-induced filaments of a fil$^+$ strain (1899NM) of *E. coli.*[1] Extracts of normal but not filamentous cells are active.[2] Recently, Hakenbeck et al.[3] have isolated components of the murein hydrolase complex in the *E. coli* membrane and suggested that an endopepidase associated with the inner membrane may function uniquely in septum formation. These observations raise two obvious questions. (1) Is the division-stimulating activity of the extracts in the same membrane fraction as the putative septum-forming enzyme? (2) Are cellular extracts which lack division-promoting activity also deficient in components of the murein hydrolase complex?

The division-promoting activity of extracts is associated with the inner or cytoplasmic membrane and is recovered in the same fractions from sucrose gradients as murein hydrolase activity. Both activities cosediment with membrane-associated dehydrogenase enzymes. Data for extracts deficient in division-promoting activity are not complete.

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FREEZING SENSITIVITY OF *NEUROSPORA CRASSA* AS A FUNCTION OF HYDRATION AND LIFE CYCLE

J. L. Leef* and Peter Mazur

Air-dried conidia of *Neurospora* are quite resistant to freezing, but when suspended in water they become sensitive within 2 min, especially to the sequence of rapid cooling and slow warming. Survivals with this sequence were below 50%. Measurements of spore diameters showed that their volume doubled in this time period; thus, the increased sensitivity seems to be associated with hydration of the spore. If spores are placed in Vogel's minimal medium (VMM) without sucrose, they hydrate, but their respiration remains low and they do not germinate. Their sensitivity to freezing remains the same for at least 12 hr as that of spores suspended in water for only 2 min. On the other hand, when the spores are suspended in VMM with sucrose, respiration increases exponentially, and some 4–6 hr later they germinate. Associated with germination is a further increase in the sensitivity to freezing, and, as is the case with most other cells studied, slow freezing is less damaging than rapid, and rapid warming is less damaging than slow, especially to rapidly cooled cells. In addition, in collaboration with F. H. Gaertner we have preliminary evidence that auxotrophic mutants can be selected by freezing. On deficient media, the mutants do not germinate and therefore are more resistant to freezing than the wild type, which does germinate.

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EFFECTS OF FREEZING ON PLANT TISSUE-CULTURE CELLS

L. E. Towill, Peter Mazur, and G. P. Howland

Plant tissue cultures are useful for the study of events occurring during freezing of higher plant systems, since one utilizes a fairly homogeneous suspension in which injury can be analyzed at the cellular level. The efforts to date have been concerned with (1) methods for assaying survival in a variety of tissue cultures, (2) studies implicating shrinkage of the cell protoplast as an

injurious factor, and (3) increasing the survival after freezing through the use of protective agents. The tissue cultures used have included *Acer, Haplopappus, Hedera, Daucus, Glycine, Petunia,* and *Sagina. Haplopappus* is the only culture which can be plated quantitatively; thus, the reduction of tetrazolium as a viability assay was used for the other tissue cultures. It produced similar results when compared to the plating assay for *Haplopappus.* Fluorescein diacetate was also used as a viability assay. Exposure to plasmolyzing agents (1 and 2 molal) produced a decrease in survival. Since these are the approximate concentrations that occur at the freeze-killing point of all the tissue cultures examined (−2 to −5°C), shrinkage of the cell seems to be potentially injurious. Survival from the plasmolyzed state can be increased if dilution from the osmoticum is slow; similarly, some increase in survival from ~−4°C can be obtained with slower thawing rates. Survival of *Acer* tissue culture, and perhaps others, at low temperatures can be increased significantly with the use of dimethylsulfoxide (DMSO). If the DMSO is added to a final concentration of 1 *M* to cells in growth medium (slow addition of the permeable DMSO, so that plasmolysis does not occur), some cells survive to −50°C if cooled at rates less than ~5°C/min. Thawing rates also affect survival, with the lower rates giving increased survival.

EFFECT OF COOLING AND WARMING VELOCITY ON THE SURVIVAL OF FROZEN-THAWED HUMAN RED CELLS

R. H. Miller and Peter Mazur

Human red cells fully equilibrated with 2 *M* glycerol were cooled to −196°C at rates ranging from 0.2 to 1200°C/min and warmed at rates ranging from 0.5 to 550°C/min. Survival was defined as absence of hemolysis. In some respects the results were quite consistent with those demonstrated with other types of cells: (1) Survival increased with increasing cooling rate to a maximum and then declined abruptly with still more rapid cooling. With warming rates of 160–500°C/min, the survival maximum occurred at a cooling rate of 200°C/min. (2) When cells were cooled at rates above optimum, slow warming and thawing were more damaging than rapid. These two findings are consistent with the view that supraoptimal cooling rates induce intracellular ice. But there were also two novel features in the results: (1) When cells were warmed slowly (0.5–1°/min), the deleterious effects of lower cooling rates became less noticeable than when warming was rapid. (2) In the case of cells cooled very slowly (0.2°C/min), warming at 26–550°C/min was considerably more damaging than warming at 0.5–1°C/min. This latter finding resembles that observed for mammalian embryos and higher plant cells, and may mean that during very slow cooling extracellular solutes enter the cells and make them susceptible to osmotic damage from the rapid dilution that accompanies rapid thawing. These two new findings also demonstrate that damage from slow cooling does not appear related to the length of time the cells are exposed to concentrated solutes during freezing and thawing.

FREEZING OF HUMAN RED CELLS IN GLYCEROL AND SUCROSE SOLUTIONS

Peter Mazur and R. H. Miller

Last year we reported that some 82% of red cells survived freezing at 50°C/min when suspended in 1.4 *M* sucrose, a solute to which the red cell is impermeable. This is about the same percentage that survive freezing at 50°C/min when suspended in a glycerol solution of the same osmolality as the sucrose for sufficient time to allow complete permeation of the glycerol. These facts suggest that survival does not require permeation of the glycerol; however, survival (lack of hemolysis) was measured in the solution in which the cells were frozen. More recently, we have made hemolysis measurements after returning the cells to isotonic saline. About 90% of the red cells frozen in glycerol survive this transfer if precautions are taken to avoid osmotic shock. The procedure is to transfer the cells from hyperosmotic glycerol to a solution of sucrose that has lower osmolality but is still hyperosmotic. One waits about 15 min to allow time for the glycerol to diffuse out of the cells (they are impermeable to sucrose), and the cells are then transferred to isotonic saline. However, in the case of cells frozen in sucrose, only about half survive the double transfer first to a more dilute sucrose and then to isotonic saline, and then only when the solutions are carefully transferred to the cells rather than vice versa. One possible explanation is that, during freezing, sucrose in fact permeates the shrunken cells and, because its molecular weight is greater than that of glycerol, is more difficult to remove from the thawed cells without causing osmotic shock.

CORRELATION OF FREEZING INJURY IN CHINESE HAMSTER TISSUE-CULTURE CELLS WITH THE CONCENTRATION OF ELECTROLYTES IN THE SUSPENDING MEDIUM

S. P. Leibo and Nicholas Rigopoulos

A commonly held belief in low-temperature biology is that cells killed by being cooled at rates less than the

optimum are killed by exposure to electrolytes whose concentrations increase as the suspending solution freezes. The evidence for this belief is somewhat circumstantial, and there are data that are incompatible with it. A hopefully definitive test of this belief will be provided by experiments to determine the survival of hamster tissue-culture cells suspended in sodium chloride solutions of DMSO, glycerol, and sucrose at various concentrations as a function of the temperature to which they are slowly frozen. Since there exist reliable phase-diagram data for such simple solutions, the results of the survival experiments can then be directly compared to the phase diagrams, permitting an accurate plot of cell survival vs electrolyte concentration in partially frozen solutions. The preliminary data indicate that, in general, hamster tissue-culture cells suspended in NaCl solutions of these various solutes are more sensitive to freezing than cells suspended in Hanks' balanced salt solutions (HBSS) of these solutes. This sensitivity is manifested by the fact that cells in NaCl solutions are killed at higher subzero temperatures than are those frozen in the corresponding HBSS solutions. Moreover, when these cells are frozen in HBSS solutions of glycerol, their resistance increases as the glycerol concentration is increased from 0.4 to 0.7 and 1.0 *M*. But when they are frozen in NaCl solutions of glycerol, their sensitivity is the same in all three concentrations of glycerol. When DMSO is used as the protective compound, cells frozen in either HBSS or NaCl solutions display increasing resistance to freezing with increasing concentrations of DMSO. And finally, cells frozen in either HBSS or NaCl solutions of sucrose also respond similarly, in that increasing the sucrose concentration confers no additional resistance to freezing at low cooling rates.

SURVIVAL OF SLOWLY FROZEN HAMSTER TISSUE-CULTURE CELLS AS A FUNCTION OF THE SOLIDIFICATION OF THE EXTRACELLULAR MEDIUM

Peter Mazur and S. P. Leibo

Increasing evidence indicates that the formation and recrystallization of intracellular ice are responsible for the death of cells cooled at supraoptimal rates. There is less understanding of the cause of injury in cells cooled at suboptimal rates. One favored hypothesis is that injury results from the concentration of electrolytes during freezing. A second hypothesis ascribes injury to the shrinkage of cells during freezing. Additives such as glycerol and DMSO are hypothesized to protect by reducing the electrolyte concentration or by reducing cell shrinkage. As discussed previously,[1] data on the survival of slowly frozen Chinese hamster tissue-culture cells vs temperature do not appear consistent with these proposed mechanisms of protection. The present experiments were designed to determine whether survival of the cells can be correlated with the extent to which the extracellular medium is solidified. The latter was estimated by measurements of the AC electrical resistivity and capacitance of solutions during slow freezing. The solutions used were 0 to 1 *M* concentrations of DMSO, glycerol, and sucrose in Hanks' balanced salt solution (HBSS). Between the freezing point and the temperature of total solidification, the resistivity increased by some five to six orders of magnitude. And at the solidification point, a sharp "break" occurred in the capacitance of the sample. Plots of the survival of hamster cells vs the resistivity and capacitance of the solution showed the following: (1) Injury after slow freezing in HBSS occurred *before* the medium completely solidified. (2) In the presence of partly protective additives, the median lethal temperature correlated quite closely with the solidification temperature of the medium. The correlation was far better than with either temperature per se or the concentration of electrolytes. It appears then that, during slow freezing, hamster cells are subjected to at least two sequential injurious events. Cells in HBSS are injured by the concentration of solutes or by cell shrinkage. Glycerol, sucrose, and DMSO protect against this source of injury but often do not protect against the second injurious event – total solidification of the medium.

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INTERACTION OF pH AND pO_2 IN THE DEVELOPMENT OF MOUSE EMBRYOS *IN VITRO*

Nicholas Rigopoulos and S. P. Leibo

To develop normally *in vitro*, mammalian embryos require rather precisely controlled culture conditions. In addition to the composition of the culture medium, other factors that may affect the *in vitro* development of mouse embryos are pH and gaseous environment in which the embryos are grown. Mouse embryos are normally cultured in CO_2-bicarbonate-buffered solutions, and the pH of such solutions can easily be altered by changing the concentration of sodium bicarbonate added to the solution. We have begun to examine the interaction of oxygen tension and media pH on the *in vitro* development of both two-cell and eight-cell embryos, since there are suggestions that the embryos'

requirements change during development. Embryos of each of these two stages have been cultured in different solutions of Brinster's BMOC-3 medium in which the pH was varied from 6.6 to 8.0 and the gaseous atmosphere was adjusted to be (1) 5% O_2:5% CO_2:90% N_2, (2) 8% O_2:5% CO_2:87% N_2, and (3) 20% O_2:5% CO_2:75% N_2. The results thus far indicate that both two-cell and eight-cell embryos grow best at a pH of 7.4 to 7.6 regardless of the pO_2, and that they develop less well at the lower and upper limits of the pH range, particularly under 5% O_2:5% CO_2: 90% N_2 atmosphere. Moreover, two-cell embryos appear to be more fastidious in their culture requirements than eight-cell embryos with respect to both pH and pO_2.

FACTORS AFFECTING THE SURVIVAL OF FROZEN MOUSE EMBRYOS

S. P. Leibo and Suzanne C. Jackowski*

We reported previously that maximum survival of mouse embryos after freezing and thawing requires that the embryos be cooled at about 0.2–1°C/min, and that they be warmed at about 2–20°C/min. We also suggested that dimethylsulfoxide (DMSO) need not permeate the embryo cells to protect them against freezing damage. Both of these aspects of the freezing sensitivity of mouse embryos have been examined in more detail. We find that rapid freezing to −196°C kills the embryos unless they have first been cooled very slowly to at least below −50°C. We also find that the damaging consequences of thawing frozen embryos too rapidly occur between −70 and −20°C. To examine the role of DMSO permeation in freezing sensitivity, we have compared the survival of embryos as a function of time in DMSO prior to freezing and thawing with the embryos' volume changes as a function of time in DMSO. The evidence indicates that DMSO need not permeate to protect the embryos against freezing damage. From these and other data obtained previously, it appears that the embryos' response to freezing and thawing is qualitatively similar to that displayed by many other cell types. Parenthetically, we have found that 80% of one sample of mouse embryos survived freezing and storage at −196°C for 11 months. This preliminary result will be tested further by examining the survival of other embryos stored in the frozen state for years rather than months. But this result already suggests that long-term preservation of the entire genome of an individual mammalian organism can be realized.

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FREEZING SENSITIVITY OF RABBIT OVA FERTILIZED *IN VITRO* AND *IN VIVO*

S. P. Leibo and B. G. Brackett*

We have previously demonstrated that mouse embryos, including one-cell ova, can be successfully frozen to −196°C.[1] Those first successful experiments with a mammalian embryo suggest others of both fundamental and applied nature: (1) What roles do embryo size and stage play in the freezing sensitivity of mammalian embryos? (2) Do the physiological changes that result from fertilization alter the freezing sensitivity of mammalian ova? (3) What changes, if any, in the procedures used to freeze mouse embryos are necessary to freeze the embryos of other species? A preliminary approach to answering these questions has been initiated using rabbit ova that have been fertilized either *in vitro* or *in vivo*. One-cell ova fertilized *in vivo* or two-cell ova fertilized *in vitro*, using the first cleavage as evidence of successful fertilization, were suspended in 1.0–1.5 *M* dimethylsulfoxide, cooled to −78°C at rates from 0.1 to 2.0°C/min, and warmed at rates from 2.5 to 15°C/min. The criterion of two or more normal cleavages *in vitro* was used as evidence of embryo survival. The very preliminary data indicate that about 25% (range 11 to 33%) of rabbit ova fertilized both *in vitro* and *in vivo* survive being frozen to −78°C when they are cooled at about 0.25°C/min and warmed at about 5°C/min. Higher and lower cooling rates and warming rates appear to be more damaging.

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SUPEROVULATION, FREEZING, AND *IN VITRO* CULTURE OF BOVINE EMBRYOS

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

Emphasis during the past year has been on the induction of superovulation in cows and the *in vitro* culture of their embryos. Factors found to have an effect on the number of embryos collected are the batch of human chorionic gonadotropin and its presence or absence, and the number of days over which follicle-stimulating hormone (FSH) is injected. Factors that do not appear to have any effect are batch of FSH, natural mating vs artificial insemination, weight of the

animal, and presence or absence of a corpus luteum from a prior estrus. To date, the mean number of ovulation sites per superovulated animal (12–30 months old) is 24, and the mean number of embryos collected per animal is 15. The maximum number collected is 33; the minimum number is 0. Our mean values are at the high range of those reported by most investigators, and the variation is about the same.

Our assay for survival is cleavage *in vitro*. With design input from T. J. Mitchell and D. G. Gosslee of the Computer Sciences Division, an experiment is under way to determine the percentage of embryos that undergo one or more cleavages as a function of pH, pO_2, and the concentration of lactate in the culture medium. The percentage undergoing one or two cleavages is above 80% for most combinations; however, the percentage developing beyond the 12-cell stage is low.

Our data on control embryos in 1 *M* DMSO for 10 to 100 min are too few to allow certain conclusions, but in the case of embryos held less than 10 min at 4°C prior to the addition of DMSO, 50% cleaved (7/14). In the case of those held >120 min at 4°C, only 2/13 cleaved.

This past fall the only animals available were initially sexually immature. The first animals treated superovulated well, but succeeding animals responded less and less well. We believe the problem resulted from the fact that the later animals were just entering puberty, resulting in competition between their endogenous hormone levels and estrous cycles and the hormones we were injecting.

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EFFECT OF CYTOCHALASIN B ON RESISTANCE OF HUMAN ERYTHROCYTES TO MECHANICAL STRESS

William Rall* and J. S. Cook

Cytochalasin B at concentrations of 10^{-5} *M* and higher disaggregates microfilaments associated with membranes of mammalian cells, including human erythrocytes. Since these cells are subjected to considerable mechanical shear in the circulatory system, we have investigated whether cytochalasin B might alter the physical properties of red-cell membranes as measured by the cells' resistance to mechanical stress. Our assay was lysis by shear forces as opposed to lysis by osmotic (membrane stretching) forces; the red-cell membrane is known to be resistant to the former but very susceptible to the latter. Our criteria for distinguishing mechanical lysis included: (1) absence of prelytic sphering, (2) absence of the prelytic K^+ loss that is a characteristic feature of colloid osmotic hemolysis, and (3) a first-order rate of hemolysis, dependent on the number of unlysed cells at the time of mechanical stress but independent of time after stress by the lytic agent. Sonication was found to be a convenient, quantifiable agent that hemolyzed red cells according to these criteria. Cytochalasin B at 5×10^{-5} *M* conferred on human red cells an increase greater than tenfold in their resistance to sonic oscillation. We conclude that microfilaments play an important role in the mechanical properties of the cell membrane; they appear to impose a structural rigidity on the erythrocyte membrane which may enhance the tensile strength of the surface[1] but reduces the flexibility in response to high-frequency shear forces.

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MEMBRANE PROTEIN TURNOVER AND SPECIFIC MEMBRANE ENZYME ACTIVITIES DURING THE HeLa CELL CYCLE

W. R. Proctor,* Emily T. Best, and J. S. Cook

The turnover parameters of plasma membrane protein have been measured in near steady-state conditions in synchronized (double thymidine block) HeLa cells in suspension. Throughout the cell cycle near steady-state conditions for leucine incorporation, used to monitor protein synthesis, are characterized by (1) rapid equilibration (>50% complete in less than 1 min and complete in less than 5 min, compared to 30-min periods used to estimate protein synthesis) of free intracellular leucine with exogenous isotopic leucine; (2) constancy of free intracellular leucine, concentrated at ~2 m*M* for cells in medium containing 0.5 m*M* leucine; and (3) less than 8% change in total protein during turnover determinations (3-hr increments, with a total cell-cycle time of 27 hr). Using a double-isotope, two-pulse technique, we have shown that the rate of total membrane protein synthesis is essentially constant at all points in the cell cycle. At mitosis, membrane protein undergoes degradation at an increased rate, resulting in an enhanced turnover. Conversely, there is a compensatory decrease in turnover during G_1. These results indicate that degradation is the major regulatory process for membrane protein levels.

Activities of several plasma membrane enzymes, transport of $^{86}Rb^+$ (K^+ analog), and ouabain binding

were followed throughout the cell cycle. The rate of $^{86}Rb^+$ transport per cell increases directly with cell growth, reaching a maximum at late G_2 and halving at division. The rate reflects continuous net synthesis of the transport enzyme (Na^+-K^+)-ATPase at a rate corresponding to the calculated increase in surface area; there is a parallel increase in [^{3}H] ouabain-binding sites, reaching 2.1×10^6 per cell in late G_2. Consistent with this, the specific activity of (Na^+-K^+)-ATPase is constant in membranes isolated at various times of the cell cycle, whereas the specific activities of 5′-nucleotidase and ouabain-insensitive Mg^{2+}-ATPase reach a marked maximum at late G_2–early M, returning to a low level in G_1.

The (Na^+-K^+)-ATPase regulates intracellular alakli cations, which double in quantity during the cell cycle. These observations fit a quantitative description of increasing cell volume as a consequence of net accumulation of ions, the latter in turn a consequence of the increasing number of transport sites on the growing cell surface.

Regeneration of ouabain-blocked transport sites is much more rapid than either the net accumulation or the turnover of total membrane protein. These results are consistent with a model of independent regulation of specific membrane proteins rather than of turnover of whole membrane patches.

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CYCLOBUTADIPYRIMIDINES *IN VIVO* AND *IN VITRO*

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The effects of uv radiation on DNA can be quantitated in terms of the number and kinds of cyclobutyl pyrimidine dimers that are formed by known fluences. A recent publication[1] indicates that (1) the number of dimers observed in DNA containing radioactive dThd (T*<>T*, C<>T*) differs from the number observed in a DNA labeled in both dThd and dCyd (T*<>T*, C*<>T*, C*<>C*), even after account is taken of the different types and specific activities of dimers,[2] and (2) the number of dimers in DNA irradiated *in vivo* may be one-half of the number of DNA irradiated *in vitro*. We have repeated these experiments using double-label techniques, and we find that the number of dimers in *E. coli* DNA is independent of the radioactive label and also independent of whether the DNA is irradiated *in vivo* or *in vitro*.

E. coli cells were grown in medium containing [^{3}H] dThd or [^{14}C] Urd. The latter procedure labels the pyrimidines of RNA and DNA to equal specific activities. The labeled cells were mixed and irradiated together with several fluences of 254-nm light. After irradiation the nucleic acids were extracted, the RNA was removed by alkaline hydrolysis, the DNA was hydrolyzed in formic acid at 175°C for 45 min, and the hydrolysate was subjected to paper chromatography to separate the pyrimidines and the three types of dimers.[1] The chromatogram was cut into strips, from which the radioactivity was eluted and counted in a scintillation counter to determine ^{3}H and ^{14}C. The numbers of T<>T and C<>T were the same (±10%) for both labels.

In a second type of experiment, [^{3}H] dThd-labeled cells were mixed with purified ^{14}C-labeled DNA, and the two were irradiated together, hydrolyzed together, and analyzed together by radiochromatography. The DNA irradiated *in vivo* contained 0.95 of the number of dimers of that irradiated *in vitro*. The small difference is expected because of the absorption of light by the cytoplasm.

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THE NUMBER OF UV-ENDONUCLEASE-SENSITIVE SITES EQUALS THE NUMBER OF PYRIMIDINE DIMERS

R. B. Setlow, W. L. Carrier, and Juarine Stewart*

The preceding article indicates that it is possible to obtain consistent values for the number of cyclobutyl pyrimidine dimers per unit length of uv-irradiated DNA. It is known that the uv endonuclease from *Micrococcus luteus* attacks dimer-containing DNA,[1] but there has been no good indication that it attacks all pyrimidine dimers. Even if every dimer were a point of attack, the number of breaks estimated by sedimentation in alkali might be low, because although the small-molecular-weight molecules contribute markedly to the number of breaks, they are usually omitted from the calculation because they are close to the meniscus.

We used two techniques to measure the number of endonuclease-sensitive sites in uv-irradiated DNA. The first utilized the covalently closed duplex DNA of phage PM2 (6.6×10^6 mol wt) labeled with dThd. A single break lowers the sedimentation coefficient of the DNA threefold and hence enables one easily to measure the fraction of molecules not broken by endonuclease.

The fluence that leaves 37% of the molecules not broken after enzyme treatment is the fluence that makes an average of one endonuclease-sensitive site per molecule. These fluences were determined for wavelengths 254, 265, 280, and 313 nm. The radioactivity in thymine-containing dimers was also measured for these fluences, and the total number of dimers per molecule was calculated assuming that 0.1 of the dimers were unlabeled C<>C. At each of the above wavelengths, an average of one endonuclease-sensitive site per molecule corresponded to 1.0 ± 0.1 dimer per molecule. The assay using PM2 DNA would not detect clustering of endonuclease-sensitive sites, since it measures only the fraction of molecules that have no breaks (or the fraction that have one or more).

Table 1. Endonuclease-sensitive sites in uv-irradiated *E. coli* DNA

Wavelength (nm)	Fluence (J/m^2)	Sites per 10^6 daltons	Dimers per site
254	50	1.37	0.94
	100	2.42	1.19
	200	4.02	0.91
280	205	3.65	0.96
313	1.93×10^5	1.46	0.94
	3.57×10^5	2.39	1.08
	4.56×10^5	3.47	0.94

The second series of measurements used purified *E. coli* DNA labeled with [^{14}C]dThd and [^{14}C]dCyd. At different wavelengths and fluences it was possible to measure directly the number of dimers per unit length (see the preceding article) and, by sedimentation in alkaline sucrose, the number of endonuclease-sensitive sites per unit length. The gradients had been calibrated previously with five standard phage DNAs, and centrifugation was for times sufficient to bring the average molecule near the center of the gradient. In computing number-average molecular weights we omitted the top fraction, corresponding to mol wt <30,000. Thus, any appreciable dimer clustering closer than 100 nucleotides would underestimate the number of breaks. The results are shown in Table 1. Within experimental error, the number of endonuclease-sensitive sites equals the number of dimers, and we conclude that clustering of dimers is not an important consideration in *E. coli* DNA.

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FURTHER STUDIES OF POSTREPLICATION REPAIR IN *E. COLI*

S. G. Sedgwick

DNA synthesized after uv irradiation of *E. coli* contains breaks, which are subsequently sealed during postirradiation incubation.[1] The sealing of these breaks occurs through a process called postreplication repair. Alkaline sucrose gradient centrifugation, coupled with certain biochemical and biological assays, has been used to show the following:

(1) The frequencies of pyrimidine dimers in parental DNA and breaks in newly synthesized DNA were equal for doses up to 10.3 J/m^2 in both repair-proficient and deficient strains of *E. coli*. This finding strengthens the original hypothesis[1] that breaks are formed opposite pyrimidine dimers which have passed through the DNA replication complex but which are unable to base-pair within the structural constraints of a double helix.

(2) Measurements of the kinetics of repair in different DNA-polymerase mutants of *E. coli* indicate that there are at least two modes of repair. One is fast and relies on DNA polymerase I. The other is two to three times slower and utilizes DNA polymerase III. The kinetics of repair in wild-type *E. coli* most resemble those present when DNA polymerase I alone is active, suggesting that normally most repair depends upon DNA polymerase I. Both processes are blocked by *exrA* and *recA* mutations. A different joining process which operates on the discontinuously synthesized DNA arising from DNA replication of undamaged DNA is at least five times as fast as postreplication repair and is not affected by *recA* or *exrA* mutations.

(3) A current hypothesis is that uv-induced mutagenesis occurs through the action of an inducible error-prone repair system.[2] Preliminary experiments, in which *p*-chloramphenicol is added at the time of irradiation to prevent *de novo* protein synthesis show that (*a*) polymerase-III-dependent repair is inhibited by chloramphenicol, and (*b*) chloramphenicol prevents mutation fixation, even in strains which have a functional DNA polymerase I and can carry out strand joining of newly synthesized DNA.

These observations suggest that the polymerase III mode of repair is responsible for uv-induced mutagenesis.

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STRAND BREAKS IN THE DNA OF *SACCHAROMYCES CEREVISIAE* AFTER ULTRAVIOLET IRRADIATION

R. J. Reynolds* and R. B. Setlow

The large number of mutants conferring sensitivity to ultraviolet light, together with the relative ease of genetic manipulation, would seem to make *Saccharomyces cerevisiae* an ideal organism for studying the response of eukaryotic organism to ultraviolet irradiation. However, the presence of a tough cell wall and the absence of thymidylate kinase[1] have hindered studies on the molecular events during the repair of irradiated DNA. We have now developed modifications of the method of McGrath and Williams[2] for detecting strand breaks in DNA and have applied this technique to both wild-type cells and mutants suspected of being deficient in the excision-repair of pyrimidine dimers. The DNA of wild-type cells was found to contain strand breaks soon after ultraviolet irradiation, but many fewer after longer postirradiation incubations. Each of the mutant cultures appeared to be normal in the patterns of strand breaking and/or strand rejoining. Data for wild-type cells are in agreement with current excision-repair models.

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ENDONUCLEASE ACTIVITIES IN EXTRACTS OF *MICROCOCCUS LUTEUS* THAT ACT ON IRRADIATED DNAs

W. L. Carrier and R. B. Setlow

Many cells are able rapidly to repair several kinds of radiation damage to their DNA. For example, gamma-ray-induced base damage, uv-induced pyrimidine dimers, and uv alterations to BrUra-substituted DNAs disappear rapidly *in vivo*. The first enzymic step in these repair sequences is thought to be an endonucleolytic attack on the altered DNA. To assay for endonuclease activity we measure, by sedimentation in alkaline sucrose, the numbers of breaks introduced into radioactive DNAs that contain several enzyme-sensitive sites in 5×10^6 daltons. We have identified three activities in extracts of *M. luteus* that act on irradiated DNA, and we have separated them into four components by chromatography on DEAE-cellulose. (1) The activity toward BrUra-containing DNA that was uv-irradiated in the presence of cysteamine (so as to inhibit the formation of alkali-labile bonds) seems to be specific for Ura residues arising as a result of debromination. The active column fractions extensively degrade PBS2 DNA – a DNA that naturally contains Ura in place of Thy. (2) The activities toward DNA containing pyrimidine dimers is found in two distinct chromatographic peaks, one of which seems to represent a breakdown product or subunit of the other. (3) A third distinct chromatographic fraction, eluting at a higher salt concentration than the dimer endonuclease, acts on gamma-irradiated DNA. (For assay we normally use DNA irradiated anoxically *in vitro*.) The existence of these three separable endonuclease activities is evidence for the generality of excision-repair and for the existence of parallel pathways capable of coping with different types of damage.

CHANGES IN DNA REPAIR WITH CELL AGE*

R. W. Hart† and R. B. Setlow

Last year we reported on measurements of unscheduled DNA synthesis (DNA synthesis during a non-S period) in uv-irradiated WI-38 cells of different ages.[1] Irradiated cells were incubated in [^{3}H] dThd plus hydroxyurea (to suppress normal synthesis), and after a 10-hr incubation radioautographs were made. Late-passage cells showed a bimodal distribution of grains per cell – one peak was at the same grain density as in young cells, the other was at a grain density that decreased with passage number. The magnitude of the peak at normal grain density decreased with age in approximately the same way as did the fraction of cells that could go through the cell cycle. Painter et al.[2] have observed a decrease in the amount of repair replication in uv-irradiated late-passage cells. We have attempted to determine whether the correlation between failure to go through the cell cycle and the decrease in repair replication and unscheduled synthesis observed as an average over a culture of many cells also holds for individual cells.

We used a double-label radioautographic technique. Cells at passage ~40 were incubated for 36 hr in [^{14}C] dThd to label those doing scheduled DNA synthesis, then irradiated with 10 J/m^2 at 254 nm and transferred to medium containing [^{3}H] dThd plus hydroxyurea for 8 hr to label those doing unscheduled synthesis. The cells were covered with a thin emulsion for detection of the β particles from both ^{3}H and ^{14}C, and after development they were covered with a thick emulsion for detection of ^{14}C decays only (Fig. 3).

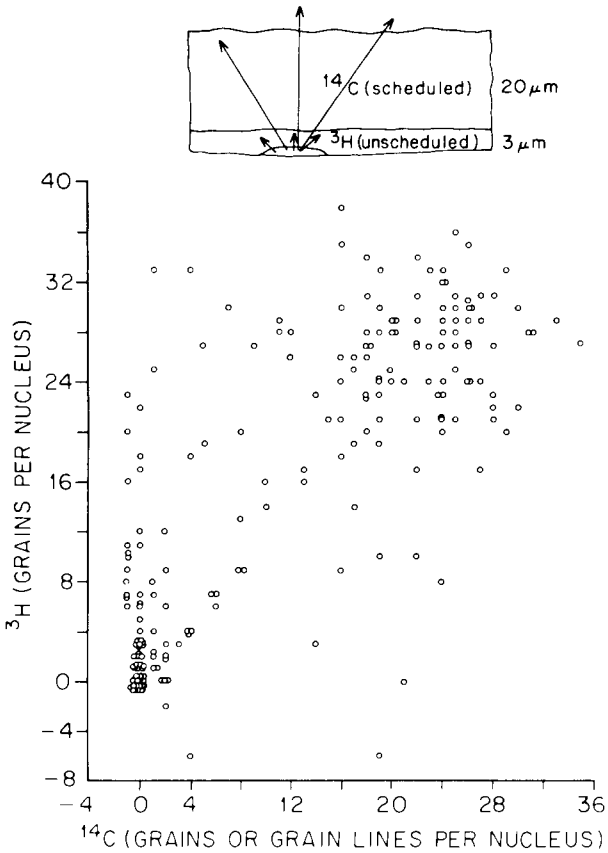

Fig. 3. Abilities of individual WI-38 cells at passage ~40 to do scheduled DNA synthesis (^{14}C grains per nucleus) before irradiation and unscheduled DNA synthesis (^{3}H grains per nucleus) after 10 J m^{-2} at 254 nm. Each of the 200 points represents a single cell. Negative numbers arise from fluctuations in the spillover corrections for double-label counting.

We consider the data in Fig. 3 to represent four cell types: (1) cells that show normal amounts of both scheduled (^{14}C) and unscheduled (^{3}H) DNA synthesis, (2) cells that show little of either type of synthesis, (3) cells that show no scheduled synthesis but some unscheduled synthesis, and (4) cells that synthesize normally but have little unscheduled synthesis. If we take fewer than six grains per nucleus as representing no synthesis, the percentages of cells in categories 1–4 are 58, 26, 14, 2. Thus only a few noncycling or slowly cycling cells show any unscheduled synthesis. The great majority of cells (84%) have their ability to do unscheduled synthesis coupled to their ability to do normal synthesis.

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EVIDENCE FOR UV-INDUCED CESSATION OF RESPIRATION IN *E. COLI* THROUGH DEREPRESSION OF AN OPERON SUBJECT TO CATABOLITE REPRESSION

P. A. Swenson and R. L. Schenley

There is evidence that a number of responses by *E. coli* cells to uv irradiation are induced through derepression of operons. All of these responses, including induction of prophage lambda, filament formation, uv reactivation, and mutagenesis (presumably involving an "error-prone" repair system) require the *recA*$^{+}$ and *lex*$^{+}$ genes (see ref. 1 for summary). We have hypothesized on the basis of experiments with inhibitors of RNA and protein synthesis that cessation of respiration by uv-irradiated *E. coli* cells grown on glycerol is an induced effect, similar to lambda induction, in which an excess of a normal respiratory-control protein is synthesized.[2] Our finding that this cessation requires the *recA*$^{+}$ and *lex*$^{+}$ genes[3] supports this hypothesis, and we suggest that many other physiological responses to radiation, including death, may be caused by induction of operons through derepression.

E. coli cells grown on glucose show less complete inhibition of respiration after uv at 254 nm than those grown on glycerol. Glucose is known to be more effective than glycerol in causing catabolite repression of the induced synthesis of enzymes such as β-galactosidase, whose gene is in the *lac* operon. Exogenous adenosine cyclic 3′:5′-monophosphate (cAMP) releases the *lac* operon from catabolite repression in glucose-grown cells and allows more β-galactosidase to be formed by induced cells. We hypothesized that in uv-irradiated cells the induced formation of the proposed respiratory-control protein is also subject to the catabolite repression action of glucose and that this is the reason that the cessation of respiration is incomplete. We predicted that addition of cAMP to uv-irradiated glucose-grown cells should cause increased formation of the respiratory-control protein and a more complete turnoff of respiration than in its absence. Figure 4 shows that this prediction was correct. Our previous work has provided strong evidence that irradiated cells die of causes associated with respiratory failure, so the role of catabolite repression in the survival response of irradiated cells is being investigated.

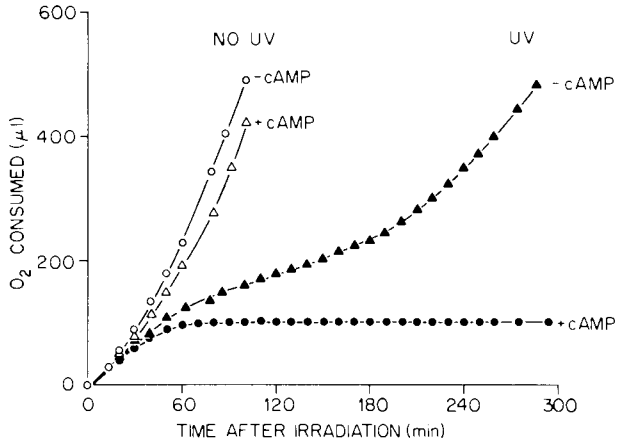

Fig. 4. Effect of cAMP on the respiration of *E. coli* B/r cells grown on minimal medium plus glucose and irradiated with uv (254 nm). The uv fluence was 52 J/m^2. The final concentration of cAMP was 5 m*M*.

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PHOTOPROTECTION OF *E. COLI* B/r: RESPIRATION, GROWTH, AND MACROMOLECULAR SYNTHESIS

P. A. Swenson, J. D. Ives,* and R. L. Schenley

Photoprotecting effects of near-uv radiations (300–400 nm, maximum at 360 nm) against far-uv radiations (primarily 254 nm) have been studied in *E. coli* B/r cells in minimal medium with glycerol as a carbon source. Near-uv light (10^5 J/m^2) has a negligible effect on survival but causes transitory inhibition of respiration and growth, DNA, RNA, and protein synthesis, and cell division. Far uv (52 J/m^2) reduces survival to about 0.5%; respiration, growth, and RNA and protein synthesis proceed for about 60 min, after which they nearly cease for several hours. Near uv given before far uv increases survival tenfold, and the kinetics of all the above processes and of DNA synthesis are characteristic of those produced by lower fluences of far uv. The viability kinetics for uv-irradiated cells with and without photoprotecting treatment are identical, except that the curve for the latter is displaced upward about one log; exponential increases (cell division) begin at 120 min in each case. Many of these data are not consistent with the interpretation that photoprotection is the result of growth or division delays.

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SELECTIVE LOSS OF PYRIDINE NUCLEOTIDES ASSOCIATED WITH THE CESSATION OF RESPIRATION OF UV- AND GAMMA-IRRADIATED CELLS

R. L. Schenley, J. X. Khym, and P. A. Swenson

The cessation of respiration by *E. coli* after uv and gamma irradiation is accompanied by the loss of more than 90% of their biologically active pyridine nucleotides to the suspending medium. We were interested in the selectivity of this loss. Levels of components of the nucleotide pools of unirradiated cells and uv-irradiated cells that had ceased respiring were compared using a column separation system described elsewhere.[1] Of all the mono-, di-, and triphosphates that were present and resolvable, AMP and CDP show the greatest losses, 40 and 60% respectively. Thus the loss of pyridine nucleotides appears to be highly selective. Respiration of irradiated cells stops even when high concentrations of pyridines are in the suspending medium, and we infer that they move out of the cells against a concentration gradient. The possibility that a transport protein is synthesized in irradiated cells will be investigated. The synthesis of some transport proteins is subject to catabolite repression (see above). We are also working with Dr. J. G. Joshi of the Biochemistry Department of the University of Tennessee on the ability of dehydrogenases of irradiated cells to bind pyridine nucleotides.

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EFFORTS TO ISOLATE A PURE POPULATION OF VIABLE *E. COLI* CELLS FROM A UV-IRRADIATED CULTURE

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

When cultures of *E. coli* B/r are uv-irradiated with 52 J/m^2 (approximately 1% survival), respiration and growth cease about 1 hr after irradiation. Treatment of the cultures with a nonionic detergent, Triton X-100, causes dissolution of the cell membranes of nonrespiring cells without effect on the viability of the culture.[1] Using velocity sedimentation through neutral sucrose, we have obtained a highly purified population of nonrespiring, dead cells and have characterized their morphological, physiological, and biochemical properties. Experiments presently in progress are directed

toward the preparative isolation and characterization of a purified population of surviving cells from irradiated cultures. Cultures irradiated with 20 J/m^2 (approximately 10% survival) are treated with Triton X-100 until dissolution of the membranes of nonrespiring cells has occurred. These cultures are suspended in neutral sucrose, from which gradients (0–5%, w/v) are made. Preparative centrifugation of the gradients (cycling) allows us to harvest a relatively pure population of normal- to large-sized cells, the majority of which are viable. When pure populations of cells are obtained, we will study the physiological and biochemical differences between the live and dead cells. Through this and other approaches (see above) we will more clearly elucidate the effects of radiation which result in death of the cells.

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X-RAY CRYSTALLOGRAPHIC STUDIES ON RICIN

J. R. Einstein and C. H. Wei

In previous studies[1] on the purification and crystallization of ricin, a phytotoxin from seeds of *Ricinus communis*, the most toxic fraction from the final CM-cellulose chromatographic separation step was crystallized in the form of large parallelopipeds from 0.005 *M* sodium phosphate buffer (pH 6.7) containing 10^{-6} *M* $CuSO_4$. In order to facilitate the preparation of isomorphous crystalline heavy-atom derivatives for x-ray structural determination, we attempted to define a protein-free solution in which these crystals dissolve to a negligible extent and do not deteriorate. We have found that the solution used in crystallization, altered to contain 12.5% by volume of 2-methyl-2,4-pentanediol (MPD), satisfies these requirements. Crystals soaked in this solution gave x-ray diffraction patterns as good as those from crystals in mother liquor, with very small changes in the intensity distributions.

A qualitative binding study (done in collaboration with Kelly Gregg*) with radioactive *p*-chloromercuribenzoate (PCMB) showed that this molecular species of ricin binds PCMB in solution. Accordingly, ricin crystals were soaked for various time periods in MPD-buffer solutions containing various concentrations of PCMB. If the crystals are soaked for two weeks in a solution containing 10^{-4} *M* PCMB, marked intensity changes occur in the diffraction patterns, while the unit-cell parameters change by less than 0.5%. PCMB-soaked crystals are therefore likely candidates as isomorphous heavy-atom derivatives. Intensity measurements for both native and PCMB-soaked crystals, followed by the calculation of a difference Patterson synthesis, should establish whether the PCMB-soaked crystals are useful. A number of other heavy-atom-containing compounds, such as uranium, lead, and platinum salts, are being screened in the same manner. If at least two isomorphous derivatives are obtained, it is planned to calculate a low-resolution electron-density function as the next stage in the structure determination of ricin.

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STUDIES ON A PRINCIPAL NONTOXIC LECTIN FROM SEEDS OF *ABRUS PRECATORIUS*: ISOLATION, PURIFICATION, AND CHARACTERIZATION

Chongkun Koh and C. H. Wei

Seeds of *Abrus precatorius* (as well as those of *Ricinus communis*) are known to contain a variety of proteins which are toxins, hemagglutinins, or have both properties. We have been particularly interested in the toxins, since they exhibit higher toxicity for certain tumor cells than for normal cells, and thus may be qualified as potent antitumor agents. On the other hand, some plant lectins (not necessarily toxic), such as concanavalin A, wheat germ agglutinin, and soybean agglutinins, have been shown to possess the remarkable property of preferential agglutination of neoplastic cells, thereby qualifying as powerful tools in studies of cell-membrane structure and neoplastic transformation. Studies on the nontoxic lectin also present in the seeds of *A. precatorius* in relatively abundant quantity may add some useful information regarding these matters. Therefore, in addition to our studies on two major *Abrus* toxins,[1–3] we have undertaken the purification and characterization of *Abrus* lectin.

A 5% acetic acid extract of the homogenized kernels of *A. precatorius* was first brought to 45% ammonium sulfate saturation. The precipitate, collected by centrifugation, was dissolved in 0.5% acetic acid and subjected to a heat treatment. Suspended impurities in the mixture were removed by ultracentrifugation, after which the supernatant was dialyzed against water and then against buffer. The crude extract was then passed through a preequilibrated DEAE–Sephadex A-50 column with 0.005 *M* sodium acetate buffer (pH 3.8) as

elutant. Fractions containing most of the *Abrus* lectin were pooled and precipitated with solid ammonium sulfate. Further purification was accomplished by affinity chromatography using a Sepharose 4B column to which the lectin was strongly bound – a binding which was abolished by D-galactose-containing buffer.

The purified *Abrus* lectin appeared homogeneous, as judged by the results of polyacrylamide disc gel electrophoresis and by isoelectric focusing experiments, which also gave a pI value of 4.7. The extinction coefficient ($\epsilon_{1\,cm}^{1\%}$) at 280 nm was determined to be 11.8. A uv spectrum indicates that the absorbancy maximum and minimum are at 280 and 251 nm, respectively. The molecular weight of the lectin was estimated by gel filtration on a Sephadex G-150 column. The estimated value of 130,000 is in agreement with the results of a subunit-molecular-weight determination by means of SDS electrophoresis, which clearly revealed two bands corresponding to subunit molecular weights of 33,800 and 32,200. Apparently, the β-mercaptoethanol treatment in the presence of SDS resulted in four subunits, of which two pairs possess approximately equal (if not identical) molecular size. The amino acid composition study is being carried out by Mary H. Welch under the direction of F. C. Hartman. Preliminary results suggest that there are no free sulfhydryl groups in the molecule.

Biological assays of the purified *Abrus* lectin were performed by Joe Meuth and Wen-Kuang Yang.[4] The lectin showed no toxicity to female BALB/c mice even up to a dosage of 160 μg per mouse (after seven days of observation). However, it strongly agglutinated human (type A^+) and rabbit erythrocytes, each at 1 μg/ml concentration level, while it agglutinated less strongly those of sheep and mouse, each at approximately 5 μg/ml concentration level.

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STUDIES ON A PRINCIPAL NONTOXIC LECTIN FROM SEEDS OF *ABRUS PRECATORIUS:* CRYSTALLIZATION AND PRELIMINARY X-RAY STUDY

C. H. Wei, Chongkun Koh, and J. R. Einstein

Initial attempts to crystallize the purified *Abrus* lectin by several methods, including dialysis against buffers of various ionic strengths and free-interface diffusion into ammonium sulfate solutions of various concentrations, were not successful. Instead, the crystals were grown by the vapor-diffusion technique. Twenty milligrams of lyophilized protein was dissolved in 1 ml of 0.01 *M* Tris-hydrochloride buffer containing 0.03 *M* NaCl. After centrifugation, the supernatant was mixed with an equal volume of 5–8% (v/v) 2-methyl-2,4-pentanediol (MPD). Droplets (50 to 100 μl) of the resulting protein solution were allowed to lose solvent slowly at room temperature (24°C) to a 100-ml solution of 30%-saturated ammonium sulfate in a glass reservoir.

The crystals usually start to appear after three days and grow to at least 0.5 mm in length within a week. Crystallization at 4°C with 40%-saturated ammonium sulfate can also be accomplished, but it takes longer to obtain crystals of usable size. The crystallization procedure is critically sensitive to a number of factors, such as the composition of the buffer, its pH value, and mechanical disturbances during crystal growth. Furthermore, the grown crystals are quite unstable: they become opaque when exposed to air of high humidity or to liquids containing too high a water content, while they soften and lose their ability to extinguish polarized light when allowed to lose too much of their water content. Nevertheless, the crystals can be transferred into 40–50% (v/v) MPD and mounted in capillary tubes for x-ray investigation.

The diffraction patterns, recorded at 24°C or 5°C with nickel-filtered CuKα radiation from an Elliott rotating-anode generator, showed the crystals to be tetragonal with unit-cell parameters a = 142 and c = 210 Å. The volume of the unit cell is 4,230,000 Å^3. The systematic absences of $h00$ for h odd and 00ℓ for $\ell \neq 4n$ indicate that the space group is either $P4_12_12$ or $P4_32_12$, with eight asymmetric units per unit cell.

The density of the *Abrus* lectin crystals was estimated as 1.177 by immersing crystals (0.5 to 1.0 mm in size) in a bromobenzene-xylene density gradient column calibrated with KBr solutions. The molecular weight of the asymmetric unit (protein *plus* solvent) can then be calculated to be 375,000. The percentage of protein in the crystal is then $35n$% by weight, and approximately $30n$% by volume (if the partial specific volume of the protein is taken as 0.74 cm^3/g), where n is the number of molecules per asymmetric unit. The value of V_M (crystal volume per unit of protein molecular weight) is $(4.07/n)$ Å^3/dalton. If n is 1, the value of 4.07 for V_M lies outside the commonly observed range (1.68 to 3.53),[1] while if n is 2, the value of 2.04 for V_M lies within the range. Thus, the latter choice appears more probable. The solvent content in the

crystal would then be 30% by weight or 40% by volume, which is close to the most frequently observed value of 43%.[1]

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PRELIMINARY CRYSTALLOGRAPHIC DATA FOR THE MOST TOXIC SPECIES OF ABRIN[1]

C. H. Wei and J. R. Einstein

As part of a systematic investigation of toxic constituents in the seeds of *Abrus precatorius,* we have succeeded in crystallizing the most toxic portion of the seed extract, abrin C,[2] by the free-interface diffusion technique, in a manner similar to that described for the less toxic abrin A.[2,3] The crystals grown at 37°C exhibit x-ray diffraction patterns of monoclinic symmetry $P2_1$ with unit-cell parameters a = 113 Å, b = 72 Å, c = 71 Å, and β = 103°. The asymmetric unit contains two protein molecules of molecular weight 63,800, and the solvent content is approximately 45% by volume. The diffraction patterns extend to 3-Å spacings, indicating that a high-resolution study could be made. Comparison of the crystallographic data for abrin C with those previously reported for two other "abrins"[3,4] suggests that this crystalline form is probably the most favorable for x-ray structure investigation.

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ISOLATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF PROTEINS (TOXINS AND LECTINS) FROM SEEDS OF *RICINUS COMMUNIS**

C. H. Wei, John Daugherty,†
Ti Ho, and Wen-Kuang Yang

In continuing work on the toxins and lectins present in the seeds of *Ricinus communis* (castor bean),[1] we have purified three proteins, designated III_H, III_L, and V-3, by chromatography on DEAE–Sephadex A-50, CM-cellulose, DEAE-cellulose, and Sephadex G-100 columns. The crystallographic data for V-3, which is similar to ricin D, have been reported.[2] Purified proteins were characterized by the following means: (1) polyacrylamide gel electrophoresis for molecular-weight determination and subunit characterization, (2) animal and tissue-culture experiments to assess toxicity and antitumor activity, (3) immunological studies to determine antigenicity-structure relationship, and (4) hemagglutination inhibition test, agarose affinity chromatography, and ^{125}I labeling for the elucidation of cell-membrane-binding specificities.

The molecular weights of III_H, III_L, and V-3 were shown by gel filtration to be 120,000, 60,000, and 60,000, respectively. Each of these three proteins was found to be composed of two types of subunits. The molecular weights of the subunits were determined by SDS disc gel electrophoresis. All three proteins had one subunit with a mol wt of 30,500, III_L and V-3 had a second subunit with a mol wt of 33,000, and the second subunit for III_H was found to have a mol wt of 34,700.

LD_{50} values (in μg per mouse) determined after 1 week for III_H, III_L, and V-3 were 22, 0.31, and 0.15, respectively. Hemagglutination activity (in μg/ml) necessary to agglutinate sheep red blood cells for III_H, III_L, and V-3 were 1.25, 40, and 160, respectively. Hemagglutinations by III_H and by III_L were inhibited by the addition of various sugars. Lactose, as well as raffinose, inhibited III_H and III_L the same amount, while *N*-acetylgalactosamine inhibition of III_L was about eight times that of III_H.

III_H and formalinized III_L were used to prepare antisera in rabbits. Immunodiffusion studies showed that antisera against III_H cross-reacted with III_L and V-3. Patterns of spurring indicated more antigenic component in III_H than in III_L or V-3, recognized by the anti-III_H antisera. Immune-absorption experiments revealed that III_H, III_L, and V-3 shared a common antigenic component. With our preparation of rabbit anti-III_L, no antigenic difference was demonstrated between III_L and V-3. Two subunits of III_H isolated by 2-mercaptoethanol treatment and CM-cellulose chromatography appeared to be immunologically distinct from each other.

The binding specificities of III_H, III_L, and V-3 were shown to be different. Sepharose 4B (polygalactose) chromatography showed that III_H and III_L bind to galactose while V-3 does not. Studies with ^{125}I-labeled III_H, III_L, and V-3 showed that initial binding of one protein to AT-1 cells inhibits subsequent binding of the other proteins by about 60–80%, suggesting common binding sites for these three proteins. However, ^{125}I-labeled protein-binding studies done in the presence of various sugars showed that the three proteins were

inhibited differently, indicating that these proteins were specific for different carbohydrate groups on the cell membrane.

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†1974 Great Lake College Association–Oak Ridge National Laboratory Science Semester Participant from Albion College, Albion, Michigan 49224.

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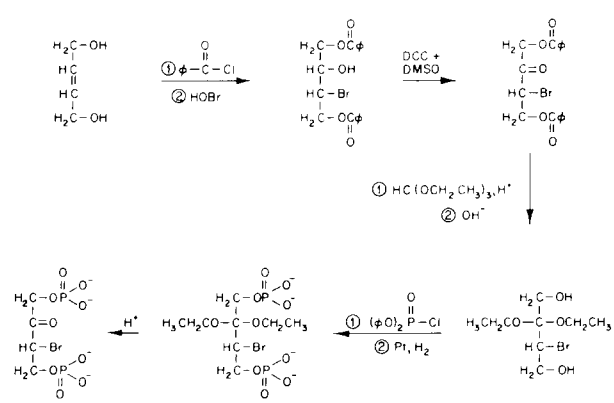
SYNTHESIS OF 3-BROMO-2-BUTANONE 1,4-BISPHOSPHATE: A POTENTIAL AFFINITY LABEL FOR RIBULOSE BISPHOSPHATE CARBOXYLASE

F. C. Hartman

The initial step in the photosynthetic carbon cycle is the carboxylation of ribulose 1,5-bisphosphate to form 3-phosphoglycerate, a reaction catalyzed by ribulose bisphosphate carboxylase. Despite the partial elucidation of the mechanism of action of the carboxylase, the functional groups of the enzyme that are essential to the catalytic process have not been identified. This probably reflects the difficulty of using general protein reagents to characterize the active site of such a complex enzyme. The enzyme from spinach has a molecular weight of 560,000 (ref. 1) and contains two kinds of subunits.[2] Since a high degree of specificity for active-site residues can be obtained with affinity-labeling reagents, I have attempted to design such a reagent for ribulose bisphosphate carboxylase. To this end, 3-bromo-2-butanone 1,4-bisphosphate, a compound that can be considered as a reactive derivative of the substrate ribulose 1,5-bisphosphate, has been synthesized. The procedures used to prepare the reagent are shown in the following scheme:



The structure of 3-bromo-2-butanone 1,4-bisphosphate has been verified by elemental analysis, base-labile bromine content, base-labile phosphate content, and total phosphate content. To provide a foundation for future studies with proteins, the reaction of the reagent with amino acids and small peptides has been studied. The reagent is most reactive toward sulfhydryl groups, but other nucleophiles found in proteins are also susceptible to alkylation.

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EVIDENCE OF ESSENTIAL LYSYL RESIDUES IN RIBULOSE BISPHOSPHATE CARBOXYLASE FROM SPINACH

F. C. Hartman and I. Lucile Norton

We recently showed that 3-bromo-2-butanone 1,4-bisphosphate is an affinity-labeling reagent for ribulose bisphosphate carboxylase.[1] To identify the essential residues that are alkylated, we have isolated and characterized peptides from tryptic digests of the modified enzyme that contain the reagent moiety. The inactivated carboxylase was incubated with $[^3H]$-$NaBH_4$ to reduce the carbonyl group of the protein-bound reagent and thereby incorporate a tritium label, carboxymethylated, and digested with trypsin. Two radioactive peptides, unique to the inactivated carboxylase, were isolated from the digest by successive chromatography on Bio-Rad AG 50W, DEAE-cellulose, and phosphocellulose. The amino acid compositions of the two peptides are His_2, Arg_1, Asx_1, Thr_1, Ser_2, Glx_2, Gly_5, Val_2, Ile_1, Leu_2 and Lys_1, Arg_1, CM-Cys_1, Thr_1, Pro_3, Gly_2, Ile_1, Leu_2, Tyr_1. In addition, hydrolysates of both peptides contain two radioactive amino acid derivatives that elute from the short column of the amino acid analyzer at 18 and 23 min, respectively. Both of these compounds are derivatives of Lys, since they are converted to Lys by periodate oxidation. The structural differences between the two derivatives have not been elucidated. Thus, inactivation of the carboxylase by the reagent results from alkylation of two lysyl residues, presumably in the vicinity of the binding site for ribulose bisphosphate.

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REACTION OF 3-BROMO-2-BUTANONE 1,4-BISPHOSPHATE WITH RIBULOSE BISPHOSPHATE CARBOXYLASE FROM *EUGLENA GRACILIS* AND *RHODOSPIRILLUM RUBRUM*

J. V. Schloss,* F. C. Hartman, and D. R. Voelker*

Based on comparative studies of analogous enzymes from diverse species, one can draw the general conclusion that the active sites of enzymes have been conserved during evolution. This fact provides a useful criterion for determining whether a given reagent, suspected to be active-site specific, is indeed inactivating the enzyme under consideration via covalent modification of a residue essential to catalysis.

A variety of data have suggested that the substrate analog 3-bromo-2-butanone 1,4-bisphosphate inactivates ribulose bisphosphate carboxylase from spinach as a consequence of alkylation of two essential lysyl residues (see the preceding article). 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 carboxylases from *Euglena gracilis,* a green alga, and *Rhodospirillum rubrum,* a purple nonsulfur bacterium. Partially purified preparations of both enzymes are inactivated by the reagent, and ribulose bisphosphate affords protection against inactivation. Efforts to identify the kind(s) of residue(s) whose modification results in inactivation are in progress.

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IDENTIFICATION OF THE HISTIDYL RESIDUE OF RABBIT MUSCLE ALDOLASE ALKYLATED BY *N*-BROMOACETYLETHANOLAMINE PHOSPHATE

F. C. Hartman and Mary H. Welch

At pH 6.5, the site-specific reagent *N*-bromoacetylethanolamine phosphate has previously been shown to inactivate aldolase by alkylation of an essential histidyl residue.[1] To determine the position of this residue in the primary sequence, we have isolated the labeled peptides in tryptic digests of aldolase after its inactivation by ^{14}C-labeled reagent. Two peptides, which contain the histidine derivative and have apparently identical amino acid compositions, are obtained. These peptides lack Arg and Lys, and thus appear to be derived from the C-terminal portion of the aldolase molecule. This assumption is confirmed by carboxypeptidase digestion of the modified aldolase and the purified peptides. Thus, the histidyl residue alkylated by the reagent is the one adjacent to the penultimate residue. The finding that two peptides arise from the C-terminal region is consistent with the previous finding that in the α subunits the penultimate residue is Asn and in the β subunits it is Asp.[2]

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FURTHER STUDIES ON THE REACTION OF RABBIT MUSCLE ALDOLASE WITH *N*-BROMOACETYLETHANOLAMINE PHOSPHATE

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

N-Bromoacetylethanolamine phosphate has permitted the identification of an active-site histidyl residue in rabbit muscle aldolase (see the preceding article). We now find that by increasing the pH of the reaction mixture from 6.5, as used previously, to 8.5, the alkylation of the essential histidyl residue is prevented and inactivation results from alkylation of some other residue. The selectivity of the reagent toward this unidentified residue and its protection from modification afforded by competitive inhibitors suggest that the residue is in the active-site region. Thus, preliminary results indicate that reaction of the affinity-labeling reagent can be modulated between two different active-site residues by the hydrogen-ion concentration. Current efforts are directed toward identifying the residue that is modified at pH 8.5 and determining whether the two sites of reaction are mutually exclusive.

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FORMATION OF ALKALI-LABILE BONDS UPON TRIPLET SENSITIZATION OF PM2 DNA

R. O. Rahn and R. S. Stafford

Triplet sensitizers such as acetone, benzophenone, and acetophenone, when irradiated at 313 nm in the presence of DNA, transfer their triplet energy to DNA, causing thymine dimerization. Sensitization is accompanied by single-strand chain breakage as deter-

mined by alkaline sucrose sedimentation.[1] To determine the extent to which the number of chain breaks observed in alkali represent alkali-labile bonds, the DNA from PM2 phage was employed. This DNA is a covalently closed circle which undergoes a change in sedimentation velocity when a single nick is present. By comparing the number of chain breaks measured under neutral and alkaline conditions, an estimate of the number of alkali-labile bonds was obtained. The number of chain breaks as determined in alkali was in general agreement with the values obtained previously with linear DNA.[1] Fewer chain breaks were observed with neutral sucrose sedimentation, and the percentage of alkali-labile bonds was estimated to be 27–39% for irradiation in the absence of oxygen and 30–60% for irradiation in the presence of oxygen. It was also found that extensive chain breaking occurred when DNA was photosensitized with methylene blue. The extent of alkali-labile bond formation was half that of clean chain breaks.

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INFLUENCE OF BOUND PLATINUM ON THE LUMINESCENCE AND PHOTOCHEMICAL PROPERTIES OF DNA

R. O. Rahn and Linda L. Munchausen*

The compound *cis*-Pt(II)$Cl_2(NH_3)_2$ has been widely studied as an antitumor agent. Its mechanism of action is thought to involve stereospecific binding to cellular DNA. We have employed optical and luminescent probes to study the binding of Pt(II) to DNA. This reaction can be followed by measuring the change in the $A_{250\ nm}/A_{270\ nm}$ ratio. This change was measured for four different DNAs, and the molar ratio of added Pt(II) to DNA base moiety (r) needed to achieve saturations varied linearly from 0.12 to 0.22 upon increasing the G + C content from 39 to 72%, respectively. The luminescence of DNA measured at 77°K indicated that Pt(II) quenched completely the fluorescence and enhanced the phosphorescence. A similar "heavy atom" effect was also observed for the homopolymers, except for poly(dT) which apparently does not bind Pt(II). The rate of thymine photodimerization in DNA is enhanced 40% by bound platinum. We conclude that this enhancement is due to localized denaturation caused by Pt(II) binding to bases other than thymine. A similar effect is noted when DNA is heat denatured.

*NIH Postdoctoral Fellow.

MEASUREMENTS OF DEFECT SITES IN DNA COMPLEXED WITH Pt(II)

Linda L. Munchausen* and R. O. Rahn

The following studies were undertaken on the physical changes induced in DNA by *cis*-Pt(II)$Cl_2(NH_3)_2$ in order to interpret the biological activity of this antitumor drug.

After reaction with the drug, DNA was assayed for regions of local denaturation by (1) the kinetic formaldehyde reaction and (2) enzymatic digestion with a single-strand-specific endonuclease, S1, obtained from *Aspergillus oryzae.* Interstrand cross-links were evaluated, following heat denaturation, by (1) equilibrium sedimentation in CsCl gradients and (2) enzymatic digestion with S1.

DNA saturated with the drug was 12% degradable by S1 and evinced an enhanced rate of reaction with formaldehyde at doses corresponding to roughly one platinum atom per piece of DNA (~10^6 daltons). After heat denaturation, the platinum-bound DNA was only 25% degradable by S1 and sedimented in CsCl at a position intermediate to that of untreated native and denatured DNAs.

From these results one concludes that this reaction of *cis*-Pt(II)$Cl_2(NH_3)_2$ with DNA induces regions of local denaturation as well as interstrand cross-links. However, the probability of occurrence is higher for denaturation defects than for cross-links.

*NIH Postdoctoral Fellow.

PROTON MAGNETIC RESONANCE STUDIES OF UV-IRRADIATED APURINIC ACID*

R. O. Rahn and Thomas Schleich†

Apurinic acid provides a good model system for studying molecular changes in pyrimidine residues when DNA is photochemically modified. In unirradiated apurinic acid, a single-stranded polydeoxyribonucleic acid easily obtained upon depurination of DNA, we have found that the proton resonances arising from the thymine and cytosine bases are readily observable in aqueous solution at 25°C. Two methylthymine resonances separated by 0.05 ppm were observed, and we have attributed these resonances to bases which have no nearest pyrimidine neighbors and to bases which have one or more base neighbors. Upon uv irradiation, the upfield methyl and H-6 thymine resonances decreased in amplitude concomitant with the appearance of two photoproduct methylthymine resonances at 1.63 and 1.52 ppm. A parallel decrease in the absorbance at 260

nm also occurred. Chromatographic analysis of an irradiated labeled apurinic acid hydrolysate revealed the presence of two photodimers, U<>T and T<>T, in the same relative amounts as the peaks located at 1.63 and 1.52 ppm. We conclude that photoinduced dimerization occurs predominantly between neighboring bases.

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PHOTOCHEMISTRY OF POLY(dA)

R. O. Rahn and R. S. Stafford

It has been shown by Pörschke[1,2] that poly(dA) and $d(pA)_2$ but not poly(rA) undergo extensive changes in their absorbance and circular dichroism following uv irradiation. It was of interest to determine whether these changes could be detected by means of radioactive labeling techniques. Poly(dA) labeled with [^{14}C]adenine was irradiated (254 nm), hydrolyzed with formic acid, and chromatographed on paper using *n*-butanol–water–acetic acid (80:30:12). Irradiation of poly(dA) with a saturating fluence of 10^5 J/m^2 resulted in the appearance of a peak in the chromatogram with an R_f of 0.3. The material eluted from this peak had an absorbance maximum at 305 nm and fluoresced strongly at 25°C with a 375-nm maximum. Preliminary results with [^{14}C]purine-labeled DNA indicate that extensive uv irradiation results in some loss of the adenine activity relative to guanine. However, doses as high as 50,000 J/m^2 at 254 nm convert less than 10% of the adenine into photoproduct. Hence, adenine photodamage is not considered to be important for biological inactivation.

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LUMINESCENCE OF MODEL INDOLE-DISULFIDE COMPOUNDS

J. W. Longworth and Claude Hélène*

Cowgill[1] found that the fluorescence of bis-(indole-3-methylene)disulfide was significantly quenched by the presence of the vicinal disulfide linkage, whereas a monocyclic heterodetic peptide, Cys Trp Cys (compound I), was perturbed much less. We have investigated the fluorescence and phosphorescence of these same compounds at 77°K. At 77°K the fluorescence is subject to a large quenching for indole compound I but not for the cyclic tripeptide. The phosphorescence of compound I is typical in its fluorescence-to-phosphorescence yield ratio, fine structure, and phosphorescence decay. The phosphorescence of bis-(indole-3-methylene)disulfide is significantly perturbed. The yield is markedly reduced, there is only diffuse structure, and the peak wavelength is shifted. The decay is shortened and nonexponential in form. A similar behavior is known for the linear heterodetic tetrapeptide, L-cystyl-bis-L-tyrosine.

Red-shifted, short-lived phosphorescence with no fine structure is known from tyrosine and tryptophan residues in certain proteins. Denaturation of these proteins restores the typical tyrosine and tryptophan phosphorescence characters. The tyrosine residues of RNase and tryptophan residues of Bence Jones proteins and lysozymes are known to be placed in contiguity with disulfide links in the molecular structure of the protein.

The nature of the interaction is not established, but it may arise from a charge-transfer interaction.

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LUMINESCENCE OF LYSOZYME-LACTALBUMIN FAMILY OF PROTEINS

V. L. Koenig* and J. W. Longworth

Hen and human lysozyme and cow α-lactalbumin are believed to fold into a homologous conformation, the lysozyme fold, even though at certain positions different amino acids occur. Our interest lies in the tryptophan residues, of which the four in cow lactalbumin are in common with four out of five in human and four out of six in the hen lysozyme. Other workers have used chemical modification to show that in hen lysozyme only two tryptophan residues, Trp_{62} and Trp_{108}, fluoresce; the human enzyme lacks Trp_{62}, and the fluorescence is consistent with emission from the single residue Trp_{108}. The fluorescence decay of hen lysozyme has two components, the human is less fluorescent and has only a single, short-lived component. The hen and human lysozymes are atypical in possessing a phosphorescence with a short decay time; the hen phosphorescence is fine structured, the human diffuse. Denaturation with guanidinium hydrochloride restores the typical tryptophan phosphorescence decay and structure. A model is proposed which accounts for the variations in fluorescence and phosphorescence and the lack of any anisotropy depolarization. The luminescence of cow lactalbumin is consistent with this interpretation of lysozyme luminescence.

The fluorescence yields of these three native and denatured proteins were studied between 100 and 350°K. The influence of temperature could be accounted for by considering a temperature-independent fluorescence and radiationless conversion and a temperature-dependent radiationless process. Fluorescence lifetimes were measured at 298 and 77°K, and the individual rates were evaluated. A linear relation was found between the preexponential and exponential terms in the Arrhenius factor. A compensation law applied to the activated internal conversion, with an isokinetic temperature of 356°K.

Phosphorescence rise times were investigated, and shortening was detected in native proteins, which was absent in denatured proteins. It is possible that the shortened phosphorescence rise times arise from a triplet photoionization in native proteins.

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MEASUREMENT OF THE LIFETIME OF PROTEIN FLUORESCENCE

J. W. Longworth and S. S. Stevens

A nanosecond light flash, with a 1-kHz repetition, was used to excite fluorescence from protein solutions. Single-fluorescence-photon events were detected with a photomultiplier. A delayed coincidence was made to analyze the time intervals between the lamp flash and single-photoelectron events. The nanoflash was formed in hydrogen at 0.5 atmosphere and produced the necessary quartz uv continuum to derive the monochromatic excitation wavelengths. Zirconium electrodes were used and permitted up to 10^9 discharges without maintenance. The lamp was enclosed within a transmission line structure beneath a coaxial capacitor and mounted upon a coaxial ceramic hydrogen-thyratron switch tube. The system response to single-photoelectron events was dominated by the photomultiplier at early times and the lamp beyond 25 nsec. Timing discrimination was achieved by forming a constant-fraction pulse at the input of a differential discriminator functioning in the low-level timing mode. This procedure eliminated serious ground loops associated with the thyratron switching.

The single-electron response was bimodal in time: at 12 nsec a later output distribution can be readily detected – late pulses. Pulses which appear at this time are clearly not preceded because a delayed coincidence is adopted. A FWHM of 1.4 nsec is routinely achieved, and late-pulse frequencies of 1.7×10^{-2} and 2.9×10^{-4} at 12 and 24 nsec with FWHMs of 3 and 6 nsec. The lamp discharge also possesses a long-lived process, 0.1% with a 42-nsec relaxation time.

We have adopted an extremely simple but laborious approach to analyze the data because of the notorious difficulties of interpretation of multiexponential data. A background of $\sim 10^{-4}$ is estimated beyond 150 nsec after the flash and subtracted. A 42-nsec slope is then fitted and subtracted from the data after the late pulse. The fluorescence decay then comprises two segments, prior to and after the late pulse. A single decay component leads to parallel line fits. Two exponentials can be resolved when 10^6 events are collected in the peak channels.

Protein decays have been determined for many examples at both 300°K and 77°K and range between 1 and 20 nsec. Examples have been found where there are two tryptophan decays and others where there is a sum of tyrosine and tryptophan. The relatively low excitation resolution, 7-nm FWHM, precludes selective excitation of tryptophan in the presence of tyrosine.

LUMINESCENCE LIFETIMES OF THYMINE- AND URACIL-CONTAINING DNA

J. W. Longworth, V. L. Koenig,* and W. L. Carrier

PBS-2, a phage of *Bacillus subtilis*, has a DNA which lacks thymine but contains uracil in its place. The luminescence features of this DNA were compared with those of calf thymus DNA and were in general similar in character. The phosphorescence of thymine DNA in its native conformation is characterized by a structureless spectrum associated with thymine residues. PBS-2 DNA possesses a similar structureless spectrum. A comparable phosphorescence is noted from the synthetic polynucleotide poly[r(A-U)·(A-U)]. Uracil in polynucleotides has a triplet energy comparable to that of thymine.

Solution of DNAs in a neutral organic solvent, ethylene glycol, creates a random-coil conformation. The phosphorescence is then predominantly from adenine residues for both DNA samples. Adenine is readily identified by its fine-structure spectrum and decay constant.

The phosphorescence decay of DNA is composed of two components in glycol–water mixtures, 0.3 sec and 2 sec. For thymine DNA it is the shorter component which predominates, for uracil DNA the longer. Phosphorescence rise times appear homogeneous and intermediate between the two components. A multiexponential decay, though faster, is observed in ice matrices.

There is clearly considerable complexity in the sites of phosphorescence in native DNA.

The fluorescence lifetime of both thymine and uracil DNA was determined at 77°K and is ~10 nsec. Denaturation with acid, alkali, or neutral organic solvent considerably shortened the lifetime (2–4 nsec) and shifted the peak from 370 nm to 335 nm. We suggest that this arises from the exciplex nature of fluorescence of native DNA.

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LUMINESCENCE OF BENCE JONES PROTEINS AND LIGHT CHAINS OF IMMUNOGLOBULINS*

J. W. Longworth, Carla L. McLaughlin,† and Alan Solomon†

Immunoglobulins are proteins with specific antibody functions that occur in sera.[1,2] They consist of two light chains (22 kg/mol) and two heavy chains (54 kg/mol). Light chains can be excreted into the urine, and when derived from patients with a monoclonal neoplasia, multiple myeloma, they are called Bence Jones proteins. Bence Jones proteins have been invaluable as a source of material for amino acid sequence studies, and these studies have established the basic primary structure of immunoglobulin light chains. Two distinct types of Bence Jones proteins or light chains, termed κ and λ, have been distinguished immunochemically and structurally. The results of amino acid sequence analyses of Bence Jones proteins or light chains[3] have shown that the amino-terminal half of the polypeptide chain is characterized by variance in sequence, the carboxyl-terminal by a constant sequence. The first ~107 residues constitute the variant region of light chains (V_1); the sequence of V_1 is unique to each protein. The remaining 107 amino acid residues constitute the constant region (C_1); the sequence of C_1 of all proteins belonging to the same basic type, κ or λ, is essentially identical. $C_1(\kappa)$ is common to all κ chains and is different from $C_1(\lambda)$, which is common to all λ chains. The variances in composition of V_1, together with the variance in the homologous variant region of heavy chains, are responsible for the antibody diversity.

Immunoglobulin molecules and their fragments are made up from independently folded domains.[4] The structure of immunoglobulin fragments has been established at atomic dimension through x-ray crystallographic studies from several laboratories.[5-7] Thus the structure[5] of an $F_{ab'}$ fragment of the myeloma protein NEW, which contains a light chain λ II NEWM together with the heavy-chain domains, has extremely similar backbone conformation to the light chain of a Bence Jones dimer λ I MCG.[6] The variant half of a Bence Jones protein V_1 κ I REI exists in the same folded-chain conformation,[7] termed the immunoglobulin fold. The immunoglobulin fold is homologous for both constant and variant halves of both light and heavy chains. Three regions in the sequence of variant halves of light chains have hypervariable sequences when individual proteins are compared.[3] In the immunoglobulin fold, the hypervariable regions occur at one end of the molecule and are fully exposed to solvent. The homologous hypervariable end of the immunoglobulin-fold domains of each light and heavy chain together constitute the antigenic binding site of immunoglobulin molecules. Each immunoglobulin has a unique pattern of amino acid side chains which are attached to a rigid and constant structural frame.

Light chains fluoresce and phosphoresce from tyrosine (Tyr) and tryptophan (Trp) residues, and the contribution by each is separable.[3] A great diversity in luminescence is found when different light chains are examined. No two proteins have the same yield, spectral peak, spectral distribution, and lifetime of decay in studies upon 75 distinct light chains. For most κ-type chains there are two Trp, one to each domain, both at conserved locations that are in homologous positions in the immunoglobulin fold. As the C_1 domain is common to all κ chains, the diversity of Trp luminescence must result from perturbation of the residue in the V_1 domain.

Fluorescence properties of Bence Jones proteins κ I ROY AG and AU are quite distinct, yet these proteins have extensive sequence homologies. Two proteins with extremely similar sequences, κ II TEW and CUM, though lacking any discernible Trp contribution to fluorescence, have distinct Tyr yields.

Phosphorescence of light chains, like fluorescence, is diverse in spectral character. All κ chains decay with two components, one short-lived (1 sec) and the other with a typical value for Trp of 6 sec. Certain κ chains phosphoresce with an atypical spectrum, one lacking in fine structure from Trp; an example is κ II LAYM. The presence of short-lived and dual components is associated with the presence in the immediate vicinity of a Trp residue of a disulfide link. The immunoglobulin fold places the intrachain disulfide link vicinal to the single Trp in both V_1 and C_1 domains.

Fluorescence lifetimes of Tyr and Trp in light chains are diverse, and the apparent natural lifetime can be

described with a series of groups within an eightfold variation. The variance derives from an alteration in internal conversion. In the future, it is hoped that variations will be associable with specific structural elements that are created within the basic immunoglobulin fold by alteration in the hypervariable and variable amino acid locations.

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ATYPICAL PHOSPHORESCENCE SPECTRA AND LIFETIMES OF BENCE JONES PROTEINS AND LIGHT CHAINS OF IMMUNOGLOBULINS*

J. W. Longworth, Carla L. McLaughlin,† and Alan Solomon†

Tyrosine (Tyr) and tryptophan (Trp) phosphorescence spectra of more than 30 κ and λ Bence Jones proteins were determined at 77°K. Spectral differences were apparent even among proteins belonging to the same subgroup. Thus κ I proteins ROY AG and AU, which have extensive variant-half sequence homologies, possess distinct spectra. The proportion of Tyr to Trp evident in the spectra differed, and so did Trp Stokes' shift and fine-structure width. Certain proteins had no measurable Trp fine structure. Lifetimes of Tyr and Trp were separately measured, and both residues in the majority of chains investigated possessed two decay components. The intensity decay was analyzed by a nonlinear least-squares procedure. Tyr had shorter components of 0.70 ± 0.14 sec that represented 0.47 ± 0.23 of intensity, together with 2.16 ± 0.13 sec components. For Trp, 0.61 ± 0.12 decayed at 1.07 ± 0.41 sec, the remainder at 7.1 ± 2.5 sec. Each protein had a distinct lifetime and fraction of components. We attribute the shorter component to residues immediately adjacent to disulfide links in the immunoglobulin fold.

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FLUORESCENCE ANISOTROPY SPECTRA OF PROTEINS AND ENERGY TRANSFER*

J. W. Longworth and C. A. Ghiron†

Transfer of singlet electronic energy between aromatic amino acids within a protein may lead to a depolarization of fluorescence, since it is unlikely that any two neighboring residues will have identical molecular coordinates. The anisotropy of the fluorescence of tyrosine depends upon the exciting wavelength, decreasing in value at shorter wavelengths, but lacks any fine structure. The corresponding spectrum for tryptophan is distinctly fine structured, but nevertheless attains a plateau value at its long-wavelength beginning. Tryptophan can be studied separately from tyrosine in a combination, as tryptophan absorbs to longer wavelengths.

Homotransfer causes an equal depolarization at all exciting wavelengths. This is observed in two proteins that lack any tryptophan residues, bovine pancreatic ribonuclease A and trypsin inhibitor. Their anisotropy spectra are uniformly depolarized between 260 and 295 nm, compared to the spectrum for tyrosine. Therefore, extensive tyrosine-to-tyrosine transfer occurs in these proteins.

In our experience, intertryptophan transfers are uncommon, and anisotropy of fluorescence between 300 and 310 nm of a variety of proteins is comparable to that found in tryptophan. Extensive depolarization can be found in the antibiotic gramicidin, which possesses a series of contiguous tryptophan residues. At acidic pH values, α-trypsin possesses a depolarized tryptophan spectrum, but this transfer is absent at alkaline pH values in the presence of inhibitor. Hen lysozyme, like human lysozyme, is not depolarized at 305 nm, yet its fluorescence is believed to originate from Trp 62 and Trp 108 (the human only from Trp 108). In hen lysozyme, residue Trp 63 is 0.5 nm distant and at an angle of 45°; we suggest that the absence of depolarization indicates extensive quenching of Trp 63 luminescence.

Between 260 and 285 nm there is little variation in the anisotropy of tryptophan. A typical spectrum for many proteins shows depolarization in this wavelength region, which we attribute to tyrosine-to-tryptophan transfers. In subtilisin BPN′, the anisotropy depolarization depends upon wavelength similarly to the fractional absorption of tyrosine.

The anisotropy at 305 nm is due only to tryptophan absorption and typically attains values equal to those for the monomer. We have studied the variation of anisotropy of hyman lysozyme as a function of temperature-dependent variations in viscosity. Fluorescence lifetimes were determined at 220 and 300°K, and fluorescence intensity was continuously monitored. The Perrin plot, corrected for the lifetime variation, disclosed two correlation times. The shorter time (approximately 2 nsec) was attributable to a side-chain relaxation.

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HETEROGENEOUS TRYPTOPHAN ENVIRONMENTS IN THE CYCLIC PEPTIDES TYROCIDINES B AND C: PHOSPHORESCENCE STUDIES*

C. F. Beyer,† W. A. Gibbons,† L. C. Craig,† and J. W. Longworth

Phosphorescence spectroscopy has been used to probe the microenvironments of the tryptophan residues of the cyclic antibiotic decapeptides tyrocidine B (which contains a single tryptophan residue) and tyrocidine C (which contains two tryptophan residues). Both peptides exhibited two distinct, overlapping tryptophan phosphorescence emission spectra under appropriate solvent conditions. The two emissions had characteristically different wavelength positions, phosphorescence lifetimes, and excitation spectra, and their relative intensities were strongly solvent dependent. In contrast, an open-chain derivative of tyrocidine B, which has no biological activity, showed only a single phosphorescence emission from tryptophan. This indicated that the environmental heterogeneity of tryptophan residues in the intact peptides arose not from their primary structures but from higher structural features. Factors which are known to increase the self-association of the tyrocidines, such as higher peptide concentrations or greater amounts of water in alcohol-water solvent mixtures, generally led to enhanced emission of the lower-energy phosphorescence component relative to the higher-energy one. We conclude that the higher-energy component of the phosphorescence emission comes from solvent-exposed tryptophan residues of monomeric peptide molecules, and the lower-energy component from tryptophan residues buried in the hydrophobic interior of peptide aggregates. These cyclic peptides reveal many of the phosphorescence complexities observed with native proteins and thus are useful as model compounds in correlating tryptophan microenvironments with emission characteristics.

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STUDIES OF THE CONFORMATION AND SELF-ASSOCIATION OF CYCLIC DECAPEPTIDES BY TRIPLET-TRIPLET ENERGY TRANSFER*

C. F. Beyer,† L. C. Craig,† W. A. Gibbons,† and J. W. Longworth

We have studied intra- and intermolecular triplet energy transfer from ionized tyrosine residues to tryptophan residues in the cyclic antibiotic decapeptides, the tyrocidines. Chromophore separations deduced from transfer efficiencies have been used to help define peptide secondary and quaternary structure. Our major results and conclusions are the following: (1) Absence of triplet transfer in monomeric tyrocidine B (in which tyrosinate and tryptophan are separated by three intervening residues in the primary structure) indicates that the peptide does not have a secondary structure that brings the donor and acceptor chromophores into close relation. A flat, antiparallel *beta* structure for the peptide backbone, which has been proposed from nuclear magnetic resonance experiments, is consistent with the observed transfer results. (2) Extensive triplet transfer in monomeric tyrocidine C (tyrosinate and tryptophan separated by two residues in the sequence) requires that the separation between the donor and acceptor be close to 8 Å. This distance can be accommodated to the *beta*-structure model for the tyrocidine backbone conformation by rotating the chromophores about their C_α–C_β bonds to bring them into closest proximity. Thus the orientations of the tyrosinate and tryptophan side chains relative to the rigid peptide backbone are established. (3) Selective triplet transfer to the red tryptophan phosphorescence component of tyrocidine B under conditions where the peptide is partially aggregated is interpreted as intermolecular transfer between chromophores of peptide

molecules within the aggregate. This suggests that the separation between aromatic rings of different peptide molecules in an aggregate can be quite small, and lends support to the proposed micelle-like structure for the peptide aggregate.

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LUMINESCENT PROPERTIES OF ANGIOTENSIN II AND ITS ANALOGS*

C. A. Ghiron,† F. M. Bumpus,‡
and J. W. Longworth

The solution conformation of angiotensin II (AII) has been studied extensively, but the question whether AII has a single preferred and biologically active conformation in solution has not as yet been resolved. On the basis of structure-activity studies, it has been proposed that the Tyr_4 and Phe_8 residues of AII, necessary for pressor activity, are contiguous in the biologically active conformation of this hormone. This proposal implies efficient Phe → Tyr energy transfer, which should be demonstrable by fluorescence measurements. Both residues fluoresce in the octapeptide, and the Phe → Tyr energy transfer is less than 50% efficient. By contrast, at 230°K in an equivolume mixture of glycerol and water, fluorescence anisotropy measurements indicate an almost complete (100%) transfer of electronic energy from Phe to Tyr. The C-terminal penta-, hexa-, and heptapeptides and the Ala_6 analog of AII were found to give similar results. Equally small transfer was found at 300°K when AII was studied in various other solvent mixtures. Very little Phe → Tyr transfer was detected even at 230°K with the analog Phe_3-Tyr_8 AII. The $TyrOMe_8$ and Trp_8 analogs of AII showed efficient transfer of electronic energy between their aromatic residues both in water at 300°K and in glycerol-water at 230°K. Triplet-triplet ($Trp^- \rightarrow Trp$) energy transfer in Trp_8 AII was readily detected and was only partially abolished by the addition of saturating amounts of guanidine hydrochloride. These results support the hypothesis that AII does not possess a preferred conformation in water at 300°K. However, as the size and hydrophobicity of the side chain at position 8 are increased, this molecule is able to attain an ordered conformation characterized by a close association of the side chains at positions 4 and 8. At low temperatures it appears that a stable, ordered conformation is attained by all the peptides studied except Phe_3-Tyr_8 AII. Since, under these conditions, the Phe and Tyr residues are contiguous in the ordered structure of AII, we speculate that this conformation may approximate the biologically active one in the receptor.

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ELECTRONIC ENERGY TRANSFER STUDIES UPON LUTEINIZING HORMONE RELEASING FACTOR AND A COMPONENT PEPTIDE AND AN ANALOG

V. L. Koenig,* J. W. Longworth,
and Karl Folkers†

Since the end of the past century it has been understood that an organism maintains homeostasis through two major systems, the central nervous system and the endocrine glands. The anterior lobe of the pituitary gland, or adenohypophysis, controls the secretion of other peripheral endocrine tissues. In 1937, it was shown that the secretion of the adenohypophysis is controlled by the hypothalamus. Chemical transmitters are released at nerve endings in the hypothalamus and transported by a system of portal blood vessels to the anterior pituitary. These releasing factors of hypophysiotropins cause secretion of appropriate pituitary hormones into general circulation. In 1971, after several years of arduous work requiring the collection of several millions of sheep and pig hypothalamic fragments, the factor controlling the release of luteinizing hormone (LHRH) was identified by two laboratories as < Glu His Trp Ser Tyr Gly Leu Arg Pro Gly NH_2. The decapeptide has been chemically synthesized, along with many structural variants, and their biological actions have been examined to aid in defining the mode of action of the hormone. The decapeptide will also cause the release of follicle-stimulating hormone. Folkers and his colleagues have suggested that a stacking of indole and phenolic rings in a coplanar conformation is a crucial feature of the active conformation of LHRH. Two groups using both proton and carbon magnetic resonance have found no evidence for such a ring stacking in aqueous solutions. Rather, the conclusion reached is that the hormone and an amino-terminal hexapeptide fragment have a randomly coiled conformation.

We have examined the luminescence of synthetic LHRH and compared the spectra with those obtained from a derivative, Tyr_3-Trp_5 LHRH, that is a very poor

agonist but has no antagonist property. The spectra of these decapeptides are compared with spectra obtained from the hexapeptide < Gly His Trp Ser Tyr Gly, which is devoid of any agonist or antagonist activity. We find that in aqueous solution, or in methanol, there is tyrosyl fluorescence for LHRH comparable to that found from the hexapeptide, but there is more from the Tyr_3-Trp_5 LHRH. At 77°K the situation alters, and neither the hexapeptide nor LHRH shows any tyrosyl phosphorescence, but there is a tyrosyl contribution to the phosphorescence of Tyr_3-Trp_5 LHRH. In basic methanol, complete absence of ionized tryosyl phosphorescence is observed, consistent with triplet transfer to tryptophan, in both LHRH and the hexapeptide. Again, Tyr_3-Trp_5 LHRH shows ionized tyrosyl phosphorescence. Fluoroalcohols significantly influence tryptophyl fluorescence, and these effects are comparable for the hexapeptide and LHRH, and distinct from the influence found on the spectra of Tyr_3-Trp_5 LHRH. We consider these observations to be consistent with the NMR studies. The influence of low temperature may modify the conformational distribution, and a speculation is to suggest that this is more relevant to the conformer bound by the receptor.

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DATA-ACQUISITION SYSTEM

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

The Biology Division's digital data-acquisition system has been in routine use for more than two years. More than 75 Division staff members, representing every Section, currently hold valid system-user passwords. Fourteen analog data lines are wired to experiment stations in three buildings, and eight terminals (also in three buildings) are presently in use for manipulation of data files and monitoring data collection. Hardware has been installed and successfully tested for high-speed interfaces to a scanning microdensitometer (J. R. Einstein) and a 1000-channel pulse-height analyzer.

Sixty staff members have participated in informal courses designed to familiarize the Division with capabilities of the system and procedures for its use.

Hardware has been installed to permit data transfer directly from the Division's computer to the PDP-10 computer at the Computer Science Division of ORNL (10 miles away). Software for such data transfer and the required error checking is being developed.

Equipment has been ordered to facilitate additional analog and/or digital data collection, and planning has begun for the system expansion necessary to accommodate further Division use of computers in applications such as pattern information processing and ultracentrifuge supervision.

DNA DEGRADATION KINETICS

M. L. Randolph

Models of the kinetics of degradation of DNA in bacterial cells following exposure to x rays were developed. They were then tested against experimental data obtained (with the collaboration of Jane K. Setlow) using various strains of *Haemophilus influenzae* cells in both long and stationary phase. The different mathematical models allow for different initial distributions of DNA lengths, depending on cell phase and assumed replication model, and for unilateral or bilateral degradation from initiation points, which are taken as single-strand breaks (measured by alkaline sucrose gradient techniques). The major analytical problem is essentially one of applied stratistics in the sequential determination of the distributions of strand lengths or molecular weights initially, immediately after irradiation, and during subsequent incubation and degradation. Because the experimental data cannot be fitted to our models of continuous degradation, the notion of an interruption-of-degradation probability was introduced. Random interruption can be interpreted as an enzymatic process or as the occurrence of a unique sequence in the DNA chain, either of which blocks further degradation. In either case, interruption of degradation is interpreted as an early and essential, but not necessarily sufficient, step for repair of DNA. Empirical best fits to the kinetic data were obtained by iterative calculation methods based on varying the rate parameters. Depending somewhat on the strain and cell phase, the data seem best described by assuming bilateral degradation, perhaps at different rates, whose total for log-phase wild-type cells is roughly 5×10^3 nucleotides min^{-1} and an interruption probability of about 0.03 min^{-1}. For stationary-phase cells the interruption probability is unchanged, but the degradation rate is increased. By comparing rate constants for different strains we find that (1) both the degradation rate and interruption probability are greatest for strain DB117, which is most radiosensitive, and (2) degradation seems slowest in strain DB116, which is slowest in resynthesizing DNA following x-ray exposure.

KERMA FACTORS FOR NEUTRON IRRADIATIONS

M. L. Randolph, R. S. Caswell,* and J. J. Coyne*

The ratios of kerma (kinetic energy released per unit mass) per unit neutron fluence are being reevaluated for an appendix to a forthcoming report by the International Commission on Radiological Units and measurements (ICRU). The calculations based on the latest nuclear data compilation (ENDF/B-IV) include a dozen elements, 15 compositions, and 100 energy bins extending from thermal to 30-MeV neutrons. These ratios are useful in neutron dosimetry (1) to convert from kerma measured in a detector of one composition to dose in a biological tissue of different composition, and (2) to convert from a known neutron fluence and energy spectrum to dose in a biological tissue. How well "standard tissue" is representative of a variety of soft biological tissues is also being examined.

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DNA SEDIMENTATION IN HIGH-SALT, HIGH-SUCROSE SOLUTION

M. L. Randolph

Studies on the theory of DNA sedimentation in sucrose solutions are being continued. Previously,[1] we had concluded that sedimentation under usual geometries should be almost linear under all normal salt and sucrose concentrations; but experiments at high salt (2 *M*) indicate that the sedimentation is slower in high-salt, high-sucrose solution.[2] At least qualitatively, an explanation of this discrepancy seems to lie in the assumption of additivity of viscosity increments due to salt and due to sucrose, whereas viscosity measurement shows that the viscosity of NaCl-sucrose solutions increases more rapidly.

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IMPURITY QUENCHING OF PARTIALLY COHERENT EXCITONS IN LINEAR CHAINS

R. M. Pearlstein, R. P. Hemenger, and Katja Lakatos-Lindenberg*

We wish to understand the kinetic behavior of excitons, that is, mobile electronic excitations, in the chlorophyll arrays of photosynthetic lamaellae, the base stacks of nucleic acids, and a few other biological systems. Our theoretical notions of such exciton behavior have been evolving for the past few years. Last year we reported[1] that we had had some initial, limited success upon tackling a very difficult problem: How do excitons move and become quenched (or trapped) if the influence of molecular vibrations on their motion is taken into account? This influence, called the "exciton-phonon interaction," causes the excitons to be only partially coherent – that is, the excitons have some wavelike and some hopping character. The problem is important because in real systems, including the biological ones, the exciton-phonon interaction is likely to have considerable effect on the rates at which migratory excitation energy is removed from those systems. These removal rates are upper limits to the rates of photochemistry in photosynthetic processes and to the rates at which certain types of radiation-induced damage occur or can be inhibited in nucleic acids.

This year we can report a significant advance in our understanding of exciton transport and quenching in the presence of the exciton-phonon interaction.[2] Our procedure involves combining two mathematical formalisms, one previously devised here to treat the quenching of purely wavelike excitons (no exciton-phonon interaction), and the other our modified version of a formalism devised to treat the motion of excitons without quenching but with an exciton-phonon interaction of arbitrary strength. The combined formalism is used to derive the properties of a theoretical model system, a one-dimensional molecular crystal or linear chain quenched at both ends. We find that the electronic excitation on the chain exhibits a varied kinetic behavior, depending on the relative sizes of several parameters. These parameters are the exciton interaction energy J expressed in frequency units (exciton bandwidth $\cong 4J$), rate constants F and F_0 that arise from the exciton-phonon interaction and related processes, quenching rate constant Γ, and fractional concentration of quenchers c (reciprocal of chain length).

If a singlet state near an edge of the exciton band is excited initially, the rate constant, $-\gamma_1$, for decay of electronic excitation from the entire chain due to quenching displays five distinct types or regions of kinetic behavior (see Fig. 5). In three of these kinetic regions, which we refer to as "Frenkel decay," "redistribution decay," and "uniform decay," the exciton motion is wavelike rather than hopping. In each of these three regions, one of three fundamental rate processes is rate limiting. When F_0 is small enough, exciton states other than the one initially excited remain unpopulated, and the rate-limiting process is the

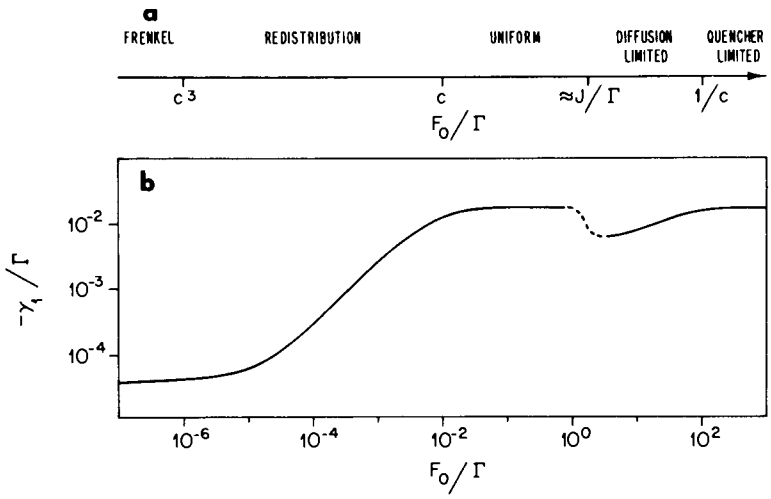

Fig. 5. (*a*) Parametric boundaries of the five kinetic regions for electronic excitation decay due to quenching of partially coherent excitons in linear chains. Symbols are explained in the text. (*b*) Relative excitation decay rate constant $-\gamma_1/\Gamma$ as a function of relative rate constant F_0/Γ (arising from the exciton-phonon interaction), for the particular case $F_0 = F$, $J/\Gamma = 5$, and $c = 10^{-2}$.

slow decay of the initially excited band-edge state (which is like that of a purely wavelike or Frenkel exciton). Excitation is randomly redistributed over all exciton states with rate constant $3F_0$, so that as the randomizing influence of molecular vibrations increases (increasing F_0), this rate constant becomes greater than the decay rate constant of the initially excited exciton state. The random redistribution process itself then becomes rate limiting until $3F_0$ exceeds the average decay rate constant of the resulting equal mixture of all exciton states. Once $3F_0$ exceeds the latter, excitation is spread uniformly and with random phases over all exciton states (and hence over all chain sites) before it becomes quenched.

Of the three wavelike kinetic regions, two, redistribution decay and uniform decay, have not been described previously. In particular, the kinetic behavior in the redistribution region, where the excitation decay rate constant is independent of quencher concentration, is quite unusual. It is also noteworthy that throughout these three regions the excitation decay rate constant $-\gamma_1$ smoothly and monotonically *increases* with F_0 independently of J. We therefore conclude that, contrary to intuitive expectation, the phase-randomizing effects of molecular vibrations actually *enhance* the rate of exciton quenching as long as the exciton motion is essentially wavelike (i.e., throughout the three regions).

There are also two regions, at still larger values of F_0, in which the exciton motion is essentially hopping rather than wavelike. These two regions, termed "diffusion-limited" and "quencher-limited" in Fig. 5, are well known in the theory of hopping excitons. There is a kinetic transition, which we do not describe in quantitative detail, that occurs when the exciton motion changes from wavelike to hopping. It is the only place where the excitation decay rate constant decreases with increasing F_0. The transition is thus the only exception to the otherwise general rule that as the degree of exciton phase randomization increases, the rate of exciton quenching is enhanced.

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SINGLET EXCITED-STATE HOMOTRANSFER IN NUCLEIC ACIDS

R. M. Pearlstein, R. P. Hemenger, and Katja Lakatos-Lindenberg*

Since the discovery of the photosynthetic unit some 40 years ago, electronic excited-state energy transfer has been a topic of considerable interest in biology. While certain aspects of such energy transfer, particularly those involving bimolecular heterotransfer, are fairly well understood, others are not. Foremost among the latter is multiple homotransfer, that is, energy transfer through a system of identical molecules or chromophores. There are a number of systems in which multiple homotransfer is feasible if not actually demon-

strated, most notably, photosynthetic systems and nucleic acids. In both of these there are apparent contradictions and unexplained phenomena.

We have reexamined these phenomena for the nucleic acid singlet states in the light of recent theoretical developments based upon a fundamental description of the interaction between the electronic excitation and molecular vibrations. This so-called "exciton-phonon interaction" crucially affects multiple homotransfer, or exciton motion, in organic molecular systems. We have adapted the exciton-phonon theory (developed for organic crystals) to biological systems and used it to explain on a common basis the phenomena of hypochromism, fluorescence depolarization, and sensitized fluorescence of bound dyes as they occur in nucleic acids, particularly poly(rA). Such a unified picture of these three phenomena is not possible with a purely hopping model of excitation transfer, which cannot explain hypochromism at all.

The results of experiments[1] on fluorescence depolarization at 210°K show that the value of the polarization for poly(rA) is about the same as that for the dinucleotide ApA, and about half that for the mononucleotide AMP. In a hopping model, one is forced to interpret these results as indicating exciton motion over no more than two bases or so in the polymer. However, in exciton-phonon theory there are several possible causes of depolarization due to exciton motion, one of which (redistribution of initially excited stationary states) is most effective in the dinucleotide. In the polymer, bending is a possible cause of fluorescence depolarization even in the presence of extended exciton motion. Our analysis implies that if the depolarization of the polymer is due primarily to bending, then an oligomer of length 10–20 would have a higher polarization than either the dinucleotide or the polymer. It also implies that fluorescence polarization values should be essentially the same over the temperature range 77–210°K, whereas hopping theory would predict much lower values in the polymer at the lower temperatures.

Room-temperature experiments[2] done with various dyes and nucleic acids show that uv excitation sensitizes the fluorescence of dye bound to the nucleic acid. In all cases the sensitized fluorescence has been interpreted as due to what is called "one-step Förster transfer" from excited nucleic acid bases directly to the dye, and not involving any homotransfer (exciton motion). From experimental data and a simple kinetic model, one deduces that as many as 10–20 base pairs can lie between the dye and the most distant "one-step transferring" base. Using exciton-phonon theory, we are able to predict that, if similar experiments were done with poly(rA) at 77°K, a substantial effect of exciton motion would be discerned with perhaps an order-of-magnitude increase in the effective number of bases quenched by each dye molecule (i.e., from ~10 to ~100). Although, generally speaking, extensive exciton motion is also possible in hopping theory, no hopping model can be made consistent with existing fluorescence depolarization data.

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MULTIPLE SCATTERING OF LIGHT

R. P. Hemenger

Several applications of multiple scattering theory to biology have been developed.

(1) The problem of finding the absorption spectrum of cells containing pigments has never been solved satisfactorily by standard methods because of the difficulty of separating scattering effects from absorption. However, by measuring the transmittance of a slab containing a dense suspension of nonabsorbing scattering particles mixed with a small concentration of pigment-containing cells, this absorption spectrum can be determined. Moreover, this requires no knowledge of the properties of the scattering particles.

(2) Last year[1] we reported the development of the "forward scattering transport equation" (FS equation), which describes multiple scattering of light in suspensions of highly anisotropic scatterers (e.g., bacterial cells and blood cells). Further work on this equation has yielded a number of results. Among these is an expression in terms of solutions of the FS equation (which are amenable to straightforward computer calculation), a natural extension of the Beer-Lambert law for cell suspensions. Thus, by making simple light-transmission measurements on suspensions of somewhat greater concentration than those for which the Beer-Lambert law is valid, we have shown how additional information about the scattering and absorption properties of these cells can be gained.

(3) We further reported last year[1] that the FS equation describes the statistics of stiff polymer chains such as DNA and polypeptides. We have shown how a particular solution of the FS equation is directly applicable to a problem of some current interest. This is

the problem of calculating the time-dependent depolarization of the fluorescence of chromophores attached randomly to a stiff polymer chain. Measurements of this depolarization can thus be related to properties of the polymer chain, the nature of chromophore binding, and excitation transfer rates among the chromophores.

1. R. P. Hemenger, *Biol. Div. Annu. Progr. Rep. June 30, 1973,* ORNL-4915, p. 56.

NANOSECOND FLUORESCENCE POLARIZATION CHRONOMETER

R. M. Pearlstein, Francis Van Nostrand, and R. P. Hemenger

We have completed the construction of an instrument to measure simultaneously the two perpendicular components of linear fluorescence polarization as a function of time in the nanosecond range. Initially, difficulties were encountered in the operation of the newly designed hydrogen discharge lamp that provides the pulses of exciting light in the instrument. However, these now seem to be resolved, and the "polarization chronometer" (PC) has been tested with alkaline sodium fluorescein solutions that have a fluorescence lifetime of 4.5 nsec. Some preliminary results – which show that the fluorescence polarized parallel to the exciting light decays faster than that polarized perpendicular, as expected – have already been reported.[1]

Much more data have so far been accumulated with the PC than have been analyzed. Moreover, much more information can, in principle, be extracted from the data already analyzed with the aid of existing data-reduction programs. A new, more reliable and effective computer program for "deconvoluting" the sample's response to the flashes of exciting light from that of the instrument (lap, photomultipliers, and electronics) has been developed in collaboration with T. J. Mitchell of the Mathematics and Statistics Research Department, Computer Sciences Division, UCC-ND. This program is now being tested on data of the fluorescence decay of tyrosine (kindly supplied by J. W. Longworth). The program uses weighted least squares to find the best fit between the sample fluorescence data and a curve constructed by the computer. The latter curve is the convolution of the experimentally determined instrument response curve with a computer solution to the actual sample fluorescence curve. The computer finds such solutions by searching through a class of curves that satisfy simple linear constraints. The program does *not* assume that the actual fluorescence decay is either exponential or any finite sum of exponentials. Initial test results on Longworth's data are quite convincing. With only the constraints that the actual decay curve be nonnegative and nonincreasing (with time), the two exponential components in the fluorescence decay of tyrosine have been clearly resolved, and their lifetimes have been accurately obtained.

1. F. Van Nostrand, *Fed. Proc.* **33,** 1303 (1974).

PHOTOSYNTHESIS

W. A. Arnold, J. R. Azzi, and Stella W. Perdue

We have continued the study of the Hill reaction on chloroplasts embedded in nuclear track emulsion. We have been trying to develop a fast way to prepare chloroplasts for these experiments. It is now clear that the nuclear track emulsion is so sensitive that we can measure the photochemistry done by a small part of one chloroplast. It may be that the activity of a single granum can be seen. We have published a paper on this work.[1]

Further work on glow curves, the light emitted by frozen chloroplasts as they are heated, has given some not-too-convincing evidence that photosystem I is involved in one of the peaks. This is surprising, as it had been believed that only photosystem II gave delayed light and glow curves.

Work is continuing on the stimulation of delayed light by electric fields.

1. W. A. Arnold, S. W. Perdue, and J. R. Azzi, *Science* **185,** 59–61 (1974).

MUTAGENESIS FOR PROTEIN COMPOSITION IN SOYBEANS

D. E. Foard, Rhonda G. Irwin, Beth C. Mullin, L. L. Triplett, Wen-Kuang Yang, and J. L. Epler

Soybean (*Glycine max*) protein is low in methionine, as is true of legumes in general. We are making an effort to increase the methionine content of soybeans by means of neutron and gamma irradiation of seeds and treatment of seedlings with ethyl methanesulfonate and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. Personnel at the UT-AEC Comparative Animal Research Laboratory have treated seeds with the first three of these mutagens and advanced the plants through the M_3 generation (the mutagen-treated seed and resulting plants are designated the M_1 generation). Our efforts in the Biology Division during the past year have been concentrated on developing a screening assay for methionine. Initially we have used an amino acid analyzer, modified so as to

accelerate the quantitative determination of methionine. This procedure is not fast enough, however, to qualify as a true screening method. Therefore we are attempting to adapt a newly modified nitroprusside technique,[1] as well as an immunological technique, to achieving our aims. The latter method requires a highly purified preparation of the 11S globulin, which contains the great majority of methionine present in the seed storage proteins. Thus far we have not discovered a completely reliable procedure for obtaining pure 11S soybean globulin.

1. C. W. Gehrke and T. Neuner, *The Determination of Methionine,* Experiment Station Chemical Laboratories, University of Missouri, Columbia, 1973.

ANALYSIS OF PLANT EXTRACTS FOR CONSTITUENTS OF NATURAL RESISTANCE

L. L. Triplett, D. E. Foard, Rhonda G. Irwin, and J. L. Epler

Much of the natural resistance of plants to pathogens and pests has its basis in the kinds of substances the plant produces (e.g., phenolics and terpenoids). We are attempting to separate and identify such compounds in plant extracts by means of high-pressure ion-exchange chromatography and spectrophotometry, gas chromatography, and mass spectral analysis. Acid-alcohol extracts of the sweet-potato leaf gave 60 to 70 peaks on the high-pressure anion-exchange chromatogram. We compared the chromatograms of a root-knot-nematode-resistant breeding line with that of a susceptible sweet-potato variety. The chromatogram of the former contained two major peaks, which the chromatogram of the latter lacked, and lacked two other peaks, which the susceptible variety's chromatogram contained. Attempts to characterize each of the individual peaks by means of gas chromatography revealed that most of the peaks contained multiple compounds, as suspected. We are identifying reference eluant volume positions by putting known compounds through the column, particularly phenolics, a class of compounds related to pathogen resistance.

REPAIR OF DNA STRAND BREAKS AFTER GAMMA IRRADIATION OF ISOLATED PLANT PROTOPLASTS

G. P. Howland, R. W. Hart,* and Margaret L. Yette

We have examined the kinetics of DNA damage and repair in cells of a higher plant after gamma irradiation. We isolated protoplasts of cultured wild carrot (*Daucus carota* L.) cells, lysed them directly on top of alkaline sucrose gradients, and measured single-stranded DNA of molecular weight 1×10^8 to 2×10^8 by velocity sedimentation. DNA sedimentation studies of gamma-irradiated protoplasts indicate that the energy absorbed in DNA per strand break (or alkali-labile bond) is 85 eV in air and 260 eV in nitrogen atmosphere.

If protoplasts are held at 28°C for various times after aerobic gamma irradiation (20 krads, 0°C), a rapid increase in the DNA molecular weight is observable while the total number of counts recovered from the gradients remains constant, indicating that the strand breaks are rejoined. The kinetics of repair show that 50% of these lesions disappear within 5 min, and by 1 hr none are detected. Thus, wild carrot cells possess the capacity to recognize and repair gamma-ray-induced DNA damage.

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PHOTOREACTIVATION AND REPAIR REPLICATION IN CULTURED HIGHER PLANT CELLS

G. P. Howland and Margaret L. Yette

Several workers have reported that some higher plant cells lack the ability to repair certain types of DNA damage: photoreactivation of survival, photoreactivation of pyrimidine dimers, and dark repair of pyrimidine dimers were not found in uv-irradiated *Haplopappus*.[1] Repair replication is apparently absent in *Vicia* root tips treated with x rays,[2] or the mutagenic chemicals 4-nitroquinoline-1-oxide and *N*-methyl-*N'*-nitro-*N*-nitrosaguanidine.[3]

We have examined our cultured *Haplopappus* cells for uv lethality and photoreactivation using the colony-formation assay.[4] The D_{37} for survival of line *Hap-a*$_2$ is 260 J/m^2 (254 nm). A 1.9×10^4 J/m^2 dose of photoreactivating near-uv light increases the D_{37} to 520 J/m^2 (254 nm).

Using isolated protoplasts of cultured wild carrot (*Daucus carota* L.) cells, we have measured thymine dimers after uv irradiation and their subsequent excision with incubation. A dose of 60 J/m^2 (254 nm) produces about 0.1% dimers or 1 dimer per 10^7 daltons DNA. Although dimer assays have given equivocal results for excision after one day of incubation, the BrUra photolysis technique[5] can detect uv-stimulated repair replication equal to about 15% of the dimers initially present. We are currently attempting to im-

prove several technical aspects of this assay procedure, and we intend to examine the response of these plant cells to certain mutagenic chemicals.

1. J. E. Trosko and V. H. Mansour, *Radiat. Res.* **36,** 333–43 (1968).
2. R. B. Painter and S. Wolff, *Mutat. Res.* **19,** 133–37, (1973).
3. S. Wolff and J. E. Cleaver, *Mutat. Res.* **20,** 71–76 (1973).
4. G. P. Howland and M. L. Yette, *Biol. Div. Annu. Progr. Rep. June 30, 1973,* ORNL-4915, p. 60.
5. J. D. Regan, R. B. Setlow, and R. D. Ley, *Proc. Nat. Acad. Sci. U.S.A.* **68,** 708–12 (1971).

THYMIDINE DEGRADATION IN CULTURED PLANT CELLS

G. P. Howland and Margaret L. Yette

The incorporation of [^{14}C]- or [^{3}H]thymidine (dThd) into the DNA of cultured wild carrot, *Haplopappus,* or *Petunia* cells was found to be very low in a 20-hr labeling period, even though the cells were growing and dividing with generation times of 1–1.8 days. Analyses of the distribution of radioactivity showed that the majority of the radioactivity from [2-^{14}C]dThd could be recovered as $^{14}CO_2$ (wild carrot, 80%; *Haplopappus,* 90%; *Petunia,* 60%). This catabolism of dThd did not take place in medium alone, even that which had been "conditioned" by the growth of cells.

Fluorodeoxyuridine (FdUrd), an inhibitor of thymidylate synthetase, has been employed to enhance the incorporation of exogenously supplied [^{14}C]- or [^{3}H]dThd into DNA. We have tested FdUrd in our plant cell cultures and have observed a tenfold enhancement of [2-^{14}C]dThd incorporation into the DNA fraction (cold-acid insoluble, hot-acid soluble) of wild carrot cells. Surprisingly, the inhibition by FdUrd of dThd degradation was just as dramatic. With increasing FdUrd concentration over the range of 5×10^{-6} to 10^{-4} *M,* less of the [2-^{14}C]dThd was catabolized to $^{14}CO_2$ (to a minimum of 7, 9, and 18% for wild carrot, *Petunia,* and *Haplopappus,* respectively), and proportionately more was recovered in the medium and in the internal cold-acid-soluble and hot-acid-soluble fractions. We do not know whether the ^{14}C recovered in the medium remains as [2-^{14}C]dThd when FdUrd is present.

GENETICS AND DEVELOPMENTAL BIOLOGY

W. E. Barnett

Mutagenesis and Cytochemistry

R. F. Kimball

Mammalian Cytogenetics

J. G. Brewen
P. Carolyn Gooch
E. A. Hiss
R. J. Preston

Human Genetic Biochemical Defects Analysis

Medical and Molecular Genetics

James D. Regan
A. A. Francis

Biochemical Analysis of Human Variants

J. L. Epler
J. X. Khym

Enzymology of Human Genetic Defects

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Somatic Cell Genetics and Biochemistry

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Yeast Genetics

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Drosophila Biochemical Genetics

E. H. Grell
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Drosophila Chromosomal Behavior

Rhoda F. Grell
J. W. Day[a]

Structure and Function of Aromatic Multienzyme Systems

F. H. Gaertner
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Cell Growth and Differentiation

J. N. Dumont*
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Tuneo Yamada

Reproductive Biology

R. A. Wallace
E. W. Bergink[a]
D. W. Jared
T. G. Hollinger[a]

Molecular Basis of Recombination and Repair of DNA

Jane K. Setlow
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Molecular Genetics of Microorganisms

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INTRODUCTION

THE FIFTEEN RESEARCH GROUPS which constitute the Genetics and Developmental Biology Section reflect quite a diversity in research interests, yet there are relevant as well as basic issues which provide a common denominator for much of their efforts.

In this regard, one can point to the increased emphasis on the effects of a variety of chemicals (particularly those related to the production and utilization of energy) on the eukaryotic, and even more specifically, mammalian genome. For example and as described in detail below, the techniques developed and in current use here for determining the genetic hazards of radiation in regard to chromosomal abnormalities, point mutation, and somatic mutation have been adapted to explore the potential genetic effects of chemicals, including gases. In addition, techniques have been developed which permit the evaluation of subtle effects of chemicals in inhibiting the *repair* of DNA lesions. The latter effect is, of course, potentially as critical as mutagen exposure per se and has received little or no attention in the past.

The newly formed Mutagenesis Testing Unit is using the *Salmonella* mutagen tester strains developed by Bruce Ames of California for rapid screening of large numbers of compounds of interest for possible mutagenicity. Similar tests are being established with yeast. The Section has also formed an Electron Microscope Facility, which serves the entire Division for both basic and programmatic research.

While it would be redundant to detail here the various research programs in the Section, it should be emphasized that programmatic research can only be as good as the basic research upon which it is based. Thus there is equal emphasis on such fundamental but closely related problems as replication, recombination, function, and repair of the genetic material in a variety of test organisms; the biochemical basis of human genetic diseases; and reproductive, developmental, and cell biology. This is reflected in the reports which follow.

INVOLVEMENT OF DNA REPLICATION AND REPAIR IN MUTAGENESIS OF *HAEMOPHILUS INFLUENZAE* INDUCED BY *N*-NITROSOCARBARYL

K. L. Beattie* and R. F. Kimball

An investigation of the cellular processes involved in mutagenesis was conducted, using the bacterium *Haemophilus influenzae* treated with the alkylating agent *N*-nitrosocarbaryl. A temperature-sensitive DNA-synthesis mutant (strain *dna*9) was treated with *N*-nitrosocarbaryl, then incubated at the permissive (36°C) and nonpermissive (41°C) temperatures. At various times, lysates were made and used to transform a second culture to mutant phenotype (novobiocin resistance). It is known that premutational lesions present in the DNA immediately after treatment with mutagen do not produce mutations in the second culture through transformation.[1] However, if the treated cells are washed and allowed to grow for a time before the DNA is extracted, the mutation yield obtained through transformation approaches that obtained when the same cells are grown for several generations and tested directly for mutation frequency.[1] This increase in the ability of DNA from lysed cells to produce mutants through transformation, known as "mutation fixation," represents conversion of premutational lesions to a form that can be detected by transformation, perhaps the ultimate change in the nucleotide sequence in the DNA.

At the permissive temperature, mutation fixation continued approximately linearly during at least half of the first round of DNA replication after treatment with *N*-nitrosocarbaryl. In the absence of DNA replication (41°C), most but not all of the mutation fixation was eliminated. The data suggest that premutational lesions occur over the entire chromosome and indicate that the bulk of the mutation fixation requires DNA replication. A similar conclusion was previously drawn from experiments on mutagenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *H. influenzae*.[2] A process independent of replication, probably an error-prone repair system, is responsible for part of the mutation fixation in cells exposed to *N*-nitrosocarbaryl.

It was previously shown that when strain *dna*9 is treated with *N*-nitrosocarbaryl and then incubated at 41°C for a time (stopping DNA replication and the bulk of the mutation fixation) before being grown at 36°C, a large decrease in the final mutation frequency is seen.[3]

This suggests that a repair mechanism still functional in the absence of DNA replication is capable of removing premutational lesions from the DNA. Repair of premutational lesions and nonreplicative mutation fixation were observed in three different strains carrying mutations affecting excision-repair of uv-induced pyrimidine dimers, indicating that the pyrimidine-dimer-excising mechanism of *H. influenzae* is not involved in *N*-nitrosocarbaryl-induced mutagenesis.

The recombination-deficient mutant *rec*1 is apparently deficient in the replication-dependent mode of mutation fixation. This conclusion is based on the following results: (1) *N*-nitrosocarbaryl-induced mutagenesis is lower in the *rec*1 strain than in *rec*$^+$ cells. (2) Repair of premutational lesions (which depends on the existence of replication-dependent mutation fixation for its detection) was not detected in the *rec*1 strain. (3) When nonreplicative mutation fixation and final mutation frequency were measured in the same experiment, about $1/4$ to $1/3$ of the final mutation yield could be accounted for by nonreplicative mutation fixation in the *rec*$^+$ strain, whereas all of the mutation in the *rec*1 strain could be accounted for by nonreplicative mutation fixation. (4) When mutation fixation in strain *dna*9 *rec*1 was followed at the permissive (36°C) and nonpermissive (41°C) temperatures, it became apparent that in the *rec*1 strain replication-dependent mutation fixation occurs at early times, but these newly fixed mutations are unstable and disappear at later times, leaving only the mutations fixed by the nonreplicative process.

The *rec*1 strain exhibits normal repair of *N*-nitrosocarbaryl-induced single-strand breaks or alkali-labile bonds in the DNA labeled before treatment, but it is slow in joining discontinuities present in DNA synthesized after treatment. After treatment of strain *dna*9 with *N*-nitrosocarbaryl, excessive degradation of DNA labeled either before or after treatment occurs, accompanied by increased lethality.

The results obtained do not indicate whether in *N*-nitrosocarbaryl-treated *H. influenzae* cells the replication-dependent mode of mutation fixation occurs by direct mispairing opposite certain alkylation-induced lesions or by error-prone joining of interruptions present in the DNA synthesized after treatment.

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

1. R. F. Kimball and J. K. Setlow, *Mutat. Res.* **14**, 137 (1972).

2. R. F. Kimball and J. K. Setlow, *Biol. Div. Annu. Progr. Rep. June 30, 1973*, ORNL-4915, p. 63.

3. K. L. Beattie and R. F. Kimball, *Biol. Div. Annu. Progr. Rep. June 30, 1973*, ORNL-4915, p. 63.

SURVEY OF MUTAGENS FOR *HAEMOPHILUS INFLUENZAE*

R. F. Kimball and Bernice F. Hirsch

Our work on mutation induction in *H. influenzae* up to now has concentrated on two nitrosocompounds, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and nitrosocarbaryl (NC).[1] It was important to expand the work to a greater variety of mutagens to find out how far the generalizations developed for MNNG and NC were of broader applicability. However, only mutagens that produce fairly high levels of mutations without much cell killing would be useful for many of the detailed studies we wish to make. Thus we have also become concerned with the problem of mutagenic efficiency.

Our own earlier work had shown that neither ultraviolet light, methyl methanesulfonate (MMS), nor bromodeoxyuridine was mutagenic for *H. influenzae*.[2] The latter substance was incorporated in sufficient amounts to increase markedly the density of the DNA. In these respects, wild-type *H. influenzae* resembles certain strains of *E. coli* (exr$^-$, lex$^-$, and recA), which appear to lack an error-prone repair system. A personal communication from another laboratory shows that *H. influenzae* can be mutated by ionizing radiation. *E. coli* strains lacking the error-prone system have been variously reported not to be mutated or to be mutated to a small extent by ionizing radiation.

A number of other mutagens have now been tested. Of these, hydrazine (HZ), hydroxylamine (HA), ethyl methanesulfonate (EMS), and hydrogen peroxide are detectably mutagenic at doses that allow at least 10% survival. Sodium bisulfite (BS), nitrous acid (NA), and tertiary butyl hydroperoxide (tBHP) are not. It becomes evident in such a comparative study that the toxicity of the compound can seriously complicate the drawing of conclusions about mutagenic effectiveness. Thus H_2O_2 is detectably mutagenic at concentrations well below those for EMS and HA, yet it is so toxic that only low levels of mutations can be produced at any practical survival level. EMS and HA can produce much higher levels of mutation at practical survivals. Thus, in the sense of the concentration required to produce detectable mutation, H_2O_2 is a much more effective mutagen than EMS or HA, but in terms of practical mutation studies, it is much poorer.

The negative compounds, MMS, NA, BS, and tBHP, are all sufficiently toxic so that none can be tested at

even the lowest concentration that will produce detectable mutation with EMS and HA. Thus there is no way to be certain that these negative results mean anything other than that the compounds cannot be tested at a high enough concentration. It is clear, however, that they are not as mutagenic as MNNG or H_2O_2, since they can be tested at concentrations that produce detectable mutations with these compounds. NA is a known mutagen for *H. influenzae* but only when single-stranded DNA is treated *in vitro*, renatured, and used for transformation.[3]

HZ represents a very interesting exception to the usual association in bacteria between mutagenic and lethal effects. It is quite mutagenic in concentrations at least as low as those that are effective for H_2O_2, yet it produces no detectable lethality in concentrations at least three orders of magnitude greater. The dose curve for mutation reaches a plateau of about 2×10^{-6} novobiocin-resistant mutants per bacterium and remains at this plateau or perhaps declines somewhat over at least a tenfold increase in concentration. This plateau is about tenfold less than the maximum that can be produced by MNNG and NC. Thus the limiting factor determining the maximum level of mutations that can be produced cannot be either differential killing or some maximum level set by the mutation detection system itself. Such maxima must also be considered in discussing the mutagenic efficiency of a compound. Other studies on HZ are discussed in the following article.

1. R. K. Elespuru, W. Lijinsky, and J. K. Setlow, *Biol. Div. Annu. Progr. Rep. June 30, 1973*, ORNL-4915, p. 95; K. L. Beattie and R. F. Kimball, "Involvement of DNA Replication and Repair in Mutagenesis of *Haemophilus influenzae* Induced by *N*-Nitrosocarbaryl," this report.

2. R. F. Kimball, J. K. Setlow, and M. Liu, *Mutat. Res.* **12**, 21 (1971); R. F. Kimball and J. K. Setlow, *Mutat. Res.* **22**, 1 (1974).

3. E. E. Horn and R. M. Hernott, *Proc. Nat. Acad. Sci. U.S.A.* **48**, 1409 (1962); E. H. Postel and S. H. Goodgal, *J. Bacteriol.* **94**, 1802 (1967).

HYDRAZINE MUTAGENESIS IN *HAEMOPHILUS INFLUENZAE*

R. F. Kimball and Bernice F. Hirsch

In the preceding article it was stated that hydrazine (HZ) is an effective mutagen for *H. influenzae* at concentrations and exposure times at which there is no detectable lethality. In this respect it differs from most other mutagens that have been used with bacteria, and this alone makes it of considerable interest. Moreover, one of its reaction products with deoxycytidine, *N*(4)-amino-2′-deoxycytidine, is mutagenic for *E. coli* and phage ϕ80, perhaps as a result of incorporation as a base analog in DNA.[1] Thus a possible chemical pathway for its mutagenesis has been identified. We have, therefore, carried out a series of studies on this compound.

Two major types of experiments have been carried out so far, the so-called "fixation" and "repair" experiments, which have been developed for work with MNNG and nitrosocarbaryl.[2] In the fixation experiment, streptomycin-resistant bacteria are treated with hydrazine, washed, and lysed either immediately or after a period of incubation. The lysate is then used to transform streptomycin-sensitive bacteria, and novobiocin-resistant mutants are looked for among the streptomycin-resistant transformants. Very few mutations were found in bacteria transformed with the zero-time lysate but many more after an incubation period of 20 to 60 min. This has been interpreted as evidence for mutation fixation.[2] Surprisingly, when lysates from still later times were used, the frequency of mutation dropped again. This secondary drop has been seen with nitrosocarbaryl-treated *rec*1 bacteria[2] but not with nitrosocarbaryl-treated wild type. Why it occurs with hydrazine-treated wild type we do not know. Tests with hydrazine-treated *rec*1 have not yet been made.

The repair experiment[2] consists of treating a temperature-sensitive DNA replication mutant with the mutagen, washing it out, and incubating for various periods of time at the restrictive temperature before returning to the nonrestrictive temperature, growing for several cell generations, and testing for mutation. DNA replication is completely blocked during the period at the restrictive temperature. The result with hydrazine is essentially the same as with nitrosocarbaryl. The mutation yield declines to about a third to a quarter of its value when DNA replication is allowed to proceed immediately. This has been interpreted[2] in the case of nitrosocarbaryl to mean that potentially mutagenic alterations in the DNA can be repaired during the interval before replication, at which time the remaining alterations become converted to fixed mutations with a reasonably high probability. The lower baseline has been interpreted, in light of other evidence, to be the fixation that occurs in the absence of replication. The hydrazine data can be interpreted in the same way, though there is less information available as yet than there is for nitrosocarbaryl.

Finally, no effect on final mutation yield has been detected for any of the three mutations in *H. influenzae* that are defective in excision-repair, nor for either of the two mutations that are defective in recombination.

1. B. C. F. Chu, D. M. Brown, and M. G. Burdon, *Mutat. Res.* **23,** 267 (1974).

2. K. L. Beattie and R. F. Kimball, *Biol. Div. Annu. Progr. Rep. June 20, 1973,* ORNL-4915, pp. 63–64; K. L. Beattie and R. F. Kimball, "Involvement of DNA Replication and Repair in Mutagenesis of *Haemophilus influenzae* Induced by *N*-Nitrosocarbaryl," this report.

DEVELOPMENT OF AN AUXOTROPHIC MUTATION SYSTEM FOR *HAEMOPHILUS INFLUENZAE*

R. F. Kimball

So far, our mutation studies on *H. influenzae* have been confined to drug-resistance mutation, primarily resistance to novobiocin. This latter system has proved useful because it has a low and stable spontaneous mutation rate and because the locus for novobiocin resistance is closely linked to that for streptomycin resistance. This linkage makes it possible to use mutagenized DNA from a streptomycin-resistant strain in a transformation assay and to obtain a high frequency of transformants with the novobiocin locus from the mutagenized DNA by selecting streptomycin-resistant transformants. It would be desirable to develop other systems for detecting mutations, and specifically those using nutritional deficiencies, to test whether the generalizations developed from the novobiocin system are of wide applicability.

A strain containing eight mapped auxotrophic mutants was obtained from another laboratory. Unfortunately, all these mutants proved to have very low rates of both spontaneous and induced mutation, if indeed they mutated at all. Moreover, none was sufficiently closely linked to any other or to any of the available drug-resistance loci to allow efficient selection by the linked-marker technique of transformants for the locus to be studied for mutation.

In the course of searching for auxotrophic mutants in a thymine-requiring strain that had been treated with MNNG, several proline-requiring mutants were found. Most of these had very low spontaneous and induced reversion, if they reverted at all, but two were somewhat better. One had a low rate of spontaneous mutation and a detectably higher rate of mutation with either MNNG or hydrazine. The induced rates are low but probably within the usable range. The other had a very high spontaneous rate but a still much higher induced rate with both MNNG and hydrazine. Both these pro^- mutants proved to be very closely linked to the *thy* locus. Thus, mutagenized DNA from a thy^+ pro^- strain could be used to transform a thy^- pro^- strain, and selection for thymine-independent transformants might be used to select transformants with a high frequency of *pro* loci from the mutagenized DNA. The required thy^+ pro^- strains have been prepared.

The high rate of reversion in one of the pro^- strains is probably not the result of the strain being a "mutator." The rate of reversion, spontaneous and induced, of thy^- to thy^+ is about the same in the strains with low and high reversion rates to proline independence. The induced rate of reversion to thymine independence is too low to use in many mutation studies but is sufficiently high to be detected.

It is uncertain, as yet, whether the high rate of reversion to proline independence in one of the strains is a consequence of true back-mutation at the *pro* locus or of suppressor mutations elsewhere in the genome. If it is the latter, the linkage to the *thy* locus will not be helpful for selection at transformation for anything except the true reversions. It may then be possible to find linkage between one or more of the suppressor loci and drug-resistance or auxotrophic mutations elsewhere in the genome.

FURTHER STUDIES OF CELL-CYCLE PARAMETERS AND CELL SIZE DURING GROWTH OF CHO CELLS WITH AND WITHOUT DIBUTYRYL CYCLIC AMP

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

A first report[1] on these studies was made last year. The conclusion has been confirmed that dibutyryl cyclic AMP [$(But)_2$ cAMP] has at most only small effects on cell-cycle parameters and cell size during the growth of cultures with daily renewal of medium. Indeed, several of the small differences mentioned in last year's report were not found in repetition.

Work has also been done with cultures which were kept without medium renewal from initiation until well into the stationary phase. The cultures with $(But)_2$ cAMP reached a somewhat lower total cell population than the cultures without, but the difference was less than a factor of 2. Stationary-phase cultures with $(But)_2$ cAMP had somewhat fewer cells still in the cell cycle, as judged by labeling with tritiated thymidine and by the number of mitotic cells, but even very late in culture history some cells remained in the cell cycle. The contact inhibition characteristic of cells grown with $(But)_2$ cAMP may decrease the number of cycling cells, but it does not totally eliminate them. The overall conclusion from all this work is that $(But)_2$ cAMP, when present continuously, has very small

effects on cell-cycle parameters and cell size compared to other factors that vary during normal culture growth.

A comparison between these studies on CHO cells and our earlier studies on another Chinese hamster cell line, V79, shows that on the whole cell-cycle parameters and cell size are less altered during culture growth of CHO than of V79 cells, but there may be other respects in which the CHO cells are more affected.

1. R. F. Kimball, S. W. Perdue, and A. W. Hsie, *Biol. Div. Annu. Progr. Rep. June 30, 1973*, ORNL-4915, p. 64.

X-RAY-INDUCED TRANSLOCATIONS IN MOUSE SPERMATOGONIA

J. G. Brewen and R. J. Preston

We have extensive data on the induction of reciprocal translocations in the spermatogonial stem cells of adult mice by acute x-ray exposures.[1] We have continued our studies to compare the yields of x-ray-induced reciprocal translocations in the spermatogonial stem cells of 13.5-day embryos (*in utero*) and newborn mice with those induced in the adult (about eight weeks old). It is clear that translocations are induced *in utero*, but that the yield is lower than in adults (Table 2). The newborn animals are about as sensitive to translocation induction as adults (Table 2). These data are incomplete but already indicate that, for the mouse at least, there is little difference in translocation yield at different stages of development.

The x-ray dose-response curve for reciprocal translocations following acute exposures is "hump-shaped" – it is quadratic up to 600 R, and then the yield decreases with increasing dose.[1] We have interpreted this shape as being due to differential sensitivities to cell killing and chromosome-aberration production in stem cells in different stages of the cell cycle. This would mean that if a split dose of radiation is given, with the time between the two doses being long enough for the cells to reassort back to a normal cell cycle with cells present at all stages at the time of the second dose, then the yield of translocations will be greater than for a single dose above 600 R. The yield should be equal to the additive yields of the two doses given separately. To test this we have given repeated 400-R doses with eight weeks between the doses, up to a total of 2800 R (Table 3). The yield of translocations is, as predicted, equal to the additive yields from the doses given separately, so that at 400 R the yield is 13.8% and, for example, at 2000 R (5 × 400 R) it is 60.2%.

We have also conducted split-dose experiments with short intervals between the two doses, with a view to determining whether the yields of translocations induced in spermatogonial stem cells, and observed in primary spermatocytes, are reduced with increasing fractionation intervals up to 3 or 4 hr, as are the yields of exchanges induced in somatic cells. A single dose of 500 R gave a yield of 17.2%. With intervals between two 250-R doses of 30, 90, or 150 min, the yields decreased to 16.2%, 14.0%, and 12.5%, respectively, exactly as predicted (Table 4).

A further split-dose experiment with two 500-R fractions separated by 3, 6, 18, 48, and 72 hr was also carried out, in order to determine what happened to the translocation yield when the total dose (1000 R) was on the decreasing part of the dose-response curve. The yield of translocations following a 1000-R single dose was 5.2%. For a fractionation interval of 3 hr the yield increased to 15.0%, as expected from the repeated 400-R dose experiment described above. The yield reached a maximum of about 38% for an interval of 18 hr, when it was equal to the additive yields of two 500-R doses given separately. The yield decreased again for the 48-hr and 72-hr intervals. This pattern of

Table 2. Yields of reciprocal translocations following x irradiation of adult, *in utero*, or newborn mice

Intrauterine (13.5 days post plug) irradiation			Newborn irradiation			Adult irradiation		
Dose (R)	No. of cells scored	% Translocations	Dose (R)	No. of cells scored	% Translocations	Dose (R)	No. of cells scored	% Translocations
0	600	0.0	0	600	0.0	0	600	0.0
50	1250	0.24 ± 0.14	50	600	0.67 ± 0.33	50	600	0.7 ± 0.3
100	1500	1.40 ± 0.31	100	850	2.47 ± 0.54	100	600	1.5 ± 0.5
150	1000	0.90 ± 0.30	150	800	2.38 ± 0.55	150	600	2.5 ± 0.6
200	1250	2.8 ± 0.47	200	1225	3.02 ± 0.48	200	600	4.3 ± 0.8
300	775	3.35 ± 0.66	300	875	6.40 ± 0.86	300	600	7.8 ± 1.1

Table 3. Yields of reciprocal translocations induced by repeated 400-R doses of x rays

Total dose (R)	No. of cells scored	% Translocations
0	1000	0
400	2150	13.78 ± 0.80
800	1450	19.72 ± 1.17
1200	900	31.11 ± 1.86
1600	600	47.83 ± 2.82
2000	600	60.17 ± 3.17
2400	600	64.5 ± 3.28
2800	600	80.67 ± 3.67

Table 4. Yields of reciprocal translocations induced by single or split doses of x rays

Dose (R)	Time interval between doses	No. of cells scored	% Translocations
0		1000	0
250		500	6.2 ± 1.1
500		600	17.2 ± 1.7
500	30 min	500	16.2 ± 1.8
500	90 min	600	14.0 ± 1.5
500	150 min	600	12.5 ± 1.4

response could be explained in terms of the killing of cells in the sensitive stage of the cell cycle by the first dose, resulting in a partially synchronous population being irradiated by the second dose. The stage of the cycle in which the population exposed to the second dose will be will vary with time between the two exposures, assuming that over the time period used in these experiments the cells are still partially synchronous. With a time interval of, say, 96 hr, we would expect the yield to return to that for 1000 R.

These split-dose experiments suggest that the explanation offered for the response of cells to high doses of x rays (over 600 R) is valid, and that we need not invoke radioresistant and radiosensitive populations, but rather a differential sensitivity of cells in different stages of the cell cycle.

With the 500 R + 500 R split-dose experiment, by varying the intervals between the doses, we also have a way of varying the yield of translocations by a factor of about 8, without changing the total radiation dose. We intend to use this fact to study the recovery of translocations in the F_1 generation following a regime which will give us a large difference in translocation yield in the spermatocytes. In this way we should be able to determine whether the translocation yield itself or the dose received causes the proportion of translocations recovered in the F_1 to vary for different doses and translocation yields, as described in an accompanying article.

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USE OF INTERACTION EXPERIMENTS TO STUDY THE MECHANISMS OF CHROMOSOME-ABERRATION FORMATION

R. J. Preston and J. G. Brewen

We have made a preliminary investigation of the effects of combined treatments with x rays and uv, *N*-acetoxyacetylaminofluorine (AAF), or methyl methanesulfonate (MMS) on the production of chromosome aberrations in human lymphocytes.

All experiments were performed on purified lymphocyte suspensions obtained by separation from whole blood using plasmagel. All irradiations or chemical treatments were carried out on lymphocyte suspensions in Puck's saline F, which is a high-phosphate saline and has proved to be necessary for recovering mitotic cells following uv exposure.

The lymphocytes of the donor used in all these experiments were somewhat more sensitive to the induction of chromosome aberrations by x rays than has been found in previous experiments with other donors, and therefore an x-ray dose-response curve was obtained so that the comparison between the effects of x rays alone and of the combined treatments could be more meaningfully made (Fig. 6, Table 5).

Ultraviolet, AAF, and MMS alone induce very few dicentrics and rings in unstimulated human lymphocytes, which is not surprising from a knowledge of the mode of action and nature of the repair of damage caused by these agents.[1]

However, when a dose of 25 or 50 ergs of uv is immediately followed by doses of x rays from 50 to 200 rads, the yields of dicentrics and rings and of acentric fragments are considerably increased (Table 5, Fig. 6). For example, 150 rads of x rays induces 0.35 dicentrics plus centric rings per cell, and 25 ergs of uv gives 0.03 per cell, whereas the combined treatment gives 0.69, an increase of about a factor of 2.

In similar experiments in which x-ray exposures were followed by 2-hr treatments with 7×10^{-6} *M* AAF, 1.2×10^{-5} *M* MMS, or 6×10^{-5} *M* MMS, there was no increase in yield over the additive yields for the two treatments given alone (Fig. 6, Table 5). It was suspected that AAF might behave similarly to uv, since it has been shown[1] that the repair of the damage

Table 5. Yields of dicentrics and rings following treatment of human lymphocytes with x rays alone or with x rays plus uv, AAF, or MMS

Treatment	Dicentrics + rings per cell after an x-ray dose of				
		50 rads	100 rads	150 rads	200 rads
X rays	0.0	0.08 ± 0.02	0.24 ± 0.03	0.35 ± 0.03	0.71 ± 0.05
25 ergs uv + x rays	0.03 ± 0.01	0.12 ± 0.02	0.42 ± 0.04	0.69 ± 0.05	1.07 ± 0.06
50 ergs uv + x rays	0.10 ± 0.02			0.72 ± 0.05	
AAF + x rays	0.013 ± 0.007	0.08 ± 0.02	0.14 ± 0.02	0.37 ± 0.03	0.51 ± 0.04
MMS (1.2×10^{-5} *M*) + x rays	0.007	0.10 ± 0.02	0.21 ± 0.03	0.58 ± 0.04	0.64 ± 0.05
MMS (6×10^{-5} *M*) + x rays	0.0	0.09 ± 0.02	0.23 ± 0.03		0.51 ± 0.04

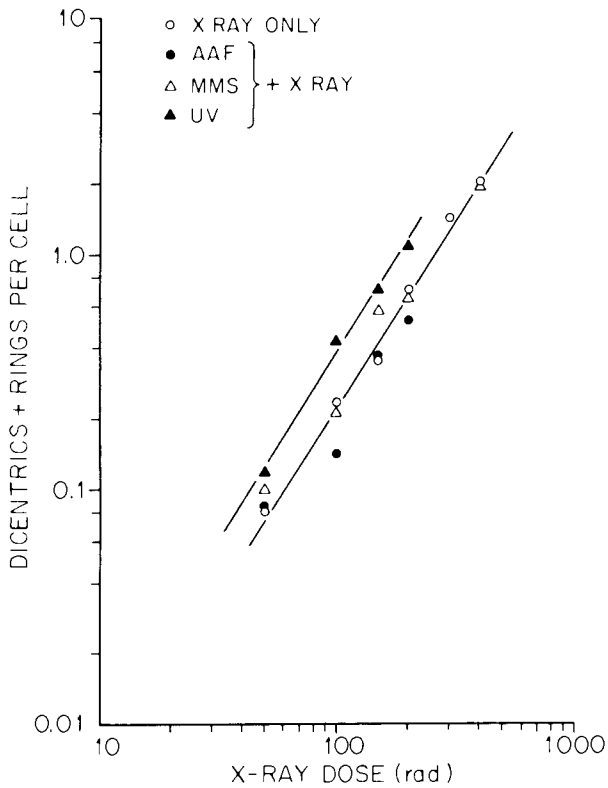

Fig. 6. Dose-response curves for human lymphocytes treated with x rays alone or in combination with uv, AAF, or MMS.

induced by the two is similar. On the other hand, MMS shows a repair pattern similar to that following x irradiation.

We intend to study the effects of combined treatments with ICR-170 and x rays, since ICR-170 also behaves similarly to uv as regards the repair of DNA damage.

One explanation of the interaction between uv and x rays is that the uv-induced single-strand breaks produced during excision-repair can interact with x-ray-induced breaks, which remain unrepaired for at least 1 hr after irradiation. The low yield following uv alone would be due to the fact that the single-strand breaks will only be present at the time of repair, and the probability of two existing at the same time within the "rejoining volume" for aberration formation is low. It must also be assumed, since we observed chromosome-type aberrations following uv exposure, that the single-strand break is converted into a double-strand break, possibly through a single-strand nuclease acting on the DNA strand opposite the excised dimer.[2] The reason why AAF, in particular, does not show an interaction with x rays is not clear, since its repair characteristics are similar to those for uv. It is possible, however, that the breaks which interact are all x-ray induced, and that uv alters the repair of these to enhance misrepair over restitution – an effect which might not be mediated by AAF. One way to consider which of these mechanisms might account for the interaction is to expose the cells to uv and x rays, allow them to incorporate BrdUrd into regions repairing uv damage, and then cleave the repaired region with 313-nm light, which will make all uv breaks available for interaction at the same time. If the interacting breaks are both uv and x-ray induced, then the yield of chromosome aberrations will be higher than for uv plus x rays in the absence of BrdUrd; but if the effect of uv is on the repair mechanism, then there will be no difference in yield for the two treatment regions.

It appears that these experiments together with others that are planned will elucidate the mechanisms of induction of chromosome aberrations by radiation and chemicals.

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MUTATION STUDIES IN CHINESE HAMSTER AND HUMAN-HAMSTER HYBRID CELLS

E. A. Hiss, R. J. Preston, Helen S. Payne, and W. F. Garrett*

The purpose of our preliminary experiments is to study comparative mutagenesis using three types of somatic cells: transformed Chinese hamster ovary (CHO) cells, human fibroblasts, and hybrids of these two lines. (The hybrid is a Sendai-virus-mediated fusion of a nonreverting hamster mutant and a normal human cell.) The goal is to evaluate the feasibility of using the human-hamster hydrid to measure mutation rates of human genes.

Human cells are not usually suitable for direct mutagenesis experiments because normal cells are diploid for the entire genome, with the exception of the sex chromosome in males, and because normal cells have a finite lifetime in culture. On the other hand, CHO cells are partially haploid for a number of loci in which mutations can be induced, and in addition, the cell line is transformed and can be perpetually maintained in tissue culture. In a human-hamster hybrid, the human chromosomes are preferentially lost. Therefore, we can select for complementation of the hamster mutation by a single human chromosome which will confer a normal phenotype on the hybrid cell.

We have chosen 8-azaguanine resistance (HGPRTase$^-$) as the system most suitable for our initial studies. This system has an advantage over others for several reasons. First, the gene for hypoxanthine-guanine phosphoribosyl transferase is hemizygous in all three lines. Secondly, forward and reverse mutations can be directly selected by altering the growth medium. Thirdly, HGPRTase can be rapidly assayed and quantitated to demonstrate that the growth of presumptive mutants is indeed caused by a mutation and is not simply a phenocopy. Using this system we can correlate forward rates in all three lines.

If the induced and spontaneous rates of the hybrid are similar to those of the human line and different from the hamster line, we will be encouraged to pursue the possibility that this system offers potential for measuring mutagenesis of human genes. Conversely, if the rate is similar to that of the hamster line and different from that of the human line, we can presume that the system is not suitable for our purposes but, in addition, that the DNA synthetic and repair enzymes of the host cell govern mutation rates and the rate is not an inherent property of each gene per se.

We have generated a series of azaguanine-resistant CHO lines by x irradiation. One of these lines was extensively characterized. This mutant was hybridized to HSBP human cells, and the hybrid was selected in THAG medium containing 10^{-7} *M* ouabain. The hybrid has been carried for approximately four months in culture and has lost most of the human chromosomes. Hopefully, the loss will continue and stabilize when the cell contains the 20 CHO chromosomes and only the X chromosome from the human parent. We have calculated a spontaneous forward-mutation rate for the CHO parent of between 1 and 2×10^{-6}. We have also attempted to determine a spontaneous rate for the human cell, and as yet no mutants have been recovered in well over 10^7 cells plated. Therefore, it seems likely that the rate of forward mutation in human cells will be significantly lower than in the hamster. X-ray-induced mutation rates are being determined for the CHO cell and will be for the human cell as well. Rates for the hybrid will then be compared with those of the two parent lines.

At the same time we have been studying reversion of EMS-induced glycine-requiring hamster cell lines by x rays. Mutants were obtained using the BrdUrd-light method described by Kao and Puck. We have been classifying these mutants by biochemical analysis and complementation tests using hybridization techniques. Using one line we have shown that the dose-response curve for reversion to glycine prototrophy is linear, and that the induction rate is about 1.2×10^{-7} per cell per R, compared to a spontaneous frequency of about 1×10^{-8}. We have obtained human-hamster hybrids, and using the method described above, whereby a line contains ideally one human chromosome, namely the one carrying the locus under study, we intend to measure mutation rates for human genes.

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X-RAY-INDUCED TRANSLOCATIONS: COMPARISON BETWEEN CYTOLOGICALLY OBSERVED AND GENETICALLY RECOVERED FREQUENCIES

J. G. Brewen, R. J. Preston, and W. M. Generoso

If the chromosomes involved in a translocation between nonhomologous chromosomes segregate randomly at anaphase I, four classes of gametes are produced. One-quarter of the gametes will carry normal chromosomes, one-half of the gametes will be duplication deficient for the translocated segments, and one-quarter will carry the translocation in a balanced form. Thus the frequency of balanced translocation heterozygotes observed in the F_1 progeny of irradiated males

is expected to be approximately one-quarter of the frequency of translocations observed at meiosis in the primary spermatocytes of the irradiated males.

In the only study done to this time, Ford et al.[1] reported that the frequency of recovery of balanced translocations in F_1 males of irradiated fathers was one-half of the expected frequency, as determined by cytological analysis of the primary spermatocytes of identically irradiated males. They interpreted this result as showing an effect on the diploid spermatogonial stem cells that is expressed in the haploid spermatids. Since Ford et al. used only one radiation regime, it was impossible to determine whether the apparent effect on translocation recovery was dose dependent.

Recently, Generoso[2] has analyzed F_1 males derived from irradiated spermatogonial stem cells for semisterility and sterility. Four acute x-ray doses were employed – 150, 300, 600, and 1200 R. Each semisterile and sterile male was cytologically analyzed for the presence of a balanced reciprocal translocation. It was determined that all but two of the semisteriles were heterozygous for a translocation and four of the steriles[3] carried translocations. The frequencies of recovered translocation heterozygotes at each dose are presented in Table 6.

At the time of irradiation, ten males at each dose were set aside and subsequently were cytologically analyzed for reciprocal translocation frequencies at diplotene–metaphase I. The results of this analysis are presented in Table 6. The ratio of translocation recovery to cytologically determined translocation frequency in the primary spermatocytes was determined, and these ratios are summarized in Table 6.

The data indicate that there is a higher probability of recovering a balanced translocation in the F_1 progeny of irradiated fathers at the lowest dose than at 300 R and above. We are currently obtaining more data for doses of 150 R and lower, to determine whether this suggestion can be substantiated. If it proves to be so, then it would suggest that higher doses of x rays induce higher frequencies of events in the diploid spermatogonial stem cells that are lethal in the haploid postmeiotic cells. It would also mean that the probability of recovering balanced translocations following low doses of ionizing radiation is what would be predicted from studies on translocation stocks rather than less than expected, as indicated by the data of Ford et al.[1]

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COMPLETE EXCISION OF ULTRAVIOLET-INDUCED PYRIMIDINE DIMERS FROM THE DNA OF HUMAN CELLS

James D. Regan and W. L. Carrier

Human cells are capable of excising ultraviolet-induced pyrimidine dimers from their DNA. However, our previous experiments[1] indicated that this capability was limited. In the dose range of 50–150 ergs/mm^2, only about 50% of the dimers were excised. Further studies by us and others confirmed this finding.[2–5]

Since our earlier experiments, refinements in the technique of detecting dimers in small amounts[6] plus the greatly increased efficiency of liquid scintillation counters has enabled us to assay dimer excision at doses as low as 25 ergs/mm^2. Under these conditions, human cells excise essentially all (93%) of the uv-induced pyrimidine dimers from their DNA. The human excision system appears to operate most efficiently at doses of 0–30 ergs/mm^2. Previous studies at higher

Table 6. Frequencies of cytologically scored and genetically recovered reciprocal translocations following irradiation of spermatogonial stem cells

Dose (R)	% Translocations scored[a]	No. F_1 males tested	% Translocations heterozygotes, minimum[b]	Ratio recovered/scored
0	0.0	2633	0.0	
150	2.5 (2.5)	993	0.60	0.25
300	7.8 (5.7)	1018	0.88	0.12
600	17.2 (16.2)	1075	1.90	0.11
1200	4.5 (3.8)	1038	0.58	0.13

[a]Numbers in parentheses are percentages of cells with a single translocation.
[b]Minimum frequency of translocation heterozygotes are the cytologically confirmed semisteriles.

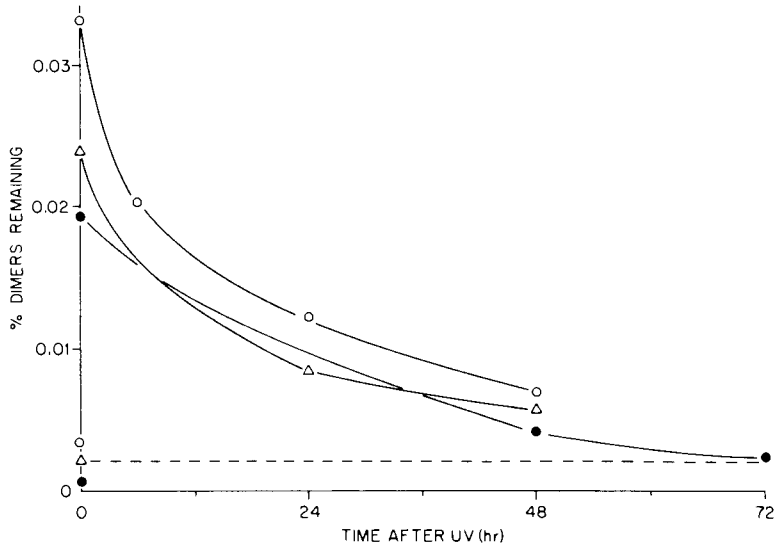

Fig. 7. Excision of uv-induced pyrimidine dimers from the DNA of human diploid fibroblasts. Dotted line indicates level of radioactivity in the dimer region of the chromatogram for unirradiated cells. HSBP (○,●) and HSWP (△) cells were irradiated with 50 ergs/mm^2 at 254 nm.

doses extrapolate to nearly 100% excision at lower doses.[3-5] Probably at higher doses the same absolute number of dimers is excised no matter what the dose. This would explain why some workers[7] failed to observe any appreciable dimer excision in cells receiving 1000–3000 ergs/mm^2.

Figure 7 shows the result of dimer excision assays in two diploid fibroblast cell lines, HSBP and HSWP, developed from human foreskin. The dimers were excised continually up to 72 hr. The level of dimers remaining in terms of radioactivity in the irradiated cells at 72 hr was equal to the level of radioactivity in the dimer region of the unirradiated cells. We interpret this result as essentially complete dimer excision. While some excision occurred over the entire 72-hr period, excision proceeded most rapidly in the first 24 hr.

Figure 8 illustrates the rate of dimer excision over the period of 0–48 hr at doses of 25, 50, and 100 ergs/mm^2. Only at the lowest dose does dimer excision approach 100% at 48 hr.

Our calculations indicate that during the first 24 hr after uv irradiation about 0.030% of the pyrimidine dimers are excised. In the first 6 hr after irradiation 0.015% of the dimers are excised. Thus, during this early period of pyrimidine dimer excision in human cells, the initial number of dimers excised is the dimer equivalent of 5 ergs/mm^2/hr; that is 0.015% dimers can be induced by 30 ergs/mm^2 of 254-nm uv.

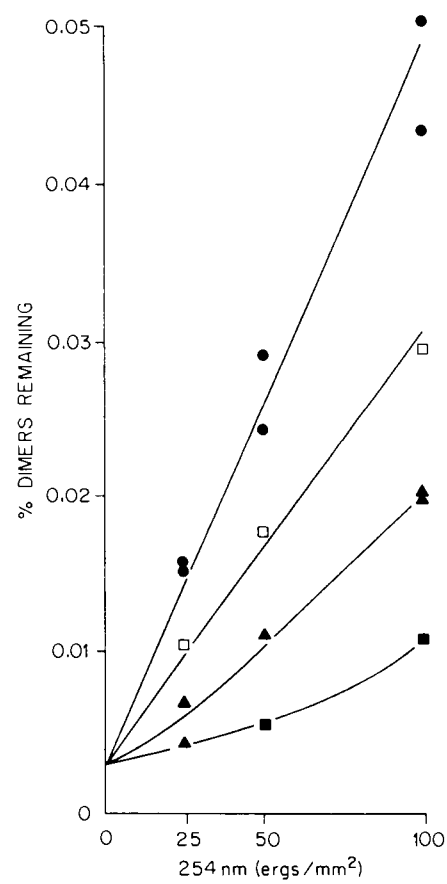

Fig. 8. Rate of dimer excision at doses of 25, 50, and 100 ergs/mm^2 of 254-nm uv in human diploid fibroblasts. Time after uv irradiation: 0 (●), 6 (□), 24 (▲), or 48 (■) hr.

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EXCISION-REPAIR IN HUMAN CHROMOSOMAL BREAKAGE SYNDROMES

James D. Regan, W. L. Carrier, and James German*

Bloom's syndrome, Fancoli's anemia, and the Louis-Barr syndrome (ataxia telangectasia) exhibit spontaneous chromosomal aberrations *in vivo*, although the predominant types of aberration differ considerably among the three disease entities. Patients with these syndromes also exhibit a certain ultraviolet sensitivity, although none of the conditions results in multiple actinic skin carcinomas as seen in xeroderma pigmentosum patients.

Considering the uv sensitivity and the spontaneous chromosomal aberrations seen in these syndromes, it is reasonable to suspect that excision-repair may be defective in Bloom's syndrome, Fanconi's anemia, and Louis-Barr syndrome. One report[1] related defective excision of uv-induced pyrimidine dimers in fibroblasts from patients with Fanconi's anemia and suggested a repair-exonuclease deficiency in this disease.

With radiochromatographic methods previously described,[2] we have examined the excision of uv-induced pyrimidine dimers in skin fibroblasts from patients with Bloom's syndrome and Fanconi's anemia. Fibroblasts from patients with these diseases exhibit 50–60% dimer excision at doses of 100–150 ergs/mm^2, and these cells also exhibit 100% dimer excision at doses of 50 ergs/mm^2 or less. Thus, Bloom's and Fanconi's cells are not defective in excision-repair, and they excise dimers completely at low doses, as do normal human cells (see the preceding article).

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DNA REPAIR IN HUMAN PROGEROID CELLS*

James D. Regan and R. B. Setlow

Progeria is a human genetic disease characterized by accelerated aging. Epstein et al.[1] used sedimentation in alkaline sucrose to study the rejoining of single-strand breaks in the DNA of gamma-irradiated cells from a progeroid patient (K.H.). Their results indicated that the cells "failed to show evidence of normal DNA strand rejoining *in vitro* after exposure to cobalt-60 gamma irradiation," suggesting to them "that an enzyme involved in DNA repair may be absent or greatly reduced in efficiency in cells from this patient with the progeria syndrome."

We have investigated the rejoining of DNA single-strand breaks in four human diploid fibroblast cell strains developed from patients with progeroid syndromes, including K.H. cells. The cells were generously supplied by Dr. R. J. Hay of Wright State University, Dayton, Ohio. Although under certain experimental conditions we can repeat the observations by Epstein et al.,[1] our results suggest that there is no inherent defect in DNA repair in progeroid cells.

Cells were treated in one of three ways: (1) as monolayers on 50-mm plastic petri dishes incubated at 37°C; (2) removed from dishes with 0.05% trypsin and irradiated and incubated at 37°C in suspension in glass centrifuge tubes, without shaking, as described by Epstein et al.;[1] and (3) removed with trypsin and irradiated and incubated in suspension in 25-ml scintillation vials, with shaking on a rotary shake table (100 excursions per min) at 37°C so as to prevent attachment of cells to the surface.

Both K.H. and normal cells in monolayer cultures and while held in suspension are able to repair gamma-ray-induced single-strand DNA breaks under the experimental conditions reported here. On the other hand, cells remaining in suspension in nonagitated cultures (2) do not repair, but degrade their DNA. The fraction of cells that do not attach is ~0.2 for K.H. and 0.1 for normal cells. This degradation by a small fraction of the initial population could account for the small changes observed between 30 and 60 min in the irradiated, shaken cultures (3).

We suggest that progeroid cells do not have an inherent reduced ability to rejoin radiation-induced DNA single-strand breaks, but that they may have a reduced and variable ability to recover from trypsin treatment. The cells remaining in suspension in nonagitated cultures after irradiation may represent either cells with a reduced ability to recover from trypsin treatment or cells that have sustained greater trypsin

damage than others. The cloning efficiencies and x-ray survivals of HeLa and Chinese hamster cells are greatly affected by the time allowed for recovery from trypsin damage. Conceivably, progeroid cells are sensitive to this damage and slow to recover. This sensitivity could be reflected in several ways, one of which could be slow DNA repair.

*Research sponsored jointly by the Carcinogenesis Program of the National Cancer Institute and the U.S. Atomic Energy Commission.

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DNA REPAIR IN *POTOROUS TRIDACTYLUS*

S. N. Buhl,* R. B. Setlow, and James D. Regan

The DNA synthesized shortly after ultraviolet (uv) irradiation of *Potorous tridactylus* (PtK) cells sediments more slowly in alkali than that made by nonirradiated cells. The size of the single-strand segments is approximately equal to the average distance between one or two cyclobutyl pyrimidine dimers in the parental DNA. These data support the notion that dimers are the photoproducts which interrupt normal DNA replication. Upon incubation of irradiated cells, the small segments are enlarged to form high-molecular-weight DNA as in nonirradiated cells.

DNA synthesized at long times (~24 hr) after irradiation is made in segments approximately equal to those synthesized by nonirradiated cells, although only 10–15% of the dimers have been removed by excision-repair. These data imply that dimers are not the lesions which initially interrupt normal DNA replication in irradiated cells. In an attempt to resolve these conflicting interpretations, PtK cells were exposed to photoreactivating light after irradiation and before pulse-labeling, since photoreactivation-repair is specific for only one type of uv lesion. After 1 hr of exposure, ~35% of the pyrimidine dimers have been monomerized, and the reduction in the percentage of dimers correlates with an increased size for the DNA synthesized by irradiated cells. Therefore, we conclude that the dimers are the lesions which initially interrupt DNA replication in irradiated PtK cells.

The monomerization of pyrimidine dimers correlates with a disappearance of repair-endonuclease-sensitive sites, as measured *in vivo* immediately after 1 hr of photoreactivation, indicating that some of the sites sensitive to the repair endonuclease (from *Micrococcus luteus*) are pyrimidine dimers. However, at 24 hr after irradiation and after 1 hr of photoreactivation, there are no endonuclease-sensitive sites, even though ~50% of the pyrimidine dimers remain in the DNA. These data indicate that not all pyrimidine dimers are accessible to the repair endonuclease. The observation that at long times after irradiation DNA is made in segments equal to those synthesized by nonirradiated cells, although only a small percentage of the dimers have been removed, suggests that an additional repair system alters dimers so that they no longer interrupt DNA replication.

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CAFFEINE AFFECTS POSTREPLICATION REPAIR IN XERODERMA PIGMENTOSUM CELLS BUT NOT IN NORMAL HUMAN CELLS

S. N. Buhl* and James D. Regan

If caffeine binds to denatured regions of DNA, it would be expected to do so in human cells at such sites as replication points, pyrimidine dimers, and single-stranded regions of DNA such as would be left around pyrimidine dimers during the next round of DNA replication (Fig. 9). We have observed a reduction in size of newly synthesized DNA of nonirradiated cells treated with caffeine.[1] This finding supports the notion that caffeine binds to the denatured regions of the replication fork.

We found an inhibition of DNA chain elongation and joining of the short segments synthesized after irradiation of xeroderma pigmentosum (XP) cells but not normal cells, and an apparent suppression in the repair system which results in synthesis of DNA in segments of normal size at long times after irradiation in cells which do not remove pyrimidine dimers, suggesting that caffeine binds to the denatured regions of DNA containing dimers.

Our results further suggest that caffeine binds more strongly to the denatured region in DNA caused by the dimers than to the denatured regions at the replication points, because DNA chain elongation is not inhibited in nonirradiated cells but is in uv-irradiated XP cells. However, the inhibition is not complete in the XP cells.

If caffeine binds to the dimer and this complex (dimer-caffeine) inhibits DNA replication in XP cells but not in normal human cells, it can be construed that the dimer-caffeine complex must be removed before DNA replication can proceed past that point. The repair endonuclease requires double-stranded DNA as template;[2] therefore, gaps are postulated opposite the dimer (b' in Fig. 9), and DNA chain elongation and

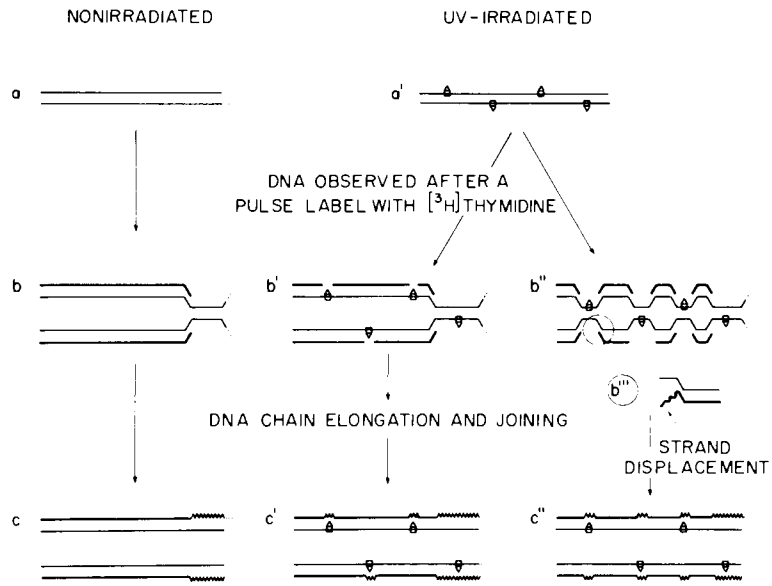

Fig. 9. DNA replication in nonirradiated and uv-irradiated human cells. Nonirradiated parental DNA (–), pyrimidine dimers (⌒), [^{3}H] thymidine-containing newly synthesized DNA (▬), newly synthesized DNA (∽). DNA synthesis in nonirradiated cells is continuous (*b*), but in uv-irradiated cells the newly synthesized DNA contains either one gap for each dimer in the strand directly opposite the dimer (*b'*) or two gaps for each dimer, with gaps in both strands of the new DNA (*b''*). The insert (*b'''*) describes the detail of the interruption in DNA replication and the existence of a strand displacement, which would occur when the parental DNA strands reform the original double helix (B. Strauss, personal communication). Completion of the DNA replication in nonirradiated cells (*c*) and in uv-irradiated cells (*c'*, *c''*) is also shown. Examination of the elongation and joining process indicates that there are two regions of different size completed by insertion of exogenous nucleotides in irradiated cells: large regions, which represent completion of the replication units present in nonirradiated cells (*c*), and small regions, which represent completion of the discontinuities caused by uv-induced pyrimidine dimers (*c'*, *c''*).[9,10]

joining should be inhibited in excision-proficient as well as excision-deficient cells. Our results[1] indicate that DNA chain elongation is not inhibited by caffeine in normal human (excision-proficient) cells, so the gaps must not be formed opposite the pyrimidine dimer during DNA replication after uv irradiation.

We previously presented a model of postreplication repair.[3] We now suggest the following modifications to this model (*b''* in Fig. 9). DNA replication proceeds until it reaches a dimer, where it stops in both strands or perhaps proceeds for a small distance in the strand not containing the dimer. A strand displacement can then result when the parental DNA anneals to form the original helix in the dimer region (B. Strauss, personal communication).

Before DNA replication can proceed, one of several things must happen. The dimer must be excised (which occurs to a great extent only in normal human cells); the dimer must be altered, as indicated by the recovery of the ability to synthesize DNA of normal size at long times after irradiation in cells which do not excise dimers; or a special polymerase must synthesize past the dimer before it is altered or excised. Caffeine does not prevent excision-repair in human cells,[4,5] but it does cause a suppression of the repair system, which results in synthesis of DNA in segments of normal size at long times after irradiation in XP cells. Neither the existence of an additional polymerase which specifically synthesizes past dimers nor its sensitivity to caffeine has been established; however, because Chinese hamster B14 cells, which have a greatly reduced excision ability[6] and lack recovery of the ability to synthesize DNA in segments of normal size at long times after irradiation, are able to elongate and join the short DNA segments synthesized after irradiation,[7] a special polymerase must be invoked to synthesize past the dimer. Completion of DNA replication would result in regions of two distinct sizes (*c'* and *c''* in Fig. 9).

The modifications in Fig. 9 indicate that two discontinuities should occur for each dimer. However, as previously indicated, the size of the newly synthesized DNA segments after irradiation of human and mouse L5178Y cells approximately equals the distance between dimers. The discrepancy between the observed data and the suggested model cannot be explained with existing data, but several points should be considered.

The size of the short, newly synthesized DNA in irradiated cells was measured with a relatively long pulse-label (usually 30 min). During this time many of the dimers could be altered or gaps could be filled (repaired), and normal DNA replication would proceed past them with no discontinuity. Hence the small segments would not represent the true result. This suggestion is supported by the observation of shoulders or skewing of the DNA profiles toward high-molecular-weight DNA in irradiated cells. Shorter pulse times may result in smaller DNA and should answer the question. Additional evidence that smaller DNA could result is that caffeine causes a reduction in the size of the already small DNA segments synthesized after irradiation. The smaller segments synthesized after uv and caffeine treatment were approximately 0.66 the size of those synthesized after uv irradiation alone (Fig. 9, ref. 8). (The ratio, determined by comparing the number-average molecular weights after corrections were made for pulse-labeling of nonirradiated cells, represents a change from $\sim 4.4 \times 10^6$ daltons to $\sim 3.0 \times 10^6$ daltons.) This indicates that DNA is made in segments smaller than the distance between dimers. However, at this time we have not been able to demonstrate whether the reduction results from a general effect of caffeine which causes DNA to be made in smaller segments in nonirradiated cells or a specific effect which results from binding to the region of uv-induced dimers, causing an interruption of DNA replication.

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HUMAN GENETIC SCREENING: HIGH-RESOLUTION CHROMATOGRAPHY AND IDENTIFICATION OF METABOLITES

J. L. Epler, C. D. Stringer, William Winton, J. X. Khym, W. E. Barnett, and James D. Regan

The research of the Biochemical Analysis of Human Variants group consists of two general efforts: (1) screening and chromatographic analysis of selected high-genetic-risk patients, and (2) isolation and identification of metabolites presumably involved in genetic lesions. After two and one-half years of operation, approximately 500 urine samples have been investigated with the uv analyzer. A number of these have yielded identification data, and possible genetic implications have been explored. A few samples have pointed to a metabolic lesion under genetic control. As an example, through a collaborative effort with Dr. L. J. Elsas of Emory University, the chromatographic approach has uncovered what appears to be a new variant of tyrosinosis. The infant in this case shows an elevated serum tyrosine, elevated urinary tyrosine, 4-hydroxyphenyllactic acid, 4-hydroxyphenylacetic acid, and, presumably, the dihydroxy (DOPA) derivatives shunted from blocked tyrosine metabolism. A concurrent urinary proline accumulation is also manifest here and may point to other metabolic involvements.

As another example, again in a collaborative effort with Dr. Elsas, we have developed and/or modified gas-chromatographic procedures to allow quantitation of branched-chain α-keto acids in serum and urine in connection with maple syrup urine disease (see the following article). In another study with Drs. George Oden (Greene Valley) and J. Mrochek (Chemical Technology), aromatic amino acid metabolism was again implicated. In a mentally retarded 20-year-old male, a number of previously unidentified urinary constituents were observed: for example, 3-hydroxy-4-glucuronosido-phenylalanine and 5-glucuronoside-6-hydroxyindole.

Obviously, many of these studies have utilized combinations of liquid chromatography and gas chromatography. The utility of the uv analyzer as a genetic screening tool is now well established. It remains to investigate specific genetic lesions in depth.

GAS CHROMATOGRAPHY OF THE α-KETO ACIDS FROM SERUM AND URINE

C. D. Stringer and J. L. Epler

In maple syrup urine disease, the enzyme system(s) involved in the decarboxylation of the keto acids of the branched-chain amino acids are defective. Thus, high serum and urinary levels of leucine, isoleucine, and valine along with the corresponding keto acids occur. Quantitation of these keto acids has been somewhat difficult.

A method for the extraction, isolation, and quantitation of the α-keto acids from serum and urine by gas chromatography was developed by modifying existing procedures from Scriver's group.

The samples were made strongly alkaline by the addition of NaOH and extracted with ether to remove impurities. Next, $NH_2OH \cdot HCl$ was added, the samples were heated to 60°C, and this temperature was maintained for 30 min. After the reaction with $NH_2OH \cdot HCl$ the samples were cooled and made acid by the addition of HCl, and the oximes of the α-keto acids were extracted into ether.

The ether extract was dried over anhydrous Na_2SO_4, and an aliquot was placed in a derivative vial. The ether was evaporated and the trimethylsilyl derivative was formed. Gas chromatography was performed on the resulting solutions with calculation of the concentration of the α-keto acids present.

Oximes of α-ketovaleric, α-ketoisocaproic, α-ketomethylvaleric, and capric acid were prepared, extracted, and then recrystallized. Utilizing capric acid as an internal standard, the branched-chain keto acids were quantitated. In this specific study the effect of thiamine therapy was under investigation.

DEVELOPMENT OF ANALYTICAL CHROMATOGRAPHIC PROCEDURES FOR THE ANALYSIS OF TISSUE NUCLEOTIDES

J. X. Khym

In two research projects of the Biology Division, there arose the need for a rapid sensitive method for the detection of tissue nucleotides and related compounds. P. A. Swenson and R. L. Schenley (Radiation Biophysics Group) are investigating the cessation of respiration in uv- and x-irradiated *E. coli* cells and would like to compare the nucleotide pools of these cells with those of unirradiated cells. E. Volkin (Nucleic Acid Enzymology Group) is investigating the pool size of nucleic acid precursors in human cell lines in tissue culture. Volkin's main interest in these studies is the effect of the nucleotide pools on the uptake of exogenous RNA and DNA.

Existing methods for the analysis of nucleotides have certain disadvantages; expensive, highly specialized equipment is needed (e.g., pellicular ion-exchange methods), or the analyses are lengthy and usually lack sensitivity. This is particularly so when the whole spectrum of the common nucleotides is considered (i.e., the ribo- or deoxyribonucleoside 5′-mono-, di-, or triphosphates). Thus, due to our experience in the use of automated chromatographic equipment for analyses of low-molecular-weight components in blood, urine, saliva, etc., our group (Biochemical Analysis of Human Variants) was encouraged to develop new methods or to

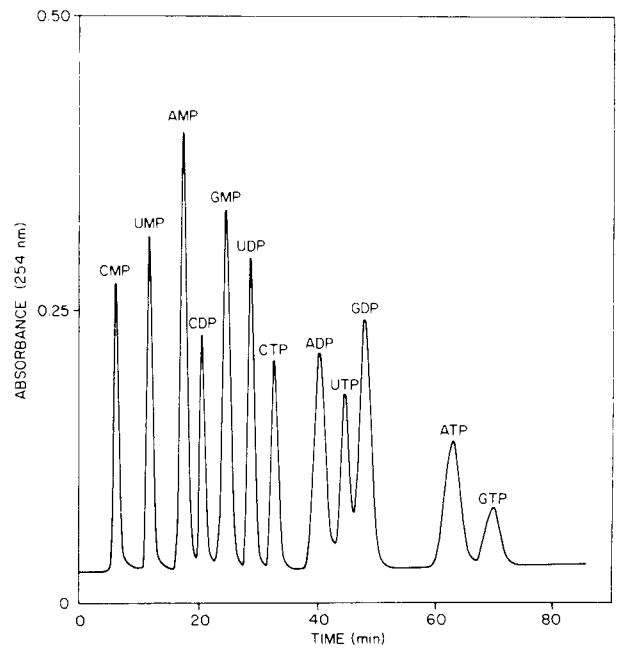

Fig. 10. Separation of the ribonucleoside 5′-mono-, di-, and triphosphates. Conditions: Column, 0.62 × 10 cm of Aminex A-27 (citrate form); elution, convex gradient formed with a closed constant-volume mixing container and an open reservoir vessel (the former contained initially 25 ml of 0.025 *M* sodium citrate solution, and the latter 0.5 *M* citrate solution, both at pH 8.3); flow rate, 0.6 ml/min; column temperature, 70°C; operating pressure, ~40 psi. Sorbed material was ~30 nmoles each of the 5′-mono-, di-, and triphosphates of cytidine, uridine, adenosine, and guanosine contained in about 30 μl of 0.025 *M* sodium citrate solution; the sample was applied to the column by an off-column septum-type injector with a Hamilton syringe without interrupting the flow of the eluent; recovery was quantitative. The column was monitored with a model UA-2 ISCO ultraviolet analyzer (10-mm path-length flow cell of 50 μl capacity). Peaks were quantitated by peak-height and peak-width measurements of the tracings from an external recorder attached to the ISCO.

improve existing chromatographic procedures so that they could be used for the analysis of nucleoside phosphates sensitive to the needs of the two groups mentioned earlier.

The system that evolved employs a conventional ion-exchange resin, Aminex A-27 (a styrene–divinyl benzene anion exchanger, 12–15 μm particle size). When used in the citrate form with alkaline sodium citrate as the eluent, some very sharp separations can be obtained with this exchanger. As seen in Fig. 10, the mono-, di-, and triphosphates of cytidine, uridine, guanosine, and adenosine can be separated on a short column (0.62 × 10 cm) of Aminex A-27 in about 1 hr. Since the column is short, it can be operated at relatively low pressure (about 50 psi). This, in turn, allows one to use readily available inexpensive chromatographic equipment.

Thus far the system is being put to practical use for evaluating means of extracting tissue nucleotides from *E. coli* cells. Some preliminary findings are reported by the Radiation Biophysics Group. Likewise, sensitivity measurements (e.g. the number of human cells per experiment that will yield reasonable absorbancy values for the nucleoside phosphates) are being carried out in Volkin's group.

Various parameters associated with the Aminex A-27 system are now under further investigation to optimize conditions for the routine analysis of samples containing tissue nucleotides as originating from either bacteria or human cell lines.

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MUTAGENESIS TESTING UNIT

J. L. Epler and Alice A. Hardigree

A mutagenesis testing unit has been established within the Biology Division. The unit furnishes the expertise necessary for testing a series of compounds of interest from the point of view of energy production, both from existing and proposed methodology of energy generation. Emphasis has been placed on "lower tier" rapid assays utilizing *Salmonella* and yeast.

The initial work has centered on the *Salmonella* tester series developed by Ames. Reversion of histidine auxotrophs representing both base-substitution and frameshift mutations can be quantitated in response to presumptive mutagens. Tests for deletion and forward mutation are also possible. The preliminary work has established the spectrum of reversion induced by a variety of proven chemical mutagens. Work is progressing on a group of structurally related heterocyclic ring compounds in an attempt to correlate structure with mutagenic activity.

DETECTION OF POTENTIAL MUTAGENS IN NATURALLY OCCURRING FOOD PRODUCTS

Florence I. Barrett,* Alice A. Hardigree,
William Winton, J. L. Epler,
and L. L. Triplett

Naturally occurring metabolites in plant material (e.g., caffeine and theophylline) have been implicated in mutagenic events. We have attempted to couple high-resolution chromatography of plant extracts and rapid, highly sensitive mutation assays in an investigation of the mutagenic properties of plant metabolites.

The *Salmonella* tester strains of Ames have been used to detect potential mutagens, initially in a group of commonly occurring plant phenolic and alkaloid compounds. Ion-exchange chromatography using the uv analyzer developed by Scott and co-workers has been used to fractionate the extracts from rich, natural sources of similar compounds. Fractions are assayed for mutagenic activity (reversion) with tester strains containing a variety of mutant histidine alleles. Thus, mutagenesis along with specificity can be detected.

The study is a prelude to the use of high-resolution chromatography in the biological investigation of a wide array of complex, potentially mutagenic effluents or consumed products in our environment.

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STUDIES ON A TEMPERATURE-SENSITIVE NUTRITIONALLY DEFICIENT VARIANT OF CHO CELLS

C. H. Schröder* and A. W. Hsie

Employing a procedure for the induction of auxotrophic variants from Chinese hamster ovary cells clone K_1 (CHO-K_1) developed by Puck and Kao,[1] which involves incorporation of 5-bromodeoxyuridine into DNA followed by irradiation with visible light, we have isolated over 30 variants that were either temperature-sensitive, auxotrophic, or temperature-sensitive auxotrophic, after standard treatment of CHO-K_1 cells with ethyl methanesulfonate. One temperature-sensitive auxotroph, TsNd-6, has been characterized. At the permissive temperature of 34°C, the TsNd-6 variant grows well both in the complete medium F12 and in the basal minimal medium F12D (in which nine compounds not required for the growth of CHO-K_1

cells are omitted). At the nonpermissive temperature of 39.5°C, the variant grows only in the complete medium. The TsNd-6 cells have been grown continuously for more than 12 months and still maintain their temperature sensitivity at a reversion rate of less than 10^{-7}. In medium F12, the TsNd-6 cells grow with a doubling time of 17–18 hr at 34°C, compared to 13 hr at 39.5°C. In medium F12D, the TsNd-6 cells have a generation time of 23–24 hr at 34°C but fail to grow at all at 39.5°C. The inability of the TsNd-6 cells to grow at the high temperature in medium F12D can be completely overcome by supplementing the medium with thymidine, hypoxanthine, and glycine. Addition of thymidine alone can only partially overcome the growth requirement, and the cells degenerate after two doublings. Hypoxanthine or glycine cannot fulfill the growth requirement. Folinic acid alone cannot, but folinic acid plus thymidine can fulfill the growth requirement. These physiological analyses suggest that this variant might be defective in the biosynthesis or utilization of tetrahydrofolate. However, we were unable to demonstrate an obvious change in the activity and temperature sensitivity of dihydrofolate reductase, a major enzyme in the synthesis of tetrahydrofolate.

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PROGRESS ON THE DEVELOPMENT AND PERFECTION OF A SPECIFIC, SENSITIVE, AND QUANTITATIVE MUTATIONAL ASSAY IN CULTURED CHO CELLS

A. W. Hsie, Patricia A. Brimer, and A. P. Li*

It has now been established that the gene for hypoxanthine-guanine phosphoribosyl transferase (HGPRTase) is X-linked in man and perhaps in other animal species. Many investigators have shown that the HGPRTase locus of cultured mammalian cells can be mutated by conventional mutagens. Recently, Beaudet et al.[1] reported that some 8-azaguanine-resistant variants of Chinese hamster cells had a defective HGPRTase protein, probably caused by a mutation in the structural genes. Sharp et al.[2] have demonstrated that in their mouse L-cell variants resistant to 8-azaguanine the affected HGPRTase has altered kinetic constants as well as heat sensitivity. Thus, this gene appears to be suitable for a detailed genetic-biochemical analysis of point mutation in mammalian cells.

We have undertaken to perfect the mutational assay of the HGPRTase in Chinese hamster ovary cells for the purpose of assessing the genetic hazards of environmental mutagens. We have largely adapted Chasin's mutagenesis protocol[3] for the production of forward mutation to 6-thioguanine resistance in CHO cells, using a CHO subclone designated as CHO-K_1-BH_4. This subclone contains a high level of HGPRTase activity and has a low spontaneous mutation frequency at this gene locus. The growth properties, physiological characteristics, and chromosome constitution of this subclone are indistinguishable from those of its parental CHO-K_1 cells.

The optimum conditions for producing the mutation to 6-thioguanine resistance has been worked out. Our routine procedure was to expose $1-2 \times 10^6$ cells in a 60-mm culture dish to a mutagen (i.e., ethyl methanesulfonate and ultraviolet light) for a known period of time. The mutagen-treated cells were allowed to express the "mutant phenotype" for seven days. Routine subculture was performed during this expression period. At the end of this period, the cells were plated in F12 medium lacking hypoxanthine and containing 10 μM 6-thioguanine. Cells at 1.4×10^5 cells per 100-mm culture dish were plated in the selective medium containing 6-thioguanine. The colony-forming ability of the 6-thioguanine-resistant cells was not affected under these conditions. The selective medium was renewed after two days, and after seven to eight days the drug-resistant colonies were developed, fixed, and stained. Mutation frequency was calculated based on the number of drug-resistant colonies per survival at the end of expression period. Colonies were also cloned and cultured for the determination of HGPRTase by (1) the incorporation of [^{3}H]hypoxanthine into the nucleic acid of the intact cell and (2) the direct assay of HGPRTase activity in the crude CHO cell homogenates.

Employing such a protocol, the quantitative induced mutation of the HGPRTase locus by ethyl methanesulfonate and ultraviolet light was studied. Preliminary results of this study are reported in the following article.

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PRELIMINARY STUDIES ON QUANTITATIVE INDUCED MUTATION AT THE HGPRTase LOCUS IN CULTURED CHO CELLS BY ETHYL METHANESULFONATE AND ULTRAVIOLET LIGHT

A. W. Hsie, Patricia A. Brimer, and A. P. Li*

The mutagenesis protocol described in the preceding article was used to study whether the induction to 6-thioguanine resistance in CHO-K_1-BH_4 cells by ethyl methanesulfonate and ultraviolet light can be quantitated, especially at nonlethal doses. The frequency of spontaneous mutation under our experimental conditions varies from less than 7.1×10^{-7} to 1.7×10^{-5}. The results of our preliminary studies can be summarized as follows.

Mutagenesis by ethyl methanesulfonate. Employing ethyl methanesulfonate at 200 μg/ml, we observed a linear increase of mutation frequency for up to 6 hr of mutagen treatment (e.g., 1.04×10^{-4} for 2 hr, 2.87×10^{-4} for 4 hr, 3.27×10^{-4} for 6 hr, and 6.65×10^{-4} for 16 hr). We chose the treatment time of 16 hr to study the dose response of ethyl methanesulfonate, that is, cell survival vs mutational frequency. Such studies demonstrated a linear increase of mutational frequency when ethyl methanesulfonate was employed between 50 to 400 μg/ml (e.g., 1.83×10^{-4} at 50 μg/ml, 3.63×10^{-4} at 100 μg/ml, 7.94×10^{-4} at 200 μg/ml, and 1.44×10^{-3} at 400 μg/ml). Most interestingly, under our mutagen treatment conditions, concentrations of ethyl methanesulfonate at 50 and 100 μg/ml did not reduce cell survival. Thus, the sensitivity of the mutational assay can detect induced 6-thioguanine resistance quantitatively at nonlethal doses of ethyl methanesulfonate.

Mutagenesis by ultraviolet light. In this set of experiments, the doses of ultraviolet light employed were from 21.6 ergs/mm^2 to 648 ergs/mm^2. Survival curves similar to many published data were observed. We did not observe a reduction of cell survival at doses up to 86.4 ergs/mm^2. However, a clear dose-response induced mutation frequency was observed in the nonlethal range of uv doses (e.g., 5.51×10^{-5} for 21.6 ergs/mm^2, 1.27×10^{-4} for 43.2 ergs/mm^2, and 2.66×10^{-4} for 86.4 ergs/mm^2). Mutation frequency increased further with higher doses of ultraviolet exposure (up to 345.6 ergs/mm^2 measured), which decreased the cell survival. These experiments demonstrate that the sensitivity of this mutational assay allows quantitative detection of induced 6-thioguanine resistance at nonlethal doses of ultraviolet light.

Biochemical determinations of the HGPRTase activity of 13 randomly isolated 6-thioguanine-resistant variants showed that all of the variants possess the enzymic activity of only 0.8–2.0% of the wild-type CHO-K_1-BH_4 cells. Incorporation of [^{3}H] hypoxanthine into the nucleic acids of the drug-resistant variants lies between 0.02 and 0.27% relative to the wild-type cells. Thus it is unlikely that the 6-thioguanine-resistant clones arose epigenetically in this system.

The experimental system described appears to offer a sensitive tool in studying quantitative mammalian cell mutagenesis. This system will be applicable in studying quantitation of mutagenicity of environmental chemical mutagens and carcinogens, as well as low doses of various sources of radiation. The high sensitivity and quantitation of this system would offer an opportunity to reinvestigate the possible role of DNA repair in mutagenesis and the relationship between cell cycle and the actions of various mutagens.

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INDUCTION AND PARTIAL CHARACTERIZATION OF AN AUXOTROPHIC MUTATION IN A DIPLOID CHINESE HAMSTER CELL LINE

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We have obtained a cell line, CHE-Y1, established from Chinese hamster embryo by George Yerganian of Children's Cancer Research Foundation, Boston. This cell line has the normal diploid 22 chromosomes. However, when the cells grown *in vitro* were implanted back into the isogenic host, fibrosarcoma developed. We employed the BrdUrd-light procedure described previously for the induction of auxotrophic mutations in this cell line. Eleven nutritionally dependent mutants, all of which require hypoxanthine for growth, were isolated after standard treatment of the cells with ethyl methanesulfonate. It is interesting to point out that, based on their genetic-biochemical analyses, Puck and Kao suggested that the genes responsible for proline requirement of the CHO-K_1 cells are at the hemizygous region, resulting presumably from partial chromosome deletion of their near-diploid cell line. Since the parent CHE-Y1 cells possess an apparently normal diploid chromosome number, it becomes important to study the genetic and biochemical basis of these mutations as well as their reversions. Parts of such studies are in progress.

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CYTOGENETIC ANALYSIS OF VARIANT CHO CELLS

J. C. Riddle* and A. W. Hsie

The karyotype of the CHO-K_1 subclone employed in this laboratory for genetic studies is 20 chromosomes similar but not identical to those reported by T. T. Puck, from whom we obtained the original CHO-K_1 cells. Treatment of CHO-K_1 cells with ethyl methanesulfonate, followed by the standard BrdUrd-light selection procedure, yielded several morphological and/or nutritional mutants whose chromosome complements are being studied. Thus far, a thymidine-requiring fibroblast-like variant was found to have only 19 chromosomes, missing in virtually every case the same small acrocentric chromosome. By examination of stored frozen samples, this particular mutant was noted to have maintained a remarkably stable karotype for the past one and a half years. Another fibroblast-like variant (which does not require thymidine) also apparently has only 19 chromosomes, having lost an acrocentric chromosome different from the one above. A third fibroblast-like variant, which has a temperature-sensitive requirement for thymidine, hypoxanthine, and glycine, has, in two-thirds of the observed cases, an acrocentric chromosome split into two segments at the centromere. Of two other morphological variants studied, one appears to have a karyotype identical to that of CHO-K_1 cells, and the other a chromosomal substitution. Further definitive statements on these mutants and their revertants must await the results of recloning and chromosome-banding studies. Such studies are in progress to establish the cytogenetic basis of the presumptive mutation(s).

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CELL CYCLE PHASE-SPECIFIC MORPHOLOGICAL TRANSFORMATION OF CHO CELLS BY DIBUTYRYL CYCLIC AMP

A. W. Hsie, J. P. O'Neill, and C. H. Schröder

We have demonstrated previously that in Chinese hamster ovary cells a high concentration of $N^6,O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphate [$(But)_2$cAMP] alone or a low concentration of $(But)_2$cAMP 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-like form.[1] Under our standard growth conditions, the generation time of these cells is approximately 13 hr. $(But)_2$cAMP 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. $(But)_2$cAMP 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 $(But)_2$cAMP is clearly recognizable after 1 hr. Only cells in both the first and second early G_1 phases converted to the fibroblast-like form within 2 hr after $(But)_2$cAMP 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 $(But)_2$cAMP only during the early G_1 phase of growth. Studies on the possible changes of cellular cyclic-nucleotide systems upon $(But)_2$cAMP exposure and of cyclic AMP receptors during the cell cycle are in process.

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MORPHOLOGICAL TRANSFORMATION OF ENUCLEATED CHO CELLS BY DIBUTYRYL CYCLIC AMP AND HORMONES[1]

C. H. Schröder* and A. W. Hsie

Treatment of cultured mammalian cells with cytochalasin B, a metabolite isolated from the mold *Helminthosporium dematioideum*, yields a population of enucleated cells. Such enucleates are excellent experimental material for the study of nucleus-cytoplasm interactions, as certain normal physiological characteristics of the intact cells can be preserved.

Previously, we have shown that in CHO-K_1 cells, $(But)_2$cAMP alone, or in combination with testosterone or prostaglandin E_1, is capable of converting cells from a compact epithelial morphology to the spindle-shaped fibroblast-like form. We found that such "morphological transformation agents" are also capable of causing morphological conversion in CHO cells enucleated with cytochalasin B (10 μg per ml). As we have reported for intact CHO cells, the morphological transformation produced by these hormonal agents in the enucleates can be prevented by compounds, such as

vinblastine or colcemid, which inhibit the assembly of microtubules from their subunit proteins.

The experiments described here demonstrate that the ability to perform three important biological processes – hormonal response, membrane alteration, and microtubule assembly – is retained in enucleates of CHO cells. Thus, these processes seem to be operative in the absence of the nucleus.

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ACTIONS OF PROSTAGLANDINS IN CULTURED CHO CELLS: EVIDENCE FOR THE EXISTENCE OF DISCRETE PROSTAGLANDIN RECEPTORS

Kohtaro Kawashima and A. W. Hsie

In our previous study of morphological transformation of CHO-K_1 cells, we found that hormones such as testosterone or prostaglandin E_1, each of which by itself possesses only a marginal morphological effect, potentiate the action of $(But)_2$cAMP so that maximal morphological conversion can be achieved by the joint action of both agents (synergism).

That synergism exists between $(But)_2$cAMP and the prostaglandins is of particular interest. In light of the generally recognized effects of prostaglandins in either elevating, as in platelets, or depressing, as in adipocytes, cellular cAMP levels by modifying mammalian adenylate cyclase activity, we have examined the effects of various prostaglandins on the formation of cAMP in intact CHO cells. We found that various prostaglandins exert various synergistic effects on the morphogenetic activity of $(But)_2$cAMP in cultured CHO-K_1 cells. When exposed to medium containing prostaglandin E_1 (PGE_1), the intracellular cAMP rose immediately, reached a maximum level of 20-fold increase within 1 min, and fell to basal level within 10–15 min. A similar profile of induced cyclic AMP synthesis was observed with other prostaglandins. The relative potency of prostaglandins to stimulate cAMP synthesis parallels their order in potentiating the ability of dibutyryl cAMP to cause morphological transformation of CHO-K_1 cells, that is, $PGE_1 > PGE_2$, $PGA_1 \gg PGF_{2\alpha}$. This observation is consistent with the hypothesis that prostaglandin receptors with some specificity may exist in CHO cells. Further studies showed that when cells were first treated with PGE_1 they then became refractory to a second PGE_1 treatment, but to a lesser degree to PGE_2 or PGA_1 treatment, in stimulating cAMP synthesis. Permutation experiments with PGE_2 and PGA_1 yield similar patterns of induced and noninduced cAMP synthesis. Moreover, the simultaneous addition of PGE_1 and PGA_1 stimulates cAMP synthesis approximately additively. The analysis of these patterns of inducibility and noninducibility suggests the existence of multiple, discrete prostaglandin receptors, possibly of E and A types, in CHO-K_1 cells.

PROSTAGLANDIN-INDUCED CYCLIC AMP FORMATION AND THE "SENESCENCE" OF CULTURED HUMAN DIPLOID FIBROBLASTS

J. P. O'Neill and A. W. Hsie

Human diploid fibroblasts WI-38 in culture show an increase of intracellular cAMP upon treatment with various prostaglandins, as CHO cells did. Treatment of WI-38 cells at early passages with prostaglandin E_1 (PGE_1) caused an immediate increase of high intracellular cAMP concentration, followed by a progressive decline. Upon addition of PGE_1 to logarithmically growing (low density) cells, the intracellular cAMP level increases slowly (200 pmoles/min/10^6 cells) and eventually reaches a high maximum induced level (100- to 200-fold). However, cells at stationary phase (high density) show a faster increase (400 pmoles/min/10^6 cells) but a lower level of maximum accumulation (10- to 20-fold) of intracellular cAMP.

Cells at later passages show a general decrease in both the rate of increase and the maximum induced level of intracellular cAMP upon treatment with PGE_1. Since our studies on the actions of prostaglandins in CHO cells suggest the existence of discrete prostaglandin receptors, it is likely that the prostaglandin receptors of the WI-38 cells approaching senescence have reduced sensitivity to hormones such as PGE_1.

RELATIONSHIP BETWEEN CYCLIC AMP, MICROTUBULE ORGANIZATION, AND MAMMALIAN CELL SHAPE: STUDIES ON CHO CELLS AND THEIR VARIANTS

Linda S. Borman,* J. N. Dumont, and A. W. Hsie

In studying morphological transformation of CHO-K_1 cells, we found that agents such as colcemid and vinblastine, which inhibit assembly of microtubules, prevent conversion from the epithelial-like to the fibroblast-like form. Therefore, it has been postulated that morphological transformation agents [1 m*M* $(But)_2$cAMP, 0.1 to 0.2 m*M* $(But)_2$cAMP plus 20

μM testosterone, or 0.1 to 0.2 mM $(But)_2$ cAMP plus 6 to 60 μM prostaglandin E_1) act by promoting the assembly of microtubules from protein monomers. It has also been shown that one of the first manifestations of the action of morphological transformation agents is to cause the disappearance of knoblike protrusions from the periphery of the epithelial-like cells. These rounded knobs, which are supported by a small stalk of cytoplasm, appear to be centers of active motion of the cell membrane.

In the course of studies on the molecular mechanisms and the possible genetic basis of morphological transformation, variants have been cloned which differ from the parental CHO-K_1 cells in their response to morphological transformation conditions after standard mutagenesis treatment with ethyl methanesulfonate. The morphological characteristics of CHO-K_1 cells and their variants are being studied in detail.

Electron microscopic studies reveal that, in the presence of morphological transformation agents, the microtubules elongate, increase in number, and become arrayed parallel to the long axis of the cell. Microfilaments remain beneath the cell membrane. In the absence of morphological transformation agents, there are but a few short microtubules randomly distributed within the cytoplasm, usually in the centriolar region.

An epithelial variant, M-7, retains its epithelial-like form in the presence of morphological transformation agents for as long as six to seven days. Despite the fact that the cells maintain their epithelial-like shape after treatment with $(But)_2$cAMP, there is an apparent increase in the number and length of randomly arranged microtubules. It appears that the morphological transformation agents have promoted the assembly of microtubules in M-7 cells but not their organization into parallel arrays and the concomitant conversion of the cells to a fibroblast-like morphology.

A fibroblast-like variant, M-6, becomes even further elongated under conditions of morphological transformation. Although an increase in the numbers of microtubules under these conditions has not been established, there is an apparent increase in the length of individual microtubules.

These studies with the CHO-K_1 cell and its variants M-6 and M-7 provide direct evidence for the role of microtubules in conferring mammalian cell form. The experiments with M-7 cells also suggest that the assembly and organization of microtubules are two distinct biological processes, since the assembly does not necessarily lead to a change in cell form. Changes in cell form appear to be more directly dependent upon the orientation of microtubules.

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AMINO ACID SUBSTITUTION FREQUENCY IN HUMAN HEMOGLOBIN

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

Introduction. We stated last year that a system was being developed to measure, by simple chemical analysis, the substitution frequency of isoleucine for other amino acids in highly purified human hemoglobin. Since human adult hemoglobin does not contain any coded isoleucine, its presence in hemoglobin must originate by errors in translation or transcription and/or somatic mutations. The isolecuine substitution frequency would be expected to increase following an exposure to agents causing base-substitution mutations. Radiation may cause base-substitution mutations in human somatic cells, but this possibility has never been established. In February of 1974, Dr. Robert Conard of the Brookhaven National Laboratory asked if we would be interested in receiving some blood samples from the Marshall Island people to analyze their hemoglobin for its isolecuine content. Some of the Marshallese were accidentally exposed to fallout from an atomic bomb test in March of 1954. This report briefly describes the analysis made on 25 samples of blood received from Dr. Conard in April of 1974.

Methods. Hemoglobin was purified by removing nonhemoglobin proteins from the lysate initially by molecular sieving on Sephadex G-200 and then by three successive chromatographic separations of the carbon monoxyhemoglobin, methemoglobin, and metcyanhemoglobin forms on carboxymethylcellulose. After removing the heme ligand, about 500 nmoles of globin was hydrolyzed in 6 N HCl at 110°C for 21 hr. The absolute amount of globin hydrolyzed was determined by using an aliquot of the hydrolysate, usually 2% of the sample, for quantitative amino acid analysis. The amino acids in the remainder of the globin hydrolysate, to which tracer amounts of [^{3}H]Ile and [^{14}C]Leu were added, were separated on a preparative column designed primarily to separate isoleucine from other amino acids rapidly and effectively. Isoleucine elutes from the preparative column between methionine and leucine. The use of the radiotracers aided both in locating the tubes containing the small amounts of isoleucine and in excluding tubes containing leucine, because small amounts of isoleucine are difficult to quantitate when large amounts of leucine are present in the sample (i.e., they do not resolve completely by

chromatography). The [^{3}H] Ile radioactivity counts were also used to calculate the amount of isoleucine eluting from the preparative column, which was actually pooled for the quantitative analysis. The isoleucine substitution frequency is the value obtained when the calculated number of nanomoles of isoleucine in the sample is divided by the number of nanomoles of other amino acids in the sample.

Results. Analyses of the substitution frequency of isoleucine for other amino acids in the 25 samples of Marshallese blood and three samples of blood taken from individuals in our laboratory are shown in Table 7. The calculated average exposure to gamma rays and the age at exposure are given for those exposed. Samples designated by a number followed by *A* and *R* came from individuals exposed to 69 and 175 R of gamma rays, respectively. Age and sex controls are samples with numbers followed by *U* or designated by numbers only. Samples from three persons from our laboratory are identified by EB, WL, and RAP.

Discussion. Except for one sample, 1547, the high isoleucine-substitution frequencies were in samples from individuals exposed to gamma rays from the atomic bomb fallout; however, substitution frequencies within the control range were found for some individuals exposed to 69 and 175 R of gamma rays. T tests for the level of significance show that groups designated by letters *A* and *R* are not significantly different, $P \geqslant 0.25$; group *R* is significantly different from controls, $P = 0.03$; and group *A* is not significantly different from controls, $P = 0.08$. Inspection of the data reveals, however, that the higher isoleucine-substitution frequencies occur among individuals who were exposed very young. But even this is not always true, since 33*R* does not show an unusually high value even though she was exposed to 175 R at one year of age. More data are

Table 7. Substitution frequency of isoleucine for other amino acids in human hemoglobin from 25 Marshall Island people and three ORNL laboratory personnel

Sample	Exposure (R)	Age at exposure	Age at present (years)	Sex	Substitution frequency ($\times 10^{-5}$)	Average ± S.E.M. ($\times 10^{-5}$)
3*R*	175	15 months	21	M	19.79	
10*R*	175	30 years	50	M	3.58	
18*R*	175	24 years	44	F	5.06	
24*R*	175	13 years	33	F	13.45	8.81 ± 1.96
33*R*	175	1 year	21	F	4.74	
35*R*	175	12 years	32	F	5.19	
42*R*	175	2 years	22	F	10.40	
71*R*	175	27 years	47	F	8.29	
6*A*	69	1 year	21	M	6.98	
8*A*	69	17 months	21	F	12.93	
44*A*	69	3 years	23	M	4.04	5.94 ± 1.92
45*A*	69	31 years	51	F	3.65	
81*A*	69	7 years	27	F	2.12	
813*U*	0		20	M	3.37	
815	0		24	M	2.17	
929*U*	0		35	F	3.47	
836	0		41	M	2.45	
839	0		46	F	1.89	
841*U*	0		41	F	3.56	3.20 ± 1.52
846	0		51	F	2.41	
867*U*	0		46	F	2.12	
868*U*	0		51	F	4.35	
944*U*	0		49	M	3.93	
1547	0		60	F	7.15	
1549	0		21	M	1.57	
EB	0		34	M	2.84	
WL	0		45	M	4.37	3.52 ± 0.45
RAP	0		43	M	3.36	

needed to prove that exposure of infants causes a higher substitution frequency than similar exposure of older children or adults. We are examining whether or not the elevated substitution frequencies are due to base-substitution mutations induced by irradiation or to other possible mechanisms. The high value obtained for sample number 1547 is interesting because she is a 60-year-old woman showing senility. One theory is that protein transcription and translation errors may accumulate with age, whereby the incidence becomes exponential with time. The control data other than sample 1547 show no significant increase in the isoleucine-substitution frequency between ages 20 and 51.

Conclusion. Chemical analysis of the isoleucine content of highly purified hemoglobin from 25 persons from the Marshall Islands indicates that persons exposed to gamma rays, especially at a young age, have a higher content of isoleucine in their hemoglobin. This observation is consistent with the notion that gamma rays induce base-substitution, point mutations in human somatic stem cells, but it does not exclude the possibility that translational errors contribute significantly to the elevated substitution frequency.

AMINOACYL-tRNA LIGASE ERRORS IN MOUSE TISSUES WITH AGING

G. P. Hirsch and R. A. Popp

One expected result of accumulated errors in aging systems is a reduced fidelity of elements of the protein synthetic apparatus. Aminoacyl-tRNA ligases which themselves contain errors would be expected to have a reduced ability to discriminate against amino acid analogs and other natural amino acids, and thereby continue to propagate errors.

The fidelity of this portion of the protein synthetic apparatus has been examined during aging in mouse tissues by the incorporation of the amino acid analog, alpha-aminoisobutyric acid, into protein. To control for possible differences in protein synthesis with age, the level of analog incorporate was related to the level of natural amino acids incorporated. This ratio is used to indicate the fidelity of ligase specificity. In liver tissue 4 hr after isotope administration, adult and chronically ethionine-treated mice had a higher ratio than young mice. Old animals, however, had a twofold lower ratio than young mice, which can be attributed to differences in transport or amino acid pool sizes among these groups.

In brain tissue 24 hr after *in vivo* alpha-aminoisobutyric acid labeling, ratios in young (2 months), adult (6 months) and old (23 months) were more similar than liver values, but ethionine-treated and aged (27 months) mice had lower ratios than young animals. The isotope present in extensively dialyzed protein was recovered from brain tissue as alpha-aminoisobutyric acid, which indicates that this analog and not its metabolites is in fact incorporated into protein.

A final resolution of the error theory of aging as applied to nondividing tissues can be expected when a more detailed study is completed on the kinetics of analog incorporation and when a correlation is established between analog incorporation and measured error rates in hemoglobin protein.

HEAT-LABILE GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN ETHIONINE-TREATED MICE

G. P. Hirsch, Ellen B. Gould,* and R. A. Popp

Because the amino acid sequence analysis method of measuring errors of protein synthesis is not generally applicable to proteins of various tissues, we have initiated work to correlate measured error rates in the hemoglobin of red cells to indirect detection of errors in glucose-6-phosphate dehydrogenase in the same cells. In lieu of making many determinations of enzyme activity after various periods of denaturation, we have examined the enzyme activity during denaturation by measuring enzyme activity continuously at high temperature. Red-cell lysates from mice treated for more than two weeks with an equivalent of 0.05% ethionine in the diet consistently show an initial level of activity which is lost within the first 3 min at 60°C. Lysates from untreated mice show constant activity. The initial activity which is lost at this temperature is considered to be more heat labile than the control enzymes and hence to contain errors. The heat-labile activity exists after partial purification of the enzyme. The interpretation of enzyme activity at the high temperature in lysates but not in partially purified enzyme samples is complicated by a dramatic non-substrate-dependent increase in enzyme activity near the denaturation temperature.

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IMMUNE RESPONSE INDUCED BY H-2D AND H-2K ANTIGENS: EFFECTS OF CORTISONE ACETATE

Diana M. Popp

Lymphocytes transferred into irradiated recipients will respond to the antigens of the host by transforming into large pyroninophilic cells (LPC) and by proliferating. When congenic mice are used as donors and recipients, the inducing antigens are the H-2 antigens; when the congenic crossover lines 2R and 5R are used with inbred C57BL/10 mice as recipients, the effects of the H-2D and H-2K region antigens can be studied. Previous studies have shown that H-2K region antigens induce an immediate appearance of LPC with a peak response on day 3–4, similar to the response induced by a complete H-2 difference. The peak response to H-2D antigens occurs on day 5. In order to determine whether different populations of cells were responding at these different times, donor mice were treated with 2.5 mg of cortisone acetate two days prior to collection of the lymph-node lymphocytes. Recipients of lymphocytes from normal donors received approximately 50 X 10^6 lymph-node lymphocytes (one donor to one recipient).

Since we were interested in assessing the contribution of subpopulations of cells by testing cortisone-resistant vs cortisone-sensitive cells, it was decided to retain the ratio of one donor to one recipient, although the number of cells injected was lower (approximately 10 X 10^6). Presumably, the number of cortisone-resistant cells in the lymph nodes of treated vs nontreated mice would be equivalent. Some recipients received 50 X 10^6 cortisone-resistant lymph-node lymphocytes to ascertain the effect of a massive dose of these cells. This is equivalent to the cortisone-resistant cells from five mice. The spleens were taken daily for six days, fixed in Carnoy's fixative, and stained with methyl green and pyronin. Three thousand to 5,000 nucleated cells were counted per spleen, scoring separately for the LPC and mitotic figures. The frequency of LPC and mitotic figures per 100 nucleated cells was calculated and expressed as % LPC and mitotic index (MI). The % LPC and MI observed in the lymphatic follicles of spleens of recipients of cortisone-acetate-treated and normal lymph-node lymphocytes that differ from the recipient at H-2, H-2K, and H-2D are shown in Table 8. The

Table 8. The effect of cortisone acetate on the cellular response of lymph-node lymphocytes infused into hosts allogeneic at different segments of the H-2 region

Days after lymphocyte injection	Antigenic[a] difference	% LPC		P^c	% Mitotic figures		P
		Normal donors	Cortisone[b] acetate treated donors		Normal donors	Cortisone acetate treated donors	
1	H-2	2.6 ± 1.1	1.5 ± 0.3	NS	0.2 ± 0.1	0.3 ± 0.1	NS
	H-2K	2.8 ± 0.9	1.6 ± 0.8	NS	0.1 ± 0.1	0.2 ± 0.1	NS
	H-2D	0.7 ± 0.5	1.7 ± 1.0	NS	0.3 ± 0.2	0.5 ± 0.1	NS
2	H-2	17.7 ± 1.4	8.7 ± 2.5	NS	1.2 ± 0.2	1.0 ± 0.2	NS
	H-2K	18.1 ± 1.3	12.0 ± 1.9	NS	1.6 ± 0.1	1.0 ± 0.2	NS
	H-2D	1.3 ± 0.2	1.4 ± 0.6	NS	0.5 ± 0.03	0.4 ± 0.2	NS
3	H-2	28.7 ± 2.9	20.4 ± 3.9	NS	3.1 + 0.3	1.4 ± 0.3	0.005
	H-2K	23.5 ± 1.1	27.4 ± 4.2	NS	3.4 ± 0.4	2.3 ± 0.6	NS
	H-2D	6.0 ± 2.2	2.8 ± 0.6	NS	1.0 ± 0.2	0.4 ± 0.1	NS
4	H-2	27.1 ± 1.6	26.2 ± 3.6	NS	3.6 ± 0.5	2.9 ± 0.6	NS
	H-2K	24.0 ± 1.5	24.8 ± 1.3	NS	3.7 ± 0.3	3.1 ± 0.4	NS
	H-2D	11.3 ± 2.1	3.9 ± 0.6	NS	1.3 ± 0.4	0.9 ± 0.1	NS
5	H-2	27.2 ± 2.8	19.2 ± 2.4	NS	2.6 ± 0.3	2.7 ± 0.5	NS
	H-2K	20.1 ± 0.9	23.0 ± 2.6	NS	2.8 ± 0.4	3.2 ± 0.6	NS
	H-2D	24.5 ± 2.5	4.6 ± 0.6	<0.001	3.0 ± 0.8	0.6 ± 0.1	<0.01
6	H-2	21.6 ± 1.1	19.8 ± 3.9	NS	2.5 ± 0.7	2.4 ± 0.5	NS
	H-2K	13.9 ± 0.5	17.8 ± 3.9	NS	2.5 ± 0.6	2.5 ± 0.1	NS
	H-2D	14.6 ± 2.1	6.1 ± 0.8	<0.01	2.3 ± 0.2	1.2 ± 0.1	<0.005

[a]H-2 = B10.A → B10; H-2K = 2R → B10; H-2D = 5R → B10.
[b]Lymphocyte donors received 2.5 mg cortisone acetate i.p. 2 days before lymph nodes were removed.
[c]NS = Not significant at the 99% level.

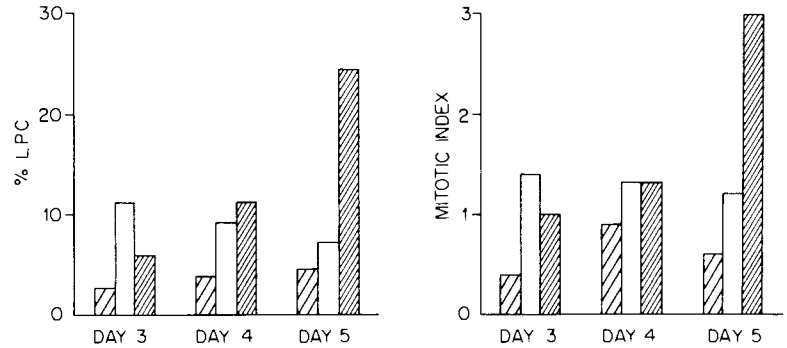

Fig. 11. Comparison of transformation and division of normal and cortisone-acetate-resistant lymph-node lymphocytes. ▨, Cortisone-acetate-resistant cells from one donor (10×10^6 cells); □, 50×10^6 cortisone-acetate-resistant cells; ▨, 50×10^6 normal cells.

figures represent the average of four to six mice per day (± S.E.M.). The data show that only the H-2D response is affected by pretreatment with cortisone acetate. Since cortisone acetate has been shown to singularly affect T helper cells, this observation suggests that the immune response induced by antigens specified by the H-2D region is dependent on the presence of T helper cells; furthermore, the cells responsible for the late peak at day 5, induced by H-2D antigenic differences, are T helper cells or T helper cells plus secondarily stimulated B cells.

That such cellular interaction is responsible for the late peak is suggested by the results of the cell dose experiment wherein 50×10^6 cortisone-resistant cells were injected. Figure 11 shows that although the response of LPC induction and MI is greater than when 10×10^6 cortisone-resistant cells are injected, the response still does not equal that when 50×10^6 normal cells are injected. The LPC response induced by 50×10^6 cortisone-acetate-resistant cells is one-third and the MI is one-half that of the responses induced when 50×10^6 normal cells are injected; 50×10^6 lymph-node lymphocytes from normal mice are composed of approximately 10×10^6 cortisone-resistant cells and 40×10^6 cortisone-sensitive cells.

These results permit the following observations to be made concerning the characteristics of the gene products that induce immune responses to transplantation antigens. (1) Different portions of the H-2 regions stimulate different lymphocyte populations; (2) products of H-2D stimulate cells that are cortisone sensitive and transform late, resembling cell types involved in humoral immunity; and (3) products of H-2K stimulate cells which are cortisone resistant and transform early, resembling cell types involved in cell-mediated immunity.

IN VIVO TEST FOR T KILLER-CELL ACTIVITY

Diana M. Popp and Mary W. Francis

Earlier studies have shown that antigenic differences controlled by the D end of the H-2 region stimulate donor lymph-node lymphocytes (LNL) to transform in the lymphatic follicles of the recipient spleen five days after exposure to antigen, whereas antigenic differences controlled by the H-2K end stimulate lymphocytes within 24 hr and reach a peak response by three days. Since T-cell proliferation induced by oxazolone begins on day 1, peaking at day 3, and since B-cell proliferation induced by T-cell-independent antigens occurs on day 5, we suggested that the two responses corresponding to separate H-2 regions represent two subpopulations of lymphocytes, perhaps T cells involved in cellular immunity and B cells (or T + B) involved in humoral immunity. The nature of the cells involved in the late response is being studied by treating the donors with cortisone acetate or thymectomizing the donors. To characterize the cells involved in the early response, we asked whether there were more killer cells in the spleens of irradiated mice that received LNL from donors that differed at the H-2K region than in the spleens of mice that received LNL from donors that differed at the H-2D region.

The test for presence of killer cells is the ability of donor LNL to reduce in the recipient's spleen the number of plaque-forming cells (PFC) to sheep red blood cells (SRBC). 50×10^6 parental LNL were injected into F_1 recipients that had been lethally irradiated three days previously. After four days, the spleens were removed, cells were suspended and washed, and 20×10^6 or 50×10^6 cells were injected into F_1 recipients. Two days later, these recipients received 0.5 ml of a 2% suspension of SRBC, and the

spleens were analyzed for PFC five days later by the slide technique. The mice used in these experiments were the inbred C57BL/10, the congenic B10.A, the congenic crossover lines B10.A(2R) and B10.A(5R), and the F_1 hybrids (B10 X B10.A)F_1, (2R X B10)F_1, (2R X B10.A)F_1, (5R X B10)F_1, (5R X B10.A)F_1. With respect to B10, 2R and 5R represent H-2K and H-2D antigenic differences, respectively; with respect to B10.A, 2R and 5R represent H-2D and H-2K antigenic differences, respectively.

In these experiments, basic information is being obtained on the relative ability of different strains of mice to produce plaques to SRBC. There is a range in the efficiency of response, even among the congenic lines used, from a minimum of 110,000 PFC per spleen for B10.A males to 240,000 PFC per spleen for 5R females. A range is also seen among the F_1 hybrids, from 102,000 PFC per spleen for (B10.A X 5R)F_1 males to 230,000 PFC per spleen for (B10.A X 5R)F_1 females. The numbers of PFC in all inbred strains and F_1 hybrids have not as yet been analyzed.

The ability to demonstrate presence of killer cells in the spleens of recipients of allogeneic LNL has been made difficult by a phenomenon known as the allogeneic effect, as well as by an unknown phenomenon we call the isologous effect. These effects are described as the ability of cells from LNL-repopulated spleens to increase the PFC capacity of F_1 recipients when certain strain combinations and cell doses are used; they are not necessarily dependent upon allogeneic stimulation.

When the lymphocytes are sensitized to an H-2D difference for four days and 20×10^6 or 50×10^6 LNL-repopulated spleen cells are used, there is no reduction in number of PFC, that is, no killing. However, when the lymphocytes are sensitized to an H-2K difference and 20×10^6 or 50×10^6 LNL-repopulated spleen cells are transferred, there is a slight increase in number of PFC per spleen in the isologous control and a threefold increase in PFC in the allogeneic combination. When the lymphocytes have been sensitized to a complete H-2 difference for four days and 50×10^6 LNL-repopulated spleen cells are transferred, there is a threefold increase in PFC in the allogeneic combination and in the isologous control as well. When the spleen cell dose is dropped to 20×10^6, however, there is evidence of killing, as the allogeneic combination shows a 40% decrease in PFC but the isologous control still shows a threefold increase in PFC.

We intend to investigate the nature of the cellular interactions that permit F_1 hybrid recipients to escape regulatory mechanisms that maintain consistent levels of PFC when isologously or allogeneically LNL-repopulated spleen cells are injected into the F_1 hybrids two days prior to injection of SRBC. A delicate balance of subpopulations of lymphocytes must regulate the efficacy of the immune response, and cell-surface antigens controlled by the H-2 region may play an important part in regulating these interactions.

ISOLATION AND CHARACTERIZATION OF AN ULTRAVIOLET-LIGHT-REVERTIBLE ALLELE OF THE STRUCTURAL GENE RESPONSIBLE FOR NITRATE REDUCTASE IN *USTILAGO MAYDIS*

J. F. Lemontt*

Nitrate reductase (NR) enzyme activity in the yeast-like, unicellular, budding corn smut fungus *Ustilago maydis* is easily quantified *in vivo* with good correlation to *in vitro* activity measured in crude extracts.[1] A mutant revertible by ultraviolet light (uv) was selected by chlorate resistance with the expectation that the "fixation" and eventual expression of uv-induced reversions could then be monitored by the appearance of active enzyme. Genetic analysis revealed that the mutant phenotype was conferred by a single recessive allele (*nar1-m*) of the structural gene (*nar1*) for NR, based upon the following observations: (1) Growth is blocked by nitrate but can be supplemented by nitrite *and* by hypoxanthine (indicating functional xanthine dehydrogenase). (2) Heterozygotes utilize nitrate for growth, are chlorate sensitive, and segregate equal numbers of mutant and nonmutant phenotypes among random haploid meiotic products. (3) Noncomplementation and tight linkage (or allelism) are exhibited when crossed to *nar1-1*. (4) Both spontaneous and uv-induced revertants are chlorate sensitive, are dominant, and map at *nar1*. (5) Normal meiotic linkage to *nir1* is observed (~2.5% recombination is expected between *nar1* and *nir1*). (6) Both spontaneous and uv-induced frequencies of mitotic reversion are approximately twice as great in homozygous diploids as in the mutant haploids.

Also revertible by gamma rays, the *nar1-m* allele was found to be unusually unstable. The spontaneous reversion rate was estimated to be approximately 3×10^{-5} per division, calculated from the median frequency of a set of parallel cultures.[2] All *nar* mutants, including *nar1-m*, exhibit considerable residual growth on nitrate minimal (NM) agar medium, on which only nar^+ revertants should grow, presumably because traces of reduced nitrogen are contained in the agar. Because of the high reversion rate, additional revertants were generated during the period of background growth, making it difficult to obtain accurate reversion frequen-

cies. To overcome this problem, a method was devised that reduced residual growth to the point where no new spontaneous revertants could arise after plating. A stable (i.e., virtually nonrevertible) *nar1* mutant from a different strain was suspended in precooled NM agar before pouring, and the plates were incubated at the usual 32°C for two to three days, during which time residual growth of this "scavenger" strain depleted the utilizable nitrogen in the agar. Subsequent plating of the *nar1-m* strain resulted in little or no residual growth, so that only preexisting or induced revertants formed visible colonies.

Both the high-reversion-rate and all other *nar1-m* phenotypes segregate together in meiosis as a single locus mapping at *nar1*. The instability thus cannot be the result of an unlinked mutator gene, but on the other hand, is a property of the allele itself; nor does *nar1-m* appear to influence mutation at other loci. Despite the fact that little data is available concerning the molecular basis for *nar1-m* or its reversion, the genetic properties observed so far suggest that a valid point-mutational change is involved.

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IN VIVO BIOCHEMICAL ASSAY FOR INDUCED MUTAGENESIS IN *USTILAGO MAYDIS*

J. F. Lemontt*

A great deal of evidence gathered for both prokaryotic and eukaryotic cells now points toward the existence of genetically controlled pathways responsible for induced mutation which require the participation of functional yet *error-prone* repair enzymes. Virtually all determinations of mutation frequency rely upon the identification of mutant clones among survivors of the mutagenic treatment. To gain a better understanding of the relation between repair and mutagenesis, it would be of great value to be able to detect induced mutations in a population of mutagenized *cells*. With such a system it should be possible to determine whether or not induced mutations can be generated in cells that ordinarily would not form a colony after treatment with the mutagen.

With this goal in mind, an *in vivo* enzymatic assay sensitive to the expression of induced mutations under conditions preventing clonal growth of survivors has been developed for the yeast-like fungus *Ustilago maydis*, which has an assimilatory pathway for inorganic nitrate. The activity of nitrate reductase (NR), the first enzyme in the pathway, inducible by NO_3^- and repressible by NH_4^+, is easily quantified *in vivo* with good correlation to *in vitro* activity measured in crude extracts.

First, a uv-revertible allele (*nar1-m*) of the structural gene (*nar1*) coding for NR was isolated and characterized genetically. Reversions to wild type induced by uv and by gamma radiation were detected by observing the kinetics of appearance of increased levels of NR activity in a nondividing, growth-blocked population of irradiated cells producing predominantly inactive mutant enzyme. Holliday[1] had first utilized this system to monitor expression of gamma-radiation-induced mitotic gene conversions in a diploid heteroallelic for *nar1*.

To carry out the assay, cells must carry *nir1* for defective nitrite reductase, so that nitrite accumulates as soon as it is formed and is excreted into a liquid medium containing nitrate as the sole nitrogen source. Cell suspensions were filtered, permitting immediate colorimetric nitrite determination of the filtrate.[2] One unit of activity was defined as one nanomole of nitrite accumulated per hour by 10^8 cells. Resnick and Holliday[3] found that a population of haploid cells carrying only the stable *nir-1* allele will exhibit full or nearly full final enzyme activity after a suitable uv exposure, which introduces a small dose-dependent time lag, despite the fact that a much larger proportion of the population will be reproductively dead if plated. Greater uv doses lead to decreased enzyme activities because of direct inactivation of the structural gene.

The needed *nar1-m nir1-1* recombinant was selected from meiotic products of the cross *nar1-m* × *nir1-1*. One nar^+ revertant from this strain was found to have fully restored *in vivo* NR activity comparable to wild type. Unirradiated *nar1-m nir1-1* cultures were found to have a significant level of enzyme activity due to the presence of a high frequency of spontaneous nar^+ revertants. After either uv or gamma irradiation in distilled water and subsequent incubation in nitrate medium in the dark, a dose-dependent delay in the appearance of enzyme from spontaneously occurring revertants was observed, as expected, followed by a rise in activity to a final dose-dependent level surpassing that observed for the unirradiated control. For example, at a low uv exposure permitting nearly 100% survival, NR activity increased from approximately 1.5 (spontaneous) to 4.0 units, while plating experiments indicated that the frequency of nar^+ revertants increased from $\sim 10^{-4}$ to 5×10^{-4}.

These preliminary results clearly demonstrate that both uv and gamma rays enhance levels of NR activity, resulting presumably from the expression of induced reversions from *nar1-m* to nar$^+$. It is hoped that this system can be used to answer fundamental questions concerning mechanisms of induced mutagenesis and their genetic control in eukaryotic organisms.

*Research sponsored by a fellowship from the European Molecular Biology Organization while on leave of absence at the National Institute for Medical Research, London, England.

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CYTOLOGICAL MAPPING OF STRUCTURAL GENES OF ENZYMES FROM HYBRIDS OF *DROSOPHILA MELANOGASTER* AND *DROSOPHILA SIMULANS*

E. H. Grell

Previously, methods have been developed in this laboratory to assign the structural locus of an enzyme to a particular chromosome arm if there is an electrophoretic difference between the enzyme of *D. melanogaster* and *D. simulans*. After the identity of the arms is known, the next task is to assign the enzyme to a position on the linear map of the arm. Because F_1 hybrids between the two species are sterile, a crossover map cannot be constructed. The most obvious alternative method is to use deficiencies of chromosomal segments of *melanogaster* and make them heterozygous with *simulans*. The limits of the deficiencies can be determined by cytological examination of salivary-gland chromosomes. If a hybrid fly does not have the *melanogaster* enzyme, the enzyme locus is in the deleted segment. The problem is that hybrid flies heterozygous for a deficiency are often nonviable.

The alternative method is to use duplications of segments of the *melanogaster* chromosome. The results are easiest to assess if the enzyme being located contains two subunits specified by the gene. In a hybrid with one *simulans* and one *melanogaster* gene, the ratio of simulans to hybrid to *melanogaster* is 1:2:1; if the ratio of *simulans* to *melanogaster* genes is 1:2, the ratio of enzymes is 1:2:2. Duplications which cause a shift in the enzymes as described are judged to contain the structural gene of the enzyme. A precise cytological location is most likely if a series of duplications are available or can be synthesized.

CHROMOSOMAL PARAMETERS OF GENE REGULATION

J. E. Tobler

The research completed during the past year involved the examination of chromosomal parameters of gene regulation in *Drosophila*, specifically, the effect of chromosomal position on the ontogenic expression and dosage-compensation properties of genes in *Drosophila*. One experiment was to examine and compare dosage-compensation properties and ontogenic expression of v^+ activity in suppressed, wild-type, and "transformed" vermilion flies, the latter of which reportedly carries the v^+ gene alongside but not incorporated into the chromosome. A spectrofluorometric assay system for tryptophan oxygenase was developed and used to determine v^+ activity in the various stocks. The results indicate that, although different stocks showed different levels of oxygenase (v^+) activity, all showed dosage compensation. The ontogenic expression of tryptophan oxygenase was observed to be different in the various genotypes. Whereas suppressed vermilion resembled wild type in pattern, the ontogenic profiles of transformed flies were different. The implications from the study are (1) that the dosage-compensation function is at least closely linked to the structural gene and indeed may be encoded in the gene itself; and (2) that the ontogenic expression function is independent of dosage compensation, and its control may somehow involve the chromosomal unit. These results and their implications have been submitted for publication.[1]

A second research effort is an extension of the above and involves testing the hypothesis that the dosage-compensation function is encoded in the structural gene. The basic design is to induce a dosage-compensation mutant and examine its enzyme properties. The experiment requires a special system in which one can easily screen for enzyme and dosage-compensation mutants biochemically. Such a system has been developed and is described in a paper currently in preparation. The paper reports a mutant for the *HAD*$^+$ gene, its location on the X chromosome, and the dosage-compensation property associated with the gene. The enzyme, "hydroxyl acid dehydrogenase," is not required for the flies' viability and is easily assayable both qualitatively and quantitatively. The biochemical characteristics of the enzyme, such as pH optima, substrate specificities, and electrophoretic patterns, are reported. These characteristics of the system, along with a spot test that has been developed to screen for the mutants, allow the testing of the above hypothesis.

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POSITIVE INTERFERENCE IN *DROSOPHILA*

Rhoda F. Grell

The ability of a reciprocal exchange to reduce or eliminate crossing-over in its vicinity is known as positive interference. The degree of reduction is a function of distance; with sufficient distance interference disappears. The operation of this force over vast molecular lengths of DNA is perplexing. Heretofore, changes in interference were considered to be directly linked to changes in crossing-over, with decreases accompanying genetic lengthening and increases accompanying genetic diminution of a chromosome or its parts. Heat treatment has provided a way to separate the two phenomena. Changes in interference induced by sequential heat treatments have been followed throughout the genome and compared with heat-induced changes in crossing-over in the same regions during identical time spans.

Analysis is simplified by establishing two categories within a chromosome arm -- (1) nearest-neighbor regions and (2) most distant regions. Employing these criteria it becomes apparent that alterations in interference are not a simple consequence of alterations in exchange. For example, two nearest neighbors (regions 7 and 8) in the right arm of chromosome 3 show a decrease in interference which coincides with increased crossing-over; but two most distant regions (5 and 8) in the same arm, normally lacking interference, now exhibit a high degree of interference, and imposition of interference here also coincides with maximal increases in exchange.

Among 18 sets of nearest neighbors in the five chromosome arms, 16 manifest decreases in interference irrespective of decreases or increases in their genetic length, both of which occur. Among five sets of most distant regions in the five chromosome arms, normally lacking interference, four exhibit heat-induced interference, again uncorrelated with changes in their genetic length. It is concluded that the effect of heat on interference is independent of its effect on exchange. A model to account for heat effects on recombination has been proposed.

REC$^-$ MUTANTS IN *DROSOPHILA*

Estela E. Generoso and Rhoda F. Grell

Isolation of rec$^-$ mutants for studying the recombination process is being pursued. Attention is focused on a chromosomal region consisting of 18 bands, within which the *rec* gene *c(3)G* lies. Newly induced mutants recovered from EMS treatment are characterized through genetic analyses, including tests for complementation, temperature sensitivity, and segregation behavior, as well as through electron microscopic examination for the presence and appearance of the synaptonemal complex.

A total of approximately 13,000 EMS-treated third chromosomes have produced 12 new rec$^-$ mutants located within the 18 bands demarcated by the sbd^{105} deficiency. Complementation tests indicate that they represent two loci, and that 11 of the mutants are allelic to *c(3)G*, whereas the twelfth constitutes a new locus. Only the latter displays temperature sensitivity and fails to abolish the synaptonemal complex.

THERMAL RECOMBINATION MAP FOR THE *DROSOPHILA* GENOME

Rhoda F. Grell

A study of the temperature-sensitive period for enhancing meiotic recombination throughout the *Drosophila* genome is now complete. The genome has been divided into 32 regions, each averaging 10 crossover units in length. The regions include five in the X chromosome, five in each arm of chromosome 2, four in each arm of 3, four each spanning the proximal portions of chromosomes 2 and 3, and one in chromosome 4. Responses in each of the 32 segments were measured during at least nine sequential time intervals, with treatments (35°C, 12 hr) spaced 6 hr apart so as to encompass the first half of the pupal period, the time to which sensitivity for the sample of oocytes employed was previously localized.

As determined for the portion of the genome examined earlier, the limits of response for the total genome fall within a 30- to 36-hr period, beginning close to oocyte formation and terminating before the stage-1 oocyte is produced. Response is therefore confined to the germarial stages and is largely coextensive with the long meiotic S phase, which occupies a 24- to 30-hr period, also beginning close to oocyte formation. Extrapolating from the coincidence with the main DNA replication, enhancement of recombination occurs during premeiotic interphase.

Within the sensitive interval, different regions display characteristic peaks, magnitudes, and durations of response that are highly reproducible. A thermal recombination map reflecting the regional variations has been constructed. If each of the five chromosomal arms is viewed as a unit, a comparison of their magnitudes of

response shows that they are separable and follow the seriation 3L > 3R > 2R > 2L > X. A comparison of the time of maximal response within each arm gives the chronological sequence 2R, X, 3L, 3R, 2R.

THE SYNAPTONEMAL COMPLEX AND DNA REPLICATION

J. W. Day and Rhoda F. Grell

Current concepts hold that the synaptonemal complex, a tripartite structure located along the longitudinal axis of synapsed homologs and revealed only by electron microscopy, first appears during the zygotene stage of meiotic prophase. Conversely, our studies have shown that the complex is already present within 6 hr of oocyte formation, and that its appearance coincides with the early phase of the main DNA replication. Thus, by definition, the time of its formation is identified as premeiotic interphase. To directly demonstrate the coincidence between complex formation and DNA synthesis, electron micrograph–autoradiographs of oocyte nuclei of appropriate age which had been incubated in [^{3}H] thymidine for 1 hr were prepared and inspected for the presence of label and complexes.

Synaptonemal complexes were located in oocytes undergoing DNA synthesis, as indicated by the presence of label within their nuclei (Fig. 12).

DISTINCT KYNURENINASE AND HYDROXYKYNURENINASE ACTIVITIES IN MICROORGANISMS AND ANIMALS

F. H. Gaertner and A. S. Shetty

Three kynureninase-type reactions are known to occur in *Neurospora crassa:* the hydrolysis respectively of *N*-formyl-L-kynurenine, L-kynurenine, or L-3-hydroxy-

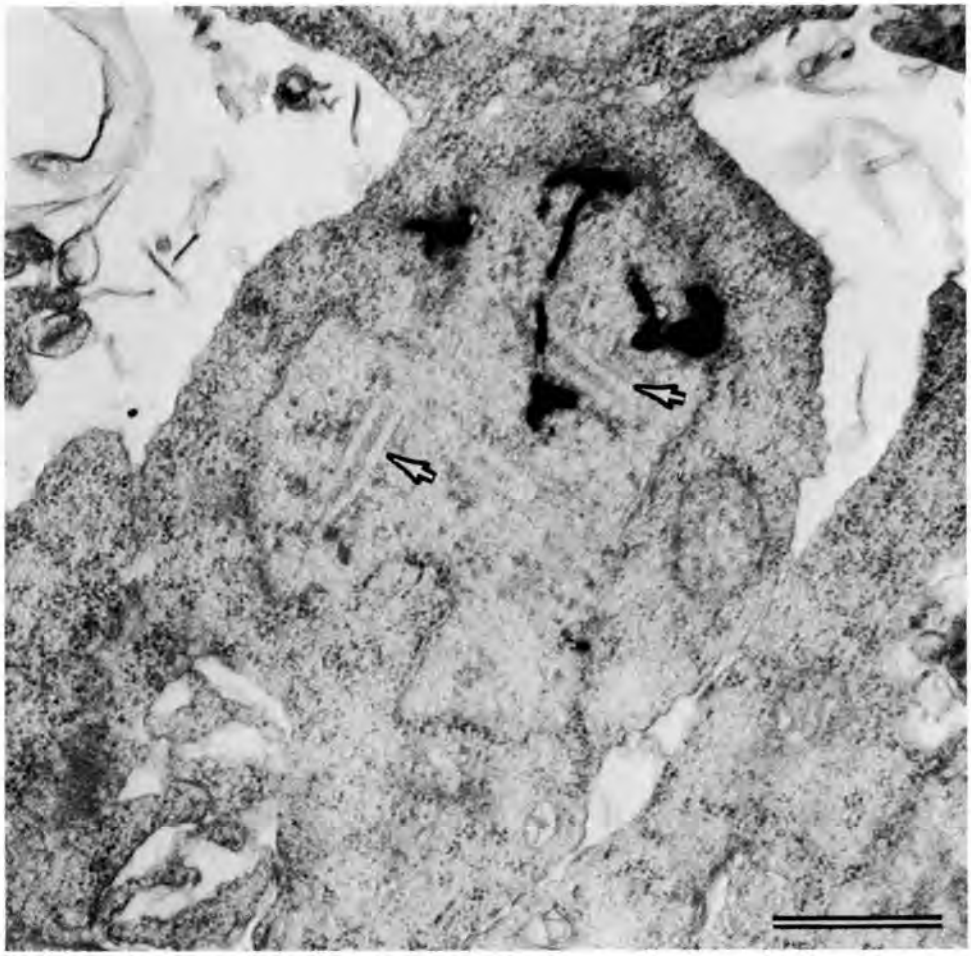

Fig. 12. Electron micrograph–autoradiograph of an oocyte nucleus from a 144-hr ovary incubated in [^{3}H] thymidine for 1 hr, showing the presence of both label and synaptonemal complexes in the same nucleus. Arrows indicate synaptonemal complexes. The bar represents 1 μm.

kynurenine to pyruvate plus *N*-formylanthranilate, anthranilate, or 3-hydroxyanthranilate. In an assay wherein the appearance of anthranilate is measured spectrophotofluorometrically in a reaction mixture containing the substrate L-kynurenine, we have observed two peaks of hydrolytic activity by DEAE-cellulose chromatography of *N. crassa* extracts.[1] One of these, the first to elute from the column, is induced over 400-fold by the addition of L-tryptophan to the culture medium. On the other hand, the second peak of activity is unaltered under these conditions. We have examined the substrate specificity of these two activities and have found that the inducible one has a preference for L-kynurenine or *N*-formyl-L-kynurenine over L-3-hydroxykynurenine. The V_{max} for all three substrates is nearly the same, while the K_m for L-3-hydroxykynurenine ($\sim 10^{-4}$ *M*) is about ten times greater than that for L-kynurenine or *N*-formyl-L-kynurenine ($\sim 10^{-5}$ *M*). This difference in K_m is even more dramatic when it is compared with that obtained for the constitutive activity found in the second peak, which has a K_m for L-3-hydroxykynurenine 50 to 100 times lower ($\sim 10^{-6}$ *M*) than that for L-kynurenine or *N*-formyl-L-kynurenine ($\sim 10^{-4}$ *M*). Hence, we propose that the inducible activity is specifically a kynureninase involved in the catabolism of L-kynurenine or *N*-formyl-L-kynurenine to anthranilate, while the constitutive enzyme is specifically an hydroxykynureninase involved in the biosynthetic conversion of L-3-hydroxykynurenine to 3-hydroxyanthranilate in the pathway leading to NAD. In further support of this notion is the fact that the constitutive enzyme exceeds the concentration of the inducible one by a factor of 5 or more in cells grown without added tryptophan. Hence, it is apparent that the constitutive enzyme is involved in the biosynthesis of NAD. In addition, the level of inducible enzyme correlates with the excretion of anthranilate in cells grown in medium supplemented with L-tryptophan.

To substantiate further our findings and to determine how specific each enzyme is physiologically, we would like to obtain mutants lacking each activity. Thus far, however, it has not been possible to isolate such mutants. In lieu of such variants, we have attempted to learn more about the "kynureninase" enzymes through a comparative study of other organisms. For example, we have partially purified the kynureninase-type activity from the livers of various animals, including a mammal, bird, and amphibian. In the mouse,[2] chicken, and frog, NAD is apparently synthesized from L-tryptophan, and in each case the liver enzymes are of the constitutive type, with K_m's approximately 100 times lower for L-3-hydroxykynurenine than for L-kynurenine ($\sim 10^{-6}$ *M* vs 10^{-4} *M*). Thus in the liver of these animals the enzyme can be characterized as an hydroxykynureninase. The question arises, then, whether the enzyme demonstrated in liver with its specificity for L-3-hydroxykynurenine can be responsible for the reported urinary excretion of anthranilate, anthranilate glucuronide, or *o*-aminohippurate. Although another enzyme possibly operating in an organ other than the liver may be responsible, we have no evidence for such an enzyme. Alternatively, the low levels of these metabolites detected may arise from dietary anthranilate. During the course of the study of the liver enzymes it was observed that the hydroxykynureninase reaction is apparently inactive in crude extracts. However, this apparent lack of activity can be attributed to the exceedingly active 3-hydroxyanthranilate oxygenase present in liver crude extracts. We have found the latter enzyme to be more than 1000 times more active than the hydroxykynureninase, a fact which may reflect the physiological lability or toxicity of 3-hydroxyanthranilate.

Our comparative studies with microorganisms have substantiated further the biological generality of the distinct kynureninase-type enzymes and have provided some evidence as to their physiological specificity. For example, the bacterium *Pseudomonas fluorescens* lacks the ability to synthesize NAD from L-tryptophan yet has the capacity to degrade L-tryptophan via the kynurenine pathway. Here the single inducible kynureninase-type enzyme has no obvious biosynthetic function, since NAD is formed by a different pathway. As expected, the enzyme has a specificity for L-kynurenine and is comparable to the inducible enzyme of *N. crassa.*

In addition to finding other examples of fungi which appear to contain two enzymes similar to those of *N. crassa* (e.g., *Penicillium roqueforti* and *Aspergillus niger*), we have found three examples which contain only the constitutive enzyme: *Mucor hiemalis, Rhizopus stolonifer,* and *Saccharomyces cerevisiae.* In the latter case, we have shown[3] that this yeast, unlike *N. crassa,* fails to degrade L-tryptophan rapidly, and rather than excreting anthranilate it excretes only 3-hydroxyanthranilate. Moreover, as predicted, the single constitutive enzyme it contains has a K_m for L-3-hydroxykynurenine 100 times lower than that for L-kynurenine.

As the above observations were made we became aware of the interest in 3-hydroxyanthranilate as a potential carcinogen. Therefore, since *S. cerevisiae* is used in the manufacture of bread, beer, and wine, we

attempted to determine whether 3-hydroxyanthranilate occurs in these products. The assay consisted of acidifying the product or its aqueous extract to pH 2.0, extracting it with an equal volume of ethyl acetate containing 0.1% 2-mercaptoethanol, separating and concentrating the organic layer 50-fold by evaporating it under vacuum, and chromatographing 1 to 10 λ of the concentrate on Whatman #1 filter paper with butanol, propanol, water, NH_4OH, and 2-mercaptoethanol (25:50:25:0.25:0.25). The resultant chromatogram was examined for fluorescence corresponding to bona-fide 3-hydroxyanthranilate. Quantitation was accomplished by reading the fluorescence of the spots directly with a Turner fluorometer. With 1 to 10 nmoles of 3-hydroxyanthranilate per spot, we could quantitatively determine 1 to 10 nmoles per ml of starting material. However, with this method we have been unable to detect any evidence of 3-hydroxyanthranilate in the foods mentioned. Unfortunately, beer and wine contain numerous extractable fluorescent substances which interfere with this assay. Further purification would be necessary for accurate quantitation, but until the carcinogenicity of exogenous 3-hydroxyanthranilate is determined, it probably is unwarranted to attempt further analyses of this type.

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TRYPTOPHAN 2,3-DIOXYGENASE IN *NEUROSPORA CRASSA:* ANSWER TO AN ENIGMA

Anthony Vitto, Jr.,* and F. H. Gaertner

Although some of the earliest work in biochemical genetics was accomplished by Beadle and Tatum on the tryptophan-niacin relationship of *Neurospora crassa,* all previous attempts to study *in vitro* the first enzyme of the pathway in this or in other fungi have been unsuccessful. As a result, many of the interesting questions concerning the structure and function of the tryptophan-to-NAD pathway of yeasts and molds either have been approached indirectly or remain entirely unanswered. Moreover, while it has been established with some certainty that the biosynthesis of NAD in fungi takes place via the oxidation of L-tryptophan to *N*-formyl-L-kynurenine, the failure to assay the enzyme responsible for the initial oxidative step has led to speculation that the reaction mechanism might be unusual, or even that the process might be non-enzymic.[1] In comparison, the enzyme known to be responsible for this reaction in both bacterial and mammalian systems, tryptophan 2,3-dioxygenase, has proven to be easily assayed.

We have found that the past failures to assay the enzyme were due to at least three factors. First, the enzyme cannot be measured in crude extracts, apparently due to the presence of an inhibitor. Thus, the enzyme must be previously purified by DEAE-cellulose chromatography. Second, the enzyme exhibits an absolute requirement for ascorbate. Third, due to an interfering reaction, which exists even in DEAE-cellulose preparations, the enzyme cannot be assayed by the usual spectrophotometric method. A fluorometric assay which we recently developed[2] avoids this latter problem. Our efforts are now directed to characterizing the tryptophan 2,3-dioxygenase of *N. crassa* and to studying its regulatory properties.

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CHORISMATE SYNTHASE OF *NEUROSPORA CRASSA:* SUBSTRATE-DEPENDENT DIAPHORASE ACTIVITY AND REQUIREMENT FOR FLAVIN MONONUCLEOTIDE

G. R. Welch,* K. W. Cole, and F. H. Gaertner

Chorismate synthase was purified from *Neurospora crassa.* The final step was accomplished by preparative polyacrylamide gel electrophoresis. The purity of the enzyme was estimated to be ≥90%. It appears to be active in at least two multimeric states, with a subunit molecular weight of ~55,000. The purified enzyme is absolutely dependent on the presence of a reducing system, which can readily be provided under aerobic conditions by NADPH plus FMN or under stringent anaerobic conditions by dithionite. The following evidence implicates a physiological role for FMN in *N. crassa* chorismate synthase activity: (1) a preferential stimulation of activity by NADPH and FMN over other pyridine and flavin nucleotides, respectively, in both impure and purified enzyme preparations; (2) an alteration of the chromatographic pattern of the enzyme on DEAE-cellulose by the addition of FMN to the elution buffer; (3) an apparent binding of FMN to the enzyme as exhibited by gel filtration in the presence of the substrate, 3-*enol*pyruvylshikimate 5-phosphate; (4)

a requirement for preliminary incubation with FMN, in concert with the substrate, to eliminate a reaction lag (i.e., to activate the enzyme); and (5) a substrate-dependent diaphorase activity exhibited by the purified enzyme preparation in the presence of FMN and NADPH. The observed activation and alteration of chromatographic behavior of chorismate synthase by FMN suggest that the flavin nucleotide influences the conformation of the enzyme. The ability to replace NADPH and FMN with dithionite suggests that FMN mediates the flow of electrons from a source of reducing power (NADPH) to some enzymic site important to the function of the enzyme. Hence, we propose that the diaphorase activity which is apparently intrinsic to chorismate synthase of *N. crassa* may be significant from thestandpoint of catalysis or may have importance as a regulatory mechanism.

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THE *arom* COMPLEX OF *NEUROSPORA CRASSA*: PHYSIOLOGICAL ROLE IN CELLULAR METABOLISM?

G. R. Welch* and F. H. Gaertner

The *arom* multienzyme complex of *N. crassa* and other organisms catalyzes a series of five consecutive reactions common to the biosynthesis of the aromatic amino acids and other important metabolic substances. Specifically, the complex converts 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) to 3-*enol*pyruvyl-shikimate 5-phosphate (DAHP), which is the immediate precursor of the important branch-point compound, chorismate. Our general goal is the determination of the possible physiological significance of multienzyme complexes in the evolution of cellular metabilism. In view of this aim, our present focus concerns the nature of the observed catalytic facilitation by the *arom* complex.[1,2] Our main experimental emphasis in this regard has been kinetic studies.

Preliminary efforts have been directed toward two projects. Firstly, we have obtained sizeable quantities of purified *arom* complex. The final step in purification was achieved by polyacrylamide gel electrophoresis. Secondly, we have developed methods for the synthesis and purification of the *arom* complex pathway intermediates – DAHP, 5-dehydroquinic acid, 5-dehydroshikimic acid, shikimate 5-phosphate, and ES-5-P – the obtainment of which is necessary for a detailed kinetic analysis of the *arom* complex.

Substantive findings concerning the *arom* complex relate to four main areas: (1) coordinated activation as a means of catalytic facilitation, (2) kinetic studies, (3) integration of the *arom* multienzyme complex into the overall enzymic scheme (viz., erythrose-4-phosphate + phospho*enol*pyruvate → chorismate) connecting glycolysis and aromatic amino acid biosynthesis, and (4) theoretical aspects. With regard to the first category, we have shown that at least two of the constituent enzymic activities (dehydroshikimate reductase and ES-5-P synthase) of the *arom* complex can be activated by the *first* substrate (i.e., DAHP). The possible role of single-ligand-induced, coordinated activation of the *arom* complex in catalytic facilitation has been suggested previously.[1,2] Further studies will examine the potential activation of other constituent *arom* complex activities.

Kinetic studies have been somewhat limited until now, pending obtainment of preparations of intermediate substrates. Preliminary results with purified *arom* complex indicate that the last enzymes (ES-5-P synthase) of the sequence possesses the lowest apparent V_{max}. Moreover, when activated by the first substrate, the overall flux (DAHP → ES-5-P) through the complex is still controlled by the (activated) V_{max} of ES-5-P synthase. Work currently in progress seeks to determine, on a purely kinetic basis, whether or not the *arom* complex facilitates catalysis over the hypothetical unaggregated enzyme system.

Thirdly, we have attempted to examine possible physical relationships between the *arom* multienzyme complex and the metabolically proximal enzymes, DAHP synthase and chorismate synthase. Initial results have revealed no *in vitro* physical association of these three components. However, studies have shown that both DAHP synthase[3] and chorismate synthase[4] are markedly activated by the substrates phospho*enol*pyruvate and ES-5-P, respectively. Thus, the picture emerging from this work may reveal that each enzymic moiety in the overall pathway (erythrose-4-phosphate + phospho*enol*pyruvate → chorismate) is subject to substrate activation *in vitro*. The possible physiological (or *in vivo*) significance of this consistency remains to be seen.

Theoretical studies to date have treated the concept of multienzyme complexes and their evolutionary development from the viewpoint of thermodynamics of irreversible processes. Results obtained from a study of the catalytic properties of various naturally occurring multienzyme complexes (e.g., *arom* complex) depict these aggregates thermodynamically as "dissipative structures."[5] "Dissipative structure" represents physi-

cochemical ordering in space and time arising under conditions far from thermodynamic equilibrium and maintained by a continuous flow of matter and energy from the surroundings. In the case of multienzyme complexes, this "structure" apparently emerged at some point along the evolutionary scale and was preserved during further evolution by the introduction of physical bonding among the respective enzymes. With this view in mind, multienzyme complexes fit into an evolutionary scheme[5] wherein the evolution of existing biological order takes place in discrete steps from a completely homogeneous system toward more and more complex and organized states ("dissipative structures").

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ESTROGEN-INDUCED CHANGES IN THE LIVERS OF MALE *XENOPUS LAEVIS*

J. N. Dumont and G. S. Spady*

The synthesis of vitellogenin, a specific yolk-protein precursor, can be induced in both male and female *Xenopus* by injections of estradiol-17β. The yolk-protein precursor molecule is synthesized in the liver and secreted into the blood where it accumulates. In females, injections of chorionic gonadotropin induce endocytotic uptake of vitellogenin by developing oocytes. The incorporated protein becomes the major constituents, phosvitin and lipovitellin, of the yolk platelets. Modifications of the ultrastructure of the liver are readily apparent in estrogen-treated frogs. The liver cells of control, nonestrogenized frogs contain large stores of glycogen, scant amounts of endoplasmic reticulum, a few mitochondria, and a nucleus with a small nucleolus. The Golgi complexes, located in the apex of the cell near the bile canaliculi, contain dense material and are presumably involved in the formation of lysosomes. In liver cells from estrogen-treated frogs there is a dramatic proliferation of ribosome-studded endoplasmic reticulum and a depletion of the glycogen stores. The nuclei become rounded and there is an increase in the size and density of the nucleoli. At the present time there is little information on the mechanism by which vitellogenin is secreted from the liver. However, in tissue from hormone-treated animals, the cisternae of the endoplasmic reticulum are enlarged and contain a dense material which may be interpreted as synthesized vitellogenin. These enlarged cisternae lose their ribosomes, become smooth-surfaced vesicles, become closely associated with the lateral and basal cell membrane, and appear to fuse with it. Consequently, the material contained in the endoplasmic-reticulum-derived vesicles appears to be secreted directly into the extracellular space of Disse, from which it eventually reaches the circulation.

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LOCALIZATION OF MEMBRANE CHARGES ON THE SURFACE OF *XENOPUS* OOCYTES

Anna R. Brummett* and J. N. Dumont

Developing oocytes and coelomic eggs of mature *Xenopus* females treated with human chorionic gonadotropin were fixed in glutaraldehyde and subsequently treated with a positively charged solution of colloidal iron at pH 1.25. Electron microscope studies revealed that the positively charged particles of iron are localized almost exclusively on the distal portions of the microvilli of the developing oocytes. No iron particles are observed in micropinocytotic pits of the oocytes. Micropinocytotic pits on follicle cells, however, are labeled with the iron. Iron localization on the oocyte is particularly striking at that stage of development when the oocyte exhibits the greatest amount of surface activity, as evidenced by the presence of many micropinocytotic pits and vesicles and a large number of microvilli. As the oocyte matures and surface activity decreases, the localization becomes less specific until, in the coelomic egg, the iron appears evenly distributed over the now essentially nonmicrovillous surface.

The presence of the negative charges on the membrane is assumed to be due to sialic acid moieties of surface mucopolysaccharides. This assumption was borne out by treatment of the unfixed oocyte with neuraminidase. Oocytes subjected to the enzyme and subsequently fixed and treated with positively charged colloidal iron showed marked reduction in the amount of iron present. The amount of reduction correlated directly with enzyme concentration and length of treatment.

Localization of anionic sites on the distal parts of the microvilli and the absence of such negatively charged sites in the micropinocytotic pits suggests that the pits on the oocyte are involved only in the uptake of negatively charged or perhaps neutral molecules. The microvilli, on the other hand, may be involved in the uptake of positively charged small molecules or ions, or they may play some other as yet unelucidated role in the developing oocyte. Experiments designed to localize positively charged sites on the oocyte membrane were also undertaken. For example, the uptake of the yolk-precursor protein, vitellogenin, by *Xenopus* oocyte has been investigated. Vitellogenin carries a strong negative charge at physiological pH. Oocytes were incubated with labeled vitellogenin (specific activity 1845 dpm/μg) for 20 and 40 min. Although no label could be detected in micropinocytotic pits on the cell membrane, label was localized over internalized yolk bodies.

The polyanionic substance, poly(sodium styrene sulfonate) was used in an attempt to localize positively charged membrane sites. Following treatment of the oocyte with this polyanion, lead nitrate was used to produce a product which would be visible with the electron microscope. The results of preliminary experiments indicate the presence of cationic sites on the microvilli of the follicle cells and at points where follicle-cell microvilli make close contact with the microvilli of the oocyte.

Negatively charged colloidal iron has been prepared, and experiments are in progress to determine whether it can be used to localize positively charged membrane sites.

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AMINO ALCOHOL ANTIFERTILITY AGENTS

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We have shown that 2-*N,N*-diethylaminoethanol (DEAE) and several other related compounds (procaine, *N*-piperidine ethanol, 2-piperidine ethanol, and iminodiethanol) are nontoxic antifertility agents in female mice (CD-1 White Swiss) and rats (Sprague-Dawley) when administered in the animals' drinking water. DEAE was totally effective in preventing pregnancy in mice in the range 0.01 *M* to 0.05 *M*. Pathological examination of these mice revealed no lesions or abnormalities. After cessation of treatment, fertility was restored in less than 24 hr, and the offspring were normal and fertile. The effective time of administering DEAE was from day 0 to day 3 from mating, designating day 0 as the day a vaginal plug was observed. Thus it appears that DEAE affects the early preimplantation embryos.

DEAE up to 0.05 *M* does not affect the estrous cycle of mice as demonstrated by daily vaginal smears, although 0.1 *M* appears to prolong the cycle in rats. DEAE at 0.05 *M* in mice does not interfere with mating, ovulation, or fertilization as demonstrated by the presence of multicell embryos in oviductal and uterine flushings. This is a good indication that DEAE does not operate by hormonal action.

DEAE was further shown to be nonestrogenic as demonstrated by ovariectomy experiments. Two groups of mice were ovariectomized, one group receiving 0.01 *M* DEAE, the other water. Two other groups were kept on similar regimes but not ovariectomized. After six weeks, uterine weights of the four groups were compared. No statistically significant differences in weight were observed either for the two ovariectomized groups or for the two nonovariectomized groups. However, the uteri of the former were severely atrophied as expected.

Studies of uptake of tritiated lysine by HeLa cells in the presence of various concentrations of DEAE showed no marked effect on the rate of protein synthesis below 0.01 *M*. These experiments suggest that DEAE probably does not interfere with normal metabolic processes. In support of this is the observation that 0.01 *M* or 0.05 *M* DEAE in drinking water over a two-month period does not interfere with the growth rates of young mice.

To determine whether DEAE and related compounds affect embryonic development directly, *in vitro* culture of mouse embryos in the presence of various concentrations of these compounds is currently under way. $BC3F_1$/Cum females are superovulated in the standard manner, and embryos are cultivated in Brinster's medium. Preliminary results indicate that compounds which are effective antifertility agents (DEAE, procaine) inhibit growth at $\sim 10^{-4}$ *M*, but that triethylamine, which is not an antifertility agent *in vivo*, has little effect.

Scanning electron microscopic studies have been performed on preimplantation mouse embryos from mothers on 0.05 *M* DEAE (experimental) and from those on normal drinking water (control). No obvious changes in microvilli shape, number, or distribution or changes in blastomere shape are observed at day 0 and day 1. However, at day 2 many of the blastomeres become irregular in shape and develop ridges or folds in their surfaces. Furthermore, the blastomeres of embryos from DEAE-treated mothers tend to separate.

Scanning electron microscope studies of embryos from *in vitro* experiments reveal that DEAE has effects similar to those seen *in vivo*, indicating that DEAE acts directly.

Transmission electron microscopic studies of preimplantation mouse embryos are also being performed in order to observe any ultrastructural changes which may occur due to the action of DEAE. This type of information may lend some insight into the mechanism of action of DEAE. Embryos have been collected from mothers on DEAE and from mothers on normal drinking water on days 0, 1, and 2. Initial studies reveal that there are no obvious ultrastructural changes due to DEAE in the blastomeres of embryos at day 1.

Once the mechanism of action of DEAE is understood, the use of this agent as a human postcoital contraceptive may be considered. If this compound is found not to be acceptable as a contraceptive, it will serve as a tool to study early embryonic development.

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CELL-TYPE CONVERSION AND GALACTOSYLTRANSFERASE IN LENS REGENERATION

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It has been demonstrated that lens fiber differentiation, which follows lentectomy in the adult newt *Triturus viridescens*, is characterized by a unique pattern of cell adhesion.[1,2] Recently it has been proposed that in cell-to-cell adhesion, complex carbohydrates and glycosyltransferases play basic roles as complementary molecules on the cell surface.[3] The glycosyltransferases which appear to be located at the cell surface might be the controlling mechanism of the pattern of intercellular adhesion. A remarkable alteration of the activity level of the enzyme during fiber differentiation demonstrated in the present work suggests that the enzyme is involved in the development of intercellular adhesion.

Endogenous galactosyltransferase activity of newt dorsal iris as a function of time after lentectomy. This series of experiments was designed to test whether changes in the endogenous activity of galactosyltransferase occur in association with the cell transformation taking place after lentectomy in the dorsal iris of the adult newt. The results shown in Table 9 indicate a progressive increase of the endogenous activity of galactosyltransferase from day 3 to day 17 after lentectomy.

Table 9. Galactosyl transferase activity as a function of time after lentectomy: endogenous acceptors

Tissue	Time	Activity	
		cpm/mg DNA/hr	cpm/mg protein/hr
Dorsal iris	Day 3	210 ± 65	16.5 ± 6.5
Dorsal iris	Day 10	768 ± 188	67.5 ± 12.5
Dorsal iris	Day 17	1424 ± 309	133.0 ± 55

Exogenous galactosyltransferase activity as a function of time after lentectomy. The increase in enzyme activity observed in the previous series could have resulted from a genuine increase in enzyme activity, an increase in the availability of the endogenous acceptors, or both. To minimize the influence of the endogenous acceptors, a constant amount of *N*-acetylglucosamine was added to the incubation mixtures containing the homogenates (according to the technique of Roth and White).[4] The results of these experiments show that for dorsal irises of days 0, 3, 10, and 17, there is a marked increase in galactosyltransferase activity as compared with the values obtained using endogenous acceptors for corresponding days. The levels of galactosyltransferase activity at different times after lentectomy indicated the following changes: first, a progressive increase, corresponding to the first three days after lentectomy; then, a period of high activity, distinguished on day 10 to day 17; and finally, a decline of enzyme activity, noticed at day 41. Contrasting with this decline in activity of the dorsal iris, the day-41 lens regenerate revealed the most marked increase of the entire series. With few exceptions, the changes of galactosyltransferase activity per milligram of protein parallel those of the previous series (Fig. 13).

Previous studies revealed that there are changes in the pattern of DNA and protein synthesis in close association with the process of regeneration of the lens following lentectomy.[5] To assess the influence of these changes on the values obtained for the previous two series, galactosyltransferase activity is expressed per unit of tissue, as shown in Fig. 14. The activity pattern observed for the dorsal iris in the previous series is confirmed. The most striking increase was again observed in the day-41 regenerate. In subsequent experiments not depicted in the figures, the day-150 regenerates revealed activity values approaching that of the adult lens.

The changes in activity pattern are closely associated with the changes in intercellular adhesion occurring during lens regeneration. When the iris cell loses its phenotypic expression and acquires a new develop-

mental pathway, there is an increase in intercellular contact associated with an increase in galactosyltransferase activity. The activity remains high with the onset of fiber differentiation, to decline subsequently when a considerable loss of cell membrane and of intercellular contact occurs. The results are therefore consistent with the concept that galactosyltransferase is involved in the development of intercellular adhesion.

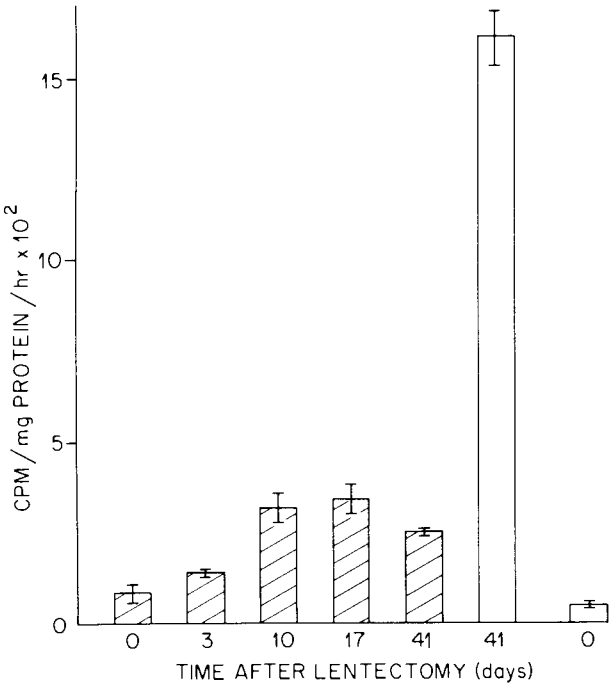

Fig. 13. Galactosyltransferase activity as a function of time after lentectomy. Bars indicate standard error of the mean.

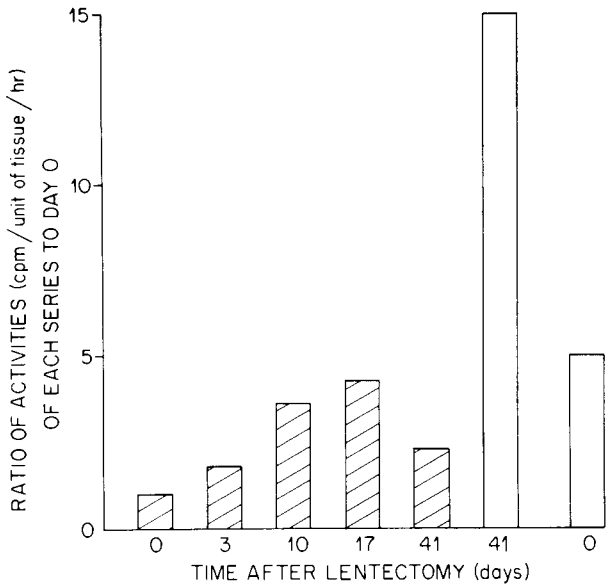

Fig. 14. Galactosyltransferase activity per unit of tissue as a function of time after lentectomy.

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INDUCTION OF VITELLOGENIN SYNTHESIS AND SECRETION *IN VITRO*

R. A. Wallace and E. W. Bergink*

Over the past few years we have made numerous attempts to induce vitellogenin synthesis *in vitro*. These attempts have included the incubation of male liver slices in a complete medium supplemented with "normal" serum or serum from hormone-treated males and females and containing estradiol either alone or in combination with other steroids and gonadotropins. We monitored the medium over at least a three-day period, and in no case did we observe the appearance of significant amounts of phosphoprotein.

Within the past year, we have achieved a modest success in this effort by changing to liver slices from males given estrogen at least once previously. We had previously found that when adult males were given 1 mg estradiol, vitellogenin synthesis and secretion into the bloodstream were induced for about 18 days, after which they appeared to end. Yet if liver slices from such animals were cultured as much as six months after the original injection of estrogen, a small amount of phosphoprotein was found to be released into the medium with time. Furthermore, in the presence of estrogen, a distinct increase in the release of labeled phosphoprotein occurred. Electrophoresis of samples of the culture media on SDS-polyacrylamide gels indicated that a small amount of vitellogenin polypeptide had been secreted in the absence of estrogen and a much larger amount had been secreted in the presence of estrogen. These events have never been observed in liver slices from unprimed males.

It would appear that estrogen has several types of action: (1) in unprimed liver (males), which apparently synthesizes *no* vitellogenin, a differentiation step is made whereby the liver cells become more sensitive to the presence of estrogen; and (2) once differentiated, a basal level of vitellogenin synthesis takes place which can be modulated by the presence of estrogen. The differentiation step (possibly involving a cell-cycle phenomenon or the synthesis of estrogen receptors)

cannot as yet be induced *in vitro,* whereas the modulation can to some extent.

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STEROID INHIBITION OF PROTEIN INCORPORATION BY ISOLATED AMPHIBIAN OOCYTES

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To examine the effect of steroids on protein incorporation by oocytes, we incubated fully grown *Rana pipiens* oocytes *in vitro* with radioactively labeled yolk precursor ([^{3}H]vitellogenin) isolated from the serum of estrogen-treated *Xenopus laevis.* Incorporation of [^{3}H]-vitellogenin into oocytes proceeded in a linear manner over a 24-hr period in the absence of steroid. Oocytes simultaneously exposed to deoxycortisone acetate and to [^{3}H]vitellogenin exhibited a stimulation of nuclear maturation and cortical changes. Inhibition of [^{3}H]-vitellogenin incorporation was observed after approximately 9 hr of incubation. Preincubation of oocytes in steroid for 9 hr essentially abolished subsequent [^{3}H]-vitellogenin incorporation.

These results demonstrate that a macromolecular transport system remains operative in fully grown oocytes, and that a steroid hormone can directly regulate this transport system. Additional electron-microscopic evidence suggests that the mechanism of steroid inhibition involves the suppression of the micropinocytotic process in the oocyte cortex.

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DNA POLYMERASE ACTIVITY IN THE GERMINAL VESICLE OF THE *XENOPUS* OOCYTE

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During oogenesis, the amphibian oocyte accumulates and stores many different materials. Some of these are 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.

With the exception of nucleolar and mitochondrial DNA, there is no measurable DNA synthesis and storage during oogenesis. The lack of synthesis could simply reflect the absence of specific DNA polymerases in the oocyte. In fact, it has been reported that DNA-synthesizing activity is absent in the oocyte (both nucleus and cytoplasm) and appears only a few hours prior to fertilization.[1] Since this situation appeared to be very different from that known for RNA polymerases, which are made during oogenesis and stored in the oocyte,[2] we looked for DNA-polymerizing activity in the *Xenopus* oocyte.

We found enough DNA-polymerizing activity in a single oocyte to incorporate about 100 pmoles of [^{3}H]dAMP when assayed with poly(rA)·poly(dT) template using the assay procedures of Brun.[3] Incorporation was linear for at least 30 min on the 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 template or when poly(rA) was used in place of the poly(rA)·poly(dT).

The DNA-polymerizing activity appears similar to DNA polymerase I from chick.[3] The incorporation of [^{3}H]dTMP is only 6% that of [^{3}H]dAMP when the poly(rA)·poly(dT) template is used. The activity sediments in both sucrose and glycerol gradients in a manner similar to chick and mammalian DNA polymerase I. The enzyme activity increases during oogenesis. Stage-VI oocyte germinal vesicles[4] have almost 70 times more activity than those from stage-III oocytes. This represents about a tenfold increase in nuclear concentration (activity per nl) during oogenesis. In the full-grown oocyte, about 95% of the enzyme activity was found in the germinal vesicle (oocyte nucleus). No evidence for substantial amounts of DNA polymerase II activity has been seen in either the nucleus or cytoplasm; the possibility that this type of activity is present has not been eliminated. Taken together, this preliminary evidence indicates that DNA polymerase activity is made during oogenesis and stored for use after fertilization. Control of DNA synthesis is probably through mechanisms other than new synthesis of DNA polymerase.

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FACILITATION OF PROGESTERONE-INDUCED MATURATION IN *XENOPUS* OOCYTES BY OUABAIN AND POTASSIUM DEPRIVATION

Anthony Vitto, Jr.,* and R. A. Wallace

We have found that ouabain, at a concentration (10^{-5} *M*) which effectively suppresses Rb^+ transport into isolated, full-grown oocytes, facilitates progesterone-stimulated maturation: germinal-vesicle breakdown occurs several hours sooner in maximally stimulated oocytes or occurs with increased frequency in marginally stimulated oocytes. This facilitation does not involve an increased uptake of progesterone and can be mimicked by placing stimulated oocytes in a K^+-depleted medium. The effect of ouabain seems fairly specific, since a variety of other metabolic inhibitors were found simply to inhibit progesterone-induced maturation.

We therefore propose that (1) progesterone stimulation initiates a chain of reactions which gives rise to a cessation of active K^+ transport and a concomitant increase in Na^+ influx, (2) the resulting alteration in the intracellular ionic environment is a prerequisite for the subsequent events of oocyte maturation, and (3) the attainment of this condition is normally the rate-limiting process for oocyte maturation.

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PROTEIN PHOSPHORYLATION DURING OOCYTE MATURATION

R. A. Wallace

A recent report by Morrill and Murphy[1] suggested that the "release of prophase block at ovulation is associated with intense protein phosphorylation." They further found that "the principal protein species phosphorylated between meiotic prophase and the second metaphase appears to be the phosphoprotein phosvitin." Their study was done by injecting $^{32}P_i$ into intact, gonadotropin-stimulated female *Rana pipiens* and subsequently analyzing nonovulated oocytes and ovulated eggs.

The macromolecular precursor for phosvitin is vitellogenin. Our own experience has indicated that (1) vitellogenin is synthesized, phosphorylated, and secreted by the liver into the bloodstream, (2) all normal females do this to some extent, (3) circulating vitellogenin is selectively sequestered by growing oocytes and converted by proteolysis to lipovitellin and phosvitin, (4) even fully developed oocytes sequester vitellogenin to some extent, and (5) ovulation of fully developed oocytes follows gonadotropin administration by a time period which is sufficient to allow simultaneously administered $^{32}P_i$ to become incorporated into newly synthesized vitellogenin and for the latter to be subsequently transported to the oocyte and converted to [^{32}P] phosvitin.

It thus appeared desirable to repeat Morrill and Murphy's study, but with oocytes *in vitro*. Accordingly, isolated oocytes were preincubated in $^{32}P_i$ for several hours, after which half were stimulated to undergo maturation by adding progesterone (5 mg/ml) to the medium. Analysis of progesterone-stimulated oocytes indicated that there was an increased rate of protein phosphorylation relative to control oocytes. High-salt extracts of oocytes were then chromatographed on TEAE-cellulose to indicate labeled protein. The increased amount of labeled material in progesterone-treated oocytes was found (1) initially percolating through the column, (2) between the lipovitellin and phosvitin peaks, and (3) eluting with the wash solution at the end of the run. Phosvitin was unlabeled in both control and progesterone-stimulated oocytes.

We thus conclude that there is indeed an increase in the amount of protein phosphorylation during oocyte maturation, but that the specific proteins phosphorylated do not appear to involve phosvitin and remain to be identified.

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STRUCTURE OF DNA SYNTHESIZED IN COMPETENT *HAEMOPHILUS INFLUENZAE* CELLS

J. E. LeClerc* and Jane K. Setlow

One of the puzzling aspects of transformation in *H. influenzae* has been the question of how specific pairing between donor and recipient DNA can occur between two double-stranded DNA molecules, since donor DNA is not found in single-strand form inside the cell. A partial answer to this question is provided by our discovery that the DNA synthesized by the competent cells of most strains of *H. influenzae* contains single-strand regions. The exception is the very recombination-defective mutant *rec2*, in which transforming DNA taken up by the cell does not interact with the cell genome.

Cell DNA has been labeled with [^{14}C] dThd before the competence regime, and then pulse-labeled with [^{3}H] dThd at the peak of competence. The cells were

then either lysed directly on top of an alkaline sucrose gradient, or lysed before being placed as native DNA on a benzoylated naphthoylated DEAE-cellulose column, and eluted with a caffeine-NaCl gradient, which permits the separation of double-stranded DNA containing single-strand regions from completely double-stranded DNA. As judged by sedimentation of the DNA in alkali, the single-strand molecular weight of the newly synthesized DNA in competent wild-type cells is about 5×10^7, but the DNA strand synthesized before the competence regime is about four times bigger. These data indicate that the DNA synthesized in the competent state contains gaps or nicks. The BND chromatography showed that about 30% of the prelabeled DNA and about 75% of the pulse-labeled DNA elutes in the position of DNA containing single-strand regions.

During the competence regime, the DNA was found to undergo slightly less than one round of replication. Thus the ^{3}H pulse-label is mostly in a small part or parts of an unlabeled strand opposite the ^{14}C-labeled strand. Experiments in which cells at the peak of competence were pulse-labeled with [^{3}H] BrdUrd and the DNA was sedimented in alkaline CsCl suggest that most of the pulse-label is distributed in a series of short, dispersed regions in the strand synthesized during the competence regime, as though this synthesis were a type of repair replication.

Further information on the structure of the DNA in competent cells has been obtained with the use of SI deoxyribonuclease that we have partially purified from *Aspergillus oryzae*. This enzyme is specific for single-strand DNA but does not degrade native *H. influenzae* DNA. The DNA containing single-strand regions that was isolated from a BND column was treated with the enzyme, and 23% of the ^{3}H-labeled and 15% of the ^{14}C-labeled material was degraded, suggesting that there are single-strand gaps opposite the ^{14}C strand, and also single-strand tails containing ^{3}H.

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MOLECULAR MECHANISM OF TRANSFORMATION IN *HAEMOPHILUS INFLUENZAE*

J. E. LeClerc* and Jane K. Setlow

To investigate the molecular basis of recombination in transformation, in which a single strand of transforming DNA is inserted into the recipient cell genome, we have performed the following types of experiments with wild-type, transformation-deficient, and temperature-sensitive mutant recipient cells. (1) ^{32}P-labeled transforming DNA taken up by competent cells has been extracted and tested for single-strand regions by digestion with SI nuclease. (2) Competent cells either labeled with [^{3}H] dThd before the competence regime or pulse-labeled at the peak of competence have been exposed to ^{32}P-labeled transforming DNA, incubated for various times, and either lysed directly on top of an alkaline sucrose gradient for sedimentation or lysed and the DNA then sedimented in neutral gradients. (3) Competent cells labeled with [^{14}C] dThd before the competence regime and pulse-labeled with [^{3}H] dThd at the peak of competence have been exposed to unlabeled transforming DNA, and the release of the two labels into the medium has been measured as a function of time of incubation of the transforming cells.

The results have provided the following new information on the mechanism of transformation. (1) The transforming DNA after it enters the cell, whether or not it can interact with the cell's DNA, becomes 10–15% degradable with the single-strand-specific nuclease. We assume that the transforming DNA becomes single-stranded at its ends. The enzyme responsible for the conversion is not the ATP-dependent nuclease, since the transforming DNA also becomes partly degradable in a strain which lacks this enzyme. (2) The transforming DNA interacts and forms covalent bonds with recipient DNA in all of our strains except *rec2*, whose DNA does not contain single-strand regions when the cells are competent. Thus the single-strand regions in the DNA of competent cells are essential for an early stage in recombination, presumably for pairing with the single-strand regions of transforming DNA. (3) When the transforming DNA interacts with cell DNA, the strand labeled before the competence regime is broken, except in the strain which lacks the ATP-dependent nuclease. We conclude that the small (around 50%) loss of transformability in this strain results from its inability to insert transforming DNA into the strand synthesized before the competence regime, whereas in the wild type there can be recombination between transforming DNA and either of the two recipient strands (and the label from either strand is found in the medium during transformation). (4) There is an intermediate stage in interaction of transforming DNA and cell DNA in which there is no covalent bonding between the DNAs, as judged by the difference between sedimentation patterns in neutral and alkaline sucrose. (5) Transforming DNA first becomes covalently linked with cell DNA of intermediate single-strand length. The sealing of the DNA into high-molecular-weight recipient DNA is more rapid

when DNA synthesis is not occurring (in a mutant temperature-sensitive for DNA synthesis, at the restrictive temperature). (6) In the *rec1* mutant, there is degradation of recipient DNA at the site of the interaction between the DNAs, which prevents the sealing of transforming DNA into high-molecular-weight DNA.

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ULTRAVIOLET SENSITIVITY OF COMPETENT *HAEMOPHILUS INFLUENZAE* CELLS

M. E. Boling and Jane K. Setlow

A number of years ago we found that wild-type cells in the competent state are considerably more sensitive to ultraviolet radiation than exponentially growing or stationary cells. Since the recent discovery that the DNA of competent wild type as well as the mutant *rec1* contains single-strand regions, but not *rec2*, it was of interest to determine whether the enhanced ultraviolet sensitivity of competent cells was correlated with the single-strand regions in the DNA.

Comparison of the ultraviolet sensitivities of competent and growing *rec1* and *rec2* cultures has shown that *rec1* is more sensitive in the competent state, whereas *rec2* has the same sensitivity. We conclude that the existence of single-strand regions in the DNA of competent cells can account for the enhanced sensitivity, and that the excision mechanism is unable to repair damage in single-strand regions of DNA, in accord with the fact that only excision-proficient cells show enhanced ultraviolet sensitivity.

INACTIVATION OF TRANSFORMING DNA BY LONG-WAVELENGTH UV RADIATION

Emiliano Cabrera-Juárez, Jane K. Setlow, and P. A. Swenson

Four aspects of the inactivation of transforming DNA by long-wavelength irradiation have been explored: (1) the action spectrum, with comparisons of the efficiency of inactivation by 254-, 290-, 313-, 334-, 365-, 405-, and 460-nm radiations; (2) the photoreactivability of DNA inactivated by these wavelengths, which is a measure of the fraction of the biological damage that results from pyrimidine dimers; (3) the ability of the cell to repair the damage to transforming DNA by the excision mechanism, as judged by differences in survival of transforming ability in wild-type and excision-defective hosts; and (4) the effect of 0.2 *M* histidine during irradiation on the biological inactivation.

Since very large doses are needed for inactivation of transforming DNA at the long wavelengths, it was necessary to use the radiation facilities at the Argonne National Laboratory to obtain sufficiently high intensities. Transforming DNA from *Haemophilus influenzae*, purified to eliminate photosensitizing contaminants, has been exposed to monochromatic radiation, with the help of Dr. M. J. Peak at the Argonne Laboratory. At all the wavelengths, the inactivation kinetics fitted a square-root relationship between survival and dose. The sensitivity of the DNA to inactivation varied over a factor of 10^6 from 254 to 460 nm. The action spectrum dropped steeply from 254 to 334 nm, and then declined gradually. Photoreactivation with yeast photolyase was not observed following inactivation at wavelengths longer than 313 nm, indicating that one or more lesions other than pyrimidine dimers are responsible for inactivation at these wavelengths. Lower survival of the transforming activity in the excision-deficient mutant compared to the wild type was observed at all wavelengths, but there was a larger difference following 254-nm irradiation than at the longer wavelengths.

MUTATION OF TRANSFORMING DNA BY LONG-WAVELENGTH UV RADIATION

Emiliano Cabrera-Juárez and Jane K. Setlow

Since we have over the past six years learned a great deal about the mechanisms of inactivation of DNA by ultraviolet radiation and its repair by the *Haemophilus influenzae* cell, it has been of interest to attempt to develop an *in vitro* mutation system with uv irradiation of transforming DNA. However, we have been unable to detect mutations in *H. influenzae* cells irradiated at 254 nm, and all attempts to mutagenize transforming DNA by irradiation at this wavelength have failed. Thus it has been very satisfying to discover that *H. influenzae* transforming DNA is mutated by long-wavelength uv radiation.

Transforming DNA bearing a streptomycin-resistance marker was irradiated and then used to transform wild-type cells, and streptomycin-resistant transformants were selected. If mutations have been induced in the novobiocin locus, which is closely linked to the streptomycin marker, there is a high probability that these mutations would be cotransformed along with the streptomycin markers.

The largest amount of mutation was observed after 334-nm irradiation. There was less at 313 and 365 nm,

and none at 290, 405, or 460 nm. When the DNA was irradiated in the presence of 0.2 *M* histidine, the observed mutation was enhanced, whereas there was less inactivation of transforming ability. Thus it is clear that the photochemical alteration responsible for the mutation induced at long wavelengths is different from the inactivating lesion, and probably does not involve radical formation. Thus the excision mechanism operates on repair of the nondimer damage at long wavelengths but less efficiently than on the dimer damage at the short wavelengths.

Histidine during irradiation has little or no effect at the short wavelengths but protects transforming DNA from biological damage at 365 nm and longer wavelengths. Since histidine does not absorb at the longer wavelengths, we conclude that it protects by radical scavenging, and thus it is probable that some of the biological damage at the long wavelengths involves radical formation.

CHEMICAL AND BIOLOGICAL EFFECTS OF *CIS*-DICHLORODIAMMINEPLATINUM(II), AN ANTITUMOR AGENT, ON DNA

Linda L. Munchausen*

cis-Dichlorodiammineplatinum(II) binds irreversibly to the bases in DNA, and the amount of platinum complex bound can be determined from changes in the ultraviolet absorption spectrum. The maximum amount bound at saturation increases linearly with increasing G + C content of the DNA. As the ratio of platinum to phosphate is increased, an increasing inactivation of transforming DNA from *Haemophilus influenzae* is observed. At a ratio which corresponds to spectrometric saturation, transforming activity is inactivated more than 10^5-fold. The *trans* isomer of the platinum complex, which is not effective against tumors, induces a similar inactivation of transforming DNA but with half the efficiency, indicating a different mode of binding. The sensitivity to inactivation by *cis* isomer varies slightly with the genetic marker assayed but is not dependent on the excision-repair system. Uptake of DNA by competent cells is unaffected by bound platinum complex; however, integration of platinum-bound transforming DNA into the host genome decreases as the mole fraction of platinum increases. This loss of integration parallels the decreased transforming activity of the DNA. Although the drug induces interstrand cross-links in DNA *in vitro*, these cross-links are relatively rare events and cannot account for the observed inactivation of transforming ability.

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ON THE MECHANISM OF MUTAGENESIS BY NITROSO COMPOUNDS

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The temperature dependence of mutation of *H. influenzae* by nitroso compounds has been investigated. There is no observable mutation after treatment at 4°C and there is an increase in induced mutation between 25 and 45°C. The mutation frequency can be considerably increased by leaving the compounds in basic solution at 0°C, room temperature, or 37°C before the cells are treated at 37°C. As a function of time of "activation" of the compounds, regardless of temperature, the mutation frequency and the lethal effect increase to a maximum and then decrease. These data suggest that the active component of nitroso compounds for mutagenesis and killing is not the original compound but a derivative formed in the presence of hydroxyl ions.

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OSMIUM TETRAOXIDE INACTIVATION OF TRANSFORMING DNA

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DNA treated with osmium tetraoxide contains an alteration in the thymine residues which is chromatographically similar to an alteration made in DNA by ionizing radiation. However, unlike ionizing radiation, OsO_4 does not form strand breaks in DNA. Thus it becomes of interest to correlate the OsO_4 damage with biological inactivation of transforming DNA from *Haemophilus influenzae* and to determine whether such damage can be repaired by the cell.

Tritium-labeled transforming DNA has been treated with OsO_4 for various times, and the number of thymine alterations has been determined, on the same samples used for measurement of transforming ability with two genetic markers on the DNA. The results show that there is a dose-dependent inactivation, slightly greater for the uv-sensitive streptomycin marker than for the uv-resistant novobiocin marker, and that conversion of 2.4% of the thymine to product causes around 80% inactivation. There is slightly more apparent inactivation of the transforming DNA as assayed on a strain deficient in excision of pyrimidine dimers than on the wild type. It is concluded that the excision

mechanism can repair a small fraction of the OsO_4 damage to transforming DNA.

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HOST-CELL RESTRICTION AND MARKER RESCUE

M. E. Boling and Jane K. Setlow

Marker rescue is a type of recombination between transfecting phage DNA which enters a competent cell and DNA injected from mutant phage, in which wild-type markers from the transfecting DNA are rescued by the injected DNA, resulting in wild-type recombinant phage. We earlier discovered a host-cell restriction-modification system in *Haemophilus influenzae*. Some strains (restricting) inhibit growth of phage originally grown on other strains (nonmodifying and nonrestricting), but phage grown on restricting strains can be modified so that they are immune to restriction. These phenomena result from restriction deoxyribonucleases and methylation enzymes that methylate specific sites in phage DNA, so that they are no longer recognized by the restriction enzymes.

To investigate the effect of host-cell restriction on marker rescue with nonmodified transfecting DNA and modified superinfecting DNA in a restricting host, it was necessary to obtain a restricting host that could be used at 34°C, the permissive temperature for the temperature-sensitive phages. Our standard restricting strain would not support wild-type or mutant phage growth at this temperature. Although plaques were produced at 37°C on this host, the efficiencies of the temperature-sensitive phage are markedly reduced at this temperature. A large number of single-colony isolates from our various *H. influenzae* stocks were tested for restriction and ability to grow phage at 34°C. The results showed that these two properties involve independent mutations, each occurring spontaneously in the population with a frequency of 2–7%, and each reverting with a similar frequency. A culture which was predominantly restricting and could also grow phage at 34°C was used for determination of the effect of restriction on marker rescue. The results show that host-cell restriction of the transfecting DNA carrying the wild-type markers has no effect on marker rescue, suggesting either that recombination between injected and transfecting phage DNA is much more rapid than the action of restriction enzymes, or that there are only a small number of nucleolytic cuts made in transfecting DNA by these enzymes, which do not appreciably affect the probability of wild-type recombinants between transfecting and injected phage DNA.

MINICELLS IN *HAEMOPHILUS INFLUENZAE*

Barbara Sedgwick, Jane K. Setlow, and M. E. Boling

Last year we reported the isolation of a minicell-producing strain of *H. influenzae*, LB11, which is to large extent killed when it takes up transforming DNA, because of induction of the defective prophage present in most of our strains of *H. influenzae*.

We have studied the genetic basis for minicell production by two methods. (1) Strain LB11 was transformed with wild-type DNA also containing seven drug-resistance markers. Selection was made for about 600 different clones resistant to these drugs, singly and in combination. These clones were then examined individually for the presence or absence of minicells. (2) Wild-type cells were transformed with DNA from strain LB11, carrying various drug-resistance markers, and transformants were processed as in method 1. The results showed clearly that two mutations are responsible for maximum minicell production. One of these mutations is the same as the thymidine requirement in the parent strain from which LB11 was derived by mutagenesis. After developing more sophisticated methods of looking for minicells, including staining with crystal violet, we found that some minicells were also produced by the parent strain, grown under conditions of limiting nutrients. This strain was isolated as a spontaneous aminopterin-resistant mutant. To investigate further the relationship between thymidine requirement and minicell production, we isolated 71 more spontaneous mutants resistant to aminopterin or trimethoprin, drugs routinely used for isolation of thymidine-requiring strains of other bacterial species. All of these were thymidine-requiring and all formed some minicells. We do not know why minicell production should be associated with thymidine requirement. However, it may be that any disturbance in nucleic acid metabolism results in some minicell production, since even the wild-type strain treated with mitomycin C forms some minicells.

Mapping of the two mutations causing a large production of minicells is complicated by the fact that both mutations are apparently in the region of the defective phage, so that transformation in this region frequently causes induction of the phage, and the potential transformant is lost. Nevertheless, our data suggest that the loci are close to one of the erythromy-

cin markers. Both mutations have been moved to the wild-type strain, singly and in combination. The double mutant is killed by transforming DNA to the same extent as the original strain LB11, and the thymidine requirement alone causes an intermediate level of killing by transformation and defective-phage production.

A purification procedure for minicells has been developed, with differential centrifugation followed by infection with phage, which do not attach to the minicells. There does not appear to be any DNA in the minicells.

REPAIR SYNTHESIS AND THE ROLE OF DNA POLYMERASE I IN X-IRRADIATED *BACILLUS SUBTILIS* MADE PERMEABLE TO NUCLEOTIDES*

Daniel Billen and G. R. Hellermann†

Many studies of DNA synthesis in living cells exposed to x or gamma rays have shown that DNA replication can be inhibited (for review see Okada[1]). Stimulation has been observed *in vitro*,[2] as have regulatory abnormalities,[3] along with little or no inhibition of the DNA-synthesis rate. Unscheduled DNA synthesis can be induced in mammalian cells by x irradiation.[4]

While it is generally accepted that the lesions produced in the DNA are similar in a variety of cellular systems, it is apparent that the results reflect different repair capacities and cell-cycle variations, as well as replicative response to unrepaired damage to the template and/or replicative apparatus.

Toluene treatment of bacteria causes a nonselective permeability of such cells to substances of low molecular weight. Toluene-treated bacteria are capable of limited semiconservative as well as repair-type DNA synthesis when incubated in an appropriate reaction mixture which provides the four precursor deoxynucleoside triphosphates. The effects of x irradiation on the DNA-synthesizing capacity and the role of polymerase I in the response were investigated. Wild-type cells were compared with a polymerase-I-deficient ($PolI^-$) mutant.

Our results show that x-ray exposure at "physiological" doses (i.e., equal to or less than a D_{37} dose) stimulated a partially ATP-dependent repair-type synthesis which was greatly reduced in the $PolI^-$ strain. We believe that this stimulated DNA synthesis reflects the production of 3′-OH termini of DNA either directly or, more likely, by the subsequent action of appropriate enzymes, especially endonucleases, responding to chemical changes in the DNA. This response reflects an abnormal amplification of the initial steps involved in repair and perhaps replication as expressed under the pathological conditions caused by toluenization.

Nicotinamide adenine dinucleotide (NAD) prevents DNA-polymerase-I-dependent repair-type synthesis in x-irradiated, toluenized *Bacillus subtilis*. In unirradiated preparations, NAD had little effect on an ATP-dependent, semiconservative synthesis but partially inhibited a repair-type synthesis. In a mutant lacking polymerase I (Pol^-), the presence of NAD did not affect TTP utilization in DNA synthesis. The presence of NAD reduced strand breakage in the Pol^+ strain during postirradiation incubation in the assay mix. Nicotinamide mononucleotide (NMN) partially reverses the NAD inhibition of repair-type DNA synthesis. At 10^{-4} *M*, NADH was as effective as NAD, in contrast to NADP, NADPH, and flavin adenine dinucleotide, which were essentially ineffective as substitutes for NAD. These findings are most reasonably interpreted as showing that x-ray induction of the repair-type synthesis results from enzymatic nicking at damaged DNA sites leading to 3′-OH termini production. These ends may then either undergo rejoining in the presence of NAD (ligase cofactor) or serve as a primer site for polymerase activity. In this system the extensive polymerase-I-dependent repair-type synthesis would reflect a departure from the normal incision-repair process seen *in vivo*.

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*Part of this work was done at The University of Florida.

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REGULATION OF DNA SYNTHESIS IN *BACILLUS SUBTILIS* AFTER X IRRADIATION*

Daniel Billen

DNA has been implicated as the target of the primary biologic lesion induced by ionizing irradiation. The precise nature of the total damage to the DNA is as yet unknown, although strand breakage and chemical alterations occur. The bacterial cell, utilizing various repair systems, must be capable of rescuing at least one complete and functional genome to insure viability.

Failure to restore the damaged DNA to its original state and/or induced structural modifications of com-

ponents regulating DNA function and replication could be reflected in an aberrant replication of the DNA. This may take the form of either a regulatory abnormality or some alteration in the synthetic process itself. The cause of death in many organisms therefore will be a failure to maintain the minimal integrity of a replication system required for normal duplication of DNA.

The effects of x rays on the programming of sequential replication of genetic markers have been studied in *Bacillus subtilis.*[1] Density-transfer and transformation techniques were employed to study the replication sequence of eight genetic markers distributed along the chromosome. It was observed that x rays caused premature gene replication independent of marker position in both stationary and growing cells.[2] The *metA* region was observed to be particularly sensitive to premature induction of replication. The loss of control of chromosome cycling is best explained by the induction of new starts scattered through the chromosome. These new initiation sites allow limited semiconservative replication and could be the result of x-ray-induced primer sites specific for polymerase(s).

*This work was done at the University of Florida.

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STUDIES ON UVR⁻ MUTANTS

C. T. Hadden

Our studies on Uvr⁻ mutants have met with mixed success. We have studied strains carrying mutations in two genes controlling pyrimidine-dimer excision, *uvrA1* (ref. 1) and *uvs-42* (ref. 2). These mutations confer almost identical sensitivities of colony-forming ability to uv. Logarithmically growing *uvrA1* cells are slightly more sensitive to ionizing radiation than Uvr⁺ cells, but *uvs-42* are more resistant. UvrA cells carry out repair synthesis at about one-half the rate of Uvr⁺ cells. In contrast, *uvs-42* cells carry out very little repair synthesis.

Our working hypothesis is that the *uvs* gene product is required for incision at dimer sites, and that the *uvrA* product is required for excision. Thus the *uvrA* mutant can nick and make primer for repair synthesis, while *uvs* cells cannot. The response to ionizing radiation would be explained if the excision-repair system repairs a part of the damage done by ionizing radiation but with a certain probability of inactivation by a competing system. Thus *uvs-42* cells would shunt all damage to another repair pathway, while in *uvrA* cells, a non-repairable nicked intermediate could accumulate.

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ROLE OF DNA POLYMERASE IN UV REPAIR

C. T. Hadden and Daniel Billen

We have studied some of the responses of DNA-polymerase-I-deficient (PolI⁻) mutants to irradiation, and have concluded that DNA polymerase plays an important role in the repair of uv damage. PolI⁻ strains (for example, HA160) are as resistant as wild-type strains to low doses of uv (below about 200 ergs/mm^2). However, at high doses the sensitivity of HA160 cells approaches that of Uvr⁻ cells. At high doses the rate of repair synthesis in PolI⁻ strains is about 30% of the wild-type rate, and uv causes degradation of both the preexisting DNA and DNA synthesized after the irradiation. Apparently one of the functions of PolI is to prevent degradation of DNA made susceptible to nucleases by the dimer-excision system, as has been concluded for *E. coli.*[1]

Although PolI⁻ mutants show no reduction in transforming ability, there must be some dependence of recombinational repair on PolI. Strain JB1-49(59) is both PolI⁻ and RecD⁻. This strain is almost identical to HA160 in uv sensitivity and post-uv repair synthesis. A RecD⁻ strain, BD193, has been reported to be blocked in the formation of covalently linked recombinational intermediates.[2] This step must also be involved in uv repair, since BD193 is sensitive to uv: at 100 ergs/mm^2 the surviving fraction of JB1-49(59) cells is about 90%, but only about 4% for BD193. PolI may act at some step of the recombination pathway prior to that of the *recD* gene product. If so, then the lack of DNA polymerase in JB1-49(59) could shunt damage into another pathway, preventing abortive attempts at repair involving *recD.* These results are not conclusive, since JB1-49(59) is not isogenic with HA160 or BD193, and this explanation does not account for the transformability of PolI⁻ cells. We are currently attempting to construct isogenic strains carrying the *polA* mutation along with other *rec* mutations, to determine whether PolI deficiency protects cells against other recombination defects.

We have also used the DNA-polymerase activity of freeze-thawed cells as a probe for some of the steps in

excision-repair.[3] We have concluded that dimer excision (or perhaps only the incision step) creates primer sites for DNA polymerase I. The appearance of new primer sites in irradiated cells is inhibited by *N*-ethylmaleimide (as is dimer excision) and requires ATP (which dimer excision does not).

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MAMMALIAN GENETICS

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Genetic Effects of Radiation in Mice

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Elizabeth M. Kelly

Mammalian Cytogenetics and Development

Liane B. Russell
N. L. A. Cacheiro

Mammalian Cytochemistry and Mutagenesis

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Effects of Radiation on Mammalian Gametogenesis

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Mammalian Comparative Mutagenesis

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INTRODUCTION

Radiation mutagenesis and related work. Our first series of experiments on tritium-induced specific-locus mutation rates in mice is now complete. It provides the only data available on such gene mutations in any mammal. This furnishes a basis for estimation of the genetic hazards to be expected from tritium releases from current technological operations. Since the research on controlled thermonuclear reactors is moving rapidly, the results we are accumulating may soon be needed for an adequate evaluation of their environmental impact. Although more testing is needed, our findings suggest two possible departures from expectations based on work with external x and gamma radiation: the genetic damage per unit dose in the stem-cell spermatogonia may be greater than expected, and the spectrum of mutational events may differ from that induced by x and gamma rays.

In a joint project with two other sections, we have been scoring for radiation-induced mutations affecting a clearly defined biochemical trait, hemoglobin type. Six variants have been found. Although genetic and biochemical tests of these are far from complete, it is clear that at least some of them are anomalies of a kind that we had not anticipated finding with the methods used.

Two gaps in our knowledge on transmitted chromosomal aberrations induced by radiation in spermatogonia have been filled in. Because of the importance of the spermatogonial stage in determining human risk, information was needed on the frequency with which transmitted sex-chromosome loss could be induced in spermatogonia. A large-scale experiment with high radiation doses has revealed no significant increase over control frequency. Since radiation-induced chromosome breakage is known to occur in spermatogonia, the absence of transmitted X-chromosome loss is of considerable basic interest.

Information was also incomplete on the dose response of translocations induced in spermatogonia. Although the total frequency of transmitted translocations is considerably below that expected from a parallel cytological study within the Division, even the lowest dose tested (150 R) has shown a significant increase over control frequency. Long-term studies will continue.

The actual nature of induced genetic changes, which is of fundamental importance in evaluating human hazards, continues to be investigated by several routes. Thus, genetic male sterility, a hitherto poorly explored subject, has been studied by new chromosome-banding techniques as well as other methods. The results show that sterility in F_1 males derived from treated postgonial-stage germ cells is associated with translocations having breakpoints near the ends of chromosomes, and that it may result from position effects; the much rarer sterility in F_1 males derived from irradiated spermatogonia probably has predominantly other causes.

Further examination of the induced mutations recovered at the *c* locus in specific-locus mutation experiments has shown that certain mutants involve well-defined biochemical defects; the time of death of some of the prenatal lethals has been determined; additional complementation groups have been successfully defined; and the complementation map has been oriented with respect to the genetic map. An ingenious new approach, combining *c*-locus lethals with translocations involving the X chromosome and the autosome carrying the *c* locus, should permit a determination of whether the lethal action operates at the cellular or the organismic level.

Chromosomal aberrations, as well as mutations, recovered in the mutagenesis work provide useful tools for other genetic investigations. Thus, the rare X-autosome translocations are valuable material for continued study of X-chromosome inactivation; and the breakpoint positions in certain autosomal translocations, determined by genetic and chromosome-banding methods, have shown that the commonly accepted genetic map for the mouse needs revision.

In the male, the germ cell of primary concern in the genetic hazard of radiation is the spermatogonial stem cell. For the interpretation of mammalian mutagenesis experiments, it is important to know whether the cell histologically identified as playing this role is really a continuously dividing cell or whether, as some have claimed, it is a resting, reserve cell, stimulated to divide only after depletion of other spermatogonia by radiation killing. Labeling with [^{3}H]thymidine prior to irradiation has now clearly demonstrated that the spermatogonia identified as stem cells are in continuous cycle; there is no evidence for a noncycling cell.

Perhaps the most dramatic and important biological factor affecting radiation-induced mutation frequency is the oocyte cell stage. Conceptions occurring within 6 weeks after irradiation of female mice have a high frequency of mutation, but in conceptions occurring

later, the mutation frequency drops to control levels. Understanding of this phenomenon, particularly of its applicability to humans, has been hampered by inability to time the development of the oocytes for identification of the stages of high and low mutational sensitivity. A technique for labeling the zona pellucida of the developing oocyte with *N*-[^{3}H]acetyl-D-glucosamine has now been developed and should solve the problem. A requisite which has already been established for this type of experimental procedure is that it must not disturb the normal process of oocyte growth and development.

Chemical mutagenesis. Unexpected results in mammalian chemical mutagenesis work are further evidence of the complexity of the problem; on the other hand, particularly when the mammalian results differ from those of lower organisms, they open exciting possibilities of reaching a basic understanding of at least some of the mechanisms involved in mammals. Such understanding will be essential for a realistic evaluation of the genetic risks from environmental chemicals, which even now are too numerous for individual testing.

Results with triethylenemelamine (TEM) and 6-mercaptopurine exemplify the complexity and perhaps the futility of looking for a single best test, even for induction of chromosomal aberrations in mammals. With TEM, as with earlier data from EMS, reciprocal translocations proved to be much more sensitive and reliable than dominant lethals as a measure of chromosome breakage. With 6-mercaptopurine, on the other hand, which shows a peak effect at an entirely different stage in spermatogenesis, results indicate that dominant lethals will be a more sensitive end point.

Extensive data have been collected on the fertility of translocation heterozygotes to permit improvements in the sequential procedure for translocation detection being developed by us for use by other laboratories in large-scale screening for mutagens.

Our earlier finding that the zygote immediately following sperm entry, and for a few hours thereafter, is highly sensitive to x-ray-induced dominant lethality and sex-chromosome loss suggested the use of this stage for a sensitive test of chemical mutagens. The test requires treatment of the female shortly after mating. The first experiment, using isopropyl methanesulfonate, has indeed shown a dominant-lethal frequency two to three times higher than effects on dictyate oocytes or spermatozoa alone (females and males treated prior to mating).

Research has started on potential mutagenesis by compounds involved in nonnuclear energy production, and work on dominant-lethality and fertility effects of two mercury compounds is reported this year. Although there is not yet an individual report on our studies of the effects of SO_2, we have begun extensive tests for measuring heritable chromosomal aberrations following multiple injections of sodium bisulfite.

Results of a preliminary test of the effect of the schistosomicidal drug hycanthone on X-chromosome loss in female mice apparently parallel those of our earlier work on dominant lethals in females, showing some effect in offspring from matings made in the first week after injection of the drug. These surprising results – the limitation of the effect to this period, and the finding of *any* effect when there was none in extensive dominant-lethal tests on male mice – suggest that the damage may not be a direct effect on chromosome breakage. Therefore, resolution of the mechanism involved could be important both in the general problem of the mutagenic action of chemicals and for a full understanding of what the X-chromosome-loss test may sometimes be measuring. (Although some effect of hycanthone has apparently been detected with the massive dose used in this experiment, the risk of using the drug at the clinical dose level, one-fiftieth of that employed here, is estimated to be only a small fraction of the spontaneous frequency, even when maximizing assumptions are made.)

Analysis of heritable translocations induced by cyclophosphamide indicates a more random pattern of chromosome breakage than has been found with other chemicals – similar to the pattern induced by x rays.

Work at the molecular level has yielded two highly important discoveries. First, unscheduled DNA synthesis is clearly demonstrable in a postmeiotic stage of the germ cells of male mice exposed to EMS. This offers the exciting possibility of testing other mutagens for their capacity to induce unscheduled DNA synthesis, and perhaps of relating the results, through stage sensitivity, to a measurable part of the genetic damage, thereby gaining insights into what may be happening at the molecular level.

Second, it now appears that the dominant-lethal effect produced by EMS may be due, at least partially, to ethylation of a non-DNA component of the germ cells. Sperm protamine is currently being investigated as one possible component which, when ethylated, might lead to chromosome breakage and dominant lethality. It has been clear for some time that the genetic effects of a chemical agent in a mammalian germ cell cannot be reliably predicted from results with nonmammalian systems. The new evidence supports the view that part of the lack of parallelism may be due not to differences in intermediary metabolism and similar factors but to

fundamental differences in primary damage at the molecular level. This has far-reaching implications for mutagenesis testing programs.

PRELIMINARY TEST OF THE EFFECT OF HYCANTHONE ON X-CHROMOSOME LOSS IN MICE

W. L. Russell, Patricia R. Hunsicker, Elizabeth M. Kelly, Carolyn M. Vaughan, and Georgia M. Guinn

Generoso et al.[1] have reported 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 by a dominant-lethal-type experiment showed that the fertility effect is attributable to an increase in the frequency of dead implantations. The results are of special interest for two reasons: first, no dominant-lethal effects had been found in extensive experiments on male mice, even with massive doses of hycanthone;[1] and second, the effect in females was restricted to matings occurring within a week after injection of the drug. Together, these responses suggested the possibility that the damage might not be a true dominant-lethal effect, such as chromosome breakage from direct action of the chemical in the oocytes, but might be an indirect result of toxicity in the mother.

To explore the possible mechanisms further, we have started experiments to test whether hycanthone has any effect on X-chromosome loss in treated female mice, using the method already established for radiation work.[2]

The females are injected intraperitoneally with 150 mg/kg of 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. Chromosome counts of the mothers of these females are also made, to exclude any cases where the parent is already X/0.

When the total data from all weeks of mating are considered, there is no significant difference between the experimental and control groups. From the hycanthone-treated females there are six X/0's in the 4110 classified female offspring. The contemporary control frequency is five X/0's in 2844, and the frequency in controls from all experiments combined is 17 or 18 in 26,545. However, three of the six X/0's in the treated group 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, is significantly above the frequency in the total controls ($P < 0.01$).

More data are being collected, but it already appears likely that, as with litter size and implantation death, X-chromosome loss may also show an effect of hycanthone in matings made shortly after treatment. If this proves true, attempts will be made to analyze the nature of the damage. In view of the fact that the time of action appears to be sharply limited, particularly in comparison with radiation damage, it is still possible that the X-chromosome loss in this case is not a result of chromosome breakage. Therefore, resolution of the mechanism involved could be of great importance both in the general problem of the mutagenic action of chemicals and for an understanding of what the X-chromosome-loss test is really measuring.

Although some effect of hycanthone has apparently been detected in this test, the risk estimated at the clinical dose level (one-fiftieth of that used here) is only a small fraction of the spontaneous frequency, even when maximizing assumptions are made, such as linearity of response with dose all the way down to the clinical dose.

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RADIATION-INDUCED HEMOGLOBIN VARIANTS IN THE MOUSE

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

Electrophoretic scoring for mutations at the hemoglobin loci in the mouse has continued. The work consists of screening blood samples of F_1 progeny from experiments in which either 101 or SEC strain mice are irradiated and mated to nonirradiated mice of the opposite strain. The parent types have distinctly different electrophoretic banding patterns, and in the F_1 hybrid, variations in the parental-type bands can be detected easily with the electrophoretic technique previously described.[1] The same blood samples are subsequently screened by R. A. Popp, employing phosphate-solubility and crystal-pattern techniques. The main purposes, as described earlier,[2] are, first, to determine the radiation-induced mutation rate for a clearly defined biochemical variant and, second, to

obtain enough mutants to determine the relative frequencies of deficiencies and point mutations.

A total of 6553 F_1 offspring from experiments at various radiation doses have now been screened. Six hemoglobin variants have been detected in this total number by one or more of the screening tests applied. Breeding procedures are in progress to establish stocks from each of the variants so that more elaborate genetic and biochemical testing can be performed to determine the exact nature of the variants. Four of the variants, 300 $FHGB_{as}$ ♀ 86, $SPBB_b$ ♂ 72, $SPBB_b$ ♂ 352, and $SPBB_c$ ♀ 27, were detected by all three screening criteria. A fifth, $SPBB_b$ ♂ 122, exhibited solubility and crystal-pattern anomalies but showed no unusual electrophoretic effect. The sixth mutant, $HGB_{Fo\text{-}a}$ ♂ 81, was identified by incomplete lysing of the red blood cells, but its electrophoretic, solubility, and crystal-pattern characteristics appeared normal.

At present, three of the six variants, 300 $FHGB_{as}$ ♀ 86, $SPBB_b$ ♂ 352, and $HGB_{Fo\text{-}a}$ ♂ 81, are transmitting their particular hemoglobin anomalies to their progeny. Two others, $SPBB_b$ ♂ 122 and $SPBB_c$ ♀ 27, are fertile, and their offspring are scheduled for testing in the near future. The first variant, 300 $FHGB_{as}$ ♀ 86, was originally reported as a probable spontaneous mutation,[3] but, following the completion of a recent series of breeding tests, it now appears more likely that it was radiation induced.

Information on the actual nature of the variants should be forthcoming as genetic and primary amino acid sequence studies are completed.

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INCIDENCE OF SEX-CHROMOSOME ANOMALIES FOLLOWING IRRADIATION OF MOUSE SPERMATOGONIA WITH SINGLE OR FRACTIONATED DOSES OF X RAYS

Liane B. Russell and Clyde S. Montgomery

Since X/0 mice are viable, while monosomy for any autosome is probably lethal, the induction of sex-chromosome losses has been used to obtain some measure of sensitivity to chromosomal damage, particularly in comparisons of different germ-cell stages of both sexes.[1] The spermatogonial stage, which is of prime importance for questions of human risk, was not included in our own earlier work on induced sex-chromosome loss. An experiment by Léonard and Schröder[2] had produced negative results in a somewhat limited sample of progeny which, furthermore, yielded slightly puzzling spontaneous rates.

Studies of translocations induced in spermatogonia have clearly shown that chromosome breakage can be induced in that germ-cell stage.[3] It consequently seemed important to check for the recovery of sex-chromosome loss induced in spermatogonia in a relatively large experiment. Since 24-hr fractionation of an acute dose to spermatogonia has earlier been shown to augment the frequency of induced specific-locus mutations to levels above linear extrapolation[4] and to yield an approximately linear dose-response curve for translocations in the dose range in which single doses produce a "humped" curve,[5] the experimental setup chosen included a fractionated-exposure group as well as a single-exposure group. A total dose of 600 R was chosen, since, with single acute exposures, maximum effects are obtained at approximately this level. The fractionation scheme selected was 100 R + (24 hr later) 500 R. Because of the great variability in reported rates of spontaneous loss of paternal sex chromosomes, great care was taken to make controls strictly comparable and contemporary. The breeding scheme, and the cytological and genetic testing of all presumed exceptional offspring as well as the mothers of certain ones, also made it possible to accumulate additional information on various classes of spontaneous sex-chromosome anomalies, on which suggested explanations as to their origin can be based.

Materials and methods. Females homozygous for "greasy" (*Gs*) were mated to wild-type (101 × C3H)F_1 males. Among progeny from such matings, loss of a paternal sex chromosome, X or Y, by any mechanism or nondisjunction of X and Y is easily recognizable by external phenotype. Of the maternal changes that might occur spontaneously, half of the cases of X loss would be detectable (the other half leading to nonviable 0/Y's), but cases of nondisjunction would not be recognized. Translocations involving the X chromosome could be phenotypically detectable if they brought about – by one mechanism or another – expression of only one of the X's. This single-X phenotype could also be produced by a variety of other mechanisms. Presumptive exceptional offspring were therefore tested both genetically and cytologically. Furthermore, mothers of 0/+ progeny were also tested to rule out preexisting spontaneous events.

The three groups – 600 R, 100 + 500 R, and controls – were handled concurrently and were strictly com-

Table 10. Incidence of sex-chromosome anomalies following irradiation of male mice

	Mature spermatozoa			Spermatogonia			Total
	600 R	100 + 500 R	0 R	600 R	100 + 500 R	0 R	
No. of offspring							
Total classified	223	198	446	4173	3982	4994[a]	14016
Exceptional							
Gs/0	0	2	0	8+1[b]	7[c]	11+1[b]	30
Gs/+/Y	0	0	0	0	0	1	1
0/+	0[d]	0	0	1	2	0	3
% Exceptionals							
Gs/0	0.48		0	0.20		0.24	
Gs/+/Y							0.007
0/+							0.044

[a]Includes 844 conceived during the period in which the irradiated males were sterile.
[b]Presumed X/0///X/X mosaic.
[c]Includes one animal that died before genetic or cytological testing could be done.
[d]One animal classified 0/+ probably carried a translocation.

parable in all respects. Since matings were made immediately after treatment, a small part of the progeny derived from irradiated mature spermatozoa, but the bulk came from irradiated spermatogonia. Altogether, 14,016 offspring were scored.

Results and Discussion. Results are shown in Table 10. Of 32 $X^M/0$-appearing mice found, two were proved to be *Gs*/+ and excluded. Two others were probably $X^M/0///X^M/X^P$ mosaics, with the integument predominantly X/0 and the germinal tissue predominantly X/X. Tests of the mothers of presumed $0/X^P$ exceptionals revealed that in 9 of 14 cases there was a preexisting X/0 condition.

The frequency of paternal-sex-chromosome loss was 2.4×10^{-3} in the controls and 2.0×10^{-3} in the two irradiated groups, where $X^M/0$'s must therefore be assumed to be of spontaneous origin. Since translocation experiments provide evidence that chromosome breaks are induced by irradiation of spermatogonia, the failure to recover X/0's is explained in one of two possible ways. (1) Breakage in spermatogonia does not lead to recoverable single-chromosome loss, either because no sister-chromatid joining occurs, or, if it does, because this leads to cell-division failure. (2) Alternatively, sex-chromosome loss does occur, but resulting X/0 and 0/Y cell progeny are inviable in the testis – a suggestion supported by evidence from natural and artificial mosaics.

Incidental to its main purpose, the experiment yielded limited data on sex-chromosome loss induced in mature spermatozoa.[6] Combined with our earlier more extensive data, the adjusted induced frequency is 1.5×10^{-5} per R.

The results of the present experiment lend further support to our earlier suggestion that most spontaneously occurring $X^M/0$ mice are the result of events occurring after sperm entry into the egg. The spontaneous frequency of paternal sex-chromosome loss has ranged over two orders of magnitude in various reports.[1,7–11] On the other hand, the frequencies of spontaneous X^M loss ($0/X^P$ daughters of X/X females per total daughters) and of paternal nondisjunction ($X^M/X^P/Y$ frequency $\times$ 2) were 4.4×10^{-4} and 1.4×10^{-4}, respectively, in the present experiment – both similar to findings in several other investigations.[1,7–9,12] It is suggested that spontaneous $0/X^P$'s, unlike most $X^M/0$'s, may be the result of nondisjunction.

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ANALYSIS OF 30 *c*-LOCUS LETHALS BY VIABILITY AND BIOCHEMICAL STUDIES

Liane B. Russell, D. L. DeHamer,* and Clyde S. Montgomery

Detailed analysis of mutants recovered in the course of specific-locus mutation-rate experiments is being carried out for certain loci to gain a better understanding of the nature of induced genetic changes and of how this is related to the conditions of mutagenesis. Complementation analysis, used for this purpose, has also led to the discovery of a number of functional units in close proximity to the loci at which mutation rates were being measured. Preliminary data for *c*-locus lethals[1] indicated that each involved three or more of six to seven functional units and could be grouped into one of five groups according to complementation patterns. In continuing work with *c*-locus lethals this year we found that certain of these mutations involved one or two biochemical defects, succeeded in defining an additional group, and were able to orient the complementation map with respect to the genetic map.

Materials and methods. From 115 mutations involving the *c* locus which have occurred over the years in a large number of different mutation-rate experiments, we chose 30 lethals for complementation studies. In five of these stocks, death of homozygotes occurs around the time of birth; in the remainder, early in embryonic life (see the following article).

In continuing work aimed at producing all of the 435 possible combinations of the 30 independent mutations, crosses of the type $c^{ch}/c^{l1} \times c^{ch}/c^{l2}$ were made, and litters were either killed on the day of birth for analysis of glucose-6-phosphatase and tyrosine aminotransferase, or they were checked weekly for survival to the age of one year. In addition, an analysis for mitochondrial malic enzyme (malate oxidoreductase decarboxylate) was made in young adults of all 30 stocks, since the *Mod-2* locus, which controls this enzyme, is known to be very close to the *c* locus, recombination being only about 1%.[2] For the purpose of this test, we extracted hearts from young adults and compared c^{ch}/c^{ch} and c^{ch}/c^{l} litter mates within each stock with respect to the intensity of the malic enzyme reaction in starch gels. Crosses of c^{ch}/c^{l} are also being made with a strain homozygous for a fast-moving

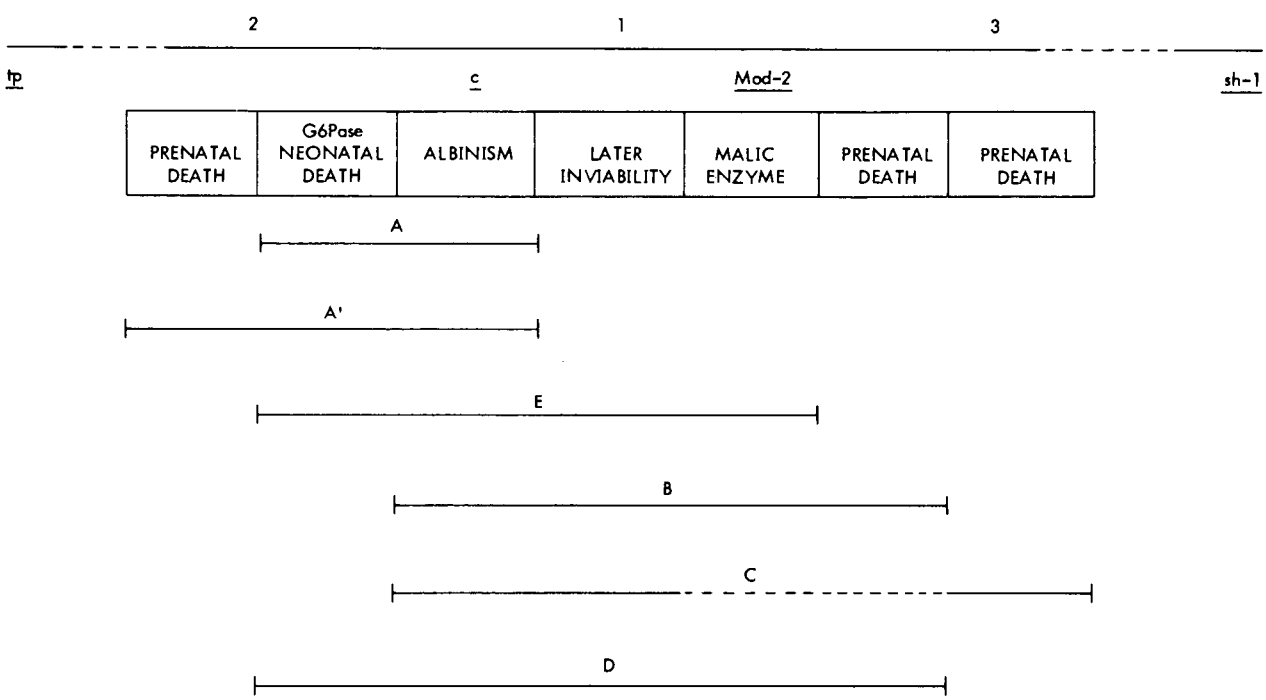

Fig. 15. Complementation map based on analyses of 30 *c*-locus mutants.

variant of *Mod-2,* and the two types of heterozygotes are being compared in starch gels.

Results and discussion. In the tests for intensity of malic enzyme reaction, heterozygotes for eight of the mutations showed a normal reaction, while 22 showed a diminished one. The latter group included all lethals of the B and D categories, as classified by complementation for viability; the former included A′, C, and A mutants – with one member of the last category being an exception. That this mutant does, in fact, yield a diminished reaction was confirmed in heterozygotes with a fast-moving *Mod-2* variant. The mutant was consequently reclassified from A to the new E category. A complementation map based on these results is shown in Fig. 15.

It is of interest that combinations of the E mutant with any of ten B-group mutants with which it has been tested have survived considerably past birth. The oldest death age has, to date, ranged from 5 to 201 days in the different combinations, with data in some of them still being meager. Since c^{lB}/c^{lE} combinations are presumably doubly deficient for *Mod-2,* this seems to indicate that malic enzyme is not a requirement for prenatal or neonatal survival.

The results also make it possible to orient the complementation map with respect to the genetic map. Since *Mod-2* is known to be on the *sh-1* side of *c,*[2] and since B-group mutants – which are presumably small deficiencies spanning *c* and *Mod-2* – were found to complement certain other groups for the so-far inseparable neonatal death and glucose-6-phosphatase deficiency, it can be concluded that the functional unit for the latter is on the *tp* side of *c.*[3]

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EMBRYONIC STAGE OF DEATH OF PRENATALLY LETHAL *c*-LOCUS MUTATIONS

Liane B. Russell and Linda S. Borman*

In a program of analysis of mutant stocks derived from specific-locus mutation-rate experiments, 25 of 103 tested *c*-locus mutations were found to be prenatally lethal in the homozygous condition.[1] On the basis of complementation tests and related investigations involving 24 of these lethals (see the preceding article), they can be grouped into four categories, A′, B, C, and D, containing 2, 12, 1, and 9 mutants, respectively. In an effort further to elucidate the nature of these mutations, we are attempting to analyze the stage of death as a prerequisite to looking for causes of death.

Materials and method. To date, preliminary embryological analysis has been done in 14 prenatally lethal *c*

Table 11. Embryonic stage of death of *c*-locus mutants

Group	Mutant symbol	c^{ch}/c^{l}♀ × c^{ch}/c^{l}♂				c^{ch}/c^{l}♀ × c^{ch}/c^{ch}♂				Pre- vs postimplantation death
		No. ♀♀	Av. no./♀			No. ♀♀	Av. no./♀			
			Died >10 days	Resorbing moles	Preimplantation losses		Died >10 days	Resorbing moles	Preimplantation losses	
A′	1 $DThW_b$	10	0.2	1.3	1.0	10	0.2	0.4	0.9	Post
		4	0	3.8	0.8					
B	1 $FDFoHr_c$	11	0.3	0.7	2.0	4	0.3	1.7	0.3	Pre
	1 $FR60H_b$	10	0	0.4	4.9	6	0.3	3.2	1.3	Pre
	14 $FR60H_b$	6	0.2	1.0	2.8	4	0	1.5	0.5	Pre
	12 $FR60H_b$	5	0.4	0.6	3.2					Pre
	3 R60L	6	0.2	1.5	3.8					Pre
	9 $FR60H_b$	7	0.1	1.9	1.9	8	0.1	0.8	1.3	Post(?)
	4 $FR60H_d$	8	0.3	2.8	1.1	6	0.2	1.0	1.8	Post
D	146G	8	0	1.3	2.6	10	0.3	1.4	0.8	Pre
	7 R250H	4	0	3.3	0.8					Post
	24 R145L	4	0	2.3	2.0					Post(?)
	19 DTR	6	0.2	3.0	1.0	5	0.6	2.6	3.2	
	68G	4	0.1	2.3	1.0					Post(?)
	202G	8	>0.7	2.9	1.1	7	0.3	0.6	1.4	Post

mutants – 1, 7, and 6 in groups A′, B, and D, respectively. Within any one stock, c^{ch}/c^{l} females are mated with either c^{ch}/c^{l} or c^{ch}/c^{ch} males. (Our aim of making these two sets of matings contemporary and in all ways comparable has not yet been truly achieved.) The first (experimental) set of matings should yield approximately 25% lethal c^{l}/c^{l} embryos, the second (control) set none; the maternal environment in the two sets is genetically the same. Females are killed about day 13.5 of gestation (range, days 11 to 16), the uterine contents are examined, and corpora lutea are counted. At that age, it is possible to distinguish between c^{ch}/c^{ch} and c^{ch}/c^{l} embryos by eye color; and if any c^{l}/c^{l} are present, they should be recognizable by the lack of eye pigment, assuming that they live beyond day 11. Altogether, 161 uteri have been examined to date.

Results and discussion. Results are shown in Table 11. In none of the uteri were there any embryos without eye pigment. Further, it was found that the frequency of embryos dying late (i.e., after day 10), was small and about equal in experimental and control matings. All of the lethals therefore apparently cause death prior to day 10. Whether death occurs before or after implantation can be judged by whether there is an excess of preimplantation loss (corpora lutea minus total implants) or of resorbing bodies when experimental matings are compared with controls. In some of the stocks, this judgement could be made with a fair degree of certainty (last column of Table 11), while in others the data are still insufficient.

The single A′ mutant and most D-group mutants probably die after implantation (but prior to day 10); in the case of most B-group mutants, the homozygotes do not even implant. It is of interest, however, that both the B and D groups contain mutants of both types, indicating that it may be possible, later, to define further functional units in the complementation map (see the preceding article). Tests of whether the various mutations act as cell or organismic lethals are being initiated, using the device of making the mutations heterozygous with T(X;1) translocations.[2]

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ATTEMPTS TO LOCATE THE X-CHROMOSOME INACTIVATION CENTER THROUGH THE STUDY OF NEW TRANSLOCATIONS

Liane B. Russell, N. L. A. Cacheiro,
Jean Bangham, and Margaret S. Swartout

X-autosome translocations continue to be the most valuable genetic material for the elucidation of problems in X-chromosome inactivation. Recent labeling studies[1] have confirmed our earlier suggestion that inactivation proceeds from one center or region. Those studies indicated that in four different T(X;A)'s, only one of the translocation products (in each case the longer one) was alternative to the intact X with respect to late replication, while the other translocation product was unaffected. The postulated inactivation center must therefore lie within that portion of the X that is common to the long translocation product of all four of these translocations, namely, the region between the R2 and R6 breakpoints, a relatively long distance. For finer definition, it would be necessary to study other T(X;A)'s with X breakpoints between R2 and R6; and the search for new translocations therefore takes on extra meaning.

This year, we discovered one new T(X;A) and began genetic studies on another one found the year before, as well as continuing our efforts to map X breakpoints in all available T(X;A)'s. We also sharpened our cytological localization of breakpoints by using Giemsa banding techniques which allow more detailed characterization than quinacrine mustard fluorescence.

The new T(X;A), designated R9, was discovered in a stock descended from an x-irradiated postspermatogonial germ cell. The discovery was not by virtue of a variegated phenotype [as in all past T(X;A)'s] but through the finding that about half the females in the stock were small, and about half the sons of the small females were sterile. Quinacrine mustard banding studies indicate that the autosome in R9 is probably chromosome 12 (= LG XVI), one not heretofore found translocated with the X. The break position (see Table 12) yields translocation products which are both of medium size – instead of one very long and one short, as in our other T(X;A)'s – and this should be useful for labeling studies.

Genetic studies on R8, the new cyclophosphamide-induced T(X;8), indicate in their early stages that breakpoint positions in both chromosomes are rather similar to R7. Giemsa banding techniques indicate the

Table 12. Cytological position of breakpoints in X-autosome translocation

Translocation	Breakpoint location	
	in X	in autosome
R1	XF2	4A5
R7	XA2	4D1
R8	?	?
R2	XA4	7D3
R3	XA2	7F1
R5	XF1	7A3
R6	XF1	7B3
R9[a]	middle of prox. bright region	near centromere

[a]Quinacrine mustard; all others determined by Giemsa banding.

breakpoint positions to be located as shown in Table 12.

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CAUSES OF STERILITY IN MALE MICE DERIVED FROM IRRADIATED SPERMATIDS OR SPERMATOGONIA

N. L. A. Cacheiro, Margaret S. Swartout, and Liane B. Russell

Sons of mice treated with irradiation or certain chemical agents fall into three broad categories: fertile, partially sterile, and completely sterile. "Partial sterility" has long been known to be independent of the reproductive capacity but to be caused, instead, by embryonic death of chromosomally unbalanced segregants resulting from reciprocal autosomal translocations for which the "partially sterile" animal is heterozygous. The causes of complete sterility of F_1 males had, until recently, not been investigated. In a cytological study of 43 sterile sons of males that had received x rays, EMS, or BHT + EMS to postspermatogonial stages, we showed that the majority carried reciprocal translocations and that most of these involved at least one break near one or the other end of a chromosome.[1]

We have now studied further groups of sterile F_1 males using quinacrine mustard and Giemsa banding techniques that permit identification of the chromosomes involved and localization of breakpoints. This new information is of particular importance in the case of steriles in which the spermatogenic block occurs prior to diakinesis, thus preventing analysis of meiotic chromosomes. The new experiments have also extended the study to F_1 males derived from treated spermatogonia, a germ-cell stage that gives an extremely low yield of sterile sons.

Materials and methods. Thirty sterile F_1 sons of x-irradiated males[2] were studied cytologically and histologically. Twelve were derived from treated spermatids (158 F_1 tested) and 18 from treated spermatogonia (4286 F_1 tested). Analyses of testis sections and epididymal content were carried out to determine the latest spermatogenic stage present and to check whether there were abnormalities in the morphology and motility of any spermatozoa that might be formed. Cytological analysis was carried out in mitotic metaphases from dividing spermatogonia (Orcein staining) and kidney cultures (banding techniques), and in meiotic cells at diakinesis wherever spermatogenesis proceeded to that point or beyond.

Results. *Sterile sons derived from irradiated spermatids (Table 13, Fig. 16).* Of 12 sterile F_1 males, 10 were found to carry reciprocal translocations as determined by banding techniques, with 7 of these also revealed by conventional staining through the presence of a small chromosome. Of the ten chromosomally aberrant males, two had Y-autosome translocations, and two others had two autosomal translocations each. Except where the Y was involved, at least one of the breaks in each male was close to the centromere (c) or telomere (t), and all but two of these males had, in fact, c/t translocations. Wherever diakinesis could be studied, it was found that only a small number, if any, of the multivalent configurations were of the ring-IV type, in keeping with the breakpoint locations near chromosomal ends.

Of the 12 steriles, 7 had spermatids, and 4 of these had small numbers of epididymal sperm, which were, however, nonmotile and morphologically abnormal. The two males in which no chromosomal aberrations could be detected were of this type. These two were found to have occluded ducts. In the remaining five steriles, spermatogenesis stopped in pachytene or diakinesis.

Sterile sons derived from irradiated spermatogonia (Table 14, Fig. 17). Only 4 of the 18 steriles had translocations, and though at least one break was near a chromosomal end in 3 of these, none was of the c/t type. In keeping with this, none was detectable by the presence of a small chromosome in the Orcein-stained material, but one of the translocations had produced a metacentric chromosome. In two of the translocation males, spermatogenesis stopped in pachytene, and two

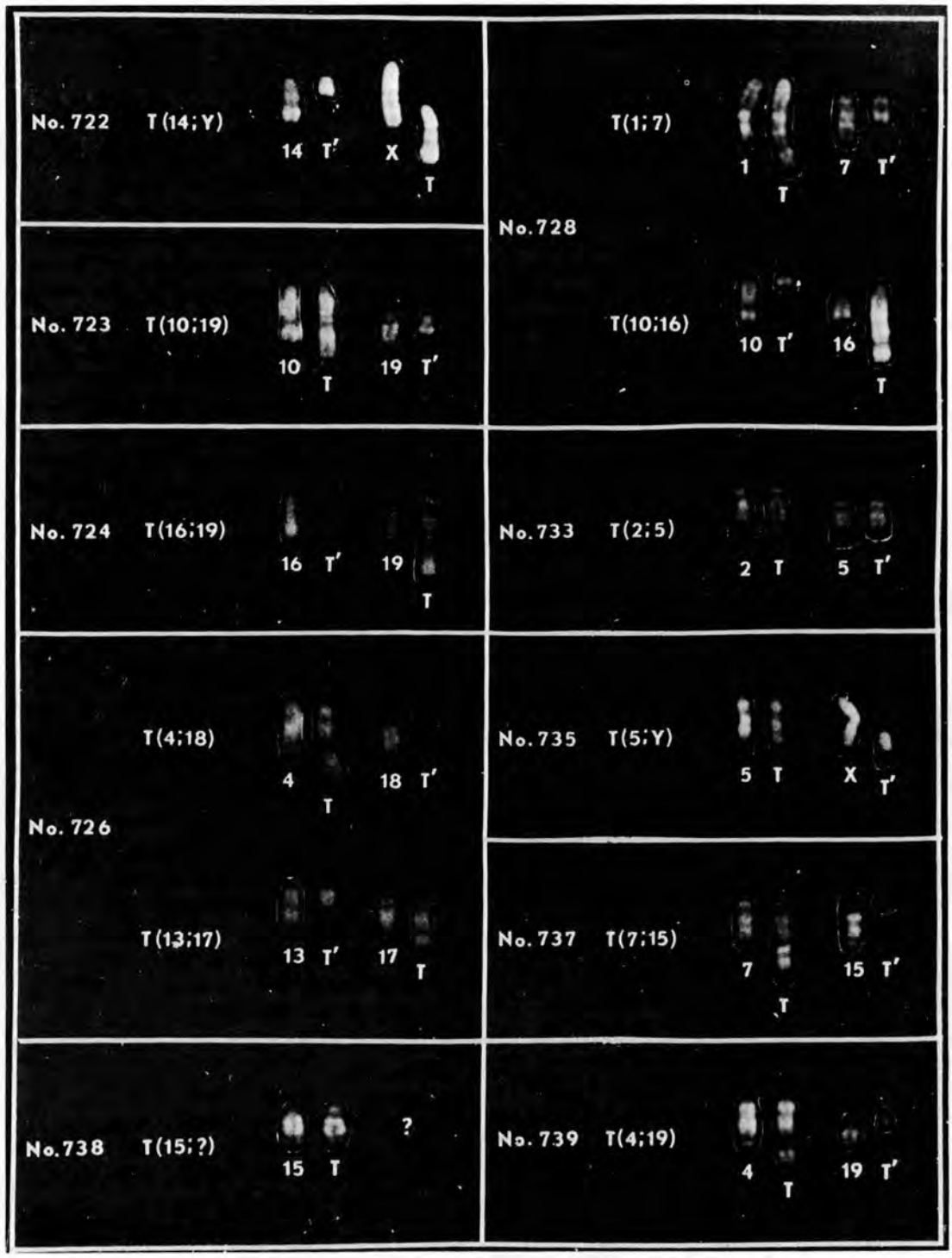

Fig. 16. Abnormal configurations found in 10 of the 12 spermatid-derived F_1 steriles. In this partial karyotype from quinacrine-mustard-stained metaphases, the normal chromosome is to the left and the abnormal one to the right of each grouping.

Table 13. Results of morphological and histological analyses of testis and spermatozoa in sterile sons of males irradiated in spermatid stage

F_1♂ #	Testes weight (mg)	Most advanced stage in testis	Epididymal sperm analysis			Chromosome abnormality		
			Number	% Motile	Morphology[a]	Orcein, spermatogonial metaphase[b]	Quinacrine mustard, kidney tissue culture	Break location
729	110[c]	Spermatids	+	0	S	–	–	–
736	110[c]	Spermatids	+	0	H	–	–	–
726	80	Spermatids	+	0	H	SC	T(4;18) T(9;17)	t/c; m/m
728	80	Spermatids	+	0	H	SC	T(1;7) T(10;16)	t/c; c/t
723	60	Spermatids	0	–	–	SC	T(10;19)	t/c
724	50	Spermatids	0	–	–	SC	T(16;19)	c/t
739	60	Spermatids	0	–	–	SC	T(4;19)	t/c
733	70	Diakinesis	0	–	–	–	T(2;5)	t/mt
735	70	Diakinesis	0	–	–	–	T(Y;5)	mt/mt
722	70	Pachytene	0	–	–	SC	T(Y;14)	mt/c
737	40	Pachytene	0	–	–	SC	T(7;15)	t/c
738	60	Pachytene	0	–	–	–	T(15;?)	c/?

[a]S, separated heads and tails; H, abnormal heads.

[b]SC, one small or very small chromosome.

[c]Occluded ducts.

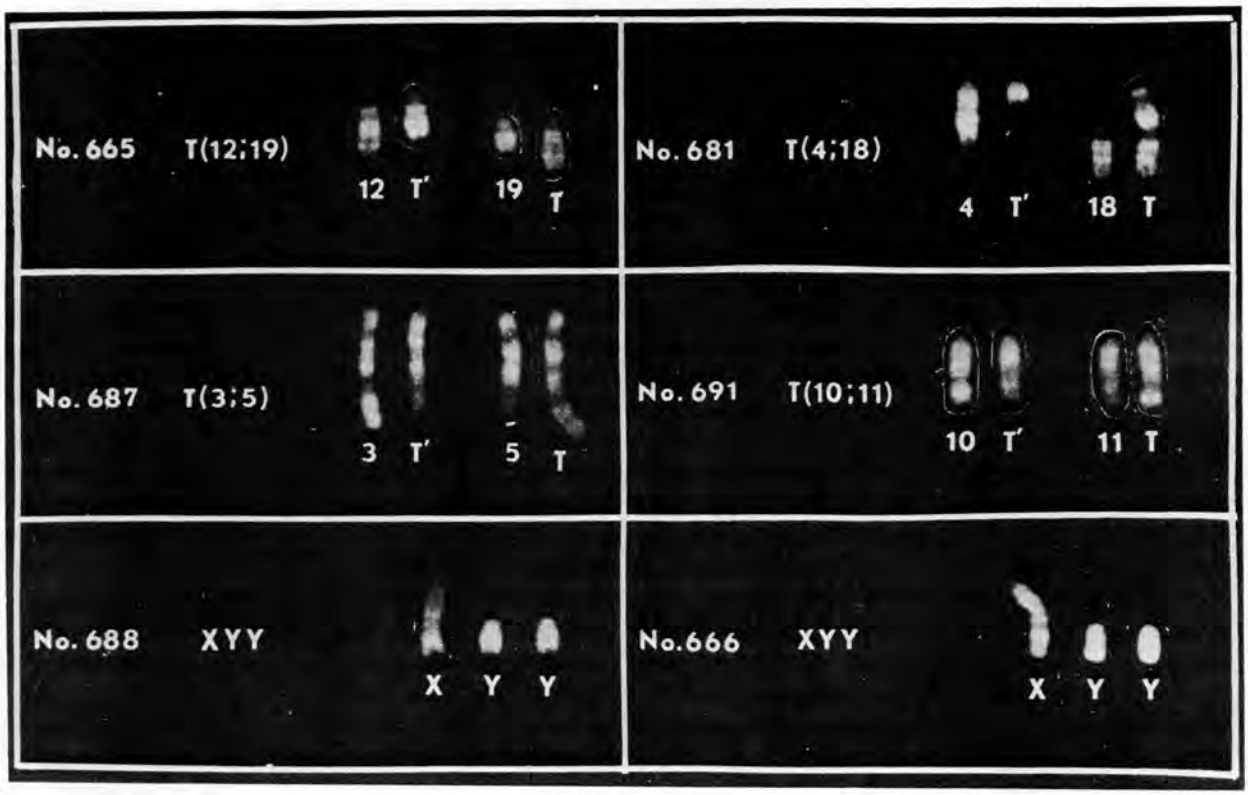

Fig. 17. Abnormal configurations from the six spermatogonia-derived F_1 males that were found to have chromosome aberrations. Four of these were reciprocal translocations and two were numerical chromosome abnormalities.

Table 14. Results of morphological and histological analyses of testis and spermatozoa in sterile sons of males irradiated in spermatogonial stage

F_1 ♂ #	Testes weight (mg)	Most advanced stage in testis	Epididymal sperm analysis			Chromosome abnormality		
			Number	% Motile	Morphology[a]	Orcein, spermatogonial metaphase	Quinacrine mustard, kidney tissue culture	Break location
670	200	Spermatids	+++	75	Normal	–	–	–
685	240	Spermatids	+++	50	Normal	–	–	–
686	260	Spermatids	+++	50	Normal	–	–	–
694	210	Spermatids	+++	50	T	–	–	–
684	190[b]	Spermatids	+++	50	H,T	–	–	–
690	260	Spermatids	+++	10	T	–	–	–
682	270[b]	Spermatids	+	0	H,T	–	–	–
689	155[b,c]	Spermatids	+	0	H,T	–	–	–
692	90[b,c]	Spermatids	+	0	S	–	–	–
698	310[b]	Spermatids	+	0	S	–	–	–
683	480[b]	Spermatids	0	–	–	–	–	–
693	170[b]	Spermatids	0	–	–	–	–	–
666	50	Spermatids	+	0	T	41	X/Y/Y	–
688	80	Spermatids	+	0	T	41	X/Y/Y	–
687	80	Spermatids	+	0	H,T	–	T(3;5)	mt/mt
691	90 + 220[b]	Spermatids	+	0	H,T	–	T(10;11)	c/c
665	40	Pachytene	0	–	–	–	T(15;19)	m/t
681	70	Pachytene	0	–	–	Metaphase	T(4;18)	c/c

[a]T, abnormal tails; H, abnormal heads; S, separated heads and tails.

[b]Occluded ducts.

[c]Only one testis.

others had small numbers of abnormal epididymal sperm. In addition to the four males bearing autosomal translocations, two had numerical abnormalities in the form of an extra Y chromosome. Sperm cells were sparse and abnormal in both of these. Among the 12 males in which no chromosomal abnormalities could be detected were 6 with apparently normal numbers of epididymal sperm. In all of these, however, sperm motility and/or morphology were abnormal.

Conclusion. Use of the cytological banding techniques has not only revealed the presence of translocations in cases in which these would not have otherwise been detectable but also strongly confirmed our earlier suggestion that complete sterility in F_1 males derived from treated postgonial stages is associated with breakpoints near the ends of chromosomes. We have proposed that male sterility may be caused by some type of position effect resulting from such rearrangements.

Sterility in sons derived from treated spermatogonia appears to be predominantly due to causes other than reciprocal translocations with near-end breaks. Small deficiencies or point mutations could play a role.

1. N. L. A. Cacheiro, L. B. Russell, and M. S. Swartout, *Genetics* 76, 73 (1974).

2. The animals came from experiments of W. M. Generoso in our laboratory. Fertility tests were carried out by Generoso.

THE USE OF STEELOID TRANSLOCATIONS IN REASSIGNING LINKAGE GROUPS TO CHROMOSOMES

N. L. A. Cacheiro and Liane B. Russell

Over the years, over 30 independent new "Steeloid" mutations have been found in irradiated and unirradiated stocks at this laboratory. These resemble the mutation "Steel" (*Sl*), also discovered here,[1] and are allelic or, possibly, very closely linked to it. A number of these Steeloids have proved to be reciprocal translocations.

We have now cytologically examined four such translocations, using quinacrine mustard banding techniques. The chromosomes involved are 10 and 5, 10 and 12, 10 and 17, and 10 and 18. This would strongly indicate that the *Sl* locus is on chromosome 10.

However, *Sl* has previously been assigned to LG IV (ref. 2), which has not yet been matched with a chromosome of the standard karyotype, while chromosome 10 has been matched with LG X. We thus considered it necessary to test the possibility that all or part of LG IV, as well as LG X, is located in chromosome 10. A linkage test for *Sl* (LG IV) and "grizzled" (*gr*) (LG X), while just beginning to yield data, already indicates that one gets far less than the 50% recombination expected for independent assortment. In the first 29 backcross young classified to date, there was only one recombinant (3%), indicating either that "two" linkage groups are, in fact, but one, or that *Sl* has been wrongly assigned in the past.

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STUDIES ON PROTAMINE SYNTHESIS AND POSSIBLE PROTAMINE PHOSPHORYLATION IN MOUSE SPERM: RELATIONSHIP TO GENETIC DAMAGE CAUSED BY ETHYL METHANESULFONATE*

G. A. Sega, R. B. Cumming, and J. L. Armistead†

In late spermiogenesis in the mouse (around spermatid stage 11), histones associated with nuclear DNA are replaced by an arginine-rich protamine.[1] Protamines are also synthesized during spermiogenesis in fish such as trout, and in these cases it is also known that the protamines are first phosphorylated and then later dephosphorylated.[2] If mouse-sperm protamine is phosphorylated after it binds to DNA, this might produce a more extended chromatin configuration that could lead to an increased susceptibility to attack by alkylating agents such as ethyl methanesulfonate (EMS). Since there is little information concerning phosphorylation of mammalian protamine, experiments were performed to determine specifically the time of protamine synthesis in the mouse relative to the time of peak EMS sensitivity, and to determine whether protamine phosphorylation might also be occurring.

Male mice were injected with L-[^{3}H]arginine and $^{32}PO_4$. Both isotopes were injected within a 1-hr time span. Four males were killed daily for two weeks, and the sperm from the caput epididymides, the caudal epididymides, and the vasa deferentia were recovered separately. ^{3}H and ^{32}P activities in the sperm were assayed with a liquid scintillation counter.

The results of the experiment showed that the ^{3}H activity reached a peak in the sperm of the caput epididymides six days after injection. Associated with this ^{3}H peak was a ^{32}P peak occurring on the same day. The association of the ^{32}P peak with the ^{3}H peak is, at present, strong presumptive evidence that at or near the time of protamine synthesis phosphorylation of the protamine is occurring.

The ^{3}H activity associated with the labeled protamine can be traced through the caudal epididymides and the vasa deferentia, with the peak activity occurring in the former 9 days after injection and in the latter 12 days after injection. However, no significant ^{32}P activity was detected in the sperm from these last two regions of the reproductive tract. This result indicated that as the sperm were maturing and moving from the caput epididymis to the caudal epididymis, dephosphorylation (presumably of protamine) was occurring.

The germ-cell stage in the testis labeled with ^{32}P has been tentatively established as preceding that stage of spermiogenesis most sensitive to the induction of chromosomal aberrations by EMS. Since the peak of ^{3}H activity due to incorporation of L-[^{3}H]arginine into protamine occurred in vas sperm 12 days after injection, while ethylations from EMS reach a peak in the vas sperm 9–10 days after injection and the dominant-lethal frequency is highest between 7 and 9 days after injection, it was concluded that protamine synthesis occurs 2 or more days prior to the germ-cell stage most sensitive to EMS. Protamine is therefore present in the late spermatids most sensitive to EMS.

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†Southern College University Union participant from the University of the South, Sewanee, Tennessee 37375.

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ETHYLATION PATTERN IN MOUSE SPERM AS A FUNCTION OF DEVELOPMENTAL STAGE AND TIME AFTER EXPOSURE TO ETHYL METHANESULFONATE*

G. A. Sega, R. B. Cumming, and Marva F. Walton

A single intraperitoneal injection of 200 mg/kg of [2-^{3}H]ethyl methanesulfonate ([^{3}H]EMS) was given to (C3Hf X 101)F_1 hybrid males 10–12 weeks old at the time of treatment. At 11 time points from 4 hr to 16

days after [^{3}H]EMS injection, four mice were killed and the sperm from the caput epididymides, caudal epididymides, and vasa deferentia were recovered separately. Sperm ethylations were determined from the specific activity of the [^{3}H]EMS and from [2-^{3}H]ethyl activity measured in samples of purified sperm heads by liquid scintillation counting.

The ethylations per sperm reach a peak first in the caput epididymis about 2 days after injection, followed by a peak in the caudal epididymis at about 6 days, and finally an ethylation peak occurs in the sperm of the vas at 9–10 days. The ethylation pattern for the vas sperm over the 16-day period studied closely parallels the dominant-lethal pattern measured for the offspring of treated males; that is, when the dominant-lethal frequency is low the ethylations per sperm cell are low, and when the dominant-lethal frequency is high the ethylations per sperm cell are high.

The pattern of ethylation indicated that the sperm most heavily ethylated were late spermatids still in the testes at the time of treatment. As these heavily ethylated spermatids matured they could be traced moving through the caput epididymis, the caudal epididymis, and the vas in sequence.

After the number of ethylations per sperm in the vas had been determined as a function of time after injection with [^{3}H]EMS, the DNA was extracted from these sperm heads. The extraction procedures that were used assured the removal of at least 99% of the protamine and other proteins present and resulted in no preferential recovery of either (G + C)-rich or (A + T)-rich regions of the mouse genome.[1]

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 $2-3 \times 10^{-5}$ ethylations per nucleotide from 4 hr after injection through ten days after injection. Since the increased number of ethylations found in the vas sperm by nine to ten days after injection is apparently not due to increased DNA ethylation, it is possible that another chromosome component, such as protamine, may account for the ethylation peak in the vas at nine to ten days. As dominant lethals are generally attributed to chromosomal aberrations, it remains possible that ethylated protamine may lead to dominant lethals through a weakening of the chromatin structure that results in chromosomal aberrations. The relation between protamine ethylation and the dominant lethals produced by EMS in the mouse will be studied next.

*Research sponsored jointly by the National Center for Toxicological Research and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

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UNSCHEDULED DNA SYNTHESIS IN POSTMEIOTIC GERM-CELL STAGES OF MALE MICE AFTER TREATMENT WITH ETHYL METHANESULFONATE*

G. A. Sega

Experiments have been performed to test the hypothesis that ethylation of postmeiotic-germ-cell DNA could result in unscheduled DNA synthesis. Two groups of males were both given testicular injections of [^{3}H]thymidine ([^{3}H]dThd), and one of the groups was also given an intraperitoneal injection of between 200 and 250 mg/kg of ethyl methanesulfonate (EMS). Three or four animals were killed from both the control ([^{3}H]dThd only) and the EMS-treated ([^{3}H]dThd + EMS) groups at various times from 9 days to 39 days after treatment. Purified sperm heads were recovered separately from the caput epididymides, the caudal epididymides, and the vasa deferentia.

The [^{3}H]dThd activity from several million sperm taken from each of the three regions of the male reproductive tracts from both the control and EMS-treated groups was measured in a liquid scintillation counter for all of the time points. The control group showed no [^{3}H]dThd activity until sperm that had been preleptotene spermatocytes undergoing DNA synthesis at the time of treatment first reached the caput epididymis about 24 days, the caudal epididymis about 27 days, and the vas about 30 days after injection. For the EMS-treated group there was [^{3}H]dThd activity present in the sperm of the caput epididymis about 9 days, in the caudal epididymis about 12 days, and in the vas about 14 days after injection. The levels of [^{3}H]dThd uptake by the EMS-treated postmeiotic cells remained at relatively constant values over a 10- to 12-day period.

Compared to the amount of preleptotene DNA synthesis that occurred during the time of [^{3}H]dThd incorporation, there was about $1/200$ as much unscheduled DNA synthesis occurring in the EMS-treated postmeiotic cells. Using 1 hr as an estimate of the time [^{3}H]dThd was available for incorporation into DNA, the amount of unscheduled DNA synthesis amounted to nearly 0.01% of the entire mouse genome. This data demonstrates, for the first time, that unscheduled DNA synthesis can occur *in vivo* in mammalian cells exposed to a chemical agent.

When the time of unscheduled DNA synthesis is compared to the time of maximum sensitivity to EMS,[1,2] it is found that the germ-cell stages most

sensitive to EMS show no unscheduled DNA synthesis. The unscheduled DNA synthesis starts to occur only in germ-cell stages earlier than those which show a sensitivity to EMS. When a comparison is made between the time of protamine synthesis in mouse spermatids[3] and the beginning of unscheduled DNA synthesis, it is found that the unscheduled DNA synthesis, again, starts to occur only in earlier germ-cell stages. This suggests the possibility that once protamine has replaced the chromosomal histones no unscheduled DNA synthesis is possible.

With the same techniques described here for measuring unscheduled DNA synthesis in postmeiotic germ cells of the mouse after treatment with EMS, it is possible to test a vast variety of agents that produce different patterns of genetic damage in the mouse. X rays and cyclophosphamide are two such agents currently under investigation.

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*Research sponsored jointly by the National Center for Toxicological Research and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

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TRITIUM-INDUCED SPECIFIC-LOCUS MUTATIONS AND RADIATION DOSE IN THE MALE MOUSE FROM INJECTED TRITIATED WATER

R. B. Cumming, W. L. Russell, and G. A. Sega

The first series of specific-locus mutation experiments using tritiated water as the radiation source, which has been partially described in previous reports,[1-4] has now been completed, except for testing of some of the mutations. There are no additional data from postspermatogonial germ-cell stages since last year's report.[3] An additional 1514 offspring with two mutations have been scored from germ cells treated primarily as spermatogonia, and this brings the total to 20,626 offspring with 16 mutations for this germ-cell stage. Thus the mutation rates have not changed significantly since last year's report.[3,4]

Substantial additional data are now available on the radiation dosimetry from a single injection of tritiated water. New calculations reflecting these data on the mean absorbed dose to the postspermatogonial cells in these experiments give a value of 430 rads. The figure is the same as estimated last year,[3,5] but it is based on more substantial data. The mean dose to germ cells irradiated primarily as spermatogonia is now calculated to be 700 rads. Again the confidence on this dose estimate is greatly increased from last year, though the value is within the range of 690 to 790 rads estimated at that time.[5] Thus, if absorbed dose to the testis is accepted as meaningful for purposes of comparison with gamma or x rays, the major conclusions of last year's report[3,4] are reinforced. The point estimate of relative biological effectiveness (RBE) for post-spermatogonial germ-cell stages is close to 1, with fairly wide confidence intervals. The point estimate of RBE for spermatogonia is slightly above 2, with confidence intervals which include 1, and there remains the suggestion that the distribution of mutants among the seven loci may differ from that produced by gamma rays. It is clear that the last two points should be clarified by additional data.

The question of whether whole-tissue absorbed dose is meaningful for tritium in view of microdistributional factors and incorporation into biological macromolecules cannot be answered at this time. However, our dosimetry studies have provided some information on the extent of incorporation of tritium into specific molecules following a single injection of tritiated water. Tritium is incorporated into testicular DNA from tritiated water, and peak levels of radioactivity are reached from 3 to 9 days following injection. Radioactivity is then lost from the DNA, presumably largely by dilution due to new DNA synthesis. At 95 days after injection there remains about 0.5% of the radioactivity that was present in the DNA at the peak. The stable incorporation of tritium into trichloroacetic-acid-insoluble materials is about 75% in protein and 25% in nucleic acids at all time periods following injection. Thus the incorporation into protein follows a pattern similar to that of DNA, with peak activities reached 3 to 9 days following injection. The percentage of the total radioactivity incorporated into macromolecules is always low, but measurable radioactivity is incorporated within a few minutes after injection. The ratios of radioactivity incorporated into macromolecules to total tissue radioactivity for various times after injection are approximately: 1 hr, 2×10^{-4}; 12 hr, 3.3×10^{-4}; 24 hr, 1×10^{-3}; 6 days, 1×10^{-2}; and 95 days, 2.5×10^{-1}. The total percentage of the dose delivered from ^{3}H incorporated into macromolecules is small – less than 0.5%.

The questions raised by these studies make it clear that additional work should be done, particularly in

view of possible levels of tritium release not only from existing nuclear installations but also from contemplated controlled thermonuclear reactors.

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DOSIMETRY OF METHYLMERCURIC HYDROXIDE IN THE GERM CELLS OF MALE MICE*

G. A. Sega and Marva F. Walton

It is estimated that a single 700-megawatt power plant burning coal will emit 1500 pounds of mercury annually. Once this mercury is converted to an organic form such as methylmercuric hydroxide, it poses a potential mutagenic threat to human populations. It is, therefore, relevant to be studying the effects of organic mercury compounds on the germ cells of a mammal.

Preliminary experiments on the dosimetry of methylmercuric hydroxide in the germ cells of male mice have been conducted to parallel genetic work being done on possible dominant-lethal effects from this compound. Using ^{203}Hg, a β^- emitter, to trace incorporation of the methylmercuric hydroxide into sperm cells, we have found that by one day after a 10-mg/kg injection of the chemical, there is considerable labeling of the vas sperm. The relative levels of mercury in the vas sperm gradually decrease through 17 days after injection, with only about $1/35$ as much mercury present in the sperm at 17 days as at 1 day after injection. (Only relative levels of the mercury present in different samples can be determined at present, as we do not yet have a ^{203}Hg standard to convert sample counts to sample disintegrations.) At 32 days after injection the relative level of mercury found in the vas sperm appears to rise again, increasing to more than ten times the level found at 17 days, but then by 37 days it drops to the low levels found at 17 days. The sperm in the vas at 32 days after injection were primary spermatocytes in or near the preleptotene S period at the time of injection. It may be that primary spermatocytes take up more mercury than do some of the later germ-cell stages in the mouse.

*Research sponsored jointly by the National Center for Toxicological Research and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

POSSIBLE RELATION BETWEEN CYSTEINE IN THE GERM CELLS OF MALE MICE AND THE GENETIC DAMAGE PRODUCED BY ETHYL METHANESULFONATE*

G. A. Sega

There have been reports that, at least in some mammals, the mature spermatozoa are more resistant to reducing agents than are the immature sperm. This has been found here to be true in the mouse and is believed to be a consequence of the formation of disulfide bridges between cysteine residues in sperm protein. Such sulfur-containing proteins should also be potential sites for attack by alkylating agents such as ethyl methanesulfonate (EMS). Previous work with the rat, for example, has shown large amounts of ethylcysteine in the urine following injections of EMS.[1]

The pattern of incorporation of L-[^{3}H]cystine into the developing sperm has been studied. In particular, the movement of labeled cystine has been traced from the sperm of the caput epididymis to the sperm of the caudal epididymis and finally to the sperm of the vas. (C3Hf × 101)F_1 male hybrids, 10–12 weeks old at the time of treatment, were injected with L-[^{3}H]cystine, and three animals were killed at each of ten time points from $3/4$ day to 18 days after injection.

Except for a small "blip" of ^{3}H activity in the vas sperm at one day after injection, the labeling was first picked up in the sperm of the caput epididymis, then the caudal epididymis, and finally in the vas. The labeling pattern indicates that, by far, most of the cystine is incorporated into testicular sperm, and little if any cystine is incorporated into sperm of the epididymides or vas. Therefore, it does not appear that the insensitivity of the most mature spermatozoa (found in the vas) to genetic damage by EMS (as measured by dominant lethals) results from higher levels of cysteine in these sperm.

Although Lam and Bruce[2] did not report the presence of cysteine in mouse sperm protamine, the pattern of sperm labeling after injection of L-[^{3}H]cystine is similar to that obtained after injection of L-[^{3}H]arginine,[3] indicating that cysteine is probably also present in protamine.

The germ-cell stage most sensitive to EMS is near the time of protamine synthesis.[4–6] It is possible that if

EMS were to ethylate the sulfur atoms of cysteine before the disulfide bridges were formed this might prevent proper chromatin condensation, which could lead to chromosome damage and dominant lethals. Future research will attempt to study the pattern of ethylation of cysteine in developing germ cells and relate these ethylations to genetic damage.

*Research sponsored jointly by the National Center for Toxicological Research and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

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INDUCTION OF TRANSLOCATIONS BY CYCLOPHOSPHAMIDE IN MICE: CYTOLOGICAL CHARACTERIZATION AND TRANSMISSION

R. E. Sotomayor* and R. B. Cumming

Cytological and fertility tests were performed in F_1 male mice derived from different germ-cell stages of male parents treated with cyclophosphamide (350 mg/kg body weight). The objectives of the present study were (1) to determine the sensitivity of the male germ-cell stages to the induction of translocations by the compound, (2) to characterize cytologically different types of translocation configurations in F_1 and F_2 males, and (3) to obtain information about the pattern of chromosome breakage induced and its transmission to subsequent generations.

From a total of 508 F_1 males studied, 39 were partially sterile and nine were fully sterile. The group of males conceived 8–21 days after treatment contained by far the highest proportion of partially sterile animals, namely, 30%. It was also the only group in which totally sterile animals (11%) were found. Twenty-four out of twenty-five semisterile males from this group gave evidence of translocations when spermatocytes were scored at diakinesis. Of the nine sterile males, only five could be studied cytologically, and they were found to be translocation heterozygotes. Of the other four, two died before the cytological analysis could be performed and two did not show chromosomes in diakinesis, indicating either spermiogenic arrest or failure to find very rare cells. No translocations were detected cytologically in six semisterile males derived from treated spermatogonial stages (i.e., conceived 36–90 days after treatment).

These findings appear to indicate that spermatid stages are especially sensitive to the mutagenic action of cyclophosphamide.

No attempt to characterize different translocation configurations was made in sterile males because of the low yield of cells at diakinesis. Only semisterile males carrying translocations were used for extensive cytological analysis of their meiotic chromosomes. Most of these showed that over 60% of the spermatocytes contained 18 bivalents plus a ring-of-four configuration. About 30% of the cells showed 18 bivalents plus a chain-of-four configuration. Two types of chains were detected according to the position of the centromeres. In type A all centromeres were at intermediate position in the chain, and in type B two pairs were at the ends and two pairs at the center of the chain. Most males showed preponderantly or exclusively one type or the other. Other configurations observed were 18 bivalents plus either 2 unequal bivalents or a chain trivalent plus a univalent. The latter two made up about 8% of the cells analyzed. Associations of more than one translocation were observed in 11 out of 31 translocation males, but in a very low frequency (about 1% of the spermatocytes).

Comparisons with published data on x-ray-induced translocation configurations in partially sterile sons show that cyclophosphamide-induced configurations resemble x-ray-induced configurations more than chemically induced ones. TEPA, for instance, induces an average of 70% chains and 20% rings in partially sterile males derived from treated postspermatogonial stages. This pattern contrasts with that induced by cyclophosphamide, which shows a more random pattern of chromosome breakage.

With regard to the transmission of the configurations, the pattern observed in F_1 was in general transmitted to F_2. This result, which may be expected on *a priori* grounds, has not been demonstrated before. As expected, gametes from F_1 translocation heterozygotes carrying normal and balanced genotypes produced a ratio of 1:1 normals and translocation heterozygotes in F_2.

At present, studies on the detection of *in vivo* unscheduled DNA synthesis after incorporation of

[^{3}H] thymidine and treatment with cyclophosphamide are in progress.

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EFFECT OF SUPEROVULATION ON NUMBER OF OVARIAN OOCYTES AND FERTILITY OF FEMALE MICE

E. F. Oakberg, Dilip Bhattacharjee,* and Patricia D. Tyrrell

These experiments were designed to provide information on the oocyte stages involved in the dramatic shift from high to low mutation sensitivity observed with time after irradiation.[1] Seventy virgin 101 × C3H female mice about eight weeks old were randomized into five groups of 14 mice each. Group A served as controls. Group B received 5 IU of pregnant mare's serum (PMS) and, 48 hr later, 5 IU of human chorionic gonadotropin (HCG). Group C was given 50 R of x rays. Group D was given 50 R of x rays and 1 hr later injected with 5 IU of PMS, followed by 5 IU of HCG 48 hr later. Group E was given 5 IU of PMS followed by 5 IU of HCG and irradiated with 50 R 18 hr later. Three females from groups C and E were killed 72 hr after irradiation, and three females from groups B and D were killed 18 hr after injection of HCG. Three controls (group A) were killed at the same intervals. Ovaries were fixed in Bouin's, sectioned at 7 μm, and stained with hematoxylin and eosin. Classification of oocytes was the same as used previously.[2] For groups A and B, stage-1, 2, 3, and 4 follicles were scored in every tenth section; stages 5, 6, 7, and 8 were counted in every section. A count of all oocyte stages in all sections was made for irradiated mice (i.e., groups C, D, and E). The remaining 11 mice in each group were pair-mated with T-stock males four days after superovulation.

Oocytes in follicle stages 1, 2, and 3 were reduced to 0.08, 1.26, and 11.67% of control by 50 R (group C). Superovulation after x irradiation (group D) had no effect, with survival percentages of 0.09, 1.07, and 10.14 for the same stages. Superovulation before irradiation (group E), however, increased survival to 0.19% for stage-1, 4.4% for stage-2, and 20.9% for stage-3 oocytes. The data, although not extensive, suggest that hormone treatment prior to x-ray exposure increases the survival of early oocyte stages.

The fertility data are not yet complete, but they suggest that fertility, both in number of litters and number of young per female, is slightly increased by superovulation prior to x irradiation. Exposure to 50 R of x rays reduced the number of litters to 4.4 and the number of young to 20.4 per female. Superovulation following x-ray treatment reduced these values to 3.5 and 16.2, or about one litter per female. The data are not extensive, and the observed differences are small, but it appears that superovulation depletes the supply of older, radiation-resistant follicles.

*IAEA Research Fellow.

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RADIATION RESPONSE OF SPERMATOGONIAL STEM CELLS

E. F. Oakberg, C. Huckins,* and Patricia D. Tyrrell

On the basis of tubule whole mounts scored at 8 and 13 days after irradiation, Dym and Clermont[1] concluded that all spermatogonia except the A_0 were killed, and that depletion of the differentiating population stimulated mitosis among the A_0 survivors. However, our data on the mouse indicated that when [^{3}H] thymidine was injected prior to irradiation, a significant proportion of the survivors were labeled,[2] suggesting that the stem cells were in cycle at the time of irradiation. Further studies by Huckins[3] and by Oakberg[4] concluded that there was no evidence for an A_0 (noncycling) spermatogonial population. The radiobiological, and especially genetic, implications of this interpretation are discussed elsewhere.[2] In spite of the extensive data which show the stem cells to be in continuous cycle, there has been equivocation over this point in recent papers on radiobiology of the testis.

To obtain more precise data than previously had been available, we decided to repeat the [^{3}H] thymidine labeling and irradiation experiment in the mouse. Twenty-four hybrid (101 × C3H) male mice, 12 weeks old, were randomized into two groups. One group received six injections of 17.5 μCi of [^{3}H] thymidine per mouse at 12-hr intervals, and one group received a single injection of 17.5 μCi of [^{3}H] thymidine. At 159 hr after the last injection, half of the mice in each group were given 150 R of x rays, and they were killed 48 hr later (207 hr after the last [^{3}H] thymidine injection). This should allow the cells to complete one cycle of the seminiferous epithelium; that is, the cells would be in the same stage of the cycle of the seminiferous epithelium as at the time of labeling. Tissues fixed in Zenker Formol were processed by the method of Kopriwa and Huckins[5] and scored for labeled vs unlabeled stem cells.

Table 15. Percentage of labeled A_s spermatogonia 207 hr after 150 R

Stage of cycle	6 × 17.5 μCi [^{3}H] thymidine		1 × 17.5 μCi [^{3}H] thymidine	
	Control	150 R	Control	150 R
1	51	76	17	23
2	49	56	21	30
3	36	55	13	22
4	48	51	9	5
5	42	27	6	4
6	32	33	0	1
7	24	21	8	3
8	36	17	3	9
9	29	24	14	12
10	29	16	8	4
11	39	33	11	22
12	38	41	14	19

The data of Table 15 reveal a high labeling percentage at all stages of the cycle for both irradiated and control mice after multiple injections. The single injection of [^{3}H] thymidine showed the highest labeling at stages 1, 2, 3, 9, 11, and 12 of the cycle of the seminiferous epithelium for both control and irradiated groups. Thus the survivors are from a cycling and not a noncycling population as required by the A_0 (reserve-stem-cell) concept. This is conclusive evidence that the stem cells are in continuous cycle; if accelerated division rates do occur after irradiation, it is enhancement of a naturally occurring process. In fact, the data suggest that the behavior of A_s spermatogonia is not perturbed by 150 R.

The importance of these results in the radiobiology of the testis cannot be overemphasized. The demonstration of the A_s spermatogonia as stem cells[3,4] with no reversal in the developmental sequence makes them the most important cell with respect to fertility and genetic damage.[2] All subsequent stages have a short life span, also can be sensitive to cell killing, and are rapidly cleared from the reproductive tract. The A_s spermatogonia, however, with the capacity to replenish their own numbers and to differentiate, persist throughout the reproductive life of the individual. Thus they are the most important cell type regardless of total dose, dose rate, or dose fractionation.

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EFFECTS OF DOSE ON THE INDUCTION OF DOMINANT-LETHAL MUTATIONS AND HERITABLE TRANSLOCATIONS WITH TRIETHYLENEMELAMINE IN MALE MICE*

W. M. Generoso, Sandra W. Huff, and Katherine T. Cain

We are presently studying the effects of dose on the induction of chromosome breakage at postmeiotic stages in male mice with a number of mutagenic chemicals, using dominant-lethal mutations and heritable translocations as end points. This study allows us to make two comparisons. First is the comparison between chemicals in their effectiveness in inducing chromosome aberrations at high and at low doses, and second is a comparison in efficiency between dominant-lethal mutations and heritable translocations as indicators of chemically induced chromosome breakage in male germ cells. As we gain better understanding of the relationship between dominant-lethal mutations and heritable translocations for a variety of chromosome-breaking agents, it is obvious that we should also be able to improve our methodology for evaluating chromosome-breaking ability of chemicals.

Dose effects of triethylenemelamine (TEM) in the induction of dominant-lethal mutations were studied at spermatocyte, midspermatid, and early spermatozoa stages, with doses ranging from 0.035 to 0.4 mg/kg. The comparative translocation experiment was conducted only for the midspermatid stage (the most sensitive stage for dominant-lethal induction with TEM). In both experiments, (101 × C3H)F_1 males 12–15 weeks old were used. In the translocation experiment, treated males were mated to (SEC × C57BL)F_1 females and male progeny were tested for sterility and partial sterility using the sequential procedure described earlier (ref. 1 and the following article).

The pattern of effects on spermatocytes, unlike midspermatids and early spermatozoa, indicated possible cytotoxic damage, so for determination of dose-response curves in the induction of genetic damage only the data for midspermatids and early spermatozoa were used (these data are now in press[2]). The TEM dominant-lethal dose-effect curves were tested for linearity by the method of least squares. The slope parameter in the model is $P_i = 100 - bD_i$, where P_i is the average number of living embryos at dose D_i, divided by the average number of living embryos in the control group times 100. For both spermatozoa and

Table 16. Dose effects of TEM in the induction of heritable translocations

Dose (mg/kg)	Number of male progeny tested	Number of translocations		
		Number of sterile males	Number of partially sterile males	Frequency (%)
0.2	204	23	36	28.9
0.1	466	22	48	15.0
0.05	597	10	25	5.9
0.025	732	5	5	1.4
0.0125	927	3	5	0.86[a]
Control	2633	3	0	0.11

[a] $P < 0.01$ for a significant difference from control.

spermatids, the deviations from this model were significant ($P < 0.01$ in both cases), indicating that the linear model gave a poor fit. Nevertheless, there is a marked difference between the shapes of the dominant-lethal dose-response curves for EMS[3] and TEM. Beginning with the dose points at which a significant effect was first observed, there is a considerably more rapid increase in dominant-lethal effects with dose of EMS than of TEM. For both spermatozoa and spermatids there is a slight shoulder at the lowest dose levels of TEM, but this shoulder is not nearly as pronounced as that observed for EMS.

The frequency of translocations (as indicated by sterile and partially sterile animals) is shown in Table 16. The frequency induced by the lowest dose of TEM studied (0.0125 mg/kg) is significantly higher than the control ($P < 0.01$). On the other hand, in the dominant-lethal study, increase in dominant-lethal mutations was detected beginning with the dose of 0.05 mg/kg. Thus, as in the EMS results, reciprocal translocations proved to be a much more reliable measure of induced chromosome breaks.

Progeny testing and cytological examination of partially sterile males are now under way. Data so far indicate that, as with EMS, partial sterility is always associated with transmissible translocations. From the practical standpoint, the study of transmissibility and cytology of partial sterility is important, as it is becoming increasingly clear that partial sterility alone can be taken as an unequivocal indicator of a reciprocal translocation. For details on the fertility of TEM-induced translocations, see the following article.

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FERTILITY OF X-RAY- OR TEM-INDUCED TRANSLOCATIONS IN MALE MICE*

W. M. Generoso, Katherine T. Cain, and Sandra W. Huff

In male mice our results have proved that heritable translocations are a more efficient end point in measuring low chromosome-breakage effects of EMS and TEM than are dominant-lethal mutations. These findings clearly point to a more extensive use of the heritable translocation procedure in the evaluation of the ability of chemicals to induce chromosome breakage. To this end, we are developing a sequential procedure for the detection of translocation heterozygotes in male mice.[1] The development of this procedure requires a good understanding of the relative fertility of translocation heterozygotes induced by many different agents as compared with normal animals. Thus we found that about one-third of EMS-induced translocations were completely sterile, while two-thirds were partially sterile.[2] The average fertility of the EMS-induced partially sterile males was about 42–43% that of normal males, and analysis of litter size sired by them on (SEC × C57BL)F_1 females showed that only 0.0195% of the litters included ten or more offspring.

In the present report, the translocation heterozygotes studied were derived from x-ray- or TEM-treated postmeiotic male germ cells. For details of the TEM experiment see the preceding article. In the x-ray experiment, (101 × C3H)F_1 males 12–15 weeks old were exposed to 700 R of acute x rays and caged individually with (SEC × C57BL)F_1 females immediately afterwards. After one week and after each of the next two weeks, the females were removed and replaced with fresh females. Male progeny were tested for fertility as follows. Each male to be tested was caged with an (SEC × C57BL)F_1 female. The cages were checked for newly born mice when they were expected, and young were discarded immediately after they were scored. Each male was kept with the female until a maximum of two litters were scored, and the males were grouped according to the following fertility classifications: class A, no young produced within 90 days; class B, at least one litter of ten or more; class C, neither litter of ten or more. Class A males were caged

Table 17. Average fertility of partially sterile sons from x-ray- or TEM-treated male mice[a]

Treatment	Class	Number of males	Implants (av)	Living embryos (av)	Dead implants (%)
X rays	Partially sterile	30	9.1	3.9	57
	Normal	39	9.6	9.0	6
TEM	Partially sterile	119	9.2	4.2	54
	Normal	69	10.0	9.5	5

[a]Six pregnancies were analyzed for each partially sterile male and three for each normal male.

with three or more virgin (C3H × C57BL)F_1 females which were killed for uterine analysis 14 days later. All of the class C males and 93 class B males were caged individually with virgin (SEC × C57BL)F_1 females 12–15 weeks old, and at least four litters from each pair were scored for the number of offspring. If the total number of young in four litters was 40 or more, the male was classified as fertile; otherwise the male was given three virgin (C3H × C57BL)F_1 females which were killed during pregnancy for uterine analysis. Obvious partially sterile males and those in which fertility was still inconclusive at this point were given at least three more (C3H × C57BL)F_1 females. Thus, a total of six pregnancies per partially sterile male were analyzed. The extensive fertility testing procedure conducted for sons of x-irradiated males was designed to show whether the sequential procedure we are currently using on a large scale is detecting all the translocations induced by another agent, since it was developed on the basis of the fertility of EMS-induced reciprocal translocations.[2]

In the TEM experiment, 119 male progeny were partially sterile and 63 were completely sterile, while in the x-ray experiment there were 30 partially sterile and 18 completely sterile. Thus, as for EMS,[2] the ratio of partial to complete steriles is about 2 to 1. The average fertility of x-ray- and TEM-induced partially sterile translocations, as determined from uterine analysis of females (Table 17), is 43% and 44%, respectively, that of normal animals. In both cases, the great majority of unbalanced gametes were expressed as dead implantations, and a small proportion of them resulted in preimplantation losses. In all respects, the fertility data for partially sterile translocations induced by EMS,[1] TEM, or x rays were remarkably similar.

All partially sterile males in the x-ray experiment were derived from class C. On the basis of live births, the average fertility of these partially sterile males was only 39% that of normal. Of the 177 litters sired by 30 partially sterile males, only one was bigger than nine (0.56%). This frequency is slightly lower than that for EMS-induced partially sterile males, which is about 2%. Thus the sequential procedure for detecting translocation heterozygotes,[1] used extensively in our lab, in which litter size of ten or more sired by a male in his first or second litter is used as the indicator of full fertility, clearly appears to be efficient in detecting translocation heterozygotes induced by the three agents studied. It is likely that this situation is also true for all inducers of transmissible translocations.

Our effort in developing the translocation procedure is continuing. We have so far studied the fertility of male translocation heterozygotes and developed the procedure for detecting them. We are presently studying the fertility of female translocation heterozygotes, hoping that a procedure for detecting them can also be developed.

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6-MERCAPTOPURINE, AN INDUCER OF DOMINANT-LETHAL MUTATIONS IN PREMEIOTIC AND EARLY MEIOTIC GERM CELLS OF MALE MICE*

W. M. Generoso, R. J. Preston, and J. G. Brewen

Chromosome breakage induced by mutagenic chemicals in the germ cells of male mice can be detected and quantitated by dominant-lethal and heritable-translocation tests, and by direct cytological analysis of meiotic and premeiotic germ cells of treated animals. From the results reported in the literature, two generalizations can be made. First, chemicals which clearly produce chromosome aberrations in male mice either are alkylating agents or are known to be transformed *in*

vivo into alkylating forms. Second, chromosome-breakage effects induced by chemicals and detected by dominant-lethal or heritable-translocation tests are found only when postmeiotic germ cells or late spermatocytes are treated. It has also been observed[1,2] that when spermatogonia are treated with chemicals that are known to induce chromosome aberrations in postgonial stages, it is not possible to recover translocations in primary spermatocytes of the treated animals.

These generalizations have subsequently been found to be oversimplified, as shown by the recent cytological data of Adler,[3] Schleiermacher,[4] and Manyak and Schleiermacher[5] with mitomycin C, and the dominant-lethal results of Ray and Hyneck[6] with 6-mercaptopurine. Mitomycin C was shown, by direct observation of spermatogonial metaphases, to cause a high incidence of chromatid aberrations in spermatogonia that are largely in differentiating stages. The antileukemic purine analog 6-mercaptopurine was shown to induce a detectable, but relatively low, frequency of dominant-lethal mutations in cells that were either in very early meiotic or late differentiating spermatogonial stages. The data presented here confirm the dominant-lethal mutation results of Ray and Hyneck[6] with 6-mercaptopurine and shed light on the chromosomal basis for the dominant lethality induced by this compound, as well as showing that treatment of cells in late spermatogonial or early meiotic stages results in chromosome aberrations observable in diakinesis–metaphase I.

Male (101 × C3H)F_1 hybrid mice 10–12 weeks old were injected intraperitoneally with 6-mercaptopurine dissolved in 0.03 *N* NaOH. Doses ranging from 150 to 482 mg/kg were adjusted to the weight of each mouse, with a maximum of 1 ml solution given to any animal. In the dominant-lethal experiments, each male was caged with two virgin (101 × C3H)F_1 females 10–12 weeks old at the beginning of the respective mating periods. Females were checked for presence of vaginal plugs every morning, and each mated female was replaced by a fresh one. Females that did not show a plug after five days of caging with males were also replaced. Mated females were killed for uterine analysis between the twelfth and fifteenth day after the plug. Males that were used for cytological analysis of the testes were killed 9 to 16 days after treatment. This time interval permits cytological analysis of the germ cells that are most sensitive to dominant-lethal induction with 6-mercaptopurine as they pass through the diakinesis–metaphase I stage. Cytological preparations from the testes were made by the method of Evans et al.[7] with the modification previously published.[8]

Dominant-lethal data presented in Table 18, which were obtained to determine the sensitivity of the entire spermatogenic cycle, showed that effects of the 150-mg/kg dose were restricted to matings that occurred 32.5–35.5 days after treatment. At higher doses, the most sensitive period appears to begin also at 32.5 days after treatment but with apparent spreading of effects up to 39.5 days.

The corresponding cytological analysis of the dominant-lethal effects of 250 mg/kg or 150 mg/kg of 6-mercaptopurine revealed very few chromatid aberrations in cells sampled 9–13 days after treatment. Following 150 mg/kg there was a slight increase in chromatid and isochromatid deletion frequency on day 14 and a substantial increase in isochromatid frequency on day 15. The yields of both chromatid and isochromatid deletions decreased again on day 16. Following

Table 18. Sensitivity of germ cells of adult (101 × C3H)F_1 male mice to dominant-lethal induction with 6-mercaptopurine

Treatment	Dose (mg/kg)	Treatment-to-fertilization interval (days)	No. of mated females	No. of pregnant females	Total implants among fertile females (av)	Living embryos among fertile females (av)	Dead implants (%)
6MP	150	0.5–3.5	19	19	7.4	6.8	7.9
		4.5–7.5	25	19	7.0	6.7	4.5
		8.5–11.5	22	14	6.9	6.4	6.3
		12.5–15.5	31	26	7.4	6.7	8.9
		16.5–19.5	32	26	7.3	6.7	8.4
		20.5–23.5	32	21	6.8	6.4	6.3
		24.5–27.5	14	13	7.3	6.8	7.4
		28.5–31.5	18	16	7.1	6.4	9.6
		32.5–35.5	14	10	8.4	5.9	29.8
		36.5–39.5	11	9	7.0	6.4	7.9
		40.5–43.5	19	16	6.4	5.8	9.8
		44.5–47.5	34	31	6.9	6.6	5.1

250 mg/kg there was a very large increase in chromatid and isochromatid deletion frequency on day 15 alone. The yield of interchanges was low and relatively constant on all days of sampling and at both doses used. This observation of predominantly deletion-type aberrations agrees with the results obtained in mouse bone marrow.[9]

The mating interval that is most sensitive to dominant-lethal induction with 250 or 150 mg/kg of 6-mercaptopurine is that between days 32.5 and 35.5 after treatment. The actual germ-cell stage treated that corresponds to this sensitive period cannot be determined with high precision because of the possible mixing of cells from different spermatogenic stages between the time of treatment and the time they appear in the ejaculate. There is also the possibility, considered likely, that the treatment may cause some slowdown in spermatogenesis. If time relations are taken at face value, it would appear from the data of Oakberg[10] that the cells most sensitive to dominant-lethal induction by 6-mercaptopurine are late-differentiating spermatogonia and spermatocytes in the preleptotene or leptotene stages of meiosis. On the other hand, if no slowdown of spermatogenesis has occurred, the 14- to 15-day interval between treatment and cytological observation at which most chromatid and isochromatid aberrations were observed would indicate that only late-differentiating spermatogonia were the sensitive cells. Thus, the data of Oakberg[10] and Monesi[11] suggest that cells sampled 14 or 15 days after treatment may have been treated as intermediate spermatogonia, including those undergoing DNA synthesis. However, these estimates were based on the timing in animals that were not chemically treated, and we feel for a number of reasons that the cells sampled 14 or 15 days after 6-mercaptopurine treatment may not have been in this stage at the time of treatment but in early meiosis (preleptotene), with some in late-differentiating spermatogonial stages.

The present results and the finding by Ray and Hyneck[6] clearly establish 6-mercaptopurine as an inducer of dominant-lethal mutations in male mice. The most conclusive evidence of dominant lethality in this case is the clear-cut increase in the frequency of dead implants in pregnancies derived from a specific mating interval. The effects of 6-mercaptopurine differ from those of all other known dominant-lethal inducers in male mice in two respects. First, the late meiotic and postmeiotic cell stages are not affected; and second, it is the only compound that has been demonstrated to induce dominant-lethal mutations in the very early meiotic or differentiating gonial stages. The rough agreement in sensitive cell type for the induction of dominant lethality and chromatid aberrations suggests chromatid deletions as the cause of dominant lethality in this study. Conservative estimates of the frequency of dominant lethality expected from the chromatid-aberration frequencies tend to substantiate this suggestion.

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DOSE EFFECTS OF ACUTE X RAYS ON INDUCTION OF HERITABLE RECIPROCAL TRANSLOCATIONS IN MOUSE SPERMATOGONIA*

W. M. Generoso, Katherine T. Cain, and Sandra W. Huff

This report presents the first information on the effects of dose of radiation on the induction of heritable translocations in mouse spermatogonia. Together with the simultaneous cytological study by Brewen et al.,[1] the relationship between the frequencies of heritable and cytologically scored reciprocal translocations induced in this germ-cell stage has become clearer.

Our ability to measure chromosome-breakage effects of radiation on mammalian spermatogonia has been greatly improved as a result of the development by Evans et al.[2] of the air-drying method of preparing diakinesis–metaphase I chromosomes. This method has been widely used, and a good number of papers on radiation effects on spermatogonia studied by this technique have been published. (A thorough review for work published up to 1972 was prepared by the United Nations Scientific Committee on the Effects of Atomic Radiation.)[3]

Despite this great progress on the cytological measurement of radiation-induced reciprocal translocations, our ability to estimate genetic risk from radiation-induced reciprocal translocations has improved only slightly. This situation is attributable to the fact that data on the most pertinent end point for risk estimation, heritable reciprocal translocations, are still highly deficient. Consequently, the use of the vast amount of cytological data for risk estimation for spermatogonia is limited by the fact that we know very little about the relationship between the frequency of reciprocal translocations scored cytologically and the proportion of balanced exchanges actually transmitted to progeny. This relationship has been determined for only one condition of radiation exposure – two 600-R exposures to x rays given eight weeks apart to male mice.[4] It was found that the frequency of transmissible reciprocal translocations was only about one-half of that expected on the basis of the cytological data.

We have long felt that there is considerable uncertainty as regards the actual rates by which heritable reciprocal translocations are induced by ionizing radiations, and that the relationship between the frequencies of cytologically scored reciprocal translocations and those transmitted may vary, depending upon certain variables in radiation exposure such as dose, dose rate, and dose fractionation. Thus, for purposes of risk estimation and better use of the cytological data, extensive information on the effects of various conditions of radiation exposure on the induction of heritable reciprocal translocations is needed.

Male (101 × C3H)F_1 mice 12 to 15 weeks old were given partial-body irradiation of 150 R, 300 R, 600 R, or 1200 R x rays delivered at 96 R/min. The radiation source was a G.E. Maxitron 250 operated at 197 V, 250 kVp, and 30 mA. Irradiated males were caged individually with young virgin (SEC × C57BL)F_1 females 42 days after irradiation. The first six males in each irradiated group that sired a litter were killed as soon as the sixth female had given birth, and the testes were prepared for cytological scoring of reciprocal translocations in diakinesis–metaphase I spermatocytes.[1] Each breeding pair was allowed to produce young continuously, except for the few in which the irradiated males were killed for cytological analysis of diakinesis–metaphase I spermatocytes soon after the first litters were born. Thus, both the irradiated males and their mates were progeny-tested automatically for any preexisting translocations.

Only male progeny born 69 days after irradiation or later were tested for translocation heterozygosity when they were eight weeks old or older as follows. Each male to be tested was caged with a female of the (SEC × C57BL)F_1 stock. The cages were checked for newly born mice when they were expected, and young were discarded immediately after they were scored. For each male, if the first litter sired was ten or more, the male was declared fertile and discarded immediately after the litter was scored. If the first litter was fewer than ten, a second litter was produced. If the second litter was ten or more, the male was declared fertile and immediately discarded. When neither the first nor the second litter sired was ten or more, or when no young were produced within 90 days, the male was classified as suspect and was tested further by being caged with three or more virgin (C3H × C57BL)F_1 females until the females were killed for uterine analysis, beginning 14 days after the start of caging. A minimum of three females were analyzed before a decision on fertility was made.

Males confirmed as partially sterile were progeny-tested and finally killed for cytogenetic analysis of diakinesis–metaphase I spermatocytes. In a few cases, when the first-generation male progeny died prior to cytological analysis or when cytology of these males was inconclusive, cytological analysis was performed on the second-generation sons that were proven to be partially sterile. Sterile males were killed after the fertility test was finished. The two epididymides were examined for presence and quality of sperm, and the testes were weighed. For histological and cytological analysis of the sterile male progeny from this study see the article by Cacheiro et al.[5]

The frequencies of heritable reciprocal translocations recovered after various doses of x rays are shown in Table 19. In the irradiated group, the frequencies were based on all partially sterile males plus the steriles that were cytologically confirmed as translocation heterozygotes. All the partially sterile males, with the exception of two, had produced partially sterile sons. One of the two partially sterile males became completely sterile before a progeny test could be done, but the cytology unequivocally proved translocation heterozygosity. The other died before the progeny test and cytology were done, but the fertility data on three females mated to him are conclusive indication of translocation heterozygosity (58% of the 10.3 implants per female were dead, as compared to about 5–8% dead implants for normal animals). The first translocation recovered in this experiment was born 87 days after irradiation.

The frequencies of reciprocal translocations at all doses of x rays studied were significantly higher than the spontaneous level. The shape of the dose-effect curve is clearly "humped," as the highest frequency,

Table 19. X-ray induction of transmissible reciprocal translocations in mouse spermatogonia

X-ray exposure (R)	Number of male progeny tested	Number of sterile males	Number of partially sterile males	Frequency of translocations (%)[a]
0	2633	3[b]	0	0.11
150	993	4	6	0.60
300	1018	5[c]	8	0.88
600	1075	7[d]	18	1.86
1200	1038	4[c]	5	0.58

[a]The frequencies for the irradiated groups were based on all partially sterile males plus the steriles that were cytologically confirmed.

[b]No cytology was done on the three sterile males, but all had small testes typical of sterile translocations, and in the tests of significance these animals were assumed to be translocation heterozygotes.

[c]One sterile male was cytologically confirmed as a translocation heterozygote.

[d]Two sterile males were cytologically confirmed as translocation heterozygotes.

which was observed at 600 R, was significantly higher than those observed at 300 or 1200 R. The frequency observed at 300 R appears to be slightly higher than that observed at 150 R, but this difference is not significant. The "humped" characteristic of this dose-effect curve is in general agreement with the cytologically scored reciprocal translocations induced in mouse spermatogonia found in the simultaneous study by Brewen et al.[1] and in several earlier published studies.

Brewen et al.[1] estimated the frequencies of transmissible translocations expected on the basis of cytological observation to be 0.6, 1.9, 4.2, and 1.1% for 150, 300, 600, and 1200 R, respectively. With the exception of 150 R, for which the observed frequency is similar to that expected, the frequencies observed for all doses are about one-half of those expected. The observed frequency of 6/993 for 150 R is not significantly higher than one-half of that expected, and for the three higher doses the observed frequencies are all significantly lower than the respective expected values. Thus, these results seem to indicate, as with the data of Ford et al.,[4] that, indeed, the number of heritable translocations induced by radiation is only about one-half of that expected on the basis of cytological data. This study is continuing, to obtain more data at 150 R and to find out whether the above situation holds true also for other conditions of radiation exposure.

*Research sponsored jointly by the National Center for Toxicological Research and by the U.S. Atomic Energy Commission under contract with the Union Carbide Corporation.

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HIGH FREQUENCY OF PRESUMED DOMINANT LETHALS IN MICE TREATED WITH ISOPROPYL METHANESULFONATE (IMS) AFTER MATING*

K. E. Suter†

Complete information on the sensitivities of various germ-cell stages in male and female mice to induction of chromosome aberrations by chemicals is important in our understanding of the mutation process in mammals and in the evaluation of genetic hazard to humans. The development of germ cells from the time they are contained in the gonads until they participate in syngamy is generally described as follows. In adult males, differentiation of spermatogonial stem cells, meiosis, and spermiogenesis occur in the testes. and the resulting sperm undergo some maturation in the epididymis and vas. After ejaculation, the process of sperm maturation continues in the female reproductive tract, followed by a developmental change of the sperm within the oocyte which is characterized by the

appearance of the male pronucleus. In the adult ovary, all germ cells are "arrested" in a diffused diplotene stage of meiosis I, with the exception of a few that will be ovulated within a short time. The latter oocytes are in various postdictyate stages – they complete first meiotic division in the ovary and are ovulated with the chromosomes in the metaphase of the second division. The oocytes remain in this state until stimulated by sperm entry. The oocyte stage at the time of mating may vary, depending upon the strain of mice and the environmental conditions.

So far, all the information available on the responses of various germ-cell stages in male and female mice to chemical induction of chromosome aberrations has been on stages treated prior to mating. Because of the earlier finding by L. B. Russell,[1,2] which showed that certain pronuclear stages, compared with the dictyate stage, had high sensitivity to x-ray induction of dominant-lethal mutations and sex-chromosome loss, it is mandatory to conduct a similar study with chemical mutagens. Thus, the present work is the first in a series in which the response of the postmating-presyngamy germ cells to chemical induction of chromosome aberrations will be investigated. In this study, presumed dominant-lethal effects of isopropyl methanesulfonate (IMS) on postmating treatment of both sperm and oocytes are compared with those on the most sensitive stage in male (sperm in vas and epididymis) and female (oocytes in the advanced stages of follicular development) mice.

Females from (C3H × 101)F_1 and a mixed stock (X^{Gs}/X^+) were injected intraperitoneally with either 25 or 50 mg/kg of IMS or of Hanks' solution 2 hr after the end of the dark period during which mating occurred. Ovulation was partially synchronized by shortening to 5 hr the daily dark period to which females were exposed for four weeks prior to treatment. It was determined that at the time of treatment the great majority of oocytes were in first meiotic telophase or later stages, and sperm penetration was detected in only a few. For comparison, the same doses of IMS were given to females treated within 3.5 days prior to mating (dictyate oocytes) or to males which were mated within 4.5 days after treatment (sperm in vas and epididymis). In all experiments, females were killed for uterine analysis 12–13 days after mating. Presumed dominant-lethal effects were calculated as [1 – (living embryos per female in experimental group)/(living embryos per female in control group)] × 100.

The frequencies induced by 50 mg/kg IMS in dictyate oocytes, germ cells in mated females, and spermatozoa treated in epididymis and vas are shown in Table 20. Clearly, in both stocks, effects in mated females, in which both female and male germ cells were treated, were relatively much higher than the added effects on dictyate oocytes and spermatozoa and 2–3 times higher

Table 20. Comparison between pre- and postcopulation germ cells of mice in sensitivity to dominant-lethal effects of IMS

Stocks of mice	Germ cells treated	Dose (mg/kg)	Number of copulated females	Number of females with implants	Total implants per female	Living implants per female	Dead implants (%)	Dominant lethals (%)
(C3H × 101)F_1 × (C3H × C57BL)F_1	Control	–	66	63	7.33	7.08	3.4	–
	Dictyate oocytes	25	62	56	8.09	7.57	6.4	–6.9
		50	67	57	7.19	5.74	20.2	18.9
	Oocytes and sperm in copulated females	25	69	65	7.63	5.75	24.6	18.8
		50	69	64	5.98	1.51	74.7	78.7
(C3H × 101)F_1 × (C3H × 101)F_1	Control	–	40	38	6.66	6.24	6.3	–
	Sperm in vas and epididymis	25	38	34	6.82	6.09	10.5	2.4
		50	38	34	6.76	4.85	28.3	22.3
X^{Gs}/X^+ × (C3H × C57BL)F_1	Control	–	61	54	8.11	7.67	5.5	–
	Dictyate oocytes	25	64	50	8.50	7.62	10.4	0.6
		50	63	46	7.74	5.43	29.8	30.0
	Oocytes and sperm in copulated females	25	61	55	8.02	5.78	27.9	24.6
		50	61	41	5.85	1.80	69.2	76.5
X^{Gs}/X^+ × X^+/Y	Control	–	32	27	8.55	8.22	3.9	–
	Sperm in vas and epididymis	25	51	43	9.07	8.12	10.5	1.2
		50	40	34	8.09	6.09	24.7	25.9

than either. This situation is also true for the 25-mg/kg dose. The much higher frequency of presumed dominant lethals in mated females compared with that of the most sensitive stage in both sexes separately stresses the importance of studying all stages in screening chemicals for hazard evaluation.

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†Postdoctoral investigator supported partly by Subcontract No. 3322 from the Biology Division of Oak Ridge National Laboratory to the University of Tennessee and partly by the government of the Canton of Zurich (Switzerland).

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STUDIES ON THE DOMINANT-LETHAL AND FERTILITY EFFECTS OF THE HEAVY-METAL COMPOUNDS MONOMETHYLMERCURIC HYDROXIDE, MERCURIC CHLORIDE, AND CADMIUM CHLORIDE IN MALE AND FEMALE MICE*

K. E. Suter†

Preliminary studies have been completed that were necessary before the experiment on X-chromosome nondisjunction and loss in male and female mice could be carried out. These include (1) the toxicity effects of single intraperitoneal injections of monomethylmercuric hydroxide, mercuric chloride, and cadmium chloride to establish the doses that will be used in the genetic study[1] and (2) effects of these metal compounds on dominant-lethal induction in males and females and on the reproductive capacity in females.

In all experiments, the chemicals were dissolved in distilled water and given in a single intraperitoneal injection. The dose used for monomethylmercuric hydroxide was 10 mg/kg and for mercuric chloride and cadmium chloride 2 mg/kg. Control animals were injected with distilled water. In all experiments, the number of experimental and control animals was the same.

Only monomethylmercuric hydroxide was tested for dominant-lethal effects in males. Injected males of the two stocks (101 × C3H)F_1 and (SEC × C57BL)F_1 were mated serially with young virgin (C3H × C57BL)F_1 females for 48 days. This period covers all stages of the spermatogenic cycle. Every day the females were removed and replaced by fresh females, and they were subjected to uterine analysis 12 to 15 days later. Data were pooled into two-day intervals. For each interval an average of 12 pregnant females were analyzed.

Presumed dominant-lethal effects of all three compounds were studied only in (101 × C3H)F_1 females that mated 0.5 to 4.5 days after injection. As in the male dominant-lethal experiment, copulated females were removed every morning and killed 12 to 15 days later. For each chemical, the dominant-lethal experiments were repeated three times. The results of all three trials were pooled together, since there was no indication of heterogeneity.

In the study of the effects of the three compounds on the reproductive performance of females, females from (101 × C3H)F_1, (SEC × C57BL)F_1, and a mixed stock were used. Immediately after treatment, the animals were caged individually with a young male. The breeding pairs were checked for the presence of a litter when expected, and to maximize the number of offspring from each female, the newborn animals were discarded soon after scoring. Effects of monomethylmercuric hydroxide were studied for 404 days and those of mercuric chloride and cadmium chloride for 366 days.

Results in males indicate that monomethylmercuric hydroxide did not induce a detectable increase in dominant-lethal mutations at any stage in spermatogenesis. In the two stocks studied, no significant effects were found in the numbers of fertile matings, implantations, or living embryos, or in the incidence of dead implantations throughout the 48-day test period after treatment. The number of females mated and analyzed for the (101 × C3H)F_1 stock was 264 in the experimental and 240 in the control group and, for the (SEC × C57BL)F_1 stock, 230 in the control and 259 in the experimental group.

In the female dominant-lethal study, two of the chemicals, monomethylmercuric hydroxide and mercuric chloride, significantly reduced the average number of living implantations, while the other, cadmium chloride, had a diametrically opposite effect. With monomethylmercuric hydroxide and mercuric chloride, the average number of living implantations was reduced from 7.2 to 6.4 and 6.7 to 6.0, respectively, with P values less than 0.005 in both cases. In the case of monomethylmercuric hydroxide there was no significant increase in the incidence of dead implantations, while in the case of mercuric chloride there was a slight but significant increase ($P < 0.025$). With both chemicals, there was no significant reduction in the rate of ovulation (as determined by corpora lutea counts). The increase in the incidence of dead implantations with mercuric chloride only partially accounts for

the reduction in the number of living implants. Thus the effects of both compounds are attributable largely to preimplantation losses. With cadmium chloride, the increase in the number of living implants [6.9 vs 6.6 in the control ($P = 0.05$)] is attributable to an increased ovulation rate. The average number of corpora lutea among cadmium-chloride-treated females was 8.2, while for control females the value was 7.6 ($P < 0.001$), and there was no detectable increase in the incidence of dead implantations.

In the reproductive-capacity test the average number of animals born per female was reduced by the chemicals monomethylmercuric hydroxide and mercuric chloride in $(101 \times C3H)F_1$ females but not in the other two stocks. In all three stocks there was no detectable effect with cadmium chloride. The reduction of fertility induced by monomethylmercuric hydroxide and mercuric chloride was due to reductions of the average litter size between days 139 and 290 (monomethylmercuric hydroxide) and days 101 and 271 (mercuric chloride). During this period presumably follicles of stage 1 were treated. When sizes of the first litters were compared, there was significant reduction in mercuric-chloride-treated females of the two stocks $(101 \times C3H)F_1$ and $(SEC \times C57BL)F_1$. Although not significant, the general tendency of the results in the other stocks and other chemicals was in agreement with the reduction or increase of the average number of living implantations found in the dominant-lethal experiments with $(101 \times C3H)F_1$ females.

There is the question whether the results of the dominant-lethal experiments with males and females and the results of the reproductive-capacity test with females could give any indication whether nondisjunction is induced by monomethylmercuric hydroxide, mercuric chloride, or cadmium chloride. Nondisjunction leads either to polyploid or aneuploid chromosome conditions. Aneuploidy of an autosome predominantly causes the early death of the embryo that should show up in a dominant-lethal experiment as an increase in the number of dead implantations and in a reproductive-capacity test as a decrease in the average litter size. A negative result in these tests does not necessarily imply that no nondisjunction is induced. Neither the dominant-lethal test nor the reproductive-capacity test is sensitive enough to detect very low frequencies of aberrations, as is the case with large-scale experiments in which a specific aberration type is tested (e.g. sex-chromosome nondisjunction and loss). In the male dominant-lethal experiment, monomethylmercuric hydroxide showed in all stages no effect, and in the female experiments the best indication for a possible genetic effect was the slight increase of dead implantations in females treated with mercuric chloride. These findings suggest that if any of the three compounds induces nondisjunction, the frequency is low.

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†Postdoctoral investigator supported partly by Subcontract No. 3322 from the Biology Division of Oak Ridge National Laboratory to the University of Tennessee and partly by the government of the Canton of Zurich (Switzerland).

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PATHOLOGY AND IMMUNOLOGY

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Radiation Recovery of Hemopoietic Cells

L. H. Smith

Experimental Hematology

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Cellular Immunology

E. H. Perkins
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Pathology

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Laboratory Animal Science and Microbiology

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Mammalian Radiobiology

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INTRODUCTION

THE RESEARCH ACTIVITIES of the Pathology-Immunology Section encompass four main program areas: (1) the effects of dose, dose rate, and radiation quality on the induction of late-occurring disease (principally neoplasms); (2) the role of the immune system in the aging process; (3) mechanisms of radiation carcinogenesis; and (4) factors controlling proliferation and differentiation of blood cells. Certain other ancillary studies are performed which cannot be neatly categorized under these program areas but which are highly relevant to our research objectives. Summary descriptions of the individual research projects within these programs are provided on the following pages.

It is not entirely fair to single out any particular research project as representing a research "highlight," because every researcher should believe his work to be worthy of special mention. To illustrate the diversity of work within the section, however, we call attention to the following projects. In preliminary studies Goodman and Smith have shown that thymic lymphomas of mice (which appear so monotonously alike at autopsy or under the microscope) are actually extremely variable with respect to other characteristics. They have assayed for the presence of the theta antigen in these cells and have evaluated the ability of the cells to augment hemopoiesis from transplanted marrow. Great variations in these measures were found from one tumor to another. The large-scale experiments on late somatic effects of radiation reported by Storer and his colleagues have finally reached a stage at which definitive information is emerging. The close agreement between the results reported for life-shortening effects and the results predicted from physical theory is particularly gratifying. Studies by Otten and his colleagues on the radiation activation of mouse leukemia viruses show promise of giving insights into radiation leukemogenesis and may provide explanations for differences in the responses of intact animals to radiations of different qualities (neutrons vs gamma rays). Finally, Yuhas and his co-workers have extended their investigations on the potential usefulness of the radioprotectant WR-2721 in the radiation therapy of human cancers. Toxicity and radioprotective efficacy have been studied in additional animal species. The extrapolated dose of the drug for man (10–20 mg/kg) to obtain radioprotection is reasonable in terms of the logistics of drug administration. Collaborative arrangements have been made with outside investigators for a clinical trial in dogs with spontaneous tumors.

As the large-scale experiments on somatic effects gradually wind down, we expect to place increased emphasis on studies of mechanisms of carcinogenesis. Specifically, we will increase our efforts in the areas of the role of (1) viruses, (2) the immune system, (3) the endocrine system, and (4) cell killing and regeneration in cancer induction and progression.

BASIS OF LYMPHOCYTIC CONTROL OF HEMOPOIESIS

Joan Wright Goodman, Nancy L. Basford, and Sarah G. Shinpock

When lymph-node lymphocytes are transplanted along with marrow cells to a nonisogenic irradiated host, the resulting splenic hemopoietic foci a week or so after transplantation contain an abnormally high number of granulocytic colonies.[1] Moreover, the total number of colonies of all kinds is greater as a result of lymphocyte transplantation. This is a real augmentation of marrow growth and not merely the result of the few additional granulocytic colonies that may be derived from the injected lymphocytes themselves. There are at least two possible ways in which lymphocytes could be operating: (1) by "trapping" additional multipotential stem cells in the recipient's spleen in the early period of "seeding" (additional, that is, to the number ordinarily settling there from a similar marrow inoculum in the absence of lymphocytes) and then influencing their direction of differentiation by subsequent events or (2) by selectively trapping a larger than usual number of "committed" stem cells, those irreversibly differentiated one step beyond the multipotential stage.

We sought to resolve this issue by a two-step transplantation experiment. Recipient F_1 hybrid mice were heavily irradiated, then administered (intravenously) either marrow alone or the same dose of marrow plus lymphocytes. Donors were parental-strain mice whose marrow had previously been shown to grow poorly in the hybrid. This relatively poor growth situation affords a sensitive experimental model for studying factors capable of augmenting or controlling marrow proliferation and differentiation.[2] Twenty-four hours after the initial transplantation, primary hosts' spleens were removed and made into single-cell suspensions for injection into a second group of irradiated recipients. This time recipients were parental strain,

isogenic with the original donor cells, to afford an optimal environment for proliferation of whatever stem cells had settled (in 24 hr) in the primary hosts' spleens.

Eight days after the second transplantation, recipients were killed, their spleens were removed and fixed in Tellyesniczky's solution, and the surface colonies were counted under a dissecting microscope (9X magnification).[3] Histologic preparations were made of each spleen, and colonies were then counted microscopically and classified as to differentiation pathway as previously described.[1] The group that received spleen from marrow-plus-lymphocyte recipients had more than twice as many hemopoietic colonies as the comparable (marrow only) group. Distribution of colony types was the same in both groups and did not differ from the normal pattern. The second possibility – that lymphocytes cause granulocytically committed stem cells to settle preferentially in the recipient's spleen – appears to be ruled out by these findings.

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AUGMENTATION OF MARROW GROWTH BY THYMOCYTES SEPARATED BY DENSITY GRADIENT CENTRIFUGATION

Linda L. Pritchard,* Sarah G. Shinpock, and Joan Wright Goodman

Thymocytes from B6 mice augment the growth of B6 marrow cells in lethally irradiated $B6D2F_1$ recipients.[1] Very large numbers of thymocytes must be administered to obtain maximal augmentation of marrow growth.[2] We hypothesized that effective cells represent a small fraction of the total thymocyte population, and we undertook experiments to determine whether the presumptive "effective cell" could be separated from the total thymocyte population on the basis of buoyant density. Thymocytes were separated into subpopulations using a 17–25% albumin gradient (2% steps) as described by Dicke.[3] $B6D2F_1$ mice were exposed to 900 R of x radiation; the next day they received intravenous injections of appropriate thymocyte and/or marrow cell suspensions. Eight days later the animals were killed, and their spleens were removed and fixed in Tellyesniczky's solution. Hemopoietic spleen colonies, counted with the aid of a dissecting microscope (9X), were enumerated to determine the extent of marrow growth. When thymocytes from the various gradient fractions were compared with equivalent numbers of thymocytes from an unfractionated suspension, it was found that thymocytes from the unfractionated suspension augmented marrow growth as well as did thymocytes from each of the gradient fractions. Hence it appears that the presumptive "effective thymocyte" does not have a characteristic buoyant density.

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PRELIMINARY CHARACTERIZATION OF SOME RADIATION-INDUCED THYMIC LYMPHOMAS

Joan Wright Goodman and L. H. Smith

Considerable interest has been developing recently in aberrations of differentiation (e.g. chemical, morphologic) that have been reported for malignant cells. These may result from permanent changes in genetic constitution or from abnormal patterns of gene expression or from both. It is clear, however, that cancer cells retain many characteristics of normal cells. A knowledge of similarities and differences between malignant and analogous normal tissues is of paramount importance to an understanding of the neoplastic process.

We have been studying thymic lymphomas found in over 70% of C57BL/6 mice exposed early after weaning to 170 R of 250-kVp x rays each week for four consecutive weeks. Each of six tumors has been studied separately. Pooling of donor tissue has not been necessary, as a single malignant thymus may yield more than 10^9 cells. So far we have examined two parameters: one is immunochemical, expression of the cell surface marker θ, an antigen characteristic of differentiated thymocytes; the other is physiological, ability to augment hemopoiesis from transplanted marrow.[1]

In all studies, thymocytes pooled from several normal C57BL/6 donors were studied in parallel with malignant cells. Standard twofold serial dilutions, starting at 2 or 4×10^7 cells per tube, were made and washed. To each of the drained pellets was added 100 μl of anti-θ serum (AKR anti-θ C3H), and the absorption took place at 4°C over the period of 1 hr, after which cells were again pelleted by centrifugation. Then, 50 μl of supernatant

Table 21. Capacity of thymic lymphoma cells to augment hemopoiesis in irradiated $B6D2F_1$ hybrid recipients of C57BL/6 cells

Expt	Marrow cell dose	Thymocytes			Mean number of nodules per spleen	Relative θ content
		Normal	Malignant	Tumor		
A	2×10^5	–	–	–	3.8	–
		4×10^7	–	–	16.2	+ 5
		–	4×10^7	1	13.1	+ 3.5
		–	4×10^7	2	7.7	+ 2
B	4×10^5	–	–	–	0.8	–
		2×10^7	–	–	13.9	+ 5
		–	2×10^7	3	7.4	N.D.[a]
C	4×10^5	–	–	–	3.1	–
		2.4×10^7	–	–	22.3	+ 5
		–	2.4×10^7	4	6.4	+ 6
D	4×10^5	–	–	–	5.0	–
		2.4×10^7	–	–	25.4	+ 5
		–	2.4×10^7	5	25.3	+ 5.5
		–	2.4×10^7	6	11.4	N.D.[a]

[a]Not done.

was removed from each tube for testing in the usual way (by reaction with ^{51}Cr-labeled thymocytes in the presence of guinea-pig complement) for remaining (unabsorbed) anti-θ activity. Cell-surface content of θ antigen relative to that of normal thymocytes could thereby be evaluated. Ability of thymic lymphoma cells to augment hemopoiesis was measured in a straightforward transplantation experiment using the poor-growth situation of a low dose of parental C57BL/6 marrow injected with or without thymocytes into irradiated F_1 hybrid mice. Eight days later, spleens were removed from all mice, and surface colonies were counted.

It can be seen from Table 21 that the capacity to augment marrow growth was highly variable and ranged from almost no ability to values indistinguishable from those derived from normal thymocytes. Theta content was also variable but was not obviously correlated with ability of the cells to promote blood formation. These data suggest that radiation-induced thymic lymphomas are highly individualistic, despite their uniform histologic appearance.

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RADIOPROTECTION BY DRUG-DAMAGED ERYTHROCYTES

L. H. Smith

Results of preliminary experiments[1] showed that injection of isogenic erythrocytes (RBC) from mice given phenylhydrazine ($PhNHNH_2$) increased 30-day survival and hemopoiesis in the spleens of lethally x-irradiated mice. This type of protection is different from that obtained with chemicals such as cysteine and AET, in that chemicals increase cell radioresistance (D_0), whereas it is likely that RBC damaged by $PhNHNH_2$ are radioprotective because of their ability to alter proliferative states of tissues. The tissues most likely involved are those of the hemopoietic and immunologic systems, including the reticuloendothelial elements. $PhNHNH_2$-damaged RBC may belong to a class of radioprotectors which includes diverse materials such as bacterial lipopolysaccharides, inorganic particles, and certain bacterial or viral antigens. There is marginal evidence that these materials protect by a similar mechanism(s), and their actions are usually considered, perhaps incorrectly, to be nonspecific.

The basic experiment is as follows. Mice are injected with 3.0 mg of $PhNHNH_2HCl$; 24 hr later they are bled, and the RBC are washed and injected into recipient

mice. The recipients are exposed to x radiation, and various end points of recovery are determined. Mice injected with normal RBC are used for controls.

Injection of 0.5 ml of $PhNHNH_2$-damaged RBC increased the $LD_{50/30}$ about 100 R, and survival was proportional to RBC dose up to 0.5 ml. Maximum survival resulted when the interval between injection of $PhNHNH_2$-damaged RBC and irradiation was one day; as the interval was increased, survival decreased and was not significantly above controls at four days. The protective factor resides only in the formed-element fraction of the blood, not in the plasma. Hematologic indices obtained after irradiation showed that following the usual pancytopenia, there was a slow recovery of peripheral blood elements. Protection by damaged RBC appears independent of the spleen, which shows that reticuloendothelial elements in the spleen are not involved.

Protection was achieved by RBC exposed to $PhNHNH_2$ *in vitro* as well as *in vivo*, which shows that only direct contact between the RBC and the drug is involved. Using the *in vitro* system, we tested for radioprotection by RBC that were exposed to other damaging agents. Saponin, chloroform, and toluene were ineffective, as were RBC ghosts prepared by exposure of RBC to distilled water.

The results indicate that $PhNHNH_2$ alters the RBC membrane, perhaps specifically, and that this alteration is translated into a stimulus for recovery of the hemopoietic system in irradiated mice. On the other hand, the damaged RBC may stimulate the reticuloendothelial system in extrasplenic sites and provide a better defense against infection. In this case, hemopoietic recovery would originate from a small number of hemopoietic stem cells that survived irradiation.

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QUANTITATIVE COMPARATIVE STUDIES OF CELL-MEDIATED IMMUNE COMPETENCE IN AGED MICE

E. H. Perkins, Lucia H. Cacheiro, and W. J. Peterson

Quantitative studies delineating the dramatic age-related decline in immune competence have focused primarily on the humoral or B-cell immune system rather than on the cell-mediated or T-cell system, because the former is more amenable to precise quantitative evaluation. The quantitation of the T-cell immune system is difficult, since cell-mediated immunity is a collective response encompassing a variety of immunologic parameters, each with a cellular basis for its expression. Unfortunately, cell-mediated immunity during senescence has often been measured in qualitative terms. We have therefore assessed a number of reflective indices to gain better insight into and a more comprehensive analysis of the declining functionality of this system in aged BALB/c mice. We also wished to compare the relative sensitivity of these indices with changes in humoral and *in situ* cell-mediated immunocompetence.

Cell-mediated immunity was assessed by the following four methods: (1) classical splenomegaly ten days following injection of 10×10^6 young-adult or aged parental (BALB/c) spleen cells into neonatal F_1 hybrids, (2) acute graft-vs-host (GVH) disease, based on the radioactivity incorporated into the spleens of lethally irradiated young adult F_1 hybrids given a 40-min pulse of [^{3}H]dThd administered three days after injection of 25×10^6 young-adult or aged parental (BALB/c) spleen cells, (3) responsiveness of aged spleen cells to the plant mitogen phytohemagglutinin (PHA) as assessed by [^{3}H]dThd incorporation, and (4) resistance to tumor cells (P815 mastocytoma) of the same H-2 type (H-2^d). Our results demonstrate that the spleen index (classical neonatal splenomegaly) was 3.29 with young and 1.63 with spleen cells from old BALB/c mice (about a twofold decrease). A 2.4-fold reduction was seen in the acute GVH reaction as determined by *in vivo* [^{3}H]dThd incorporation in the spleen. In contrast, when aliquots of the same individual spleen-cell suspensions used to determine acute GVH by *in vivo* [^{3}H]dThd incorporation were tested for PHA blastogenic activity (T-cell proliferation), the measured decline in immunocompetence was markedly different. When the stimulation ratios ([^{3}H]dThd incorporation of PHA-stimulated cultures divided by the incorporation of nonstimulated cultures) of these same young adult and old spleen cells were compared, an eightfold decrease in activity was seen. Corroboration of this substantial decline in immunocompetence (assessed by PHA responsiveness) was noted when the cumulative 30-day mortality of young-adult mice challenged intraperitoneally with 10^5 or 10^6 tumor cells was found to be only 18 and 27%, respectively, whereas the mortality of 25-month-old mice challenged with 10^4 or 10^5 tumor cells was 87 and 100%. The humoral immune response of old BALB/c mice as measured by the number of direct plaque-forming cells in the spleen was also markedly impaired; the peak response was delayed by two days. At four days the response of old mice was 80-fold less than the four-day peak response of young adult animals, at five days it was 25-fold less,

and at six days (the peak) it was 60-fold less. From these observations it is suggested that neonatal splenomegaly and *in vivo* [^{3}H]dThd incorporation in the spleen are two relatively insensitive assays for measuring declining cell-mediated immunity, whereas *in vitro* blastogenic activity to PHA is more sensitive and relates in a more quantitative manner to the overall decline of immunocompetence, which allows unrestricted growth of transplanted tumor cells and more closely approaches the decline seen in humoral immune competence.

SIGNIFICANT RESTORATION OF PHYTOHEMAGGLUTININ RESPONSIVENESS OF SPLEEN CELLS FROM OLD MICE

Mary K. Halsall and E. H. Perkins

We have recently shown, using the T-cell-specific stimulant phytohemagglutinin (PHA) to measure immune potential, that mouse spleen cells lose their ability to respond to PHA with age.

This decreased responsiveness with age was not due to a marked drop in the relative number of T cells in the spleen,[1] greater sensitivity to cultural conditions,[2] or decreased numbers of PHA-binding sites.[3] It would, therefore, appear to be due to as yet undetermined deterioration changes in the normally responsive T-cell or supportive-cell function. It was suggested that a suppressor cell responsible for decreased T-cell reactivity in middle-aged animals was an adherent cell, since removal of this cell population from 17-month-old mouse spleen cells restored the PHA response nearly to that of cells from young adult animals. Furthermore, 2-mercaptoethanol added to unseparated spleen cells did not enhance the response as much as when it was added to cells from which the adherent cells had been removed. However, the response of spleen cells from old (30 months) animals is enhanced about fivefold with the addition of 2-mercaptoethanol, whether or not adherent cells are removed.

Macrophages were not able to restore the responses of nonadherent cells from middle-aged or old animals to that found with 2-mercaptoethanol, whereas with addition of macrophages to nonadherent cells from young adult animals there was total restoration. This suggests that, with increasing age, a second cell (supportive in nature) in or affecting the nonadherent T-cell population is deficient.

The action of 2-mercaptoethanol on the lymphocytes has been postulated to be at the cell surface, specifically on receptor sites, and it has been suggested that 2-mercaptoethanol may uncover membrane receptor sites masked during the aging process. The present observation suggests that the decline in responsiveness of spleen cells from aged animals may not be related entirely to an intrinsic T-cell defect, and that 2-mercaptoethanol may act in part by replacing deteriorating supportive-cell function.

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IMMUNOHEMOPOIETIC SENESCENCE IN RATS

C. F. Gottlieb

Senescent changes in an individual's ability to mount a humoral immune response to a foreign antigen have been studied primarily in the mouse model, and to a considerably lesser extent in other species, including humans. The few existing studies with rats have used serum titers against bovine serum albumin or thryglobulin. Because of the wide application of the rat model to research in aging, and to confirm our findings in the mouse, we have undertaken a brief study of age-related changes in the immunohemopoietic system of the rat.

We have approached the question from several directions, using rats derived from both Wistar and Sprague-Dawley strains; old rats were 20–25 months of age, young were 3 months. First, the direct plaque-forming cell response in the spleens of rats immunized with sheep erythrocytes was found to be reduced in old rats compared to young. The peak response was threefold lower and was delayed by about two days. This threefold reduction is more than accounted for by defective immunocompetent cells, because transfer of spleen cells from old or young rats together with sheep erythrocytes into lethally irradiated recipient mice resulted in about tenfold lower response from the spleen cells of old rats. Peak serum antibody titers (both total and 2-mercaptoethanol resistant) indicated an age-associated decline. The hemolysin response was only marginally decreased in old rats, although the level of 2-mercaptoethanol-resistant hemolysin produced was about tenfold higher in young than in the old rats. Both total and 2-mercaptoethanol-resistant agglutinin responses were tenfold lower in aged rats. Taken together,

these observations show a reduction in the antibody response (IgM and IgG) of old compared to young rats.

Stem-cell capacity of bone marrow from young and old rats was evaluated by the number of gross colony-forming units (CFU) expressed in irradiated xenogeneic recipient mice. The CFU frequency decreased 1.8-fold in marrow from old rats. Although the frequency was lower in the old rats, the number per individual did not change appreciably, because there was a 1.6-fold increase in cellularity of the femurs of old animals.

We conclude that age affects the immunohemopoietic system of the rat in a manner almost identical to that seen for the mouse. A delayed and decreased humoral antibody response has been observed in both, as well as cancelling changes in the CFU frequency and bone marrow cellularity, so that the number of CFU per animal remains unchanged.

T-CELL RADIATION SENSITIVITY DURING IMMUNE INTERACTION

C. F. Gottlieb

The sensitivity of mouse T cells to x irradiation has been studied, using sheep erythrocytes (SRBC) as stimulating antigen. The limiting cell in the immune response to this antigen is the T cell; therefore, the anti-SRBC response is a measure of T-cell function.

The radiation sensitivity of the T-dependent response has been measured by constructing a survival curve using the spleen-cell transfer system, wherein normal or irradiated spleen cells are cultured with antigen in lethally irradiated syngeneic recipient mice. After an initial shoulder 75 R wide (extrapolation number 2.7), the T-cell survival curve was exponential. The D_0 calculated from the limiting exponential slope was 70 R. This D_0 falls in the range expected for dividing cells, indicating that cell division is the most radiosensitive phase of this T-cell-mediated response.

Study of the temporal relationship between stimulation with a T-dependent antigen and x irradiation in intact mice has shown that enhancement, with a response greater than the unirradiated control, is observed when SRBC injection precedes irradiation by a few days. In contrast, suppression is observed when antigen follows irradiation, the maximum suppression occurring with a one-day separation. More detailed study of the recovery phase (antigen after irradiation) using the spleen-cell transfer system showed maximum suppression when the T-dependent response was assessed three days after x irradiation. An abortive rise was seen six days after irradiation, with a dip occurring about 17 days, after which recovery to twice the preirradiation response level was observed at 45 days.

Thus, we have shown that the T-dependent SRBC response of the mouse is quite radiosensitive, suggesting cell division as the critical event, and that recovery of this population of cells in the irradiated animal is complete within two months after irradiation.

TIME-DEPENDENT DETERIORATION OF THE *IN VITRO* PRIMARY ANTIBODY RESPONSE OF SPLEEN CELLS HELD AT ICE TEMPERATURE

Mary K. Halsall

When using separation techniques such as STAPUT sedimentation or Ficoll or bovine plasma albumin gradients, there is an inherent, relatively long processing time between removal of cells from an organ and their final disposition. Since experimental design often dictates that these techniques be a prelude to culturing and assessment of immunologic activity, it is necessary to maintain the functional activity of immunocompetent cells over a span which may easily extend past 6 hr. Therefore, the ability of mouse spleen cells to mount a primary immune response to sheep red blood cells (SRBC) was assessed *in vivo* and *in vitro* after cells were held on ice in various culture media for various lengths of time. This is comparable to conditions encountered using cell-separation techniques.

No loss of activity was found by *in vivo* assessment (spleen cells transferred with antigen to lethally irradiated recipients) when spleen cells were held at ~0°C for up to 6 hr in three standard media. Results obtained by *in vitro* immunologic assessment were significantly different. Cells held in CMRL1066 or MEM lost their ability to produce plaque-forming cells exponentially with time on ice, with approximately half their activity lost in 2 hr. Cells in RPMI-1640 medium followed the same kinetics of loss for 2 hr and then maintained that level for the remaining 6 hr. Neither varying cell concentration during holding on ice nor adding antigen during holding improved the response. The decreased response was not attributable to serum concentration or cell death. By separating cells by allowing them to adhere to plastic, it was determined that a plastic-adherent population was responsible for the loss in activity on ice; but this loss was not due to physical depletion of the adherent cells during processing.

Since the irradiated animal (*in vivo* assessment) could restore the loss seen *in vitro*, a radiation-resistant cell in the adherent fraction is implicated. Fresh adherent

cells, fresh peritoneal exudate cells, or adherent cells held at 37°C, when added to spleen cells which had been on ice, restored the response of the nonadherent cells, but cells held at 0°C gave nominal restoration of 10–28%. It was further determined that vitamin B_{12}, which is not present in CRML1066 or MEM but is found in RPMI-1640, could restore the response of the adherent ice-held cells in the first two media. 2-Mercaptoethanol not only restored but also replaced the adherent cell function. Thus it appears that a nutritional deficiency of some media allows deterioration of adherent cell function when the cells are held for long periods at low temperatures.

LATE SOMATIC EFFECTS OF IONIZING RADIATION IN MICE AS A FUNCTION OF DOSE, DOSE RATE, AND RADIATION QUALITY

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L. J. Serrano, M. C. Jernigan, W. C. Klima,
Lou C. Satterfield, H. C. Swann,
and Faye S. Martin

Over the past ten years we have conducted experiments with large numbers of mice to determine the effects of dose, dose rate, and radiation quality on longevity and disease incidence. Radiation with a low linear energy transfer (LET) in tissue (gamma rays) has been compared with radiation that has a high LET (fission neutrons). Both radiations have been delivered at a high dose rate and a low dose rate. We have also looked for effects at lower total doses than are usually employed. To ensure that the effects we see are not peculiar to a single mouse strain, we have studied mice of both the BALB/c and RFM strains. The radiation exposures were begun when the mice were ten weeks old. On completion of the exposures, the mice were maintained in a barrier facility for the remainder of their lives. Complete autopsies have been performed on over 95% of the dead (or killed when moribund) animals, and detailed histology has been obtained in about 40% of the cases. Types of radiation, doses, dose rates, and sample sizes are shown in Tables 22 and 23.

Except for the first experiment with RFM mice, none of these studies is yet complete, and we are unable to draw final conclusions about effects on disease incidence. Even though there are still animals alive in most of the experiments, we have been able to calculate mean survival times for all the groups by applying certain corrections for live mice. From these survival times we have evaluated the extent of life shortening in all the dosage groups. It should be noted that while life shortening is a complex end point, it is nevertheless a very useful measure of the combined deleterious somatic effects of radiation exposure. In fact it can be argued that loss of longevity is the most relevant deleterious effect.

Table 22. Experiments conducted with RFM female mice[a]

Expt no.	Initial sample size	Radiation	Dose rate	Range of total doses
1	16,200	^{137}Cs gamma rays	40 rads/min	0–400
2A	6,141	^{137}Cs gamma rays	8 rads/day	0–400
2B	1,543	^{137}Cs gamma rays	40 rads/min	50, 200
3	1,281	Fission neutrons	25 rads/min	25–200
4	1,299	^{252}Cf neutrons	1 rad/day	25–200
5	733	Fission neutrons	5–20 rads/min	0–50

[a]Total mice = 27,227.

Table 23. Experiments conducted with BALB/c female mice[a]

Expt no.	Initial sample size	Radiation	Dose rate	Range of total doses
1A	5858	^{137}Cs gamma rays	8 rads/day	0–400
1B	1725	^{137}Cs gamma rays	40 rads/min	50, 200
2	1302	Fission neutrons	25 rads/min	25–200
3	1304	^{252}Cf neutrons	1 rad/day	25–200
4	761	Fission neutrons	5–20 rads/min	0–50

[a]Total mice = 10,950.

On the basis of our analyses we have reached the following conclusions. The dose-response curve for gamma rays given at high dose rates to RFM mice is not linear over any major portion of the dose range. In the dose range of most interest, namely 0 to 50 rads, linearity can be rejected ($P < 0.01$), and a good fit is

obtained by a dose-squared equation of the type $Y = bX^2$, where Y is days life shortening and X is the dose in rads. The probability that the observed deviations from this line are due to chance is greater than 80%. We do not yet have enough data points to draw conclusions for the BALB/c strain. The points we do have agree well with those for the RFM strain. When gamma rays are delivered at a rate of 8 rads/day, there is much less life shortening than at the high rate. In this case, however, the dose-response curves for both strains appear to be linear ($P > 0.5$ in both cases). Fission neutrons delivered at either high (25 rads/min) or low (1 rad/day) rates give linear dose-response curves in the lower half of the dose range. There is little difference in effectiveness between the two rates; or in other words, the neutrons exert their effects relatively independently of dose rate. The neutrons are much more effective on a dose-for-dose basis than gamma rays in producing life shortening. Since the dose-response curve for neutrons is linear while the curve for gamma rays at high rates follows a dose-squared relationship, it is clear that the relative biological effectiveness (RBE) for neutrons increases as the dose decreases. This effect has been shown by others using simpler test systems. Figure 18 shows the dose-response curves for RFM females in the low dose region.

The above conclusions support the concept that risk estimates for low-LET radiations which are based on extrapolation from data obtained at high doses and dose rates to low doses and/or low dose rates will seriously overestimate the risk. A more realistic estimate for low doses at either a high or low rate would be obtained by extrapolating from high doses delivered at low rates (Fig. 18).

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EFFECTS OF FAST NEUTRONS OR X RAYS DURING EARLY PRENATAL DEVELOPMENT IN THE MOUSE

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and M. C. Jernigan

We are investigating the effects of neutrons and x rays on mouse embryos irradiated during early preimplantation development. Initial studies[1] were on the pronuclear zygote. The results suggested that inactivation with either neutrons or x rays is predominantly a single-event process, that the neutron RBE (which is approximately 4.5) is close to the low-dose limiting value, and that irradiation early in the pronuclear zygote stage impedes progression to a less radiosensitive phase of that stage.

More recently we have been studying the two-cell embryo, and we find it considerably more radioresistant than the pronuclear zygote. Thus an absorbed dose of 4 rads of neutrons or 19 rads of x rays given to the early pronuclear zygote will result in prenatal death for 20% of the embryos so treated. To produce the same mortality by irradiation of the two-cell embryo requires

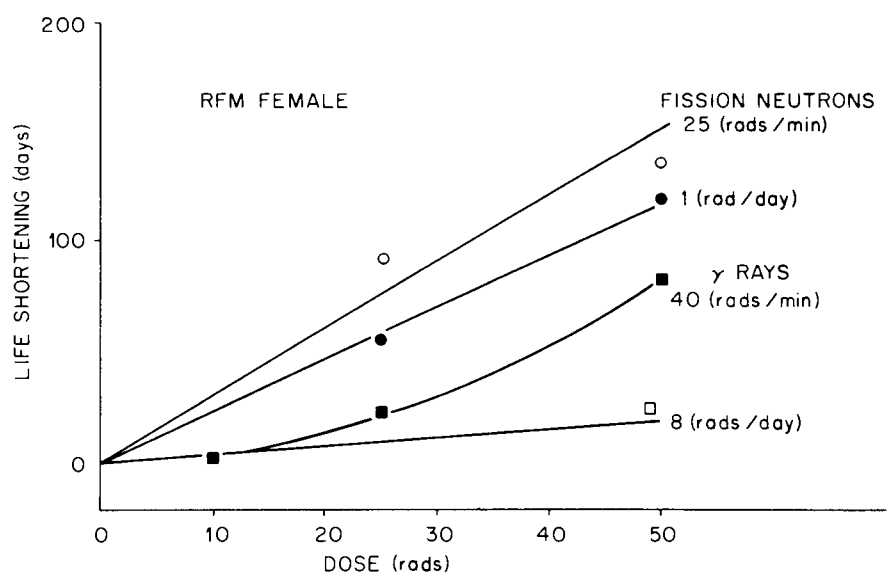

Fig. 18. Dose-response relationships in the low dose range.

a 19-fold greater dose of neutrons or x rays. We have only partially interpreted the survival curve for the two-cell embryo, but it seems to be consistent with the view that both blastomeres must be lethally injured to cause embryo death. As with the irradiated pronuclear zygotes, all or virtually all of the embryo deaths induced by irradiation during the two-cell stage occurred before day 11 of the 19-day gestation period.

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EFFECTS OF BUTYLATED HYDROXYTOLUENE, DIETHYLNITROSAMINE, AND X RAYS DURING TUMORIGENESIS IN MICE

N. K. Clapp, R. B. Cumming, C. F. Gottlieb, R. L. Tyndall,* 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 the antioxidant butylated hydroxytoluene (BHT), which is 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,[2,3] we attempted to determine whether BHT would also protect against the carcinogenic action of DEN. We found that rather than a protective effect there was some suggestion of potentiation by the BHT. Further, BHT given alone induced hyperplastic lesions in the biliary ducts, indicating that BHT may not be as innocuous as generally believed.[4] We are now expanding this preliminary investigation to determine definitively whether BHT and DEN show an interaction; whether BHT interacts with ionizing radiation; whether the two known carcinogens, DEN and radiation, interact; and the effect of each agent singly. Male and female BALB/c mice are being used. Doses of the agents are (1) 0.75% BHT in the feed throughout life, (2) DEN in the drinking water for seven weeks for an accumulated average dose of 330 mg/kg, and (3) 250 rads of x rays. Suitable control groups are also maintained. The mice will be killed at either 12 or 18 months of age for pathological examination. In addition to obtaining pathology data we will make serial observations in individual mice on serum esterase changes (Tyndall), liver microsomal enzyme changes (Cumming), immune status (Gottlieb), and viral activation (Tennant).

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EFFECTS OF ANTIOXIDANT PRETREATMENT ON LETHALITY FROM IONIZING RADIATION

N. K. Clapp and Lou C. Satterfield

Butylated hydroxytoluene (BHT) has been considered to have at least two physiological effects in mammals – enzyme inducer and free-radical scavenger. Cumming and Walton[1] observed a potentiation of radiation lethality following treatment of $C31F_1$ male mice with 725 R x rays. The evidence suggests that there was no protective effect against physical agents such as x rays, whose damage has been attributed at least in part to free-radical reactions. In contrast, BHT treatment did, in fact, protect against DEN acute toxicity, suggesting that its enzyme-inducer activity may be beneficial to animals receiving DEN.

Barrier-derived BALB/c male and female mice were maintained in an extremely clean, conventional environment. BHT was added in commercially prepared pelleted feed at 0.75% concentration and fed for 28 days prior to giving 525–750 R of x rays. Other mice received the same radiation doses with no added BHT in the feed. Lethality was determined 30 days after irradiation.

In both sexes and at all doses given (525, 560, 600, 675, and 750 R), mice receiving BHT prior to radiation received slight protection; that is, fewer mice died with BHT plus radiation than with radiation alone. The approximate $LD_{50/30}$ for males was 625 R with BHT and 580 R without BHT; for females, the $LD_{50/30}$ was 645 R with BHT and 630 R without BHT.

These observations suggest that, in contrast with $C31F_1$ male mice, BALB/c male and female mice are protected against lethal radiation doses by treatment with the antioxidant and free-radical scavenger BHT. The strain differences in response would appear to relate to some genetic difference, although environmental differences between the studies cannot be

eliminated completely. Survivors of these studies are now being observed for differences in late effects in tumors, leukemias, and other diseases of a degenerative or metabolic nature regarding dose effects.

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ESTERASE PATTERNS IN MICE AFTER BUTYLATED HYDROXYTOLUENE OR DIETHYLNITROSAMINE

R. L. Tyndall,* N. K. Clapp, and Koweta B. Davidson

We have detected serum esterase changes from a variety of aging and tumor-bearing mice as well as in DEN- and BHT-treated mice. Sera from DEN-treated tumor-bearing mice showed marked repression of activity in three specific esterases and enhanced activity in one band. Sera from mice given both DEN and BHT did not show these particular alterations but showed enhanced esterase activity in other specific bands. Thus, as interactions were found in histologic observations, serum esterase studies show an interference by BHT with DEN-induced esterase changes.

As a result of these preliminary findings, we are now examining information from serially killed BALB/c mice of both sexes to determine correlations between early esterase alterations and the possible predictability of pathological changes that occur later. We recognize esterase modifications as early as three weeks into BHT treatment, as well as specific changes associated with the DEN treatment period. We are completing the evaluation of changes which occur prior to overt tumor development. Many of the changes in DEN-treated tumor-bearing mice are in hormone-associated serum esterases, suggesting a possible source of the observed alterations.

We are assessing observations at early time points and collecting data from 12- and 18-month killing times. We are also investigating the organ or tissue sources of various serum esterases.

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OVARIAN TISSUE RADIOSENSITIVITY AS DETERMINED BY CELL TYPES IN RADIATION-INDUCED OVARIAN TUMORS

N. K. Clapp and M. C. Jernigan

We exposed over 3100 young-adult female RF/Un mice with whole-body 60-MeV protons or 300-kVp x rays at doses of 0–400 rads at 80–100 rads/min. They were observed for life-shortening effects and neoplastic and nonneoplastic diseases. Over 80% of the animals were examined histologically, and the pathological data and dose responses for the various diseases have been reported elsewhere.[1,2]

Control incidences of ovarian tumors were 3.8%, and we observed that groups of mice developing ovarian tumors exceeded 50% of the total number of mice irradiated. With the doses used in this study we were not able to detect the existence of a threshold dose, if one should exist, but there was a significant increase in ovarian tumor incidences even with the 50-rad dose. We confirmed the extreme radiosensitivity of the mouse ovary, which is essentially sterilized (no remaining Graafian follicles) at 50 rads, as well as the extreme radiosensitivity of the mouse ovary to tumor induction.

From over 2400 mice histologically examined, we evaluated the cell types that made up the ovarian tumors. Following irradiation of the mouse ovary, dying mice had a progressive peripheral proliferation and invagination of adenomatous cells downward into the remaining luteinized portion of the irradiated ovary. Very early stages of this change were described as a pretumor and not considered histologically to be tumorous. We classified tumors of the ovary into those composed of a single cell type and those of mixed cell types. Mixed-cell tumors were likewise classified by cellular components.

In tumors of a single cell type, adenomas were the most common (309/479), with granulosa-cell and luteoma and a few miscellaneous tumors in lesser incidences. Six adenocarcinomas were not highly anaplastic, and metastases even in the large numbers in this experimental group were extremely rare.

At the 100-rad dose level, we found the highest incidences of mixed-cell tumors, a majority of which were luteal adenomas. The miscellaneous tumor types again were varied and numerically rather incidental. Granulosa-cell mixed tumors were relatively few in number, even in combination with luteomas or adenomas, as most of the mixed tumors were luteal adenomas in one or both ovaries.

Figure 19 shows the percentages of animals that would be expected to develop tumors with the various cellular components in the ovary after ionizing radiation. Tumors of the adenoma elements are most frequent, showing a maximum at 100 and 200 rads and a negative dose response at higher dose levels. Luteal tumors likewise increase to a maximum at 100 rads, with a slight decrease at higher doses. Granulosa-cell tumors show little dose effect between 50 and 400 rads.

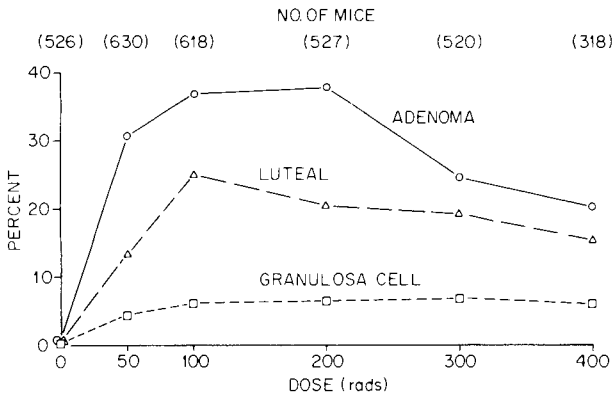

Fig. 19. Percentages of all ovarian tumors with various tumor-cell elements (no. of tumors/total no. of mice at risk).

If we consider the number of animals at risk and the number of tumors induced with the various cell types at the lowest dose (50 rads) as a parameter for evaluating radiosensitivity as reflected in radiation tumorigenesis, the adenoma-cell component is most susceptible or most radiosensitive at 50 rads, the luteal cell component is intermediate, and the granulosa cell is least sensitive to tumor induction. Tissues comprising other tumor cell types were so minimal as to be only mentioned as miscellaneous and in passing. We feel that this is a unique study because we have within a single organ a comparison of tissue response without much possible variation in dose within this particular organ, the ovary; the large number of animals enables us to evaluate radiation sensitivity as determined by radiation carcinogenesis within a single body organ.

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MEGAKARYOCYTOPOIETIC RESPONSE TO PLATELET NEED

T. T. Odell, Diane K. Beeman, and Deborah A. Boran

Some marked changes in megakaryocytopoiesis occur in rats recovering from thrombocytopenia. There is an increase in the average ploidy of cells of the recognized megakaryocyte population, an increase in the number of megakaryocytes in marrow, and possibly also an increase in the maturation rate of megakaryocytes. Earlier experiments suggested that additional megakaryocytes begin to expand the recognized population about 24 hr after induction of acute thrombocytopenia, as evidenced both by an increase in the total number of megakaryocytes in the marrow and by an increase in the proportion of the population made up by immature megakaryocytes. An increase in average ploidy of megakaryocytes was also seen at 24 hr, as measured by microspectrophotometric determinations on Feulgen-stained marrow smears. From a count of the number of mitotic figures among megakaryocytes in the marrow, an increase in their mitotic index at about 24 hr after acute thrombocytopenia was discovered. Other studies have shown that, after moderate to complete depletion of circulating platelets, the entry of new platelets into the circulation becomes very rapid by 48 hr and continues at a rapid rate for about three days. The foregoing results raise questions about the timewise contributions of each of these responses, whose ultimate function is to return circulating platelet numbers to a normal level, and the underlying mechanisms that control these responses. One question is whether the stimulus to megakaryocytopoiesis brought on by low numbers of circulating platelets acts on diploid precursor cells or cells in the late replicative stages of megakaryocyte maturation. Data on the proportion of the recognized megakaryocyte population in DNA synthesis at various times after induction of acute thrombocytopenia might distinguish between the foregoing possibilities. Therefore, rats are being injected with tritiated thymidine at intervals after injection of antiplatelet serum and killed 30 min later. Autoradiograms of marrow smears are being examined to determine the labeling index of megakaryocytes and the proportion of immature and mature megakaryocytes. Marrow sections are being examined to determine changes in the size of the megakaryocyte population. Results thus far suggest that the megakaryocyte population begins to increase by 18 hr after injection of antiplatelet serum. The labeling index of megakaryocytes, on the other hand, may be greater than in controls at 6 hr and is certainly greater at 12 hr. These observations added to others mentioned above indicate that megakaryocytes in the immature recognizable compartment are stimulated soon after induction of thrombocytopenia to undergo an additional replication of DNA beyond what they would in normal circumstances. Since the number of megakaryocytes also increases, and since polyploid megakaryocytes are not known to divide, we postulate that DNA synthesis is stimulated in the megakaryocyte line both in diploid and polyploid cells. By 60 hr after injection of antiplatelet serum, the labeling index of the compartment of recognizable megakaryocytes returned to the

control level, although the number of megakaryocytes in the marrow continued above normal for at least 72 hr. Additional experiments are in progress.

STUDIES OF A HETEROIMMUNE THROMBOCYTOPENIA MODEL IN RATS: PLATELET RESPONSE

T. T. Odell, Diane K. Beeman, and Deborah A. Boran

An experimental model of thrombocytopenia in animals, produced by injection of antiplatelet serum, has previously been used to study the thrombocytopenic condition as well as recovery from thrombocytopenia. In addition, mice in rebound thrombocytosis, after induction of thrombocytopenia with antiplatelet serum, have been used in assays of thrombopoietin. However, the recovery of circulating platelet levels after various degrees of thrombocytopenia induced with antiplatelet serum has not been systematically investigated. It is important to study such recovery for several reasons. For one, questions have been raised whether platelet response differs when thrombocytopenia is produced with antiserum or by exchange transfusion with platelet-poor blood. In addition, some discrepancies among experiments utilizing platelet antiserum remain to be explained. Moreover, it is important to quantify the megakaryocyte-platelet response to platelet antiserum if animals treated in this manner are to be used as assay subjects. A series of experiments were therefore undertaken to assess platelet recovery in groups of Sprague-Dawley-derived rats injected with different amounts of antiserum. The antiserum was made in rabbits against rat platelets. Platelet counts were made at frequent intervals, and regressions of platelet counts on time were calculated. Smaller volumes of antiserum (about 0.02 to 0.10 ml) usually produced a moderate to mild thrombocytopenia, while larger volumes (up to 2 ml) reduced the platelet count to zero or even maintained the count at zero for several days. After acute, mild thrombocytopenia, in which platelet counts were depressed to about 65% of normal levels, numbers of circulating platelets rose at a very moderate rate (about 14% of normal platelet level per day) for about four days and reached a peak on the fifth day. However, when platelets were depressed to 40% or less of normal, the platelet count rose at a similarly moderate rate for about 36 hr and then between 36 and 48 hr changed to a more rapid rate (about 20–45% of normal platelet level per day). These patterns of platelet recovery were very similar to those seen after like depressions of platelets brought about by exchange transfusion with platelet-poor blood. After larger volumes of antiserum (up to 2 ml) or daily doses for two or four days, the platelets remained depressed for two to five days. After only a brief period of increase at a moderate rate, the peripheral platelet count rose at a very rapid rate (up to 69% of normal platelet level per day). After mild thrombocytopenia, maximum platelet counts reached about 25% above normal, while the peak after more severe thrombocytopenia was 175–200% of normal. These results demonstrate that platelet response after acute thrombocytopenia brought on by injection of antiplatelet serum is variable, depending on the amount of antiserum administered, which in turn determines the level and duration of thrombocytopenia. When heteroimmune thrombocytopenic animals are used to study thrombocytopenia or as test objects, it is therefore important to understand the relation between the doses of antiserum being used and the responses of the platelet population. These platelet responses are, of course, a reflection of effects on megakaryocytopoiesis brought about by the thrombocytopenia, and in some cases perhaps by direct effects of antiplatelet serum on megakaryocytes.

MOUSE PARTIAL-ORGAN IRRADIATION

J. M. Holland and L. C. Gipson

In an effort to understand better the relationship between dose, volume of tissue irradiated, and biological response *in vivo*, we have designed a multiple-exposure device that permits simultaneous irradiation of up to eight mice with collimated x-ray beams. A feature of the device is its flexibility. Beam diameter can be continuously varied by using lead bushings of various diameter in the top shield. Performance under actual exposure conditions was evaluated by implanting 1- X 6-mm thermoluminescent glass rods in mouse carcasses. The mice were positioned so that the beam was centered on the xyphoid cartilage and irradiated using a 6-mm bushing at various doses (Table 24).

Table 24. Physical dosimetry using a mouse partial-body radiation device

Total exposure (R)	No. of mice	Absorbed dose as percentage of the total exposure		
		Pelvis	Right kidney	Throat
300	4	<1%	<4%	<1%
600	4	<1%	<4%	<1%
900	3	<1%	<2%	<1%

Background was determined from rods implanted in unirradiated animals. Rods were calibrated in air against a 250-R Victoreen. The distances of the rod from the beam axis were estimated to be 3.0 cm for the pelvis, 1–1.5 cm for the right kidney, and 4.0 cm for the throat. These distances were measured from the xyphoid, which is an excellent anatomic landmark for radiation principally of the liver.

Dosimetric measurements by this physical technique revealed very low levels of internal scatter. Back scatter did not appear to be a significant factor, primarily due to the angle of the incident beam and the presence of a 0.25-in. lead back-scatter shield. Further *in vivo* biological dosimetry experiments are in progress using the mouse ovary to estimate scatter dose. The results of these trials are encouraging, in that they suggest a means whereby large numbers of homogeneous inbred mice can be exposed to narrow beams of x rays. We anticipate investigation of the relationship between tissue volume irradiated and tumor yield, among other questions.

KINETICS OF RECOVERY FOLLOWING PARTIAL HEPATECTOMY IN MALE C3H MICE

J. M. Holland and J. W. Hall

The supposition that the sensitivity of a tissue to the tumor-inductive effects of radiation is, in general, directly proportional to the rate of turnover of the tissue forms the basis of the cell-kinetic or cell-killing theory of radiation carcinogenesis. According to this theory, tumors arising in tissues such as liver or kidney following radiation are thought to develop as the result of at least two stimuli. The first and necessary stimulus is the initiation step, the primary interaction between the protoneoplastic cell and radiation. The second step is a stimulus favoring cell division. The later stimulus may be provided directly if the dose is sufficient to kill cells outright or indirectly if endocrine glands are so disturbed that regulatory hormone levels are altered. In an effort to develop and test this model of radiation carcinogenesis, we have begun looking at the regulation of cell turnover of possible target tissues such as liver and kidney as a basis for timing radiation to the tissue.

The majority of hepatocytes in the adult mouse liver are intermitotic – that is, the level of background DNA synthesis is very low (less than 1%). Surgical partial hepatectomy induces DNA synthesis in a majority of residual hepatocytes. The kinetics of this phenomenon was the object of this study. Sixty-seven percent of the liver was removed from 60 male C3H mice. Thirty age-matched controls were anesthetized and sham-hepatectomized. Groups of ten hepatectomy and five sham control mice were killed at 1, 2, 4, 8, 16, and 32 days after surgery. Four hours prior to killing, the mice were injected with tritiated thymidine (1.0 μCi per 10 g). The mice were killed and weighed, the livers were removed and weighed, and the specific liver weights were recorded as percentage of body weight. Figure 20 shows that the rate of recovery following surgery is amazingly rapid. Eight days after surgery the liver has regained its preoperative mass. Cytologic scoring of mitotic figures and autoradiography data have not yet been completely analyzed, but preliminary indications are that DNA synthesis peaks sharply 24 hr after hepatectomy. Mitoses are numerous from day 2 through day 4. The evidence indicates that, following hepatectomy, the residual hepatocytes begin DNA synthesis as a highly synchronized population and proceed through at least one mitosis. The recovery we observed could be obtained if the cell volume increased by one-third after hepatectomy and the 33% of liver cells remaining after hepatectomy divided once during the eight-day period. In other words, depending on cell-volume changes, there may or may not be a requirement for residual cells to divide more than once following hepatectomy. Further analysis of our thymidine-incorporation data should clarify this point.

Our ultimate objective is to define the cell cycle following hepatectomy to the extent that we can administer local radiation at various critical times

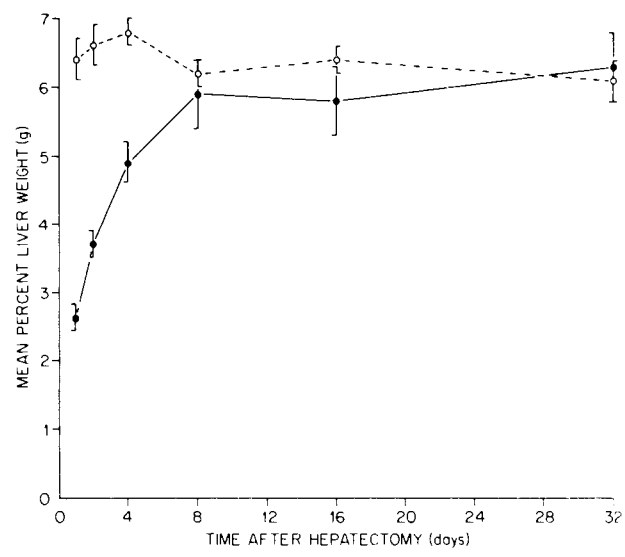

Fig. 20. Liver weight recovery after partial hepatectomy. ●–●, hepatectomized; ○--○, sham control. Vertical bars show 95% confidence intervals.

during the recovery phase. By correlating the tumor yield, latency, and histologic type with radiation at various times in the cell cycle, we will gain insight into host factors governing radiation sensitivity.

THE RELATIVE EFFICACY OF SYNGENEIC AND ALLOGENEIC THYMUS GRAFTS IN REPAIRING THYMIC DEFICIENCY IN NUDE MICE

J. M. Holland, J. W. Hall, and Diana M. Popp

BALB/c nude mice were crossed with C3H mice, and their F_1 progeny were backcrossed three times to C3H. Nude progeny from the third backcross generation were selected for homozygosity at the major H-2 locus by hemagglutination assay. The nudes selected were H-$2^{k/k}$, as are C3H mice; however, the nudes were not syngeneic with the C3H line in a genetic sense, although they were relatively more histocompatible with C3H than other strains. It has been shown previously that allogeneic thymus grafts are capable of repairing thymus deficits in nude mice, as evidenced by recovery of the immune response to heteroantigens and prompt rejection of established allogeneic skin grafts.[1,2] Workers have used a variety of approaches to reconstitute nude mice. Some have grafted thymus obtained from carrier or normal littermates of nude mice, under the assumption that these grafts would more likely persist in nude mice after reconstitution. Others have grafted allogeneic thymus from unrelated strains and reported equal success. In early experiments we observed that allogeneic thymus was more efficacious in the recovery of nude mice than were thymus grafts within the litter. The criteria used were histologic evidence of graft persistence at the site of implantation and correlative changes in the cell type observed in thymus-dependent regions of the same animal 30 days after graft implant. Since these observations were paradoxical and involved relatively few animals, we conducted the following experiment to define better the relationship between thymus donor and nude recipient.

It has been shown previously that the absolute circulating lymphocyte count in nude mice is low.[3] Therefore, we used the net change in absolute count two weeks after thymus implant as a criterion of thymus graft efficacy. In all experiments, age- and sex-matched mice were used. An increase in the relative lymphocyte count was taken as evidence of successful reconstitution of grafted nudes. The data in Table 25 reveal considerable variation as a function of the source of the thymus graft. Mean absolute lymphocyte deltas ranged from a high of 1421 in the RFM-to-nude combination to a low of −17 in the C57BL/6 combination. Only the BALB/c combination (H-$2^{d/k}$) reached significance at the 0.05% level. Both the C3H and the RFM donor combinations were significant at the 10% level. The C57BL/6 grafts were unsuccessful in altering the mean levels of circulating lymphocytes in nude mice. These data reveal that there is considerable variation between strains, which affects the efficacy of allogeneic thymus grafts implanted into weanling nude mice. Better understanding of these relationships must await the development of an inbred, preferably congenic line of nude mice.

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DEFECTIVE PLATELET FUNCTION IN THE MOUSE MUTANT "BEIGE"

J. M. Holland

"Beige" (*Bg*) is considered a homolog of the Chediak-Higashi syndrome of man. In both species the defect is

Table 25. Mean absolute lymphocyte count before and after thymus grafting in nude mice[a]

Thymus donor strain	No. of mice	Mean count (± S.E.)		Δ	T	P^b
		Day 0	Day 15			
C3H (H-2^k)	10	3275 (300)	4390 (493)	1115	1.932	<0.1
C57BL/6 (H-2^b)	10	3082 (461)	3065 (333)	-17	0.030	>0.9
BALB/c (H-2^d)	10	3127 (414)	4394 (318)	1267	2.427	<0.05
RFM (H-2^f)	12	2607 (451)	4028 (537)	1421	2.026	<0.1

[a]Nude mice were progeny derived from parents homozygous at the H-2 locus (H-$2^{k/k}$).
[b]Probability of observed difference between respective counts on day 0 and day 15 being equal to 0.

characterized by autosomal recessive inheritance and defective function of the vacuolar system, characterized by fusion or aggregation of intracytoplasmic granular organelles. Associated functional deficits are also observed and are more or less relatable to the observed abnormality of cellular vacuolar function.[1] Our recent studies have revealed that the blood platelets are also defective in *Bg* mice. In marked contrast to normal platelets, *Bg* platelets are incapable of localizing endogenous or exogenous serotinin (5HT). In mammals, the major nonneural pool of 5HT is localized in a specific platelet storage organelle, the dense body. Mammalian platelets are not capable of synthesizing 5HT but do possess specific membrane receptors for the amine and store it in dense bodies complexed with ATP. This storage is physiologically important, for it protects 5HT from degradation by ubiquitous monamine oxidase. The deficiency of platelet 5HT is absolute on the basis of filter fluorometric assays of whole-blood extracts (Table 26). The assay used is not specific for 5HT, so caution must be used in interpreting the significance of the trace amounts detected.[2] We have also demonstrated, with both *in vivo* and *in vitro* kinetic studies, impaired storage of tritium-labeled 5HT. While these studies are in progress, preliminary results reveal that 5HT initially associates with *Bg* platelets, but the association is not stable and does not result in significant storage. In other words, initial binding appears to occur, but there is a defect either in transport or storage of the amine. We plan to continue our investigations into the possible mechanism of the observed phenomena and to relate them to the several physiologic consequences one would anticipate as a result of low levels of circulating 5HT.

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Table 26. Mean whole-blood concentration of 5HT in mutant and control mice

Animal	No. of mice	μg 5HT/ml blood	S.E.
Bg	6	0.30	0.09
C57BL/6	12	3.93	0.23

RECIPROCAL INHIBITION OF MOUSE LEUKEMIA VIRUS INFECTION BY *Fv-1* CELL EXTRACTS*

R. W. Tennant, Bonnie Schluter,† Wen-Kuang Yang, and Arthur Brown†

The mouse *Fv-1* locus consists of at least two crown alleles, $Fv\text{-}1^n$ and $Fv\text{-}1^b$, which reciprocally control the susceptibility of mice to N and B host-range types of murine leukemia viruses. Resistance is dominant, and F_1 hybrids restrict both types of viruses. The *Fv-1* gene is also expressed in cultured cells, but the restriction is not absolute, and titration of the restricted virus yields two-hit kinetics. While cells of F_1 hybrids restrict both N- and B-tropic viruses, viruses of a class designated as NB-tropic are not restricted by either allele. While most mouse strains have either *Fv-1* allele, a cell line established from wild mice (SC-1) lacks the *Fv-1* gene and is sensitive to all three classes of virus.

Studies on the mechanism of the *Fv-1* restriction have shown that the inhibition is intracellular. Analysis of the fate of virus in heterokaryons of permissive and nonpermissive cells[1] indicated that the restriction functions at some step in infection early after penetration.

In our recent studies we have found that soluble extracts of mouse cells with $Fv\text{-}1^n$ or $Fv\text{-}1^b$ genotype specifically and reciprocally inhibit infection of B- or N-tropic mouse leukemia viruses in permissive cell cultures. NB-tropic virus infection was not inhibited by either cell extract. Extracts from $Fv\text{-}1^-$ cells did not inhibit infection by the three virus host-range types, but N- or B-tropic virus infection of $Fv\text{-}1^-$ cells was inhibited by extracts of the nonpermissive cells, and $Fv\text{-}1^{nb}$ cell extracts inhibited both viruses. The maximum degree of inhibition was 50–80%, as determined by immunofluorescence or plaque assays, with extracts containing up to 500 μg/ml of nonpermissive cell protein. The inhibitor(s) is relatively unstable, since activity is lost after 2 hr at 37°C or 30 min at 56°C. The inhibitor(s) is most effective if added within 2 hr before or 2 hr after infection, and it does not react with the virus directly, inhibit virus attachment, or inhibit the normal cell functions tested. These results indicate that nonpermissive mouse cells contain a product of the *Fv-1* gene which inhibits some event(s) in leukemia virus infection early after penetration.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.
†Department of Microbiology, University of Tennessee, Knoxville.

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EARLY EVENTS IN INFECTION OF CELLS BY MOUSE LEUKEMIA VIRUSES*

J. M. Quarles, Jr.,† Larry Boone,‡ and R. W. Tennant

The *Fv-1* gene-directed restriction of mouse RNA tumor viruses is apparently an active cell function which occurs after attachment and penetration of the

virus into the cell. To determine whether this restriction involves differential virus degradation in restrictive and permissive cells during or shortly after penetration but before transcription, we have begun experiments to determine the fate of labeled virus during the early events of infection in various cell types. Partially purified, [^{3}H]uridine-labeled AKR (N-type) virus and BALB/c A31 (B-type) virus were used. Previous work in this and other labs indicated that restriction was not a function of the cell membrane, and we have shown essentially identical attachment to N and B mouse cells and to cells of several other species.

For these studies, virus penetration was synchronized by attachment at 4°C and a shift to 37°C, and nonpenetrating virus was removed by treatment with trypsin. The fate of the trypsin-resistant virus was determined by assessing the distribution and state of the ^{3}H counts after cellular fractionation and sucrose density banding. Only preliminary results are available, but we have not detected differences either in the percentage of attached virus which becomes trypsin resistant or in the penetration of N-tropic virus into N and B cells. There are currently insufficient kinetic data available to determine whether the rate of degradation of virus is the same in restrictive and permissive cells. Current studies include determination of the number and specificity of sites on permissive and restrictive cells and determination of the distribution of 35S viral RNA within the cells as a function of time after penetration.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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RADIATION ACTIVATION OF MOUSE LEUKEMIA VIRUSES IN CELL CULTURE

J. A. Otten, J. M. Quarles, Jr.,*
and R. W. Tennant

Mouse RNA leukemia viruses occurring *in vivo* after exposure of mice to irradiation are linked to the subsequent development of leukemia in the irradiated animals. Both *in vivo* and cell-culture studies have shown that the host *Fv-1* gene locus controls susceptibility to leukemia virus and may be of importance in determining the fate of activated virus. In order to understand the mechanism by which these RNA tumor viruses are activated by irradiation, we have designed experiments to study activation of virus in cultured mouse cells. This approach has been applied to cells in culture derived from the BALB/c strain, which contain at least two endogenous C-type viruses, and cells from the AKR strain, which contain at least one C-type virus genome but while at low passage do not produce virus. Acute doses of x radiation followed by cell passage have been reported to activate virus from AKR cells, thus indicating the importance of cell division in this system. Other studies with iododeoxyuridine have shown the importance of host-cell DNA turnover during the virus-inducing stimulus. In our studies, BALB/c and AKR cells in tissue culture were exposed to gamma (^{137}Cs) and neutron (^{252}Cf) radiation during exponential cell growth for periods up to 40 hr to allow two to four cell divisions. Following irradiation, cells and fluids were added to selected assay cells to detect and quantitate the host-range type of the virus activated.

Results to date indicate that low levels of gamma and neutron irradiation can activate virus in these cells. Studies are continuing to increase the sensitivity of the assay system. Experiments are also under way to determine the relationship between the host range of the activated viruses and the *Fv-1* gene, and the mechanism by which irradiation causes the host cell to lose control over endogenous viruses.

*NCI Postdoctoral Fellow.

THE DOSE-RATE EFFECT IN MURINE RADIATION CARCINOGENESIS

J. M. Yuhas and Anita E. Walker

Our continuing studies on the role of dose rate in murine radiation carcinogenesis, which have been described elsewhere,[1] are now sufficiently near completion to allow preliminary analysis of the effect of this variable. Of the more than 7000 mice included in this study, more than 5000 are now dead, and we have examined the role of dose rate relative to three end points: life shortening, ovarian tumor induction, and lung carcinoma induction.

The radiation induction of ovarian tumors in BALB/c mice given total doses of 49, 98, 196, or 392 rads at rates of 1.75, 3.5, 7, 14, 28, 56, or 112 rads per day is best described by the following relation:

$$\text{Excess cases of ovarian tumors} = 0.007\, D^{2.0} T^{-0.69} ,$$

where D = total dose and T = exposure time in days. We have chosen to use the exposure time as opposed to the dose rate for two reasons: (1) use of a dose rate implies that relative time is the critical variable, as opposed to

the more realistic recognition of the importance of absolute time, and (2) the use of exposure time allows us to determine how much of the overall effect is due to age-dependent changes in resistance.

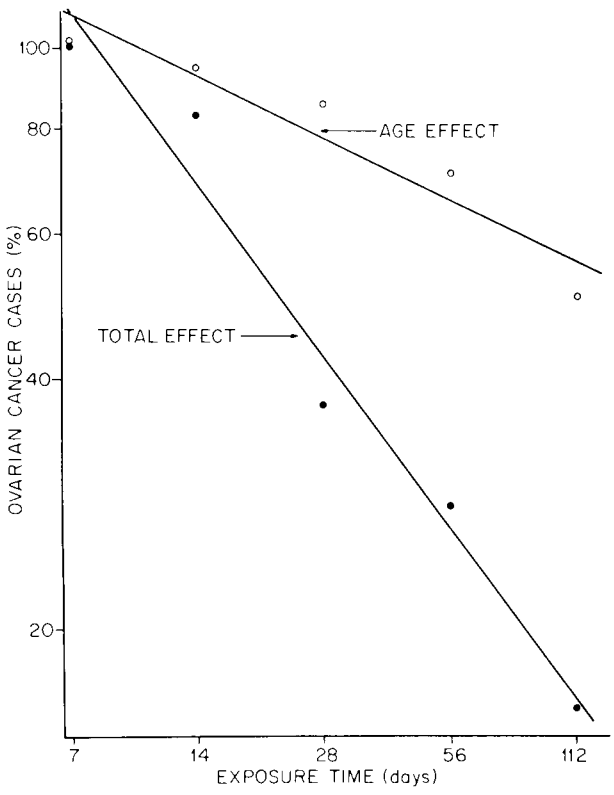

Fig. 21. Effect of exposure time (dose rate) on the induction of ovarian tumors in mice.

The latter variable is particularly critical since we already know that mice and men do not lose sensitivity to the induction of cancer at the same rate. If we relied strictly on the empirical description given above, which is heavily affected by the rapid loss of sensitivity in the mouse, we would overestimate the magnitude of the dose-rate or exposure-time effect as it might relate to other species. As part of our original design we took this factor into account, and Fig. 21 is a plot of the overall exposure-time effect compared to the exposure-time effect after correction for age-dependent loss of sensitivity. Clearly, one-third of the exposure-time effect is due to age-dependent loss of resistance, and the corrected relationship is

$$\text{Excess cases of ovarian tumors} = 0.007\, D^2 T^{-0.46} .$$

We attach no particular significance to the fact that the exponent of the dose term is 2, but we do consider the fact that it differs significantly from 1.0 ($P < 0.001$), since this establishes that the dose-response curve for this end point cannot be a linear one.

The second specific tumor system we have studied is the induction of malignant lung carcinomas. A specific description of these tumors and their etiology is given elsewhere,[2] and we concern ourselves here only with the dose-response curves for induction of this tumor. Table 27 summarizes our preliminary analysis of the induction of this tumor by gamma-ray doses of 196 or 392 rads at rates as given above. In contrast to the patterns observed for the induction of ovarian tumors (see above), the optimal dose rate for induction of these cancers is 7 rads per day, with both higher and lower

Table 27. Induction of malignant lung carcinomas by gamma rays given at various dose rates

Total dose (rads)	Dose rate (rads/day)	No. of mice exposed	Mean survival time (days ± S.E.)	No. autopsied	Lung carcinoma incidence (%)	Lung carcinoma death rate ($\times 10^{-4}$)
0	–	80	754 ± 18.0	75	2.7	0.74
196	1.75	48	765 ± 24.7	47	12.8	3.58
196	3.5	56	747 ± 21.5	51	3.9	1.08
196	7	56	719 ± 20.7	53	20.8	6.36
196	14	56	693 ± 22.9	53	9.4	3.02
196	28	48	703 ± 19.1	48	8.3	2.81
196	56	48	678 ± 19.4	47	12.8	4.48
196	112	56	689 ± 16.7	52	7.7	2.55
392	1.75	48	753 ± 19.4	47	14.9	4.32
392	3.5	40	704 ± 22.3	39	17.9	5.87
392	7	48	709 ± 19.6	46	28.3	8.94
392	14	48	672 ± 20.1	46	17.4	6.06
392	28	56	693 ± 19.8	55	14.5	4.86
392	56	56	693 ± 22.7	51	13.7	4.24
392	112	48	697 ± 22.5	43	16.3	4.91

rates being less effective. The decreasing effectiveness of the gamma rays between 7 rads per day and 1.75 rads per day is as expected, but the decreasing effectiveness at rates higher than 7 rads per day does not fit classical expectation. This latter observation is not the result of intercurrent mortality in the higher-dose-rate groups, since the death rate due to lung carcinomas is significantly higher in the group given 392 rads at 7 rads per day than in the group given the same dose at 112 rads per day – that is, an average of 1.60 ± 0.14 times higher between 500 and 900 days of age. We are currently investigating the possibility that the maximum effectiveness in the intermediate-dose-rate range is a reflection of the counterbalancing forces of malignant transformation and cytotoxicity.

Also included in Table 27 are the mean survival times for the first replicate of the experiment. It should be noted that neither total dose, 196 or 392 rads, induces significant life shortening when given at a rate of 1.75 rads per day.

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SENSITIVITY OF THE FETAL MOUSE TO THE CARCINOGENIC EFFECTS OF CHRONICALLY DELIVERED GAMMA RAYS

J. M. Yuhas and Elizabeth F. Wagner

A controversy presently exists regarding the sensitivity of fetal mammals to the carcinogenic effects of ionizing radiation. Various epidemiologists and experimentalists have investigated the problem without reaching a consensus or being able to explain the wide differences in results in the various experiments and surveys. A close examination of the studies reveals a general pattern, however, in that rapidly delivered large total doses seldom demonstrate high sensitivity, while smaller chronically delivered doses are either as effective or more effective in the fetus than in the adult, depending on the specific stage of fetal life exposed. We argued, therefore, that a definitive test of the sensitivity of the fetal mammal would involve gamma rays delivered chronically throughout fetal life.

The details of the experiment, involving 200 mice of each sex exposed to 0 or 8 rads per day through fetal life (a total of 800 mice), have been given earlier.[1] Only three mice remain alive in this experiment, and preliminary conclusions can be based on their survival and gross autopsy information. Chronic radiation exposure (8 rads per day) throughout fetal life (total dose, 168 rads) has only minimal effects on postnatal survival (–7%); it alters the shape of the survival curve without altering the mean survival time (fewer early deaths but fewer long-term survivors); and it does increase the incidence of late-appearing tumors. The critical point is that this type of exposure failed to elevate the incidence of radiation-induced thymic lymphoma. In comparing the response of these fetally irradiated mice with the response of mice given their exposures as adults (see the preceding article), it is apparent that the fetal BALB/c mouse is no more sensitive to radiation carcinogenesis than the adult.

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INTERSPECIES TOXICITY AND DISTRIBUTION STUDIES WITH WR-2721 AND PRELIMINARY TRIALS OF ITS EFFICACY IN THE RADIOTHERAPY OF SPONTANEOUS TUMORS IN DOGS

J. M. Yuhas, L. C. Washburn,* R. L. Hayes,* J. E. Carlton,* D. Biery,† C. Reid,† and L. H. Smith

The radioprotective drug *S*-2-(3-aminopropylamino)-ethylphosphorothioic acid (WR-2721) has been shown in mice to be relatively nontoxic and able to offer excellent radioprotection to normal tissues but not to solid tumors. This drug would offer an excellent means of improving the radiotherapy of cancer in the clinic, provided two conditions are met: the toxicity of the drug in man is low, and the differential protection observed in mice also occurs in man.

In an effort to predict whether both of these requirements would be met, we initiated a series of cooperative studies in four mammalian species: mice, rats, rabbits, and dogs. The objective of these studies is fourfold: (1) to determine, in the four species, the relationship between injected drug dose and tissue concentrations of the drug; (2) to determine whether similar tissue concentrations of the drug offer the same level of protection independent of species; (3) to determine the maximum drug dose each species will tolerate; and (4) to determine whether selective protection of normal, as opposed to malignant, tissues occurs in the spontaneous canine tumor system, as it has in all murine solid-tumor systems tested.

The tissue concentration of the drug in the four species is not a strict function of drug dose based on body weight or drug dose based on surface area but lies

intermediate to them. If we extend the relationship between injected drug dose and tissue concentration observed in the four species to man, it predicts that injection of 10 to 20 mg of WR-2721 per kg of human body weight would result in tissue concentrations equal to those observed in the mouse following the injection of 100 mg/kg; that is, on a body-weight basis it requires five times less to achieve similar tissue concentrations in the human. These tissue concentrations (eight to nine normal tissues studied) increase the radiation resistance of the mouse by factors of 1.5 to 1.8, and we are presently determining whether similar tissue concentrations of the drug offer similar protection to both mice and rabbits.

While our own data and those of others[1] suggest that WR-2721 will be relatively nontoxic in humans, its ability to protect normal tissues selectively has been demonstrated only in the mouse.[2] We have therefore initiated a study of selective normal tissue protection in two spontaneous canine tumor systems: squamous-cell carcinomas and fibrosarcomas of the head and neck. The dogs are being treated with injections of 50 to 75 mg of WR-2721 prior to each exposure in a fractionated regimen. These studies are currently in progress, and, should positive results be obtained, a more complete randomized trial will be undertaken.

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DISTRIBUTION OF WR-2721 IN THE NORMAL AND MALIGNANT TISSUES OF TUMOR-BEARING MICE AND RATS

L. C. Washburn,* R. L. Hayes,* J. E. Carlton,* and J. M. Yuhas

In our continuing study of the possible usefulness of WR-2721 in the radiation therapy of cancer, a series of studies have been performed whose ultimate objective is to determine whether this drug, which offers great promise in experimental systems, will ultimately prove useful in the clinic. The three experiments conducted during the past year have concerned: (1) the basis of the differential protection of normal and malignant tissues, (2) development of a means of identifying tumors that would benefit from combined treatment with WR-2721 and radiation as opposed to radiation alone, and (3) development of a means of monitoring drug purity that would be adaptable to the clinic.

The first point – the basis of the drug's differential action – was considered to be of critical importance, since a highly complicated mechanism might be species specific, whereas the simple mechanism we proposed, deficient absorption, might be expected to be species independent. In brief, we found that deficient absorption of the drug by the tumor could account for almost all of the differential protection observed. Since clinical tumors are often insufficiently vascularized as well, it would appear likely that the drug's ability selectively to protect normal tissues will not be species specific. Further discussion of this point is given in the preceding article.

While we have proposed that WR-2721 might prove to be an effective means of treating a variety of solid tumors, we recognize the fact that vascularity patterns are not "all-or-none" phenomena, and that certain solid tumors should absorb the drug quite readily. An experimental tumor was found, the Morris 7777 hepatoma, which absorbs the drug readily, and we employed this tumor system to determine whether a simple means could be developed for identifying tumors that do and do not absorb the drug. Our studies have shown that it is possible to determine this difference by comparing the drug concentration in the tumor and in the adjacent normal tissues following the injection of doses as small as 1 mg of the drug per kg of body weight in the rat. As suggested by our interspecies study (see the preceding article), the drug dose required to achieve this differentiation in man should be fivefold lower, far below toxic levels.

The last point, development of a means of monitoring drug purity, has been successful in that a colorimetric assay requiring 5 min has been developed. This assay allows determination of significant quantities of eight of the drug's major contaminants or degradation products, the free sulfhydryl, or the symmetrical disulfide. This rapid assay would allow quality control just prior to clinical use.

These studies are continuing, with a major effort being directed to the chemical nature of the drug in normal and malignant tissues.

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INDUCTION OF MALIGNANT AND BENIGN LUNG TUMORS IN THE BALB/c MOUSE

J. M. Yuhas, Anita E. Walker, C. C. Lushbaugh,*
N. H. Pazmiño,† Carole S. King, and J. N. Dumont

In addition to the normally encountered papillary adenoma of the BALB/c mouse lung, our studies on low-dose-rate radiation carcinogenesis[1] have demonstrated the existence of a malignant lung tumor in this strain, apparently derived from the same type-II alveolar cell of the lung. In autochthonous hosts, the major differences between the benign adenoma and the malignant carcinoma include the facts that the carcinoma grows markedly faster and that the carcinoma metastasizes readily (38%) while the adenoma has never metastasized in our five years of experience with it. In combination, these two facts make the carcinoma highly lethal while the adenoma is innocuous. Our first proposal was that the carcinoma was a progressive form of the adenoma. If this were valid we would expect that treatments which increase the incidence of adenomas would also increase the incidence of carcinomas. Quite the contrary, injection of 1 g of urethane per kg of body weight increased the adenoma incidence to 100% within three months but failed to elevate the carcinoma incidence during the next 15 months.

We therefore modified our proposal to state that an adenoma progresses to a carcinoma only when an additional stimulus is added. Since radiation exposure alone[1] can increase the incidence of carcinomas, we argued that radiation exposure of mice bearing urethane-induced adenomas should yield a marked increase in the carcinoma incidence. These studies are now completed and indicate that radiation exposure of adenoma-bearing mice produces the same incidence of carcinomas as does radiation exposure of normal mice. It would appear therefore that the carcinoma is not a progressive form of the adenoma and that the incidences of the adenoma and the carcinoma can be selectively increased by use of the appropriate carcinogen, urethane or radiation, respectively.

In order to pursue the dissimilarity of these two tumors further, we have adapted both types to propagation *in vivo* and *in vitro*. In brief, the adenoma is difficult to adapt to propagation *in vivo* or *in vitro*, and following its adaptation it grows very slowly, much as it does in autochthonous hosts. The carcinoma, on the other hand, adapts readily to both types of propagation and grows with an *in vivo* tumor-size doubling time of less than three days and an *in vitro* doubling time of 12 hr. The fact that it requires fewer than 100 cells to transplant the carcinoma but more than 10^4 cells to transplant the adenoma suggested to us that the immunogenicity of the two types of tumors might account for the difference in growth in autochthonous hosts. Neither has proven to be strongly immunogenic (see the following article), and it would not appear that immunogenicity alone can account for the differences observed.

We have initiated a series of studies whose objective is to determine whether either or both of the tumors contain oncogenic viruses and whether the observed virus burdens can account for the markedly different tumor characteristics. Electron microscopic examination of the two types of tumors has demonstrated that both arise from the type-II alveolar cell of the lung and that neither contains significant quantities of typical type-C viral particles. The only difference noted that was not a product of the growth rate was the presence of large numbers of intracytoplasmic and intracisternal type-A particles in the carcinoma. These particles have been observed in all forms of the carcinoma (autochthonous, transplanted, and cultured), but they may be a reflection of, as opposed to the cause of, the malignant nature of the carcinoma. In order to test the possible causative role of these particles, we are presently isolating them from the carcinoma and assaying their oncogenic potential both *in vivo* and *in vitro*.

Whether or not these type-A particles prove to have a causative role in the appearance of the carcinoma, we wished to determine whether the carcinoma expressed or could be induced to express antigens or enzymes normally associated with typical murine oncogenic RNA viruses. The carcinoma neither expresses these antigens spontaneously nor can be induced to do so by treatment with agents which activate latent oncogenic virus expression (IdUrd and BrdUrd). Further, the extracts of the carcinoma cells do not contain significant quantities of the marker enzyme for these viruses, reverse transcriptase.

We conclude, therefore, that the carcinoma is not a progressive form of the adenoma, and that neither tumor type is etiologically related to typical oncornaviruses.

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*Medical Division, Oak Ridge Associated Universities.
†Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

1. J. M. Yuhas and A. E. Walker, "The Dose-Rate Effect in Murine Radiation Carcinogenesis," this report.

THE LINE-1 LUNG CARCINOMA SYSTEM AS A MODEL FOR THERAPY OF HUMAN LUNG CANCER

J. M. Yuhas, N. H. Pazmiño,* C. C. Lushbaugh,†
R. E. Toya, Elizabeth F. Wagner, and Anita E. Walker

Most preliminary studies in cancer therapy must be conducted in lower animals, and it therefore is necessary to use the most adequate models available. Unfortunately, most if not all models suffer from the fact that metastasis is either absent or under rigorous immunologic control. Clearly, neither of these situations allows the investigator to evaluate the efficacy of his therapy except in terms of the primary tumor. Since the major problem in clinical cancer therapy is the dissemination of metastases, the model systems currently used often yield false hopes for the effectiveness of a given therapeutic modality. Based on our development of a means of effectively treating primary lung tumors,[1] we wished to extend our studies to include metastasis. The most logical candidate for the study of the therapy of metastasis appeared to us to be the highly malignant lung cancers which were occurring in our studies of radiation carcinogenesis in the BALB/c mouse.[2]

We adapted these tumors to both *in vivo* and *in vitro* propagation and initiated experiments designed to determine the relationship between the implanted tumors and the metastases the tumors shed. Quite early it became clear that the propensity of this tumor to metastasize persisted even when it was serially propagated. Relative to other murine tumors of our experience, this cancer metastasized readily, and the metastases proved to be the proximate cause of death. Immunosuppression proved to accelerate metastases, as classically expected, but not as markedly as is observed in other murine tumor systems. This suggested to us that the normal host was only marginally able to control its own metastases, which is a direct parallel of the clinical problem. This relatively weak concomitant immunity was tested and compared with other murine tumor systems. In contrast to other systems, metastases develop within the first week after transplantation of this tumor and survive ultimately to kill the host. This tumor would, therefore, appear to offer an ideal means of studying the therapy of metastases in the absence of control mechanisms that are observed only in experimental animals.

As our work on this cancer has proceeded, its parallelism to the clinical cancer problem has become more apparent: enzymatic treatment of the tumor cells fails to increase their antigenicity, immunization with dead tumor cells is essentially without effect on the growth of the primary tumor, the tumor can be transplanted with as few as ten cells, and after apparent "cures" the tumors eventually recur. On the basis of our past experience, we are presently attempting to treat the primary cancer with localized therapy (WR-2721 and radiation) and then treat the metastases with either chemotherapy, immunotherapy, or both. The results are too preliminary to discuss, but it is already apparent that the therapy of metastases is much less complex when the primary tumor is controlled, that is, when the source of new metastases is removed or at least inhibited. Additionally, immunotherapy of metastases with a variety of immunostimulants is proving more effective than chemotherapy.

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CYTOGENETIC STUDIES OF THE HIGHLY MALIGNANT LINE-1 LUNG CARCINOMA

R. E. Sotomayor* and N. H. Pazmiño*

The line-1 alveolar-cell carcinoma arose spontaneously and has been adapted to propagation both *in vivo* and *in vitro*. Although this tumor originates from the type-II alveolar cell of the lung,[1] as does the benign adenoma, it metastasizes readily both in the host of origin and in transplant recipients (see the preceding article). A number of possibilities can be proposed to account for the gross differences in behavior of these two tumor types, which arise from the same cell, including differences in the genetic constitution of the two types of tumor cells. Elsewhere we report our attempts to determine whether the two tumors cross-react immunologicially (see the preceding article), and the present study concerns itself with the possibility that the chromosomal patterns in the two tumors differ.

The objective of our preliminary studies was to determine whether the chromosomal constitution of the line-1 carcinoma deviates from the standard murine patterns ($2N = 40$). Both *in vivo* and *in vitro* samples of the line-1 carcinoma were analyzed, and the techniques involved were standard Giemsa staining for the detection of gross chromosomal alterations and trypsin-Giemsa banding for the detection of structural alterations in individual chromosomes.

Tumor cells from the eleventh *in vitro* passage were highly polymorphic, with approximately 60% of the cells possessing 68 to 73 chromosomes and 31% possessing between 110 and 400 chromosomes. By the thirty-fourth *in vitro* passage, 76% contained 68 to 73 chromosomes and only 11% possessed chromosome numbers of approximately 140. No cells in this passage possessed the extremely large constitutions. Most of the gigantic cells observed in the earlier passage demonstrated chromosomal fragmentation, especially those with chromosome numbers of 200 or more. Whether this represents nuclear fusion or lack of nuclear division remains to be determined.

Cells from the *in vivo* tumor showed quite a different pattern. More than 70% possessed 41 to 43 chromosomes, while less than 10% possessed as many as 85 chromosomes. None of the *in vivo* tumor cells demonstrated chromosome numbers in excess of 100. It would appear therefore that *in vitro* culture conditions allow the persistence of cells with aberrant chromosomal constitutions which would not survive *in vivo*, and that the frequency of these aberrant cells declines in later *in vitro* generations. Whether these two observations reflect artificial selection due to our methods or natural selection is presently under study.

A variety of chromosome markers were detected in both the *in vitro* and *in vivo* tumor samples, including metacentrics, dicentrics, minutes, submetacentrics, long acrocentrics, and nonhomologous pairing. The *in vitro* samples possessed the greatest variety of aberrations, with the eleventh passage showing, as might be expected from the data above, a higher frequency than the thirty-fourth passage. Quantitative analysis is currently in progress in these two passages as well as in others. The only specific chromosomal marker detected in the *in vivo* tumor sample was the presence of minutes. In 97% of the cells analyzed, one to four minutes were detected. We are presently comparing the frequency of these minutes in the tumor and in the adjacent normal tissues of tumor-bearing mice, and we will extend our studies to include the adenoma within the next year.

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CELLULAR AND HUMORAL IMMUNOLOGIC CONTROL OF SENSITIVITY TO TUMORS INDUCED BY MURINE SARCOMA VIRUS IN SENESCENT MICE

N. H. Pazmiño,* R. E. Toya, and J. M. Yuhas

Between birth and young adulthood, the marked sensitivity of the mouse to the oncogenic effects of murine sarcoma virus, Moloney strain (MSV-M), all but disappears, only to return again in old age.[1] Between birth and young adulthood the immunologic system develops sufficiently to allow the mice to eradicate MSV-M or the tumors it induces, and we therefore investigated the possibility that senescent decline in immunologic competence was responsible for the return of sensitivity in old age.

We have reported earlier[1,2] that the ability of mice to develop specific neutralizing antibodies against the MSV-M depended on the age of the mice at the time the MSV-M was given, and that mice which resisted tumor development (young adult through 1.5 years of age) developed much higher titers of this antibody. This antibody would be expected to exert its major influence on the virus itself, although recent evidence suggests that it can also function in destruction of the induced tumor cell.[3] Classically, one would expect that cellular immunologic reactions (T lymphocytes, B lymphocytes, and macrophages) would carry the major burden of controlling any tumor cells which developed, and we therefore extended our studies to include the role of these cellular reactions in the age-dependent return of sensitivity to MSV-M.

Figure 22 is a plot of the specific cytotoxic effects of lymph-node and spleen cells from $CD2F_1$ mice of various ages as a function of time after they received MSV-M. Cytotoxicity was determined *in vitro* by the colony-inhibition technique of the Hellstroms. For this plot we have included two age groups that resist tumor development (6 and 18 months) at least acutely[4] and two that succumb with progressively growing tumors within the first six months after virus injection (23 and 36 months). Both spleen and lymph-node cells from the highly resistant six-month-old mice become progressively more cytotoxic during the nine weeks after virus injection, while similar assays in the older (18 months) yet still resistant mice show high levels of cytotoxicity that begin to decline by the ninth week. The sensitive older ages, 23 and 36 months, show less to essentially no reactivity over the same time period. We conclude therefore that the development of specifically cytotoxic lymph-node and spleen cells in these mice correlates

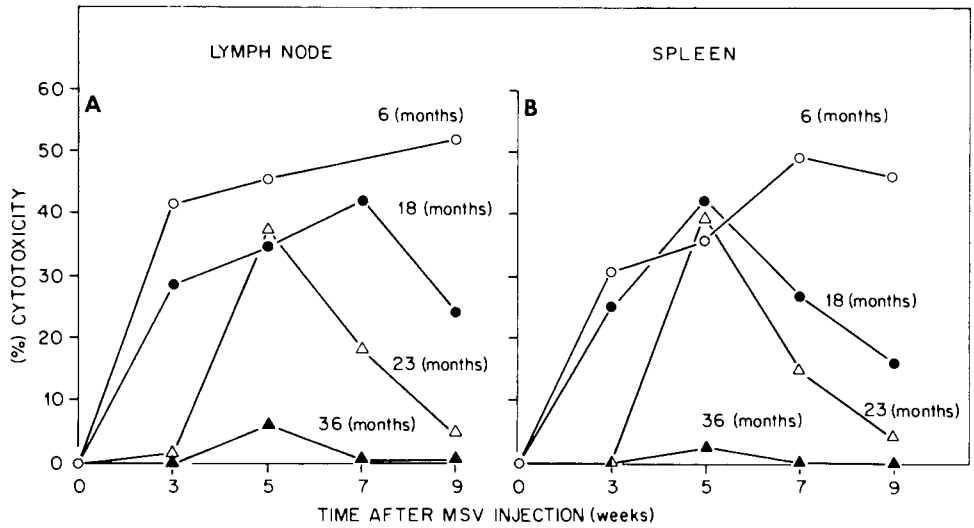

Fig. 22. Specific cytotoxic effects of (*a*) lymph-node and (*b*) spleen cells from $CD2F_1$ mice of various ages as a function of time after injection of MSV-M.

with their resistance to tumors induced by MSV-M, just as was the case for the development of neutralizing antibody.[1,2]

The cellular compositions of the lymph node and spleen differ, the former being predominantly T lymphocytes and the latter predominantly B lymphocytes. From the data presented above (Fig. 22), we cannot determine whether a single cell type or both are critical in determining resistance. We are currently investigating the relative importance of each of these cell types and of others, through selective killing of one or the other *in vitro* just prior to cytotoxicity assays and through stimulation of their development and maturation with a variety of immunologic stimulants.

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PREVENTION AND THERAPEUTIC TREATMENT OF A VARIETY OF MURINE TUMORS WITH *CORYNEBACTERIUM PARVUM*

J. M. Yuhas, Luka Milas,* and N. H. Pazmiño†

Corynebacterium parvum produces, upon injection into immunocompetent mammals, a marked stimulation of the reticuloendothelial system, and a number of investigators are presently attempting to exploit this property in the treatment of cancer both experimentally and clinically. As is usually the case, this agent is remarkably efficient in certain experimental systems, but its success in the clinic is somewhat less. The objective of the present studies is to determine both the potential and the limitations of this agent through analysis of its effectiveness and mechanism of action in a variety of tumor systems and species. Should these results prove promising, we hope to use *C. parvum* for the treatment of metastases in animals whose primary tumors have been adequately controlled by the combination of WR-2721 and radiation.[1]

In order to determine the role of the experimental system in studies of the prophylactic and therapeutic effectiveness of *C. parvum,* we are currently assessing its antitumor properties in systems involving tumors that are strongly immunogenic (methylcholanthrene-induced fibrosarcoma), moderately immunogenic (spontaneous mammary tumor virus plus mammary carcinoma), and weakly immunogenic (the line-1 alveolar cell carcinoma[2]). As might be expected, *C. parvum* is most efficient against the strongly immunogenic tumors, yet even in the systems with very weakly immunogenic tumors, *C. parvum* can generate a fivefold reduction in tumor burden. Although most human tumors are of weak antigenicity, we continue to use all three systems in our mechanism studies, but we concentrate on the last system for efficacy testing.

Another variable currently under test is the importance of the host's immune competence in determining

responsiveness to *C. parvum*. While most experimental systems involve tumors transplanted into healthy young adults, the clinical cancer patient is often older and relatively less competent than non-tumor-bearing patients of the same age. If *C. parvum* is effective only in patients with vigorous immune responsiveness, its applicability will be severely limited. We are therefore determining its therapeutic effectiveness in aged animals, whose immune competence has waned, bearing spontaneous or transplanted tumors. Again, this variable does affect the antitumor properties of *C. parvum*, the older, less competent animals being less responsive. Additionally, transplanted tumors are more responsive to the antitumor effects of *C. parvum* than are spontaneous ones.

The ability of *C. parvum* to inhibit tumor growth is thought to be mediated by its ability to stimulate macrophages, etc., which then nonspecifically attack the tumor, or its ability to stimulate the immune system in such a way that the response to specific tumor antigens is more efficient – that is, it may operate as an adjuvant. The fact that this agent was more effective against strongly immunogenic tumors suggested to us that the adjuvant properties of *C. parvum* were of major importance, and we therefore initiated studies to determine the relative importance of specific and nonspecific immunostimulation. These experiments are presently in progress, but it is already apparent that enhancement of the specific immunostimulatory properties of *C. parvum* via simultaneous injection of this agent and tumor antigen can markedly improve its therapeutic effectiveness. As an example, *C. parvum* alone is unable to alter the growth rate of the line-1 carcinoma in subcutaneous tissues; but addition of tumor antigen, which itself is unable to alter tumor growth, results in marked growth suppression.

In summary, *C. parvum* appears able to eradicate small numbers of weakly immunogenic tumor cells even when they have spread to the viscera, and therefore this appears to be an ideal agent for therapy of metastases in our studies of lung-cancer therapy.

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1. J. M. Yuhas, A. E. Walker, S. F. Echols, and M. G. Hayes, *Biol. Div. Annu. Progr. Rep. June 30, 1972*, ORNL-4817, p. 155; J. M. Yuhas, *J. Nat. Cancer Inst.* **50**, 69–78 (1973).

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LATE TUMOR APPEARANCE IN MICE WHICH INITIALLY RESIST THE ONCOGENIC EFFECTS OF MURINE SARCOMA VIRUS

J. M. Yuhas and N. H. Pazmiño*

Unless a mouse develops a tumor at the injection site within the first few months after receipt of murine sarcoma virus, it is classified as being resistant to the oncogenic effects of the virus. Two facts suggested to us that this resistance was more apparent than real: both we and others[1] were able to retrieve infective virus from these resistant mice at long periods after injection, and we already knew that resistance to the virus declined in old age (older than two years).[2] To test the possibility that the persistent viruses and/or transformed tumor cells eventually contributed to the cancer burden of apparently resistant mice, we gave intramuscular injections of this virus to six-month-old resistant mice (BALB/c or $CD2F_1$) and observed them for up to 18 months afterward. The results obtained were similar in the two genotypes, and Fig. 23 is a plot of the incidence of injection-site tumors in the BALB/c mice as a function of time after virus injection. The injection of the virus alone resulted in the eventual incidence of 70% tumors at the injection site, with a mean latency between injection and tumor appearance of 8.4 ± 0.74 months. Exposure of these virus-injected mice to 500 R of x rays one month after injection did not alter the final incidence of injection-site tumors but did significantly reduce the latent period (4.8 vs 8.4 months, $P < 0.05$). We conclude, therefore, that a major effect of oncogenic viruses has previously been ignored and that its import to viral carcinogenesis demands study.

As soon as the marked late effects of virus injection became apparent, we initiated a series of studies with a twofold objective: to elucidate the mechanisms which would allow persistence and eventual appearance of oncogenic viruses or cells, and to determine whether this appearance could be altered by pre- or posttreatment of the virus-injected mice, as suggested by the data given in Fig. 23.

These studies on the mechanisms involved and on alteration of the natural process are not complete enough to warrant final conclusions, but certain obvious preliminary conclusions are already apparent. Figure 24 shows the data from an experiment in which we compared the ability of mice exposed to 0 or 500 R at the age of six months to produce direct-plaque-forming cells in response to the injection of sheep red blood cells as a function of time after irradiation. Two effects

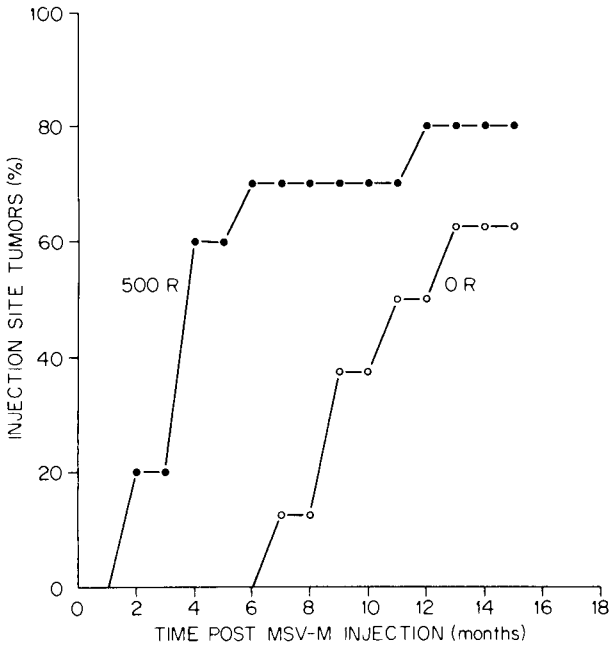

Fig. 23. Incidence of injection-site tumors in BALB/c mice as a function of time after injection of MSV.

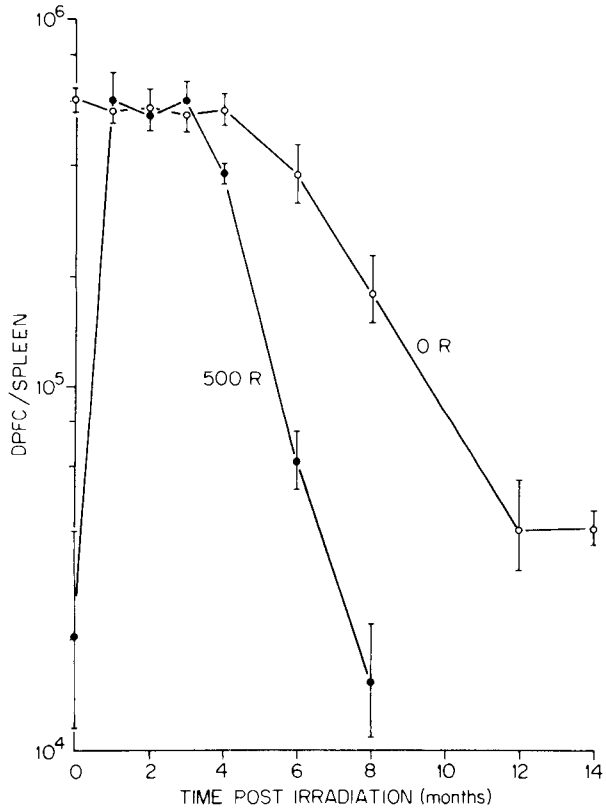

Fig. 24. Plaque-forming-cell response as a function of time after irradiation.

of radiation exposure are readily apparent: an immediate but temporary loss of immunologic responsiveness, and a premature decline of immunologic responsiveness with advancing age. This latter effect correlates with the shortened latent period for tumor appearance in irradiated mice. We are currently expanding these studies to include specific immunologic reactions to murine sarcoma virus injection, such as those we describe elsewhere for aging mice.[3]

Two types of experiment are currently in progress in our attempt to alter the course of the eventual appearance of these late injection-site tumors: attempts to stimulate immunologic competence in order to postpone or prevent tumor appearance (see the preceding article), and continuation of our analysis of the role of immunosuppression in determining the rate of tumor appearance. The data relating to stimulation of the immune system are reported elsewhere (see the preceding article), and we report here our analyses of the effects of immunosuppression on tumor appearance. We have already established that the ability of total-body irradiation to accelerate the appearance of the late virus-induced tumors is dose dependent and dose-rate dependent; acceleration is less pronounced with low doses and low dose rates. Local radiation cannot duplicate the acceleration seen with total-body exposures, and the age at virus injection is a critical variable in determining the latent period for tumor appearance: tumors appear earlier when the virus recipients are older (closer to their natural age for loss of immunologic competence). Additional studies on other immunosuppressants lend weight to our argument that these treatments accelerate the rate of tumor appearance by inducing a premature senescent loss of immunologic competence.

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CARCINOGENESIS PROGRAM*

F. T. Kenney

Respiratory Carcinogenesis

Paul Nettesheim
D. A. Creasia
R. A. Griesemer
Joseph Kendrick
J. C. Kim
Ann C. Marchok
Hans Schreiber[a]

Mechanisms of Chemical Carcinogenesis

William Lijinsky
A. R. Jones
G. M. Singer
H. W. Taylor

Repair Mechanisms in Carcinogenesis

R. B. Setlow
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R. W. Hart[a]
James D. Regan

Immunology of Carcinogenesis

M. G. Hanna, Jr.
B. L. Batzing[b]
Corazon D. Bucana[b]
J. C. Lee[b]
F. A. Salinas[b]
M. J. Snodgrass[c]
A. K. Szakal

RNA Tumor Virus – Cell Biology

R. W. Tennant
G. C. Lavelle
J. A. Otten
J. M. Quarles, Jr.[b]

Enzymology of Carcinogenesis

Wen-Kuang Yang
Ti Ho
Ching-Yuan Hung[b]
Beth C. Mullin[b]
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L. C. Waters

Regulation of Gene Expression

F. T. Kenney
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*Research sponsored by the National Cancer Institute.
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[c]Temporary appointment.

INTRODUCTION

THE CARCINOGENESIS PROGRAM is an interdisciplinary research effort with primary emphasis on chemical and viral carcinogenesis; operating funds are provided by the Division of Cancer Cause and Prevention, National Cancer Institute, via interagency agreements with the Atomic Energy Commission. The scope of the program is broad, reflecting the complexity of the problem as well as the diversity in expertise brought to bear by staff scientists with primary training in pathology, immunology, genetics, developmental biology, virology, biochemistry, biophysics, and analytical and organic chemistry.

Progress in FY 1974 is reflected in the series of individual project reports which follow. New approaches planned for FY 1975 and based on these research findings include study of differentiation of the respiratory epithelium and the effects of carcinogens, vitamin A, and other agents, using the organ culture system developed by Marchok and Cone; defined analyses of respiratory carcinogenesis in tracheal grafts (Griesemer et al.); a long-term, moderate-scale exposure of rats to significant quantities of cigarette smoke, using the devices and techniques worked out by Kendrick and Nettesheim; biochemical analysis of the mechanism of carcinogenesis by nitrosamines (Lijinsky et al.), with emphasis on the alpha-labeled compounds prepared by Jones; follow-up of the demonstration by Setlow and Hart that photoreactable uv-induced lesions in DNA (i.e., thymine dimers) are directly associated with uv-mediated cancers, extending this approach to chemical carcinogens and if possible to human cells (Regan et al.); analysis of induced mutants of HeLa cells with altered membrane properties (Cook and Best); characterization of antigens found to be present in membranes of human melanomas as well as in the cell wall of the immunotherapeutic agent BCG (Bucana and Hanna); development of rapid *in vitro* methods for bioassay of carcinogens (Hanna et al.); characterization of the antigens of RNA tumor viruses responsible for autogenous immunity to these viruses and the nature of the immune response to them (Hanna, Lee, Ihle et al.); preparation of DNA copies of the viral RNA subunits shown to be genetically distinct, and their use as probes to analyze synthesis of the viral genome in cells activated to express endogenous virus (Ihle, Farrelly et al.); analysis of the significance to gene regulation of phosphorylation of the model enzyme tyrosine aminotransferase (Lee et al.); characterization of the putative gene product shown to be capable of reversing cellular resistance to infection by tumor viruses (Tennant, Yang et al.); and isolation and characterization of the cellular 4S RNA which has a nucleotide sequence complementary to that of 70S viral RNA and study of the significance of this complementarity to viral function in infected cells (Waters, Yang et al.).

Future development of the program will involve increasing emphasis on carcinogenic agents associated with nonnuclear production of energy and expansion into areas of cancer research (e.g., diagnosis, therapy) other than carcinogenesis. Our affiliation with the developing East Tennessee Cancer Research Center in Knoxville is expected to be significant in this expansion, in providing an interface with clinical oncology and in determining the areas we are best suited to explore.

LONG-TERM GROWTH AND MAINTENANCE *IN VITRO* OF TRACHEA EXPLANTS EXHIBITING DIFFERENT TYPES OF EPITHELIA

Ann Marchok and M. Virginia Cone

The overt expression of different cell types in epithelial tissues is known to be influenced by changes in the cellular environment. In the trachea, a wide spectrum of epithelial cell types is found, including ciliated cells, brush border cells, goblet cells, and possibly a number of other kinds of secretory cells, all of which may or may not be variations of one secretory cell type, as well as basal cells, which are the stem cells. Under particular environmental conditions the secretory cell number and/or specific cell products may increase, while under other stress conditions the tracheal epithelium may undergo metaplastic changes to a keratinizing squamous metaplasia. To bring about these metaplastic changes, several levels of control may be operating. At the basal cell level, environmental factors may selectively stimulate the production of specific epithelial cell types from one common stem cell. On the other hand, these factors may stimulate the outward expression of covertly differentiated stem cells. Conversions from one cell type to another at different stages of differentiation have also been claimed. The latter would imply that epithelial cells are programmed to produce more than one specialized cell product and could be open to control at any one of a

number of points along the paths of transcription and translation.

It was our interest to develop an *in vitro* system for the long-term growth and maintenance of trachea explants and to establish environmental conditions for the production, maintenance, and replacement of different epithelial cell types in order to study the molecular and cellular mechanisms involved in normal and abnormal epithelial tissue differentiation. We have found that pieces of adult rat trachea supported on Nitex cellulose acetate or Gelman triacetate filters in conventional Falcon organ-culture dishes can be maintained for at least 75 days. The growth of the explant is characterized by a marked stimulation of cell proliferation throughout the epithelium and outgrowth of epithelial cells to the underside of the explant and onto the filter. The kind of filter used as support influences the amount of epithelial migration – on the Nitex the outgrowth is extensive while on the nonwettable Gelman filter outgrowth is limited.

The level of proliferation, measured by [^{3}H] thymidine incorporation, is cyclic in nature, being markedly higher at particular points of the culture period studied so far. These times correlate well with periods of dedifferentiation and renewal of the epithelium. In the presence of a completely defined medium, Waymouth's MB 752/1, the typical mucociliary epithelium is almost totally replaced by keratinizing squamous metaplasia after 51 days in culture. It is important to note that the migrating epithelium usually shows squamous metaplasia as early as nine days in culture, indicating that rapid proliferation may dilute out any cellular substances that control the production of mucociliary epithelium. We have found that vitamin A (2 μg/ml all-*trans*-retinol) completely inhibits this squamous metaplasia. When the Waymouth's medium is supplemented with 10% horse serum, a similar kind of cyclic proliferation is found, but in this case only a mucociliary epithelium is differentiated. Preliminary histological studies indicate that the amount of mucus produced by the goblet cells can be increased by the presence of vitamin A in the horse-serum-supplemented medium. We have also found that respiratory epithelium can be made to dedifferentiate rapidly by supplementing the Waymouth's medium with 10% fetal calf serum. Now that we have culture conditions available that select for the development of particular types of respiratory epithelium, we will be able to study the properties of the specific cells in detail.

THE EFFECTS OF BENZO[*a*]PYRENE ON THE GROWTH AND DIFFERENTIATION OF TRACHEA EXPLANTS *IN VITRO*

Ann Marchok and M. Virginia Cone

Organ-culture methods have been developed in this laboratory for maintaining pieces of rat trachea *in vitro* for several weeks. It appears that a mucociliary epithelium can be maintained with a reproducible cyclic replacement every few weeks by culturing the tissue on Waymouth's MB 752/1 medium supplemented with 10% horse serum. Growing the trachea explants on Waymouth's medium in the absence of horse serum resulted in a gradual but complete replacement of the normal mucociliary epithelium by squamous metaplasia. This is similar to the situation found in localized areas of the trachea of intact animals under conditions of vitamin A dificiency or after exposure of animals to chemical carcinogens and other toxic agents.

The availability of these culture conditions for the growth and maintenance of different types of tracheal epithelium opens up the possibility of studying the effects of carcinogens on tracheal epithelium on a long-term basis *in vitro*. As the first step in this direction, the effects of two concentrations of benzo[*a*]pyrene (1 and 5 μg/ml) were tested in the Waymouth's plus 10% horse serum system. Each concentration was tested for 6 to 21 days and 6 to 39 days in culture, and the histological changes in the explants were noted at six-day intervals for a total of 69 days. The major changes found in these cultures were (1) hyperplasia was greater than that present in the controls during the first week of carcinogen treatment; (2) the mitotic index was higher in all the treated cultures throughout the culture period; (3) for both benzo[*a*]pyrene concentrations, the reestablished mucociliary epithelium found in the control cultures at six weeks of culture was also present in the cultures treated for only 15 days, but the mucociliary epithelium present at ten weeks of culture in the controls was replaced by an undifferentiated epithelium in the treated cultures; (4) the cultures treated for 33 days with carcinogen did not reestablish a mucociliary epithelium at six weeks, but the cultures treated with only 1 μg benzo[*a*]pyrene did show a typical mucociliary epithelium by ten weeks of culture. Using 5 μg/ml benzo[*a*]pyrene for 33 days completely inhibited the differentiation of a typical mucociliary epithelium, and, at best, some ciliated low columnar and cuboidal

cells developed about midway through the culture period. These cells were replaced by an undifferentiated epithelium. The cartilage stroma also breaks down in the presence of the high carcinogen concentrations, and fibroblasts appeared to be invading this area at the late culture periods.

The second experiment that has been completed is the effect of 5 μg of benzo[*a*]pyrene per ml of Waymouth's defined medium. Treatment was for 33 days, and one group was treated starting at day 0 and a second group starting at day 6 of culture. The essential results of this experiment are (1) stimulation of hyperplasia, particularly by treatment of the culture starting at day 0; (2) a more rapid replacement of the mucociliary epithelium by squamous metaplasia – for the cultures treated from 0 to 33 days, the epithelium of most explants was completely squamous metaplasia by three weeks, and complete replacement by squamous metaplasia in control cultures was first detected at seven weeks and was found in all cultures even at ten weeks; and (3) persistence of squamous metaplasia after the treatment with carcinogen in defined medium, in contrast to experiment I, in which an undifferentiated epithelium eventually resulted from treatment with carcinogen in medium supplemented with horse serum.

It is evident from these beginning studies that the carcinogen can affect the differentiation of trachea epithelium *in vitro* on a long-term basis. The gradual changes found in the cultures indicate that we may be able to study the sequence of events during preneoplastic and possibly neoplastic changes in the trachea with this system. Experiments in progress include a detailed analysis of the cytological changes in the epithelial and submucosa cells during the following carcinogen treatment in order to detect any consistent "preneoplastic" changes in the explants. Studies in the immediate future will center on testing media supplements to find culture conditions that may support more typical kinds of "neoplastic" respiratory tissue *in vitro*.

THE EARLY STAGES OF DNA SYNTHESIS AND CELL PROLIFERATION IN TRACHEA EXPLANTS GROWN *IN VITRO* UNDER SEVERAL CULTURE CONDITIONS

Ann Marchok and M. Virginia Cone

In order to study possible relationships between the levels of cell proliferation and the subsequent differentiation of particular kinds of cells in trachea epithelium, we have begun an investigation of the initial increase in DNA synthesis and cell proliferation seen in trachea explants after culture *in vitro*. During the first week in culture, the highest levels of [^{3}H] thymidine incorporation are found in hot trichloracetic acid extracts prepared from trachea explants grown in Waymouth's MB 752/1 defined medium. Autoradiograph studies showed that this increase in DNA synthesis took place both in the epithelium of the explant proper and in epithelial cells migrating around the outside of the explant. Similar patterns of DNA synthesis were found in trachea explants grown in Waymouth's medium supplemented with 10% horse serum; however, [^{3}H] thymidine incorporation was never as great as that found in trachea pieces grown in Waymouth's medium alone. Medium supplemented with 10% fetal calf serum supported much lower levels of [^{3}H] thymidine uptake into hot trichloroacetic acid extracts. Histological preparations showed that cell migration predominated in the presence of fetal calf serum and that most of the cells synthesizing DNA were at the zone between the epithelium proper and the area of migration over the cut edge of the cartilage.

More detailed analysis of the periods of DNA synthesis in the trachea explants has demonstrated waves of DNA incorporation as well as periods of marked increase in the mitotic index, indicating induction of at least partial synchrony in the initiation of cell proliferation in the cultures. Vitamin A (all-*trans*-retinol) added to either the defined medium or horse-serum-supplemented medium resulted in an increase in the initial wave of DNA synthesis. This was particularly evident in the presence of Waymouth's medium. In this case, the appearance of the first peak of DNA synthesis was retarded but was greater than that obtained in the absence of the vitamin. Vitamin A was also found to maintain a consistently higher incorporation of [^{3}H] thymidine throughout 39 days of culture. These data support the view of others that vitamin A may stimulate DNA synthesis, but the increase in uptake could be the result of some membrane change. It must also be determined whether the vitamin A is selecting for the increase in numbers of mucus-secreting cells or acting as a nonspecific mitogenic agent. To help distinguish among these possibilities, the effects of insulin, a known mitogenic agent, are also being studied. The addition of insulin (1 μg/ml) to Waymouth's medium stimulated the incorporation of [^{3}H] thymidine at 1.5 days in culture, the same as the unsupplemented medium. However, the peak was much greater in the presence of insulin, and this was the major peak found during six days in culture.

Autoradiographs of cultures grown for three and six days in the presence of insulin, which are periods of low [^{3}H] thymidine incorporation, show a deep hyperplasia

of mostly unlabeled cells in the epithelium proper. This again is most likely a reflection of periodic and partially synchronous cell replication. The undergrowth of these cultures was extensive and highly labeled, and by six days in culture squamous metaplasia was already quite conspicuous. This is several days in advance of extensive squamous metaplasia seen in control cultures.

These experiments will be continued and will include analysis of the molecular events leading to the stimulation of DNA synthesis in the explants as well as a close assessment of the cell-proliferation kinetics in order to define pathways of differentiation of specific kinds of epithelial cells. Comparable studies will also be made on explants treated with carcinogens in an attempt to determine differences in the sequence of events leading to stimulation of DNA synthesis in the presence of these agents that may relate to the high level of cell proliferation and disturbances in patterns of tissue differentiation found in trachea explants.

ESTABLISHMENT OF TRACHEAL GRAFTS

R. A. Griesemer, Paul Nettesheim, and D. H. Martin

We have recently reported the development of techniques for successfully transplanting the trachea from one animal to the subcutis of another.[1] The grafted trachea provides respiratory tissue in which lesions can be produced and studied without interfering with respiration.

To utilize tracheal grafts as research tools it was necessary to study the effects of transplantation and to compare their structure with that of ungrafted tracheas.[2] Shortly after transplantation, the mucosal epithelium of the rat tracheas retracted but remained intact. The graft became covered with fibrin which was converted to granulation tissue in several days. New blood vessels from the host penetrated the graft, and the circulation was reestablished four days after transplantation. The renewed blood supply caused a burst of mitotic and secretory activity which gradually subsided, so that two weeks after transplantation the tracheal grafts again resembled normal tracheas. The only persistent sequela of transplantation was demyelination of autonomic nerves in the adventitia.

1. J. Kendrick, P. Nettesheim, and A. Hammons, *J. Nat. Cancer Inst.* **52**, 1317 (1974).
2. R. Griesemer, J. Kendrick, and P. Nettesheim, *Proc. Symp. Exp. Resp. Carcinogenesis* (in press).

EFFECT OF DMBA ON TRACHEAL GRAFTS

R. A. Griesemer, Paul Nettesheim, and D. H. Martin

One of the problems in experimental respiratory carcinogenesis is that when carcinogens are introduced into the respiratory airways they become distributed unevenly over a large surface area. Dose-response effects in particular are difficult to ascertain. To learn the effects of chemical carcinogens on respiratory tissues, we chose as one model the use of tracheal grafts. Tracheal grafts have the advantage of being small defined targets that lend themselves to quantitation and frequent sampling.

Tracheal grafts in rats were exposed to 7,12-dimethylbenz[*a*]anthracene (DMBA) by inserting pellets containing the carcinogen into the lumen.[1] Beeswax was selected as a vehicle for sustained release of carcinogen. Pellets that initially contained 3.0 mg DMBA still retained 77% after two months and 53% after three months.

After exposure to DMBA, the mucosal epithelium underwent squamous metaplasia within a week. Squamous keratinizing metaplasia persisted and after two months resulted in large keratinic cysts that distorted the grafts. Three months after first exposure to carcinogen, the proliferating squamous epithelium invaded the graft wall, and after four months, well-differentiated squamous carcinoma extended into the overlying skin.

Additional dose-rate experiments with DMBA in tracheal grafts are in progress. In the tracheal graft the lesions have an opportunity for prolonged development, in contrast with the ungrafted trachea, where relatively small early lesions cause suffocation.

1. R. Griesemer, J. Kendrick, and P. Nettesheim, *Proc. Symp. Exp. Resp. Carcinogenesis* (in press).

EFFECT OF VITAMIN A ADMINISTRATION INITIATED *AFTER* INTRATRACHEAL CARCINOGEN INJECTION

Paul Nettesheim and M. Virginia Cone

Previous studies in our laboratory have shown that nodular squamous metaplasias can be induced in rat lungs within weeks following intratracheal injection of 10–25 mg of 3-methylcholanthrene (MCA) and that such lesions become invasive within six to nine months. We also showed that the development of such metaplastic lesions is inhibited in rats maintained on high

Table 28. Effect of vitamin A on incidence of MCA-induced squamous-cell lesions

RA dose (μg per week)	Group	Start of RA administration[a]	Number of rats with lung squamous nodules
10,000	1	3 wks	0
10,000	2	5 wks	1
10,000	3	10 wks	1
10	4		10

[a]RA dose was changed from 10 to 10,000 μg per week at three, five, or ten weeks after administration of 4 mg MCA (groups 1–3). Group 4 was maintained on 10 μg RA throughout. Each group consisted of 24 rats.

levels of retinyl acetate (RA). It was not clear whether this inhibition was due to an effect of RA on MCA metabolism or on cell growth and differentiation in the early postinitiation stage. Rats maintained on a low vitamin A intake (10 μg/week) were given an intratracheal injection of 4 mg MCA (a carcinogen dose that turned out to induce nodular squamous metaplastic lesions but no invasive neoplasms). Three, five, and ten weeks after carcinogen administration, groups of 24 rats each were changed to a weekly RA dose level of 10,000 μg. One group continued to receive 10 μg RA per week. One year after carcinogen administration all animals were killed, and the number and size of metaplastic nodules were determined under a dissecting microscope. The data summarized in Table 28 show that the development of the MCA-induced squamous nodules was markedly suppressed in all groups receiving high levels of RA. The significance of this finding lies in the fact that the development of preneoplastic lesions can be inhibited by high RA doses *after* 80–90% of the carcinogen has been eliminated. Studies on the inhibition of MCA-induced invasive tumors are in progress.

EFFECT OF VITAMIN A ON THE GROWTH OF A TRANSPLANTABLE SQUAMOUS-CELL CARCINOMA

Paul Nettesheim and Mary L. Williams

A number of studies in our own and other laboratories indicate that vitamin A, which is a well-known "differentiation hormone" for mucous membranes, can inhibit tumor induction in surface epithelia such as skin, stomach, intestine, and lung. It was therefore important to determine whether vitamin A can affect the growth of *established* neoplasias derived from epithelium normally under its control.

A series of studies was conducted with a transplantable squamous-cell carcinoma derived from the respiratory tract of mice. The tumor was transplanted either intramuscularly or intrapulmonarily (the latter by intravenous injection of tumor cells). Tumor size was determined at different times after transplantation. The recipient mice were maintained on a semisynthetic vitamin-A-free diet, and doses of retinyl acetate (RA) ranging from 0.1 μg to 3500 μg per week were administered by gavage. No consistent effect on tumor growth was observed at any of the tested RA dose levels. The only noticeable effect was inhibition of keratin formation in the tumor by very high RA levels. This negative finding is important, since administration of high doses of vitamin A has been recommended for therapy of bronchogenic carcinoma.

EFFECT OF CHEMICAL PNEUMONIA ON RESPIRATORY CLEARANCE OF INHALED $^{51}Cr_2O_3$

D. A. Creasia

Previous studies have shown that pulmonary clearance is markedly affected by influenza infection. The defect in particle clearance from the lung first becomes measurable during the acute stage of pneumonia produced by the infection. It was decided to test whether pneumonia produced by other means than infection also has the same effect on pulmonary clearance. Nitrogen dioxide is a common air pollutant and a deep-lung irritant, which at high concentrations is known to produce chemical pneumonia. Thus NO_2 was chosen to test its effect on pulmonary clearance of $^{51}Cr_2O_3$ particles in the lungs of mice.

Five groups of eight animals each were given nose-only exposure to a $^{51}Cr_2O_3$ aerosol in an inhalation chamber described in the following article. Except for the controls, each group was then exposed to NO_2. The NO_2 exposure spanned a concentration range of 30 to 175 ppm NO_2 and was either single or multiple

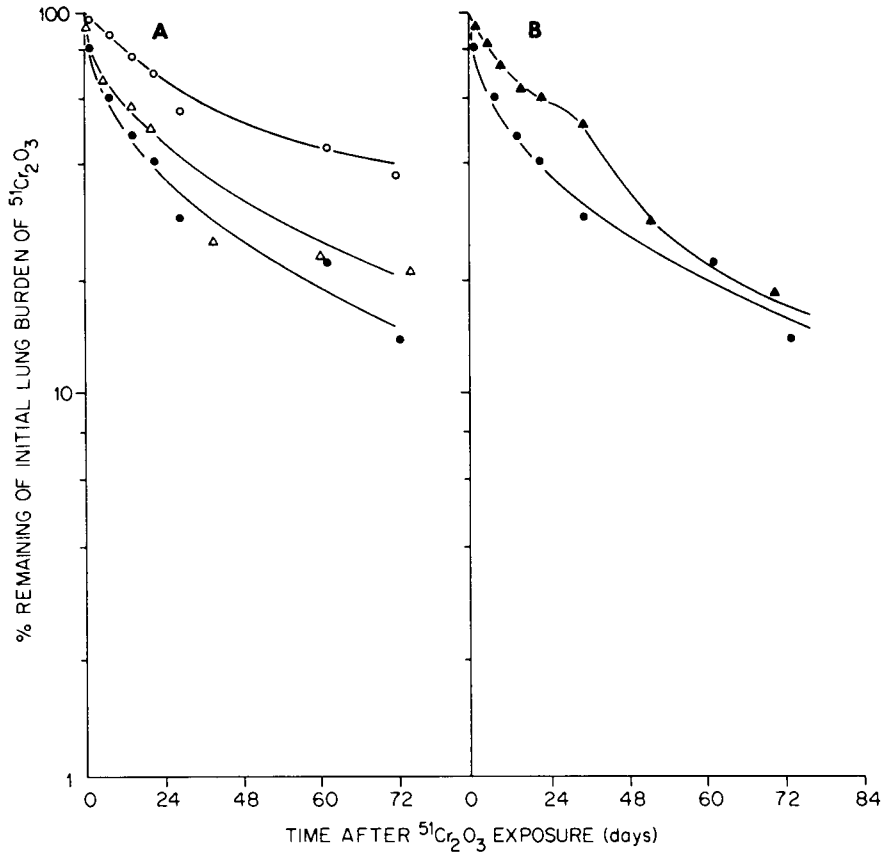

Fig. 25. Respiratory clearance of $^{51}Cr_2O_3$ from the lungs of mice exposed to NO_2. (*a*) ○–○, mice exposed to 175 ppm NO_2; △–△, mice exposed to 30 ppm NO_2; ●–●, control mice. (*b*) ▲–▲, mice exposed to 70 ppm NO_2; ●–●, control mice.

exposures. The results are summarized in Fig. 25*a* and *b*. Mice exposed one time to 30 ppm NO_2 show no significant change in their pulmonary clearance rates (Fig. 25*a*). With a single exposure to 175 ppm, however, a marked retardation of particle clearance was observed (Fig. 25*a*). The biological half-life of $^{51}Cr_2O_3$ under these conditions was approximately 50 days, while control animals showed an average biological half-life for $^{51}Cr_2O_3$ of 12 days. This increase by four of the biological half-life could be extremely significant if the inhaled material was either a carcinogen, highly toxic, or highly radioactive. With NO_2 exposure intermediate between 30 and 175 ppm, the effect, if any, on pulmonary clearance was marginal (see Fig. 25*b*). If there was an effect, it appeared to be only in the early stages of particle clearance, up to about day 24, at which time the defect (?) was subject to reversal. Even with ten daily exposures to 70 ppm, the results (not shown) were essentially identical to those observed with a single exposure to 70 ppm NO_2. Thus it must be concluded that only very high NO_2 concentrations will significantly affect pulmonary clearance of particles from the lungs of mice.

IMPAIRMENT OF PULMONARY CLEARANCE OF INHALED PARTICLES BY RESPIRATORY INFECTION

D. A. Creasia

We have previously reported on the impaired pulmonary clearance in mice of intratracheally instilled $^{51}Cr_2O_3$ caused by a PR8 influenza viral infection. A valid criticism of the previous study was the unphysiological method of administering the $^{51}Cr_2O_3$ particles, namely by intratracheal instillation. To avoid this criticism in future studies, an inhalation chamber was designed for nose-only exposure to an aerosol of $^{51}Cr_2O_3$ particles. The inhalation chamber is essentially a hexagon approximately 0.5 m^3 in volume, with four ports for nose-only exposure of mice on each of the chamber sides. The $^{51}Cr_2O_3$ aerosol is generated by

pneumatic aspiration of a suspension of $^{51}Cr_2O_3$ particles in distilled water. The heterogeneous aerosol produced in this manner is forced to maneuver through a series of baffles designed to remove the larger particles, so that respiratory-tract deposition will be mostly of small particles deposited in the deep lung.

Using the inhalation chamber described above, a small experiment was designed with inhaled $^{51}Cr_2O_3$ to compare with the previous finding of impaired pulmonary clearance of intratracheally administered $^{51}Cr_2O_3$ particles. Twenty-one mice were exposed to a $^{51}Cr_2O_3$ aerosol for 3.5 hr. Seven of these mice were infected with PR8 virus on the same day as $^{51}Cr_2O_3$ exposure, and seven others were infected 12 days before $^{51}Cr_2O_3$ exposure. The remaining animals were maintained as uninfected controls. Clearance of $^{51}Cr_2O_3$ was followed for at least 60 days after exposure. The results are summarized in Fig. 26. The usual course of the PR8 viral infection is such that a stage of acute pneumonitis is produced 10 to 14 days after exposure, with subsequent, often permanent, fibrotic scarring of the lung tissue. When the animals are infected on the same day as $^{51}Cr_2O_3$ exposure, impairment of pulmonary clearance first becomes obvious 6 to 18 days after virus infection. Thereafter, particle clearance remains slower and is drastically reduced as compared to the controls. When the $^{51}Cr_2O_3$ exposure coincides with the peak of the infection, clearance of $^{51}Cr_2O_3$ from the lungs is almost immediately impaired and remains so for at least 60 days afterward.

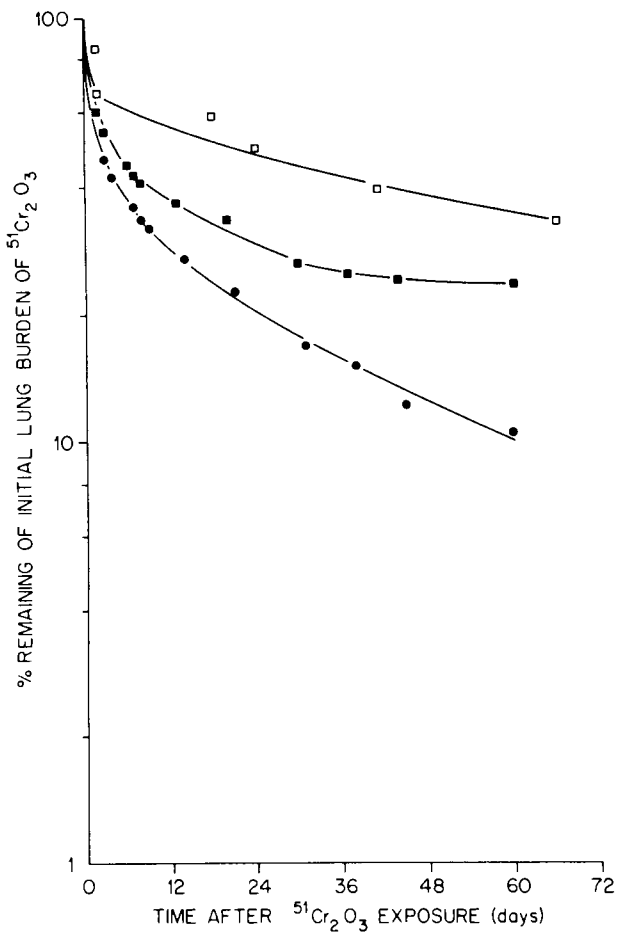

Fig. 26. Respiratory clearance of inhaled $^{51}Cr_2O_3$ by influenza-infected mice. ●–●, uninfected controls; △–△, mice infected on same day as $^{51}Cr_2O_3$ exposure; □–□, mice infected 12 days before $^{51}Cr_2O_3$ exposure.

Although the actual numbers are different, the two conclusions derived from studies on the effect of PR8 infection on pulmonary clearance of inhaled and intratracheally administered $^{51}Cr_2O_3$ are essentially the same. Both studies indicate that infection does impair pulmonary clearance and that the degree of impairment bears a relationship between time of particle exposure and time of viral infection.

SUITABILITY OF ^{103}Ru-LABELED CARBON PARTICLES FOR LONG-TERM PULMONARY-CLEARANCE STUDIES

D. A. Creasia and J. K. Poggenburg, Jr.*

Previous studies with $^{51}Cr_2O_3$ have shown that pulmonary clearance of insoluble particles can be drastically impaired by respiratory infection and exposure to an irritating gas. The particle most commonly associated with environmental carcinogens and other polyaromatic hydrocarbons (PAH), however, is carbon. The physical and surface properties of carbon particles are more favorable for the attachment and retention of PAH. In fact, a major route of exposure of the respiratory tract to environmental carcinogenesis is via inhaled carbon particles associated with PAH. Thus, when it was decided to test whether the pulmonary clearance effect seen with $^{51}Cr_2O_3$ particles could be duplicated with other particles, and whether this impaired clearance would have any effect on chemicals *carried* on other insoluble particles, it was decided to use small carbon particles labeled with a gamma-emitting isotope. The gamma radiation is necessary for *in vivo* measurements without killing the animal. The most common gamma-emitting isotopes used for tagging other particles have half-lives too short to be useful for long-term pulmonary-clearance studies. Ruthenium-103 is a gamma emitter with a half-life of 40 days and could be used for long-term studies. Since ^{103}Ru had not been used as a label for other particles, it was

necessary to determine whether ^{103}Ru remained with the carbon particle. Five hamsters were given intratracheal injections of 20 μg of 0.5- to 1.0-μm carbon particles labeled with ^{103}Ru and placed in metabolic cages. The urine and feces were collected daily, and their ^{103}Ru contents were determined. At the end of five days, 15.0% of the ^{103}Ru had been eliminated from the animals, 6.0% in the urine and 9.0% with the feces. At this time the animals were killed, and the amount of ^{103}Ru in the whole body was determined. The lungs and trachea (minus lymph nodes and extraneous tissue) were removed and measured for their ^{103}Ru content. The lungs retained an average of 96.0% (range 92 to 103%) of the ^{103}Ru found in the intact animal. Thus it was concluded that the amount of ^{103}Ru-labeled carbon measured by whole-body counting of the intact animal was representative of the lung burden, that ^{103}Ru remained attached to the carbon particles, and that the ^{103}Ru-labeled carbon particles were suitable for long-term pulmonary-clearance studies.

*Isotopes Division.

IN VIVO DISSOCIATION OF CARCINOGEN AND CARRIER PARTICLE

D. A. Creasia

In the field of respiratory carcinogenesis, no other chemical has been investigated as extensively as ferric oxide dust. These investigations have mainly been concerned with defining the role by which Fe_2O_3, when used as a carrier particle for a carcinogen, is able to enhance the response to a given dose of carcinogen. From the many investigations that have been concerned with the combination of benzo[*a*]pyrene (BP) and ferric oxide, several common features have emerged: (1) small BP crystals are cleared from the lung quickly and have low tumorigenicity; (2) large BP crystals are cleared slowly and have strong carcinogenicity; (3) BP bound to Fe_2O_3 particles is cleared slowly and is highly tumorigenic; and (4) BP with Fe_2O_3 *added* is cleared only slightly slower than BP alone and is only slightly tumorigenic. These findings strongly suggest that the major mechanism of the Fe_2O_3-mediated enhancement of the tumorigenic response of the lung to BP is related to carcinogen clearance. Subsequent studies with other carrier particles, namely aluminum and carbon, showed that carcinogen retention and tumorigenicity do not always go hand in hand. These findings suggest that other factors such as surface activity, alteration of carcinogen metabolism, or lack of sufficient release of the carcinogen from the carrier particle might also play a decisive role.

In conjunction with our other pulmonary-clearance studies, the *in vivo* release of BP from the carrier particle carbon was studied. Twenty mice were given intratracheal injections of BP attached to 0.5- to 10-μm carbon particles and carbon particles labeled with ^{103}Ru. The pulmonary clearance of the labeled carbon was measured by whole-body counting, and the elimination of BP from the lung was determined by a spectrofluorometric technique. The results are summarized in Fig. 27). The carbon was eliminated much slower from the lung than the BP. It took six days to clear 50% of the carbon, while in only two days 50% of the BP was eliminated, and by day 4 less than 20%

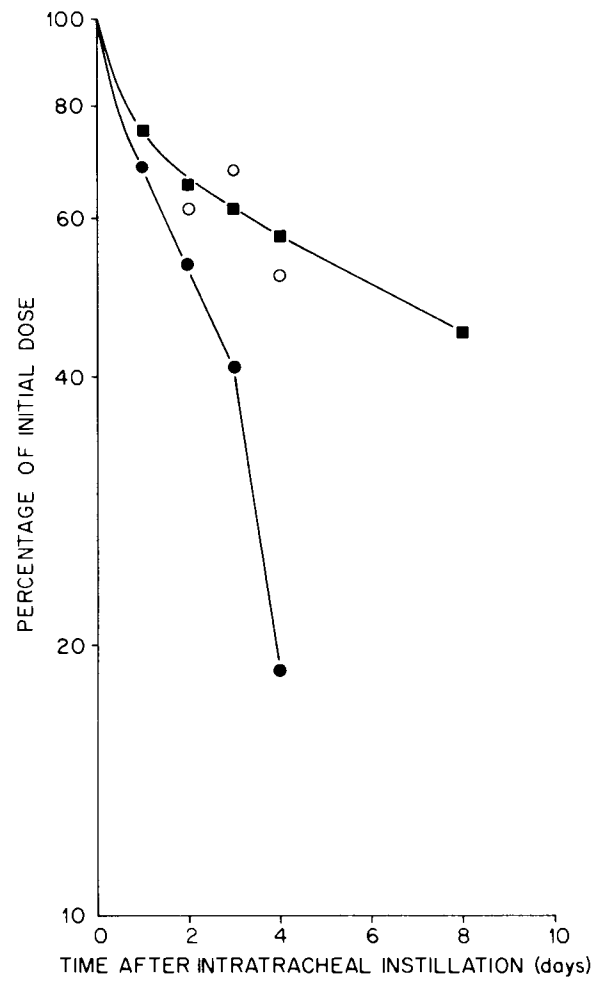

Fig. 27. Clearance of carbon and BP from the lungs of mice intratracheally injected with BP attached to carbon particles. ■–■, rate of carbon clearance; ○–○, rate of BP clearance; ●–●, rate of carbon clearance from the lungs of animals killed for BP determination.

remained. (However, this rate of elimination was slower than if small BP crystals alone had been instilled intratracheally. These crystals of BP are 100% eliminated within hours.) The data suggest a slow but steady dissociation of the BP from the carbon particles. In contrast to the low tumorigenicity and the relatively rapid elimination of small crystals of BP administered alone to the lung, the tumorigenicity of BP attached to 0.5- to 1.0-μm carbon particles is enhanced. It is believed that this slow but steady release of BP to the lung is more favorable in delivering a carcinogenic dose to the target tissue.

Whether the biological elimination of the BP from the lung or the dissociation rate of the BP from the carbon is the rate-limiting factor has yet to be determined. This becomes an important question in cocarcinogenesis studies when one considers that two such common events as respiratory infection and exposure to an irritating gas will drastically reduce pulmonary clearance of particles such as carbon. Further studies are being pursued to help answer these questions.

INHALATION COCARCINOGENESIS STUDIES WITH NITROGEN DIOXIDE, FORMALDEHYDE, AND ACROLEIN

D. A. Creasia

This study is a continuation of the inhalation cocarcinogenesis study initiated early in 1972. The objectives of this study and the techniques by which these objectives are to be reached have been previously described. Briefly, hamsters are treated intratracheally with a carcinogen at dose levels that will produce few, if any, respiratory-tract tumors. The hamsters are also exposed to *either* nitrogen dioxide, formaldehyde, or

Table 29. Exposure protocol for inhalation cocarcinogenesis studies

Formaldehyde exposure

Group	Treatment
1H1	HCHO 10.0 ppm 5X/wk life BP None
1H2	HCHO 20.0 ppm 5X/wk 17 wks BP 5.0 mg 1X/wk 15 wks
1H3	HCHO 10.0 ppm 5X/wk life (after BP) BP 5.0 mg 1X/wk 15 wks
1H7	HCHO 20.0 ppm 1X/wk 17 wks BP 5.0 mg 1X/wk 15 wks
1H8	HCHO 50.0 ppm 1X/wk 17 wks BP 5.0 mg 1X/wk 15 wks
1H9	HCHO 50.0 ppm 1X/wk 17 wks BP None
2H1	HCHO 20.0 ppm 1X/wk 17 wks BP:Fe_2O_3 1.4 mg 1X/wk 15 wks
2H2	HCHO 20.0 ppm 5X/wk 17 wks BP:Fe_2O_3 1.4 mg 1X/wk 15 wks
2H3	HCHO 50.0 ppm 1X/wk 17 wks BP:Fe_2O_3 1.4 mg 1X/wk 15 wks

Acrolein exposure

Group	Treatment
1A1	C_3H_4O 10.0 ppm 5X/wk life BP None
1A2	C_3H_4O 15.0 ppm 1X/wk 17 wks BP 5.0 mg 1X/wk 15 wks
2A1	C_3H_4O 10.0 ppm 1X/wk life BP:Fe_2O_3 1.4 mg 1X/wk 15 wks

Nitrogen dioxide exposure

Group	Treatment
1N1	NO_2 10.0 ppm 1X/wk life BP None
1N2	NO_2 10.0 ppm 1X/wk life after BP 5.0 mg 1X/wk 15 wks
1N3	NO_2 10.0 ppm 1X/wk 17 wks BP 5.0 mg 1X/wk 15 wks
1N4	NO_2 10.0 ppm 1X/wk life BP 5.0 mg 1X/wk 15 wks
1N5	NO_2 10.0 ppm 5X/wk life BP 5.0 mg 1X/wk 15 wks
2N1	NO_2 10.0 ppm 1X/wk life BP:Fe_2O_3 1.4 mg 1X/wk 15 wks

Controls

Group	Treatment
1B1	BP 5.0 mg 1X/wk 15 wks
1B3	BP 5.0 mg 1X/wk 15 wks
1B4	BP 5.0 mg 1X/wk 15 wks
2B1	BP:Fe_2O_3 1.4 mg 1X/wk 15 wks
1O1 1O2 1O3	Room controls

Table 30. Cumulative mortality of animals exposed to NO_2, CH_2O, and C_3H_4O

Group	Months after initiating exposure								
	3	6	9	12	15	18	21	24	27
1H1	0	8	13	18	28	57	77	78[a]	
1H2	0	4	14	24[a]					
1H3	0	7	15	23[a]					
1H7	0	2	9	25	25[a]				
1H8	0	4	15	33	33[a]				
1H9	0	0	9	11[b]					
2H1	0	1	6	26	46	48[a]			
2H2	0	0	0	20	24	28[a]			
2H3	0	0	13	21	28[a]				
1N1	0	0	1	6	10	21	41	69	84[a]
1N2	0	2	5	11	24	53	76	88[a]	
1N3	0	2	4	11	27	46	67	88[a]	
1N5	0	1	7	14[a]					
2N1	0	2	16	22	29	36[a]			
1A1	0	2	3[a]						
1A2	0	11	19[a]						
2A1	0	1	9	19	25[a]				
1B1	0	1	3	15	22	31	42	44[b]	
1B3	0	1	4	7[a]					
1B4	0	2	7[a]						
2B1	0	1	1	12	26	34[b]			
1O1	0	0	3	7	16	28	36	40[a]	
1O2	0	1	5	6	12	26	27[a]		
1O3	0	0	2	4	10	21	23[b]		

[a]Total number of dead animals as of July 1974. Control groups included only 44 animals, except for control group 2B1 with 88 animals. All other groups included 88 animals.

[b]For treatment of group refer to Table 29.

acrolein by inhalation techniques. With each gas, some groups of animals are exposed to the gas simultaneously while others are exposed to the gas after carcinogen treatment in a typical promotion-type scheme. The gas-exposure and carcinogen-treatment protocols are summarized in Table 29.

The groups of animals exposed to NO_2 were the first groups to be started, and consequently, at this time most have died. The survival data are summarized in Table 30. CH_2O and C_3H_4O exposures were initiated later. The formaldehyde-exposed animals are approximately at the midpoint of their life span, and it is expected that most of the animals in this experiment will have died within a year. Histological evaluation of all dead animals is incomplete, and it is too early to predict the cocarcinogenic effect, if any, of these three gases.

CHRONIC EXPOSURE OF RATS TO CIGARETTE SMOKE*

Joseph Kendrick and Paul Nettesheim

Several recent studies with chemical carcinogens suggest that the respiratory tract of the rat may be more susceptible to topical carcinogens than that of the hamster. In spite of this observation, the rat has generally not been used as extensively as the hamster in smoke-inhalation carcinogenesis studies. High susceptibility to respiratory-tract infections and nicotine toxicity are the main reasons. We have demonstrated during the past year that these objections to chronic smoke-inhalation studies in the rat can be eliminated by (1) selecting specific-pathogen-free rats for testing and exposing and maintaining these animals under barrier conditions, (2) allowing several weeks for slow adapta-

tion to cigarette smoke, and (3) allowing appropriate "air breaks" between successive cigarette exposures to decrease nicotine and carbon monoxide to nontoxic levels.

Two small-scale studies, each employing ten Fischer 344 female rats, demonstrated that these animals can be exposed safely and for extended periods to at least seven cigarettes per day. Few signs of toxicity are seen after the first four weeks of adaptation. Lesions (including alveolar aggregations of macrophages, alveolar epithelial bronchiolization, bronchial epithelial hyperplasia, and squamous metaplasia) were found in some lungs four months after exposure.

A third, life-time study, in which 100 rats are exposed to seven cigarettes per day, has been in progress for five months, and survival to date is 100%. Both the daily dose of smoke exposure and the survival rate are much greater than have been reported possible by other investigators. Our work thus far suggests that the rat might be an appropriate rodent model for testing the carcinogenicity of cigarette smoke and other carcinogenic air contaminants.

*Research sponsored by the Tobacco and Health Program of the National Cancer Institute.

VARIATIONS IN SMOKE EXPOSURE AND THE EFFECT ON LUNG DEPOSITION AND RESPIRATION IN HAMSTERS*

Joseph Kendrick and Paul Nettesheim

Efforts to develop chronic smoke-exposure protocols useful in an inhalation carcinogenesis bioassay depend largely on information gained from acute dosimetry and toxicity tests. Such tests indicate the conditions of exposure, in terms of method of smoke generation, and smoke exposure time and concentration which offer maximum lung dose with minimum toxicity.

It has generally been assumed that a given time of exposure to a given concentration of smoke, whether it is offered continuously or intermittently, will result in a given total dose of smoke deposited in the lungs. However, studies to date have shown that the relationship between smoke exposure time, concentration, and dose is substantially more complicated than expected. Respiration during exposure appears to be the main factor controlling the dose the animal receives.

Most dosimetry and toxicity studies to date at ORNL have involved exposing the conventional Syrian golden hamster to [^{14}C] dotriacontane-labeled smoke on either the ORNL intermittent smoke generator or the Hamburg II continuous smoker. Intermittent smokers are designed to introduce a metered quantity of smoke into the exposure chamber, allow a selected stand time to expose the animals, and quickly replace the smoke with fresh air. Continuous-exposure systems, on the other hand, are designed to produce an uninterrupted supply of fresh smoke throughout the exposure.

A principal finding to date using the ORNL device (set to deliver 10% smoke) is that increasing the smoke/air time from the conventional 30 sec to 40 sec more than doubles the smoke particulate dose deposited in the lungs. A similar increase in respiratory minute volume is the apparent explanation for the increase (Table 31). Both acute and chronic survival studies show no corresponding increase in toxicity. When the continuous smoker is used as described by Dontenwill, chamber smoke concentration is approximately 5%. Lung deposition under these conditions is the same as on the ORNL device at 10% smoke and 30 sec exposure time. Note, however (Table 31), that the respiratory minute volume of animals on the continuous smoker is double the volume on the ORNL device at the 30-sec exposure. All animals under smoke exposure show a substantial decrease in minute volume in comparison to the no-smoke controls.

The effects of smoke concentration (in both exposure systems) on dosimetry and toxicity will be further evaluated.

*Research sponsored by the Tobacco and Health Program of the National Cancer Institute.

Table 31. Effects of smoke exposure on deposition and respiration of hamsters[a]

Time (sec)	Machine type	Lung deposition [TPM per organ (mg)][b]	Respiration minute volume (ml/min)
controls (no smoke)			65.6
30	Intermittent (10%)	0.27	23.4
40	Intermittent (10%)	0.64	51.7
60	Continuous (5%)	0.27	47.0

[a]Data are based on ten animals per group.
[b]TPM, total particulate matter.

FORMATION OF CARCINOGENIC NITROSAMINES FROM AMINES AND NITRITE

William Lijinsky

We have continued to examine the hypothesis that nitrosamines formed *in vivo* from ingested nitrite and secondary or tertiary amines are related to human cancer. A number of drugs and agricultural chemicals having such structures have been found to react more or less readily with nitrite at mildly acid pH and at concentrations of a few mg per ml. Small but significant yields of carcinogenic nitrosamines were formed in each case. The insecticide carbaryl gave a nitroso derivative which was a potent mutagen in bacteria.

To ascertain whether sufficient nitrosamine could be formed *in vivo* to give rise to tumors, several of the secondary and tertiary amino compounds already examined in chemical systems are being given to rats in drinking water together with nitrite, and development of tumors in the animals is being assessed. So far, two compounds, aminopyrine and heptamethyleneimine, have given rise to a high incidence of malignant tumors (of liver and lung, respectively) when administered with nitrite. Neither amine nor sodium nitrite alone has given rise to any tumors; the tumors seen in these controls were only those found in untreated rats. Oxytetracycline and nitrite appear to be negative in a similar test, as does chlorpromazine. Piperidine, although very similar in structure to heptamethyleneimine, has not given rise to tumors when tested with nitrite. Experiments with the other compounds, including hexamethylenetetramine, trimethylamine oxide, tolazamide, lucanthone, cyclizine, chlordiazepoxide, dimethyldodecylamine, dimethyphenylurea, and carbaryl are still in progress. No conclusions can yet be reached about the *in vivo* formation of nitrosamines by the interaction of the latter group of compounds with nitrite.

STRUCTURE-ACTIVITY RELATIONS IN NITROSAMINE CARCINOGENESIS

William Lijinsky and H. W. Taylor

There is reason to believe that clues to the mechanism of action of carcinogenic nitroso compounds might be found in the effect (or lack of effect) on carcinogenic activity of small modifications in some basic nitrosamine structures. We have prepared a number of derivatives of nitrosopiperidine, nitrosopiperazine, nitrosopyrrolidine, nitrosomorpholine, and nitrosourea and have given equimolar doses in drinking water to groups of Sprague-Dawley rats for the same time periods.

Nitrosopiperidine, 4-methylnitrosopiperidine, nitroso-4-piperidinol, nitroso-3-piperidinol, and nitroso-4-piperidone were about equally potent, all giving rise to olfactory adenocarcinomas and tumors of the esophagus. Some liver tumors were also seen in the groups treated with the hydroxy compounds, but not in the other groups. 3-Methylnitrosopiperidine was a little less potent and 2-methylnitrosopiperidine much less potent than nitrosopiperidine, while 2,6-dimethylnitrosopiperidine 2,2,6,6-tetramethylnitrosopiperidine, and nitrosoisonipecotic acid appear to be noncarcinogenic. Quite analogously, while nitrosopyrrolidine was a potent liver carcinogen, 2,5-dimethylnitrosopyrrolidine was inactive.

In the nitrosopiperazine series, dinitrosopiperazine was a little less carcinogenic than 2,5-dimethyldinitrosopiperazine but was much less active than either its 2-methyl or 2,6-dimethyl derivatives, or than dinitrosohomopiperazine. *N*-Methylnitrosopiperazine (which is a base) was inactive.

A significant deuterium isotope effect in carcinogenesis by nitrosomorpholine was seen by replacing the four hydrogen atoms alpha to the nitrogen with deuterium. The animals given the deuterium-containing compounds developed fewer tumors and with a longer latent period than those given the unlabeled compound. On the other hand, 2,6-dimethylnitrosomorpholine was a much more potent carcinogen than nitrosomorpholine, and appeared to be the most potent carcinogen for these rats among all of the nitroso compounds tested.

Among four trialkylnitrosoureas examined, all accelerated the formation of the normally spontaneous mammary tumors in these rats. However, only three of them induced tumors of the central nervous system, and nitrosotrimethylurea did not. Nitrosomethyldiethylurea induced CNS tumors (brain and spinal cord) in 50% of the animals, while nitrosotriethylurea and nitrosoethyldimethylurea gave rise to 17% and 7% CNS tumors, respectively. These tumorigenic effects could not be correlated with the stability of the compounds in acid or alkali, ruling out chemical decomposition as a factor in the different carcinogenic potencies of the compounds.

These results point to some activity at the carbon atoms alpha to the *N*-nitroso function as the rate-limiting step in chemical carcinogenesis by these *N*-nitroso compounds.

SYNTHESES OF NITROSAMINES

A. R. Jones

Syntheses of several nitrosamines were developed in order to further the investigation of the mechanism of their carcinogenic activity.

***N*-Nitrosoguvacoline.** 3-Carbethoxy-4-piperidone hydrochloride was hydrogenated over platinum at moderate pressure to the piperidinol, which was dehydrated and deesterified by gaseous hydrogen chloride at 200°C to guvacine (1,2,5,6-tetrahydronicotinic acid). Treatment of the latter with sodium nitrite yielded *N*-nitrosoguvacine, and this with diazomethane gave the desired product.

***N*-[2-^{14}C]Nitrosohexamethyleneimine and *N*-[2-^{14}C]nitrosoheptamethyleneimine.** Since these compounds were ultimately to be synthesized with ^{14}C labeling, the starting material was barium carbonate. The synthetic schemes were identical and will be considered together. Barium carbonate was converted to sodium cyanide by the Jeanes[1] procedure, using potassium metal rather than sodium. The sodium cyanide was treated with pentamethylene and hexamethylene dibromides in ethanol-dimethylsulfoxide solution under mild conditions to produce, respectively, pimelonitrile and suberonitrile. These were hydrolyzed in basic solution, and the corresponding acids were converted to the hydrated salts barium pimelate and calcium suberate. Pyrolysis of the latter gave the cyclic ketones. Oxime formation and rearrangement were carried out in one step by adding the ketones to a solution of hydroxylamine in concentrated sulfuric acid at 100°C. The lactams were reduced with lithium aluminum hydride, and the amines formed were treated with sodium nitrite in aqueous acetic acid solution to form the final products.

1. J. K. Jeanes, *Science* **118**, 719 (1953).

STUDIES OF THE HISTOPATHOLOGY OF TUMORS INDUCED BY ADMINISTRATION OF NITROSAMINES

H. W. Taylor

From the response of Sprague-Dawley rats to (1) aminopyrine, sodium nitrite, and CCl_4, (2) aminopyrine and sodium nitrite only, or (3) dimethylnitrosamine (DMN) only, it was determined that the combination of aminopyrine, sodium nitrite, and CCl_4 is highly hepatotoxic and cirrhogenous and that this combination of chemicals induces hepatocellular tumors. All animals that received aminopyrine and nitrite or DMN without CCl_4 developed only Kupffer-cell sarcomas of the liver. From these studies, it appeared that Kupffer cells were more responsive to the action of DMN than were liver cells themselves, which is a situation previously unreported.

Attempts are being made at present to explant liver tissue from the portal circulation to a position beneath the kidney capsule and to a subcutaneous position in mammary tissue. The effects of DMN on such aberrant liver would help to determine the importance of DMN concentration in portal circulation in its action upon liver tissue. It is also hoped that such a system of aberrant positions of liver can be devised that will cause loss of hepatocytes but preserve the Kupffer cells. In such a situation, the action of DMN on Kupffer cells in the absence of hepatocytes could be determined.

Nitrosoheptamethyleneimine studies in Fischer 344 and Sprague-Dawley rats. Nitrosoheptamethyleneimine (NHMI) was tested for carcinogenicity in Fischer 344 and Sprague-Dawley rats by intragastric administration and subcutaneous injection. Cumulative doses ranged from 5.5 to 1200 mg/kg, and dosage schedules ranged from 40 divided administrations to one single injection. No difference in response was seen between sexes or strains of rats. Following the highest carcinogen doses by intragastric administration, a high incidence of squamous-cell tumors occurred in the lung. The highest incidence of squamous-cell tumors occurred in the nasal cavity, trachea, and esophagus. Following subcutaneous injection, tumors induced in the lungs were all alveologenic adenomas and adenocarcinomas. The upper respiratory and gastrointestinal tracts were again the most commonly affected sites. Intragastric administration was more effective in producing tumors than was subcutaneous injection, and administration of multiple small doses was more efficient than single large doses. The results demonstrated that the route and schedule of administration markedly influenced the tumor response to NHMI, both quantitatively and qualitatively.

DEVELOPMENT OF ANALYTICAL METHODS FOR DETERMINATION OF NITROSATABLE AMINES IN FOOD AND OTHER MATERIALS

G. M. Singer

It appears probable that nitrosamines formed *in vivo* are at least as important in human carcinogenesis as are nitrosamines ingested as part of the food supply. Analytical procedures for identifying and quantifying the precursors of these nitrosamines have been lacking, however. We have refined, simplified, and extended our

procedure for the analysis of nitrosatable amines to include a number of foodstuffs, beverages, and tobacco.

The material is concentrated or puréed while acidic and is then subjected to basic steam distillation. The acidified steam distillate, after overnight extraction to remove volatile neutrals, is concentrated and subjected to a Hinsberg-type derivatization and separation to provide separate solutions of primary toluenesulfonamides, secondary toluenesulfonamides, and tertiary amine hydrochlorides.

Analysis of the secondary toluenesulfonamides has shown the presence of dimethylamine in all substances studied. The level ranges from 71 μmoles/100 g in wine to 730 μmoles/100 g in frozen cod fillets. Other types of fish contain 40 (tuna), 150 (smallmouth bass), and 250 μmoles/100 g (ocean perch). Surprisingly, morpholine has also been found in all substances studied, in amounts ranging from 30 nmoles/100 g (beer) to 80 μmoles/100 g (one tuna sample). (The source of the morpholine is under investigation. It has not been previously reported in foodstuffs.) Other secondary amines have also been found, including pyrrolidine, piperidine (both in coffee), and a tetrahydropyridine (unburned tobacco). Quantification of these and examination of the tertiary amines is continuing.

Confirmation of the identification of the amines has been carried out in conjunction with W. T. Rainey, Jr., of the Analytical Chemistry Division on their GLC-MS system. Recoveries have been monitored by use of appropriate labeled and unlabeled tracer compounds and are generally quite good.

Efforts are still under way to effect a direct computer linkup with the Biology Division PDP-11 to simplify the data acquisition and data reduction from the gas chromatograph.

THE RATES OF DNA REPAIR IN NORMAL AND IN XERODERMA PIGMENTOSUM HETEROZYGOTES

R. B. Setlow and James D. Regan

Last year we described the distribution of the capacity for DNA repair among normal and repair-variant humans.[1] The measurements utilized the bromodeoxyuridine-photolysis assay on fibroblasts at 24 hr after a uv exposure of 20 J m^{-2} at 254 nm. Since repair is about complete in this time, the measurements estimated the extent of repair and indicated a bimodal distribution – xeroderma pigmentosum (XP) individuals in one nonrepairing peak and all other cells, including XP heterozygotes, in a repairing group. We have used the technique to estimate the numbers and sizes of the repaired regions at 3–4 hr after uv irradiation in an attempt to determine whether such a measurement – which measures the rate rather than the extent of repair – would show differences between normal and heterozygote cells. Our data on 15 cell lines, including six XP heterozygotes, indicate the following.

(1) During the early stages of excision-repair there are appreciable numbers of repaired regions that are smaller than the average of ~80 nucleotides observed at 24 hr. This conclusion is reached from the complex shape of the curves relating breaks induced in the bromodeoxyuridine-containing repaired regions to fluence of 313-nm photolyzing light.

(2) Compared to a standard normal cell (HSBP), the XP heterozygotes we examined are (10 ± 8)% *less* effective in repair. Three other normal cell lines were (11 ± 15)% *more* effective in repair. Thus the two types of cells cannot readily be distinguished from one another with respect to either the rate or the extent of DNA repair.

1. J. D. Regan and R. B. Setlow, *Biol. Div. Annu. Progr. Rep. June 30, 1973*, ORNL-4915, pp. 68–69.

TUMORS ARISING FROM TREATED FISH CELLS

R. W. Hart* and R. B. Setlow

Certain species of fish are good systems to use to evaluate the carcinogenicity of physical and chemical agents. Fish such as *Poecilia formosa* can be grown as clones. Isolated cells can be treated *in vitro* and injected back into isogenic recipients for assays of cellular transformation. Last year we reported that tumors arose from uv-irradiated cells and that if the irradiated cells were illuminated with photoreactivating (PR) light before injection, the yield of tumors was markedly reduced.[1]

We have by now had a large number of fish examined histologically by J. C. Harshbarger, Director of the Registry of Tumors in Lower Animals, Smithsonian Institution. He has observed that large numbers of fish injected with irradiated cells have thyroid carcinomas and most also contain noninfectious granulomas. We are uncertain about the origin of the granulomas. The carcinomas presumably arise from thyroid cells injected along with the predominant heart cells used. A summary of the data, showing that uv irradiation results in carcinomas and that the effect is reversed if PR follows the uv irradiation but not if it is given before, is in Table 32.

Table 32. The effects of PR illumination[a] on uv-irradiated[b] cells[c] of *P. formosa*

Treatment	Fraction of fish with granulomas	Granulomas per fish	Fraction of fish with thyroid carcinoma by	
			Gross pathology	Histology
Ultraviolet	51/63	1.8	34/34	29/29
2.5 min PR + uv	49/57	2.0	26/26	22/22
5.0 min PR + uv	37/42	1.9	48/50	22/23
uv + 5.0 min PR	15/43	0.4	1/43	0/6

[a]700 J m^{-2} min^{-1} (320–420 nm).
[b]12 J m^{-2} average incident fluence (254 nm).
[c]~3 × 10^5 cells injected per fish.

Table 33. The effect of *N*-acetoxy-AAF[a] on cells[b] of *P. formosa*

Concentration	Fraction of fish with thyroid carcinomas by	
	Gross pathology	Histology
0	0/50	
10^{-5} *M*	14/50	
10^{-4} *M*	45/51	10/10

[a]Treatment for 1 hr at the indicated concentration.
[b]~5 × 10^5 cells injected per fish.

The effects on human cells of a number of chemical carcinogens mimic those of uv irradiation.[2] *N*-Acetoxy-AAF is one of these mimetics.[3] When heart (plus presumably thyroid) cells are treated for 1 hr with this agent and injected into isogenic recipients, tumors develop. The histology has not been completed in this experiment, but the preliminary results (Table 33) indicate that *N*-acetoxy-AAF mimics uv irradiation in the fish system.

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1. R. W. Hart and R. B. Setlow, *Biol. Div. Annu. Progr. Rep. June 30, 1973*, ORNL-4915, p. 152.
2. J. D. Regan and R. B. Setlow, in *Chemical Mutagens*, vol. 3, p. 151, A. Hollaender (ed.), Plenum, New York, 1973.
3. R. B. Setlow and J. D. Regan, *Biochem. Biophys. Res. Commun.* **46**, 1019 (1972).

DNA DAMAGE AND REPAIR BY CARCINOGENIC AND NONCARCINOGENIC METHYLCHRYSENE IN HUMAN CELLS*

James D. Regan and A. A. Francis

Methylchrysenes occur in cigarette smoke. 5-Methylchrysene is a strong carcinogen; 4-methylchrysene is not carcinogenic.[1] We wished to determine the extent of DNA damage and repair in human cells after treatment with these compounds. After bulk labeling of the DNA of the cells with [^{3}H]thymidine, the cells were treated with 10^{-5} *M* 5-methyl- or 4-methylchrysene for 1.5 hr and then allowed to incubate for repair periods of 0, 18, and 42 hr. The data from alkaline sucrose gradient analyses of DNA damage indicate that both methylchrysenes can cross-link the DNA of human cells.

Preliminary data indicate that more repair occurs after 5-methylchrysene treatment than after treatment with 4-methylchrysene. This difference in DNA repair is being investigated.

*Research sponsored by the Chemical Carcinogenesis Program of the National Cancer Institute.

1. D. Hoffman et al., *Science* **183**, 215 (1974).

EFFECT OF PROFLAVINE PLUS VISIBLE LIGHT ON HUMAN CELL DNA*

James D. Regan and R. B. Setlow

Proflavine plus visible light is employed in herpesvirus therapy. Proflavine reacts with DNA when exposed to visible light. We wished to examine DNA damage and repair in human cells treated with proflavine and subsequently exposed to visible light. In particular, we wished to determine whether the sequence of molecular events in the DNA of human cells after treatment with proflavine plus light resembled any of the DNA repair events we have observed in human cells after treatment with chemical carcinogens.

Normal human cells treated with 3 × 10^{-5} *M* proflavine and exposed to 5000 ergs/mm^2 of 390- to 530-nm light sustained single-strand breaks in DNA similar to those in cells exposed to 2 krads of gamma rays. These strand breaks were eventually rejoined, but

the repair period was more protracted than in a typical gamma-ray experiment. The delay in rejoining was probably due to the repair-inhibiting effect of proflavine. Cells from patients with the ultraviolet-sensitive disease xeroderma pigmentosum (XP) were similarly treated and assayed for DNA repair. The XP cells' DNA repair sequence was like that seen in normal human cells. Bromodeoxyuridine-photolysis assays of repair in normal human cells after proflavine-plus-light treatment suggested no extensive base insertion. Thus, DNA damage induced by proflavine plus light appears to be classifiable as short or ionizing-type repair. It is, therefore, not surprising that XP cells and normal cells show similar DNA repair response after proflavine-plus-light treatment.

*Research sponsored by the Chemical Carcinogenesis Program of the National Cancer Institute.

ALKALI CATION TURNOVER AND TRANSPORT IN TRANSFORMED AND NONTRANSFORMED HUMAN CELLS IN CULTURE

J. S. Cook and Emily T. Best

It has been proposed[1] that a fundamental and characteristic distinction between normal and transformed cells is the persistent elevation of transport of certain small molecules in tumor cells. This prediction has been verified for sugars, some amino acids, nucleosides, etc. We have investigated this question for alkali cations in cultured human cells. These ions, chiefly K^+, are quantitatively the most important of cell solutes and for osmotic reasons one of the principal determinants of cell size; although total cell K^+ turns over many times in one cell cycle, its net accumulation is a major factor in cell growth. We have shown that in short-term measurements the cells are close to being in a steady state; that is, efflux and influx are nearly equal. In order to minimize problems of cell geometry in our measurements, we use the steady-state assumption to calculate K^+ *turnover* from the rate constant for leak efflux of the potassium analog ^{86}Rb; *transport* is assessed from the ouabain-sensitive fraction of the influx. For a number of types of cultured human cells – diploid fibroblasts [WI38 and HSWP (the latter kindly provided by James D. Regan)] either in log growth or confluent phase; transformed fibroblasts (SV40-transformed WI38); or cells of tumor origin (HeLa S3) – the efflux rate constant was in every case 1.0 hr^{-1} (range 0.91–1.09), and the ouabain-sensitive component of the influx also was the same among the cell types at 70–80% of the total.

In serum-depleted diploid cells (HSWP) allowed to accumulate for several days in early G_1, stimulation to resumption of growth with serum led in 9 hr to a sixfold enhancement in glucose transport and in 20 hr to a 50-fold enhancement of [^{3}H]thymidine incorporation into DNA; throughout this period, ^{86}Rb transport increased only 50% and in direct proportion to cell mass.

We conclude that K^+ (= ^{86}Rb) transport is a function of cell surface area, increasing as the cell grows; in this respect, alkali-cation transport is a concomitant but not a causal determinant of cell growth and is not elevated in transformed cells.

1. R. W. Holley, *Proc. Nat. Acad. Sci. U.S.A.* **69**, 2860 (1972).

OUABAIN RESISTANCE OF HUMAN CELLS IN CULTURE

Emily T. Best and J. S. Cook

The cardiotonic steroid ouabain binds at low concentration (10^{-7} *M*) to human cells and inhibits alkali-cation transport. Secondarily to loss of cell K^+, protein synthesis is strongly inhibited over a period of a few hours in ouabain-poisoned cells. We have initiated studies on the long-term effects of ouabain on cultured human cells.

(1) Transformed cells are more sensitive than normal diploid cells. Incubated in 10^{-7} *M* ouabain, HeLa cells become completely inviable in two days and do not recover on return to normal medium. Normal diploid fibroblasts (WI38 and HSWP) can be kept in 10^{-7} *M* ouabain for more than a week with no loss in viability, although growth ceases. On return to normal medium, growth resumes at the normal rate after a 12- to 24-hr lag. This differential long-term response can be used to recover normal cells from mixed cultures.

(2) We have isolated a series of ouabain-resistant mutants by treatment of HeLa cells with ethyl methanesulfonate. Although the characterization of these mutants is just under way, there are two preliminary observations of interest. (*a*) Data on ouabain binding and dissociation, as well as dose-response curves of $^{86}Rb^+$ transport rate vs inhibitor concentration, suggest that two classes of binding sites are present on both wild-type and mutant cells. One site has a dissociation constant of $\sim 10^{-7}$ *M*, the other $\sim 10^{-5}$ to 10^{-4} *M*. The nature of some of our mutants appears to be a shift in the quantitative distribution of these two classes of

binding sites. This finding explains the relative ease with which mutants resistant to 10^{-5} *M* ouabain can be generated, whereas we have not yet succeeded in isolating a HeLa cell resistant to 10^{-4} *M* (or higher) ouabain after a one-step mutagenic treatment. (*b*) One of our HeLa mutants in 10^{-4} *M* ouabain attaches to a surface but grows very slowly. At this inhibitor concentration the cells undergo a reversible morphological change, developing dendritic structures that resemble those of neuroblastoma cells. These mutants also show cell alignments similar to those seen during contact inhibition of movement. These phenomena are the subject of continuing investigation.

INTERRELATIONSHIPS OF HEXOSE TRANSPORT AND METABOLISM IN CULTURED HUMAN CELLS

D. W. Salter* and J. S. Cook

It has been observed in many laboratories, including ours, that the uptake of hexoses (particularly glucose) by cultured cells is a plastic property of the cell membrane and is highly responsive to such factors as growth state, transformation, nutrition of the cells, and the action of inhibitors. A convenient, simple, and commonly used assay for hexose uptake is the short-term incorporation of radioactive sugars from the medium into the cells. As part of our studies on hexose transport in cultured human cells, we have, first, assessed the validity of the radioactivity assay in terms of dissecting the transport component from altered distribution of radioactivity among hexose metabolites; second, reexamined the changes in hexose transport by human cells (chiefly, the diploid fibroblast HSWP, from a stock kindly supplied by James D. Regan) in response to a variety of conditions.

(1) At concentrations below 1 m*M*, 3-*O*-methylglucose, which is not phosphorylated in the cells, equilibrates with the cell water in less than 1 min even at 25°C. At higher concentrations ($> K_m$ for transport), uptake is linear for longer periods. This nonmetabolized sugar is useful for measuring the transport component as distinct from subsequent metabolic events. [^{3}H]Deoxyglucose is rapidly phosphorylated, although less rapidly than glucose, so that free intracellular sugar is low but slowly rises during transport measurements. At 37°C and with extracellular deoxyglucose at concentrations from 4 μM to 1 m*M*, the transmembrane gradient for the sugar changes very little in 5 min. This also is a useful analog sugar for the transport assay. [^{3}H] Glucose is efficiently phosphorylated as soon as it enters the cell, so that little free glucose is found in cells growing in normal growth medium; that is, the transmembrane sugar gradient again remains constant during assay. However, lactic (+ pyruvic) acid is formed rapidly and excreted into the medium. This effect is pronounced at 1 min, and at longer times 70% of the transported glucose can be recovered from the medium as lactate (+ pyruvate). This ratio of excreted radioactivity to incorporated radioactivity holds constant for cells in sparse cultures to cells in confluent cultures, and for [^{3}H]glucose concentrations from 4 μM to 1 m*M*.

(2) Cytochalasin B, a potent and fast-acting inhibitor of hexose transport, slows but does not prevent efflux of radioactivity taken up by prior exposure of cells to [^{3}H]deoxyglucose; to some extent the cells are leaky to free and phosphorylated sugars. Cytochalasin B has no effect on the excretion of lactate (+ pyruvate) formed from [^{3}H]glucose.

(3) The effects of a number of sulfhydryl-reactive compounds, even when acting for short times at low concentrations, appear to be more on metabolism than on transport in both HeLa and HSWP. Thus, iodoacetate (IAA) and dinitrofluorobenzene (DNFB) appear to stimulate [^{3}H]glucose incorporation threefold without affecting [^{3}H]deoxyglucose incorporation. In fact, these compounds strongly inhibit lactate formation (by inhibition of glyceraldehyde-3-phosphate dehydrogenase) and its subsequent excretion into the medium. If one allows for the latter, IAA and DNFB do not appear to affect transport. Conversely, *N*-ethylmaleimide, *p*-chloromercuribenzoate, and $HgCl_2$ appear to be potent inhibitors of [^{3}H]glucose uptake in a 5-min assay, whereas analysis of the cells treated with these inhibitors show the free intracellular glucose concentration to be nearly equal to free extracellular glucose; the block to incorporation of radioactivity apparently occurs (depending on the reagent) at steps subsequent to transport.

(4) Both glucose and deoxyglucose appear to be transported on a carrier with a $K_m \approx 10^{-3}$ *M*. A second carrier, with $K_m \approx 10^{-6}$ *M*, might exist for glucose; this carrier does not appear to transport deoxyglucose.

(5) After a 24-hr starvation for glucose, HSWP cells show a tenfold enhancement of [^{3}H]glucose uptake, an observation reported previously in chick fibroblasts and HeLa cells.[1,2] This increase is accounted for by a two- to threefold enhancement of transport plus a retention of >90% of the radioactivity in starved cells as opposed to the excretion of 70% of the radioactivity in the fed controls. The presence of pyruvate or galactose during starvation profoundly alters the distribution of metabo-

lites in a poststarvation assay of [^{3}H]glucose uptake, but in both cases we observe the twofold enhancement of transport. The enhancement is also observed in the presence of insulin or deoxyglucose. None of these four compounds prevents the starvation effect on transport per se. Two hours after restoration of 5 m*M* glucose to starved cells, their metabolism is back to normal in the sense that >70% of the incorporated glucose is being excreted as lactic acid; but transport is still twice normal. These appear to be independently regulated parameters.

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MODULATION OF THE IMMUNE RESPONSE OF GUINEA PIGS BY REPEATED BCG SCARIFICATION

Leona C. Peters and M. G. Hanna, Jr.

In experimental animals, a single vaccination of *Mycobacterium bovis* (BCG), a potent immunostimulant, has been shown to enhance humoral immunity, increase antigen clearance, and accelerate allograft reactivity. Based on these results, "systemic adjuvant therapy" protocols have been initiated to enhance host response to cancer in man, performed by repeated application of BCG by dermal scarification. These studies were performed to determine what alterations of immune competence would be derived from repeated BCG scarification in random-bred and inbred (syngeneic) guinea pigs. We also assessed the histopathological alterations of the skin site and regional and peripheral lymph nodes during the course of scarification. Peripheral-blood-leukocyte changes were monitored and compared to the changes seen in man. These studies should be relevant to future application of this particular type of adjuvant therapy in man.

The results demonstrate that in this experimental model BCG scarification depresses humoral immune competence while augmenting cellular immunity. The possibility of split-mode immunodulation in nonspecific adjuvant therapy has been suggested. An ideal condition in cancer therapy would be one in which cellular immunity could be enhanced and humoral immunity suppressed, since it is believed by some that humoral immunity results in "blocking factors" which may protect tumor cells against cytotoxic lymphocytes. Also, the number of peripheral blood leukocytes was elevated 24 hr after the second and each successive BCG scarification. This is indicative of an acute systemic effect, possibly an extrusion of bone-marrow reserves of mononuclear and polymorphonuclear cells. This hematogenous effect on guinea pigs is in agreement with one report in man. Flaring of previous scarification sites was shown, with activation and enlargement of draining nodes and progressive responsiveness of nodes draining sites of the previous scarifications.

The conditions that contribute to this split mode of immune modulation as a result of BCG challenge warrant further consideration and investigation. And, due to the correlation between results observed in guinea pigs and in humans undergoing similar therapy, these results become extremely important to clinical applications of BCG in cancer therapy.

DIFFERENTIAL EFFECTS OF TWO STRAINS OF BCG ON TRANSPLANTATION IMMUNITY TO SV40-INDUCED TUMORS IN HAMSTERS

G. W. Fortner,* M. G. Hanna, Jr., and J. H. Coggin†

Mycobacterium bovis strain BCG has been shown to augment immunity to malignancy in many experimental prophylactic and immunotherapy models. In inbred guinea pigs with transplanted intradermal, syngeneic hepatocarcinomas, BCG has proven highly effective in causing regression of established tumors as well as elimination of regional-lymph-node metastases. Immunization with BCG in combination with tumor cells is more effective than either BCG or tumor cells alone for stimulating transplantation immunity. BCG alone, by various routes of administration, can be protective, have no effect, or, on occasion, cause enhancement of tumors in experimental animals. A similar range of effects has been reported when BCG has been used for treatment of malignancies in man. Variables which have been cited in the use of BCG to stimulate immunity against malignancies in experimental models are source or substrain of bacterium, dose, number of viable organisms, and time and route of administration. A less apparent, but in our experience critical, variable is the ability of the experimental animal to control the infectious process established by the live BCG. This is extremely relevant in developing reliable experimental models for establishing alternatives for therapy in man, where BCG infection is limited or can be controlled by drugs.

In order to gain insight into the basis of protection or enhancement as a function of BCG treatment, we chose

the transplantable hamster–SV40 tumor system. This model is advantageous because the tumor immunology has been well characterized and the hamster has been used to evaluate BCG strains for their virulence in relation to their use as live vaccines for tuberculosis. A correlation is made between immunoprophylaxis and immunotherapeutic properties and the growth-culture kinetics and morphology of a lyophilized and live frozen source of BCG.

Using a transplantable SV40-tumor system in hamsters, we evaluated the immunoprophylactic and immunotherapeutic properties of lyophilized Glaxo-strain and fresh-frozen Phipps-strain BCG. The results were correlated with growth-culture kinetics, virulence, and colony morphology of the two preparations. The results demonstrate an immunoprophylactic effect with Phipps, as measured by delay of appearance and reduction of total tumor incidence (Fig. 28). An actual enhancement of tumor incidence was obtained with the Glaxo strain. In combination with irradiated tumor cells, both had synergistic effects on the development of transplantation immunity in the hamster. However, the effect of the Glaxo strain was significantly lower than that of the Phipps strain.

Our results therefore indicate that there are differences in these BCG strains which may have influenced their efficacy as immunoprophylactic or immunotherapeutic agents. These differences cannot strictly be correlated with total organism counts, but they can be associated with the kinetics of growth, virulence, and colony morphology on solid media. Our results on differences between colony morphology with Phipps and Glaxo strains confirm the results of others. We

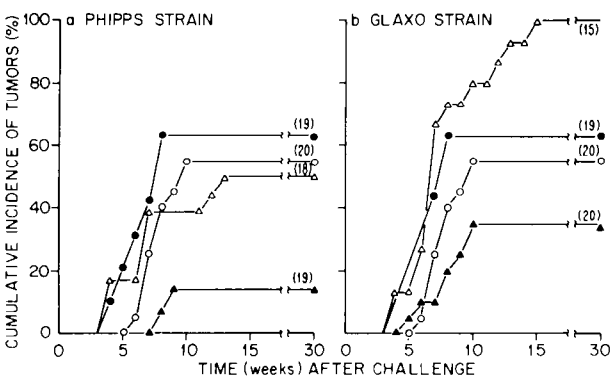

Fig. 28. Cumulative tumor incidence in hamsters challenged with 5 × 10^4 F5-1 cells after immunization with BCG alone (△–△), BCG admixed with irradiated tumor cells (▲–▲), irradiated tumor cells alone (○–○), or unimmunized controls (●–●). The number of animals in each group is noted in parentheses.

suggest that lyophilization of the Glaxo strain is probably not the single factor in its failure to stimulate immunity in this system, since other lyophilized strains have been shown to be useful for immunotherapy in man. There is ample evidence, however, that strain variability may be important in the effectiveness of BCG treatment.

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HISTOPATHOLOGY OF TUMOR REGRESSION AFTER INTRALESIONAL INJECTION OF BCG: DEVELOPMENT OF IMMUNITY TO TUMOR CELLS AND BCG

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B. Zbar,† and H. J. Rapp†

Evidence from several systems indicates that inhibition of tumor growth by the granulomagenic agent *Mycobacterium bovis* strain BCG requires the development of specific bacterial immunity. Recent results of our investigation of the mechanism of BCG-mediated therapy of established tumors with regional metastases in inbred guinea pigs suggests that activated cells of the macrophage-histiocyte compartment play a primary and essential part in the destruction of tumor cells. In this immunotherapy model, which utilizes a transplantable syngeneic hepatocarcinoma and BCG, it is reasonable that tumor inhibition may not require the development of specific tumor immunity; evidence is lacking to substantiate that the activated histiocytes are specifically sensitized to the tumor cells. Although these arguments relate to local reactivity, an important issue is control of any metastasis that may exist beyond the regional lymph node. Highly relevant to this point is the finding that guinea pigs which have undergone BCG-mediated regression of an established transplanted tumor can reject a subsequent challenge with the same syngeneic tumor.

These aspects of the BCG-mediated therapy model in inbred guinea pigs have led to the conclusion that the regression of tumor and regional metastases involves several steps. The first step requires specific immunity to the antigenic BCG. The second step involves the accumulation of lymphocytes and cells of the macrophage-histiocyte series in the skin and regional lymph nodes. The histiocytes have been demonstrated to be essential for tumor cell destruction. The third step is the ultimate development of systemic tumor immunity.

It is particularly important to obtain more information on the function of specific immunity either to BCG or tumor cells and to determine whether lymphocyte cooperation with effector cells of the macrophage-histiocyte compartment is a requirement in this tumor immunotherapy model of guinea pigs, where BCG treatment is administered to established syngeneic tumors with regional metastases.

In the present study, transplanted syngeneic tumors established in the skin of inbred guinea pigs regressed, and regional metastases were eliminated, after intralesional injection of BCG. Specific immunity to tumor cells and BCG was evaluated in this therapy model. The results demonstrated that BCG-mediated regression of established tumors had both local and systemic features, both of which involved host-specific immunity. Early sensitization to BCG antigens was required in guinea pigs whose peripheral and central lymphocyte compartments were depleted as a result of pretreatment with antilymphocyte serum. The ability to develop delayed sensitivity to the purified protein derivative of BCG was correlated with tumor regression after injection of BCG into established tumors. The development of specific tumor immunity was an aspect of this experimental model. Guinea pigs with established tumors developed humoral and cell-mediated immunity during BCG-mediated tumor regression. Antibody with specificity for line-10 tumor cells was demonstrated by the positive immunofluorescence and C1 fixation and transfer test of plasma from BCG-treated, tumor-bearing guinea pigs. Systemic cell-mediated immunity was shown by the classic lymphocyte-mediated rejection of line-10 tumor challenge in guinea pigs that had undergone BCG-mediated regression of line-10 tumor cells.

Therefore, it can be concluded that during BCG-mediated regression of tumor in the skin, if metastasis does occur beyond the draining chain of lymph nodes, it likely could be controlled by the developing specific tumor immunity. More importantly in this particular experimental model, regression of metastases in the distal lymph nodes (axillary and subcostal) possibly is mediated by tumor-specific immunity.

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IMMUNOELECTRON MICROSCOPIC ANALYSIS OF SURFACE ANTIGENS COMMON TO BCG AND TUMOR CELLS

Corazon Bucana* and M. G. Hanna, Jr.

Tumor-specific cell-surface antigens have been demonstrated by various direct and indirect techniques. Immunoelectron microscopy is one of the most sensitive direct assays, and it has been used to show that tumor-specific antigenic sites of various sizes occur in discrete areas of the cell surface. This technique provides a valuable means of determining specificity of reactivities as well as the topographical distribution of antigens on the cell surface.

A guinea pig immunotherapy model has been developed which utilizes transplantable syngeneic hepatocarcinoma cell lines (line 1 and line 10) and *Mycobacterium bovis* (BCG). In this immunotherapy model, the natural or BCG-mediated development of transplantation immunity against line-1 or line-10 tumor-specific antigen has been detected by delayed hypersensitivity reaction and transplantation rejection. Formation of xenogeneic antibodies against tumor-specific antigens of both line-1 and line-10 cells was demonstrated by complement fixation and transfer tests and immunofluorescence tests. Also, antibody development to these two hepatoma cell lines can be detected in syngeneic hosts.

More recently, Borsos and Rapp[1] reported an antigenic relationship between BCG organisms and line-10 cells. This is a very significant observation with relevance to immunotherapy. We have attempted to confirm and expand this finding by immunoelectron microscopic assay against guinea pig and human tumor cells. We have demonstrated by immunoelectron microscopy the presence of cell-surface antigens on guinea pig line-10 hepatocarcinoma cells, using syngeneic and xenogeneic tumor-specific antibody. Furthermore, we have confirmed the fact that these cells also possess a cell-surface antigen that is common to BCG cell walls. The immunologic cross-reactivity of line-10 or BCG immune sera with respect to both anti-line-10 and anti-BCG reactivity was determined by quantitating labeled cells at the ultrastructural level. This observation was supported by complement-dependent serum cytotoxicity tests.

Experiments to demonstrate whether the known tumor-specific antigens (TSA) are distinct from the shared BCG antigenic site of the line-10 tumor cell were performed using sequential incubation of the tumor

cells with guinea pig anti-BCG serum and mouse anti-line-10 serum. Results from these experiments indicated that the line-10 TSA sites may be distinct from other antigens shared with BCG.

These results confirm and supplement previous observations indicating the presence of a common antigen between the line-10 tumor cells and BCG. It was also observed that line-1 tumor cells, which are highly antigenic, do not show reactivity with the anti-BCG serum. This finding is of interest because line-1 tumor cells, although syngeneic, are rejected when transplanted to strain-2 guinea pigs. The demonstration of common antigens between the weakly antigenic transplantable line-10 hepatocarcinoma and BCG organisms is of special interest in the elucidation of the biological aspects of the BCG-mediated regression of established tumors and metastases in the strain-2 guinea pig immunotherapy model.

Based on the above observations, we performed experiments to determine the cross-reactivity of anti-BCG serum with human melanoma cells. Melanoma cells were chosen specifically because BCG scarification or intralesional injection of BCG is a mode of immunotherapy of melanoma in man. Results from this experiment showed that there is indeed a strong cross-reactivity of anti-BCG serum with a primary human melanoma cell culture now maintained in our laboratory. We feel that this is an immunologically specific reaction, since, under identical experimental conditions, two control human cell lines had negative reactivity with BCG-specific antibody (Fig. 29).

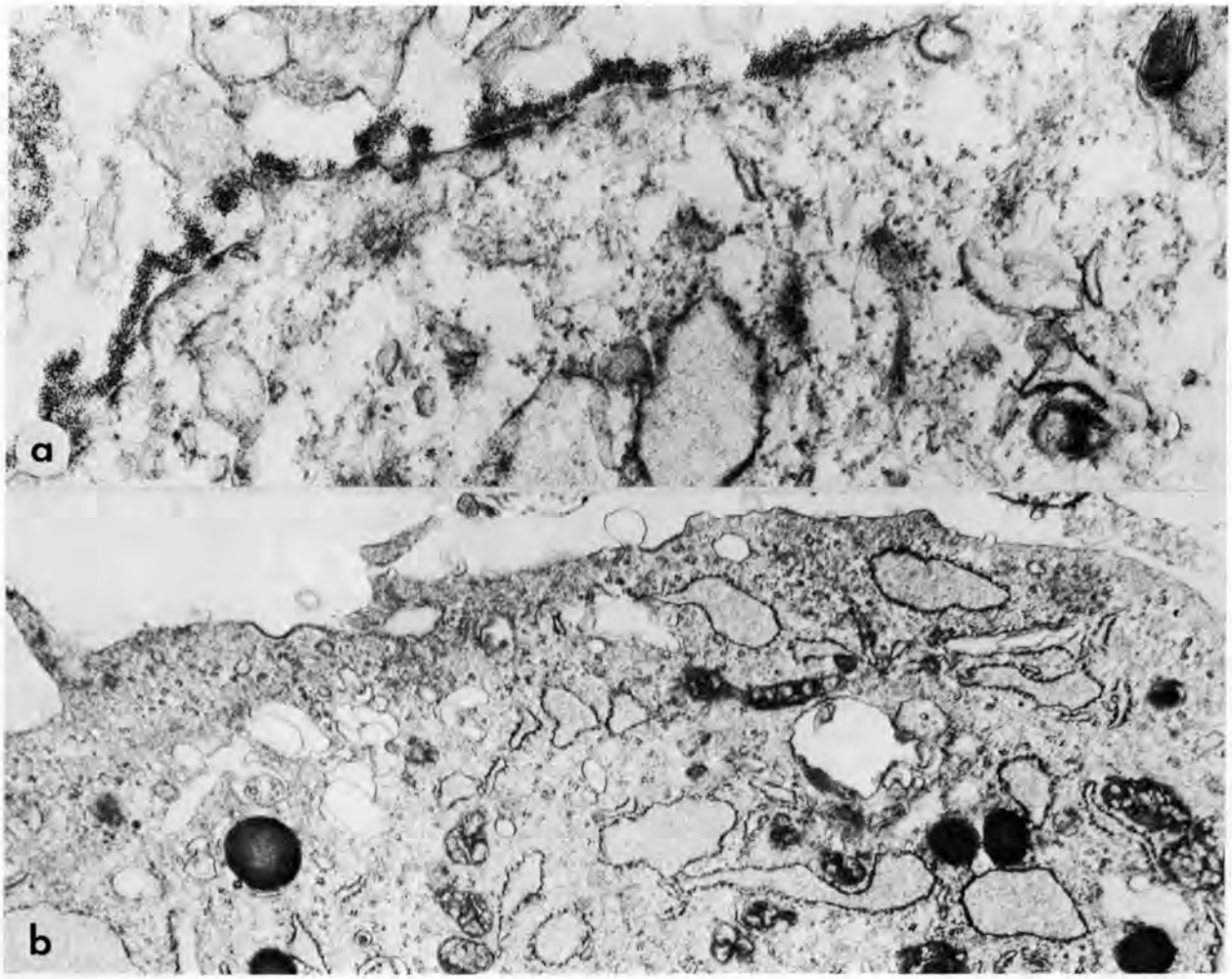

Fig. 29. (*a*) A portion of the melanoma cell surface, showing extensive labeling after incubation with guinea-pig anti-BCG serum. ×54,000. (*b*) Electron micrograph of a melanoma cell after incubation with BCG-adsorbed anti-BCG serum. ×22,800. Reduced 8%.

Although we have verified the presence of common antigens between BCG organisms, line-10 hepatocarcinoma cells of strain-2 guinea pigs, and an early human melanoma cell culture, we feel it is important to investigate further the nature of immunologic cross-reactivity between tumors of different histological types and BCG or other organisms. These studies are highly relevant to future immunotherapy protocols utilizing immunostimulants or immune potentiators.

It is known that complex oligosaccharides are important constituents of cell-surface antigens, and specific cell-surface saccharide residues have now been identified and localized by using ferritin-conjugated plant agglutinins. The most widely used agglutinins are concanavalin A and *Ricinus communis* agglutinin, which are specific for the α-D-mannopyranosyl and β-D-galactopyranosyl configurations, respectively. It has been reported that *N*-acetylglucosamine is an important part of the tumor cell-surface receptor that interacts with wheat-germ agglutinin. Thus it would be important now to determine further the correlation between the cross-reactivity and the chemical nature of the antigenic sites of the tumor cells and BCG or other types of organisms now being used as "nonspecific" immune potentiators.

*Supported by a United Order True Sisters, Inc., Postdoctoral Fellowship in Cancer Immunology.

FUNCTIONAL ASPECTS OF IMMUNITY TO AUTOCHTHONOUS PAPILLOMAS AND CARCINOMAS DURING CHEMICAL CARCINOGENESIS

A. K. Szakal and M. G. Hanna, Jr.

The hypothesis that the successful establishment of chemically induced neoplastic clones requires associated carcinogen-mediated immunosuppression is based on a series of studies that demonstrated: (1) the antigenicity of tumors induced with methylcholanthrene (MCA), (2) the immunogenicity of MCA-induced sarcomas in their original host, and (3) the immunosuppressive properties of various chemical carcinogens. The causal relationship between suppression of humoral immunity or, more importantly, cell-mediated immunity and tumor development remains equivocal. To reevaluate the significance of cell-mediated immunity in terms of *in situ* tumor development we performed a comparative study of the cell-mediated immune competence to non-tumor-related alloantigens and neoantigens of isologous and autochthonous origin in DMBA-treated hamsters during the induction and development of skin tumors.

The temporal relationship between depression of cell-mediated immunity and papilloma and carcinoma development during treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA) was studied. The test antigens used were non-tumor-related allografts and DMBA-treated isografts and autografts containing clones of neoplastic cells. Allografts were invariably rejected by DMBA-pretreated recipients, and the allograft survival times increased as a function of tumor burden on animals receiving optimal carcinogen treatment. The survival of carcinogen-pretreated isografts (with early stages of tumor development) was found to depend on immunological competence, as indicated by the prolongation of graft survival on recipients treated with antithymocyte serum. The survival of isografts also increased with the number of DMBA pretreatments and the tumor burden of recipients. The association of tumor burden with depression of the immune response to non-tumor-related and tumor-related antigens is interpreted to be the result of competition between the test antigens and the antigens of developing autochthonous tumors. This interpretation is supported by the finding that DMBA pretreatments of animals did not depress the immune response to autografts containing clones of neoplastic cells. Autograft survival, in contrast to the survival of allografts and isografts, was reduced. The survival was inversely related to the extent of tumor development on autografts prior to grafting. Because of the distinctive host response to autografts, it is concluded that the available pool of immunologically uncommitted cells may be the limiting factor in the response against alloantigens and the neoantigens present in isografts. Furthermore, competition for immunologically uncommitted cells may also be functional among autochthonous clones of neoplastic cells of different antigenicities early in DMBA-induced tumor development.

To obtain more direct evidence for the commitment of cell-mediated immunity to autochthonous DMBA-induced papillomas, we attempted passive transfer of tumor immunity. To assess systemic tumor immunity during the stages of tumor progression in DMBA-induced carcinogenesis, we also utilized an assay for *in vitro* cytotoxicity. We approached the study of systemic immunity to autochthonous carcinomas by examining the influence of increasing tumor mass and antigenic heterogeneity on lymphocyte cytotoxicity.

As indicated by a reduced survival of papilloma-bearing isografts, specific cell-mediated immunity to autochthonous neoantigens of papillomas was transferable to DMBA-treated recipients by lymphoid cells of the graft donor. Passive transfer of normal lymphoid

cells did not confer immunity to recipients of papilloma-bearing isografts. The presence of systemic immunity to autochthonous tumors in DMBA-treated animals was also shown by the *in vitro* cytotoxic effect of autologous lymph-node cells on target cells derived from papillomas or carcinomas. Analysis of the data showed no significant correlation of cytotoxicity with carcinogen dose. There was a tendency toward an inverse relationship between the average tumor mass and the percentage of animals with cytotoxic lymph-node cells. Animals with multiple antigenic malignancies had no lymphoid cells with demonstrable cytotoxicity. The effect of tumor multiplicity appeared to be independent of tumor mass. These studies illustrate that the development of tumors induced by chemical carcinogens is accompanied by an early development of tumor-specific immunity, and that there is a balance between immunity and the multiplicity of uniquely antigenic malignancies in the late phase of tumor development.

HOST-MEDIATED *IN VIVO–IN VITRO* ASSAY AS A RAPID TEST FOR CARCINOGENICITY OF CHEMICALS

M. G. Hanna, Jr., Cynthia K. Schenley, R. W. Tennant, and J. M. Quarles, Jr.*

An important aspect of cancer research is to identify potential carcinogens. In order to screen the large number of chemicals introduced into the environment, a rapid and efficient test is imperative. Morphologic changes indicative of carcinogenicity usually appear in cells cultured *in vitro* within two weeks after treatment with the test chemical. This is much more rapid than the months or years required for tumors to develop as a result of *in vivo* carcinogenesis experiments. However, most chemical carcinogens must be metabolized by the host before they can transform cells to produce a tumor. *In vitro* carcinogenesis assays lack this essential step for identification of potential carcinogens.

An experimental approach that overcomes these difficulties has been developed by Dr. J. A. DiPaolo of the National Cancer Institute. In this assay, the test chemical is injected into a pregnant hamster at approximately ten days of gestation. The fetuses, which have been exposed to both the chemical and its metabolites, are removed three days later, and the fetal cells are cultured *in vitro*. During the course of continuous passage, the presence of cells transformed by the carcinogen is determined, as in standard *in vitro* systems, by colony morphology. This system is as rapid as the standard *in vitro* assay and can detect the carcinogenicity of chemicals which must be metabolized *in vivo* to produce the ultimate carcinogen.

Dr. DiPaolo has tested 12 carcinogens and 5 noncarcinogens with this assay and has found no false positives or false negatives. Our laboratory has been asked to perform a double-blind study using this assay to confirm the reliability and speed of the procedure. We are currently completing preliminary studies using untreated hamsters and hamsters injected with three known carcinogens. Ultimately we will screen 50 unknowns in a double-blind assay.

*NCI Postdoctoral Fellow.

HOST RESPONSE TO TUMOR-ASSOCIATED FETAL ANTIGENS*

F. A. Salinas† and M. G. Hanna, Jr.

It has been adequately demonstrated in a broad spectrum of spontaneous, oncogenic-virus-transformed, and chemically transformed tumor systems that potential antigenic determinants normally present at the membrane surface of embryonic antigens are reexpressed during transformation and can be detected by a variety of immunologic techniques. Antibody specific for tumor-associated fetal antigens has been adequately demonstrated, but the contribution of the host response with respect to transplantation immunity has not been fully documented. It has been demonstrated in some but not all systems that immunization with fetal cells confers protection against tumor challenge. This disagreement can be attributed in part to the use of different techniques; that is, irradiated fetal cells were used in most of the studies where positive host reactions were found but not in those where no protection was conferred. With respect to the *in vivo* studies, one question that still may be valid is whether the host response can be attributed in part to a nonspecific and even nonimmunologic reaction rather than to true immunologic cross-reactivity. One approach for determining *in vivo* whether there is immunologic cross-reactivity would be to demonstrate that immunization with tumor cells or fetal cells can adversely affect embryoma or embryo development. We have developed a quantitative *in vivo* assay involving a modified colony-forming-unit technique which can be used to test for cytotoxic or cytostatic antibodies cross-reactive for tumor and fetal cell-surface antigens (Fig. 30). Fetal liver cells are used as both immunogenic and test or target-cell populations, because this population of cells is primarily hematopoietic and upon adoptive transfer to irradiated recipients proliferates in

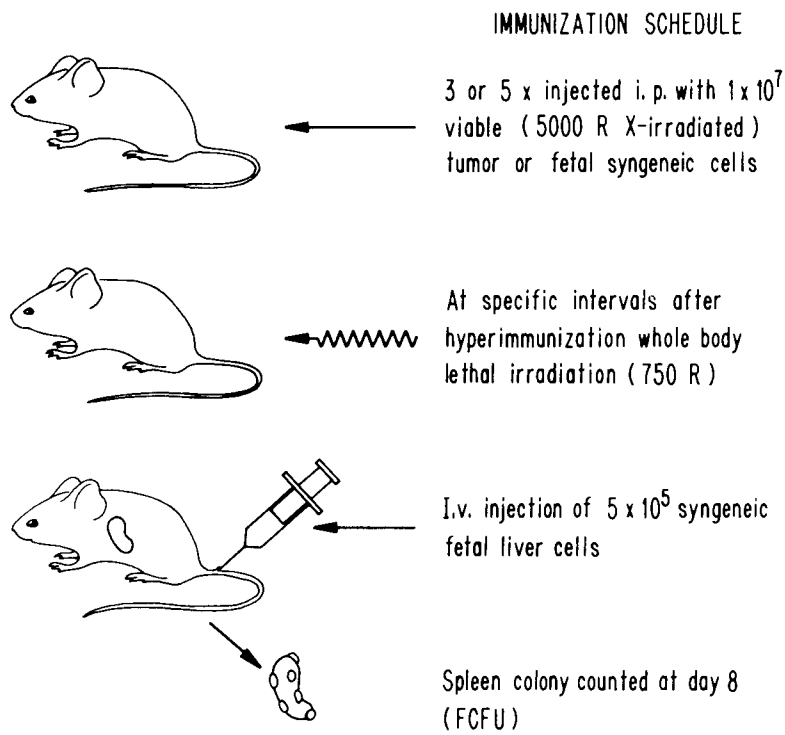

Fig. 30. Protocol for a quantitative *in vivo* assay to evaluate the host response with respect to transplantation immunity to tumor-associated fetal antigens.

the spleen to form colonies derived from fetal colony-forming units (FCFU) (Fig. 31).

We have evaluated the contribution of the host response with respect to transplantation immunity to tumor-associated fetal antigens by this colony-forming-unit technique with fetal liver test cells as a source of FCFU. To measure host immunity we evaluated the effect of immunization with tumor cells on the development of fetal colonies in spleens of immunized, irradiated recipients. It could be demonstrated that immunization with syngeneic (plasmacytoma, MSV-BALB/3T3), allogeneic (E14), or xenogeneic (SV40-hamster) tumor cells was capable of generating a host response in BALB/c recipients that was at least cytostatic to adoptively transferred fetal liver cells.

In an *in vitro* modification of the FCFU assay, fetal liver cells were first incubated with serum from mice hyperimmunized with fetal liver cells or plasmacytoma cells and then transplanted into irradiated recipients. Colony-forming ability was reduced in both cases, indicating that humoral antibody is an effective component in the host response to tumor-associated fetal antigens. A marked suppression of FCFU was also observed with transplanted fetal liver cells that had been incubated with sensitized lymphocytes (lymphocytes from mice immunized with fetal liver cells or plasmacytoma cells).

Maternal immunity to transplanted syngeneic fetal liver cells was also evaluated by the *in vivo* FCFU assay. A strong, short-lived postpartal immunity was seen in primiparous females; a weak immunity was seen after second litters but was detectable only several weeks postpartum; and a strong immunity, which increased as a function of time, was seen after five or more litters. Furthermore, we found that an injection of 5×10^5 syngeneic fetal liver cells is radioprotective in adult BALB/c mice irradiated with 900 R x rays. At this level of x irradiation there is 100% mortality in 30 days, and complete protection if the mice are injected with 5×10^5 fetal liver cells 24 hr after irradiation. We then tested the repopulation potential and thus the radioprotective effect of 5×10^5 fetal liver cells in BALB/c mice irradiated with 900 R x rays after immunization with fetal liver cells according to the regimen used for the *in vivo* assay. The results are shown in Table 34.

Thus, the 65% reduction in survival supports the contention that antibody generated against a fetal antigen is capable of exerting at least a cytostatic effect on fetal liver cells, such that not only the colony-forming potential but also the repopulation (radiopro-

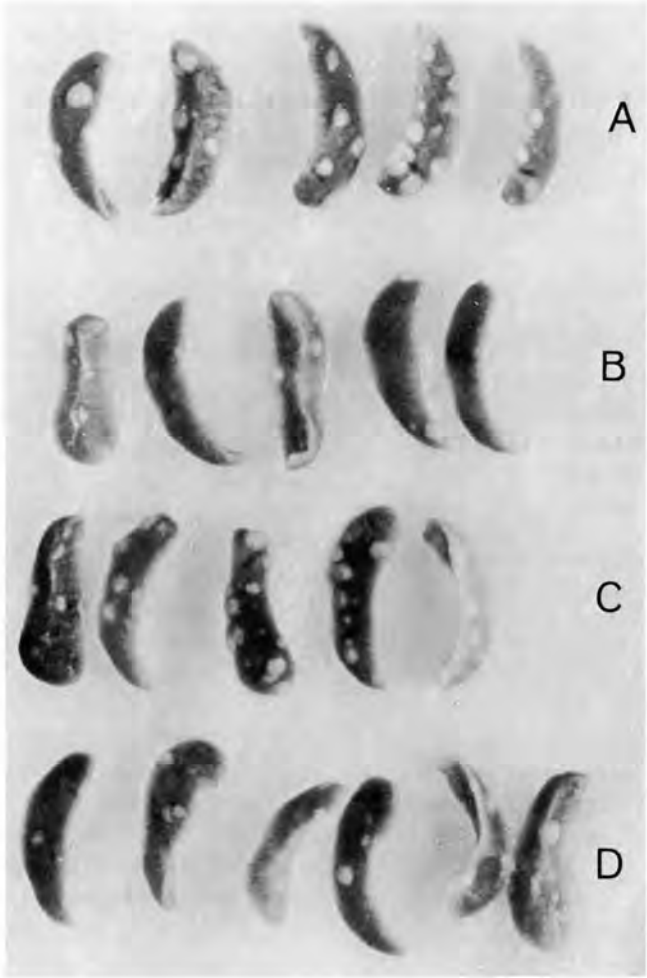

Fig. 31. Comparison of macroscopic FCFU on the spleens of mice, demonstrating a two- to threefold suppression of FCFU on experimental groups as compared to controls. Control groups: Row *A* (untreated recipient spleens) and Row *C* (immunized with adult hamster spleen cells). Experimental groups: Row *B* (immunized with plasmacytoma cells) and Row *D* (immunized with SV40 hamster tumor cells).

Table 34. Radioprotection by fetal liver cells injected after 900 R x irradiation

Immunization	Irradiation (R)	Treatment (no. of fetal liver cells)	Survival after 30 days[b] (%)
None	900		0
None	900	5×10^5	100
Fetal liver cells[a]	900	5×10^5	35

[a]Immunization five times in two weeks with 10^5 cells previously irradiated with 5000 R.

[b]Twenty mice per group.

tective) potential of these cells is markedly impaired in immunized recipients. These studies definitely indicate that transplantation immunity can be achieved as a result of cross-reactive antigens on fetal and neoplastic cells.

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AUTOGENOUS IMMUNITY TO ENDOGENOUS RNA TUMOR VIRUS: ASSESSMENT OF REACTIVITY TO VIRUS ENVELOPE ANTIGENS AND VIRUS-INDUCED CELL-SURFACE ANTIGENS*

M. G. Hanna, Jr., J. N. Ihle, B. L. Batzing,†
R. W. Tennant, and Cynthia K. Schenley

There is now considerable evidence contradicting the concept that mice are immunologically tolerant or hyporesponsive to their endogenous RNA tumor virus(es). Earlier studies of naturally acquired immunity to antigens associated with endogenous murine leukemia virus (MuLV) indicated that the major detectable immune response is directed to virus-induced cell-surface antigens (VISA) rather than virus-envelope antigens (VEA). A unique and important aspect of our work has been the demonstration of widespread occurrence of natural antibody specific for VEA.[1,2] The interaction between natural immunity to VEA and to VISA and the significance of these reactivities in host control of viral expression and virus-mediated pathogenesis have not been resolved. With respect to the biologic nature of the spectrum of natural antibody with specificity to MuLV-associated antigens, it would be important to determine whether virus-neutralizing and anticellular (cytotoxic) antibodies coexist in autogenous immune serum.

The purpose of one of our studies this year was to correlate the reactivities of the natural antibodies to MuLV-associated VEA and VISA. Natural reactivity to VEA was evaluated by a radioimmune-precipitation assay of intact and disrupted virus and by virus-neutralization tests, and the specificity of the natural antibody for VEA was determined by immunoelectron microscopy and radioimmune precipitation. Reactivity to VISA was evaluated by immunoelectron microscopy and complement-dependent serum-cytotoxicity tests.

The strains of mice selected for the study were C57BL/6, C3H/Anf, and the $B6C3F_1$ hybrid. The $B6C3F_1$ hybrid has a mean survival time of approximately 884 days and a low natural incidence of thymic lymphoma; a similar hybrid from a reciprocal mating has at birth a much lower susceptibility than the parent strains to Gross virus passage A. This resistance has been associated with the *Rgv-1* locus, which is proximal to or within the K region of H-2, as is the *Ir-1* gene, which determines immune responsiveness to certain antigens. Thus we felt it important that this strain combination be studied, since in this genetic combination the *Ir-1* and *Rgv-1* genes may be identical and function in the immune control of MuLV-associated antigens.

Although there were quantitative differences in antibody levels within the three strains, the naturally recognized antigenic determinants of the virus were consistent – gp-71, gp-43, and gp-15 (ref. 3). High levels of neutralizing antibody were detected in the normal sera against xenotropic BALB:virus-2 in the focus-reduction assay. Marginal levels of neutralizing activity, however, were detected in the XC test of virus neutralization using the Moloney leukemia virus. No anticellular antibody could be detected in the normal sera by complement-dependent cytotoxicity assays.

These results clearly show, within the strain combination used, natural antibody specific for VEA of endogenous MuLV (Fig. 32). There appear to be genetically determined quantitative differences in antibody levels among the three strains, but naturally recognized antigenic determinants of the virus are a consistent aspect of autogenous immunity. The consistent recognition of naturally occurring VEA by all three strains is borne out by the quantitative differences in the levels of intact virus-precipitating antibody detected in normal and anti-K36 hyperimmune C57BL/6 sera, and by the similarity of the specific VEA recognized by these two sera. It is also interesting that antibody specific for VEA generated in N-, B-, and NB-tropic systems reacts comparably with N- and X-tropic viruses with respect to both specific immunologic recognition and biologic function.

Since BALB:virus-2 has been shown to be an endogenous C-type virus of mice but has not yet been shown to be infectious or to have any contribution to MuLV pathogenicity in mice, it is difficult to implicate autogenous immunity as a beneficial immune component with respect to spontaneous viral carcinogenesis on the basis of these results. It still remains to be determined, however, whether virus neutralization is a primary requisite for immunologic control of virus burden and virus-mediated carcinogenesis. Nevertheless, natural recognition of VEA of endogenous leukemia viruses has been demonstrated to be a general phenom-

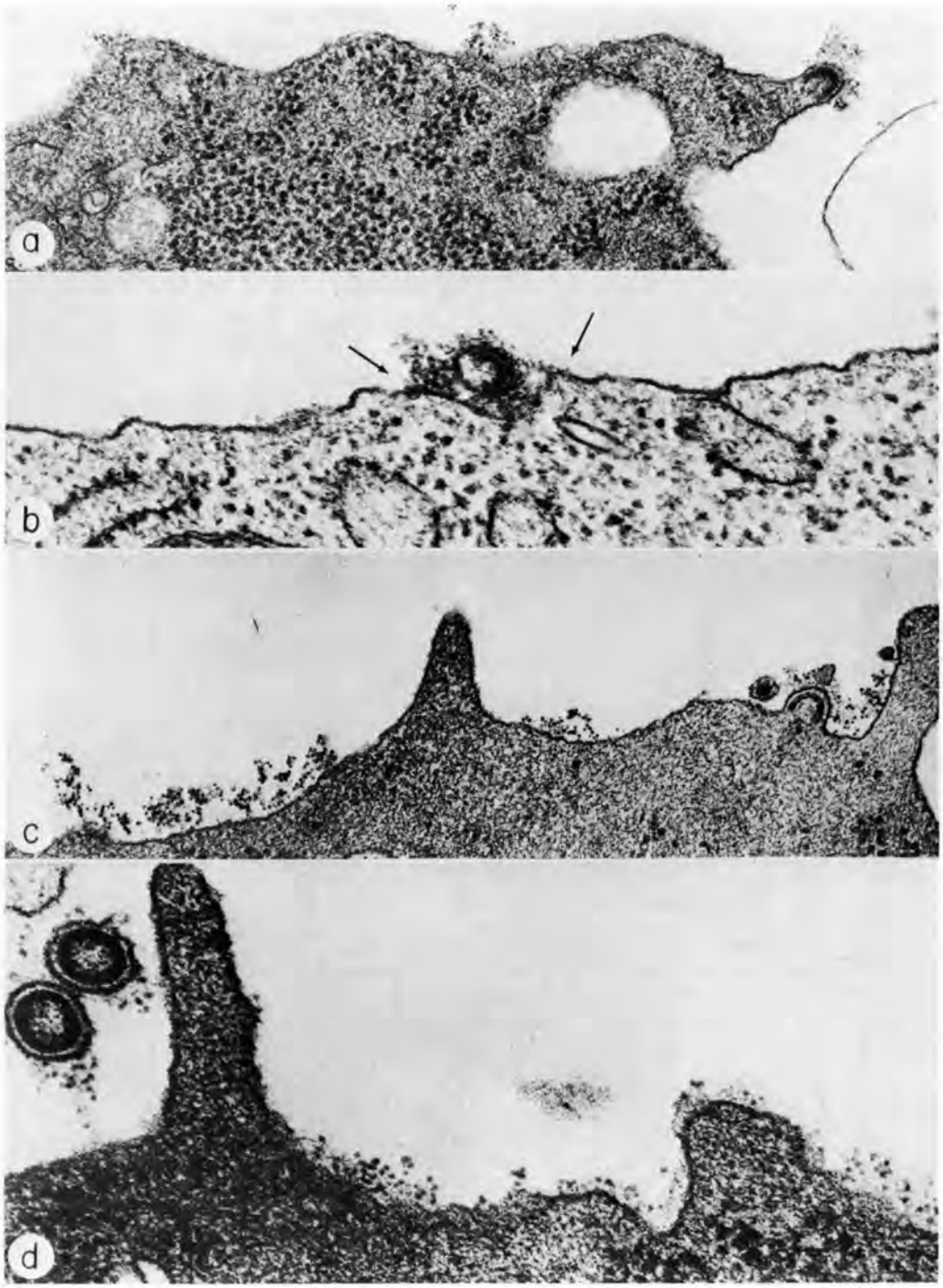

Fig. 32. Immunoelectron microscopic evidence for natural antibody specific for virus envelope antigens. (*a*) E♂G2 leukemia cells incubated with serum and labeled with ferritin-conjugated rabbit anti-mouse gamma globulin. A selected region of an E♂G2 cell is shown, with the entire envelope of a budding C-type virus and two small sectors of nonvirion cell surface labeled. ×70,000. (*b*) B6C3F$_1$ serum reacted with a BALB/c plasma-cell tumor cell. The entire envelope of a free virus that apparently has just completed budding from the cell is labeled. ×83,000. (*c*) E♂G2 leukemia cells reacted with serum from female C57BL/6 retired breeders and labeled with ferritin-conjugated rabbit anti-mouse gamma globulin. Extended regions of the cell surface and the entire envelope of a budding C-type particle are labeled. ×70,000. (*d*) Immunoferritin labeling of E♂G2 leukemia cells reacted with serum from C57BL/6 mice hyperimmunized with K36 cells. An extended region of the cell surface and the envelopes of free viruses are labeled. ×130,000. Reduced 20%.

enon, while natural antigenic recognition of VISA appears to be a more selective or restricted autogenous immune function.

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AUTOGENOUS IMMUNITY TO ENDOGENOUS RNA TUMOR VIRUS: DIFFERENTIAL REACTIVITIES OF IgM AND IgG TO VIRUS ENVELOPE ANTIGENS*

J. C. Lee,† M. G. Hanna, Jr.,
J. N. Ihle, and S. A. Aaronson‡

Previous studies have provided ample evidence for the existence of an autogenous humoral immune response in mice that may be important in the regulation of endogenous type-C virus expression *in vivo*. Earlier findings, using radioimmune-precipitation assays and immunoelectron microscopy, indicated that the regulation of virus-mediated pathogenesis by this autogenous immunity may be a function of the quality and/or

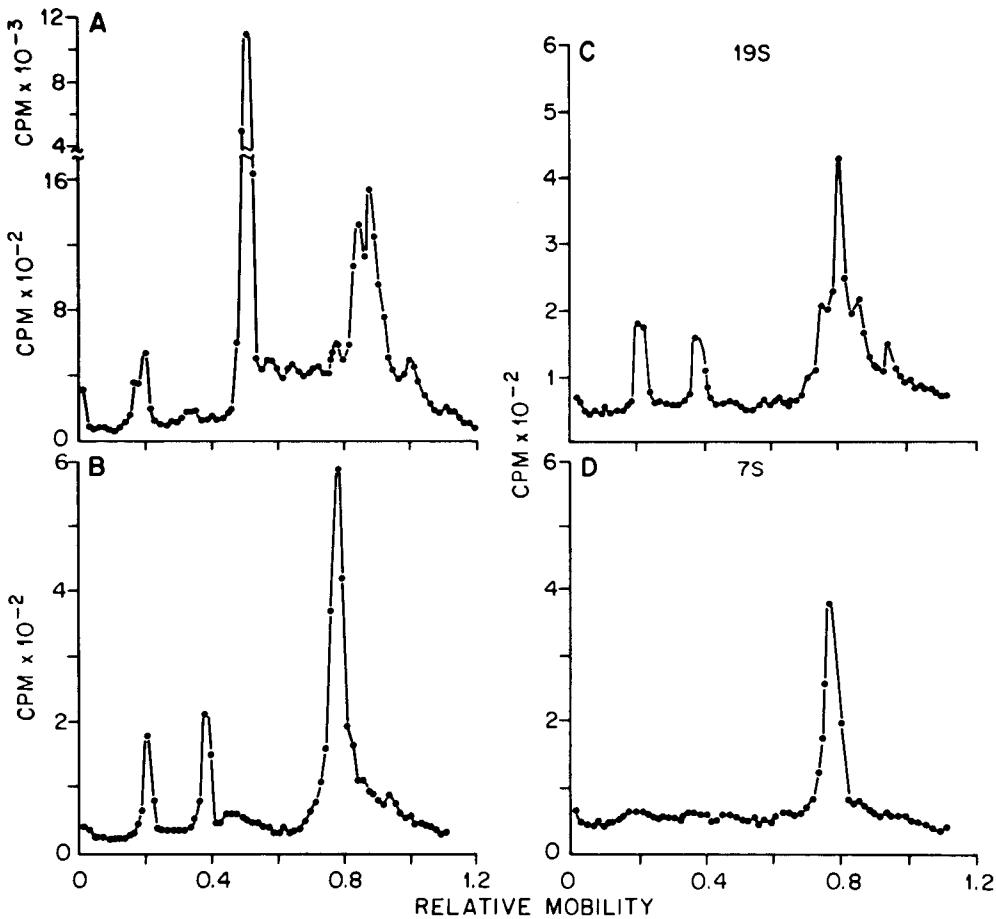

Fig. 33. SDS-polyacrylamide gel electrophoresis profiles of (*a*) Triton-disrupted [^{3}H]leucine-labeled AKR virus, (*b*) an immune precipitate of 1.5-year-old B6C3F$_1$ whole serum with Triton-disrupted AKR virus, (*c*) an immune precipitate of purified 19S fraction of 1.5-year-old B6C3F$_1$ serum with Triton-disrupted AKR virus, and (*d*) an immune precipitate of purified 7S fraction of 1.5-year-old B6C3F$_1$ serum with Triton-disrupted AKR virus.

quantity of the humoral response. The purpose of this study was to evaluate the reactivities of the individual classes of antibody in serum of normal $B6C3F_1$ mice, a strain which we previously have found to have high levels of antibody reactivity to endogenous leukemia virus and a low incidence of leukemia.

Whole serum and the IgM and IgG fractions of serum from $B6C3F_1$ mice of different age groups were compared to radioimmune-precipitation assays and viral infectivity neutralization assays. Both IgM and IgG fractions were able to precipitate virus, although not as effectively as whole serum. Virus-specific antibody levels, as well as total antibody concentrations, in whole serum appeared to increase with age.

Sodium dodecyl sulfate gel electrophoresis analyses were performed with immune precipitates obtained when whole serum or 19S or 7S fractions from animals of different age groups were reacted with disrupted virus. Both whole serum and the 19S antibody fraction reacted with three antigenic determinants on the viral envelope (Fig. 33). These antigens have apparent molecular weights of 17,000, 43,000, and 68,000. The latter two higher-molecular-weight antigens are glycoproteins and correspond to M_1 and M_2 antigens. In contrast, the 7S fraction reacted only with the 17,000-molecular-weight protein, which has since been shown to be p15. Neutralization tests using focus-reduction assays against BALB:virus-2, a xenotropic endogenous mouse type-C virus, revealed that the 19S fraction and whole serum but not the 7S fraction possessed neutralizing activity.

These findings indicate that there are differential reactivities of IgM and IgG antibodies in normal serum of $B6C3F_1$ mice with respect to both recognition of viral envelope antigens and neutralization of endogenous MuLV. The relationship of host immune response and the genetic regulation of viral pathogenesis in the mouse is a complex one. Although the present finding does not represent a generalization among mice of different strains, it provides a basis for further investigation and understanding of the role of autogenous host immune response in the regulation of the expression of endogenous RNA tumor virus.

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AUTOGENOUS IMMUNITY TO ENDOGENOUS RNA TUMOR VIRUS: IDENTIFICATION OF ANTIBODY REACTIVITY TO SELECT VIRAL ANTIGENS*

J. N. Ihle, M. G. Hanna, Jr., L. E. Roberson, and F. T. Kenney

The expression of endogenous murine leukemia virus (MuLV) during development and throughout life of normal mice has been demonstrated serologically and ultrastructurally. In addition, experiments have indicated that the consequence of this natural virus expression , the host is an autogenous humoral immune response. To evaluate this response quantitatively, we have developed a radioimmune-precipitation assay.[1] With this technique it has been demonstrated that natural antibodies to MuLV are present in mice and that the level of specific antibodies to MuLV in each strain varies inversely with the natural incidence of leukemia and lymphoma. These data suggest that a humoral response to the virus may be functional in restricting pathological expression of the virus. In the present studies we have attempted to determine which viral proteins are naturally antigenic and induce an autogenous humoral response, and whether various strains of mice recognize common or different viral antigens.

For these experiments, [^{3}H]leucine-labeled leukemia virus was isolated from a line of AKR mouse embryo fibroblasts which had spontaneously initiated viral expression. The virus was purified by isopycnic banding and sedimentation on sucrose gradients. To determine the viral proteins recognized by autogenous immune sera, the virus was disrupted with Triton and high salt and reacted with an appropriate serum. Immune complexes were subsequently isolated by adding a specific antiglobulin. After the immune precipitates had been extensively washed, the viral proteins remaining in the immune precipitate were identified by SDS-polyacrylamide gel electrophoresis. Under these conditions, normal $B6C3F_1$ [(C57BL/6) × (C3H/Anf)F_1] serum can be shown to react primarily with three viral proteins having molecular weights of approximately 68,000, 43,000, and 17,000. In addition, minor reactions with viral proteins having molecular weights of 19,000 and 15,000 are occasionally demonstrable. The specificity of these techniques is illustrated by the ability of specific rabbit antisera to the viral group-specific antigen and to p15 to yield gel profiles with only the appropriate antigens.

Although the above data demonstrate which viral antigens are recognized, they do not indicate which antigens are viral envelope antigens and which are primarily responsible for the titers observed with the radioimmune-precipitation assay. To examine these questions, we precipitated intact virus as in a radioimmune-precipitation assay and subsequently solubilized the virus from the immune complexes with Triton and high salt. Under these conditions immune complexes formed initially are not dissociated, but formation of new complexes is inhibited. The same viral proteins as above were observed following gel electrophoresis, suggesting that the antigens we have observed are formed during the reaction with intact virus and therefore are viral envelope constituents.

We have attempted to titer each of the individual antigen-antibody reactions by quantitating the amount of antigen precipitated at various serum dilutions after SDS-polyacrylamide gel electrophoresis. The results suggest that, with $B6C3F_1$ serum, the highest antibody titer is against the 68,000-molecular-weight antigen, and this titer appears to correspond to the radioimmune-precipitation titer observed with intact virus. The titer against the 17,000-molecular-weight antigen is somewhat lower, and the results demonstrate a very low titer against the 43,000-molecular-weight antigen.

The relationship of the antigens we have observed to viral proteins observed by other laboratories has been examined using [^{3}H] glucosamine-labeled virus. The major viral envelope antigens (M1 and M2) are known to be the primary glycoproteins of the virus. Using [^{3}H] glucosamine-labeled virus, only the 68,000- and 43,000-molecular-weight antigens are detected in immune precipitates, so they probably correspond to the viral envelope glycoproteins M2 and M1, respectively. The relationship of the 17,000-molecular-weight antigen to known viral antigens is reported elsewhere in this report.

We have examined a number of additional mouse strains to determine whether they consistently recognize common antigens. To date we have examined BALB/c, C57BL/6, C3H/Anf, AKR, and NIH; all these strains appear to recognize the same antigens as those given above for $B6C3F_1$ mice. In addition, we have examined C57BL/6 mice immunized with an AKR leukemia cell line to determine whether active immunization might alter the immune response. Although these sera had higher titers of antibody against the virus, the pattern of antigens recognized was not altered.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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POLYPEPTIDES OF MAMMALIAN ONCORNAVIRUSES: LOCALIZATION AND REACTIVITY OF p15 WITH NATURAL ANTIBODY*

J. N. Ihle, M. G. Hanna, Jr., W. Schäfer†
G. Hunsmann,† D. P. Bolognesi,‡ and G. Hüper‡

The expression of endogenous murine leukemia virus (MuLV) during development and throughout life of normal mice has been demonstrated serologically and ultrastructurally. The result of this chronic viral expression is an autogenous humoral immune response to the virus.[1] Furthermore, this response has been shown to be directed primarily against three viral proteins,[2] two of which correspond to the two viral envelope glycoproteins M2 and M1, with apparent molecular weights of 68,000 and 43,000, respectively, as indicated by SDS-polyacrylamide gel electrophoresis. In these studies we have examined the third naturally antigenic viral progein – which has an apparent molecular weight of 17,000 – with respect to its localization on the viral particle and its relation to the p15 antigen of Friend leukemia virus.

The isolation and physical properties of the p15 viral protein of Friend MuLV has been described.[3] Serological analysis has demonstrated that this component possesses a strong interspecies reactivity, similar to that observed with the viral glycoprotein M2 and the group-specific antigen. However, these studies have not indicated the localization of p15 on the viral particle. Using specific anti-p15 serum, we have examined the ability of this serum to interact with intact viruses in a radioimmune-precipitation assay. When this serum was titered against intact [^{3}H] leucine-labeled AKR MuLV, Moloney MuLV, or Rauscher MuLV, typical radioimmune-precipitation titration curves were obtained. Each virus gave essentially identical curves, supporting previous conclusions that p15 from various viruses can cross-react. The ability of this antiserum to precipitate intact virus suggests that p15 is an envelope antigen. The serum was subsequently examined as previously described[2] to determine what viral antigens react, and a single major peak was obtained with an apparent molecular weight of approximately 17,000.

We next attempted to correlate this antigen to the antigen recognized by normal $B6C3F_1$ mouse serum. Since the 7S antibody of normal serum reacts only with the 17,000-molecular-weight antigen,[4] we examined the ability of p15 antiserum to block precipita-

tion of intact virus by 7S antibody in a radioimmune-precipitation assay. As would be expected if the antigens were identical, the results demonstrated significant competition. Similarly, when purified, ^{125}I-labeled p15 was coelectrophoresed with immune precipitates of normal B6C3F$_1$ serum reacted with ^{3}H-labeled AKR virus, the ^{125}I-labeled p15 coincided with the 17,000-molecular-weight peak. Disrupted virus was then reacted with p15 antiserum, the immune complexes were isolated, and the remaining soluble antigens were reacted with normal B6C3F$_1$ serum. The immune precipitate of the mouse serum lacked the 17,000-molecular-weight protein but still contained the M1 and M2 glycoproteins. These data, taken together, show that the 17,000-molecular-weight antigen recognized by normal mouse sera is identical to the p15 viral protein isolated and characterized by Schäfer[3] from Friend MuLV.

Finally, the efficacy of an autogenous humoral immune response to the p15 viral protein was examined by testing the ability of specific p15 antiserum to neutralize MuLV in tissue-culture experiments. Unlike antiserum to M2 (which generally has high neutralizing titers), p15 antiserum was only effective at low dilutions (1:40) and was at least 20-fold less effective than M2 antisera in neutralization of Friend MuLV. These results suggest that the biological significance of the humoral response to p15 in autogenous immune sera is not neutralization of infectious virus.

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DIRECT EVIDENCE FOR UNIQUE VIRAL RNA SUBUNITS IN MURINE LEUKEMIA VIRUSES*

J. G. Farrelly, J. N. Ihle, and F. T. Kenney

The genome of RNA tumor viruses is composed of a 70S RNA complex which, when denatured by heat or DMSO, can be shown to consist of three to four 34S RNA molecules. In addition, several groups have reported that the viral genome contains stretches of polyadenylate [poly(A)]. We examined the possibility of fractionating the 34S RNA subunits on the basis of the presence or absence and length of poly(A) sequences, using polyuridylate-Sepharose columns. Using techniques described elsewhere,[1] we have demonstrated that the murine-leukemia-virus 34S RNA can be fractionated on poly(U)-Sepharose columns into a poly(A)-containing fraction and a non-poly(A)-containing fraction, which generally constitute 65–70% and 30–35% of the total 34S RNA, respectively. These data suggest that the viral RNA subunits are unique and are compatible with a model of the viral genome composed of one subunit not containing poly(A) sequences and two subunits containing poly(A) sequences.

To determine whether the poly(A)-containing and non-poly(A)-containing fractions contain unique sequences, we have examined their sequence homology by hybridization. For these experiments, [^{3}H]DNA was made in an endogenous reverse transcriptase reaction using AKR leukemia virus under conditions which have been shown to result in complete transcription of the viral RNA. To eliminate the possibility of DNA with internally complementary sequences, actinomycin D was used in the reaction, the purified DNA from the reaction was self-reannealed, and any DNA·DNA hybrids were removed by hydroxylapatite chromatography. Under the conditions of the reaction, only ~5% of the DNA self-annealed. This DNA was subsequently hybridized to excess 34S RNA fractions purified as above on poly(U)-Sepharose columns. In the presence of excess total RNA, more than 90% of the DNA is capable of hybridizing to the viral RNA. In contrast, when DNA is hybridized to poly(A)-containing or non-poly(A)-containing RNA, only approximately 50% of the DNA is capable of forming hybrids with each fraction of RNA. Since the non-poly(A)-containing RNA represents only approximately 35% of the total RNA, these results suggest that in the endogenous reaction relatively more transcription of this RNA has occurred. Although these results suggest that each fraction of RNA contains unique sequences, we have examined the ability of the DNA isolated from each of the hybridized fractions to rehybridize with its original RNA fraction as well as its ability to hybridize with the other fraction. DNA isolated by hybridization to non-poly(A)-containing RNA will rehybridize completely to non-poly(A)-containing RNA but not to poly(A)-containing RNA. Similarly, DNA isolated from poly(A)-containing RNA hybrids will completely hybridize back to poly(A)-containing 34S RNA but fails to hybridize to non-poly(A)-containing RNA. Thus, the poly(A)-containing and non-poly(A)-containing 34S

RNA subunits from murine leukemia virus contain unique sequences.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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AFFINITY CHROMATOGRAPHY OF RNA-DEPENDENT DNA POLYMERASE*

J. G. Farrelly and F. T. Kenney

We have previously reported that polyribonucleotides are competitive inhibitors of RNA-dependent DNA polymerase (reverse transcriptase), and that poly(G)-Sepharose and poly(2′-*O*-methyluridine)-Sepharose [poly(Um)-Sepharose] are excellent supports for affinity chromatography of this enzyme. We have used these two materials to prepare large quantities of reverse transcriptase from spleens of mice infected with Rauscher leukemia virus. The spleens are minced and homogenized in a buffer containing high salt and a nonionic detergent. The insoluble material is removed, and the supernatant solution is placed on a poly(G)-Sepharose column. When the column is eluted with a salt gradient, the cellular DNA polymerases elute before the reverse transcriptase. It is difficult to calculate the recovery of activity, since the enzyme is partially inhibited in the crude supernatant. Nevertheless, a purification factor of 150- to 200-fold is estimated, with apparently complete recovery of enzymatic activity. The enzyme activity from poly(G)-Sepharose is subsequently placed on a poly(Um)-Sepharose column. This activity is again eluted with a salt gradient. There is a further substantial purification of reverse transcriptase, with an excellent recovery of activity. The results of purification of reverse transcriptase from 10 g of spleen from RLV-infected mice are shown in Table 35.

Table 35. Purification of DNA polymerase from RLV-infected spleens

Fraction	Total protein (mg)	Specific activity[a] (units/mg)	Purification	Yield (%)
Crude homogenate	667	0.56	1	100
Poly(G)-Sepharose	4.2	125	223	141
Poly(Um)-Sepharose	0.64	753	1345	129

[a]Specific activity is expressed as pmoles of TMP incorporated per 30 min per mg protein, with poly(rA)·dT_{10} used as template.

Although this procedure gives greater than a 1000-fold purification of enzyme, the preparation is not yet homogeneous. SDS-acrylamide electrophoresis shows that three protein bands are present in the final preparation. One of the bands probably represents the cellular enzyme described by Weissbach et al.[1] We have found this activity to be present in all tissues examined to date. At the present time we are attempting to purify the reverse transcriptase to homogeneity, using a variety of biochemical procedures.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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EVIDENCE FOR A STABLE INTERMEDIATE IN LEUKEMIA VIRUS ACTIVATION IN AKR MOUSE EMBRYO CELLS*

J. N. Ihle, F. T. Kenney, and R. W. Tennant

Temin[1] has previously shown that, during infection of chick embryo cells with avian sarcoma virus, cellular DNA synthesis and/or cell division is required to initiate viral expression. In these studies we have shown that a similar requirement exists when mouse fibroblasts are infected with murine leukemia virus. When cells are deprived of serum, cellular replication ceases within 24–36 hr. If these cells are subsequently infected with murine leukemia virus, no viral expression is evident by fluorescent-antibody techniques for up to five days. However, the readdition of serum at any time during this interval, which allows the cells to reinitiate cellular replication, results in the expression of viral antigens. This expression can be completely blocked by the addition of cytosine arabinoside with serum but cannot be blocked by the addition of poly(2′-*O*-methyladenylate), which specifically inhibits the viral reverse transcriptase. These results suggest that DNA synthesis and/or cell division, perhaps unrelated to viral DNA synthesis, is required to initiate viral expression.

A similar requirement can also be demonstrated during 5-iododeoxyuridine (IdUrd) activation of endogenous leukemia virus in AKR mouse embryo cells. Since activation by IdUrd requires DNA synthesis for incorporation of IdUrd, and since this incorporation is required for activation, we reasoned that activation might require two cell cycles for complete viral expression. Such a requirement can be demonstrated by adding IdUrd at various times after the removal of

serum from the cells, thus allowing the cells to undergo one or two cycles in the presence of IdUrd prior to the cessation of cellular replication. When IdUrd is added after the removal of serum, at a time such that the majority of the cells will only complete one cell cycle, no activation is observed. However, when serum is added back and the cells are allowed to divide, viral expression is detectable by fluorescent-antibody techniques. The first cell cycle requires IdUrd but the second does not. In contrast, both phases of activation are inhibited by cytosine arabinoside. The "activation intermediate" formed after the incorporation of IdUrd is stable and can be induced to express viral functions up to four days after IdUrd incorporation by the readdition of serum. These results taken together suggest that IdUrd activation initially involves the incorporation of IdUrd into DNA and results in the formation of a stable "activation intermediate" resembling the provirus formed during infection of stationary cells with exogenous leukemia virus. The second event, resulting in expression of the activation intermediate as synthesis of virus proteins, requires DNA replication but not IdUrd.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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EFFECT OF GLUCOCORTICOIDS ON MURINE LEUKEMIA VIRUS ACTIVATION AND REPLICATION*

J. N. Ihle, S. E. Lane,† F. T. Kenney, and J. G. Farrelly

Paran et al.[1] have described an enhancement by glucocorticoids of leukemia virus synthesis during activation of mouse cells by 5-iododeoxyuridine (IdUrd). Since activation can be dissociated to two cell cycles, the first requiring IdUrd incorporation and the second related to a cellular cell-cycle-dependent function to initiate virus expression,[2] we have examined the effects of glucocorticoids on these processes in relation to the presence of a cytoplasmic receptor protein for the hormones.

Dexamethasone or hydrocortisone at optimal concentrations enhances virus synthesis during IdUrd activation of AKR mouse embryo cells two- to threefold. Using techniques previously described,[2] it is possible to show that the hormones do not affect the activation process per se but only alter the extent of viral replication once activation of the viral genome has occurred. This enhancement is evident by fluorescent-antibody techniques, quantitation of virus synthesis by [^{3}H]leucine incorporation and specific immune precipitation, or by assay of reverse transcriptase activity released into the media. Curiously, however, although the fluorescent-antibody assays suggest that the intracellular concentration of the major viral antigens increases in the presence of hormones, assays for intracellular reverse transcriptase show that no increase of this enzyme occurs intracellularly. This may suggest that this protein is one of the limiting factors in virus-particle formation, and thus enhanced synthesis of the enzyme is immediately reflected in increased virus-particle release.

Enhanced virus synthesis by hydrocortisone or dexamethasone appears to be mediated by a specific cytoplasmic receptor for these hormones. The receptor protein is comparable to other such proteins. The apparent dissociation constant at 4°C is approximately 2.4×10^{-8} *M* for dexamethasone, and there are approximately 4000 receptor molecules per cell. Analysis by sucrose-gradient centrifugation shows that the receptor sediments with an S value of approximately 4. To correlate the presence of a receptor protein with enhanced virus synthesis, we have examined the ability of several steroids to compete for dexamethasone binding *in vitro* and for stimulation of virus synthesis in tissue culture. The results show that compounds which compete *in vitro* and do not themselves induce *in vivo* also compete in tissue culture. Similarly, compounds which do not compete *in vitro* do not compete or induce in tissue culture. These results suggest that the hormonal stimulation of virus synthesis proceeds via interaction with a receptor protein and involves modulation of the expression of active genetic components. Stimulation of virus synthesis may reflect a direct effect on transcription of viral mRNA, but we cannot exclude the possibility that glucocorticoids promote synthesis of an unidentified protein that facilitates transcription of the viral genome.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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PHOSPHORYLATION OF TYROSINE AMINOTRANSFERASE *IN VIVO**

Kai-Lin Lee and Joanne M. Nickol†

Phosphorylation of protein is known to be an important process in enzyme regulation, but this process has not hitherto been implicated in the sensitivity of hepatic tyrosine aminotransferase to a variety of hormonal regulators of gene expression, in the unusually rapid turnover rate of this enzyme, or in its posttranscriptional modifications, which result in the formation of three chromatographically distinguishable forms. It is possible that one or more of these unusual properties is related to phosphorylation or dephosphorylation of the enzyme protein. We have initiated some experiments aimed at testing this possibility. Incorporation of $^{32}P_i$ into tyrosine aminotransferase was observed when the isotope was either administered into rats or added to the medium of cultured Reuber H-35 hepatoma cells. *O*-[$^{32}P_i$] phosphoserine was identified in aninotransferase subunits isolated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of immunoprecipitates. Treatment of partially purified ^{32}P-labeled aminotransferase with alkaline phosphatase had no effect on aminotransferase activity assayed in the presence of excess pyridoxal phosphate but did greatly reduce the amount of phosphate present in the aminotransferase. On the basis of these results, we conclude that tyrosine aminotransferase is a phosphoprotein. The significance of this finding in regulation of this enzyme is currently being studied.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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PYRIDOXAL PHOSPHATE CONTENT AND SUBUNIT STRUCTURE OF TYROSINE AMINOTRANSFERASE*

Kai-Lin Lee, J. G. Farrelly, Joanne M. Nickol,† and F. T. Kenney

It has been reported that hepatic tyrosine aminotransferase contains 4 moles of pyridoxal phosphate per mole of holoenzyme.[1] Consequently, it has been suggested that each mole of this enzyme contains four catalytic subunits with molecular weight about 25,000–30,000. However, recent reports from the same group of investigators suggest that the molecular weight of the enzyme subunit is 52,000.[2] These conflicting results and the importance of such information with regard to evaluation of the isolated presumptive tyrosine aminotransferase mRNA[3] pointed out the need for reexamination of the subunit structure of this enzyme, which is widely used in studies of mammalian regulation.

By fluorescence spectroscopy we found that only 2 moles of pyridoxal phosphate per mole of enzyme are involved in catalysis of the transamination reaction. Direct measurements of coenzyme content in purified holoaminotransferase which had not been subjected to heat treatment yielded the value of 2 moles per mole of enzyme. However, 4 moles of coenzyme were found per mole of holoenzyme when a heat-treatment step, wherein excess coenzyme is added for protection, was included in the procedure of purification. When the homogeneous aminotransferase (purified either way) was first incubated with SDS and β-mercaptoethanol and then subjected to SDS gel electrophoresis, one protein band with molecular weight about 50,000 was observed. Identical results were observed with performic acid oxidation products of the same enzyme preparation. Analysis of the number of CNBr-cleaved fragments of homogenous aminotransferase was carried out by gel filtration and subsequent analysis of the isolated fractions by urea gel electrophoresis. At least ten fragments could be identified. These results demonstrate that each molecule of native tyrosine aminotransferase contains two catalytic subunits, with one pyridoxal phosphate per subunit. The fact that only one species was observed when the homogeneous enzyme was subjected to either SDS gel electrophoresis or urea gel electrophoresis suggests that the two subunits are identical. Furthermore, a reducing agent was not required to produce subunits from the native enzyme, indicating that the two subunits are not held together by disulfide linkages in the native state.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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DEGRADATION OF TYROSINE AMINOTRANSFERASE DURING THE EARLY PHASES OF HYDROCORTISONE INDUCTION*

Nicholas Pomato, Kai-Lin Lee, and F. T. Kenney

Levitan and Webb[1] have shown that degradation of tyrosine aminotransferase does not occur during the

first 4 hr of hydrocortisone induction in rat liver. Extending this study to the H35 hepatoma cell line, we have shown by immunochemical techniques that tyrosine aminotransferase degradation is prevented from 4 to at least 6 hr after hydrocortisone induction is initiated.

It has been demonstrated in this laboratory that the addition of cycloheximide to cultured hepatoma cells causes an immediate cessation of protein synthesis but has little effect on tyrosine aminotransferase degradation for approximately 2 hr. When cycloheximide was added to the cultured cells, a cessation of degradation was still observed from 4 to 6 hr after the initiation of hydrocortisone induction. This eliminated the possibility that the effect on degradation which is observed is due to the reutilization of radioactive amino acids obtained from continuous protein degradation.

In addition to this, we have shown that tyrosine aminotransferase activity decreased during the time interval in which the cycloheximide was present. This indicates that a rapid increase in tyrosine aminotransferase synthesis is not necessary for the prevention of degradation under these conditions. Further studies are now in progress with regard to the mechanism by which degradation is prevented during the early phase of induction of tyrosine aminotransferase by hydrocortisone.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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EFFECTS OF POLY(2′-*O*-METHYLADENYLIC ACID) ON SUSCEPTIBILITY AND AUTOGENOUS IMMUNITY TO RNA TUMOR VIRUS ONCOGENESIS *IN VITRO**

R. W. Tennant, M. G. Hanna, Jr., and J. G. Farrelly

Poly(2′-*O*-methyladenylic acid) [poly(Am)] has been shown to inhibit murine leukemia virus reverse transcriptase competitively *in vitro* and to inhibit virus infection and cell transformation in culture. When added to cells after infection, poly(Am) inhibited only within 4 hr, indicating an effect on some early, specific virus function such as the viral reverse transcriptase. To test the effects of poly(Am) on RNA-tumor-virus oncogenesis *in vivo*, experiments were performed using the Moloney pseudotype of mouse sarcoma virus, which induces solid tumors at the site of inoculation and also includes an excess of helper Moloney leukemia virus. Poly(Am) inhibited tumor development and death induced by the Moloney sarcoma virus–leukemia virus complex in newborn mice. The compound was effective at 10 μg per mouse when given at least 1 hr before inoculation of virus, but the greatest inhibition was seen in mice treated at least 4 hr before infection. Poly(2′-*O*-methyluridylic acid) and poly(vinyl-adenine) also inhibited sarcoma development and death but were less effective than poly(Am). Poly(Am) also enhanced the antibody response of newborn mice to endogenous leukemia virus envelope antigens, which we refer to as autogenous immunity. The results of these preliminary studies suggest that poly(Am) alters the oncogenic potential of the Moloney sarcoma–leukemia virus complex *in vivo*, and the effect appears to be mediated through an enhancement of the immune response of the treated animals.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

EFFECTS OF NITROSOCARBARYL ON BALB/c 3T3 MOUSE EMBRYO CELLS*

J. M. Quarles, Jr.,† and R. W. Tennant

Nitroso compounds or their precursors are found throughout the environment and are highly active carcinogens in test animals. Testing potential carcinogens in tissue culture provides a more practical means of assaying for activity than *in vivo* methods and makes possible screening for both transforming ability of chemicals and activation of endogenous viral information. We chose the BALB/c 3T3 mouse cell line as a basic test system because of its successful use with polycyclic hydrocarbons[1] and the fact that these cells contain endogenous viruses which are known to be activated by chemical agents such as iodo- or bromodeoxyuridine. Therefore this assay system provides a means of studying the role of viral activation in cellular transformation and determination of whether expression of viral information may be a necessary or sufficient condition for ultimate transformation to malignancy. Cells are exposed in tissue culture to chemicals and monitored immediately and after passage for changes in morphology or growth conditions and for expression of viral proteins and enzymatic activity.

The nitroso derivative of carbaryl, which is similar in structure to other known carcinogens, was obtained from William Lijinsky and tested in this system. We found that nitrosocarbaryl transforms cells in tissue culture, at doses of low toxicity, to tumorigenic cells with altered growth characteristics. As compared to the untransformed parent culture, transformed cells grow more rapidly and to a higher cell density, show a loss in

contact inhibition, grow in soft agar, and form tumors when injected into newborn BALB/c mice. Transformed clones tested to date have not shown expression of endogenous viral antigens (by fluorescent-antibody tests) or reverse transcriptase activity. Now that the feasibility of the cell system has been verified and the activity of nitrosocarbaryl has been demonstrated, studies are in progress to increase the efficiency of transformation for an investigation of the mechanism of action of the chemical. Other nitroso compounds are also currently under screening for activity in tissue culture.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

†NCI Postdoctoral Fellow.

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RECIPROCAL INHIBITION OF MOUSE LEUKEMIA VIRUS INFECTION BY *Fv-1* CELL EXTRACTS*

R. W. Tennant, Bonnie Schluter,†
Wen-Kuang Yang, and Arthur Brown†

Soluble extracts of mouse cells with $Fv\text{-}1^{n/n}$ or $Fv\text{-}1^{b/b}$ gene alleles specifically and reciprocally inhibited infection of B- or N-tropic mouse leukemia viruses in permissive cell cultures. NB-tropic virus infection was not inhibited by either cell extract. Extracts from $Fv\text{-}1^{-/-}$ cells did not inhibit infection by the three virus host-range types, but N- or B-tropic virus infection of $Fv\text{-}1^{-/-}$ cells was inhibited by extracts of the nonpermissive cells, and $Fv\text{-}1^{n/b}$ cell extracts inhibited both viruses. The maximum degree of inhibition was 50–80%, as determined by immunofluorescent or plaque assays, with extracts containing up to 50 μg/ml of nonpermissive cell protein. The inhibitor(s) is relatively unstable, since activity is lost after 2 hr at 37°C or 30 min at 56°C. The inhibitor(s) was most effective if added 1 hr before or within 2 hr after infection and does not react with the virus directly, inhibit virus attachment, or inhibit the normal cell functions tested. These results indicate that nonpermissive mouse cells contain a product of the *Fv-1* gene that inhibits some event(s) in leukemia virus infection early after penetration.

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REVERSED-PHASE CHROMATOGRAPHIC STUDIES ON THE RELATIONSHIP OF CELLULAR 4S RNA TO THE 4S RNA OF RNA TUMOR VIRUSES*

Wen-Kuang Yang, Ti Ho, Chongkun Koh,
John Daugherty,† Beth C. Mullin,
L. G. Hardin, and L. C. Waters

As a continuing study of the 4S RNAs found in the RNA tumor viruses,[1] the present project aims at collecting information relating to the question of whether the 4S RNAs found in the RNA tumor viruses are selective 4S RNA species of the infected host cells or distinctly different from the cellular 4S RNA. Our results support the conclusion that most of the 4S RNAs found in the RNA tumor viruses, including those associated with the virus-genome 70S RNA, are of host-cell origin. The specific experimental results are summarized as follows.

(1) Two sublines of AKR mouse embryo cells were used. One is a high-passage subline which produces a large quantity of MuLV, and the other is a low-passage (30–40 passages) subline which does not produce MuLV. 4S RNA was isolated from both AKR sublines after exposure to labeled uridine for 36 hr. Cochromatography of the two cellular 4S RNA preparations showed completely identical profiles. Since the reversed-phase chromatographic system is generally very sensitive in detecting qualitative and quantitative changes, this finding of no difference between producer and nonproducer AKR cells indicates that active production of RNA tumor virus does not induce detectable alterations in 4S RNA synthesis. Similar studies were done with producer RD-114 cells and nonproducer RD cells, with the same result – that is, no difference was observed.

(2) We have found that BALB SV3T3 cells are highly responsive in "C-particle" activation upon exposure to IdUrd. We labeled and isolated the 4S RNA from SV3T3 cells before and two days after addition of IdUrd to the culture medium. Although the IdUrd-treated SV3T3 cells showed active production of C particles, they did not synthesize a different quality or quantity of 4S RNA, as detected by cochromatographic analysis.

(3) One possibility is that, since the virus particles are actively secreted by the cells, the 4S RNA species found in the virus may undergo rapid turnover and also constitute only a very small pool mass within the cells. We therefore labeled the producer AKR cells and also the producer RD-114 cells for a short period (1 hr and 3 hr) to obtain radioactive 4S RNA preparations for comparison with those isolated from long-term (24–48

hr) labeling of cells. The 4S RNA from the same cells labeled for short and long periods gave markedly different chromatographic profiles. However, the 4S RNA preparations from the producer cells labeled for the short period showed no particular enrichment of the chromatographic peaks observed in the virus 4S RNA preparations.

(4) Comparison of the "free" 4S RNA and the 60–70S-associated 4S RNA preparations from the same virus usually reveals considerable similarity, except that the "free" 4S RNA preparation shows a distinct prominent peak that either is absent or is a minor component in the associated 4S RNA. This peak was found to coincide with a markedly prominent peak observed in the 4S RNA preparation from the membrane fraction of the cells. Thus, we consider that this prominent peak of the "free" 4S RNA preparation is due either to contamination of the virus preparation with cell membranes or to its inclusion in the virion by the special compartmentalization of this 4S RNA species within the cells.

All these results, together with the hybridization data in the following article, indicate that the selective 4S RNA species of host cells are associated with the genomic RNA (35S or 70S RNA) or RNA tumor viruses by complementary base sequence. This implies complementarity between the host-cell DNA (coding for these 4S RNAs) and the DNA copies of 35S RNA (provirus). This may offer a positive recognition mechanism for viral genomic DNA to be inserted into specific sites of the host-cell genome, which is a critical step for virus-induced cell transformation. Studies are in progress to investigate the possibility of this mechanism of virus DNA integration into host-cell DNA during oncogenesis.

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DEMONSTRATION BY REVERSED-PHASE CHROMATOGRAPHY OF UNIQUE 4S RNA SPECIES ASSOCIATED WITH THE 70S RNA OF TUMOR VIRUSES*

L. C. Waters, Beth C. Mullin, Ti Ho,
L. G. Hardin, and Wen-Kuang Yang

The 4S RNAs most intimately associated with the AKR murine leukemia virus 70S RNA consist mainly of a few chromatographically distinct peaks. The chromatographic pattern of 4S RNA obtained by heat dissociation of 70S RNA is characterized by multiple peaks, similar to that of "free" 4S RNA from the same virus, but is less complex than that of the 4S RNA isolated from infected cells. Stepwise thermal dissociation of the 4S RNA from the 70S RNA shows 80–85% dissociation at 55–60°C, with a chromatographic profile like that of total 70S-associated 4S RNA. The 15–20% released by further heating to 80°C consists of only three or four chromatographically distinct species.

These species, because of their strong association with viral 70S RNA, are likely of significant structural or functional importance in the replication and propagation of the virus within a cell. However, the amount of material one can obtain by labeling murine viruses is very small, often only enough for one reversed-phase chromatographic analysis. In an effort to get a sufficient quantity of material showing a high degree of association with the viral 70S RNA, we have demonstrated, by RPC-5 chromatography and preliminary oligonucleotide fingerprinting (J. L. Nichols, personal communication), that unique species of 4S RNA (likely tRNA) from cells grown in tissue culture will hybridize with viral 70S RNA. Specific 4S RNAs from AKR (virus-producing and nonproducing) mouse fibroblasts, BALB/c embryo cells, and RD human cells hybridize with both AKR and avian myeloblastosis virus (AMV) 35S RNA. The conditions of hybridization suggest substantial base complementarity of these 4S RNAs with viral 35S RNA. We are currently doing experiments to determine whether these molecules can serve as primers for viral DNA synthesis and/or have some other biological role in viral replication. If they are biologically important, this system will allow us to prepare the material in sufficient quantity for extensive study.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

IN VITRO DNA SYNTHESIS BY LOW-MOLECULAR-WEIGHT DNA POLYMERASE: INCREASED INFIDELITY ASSOCIATED WITH AGING*

R. W. Barton,† L. C. Waters, and Wen-Kuang Yang

The low-molecular-weight DNA polymerases from young-adult (6 months old) and aged (24–30 months old) BALB/c mouse livers were partially purified by salt extraction of liver homogenates followed by phosphocellulose chromatography. Using $(dA)_n \cdot (dT)_6$ as

the template, we determined the replicational fidelity of the polymerases by measuring the amount of incorporation of incorrect nucleotide, dGMP or DCMP, in relation to the amount of correct nucleotide, TMP, incorporated. Such a determination revealed that the polymerase from the senescent mouse livers incorporated 1.7 to 2.1 times as much dGMP as the enzyme from the young-adult mice. The incorporation of dCMP by both of the polymerases was greater than that of dGMP. However, the enzyme from the aged mouse livers incorporated 1.5 to 1.9 times that incorporated by the polymerase from the young-adult mouse livers. The incorporation of the incorrect nucleotide was proportional to reaction time and enzyme concentration. Similar qualitative results have been observed using the template $(rA)_n \cdot (dT)_n$.

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

AGE-RELATED DECLINE IN PHA-INDUCED LYMPHOCYTE TRANSFORMATION: I. COMPARABLE SENSITIVITY OF SPLEEN CELLS FROM YOUNG AND OLD MICE TO CULTURAL CONDITIONS*

Ching-Yuan Hung,† E. H. Perkins, and Wen-Kuang Yang

Recent investigations have demonstrated that, with advancing age, mouse spleen cells in culture exhibit a much depressed DNA-synthesis response (thymidine incorporation) to phytohemagglutinin (PHA) stimulation. To explore possible fundamental cellular mechanisms that could be responsible for this marked depression, experiments were designed to answer the following specific questions: (1) Do spleen cells of old mice survive as well as those of young mice *in vitro*? (2) Do spleen cells of young and old mice show different PHA-dose responses? (3) Does lower thymidine incorporation by the old spleen cells represent fewer DNA-synthesizing cells or less incorporation by the same number of DNA-synthesizing cells? (4) Do the old spleen cells show a delayed time course of response?

Experiments demonstrated that decreased DNA synthesis by spleen cells from old mice following PHA stimulation is not related to the poorer survival of these spleen cells *in vitro*. Total nucleated cell numbers and percentage of viable cells determined after 5, 24, 48, and 72 hr *in vitro* were comparable for spleen cells from both young and old animals. DNA synthesis by spleen cells from both young and old animals was maximal at the same PHA concentration. Autoradiographic analyses performed for both young and old mouse spleen cells at various times of incubation also revealed that there are fewer cells responding to PHA in the spleen-cell population of the old animals than in that of the young animals. Furthermore, the relative number of large blast cells was significantly higher in the young than in the old spleen-cell cultures after PHA stimulation. "Conditioned" media derived from 44-hr cultures of spleen cells from young and old mice in the absence of PHA were also tested with PHA on spleen cells from both young and old mice for stimulatory or inhibitory effects. No enhancing or deleterious effects were found, indicating that the observed differences in DNA synthesis by spleen cells from young and old animals could not be attributed to soluble promoters or inhibitors secreted by the respective spleen-cell populations. Studies of thymidine-incorporation rate at various times following PHA stimulation revealed that both young and old spleen cells gave a maximal response at 72 hr.

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†UT–Oak Ridge Graduate School of Biomedical Sciences Aging Training Program Postdoctoral Fellow.

AGE-RELATED DECLINE IN PHA-INDUCED LYMPHOCYTE TRANSFORMATION: II. [^{125}I]PHA-BINDING SITES ON SPLEEN CELLS FROM YOUNG AND OLD MICE*

Ching-Yuan Hung,† E. H. Perkins, and Wen-Kuang Yang

In the previous experiments we demonstrated that the declining PHA responsiveness of spleen cells from old animals could not be attributed to a difference in the sensitivities of young and old spleen cells to *in vitro* conditions. Preincubation for 24 hr or 44 hr without PHA to eliminate the dividing-cell population made no difference in subsequent PHA response. The question was asked, therefore, whether age-related differences in blastogenic activity might be due to cell-membrane alteration with advancing age, and specifically, whether there is a decrease in PHA-binding sites on the cell membrane of spleen cells from old animals.

PHA was iodinated with ^{125}I by the iodine monochloride method. At 4°C, spleen cells of young (4–6 months) and old (30–32 months) $BC3F_1$ mice showed the same extent of binding of various concentrations of [^{125}I]PHA, indicating that there were similar numbers of membrane PHA-binding sites per spleen cell in the

young and old mice, and also that the binding affinity for PHA was not altered in the old mouse spleen cells. Most of the [^{125}I]PHA initially bound to the spleen cells at 4°C was released from the membrane into the medium when the cells were subsequently incubated in serum-containing culture medium at 37°C. Binding reactions performed at 37°C gave a considerable amount of irreversibly bound PHA, which presumably represented the portion of PHA passed through the membrane into the cytoplasm of the cells. However, these experiments did not disclose any difference between young and old mouse spleens. It was concluded that the declining response to PHA stimulation in the aged $BC3F_1$ mice was not due to a decrease in the number of PHA-receptor sites on the spleen-cell membrane.

*Research sponsored in part by the Virus Cancer Program of the National Cancer Institute.

†UT–Oak Graduate School of Biomedical Sciences Aging Training Program Postdoctoral Fellow.

ISOLATION OF LECTINS FROM SOYBEAN*

Wen-Kuang Yang, J. Meuth,† C. H. Wei,
Ti Ho, and D. E. Foard

The presence of lectins in the soybean (*Glycine max*) has been well known. These lectins are characterized by their *N*-acetylgalactosamine specificity of cell-membrane binding and by their preferential affinity to neoplastic cells. Purified soybean lectins would thus be useful in cancer research and membrane studies. Also, they may be of considerable importance in the physiology of nitrogen fixation, in view of the possible binding specificity of the lectins to the nitrogen-fixing microorganisms. This collaborative project was initiated with the primary purpose of isolation and preliminary characterization of various lectins from the strains of soybean species that are used for genetic studies by one of us (D.E.F.).

Isolation of lectins was performed by initial acid extraction of defatted soybean meal and ammonium sulfate differential precipitation, followed by stepwise chromatography on DEAE–Sephadex A-50 or DEAE-cellulose columns. Different chromatographic fractions were studied for hemagglutination activity against various mammalian red blood cells, toxicity to mice, ability to inhibit the growth of normal and transformed cells, and suppression of transplantable tumors. The purity of the fractions was assessed by polyacrylamide disc gel electrophoresis. A few isolated lectin fractions appeared to be identical to the four known soybean isolectins and showed selectively high hemagglutination activity to rabbit erythrocytes and no detectable toxicity in mice at an intraperitoneal dose of 50 μg per mouse. Our fraction C, containing some hemagglutination activity, was found to inhibit growth of plasma-cell tumors in BALB/c mice without apparent toxicity to the mice. The apparent selective toxicity of fraction C to tumor cells was also demonstrated by tissue-culture studies. While fraction C and the known soybean lectin fraction showed similar toxicity to primary cultures of BALB/c mouse embryo cells, fraction C was far more effective in killing BALB/c embryo cells transformed by Kirsten murine sarcoma virus than were the other lectins. Thus we have been concentrating on fraction C and the known soybean lectins. By chromatography of lectin fractions with a narrow, linear salt gradient on DEAE-cellulose, we obtained from fraction C a fraction IV, having higher hemagglutination activity, and a homogeneous protein, fraction VIII. We are now in the process of preparing specific rabbit antisera against fraction IV and fraction VIII.

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