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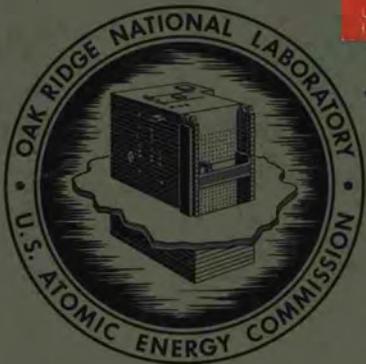
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Biology Division Annual Progress Report for period ending June 30, 1971,
page 57, left column, 3rd line from bottom

Now reads: "above, it provides an estimate for D of 14 r. This value is..."

Should read: "above, it provides an estimate for D of 144 r. This value is ..."

Please change 14 r to 144 r.

Craig Whitmire, Jr.
Editor, Biology Division

*her
errata
11/19/71*

Contract No. W-7405-eng-26

BIOLOGY DIVISION
ANNUAL PROGRESS REPORT
For Period Ending June 30, 1971

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

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The primary purpose of this document is to provide to the Oak Ridge National Laboratory short summaries of research projects in the Biology Division. In addition, it should be useful to the agencies that sponsor the Division's research and to individuals who want to have a general view of the Division's research activities.

This year we have decided to omit from the Progress Report detailed presentation of the many lecture programs, conferences, and educational activities in which the Division is deeply involved. This is done with some hesitation, since these activities are an integral part of the Division and shape the research programs in very important ways. However, in order to minimize duplication of effort on the part of our research and editorial staffs, a detailed presentation of such activities will be made only in the Biology Division Annual Bulletin, a publication that is widely distributed.

In the future we intend to experiment further with the contents of the Progress Report in order to produce a volume that will be as useful as possible. Suggestions from the users of this document will be appreciated.

Howard I. Adler
Director, Biology Division

BIOCHEMISTRY SECTION

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Chemical Protection and
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F. D. Hamilton

Developmental Biochemistry

F. J. Finamore

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CHEMICAL PROTECTION

D. G. Doherty, Kathy Miller, and Margaret A. Turner

The *in vitro* bone marrow test system results obtained with a wide variety of compounds substantiate the structural requirements for protective activity in the whole animal. They also give additional indications of selective binding at radiation-sensitive sites. The most active compounds all contain basic groups, opening the possibility of charge binding to the primary phosphate group of the DNA molecule. We are engaged in the synthesis of a series of diamino monothiols and diamino dithiols with various spacings between the amino groups. Such structures are complicated by the fact that many exist as both optical and geometrical isomers and that few of the necessary intermediates have been prepared to date. We have devised synthesis for most of the intermediates and have succeeded in preparing one compound, DL-2,5-diamino-4,6-phosphorothioate. This compound is protective at a single lethal (900r) level in mice, and further tests are in progress. Compounds of this structure should bring the protective thiol groups into close proximity to the site of radiation damage and thus give improved protection to the essential biological molecules.

IN VITRO CHEMICAL PROTECTION OF X-IRRADIATED MOUSE BONE MARROW CELLS

D. G. Doherty and L. H. Smith

We have applied the *in vitro* isolated bone marrow system that we have developed (1) to the examination of a wide variety of thiols, disulfides, masked thiols, and radical-trapping agents that exhibit various degrees of protection in the whole animal. The dose reduction factors (DRF) of mercaptoethylamine and mercaptoethylguanidine (AET) are proportional to the logarithm of the concentration, and, since higher concentrations can be achieved in the isolated marrow system, DRF's of 3.6 and 3.3 can be obtained. L-Cysteine is very effective in the isolated marrow, in contrast to the whole animal, yielding a DRF of 3.45 at a concentration of 15 mM. D-Cysteine at an equal concentration gave a DRF of only 2.0. D-Glutathione was inactive, while the DRF for the L-form was 2.5. A series of aminothiols masked as the thiosulfates or phosphorothioates, which are protective in the animal, are ineffective in the marrow system. However, the corresponding free thiols, especially 3-aminopropylaminoethane thiol, gave excellent protection with a DRF of 4.6 for this compound. Uncharged thiols such as mercaptoethanol and dithiothreitol, at higher concentrations than can be achieved *in vivo*, yield DRF's on the order of 2. Disulfides are ineffective, indicating that the marrow cells cannot reduce them to the corresponding thiols. Serotonin and p-aminopropiophenone, which oper-

ate by a physiological mechanism in the animal, were also ineffective in the marrow system. Several radical-trapping dyes, which penetrated the cells very well, gave no protection to the marrow cells. In order to demonstrate chemical protection to marrow stem cells, a sucrose gradient separation of marrow cells was prepared, and a fraction enriched fivefold in stem cells was obtained. This cell preparation gave the same D_{37} as the whole marrow and when incubated with AET prior to irradiation gave the same DRF as the whole marrow. We have also used this *in vitro* system to determine the degree of protection that might be obtained against fission neutrons from the Health Physics Research Reactor. The D_{37} for fission neutrons was approximately 37 rad for untreated marrow cells and 75 rad for AET-treated cells, for a DRF of 2. Thus, the DRF was comparable to that obtained with X-radiation. These results show that this system is most effective for compounds that undergo no metabolic transformation to an active form. In addition, the same structural requirements needed for good protection *in vivo* — e.g., basic group separated by not more than three carbons from a thiol, L-configuration — are also essential in this *in vitro* system.

1. D. G. Doherty and L. H. Smith, Biol. Div. Ann. Progr. Rept., Dec. 31, 1969, ORNL-4535, p. 52.

TOXICOLOGY INFORMATION QUERY RESPONSE CENTER

D. G. Doherty, Kathy Miller, and Deanna Oliveira

The National Library of Medicine, NIH, Toxicology Information Program, being interested in establishing a Toxicology Information Query Response Center at ORNL, entered into an interagency cooperative agreement between the AEC Division of Biology and Medicine and the NLM to pursue this objective. The goals of the center are to respond to any questions submitted to it with the appropriate specific toxicology data, literature searches, and bibliographies, as well as to develop specialized bibliographies, critical reviews, and current awareness products. In addition, the center will create machine-searchable data bases containing both toxicology information and bibliographic material. The initial emphasis will be in the area of pesticides and food additives. Since the major source of information is in FDA Pesticide and Food Additive Petitions, arrangements are being made to secure and abstract these petitions, develop the appropriate programs, and include the data in the ORLOOK program at ORNL. Accordingly, we have acquired a library of toxicology books and the necessary hardware (teletype and IBM 2741 Conversational Remote Batch Entry Terminals) to store and access on line various computer information bases both here and outside ORNL. On a trial basis we have abstracted six pesticide petitions and two food additive petitions to gain some insight into the nature of the work. At this time we have also answered some twenty questions submitted to us with either specific information or bibliographies ranging from ten to over one hundred references.

RATE OF α -CRYSTALLIN SYNTHESIS IN RELATION TO CELLULAR DIFFERENTIATION

Jean Delcour* and John Papaconstantinou

The vertebrate lens is a highly differentiated tissue composed of two morphologically distinct cell types, the epithelial cell and the fiber cell. α -Crystallin, one of the three major groups of tissue-specific structural proteins of the lens, is synthesized by both epithelial and fiber cells. We recently completed a series of studies on the rate of α -crystallin synthesis in cells at different stages of differentiation, i.e. in fiber cells and in epithelial cells, which are precursors to the fiber cells. Previous studies have shown that the differentiation of an epithelial cell to a fiber cell (fibrogenesis) is accompanied by a series of biochemical modifications of protein and nucleic acid synthesis. There is, for example, a stabilization of mRNA templates in the fiber cell as well as an apparent decrease in the rate of protein synthesis. In these studies we attempted to determine whether there is a change in the absolute rate of protein synthesis accompanying the transition to stable mRNA templates in the lens epithelial cell and fiber cell, respectively. Since both cell types synthesize α -crystallin, we were able to use this well-defined structural protein for our studies. In addition, we are able to report on the high degree of biochemical specialization these cells exhibit with respect to α -crystallin synthesis.

The rates of synthesis of total protein and of purified α -crystallin in both epithelial and cortex fiber cells from adult bovine lenses were calculated from the kinetics of incorporation of ^3H -leucine into organ-cultured lenses. A summary of these data is shown in Table I. It can be seen that (1) the rate of α -crystallin synthesis is about seven times higher in epithelial cells than in cortex fiber cells, while for non- α -crystallin protein the corresponding ratio is approximately 24; and (2) both epithelial and cortex fiber cells are highly specialized for the synthesis of α -crystallin, 60% of newly synthesized protein in epithelial cells being α -crystallin, whereas in cortex fiber cells the proportion is 75%.

TABLE I. Rates of protein synthesis

Cell type	Total protein	α -Crystallin	Non- α -protein	$\frac{\alpha\text{-Crystallin}}{\text{Non-}\alpha\text{-protein}}$
— (pmoles leucine/mg protein/hr) —				
Epithelial cells	264.8	396.0 (40.3)*	176.2	2.2
Cortex fiber cells	21.6	57.8 (28.2)*	7.4	7.8
Epithelium cortex	12.3	6.9	23.8	

*Percentage of α -crystallin within total protein.

The main purpose of this study was to compare the rates of protein synthesis in lens cells at different stages of cellular differentiation. In the vertebrate lens we find cells which exhibit active mitosis (fetal epithelial cells), extended

G₁ stationary phase of the cell cycle (adult epithelial cells), and terminal cellular differentiation (fiber cells). At the molecular level these cells carry out the synthesis of α -crystallin either on rapidly turning over mRNA templates (fetal epithelial cells) or on progressively more stable templates (adult epithelial cells and fiber cells). Thus, lens cells are particularly convenient for studies in the rate of protein synthesis at different levels of differentiation and with various degrees of template stability. The data show a significant difference between the rates of protein synthesis in adult epithelial cells and adult fiber cells, and it can be concluded that fibrogenesis is accompanied by a sharp decrease in the absolute rate of protein synthesis.

The degree of biochemical specialization for α -crystallin synthesis in the lens appears more striking when the ratio of leucine incorporated into α -crystallin to that incorporated into total protein is considered. This ratio is about 60% for epithelial cells and 75% for cortex fiber cells. Corresponding ratios for collagen synthesis in fibroblasts show that the most "specialized" fibroblast strains synthesize only 10–15% of their protein as collagen. We see, therefore, that lens cells have reached one of the highest levels of biochemical specialization, comparable to erythrocytes, in which 80–90% of the protein is synthesized as hemoglobin. This biochemical specialization is very high even in the less morphogenetically specialized epithelial cells, suggesting that even at this morphogenetic stage biochemical specialization for α -crystallin synthesis has been achieved.

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THE REGULATION OF SYNTHESIS AND ASSEMBLY OF α -CRYSTALLINS IN ADULT LENS EPITHELIAL CELLS

Jean Delcour* and John Papaconstantinou

Most differentiated cells are characterized biochemically by their ability to synthesize tissue-specific proteins. Many of these proteins are high-molecular-weight aggregates of identical polypeptides, or of qualitatively different polypeptides that have significantly lower molecular weights than the polymeric molecule. Because of the important role these polymeric proteins play in the structure and function of differentiated cells, questions arise with respect to the regulation of synthesis of their component subunits; with respect to the regulation of aggregation of these subunits, and whether subunit pools are present; and with respect to the precision involved in the formation of aggregates in which relative subunit concentrations remain constant, or in which one or more of the subunit concentrations vary during the life cycle.

α -Crystallin, a tissue-specific structural protein of the vertebrate lens, is a polymeric molecule that lends itself to studies on the regulation of subunit synthesis and aggregation. α -Crystallin has a molecular weight of approximately 1×10^6 and is an aggregate of polypeptide subunits whose molecular weights are approximately 25,000. In addition, the polymeric

TABLE I. Analysis of α -crystallin subunits with respect to ^3H -leucine incorporation

Subunit	I		II		III		IV	
	Rate of leucine incorporation into polymeric α -crystallin via each subunit		Relative rate of leucine incorporation		Relative subunit concentration within the polymeric α -crystallin		Specific rate of aggregation	
	(pmoles/mg of α -crystallin/hr)		(%)		(%)		(II/III)	
	Experiment		Experiment		Experiment		Experiment	
	1	2	1	2	1	2	1	2
Polymeric α	396.0	183.4	100	100	100	100	1.00	1.00
αB_2	118.8	58.0	30.0	31.6	26.9	27.9	1.12	1.13
αB_1	17.6	6.8	4.4	3.7	2.1	1.7	2.09	2.13
αA_2	259.6	118.6	65.6	64.7	59.0	64.4	1.11	1.01
αA_1	0	0	0	0	12.0	6.0	0	0

molecule has a sedimentation coefficient ($S_{20,w}$) of 20 and thus is easily separated from a mixture of proteins by sucrose density gradient centrifugation. We recently completed a series of studies on the kinetics of aggregation of the individual subunits forming the polymeric α -crystallin molecule in epithelial and fiber cells from adult bovine lenses. These data, together with information concerning the subunit composition of the polymeric molecule, will provide some insight into the mechanisms that govern the assembly of α -crystallin subunits.

α -Crystallin is synthesized by lens epithelial cells and fiber cells. It is a tissue-specific structural protein composed of four qualitatively different subunits: two acidic subunits (αA_1 and αA_2) and two basic subunits (αB_1 and αB_2). The relative proportions of these subunits within the aggregate depend on the state of cellular differentiation and aging.

The following major points emerge from our studies on the kinetics of aggregation of subunits to form the polymeric α -crystallin molecule in adult lens epithelial cells (Table I): (1) About 60% of the leucine incorporated into α -crystallin is accounted for by subunit αA_2 and about 30% by subunit αB_2 . Since the relative concentrations of αA_2 and αB_2 within the aggregate are 60% and 30%, respectively, the incorporation data indicate that in adult epithelial cells the composition of newly formed α -crystallin with respect to these subunits is identical to that of the previously aggregated molecule. (2) About 4% of the leucine incorporated is accounted for by subunit αB_1 , whereas this polypeptide chain constitutes only 2% of the polymeric molecule. This indicates that newly aggregated α -crystallin molecules in adult

epithelial cells are enriched in αB_1 subunit. (3) No significant uptake of radioactive leucine in the polymeric molecule by subunit αA_1 is detectable in either epithelial cells or fiber cells. This confirms our previous report that this subunit is not a product of direct genetic translation.

Subunits αB_2 and αA_2 are characterized by a specific rate of aggregation close to 1. This indicates that the relative concentration within the polymeric molecule remains constant throughout the life of the animal. Analysis of the subunit composition of α -crystallin from epithelial cells at various stages of development and aging has revealed that this is indeed the case. On the other hand, the specific rate of aggregation of subunit αB_1 is 2. This high value indicates that the subunit should accumulate in aging epithelial cells, which has also been found to be the case (unpublished observations).

The underlying mechanisms that induce such changes in a complex polymeric protein are not known. The changes may reflect a modification of the rates of synthesis of individual subunits. In this respect, the stabilization of mRNA templates (in adult stationary cells) may affect the synthesis of individual subunits. Our preliminary studies indicate a differential stability for αA_2 and αB_2 subunits and that the coordinated synthesis seen for αA_2 : αB_2 subunits proceeds by a mechanism that does not involve a polycistronic message.

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STUDIES ON THE STRUCTURE OF POLYTENE CHROMOSOMES

E. T. Chin* and John Papaconstantinou

Our long-range goal in this program is to study the assembly of the polytene chromosome through an approach that will yield information on the size of DNA synthesized and assembled as chromosomal units during polytenization. These studies will be carried out with the polytene chromosomes of the salivary gland of *Rhynchosciara*. Radioautographic analysis has shown that there are specific stages in which DNA synthesis is continuous throughout the chromosome and in which synthesis is discontinuous and found only in specific regions of the chromosome. In addition, we also find the type of disproportionate DNA synthesis seen in DNA puff formation. Spectrophotometric scanning analysis indicates that some regions or bands of the polytene chromosome contain more DNA than others. This is especially true in the puff regions where amplification occurs.

Our first experiments were designed to determine whether the many strands of DNA in the polytene chromosome can be easily dissociated and to determine the molecular weight of the DNA released. The experiments were done by gentle lysis of salivary glands at alkaline pH and subsequent analysis on alkaline sucrose gradients to determine the molecular weight of the DNA released. Salivary-gland DNA was labeled for 24 hr with ^3H -thymidine to ensure that most of the DNA labeled had a high molecular weight. The salivary glands from ten larvae were removed and lysed in alkaline buffer for 1, 4, 6, and 24 hr. The lysate was then centrifuged in an alkaline sucrose gradient to determine the size of single-stranded DNA released by lysis. This treatment resulted in a rapid release of single-stranded DNA with an $S_{20,w}$ ranging from 113S to 54S, with a peak at approximately 80S. After 3 hr of lysis, 80% of the total radioactive DNA was accounted for in this peak. This rapid release of DNA, in replicon sizes (double-stranded molecular weight = $100-200 \times 10^6$), is not detectable in vertebrate (diploid) chromosomes treated under similar lysis conditions. The DNA of a vertebrate diploid chromosome under the same experimental conditions is extracted as a very stable 208S species, which is large enough to contain many replicating units.

It appears that during the formation of polytene chromosomes the DNA is not "ligated" into larger structural units as is seen in the diploid chromosome. Thus, the DNA of the polytene chromosome reaches its maximum size as a replicon, whereas in the diploid chromosome the replicon units can be joined to form much larger structural units. If the DNA of a diploid chromosome is a unimer, then polytenization may involve unequal replication of segments of this chromosome, and these segments (replicons) remain attached to the chromosome via protein-DNA complexes. In addition, it is interesting that the DNA in "bands" characteristic of a polytene chromosome is reported to have a molecular weight in the range we report above. We are now carrying out similar analyses with diploid *Rhynchosciara* cells to substantiate our conclusion that "structural units" of polytene chromosomes may vary in size from those seen in diploid chromosomes. In

addition, we are presently developing procedures for the isolation of double-stranded DNA segments from polytene chromosomes for further studies on the arrangement of these molecules in the chromosome.

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GENE AMPLIFICATION IN DIFFERENTIATED SOMATIC CELLS

E. F. DuBrul, John Papaconstantinou, and A. A. Francis

It is the purpose of these studies to determine whether gene amplification can occur in somatic cells such as reticulocytes, lens cells, muscle cells, etc., which represent a class of highly differentiated cells committed to the synthesis of large quantities of tissue-specific proteins. The reticulocyte system with its well characterized and relatively abundant 9S globin mRNA was chosen to initiate these studies.

To determine the degree of redundancy of globin genes, specific mRNA was isolated from reticulocytes of anemic rabbits. The DNA-RNA hybridization kinetics of this mRNA were determined. Prior to hybridization the mRNA was labeled with dimethyl sulfate to a specific activity of 2400-3000 cpm/ μg RNA. Trace amounts of this RNA were added to a 2000-fold excess of reassociating rabbit marrow DNA. The DNA reassociation for rabbit bone marrow DNA shows the typical second-order kinetics that result in the rapid reassociation of repeating sequences and the slower reassociation of unique sequences. The "unique" sequences of bone marrow DNA reassociate over a C_{0t} range of 10^3-10^5 . In the presence of trace amounts of radioactive globin mRNA, reassociation of this mRNA with its homologous DNA occurs over a C_{0t} range of $3 \times 10^1-3 \times 10^3$. The difference in one-half C_{0t} values between the RNA hybridization and the "unique" DNA reassociation indicates that the globin genes consist of approximately 800 repeats of a nucleotide sequence necessary to code for 680 nucleotides (which is the size of the globin message). In addition, the RNA hybridization data (total RNA bound per genome) indicate that approximately 0.01% of the genome (i.e. 800 repeating units) is homologous to the globin mRNA.

These data were obtained from experiments in which DNA from marrow, a hematopoietic tissue, was used. To determine whether gene amplification occurs during hematopoiesis, similar kinetics with globin mRNA must be done with DNA from a nonreticulocyte-forming tissue. These studies are presently being carried out using thymus DNA. In addition, studies have been extended to include the isolation of mRNA from myosin-synthesizing polysomes and a large, unspecified polysome from chick lenses. Pure mRNA's to be used for hybridization studies have been isolated by the method of Blobel (1), which involves the dissociation and release of mRNA from polysomes by puromycin treatment.

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CHARACTERIZATION OF A SATELLITE DNA SYNTHESIZED DURING DEVELOPMENT OF RHYNCHOSCIARA TESTES

Mary Ann Handel, Emilia M. Julku, E. T. Chin,*
D. P. Allison, and John Papaconstantinou

Previous studies from this laboratory have shown that the synthesis of a satellite DNA ($\rho = 1.680 \text{ gm cm}^{-3}$) is associated with the development of spermatocytes in fourth-instar Rhynchosciara larvae. Analysis of satellite DNA on ethidium bromide-CsCl gradients revealed two components, one linear ($\Delta\rho = 0.125 \text{ gm cm}^{-3}$) and one that behaves like circular DNA ($\Delta\rho = 0.075 \text{ gm cm}^{-3}$). This circular DNA makes up 35-40% of the total satellite synthesized, and its presence suggests the mitochondria as the site of synthesis. A temporal analysis indicates that the synthesis of this DNA increases relative to the synthesis of main band DNA ($\rho = 1.695 \text{ gm cm}^{-3}$) during the developmental period prior to meiosis. During this period the mitochondria of the spermatocytes increase in size due to the development of a large proteinaceous inclusion. Since there is a close temporal correlation between mitochondrial development and synthesis of satellite DNA, experiments were performed to determine whether this DNA is associated with mitochondria. Because of their atypical morphology, the mitochondria cannot be isolated by standard procedures. By a new method, we have obtained a fraction which analysis by electron microscopy shows to be enriched for mitochondria with a small amount of membranous contamination. Ethidium bromide-CsCl gradient analysis of DNA prepared from this fraction reveals an enrichment of the circular DNA. The data indicate that this satellite DNA is probably associated with mitochondria. Electron microscope studies of this DNA and determination of its properties upon thermal and alkaline denaturation are in progress.

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STUDIES OF ALTERATIONS IN RNA POLYMERASE OF E. COLI FOLLOWING T4 PHAGE INFECTION

Audrey Stevens

Upon infection of E. coli with T4 phage, a new program of transcription takes place. Genetic studies in other laboratories suggest that new proteins are formed to control the transcription program. The new proteins may be parts of an altered DNA-dependent RNA polymerase.

Using the T4 phage infection system, alterations in purified RNA polymerase are being investigated. The protein of E. coli is labeled with ^3H -amino acids before infection and with ^{14}C -amino acids following infection with T4 phage. The RNA polymerase then is purified from the infected cells, and its subunits are examined by chromatography and electrophoresis. No change in the ^3H content of the subunits of RNA polymerase is found following infection.

^{14}C label is found in purified RNA polymerase following infection. About 95% of the label is found in three new protein bands, as detected by SDS gel electrophoresis of de-

natured holoenzymes. Their close association with the enzyme, even in the presence of low concentrations of urea, and their presence in amounts equivalent to the old subunits suggest that they are new subunits of the enzyme. They are small proteins with molecular weights of 9, 11, and 21×10^3 . The smallest one is lost on phosphocellulose column chromatography of the enzyme. When ^{14}C label incorporation into the two subunits was examined at intervals of 1-2, 5-6, and 10-11 min after T4 phage infection at 28°C , incorporation into all three was found to be highest in the 10- to 11-min interval. All are detectable at 5-6 min. The new protein bands are present in RNA polymerase purified following infection with T4 am 82, a typical early amber mutant. They are found in the gene 33 maturation-defective amber mutant, N 134, but the 21,000 molecular weight band is not found in two gene 55 maturation-defective amber mutants.

Further studies of the role of the new subunits in the infection process are underway.

TO DETERMINE WHETHER CERTAIN SUPPRESSOR GENES INTRODUCED INTO BACILLUS SUBTILIS CODE FOR NEW SPECIES OF TRANSFER RNA

Shigemi I. Simms and G. David Novelli

It is now well established that in certain strains of bacteria some suppressor genes code for new species of transfer RNA that enable the cells to read missense or nonsense codons.

Dr. M. Mandel of the M. D. Anderson Hospital has constructed a strain of Bacillus subtilis (NB 230) in which a number of suppressor genes have been introduced. He is looking at the proteins in this strain by gel electrophoresis and comparing these with proteins from the wild-type strain. We decided to take advantage of the excellent resolving capabilities of the reversed-phase column chromatographic systems developed at ORNL to see whether any of these suppressor genes code for new species of tRNA.

We have isolated tRNA from B. subtilis NB 230 and from an asparagenous mutant of B. subtilis. Each of these tRNA preparations is aminoacylated with the same amino acid, except that one carries a ^3H label, the other ^{14}C . The two samples are mixed and cochromatographed over a Plaskon RPC-5 column (1). The tRNA's are eluted with a linear gradient of NaCl. One-ml samples are collected, precipitated on millipore filters, and counted with a liquid scintillation spectrometer.

Thus far, we have made repeated chromatographic runs with the two tRNA preparations aminoacylated with Phe, Arg, Ser, Lys, and Leu. In the wild type, two peaks of Phe-tRNA are observed, whereas the mutant has four Phe-tRNA's. This holds true when the labels are reversed. With Arg, the mutant has a greatly diminished quantity of the first peak. With Ser, Lys, and Leu, the profiles of the two tRNA preparations appear to be identical.

1. R. L. Pearson, J. F. Weiss, and A. D. Kelmers, Biochim. Biophys. Acta 228, 770 (1971).

STUDIES ON THE PURIFICATION AND
CHARACTERIZATION OF tRNA
PYROPHOSPHORYLASE

Audrey N. Best and G. David Novelli

We have completed one phase of our studies with tRNA pyrophosphorylase [tRNA adenylyl(cytidylyl)transferase] (1), and the results have been published in *Arch. Biochem. Biophys.* 142, 527 (1971) and 142, 539 (1971) under the titles "Studies with tRNA Adenylyl(cytidylyl)transferase from *Escherichia coli* B. I. Purification and Kinetic Properties," and "II. Regulation of AMP and CMP Incorporation into tRNA_{pCpC} and tRNA_{pC}." We intend to explore various aspects of cellular control mechanisms involved in the regulation of the activity of this enzyme.

1. A. N. Best and G. D. Novelli, *Biol. Div. Ann. Progr. Rept.*, Dec. 31, 1969, ORNL-4535, p. 175.

TRANSFER RNA FROM *E. COLI* TREATED WITH
CHLORAMPHENICOL

L. C. Waters

It was previously reported (1, 2) that physiologic conditions which stop *E. coli* protein synthesis and allow RNA synthesis to continue result in an accumulation of tRNA's which show markedly different chromatographic properties when compared to normal. This tRNA appears to have full amino-acid-accepting capacity. The effect is most easily demonstrated with chloramphenicol.

We have investigated this phenomenon using a very simple hypothesis as a guideline; that is, that we are observing a failure of the normal tRNA modification process. Jacobson and Hedgcoth (3) have reported that only about half of the normal amount of dihydrouridine is being made in the tRNA after 1 or 2 hr in the presence of the drug. Our own work has shown, by labeling and direct chemical methods, that the extent of thiolation is drastically decreased. This can be seen from the level of 4-thiouridine present after treatment as well as from the level of 2-thiomethyl-6-isopentenyl-adenosine (Dr. Robert Bock, personal communication). Methylation appears to be quantitatively and qualitatively unaltered.

Samples of treated tRNA that contain 50-75% altered tRNA (as determined chromatographically) support hemoglobin synthesis in an *in vitro* system as well as normal *E. coli* tRNA. Whether there is an increased infidelity with this tRNA remains to be determined.

1. L. C. Waters, *Biochem. Biophys. Res. Commun.* 37, 296 (1969).
2. L. C. Waters, *Biol. Div. Ann. Progr. Rept.*, Dec. 31, 1969, ORNL-4535, p. 174.
3. M. Jacobson and C. Hedgcoth, *Biochemistry* 9, 2513 (1970).

T4-SPECIFIC LEUCINE tRNA

L. C. Waters

In 1967 (1), we reported that after infection of *E. coli* with T2 phage (subsequently shown also with T4) a new peak of leucyl-tRNA could be detected chromatographically. This peak of activity was distinct from the normal profile. Many experiments appeared to rule out artifacts. Two possible explanations, both implying phage specificity, were reported: (1) phage-mediated modification of normal *E. coli* tRNA and (2) *de novo* synthesis from the phage genome.

Studies from this and other laboratories (notably S. B. Weiss at the University of Chicago) have led us to conclude that the new leucine tRNA is coded for by the phage genome. Experiments using ³⁵S labeling, DNA-RNA hybridization, etc., lead to this conclusion. Recent results in this lab have shown that (1) mutants defective in DNA synthesis do not produce the new leucine tRNA; (2) rifampin prevents its accumulation to detectable levels; and (3) the appearance of this tRNA does not appear to correlate with whether the phage strain is capable of causing lysis inhibition or not.

A recent report from John Abelson's laboratory gave the complete primary sequence of the phage-specific leucine tRNA. Its size and nucleotide sequence show conclusively that it is different from any of the normal *E. coli* leucine tRNA's. A biological role for this tRNA has yet to be determined.

1. L. C. Waters and G. D. Novelli, *Proc. Nat. Acad. Sci. U.S.A.* 57, 979 (1967).

STUDIES ON THE STRUCTURAL MODIFICATIONS
OF tRNA CAUSED BY EXPOSING *E. COLI*
TO CHLORAMPHENICOL

Audrey N. Best, L. C. Waters, and G. David Novelli

Previous studies by L. C. Waters (1) have demonstrated that tRNA from *E. coli* cells that have been exposed to chloramphenicol (CAP) for several hours yield altered profiles when chromatographed on reversed phase columns. If such cells are allowed to grow further in the absence of CAP, their tRNA exhibits a normal profile. We propose to isolate and to purify some of these altered species of tRNA, and to determine the nucleotide composition in order to ascertain the nature of the alteration.

The results reported previously (1) suggest that CAP inhibits the synthesis of some of the enzymes involved in base modification of tRNA and that such enzymes have a short half-life, resulting in the synthesis of tRNA without the required base modification. We are investigating the thiolation patterns of these tRNA's at the present time.

1. L. C. Waters, *Biol. Div. Ann. Progr. Rept.*, Dec. 31, 1969, ORNL-4535, p. 174; *Biochem. Biophys. Res. Commun.* 37, 296 (1969).

IN VITRO SYNTHESIS OF BIOLOGICALLY ACTIVE PROTEINS

Helen Sellin and G. David Novelli

Our aim is to develop an *in vitro* message-dependent and tRNA-dependent bacterial system capable of synthesizing biologically active proteins when natural messengers are supplied.

Prior to 1970 our efforts were directed toward purification of the system's components using poly(U)-directed polyphenylalanine synthesis as a monitor. Ribosomes and enzyme fraction were obtained that were completely messenger and tRNA dependent. Since January 1970 we have turned toward use of natural messengers. Both RNA from T4-infected cells and T4 DNA direct amino acid incorporation in our system. However, these preparations were incapable of directing lysozyme synthesis.

Preparation of active lysozyme substrate by killing *E. coli* cells with chloroform proved to be unreliable at first but can now be done reproducibly. Then, preparation of RNA from T4-infected cells (mRNA) that could direct lysozyme synthesis in a crude S30 system was worked on. This mRNA preparation is also capable of directing lysozyme synthesis using enzymes and ribosomes prepared according to Gold and Schweiger (1) or enzymes passed over DEAE- and puromycin-treated ribosomes.

As might be expected, since mRNA is total RNA from infected cells, mRNA-directed lysozyme synthesis in the fractionated system is not really tRNA dependent. Therefore, we are now working on T4-DNA-directed lysozyme synthesis which should be tRNA dependent.

1. L. M. Gold and M. Schweiger, Proc. Nat. Acad. Sci. U.S.A. 62, 892 (1969).

ISOLATION OF A HEMOGLOBIN mRNA FOR GENETIC ENGINEERING STUDIES

Peter Pfuderer, Andrew Francis, and Stanfield Rogers

There are many genetic deficiency diseases in man and animals which are caused by the lack of a single enzyme. With the rapid progress in molecular biology, especially the recent development of several new nucleic acid enzyme systems, we are now in a position where it is feasible to start thinking about the transfer of genetic information into an organism to code for a missing enzyme. The first step in such a direction must be the isolation of an mRNA.

For our mRNA we propose to use the mRNA for rabbit hemoglobin, and to introduce this mRNA into tobacco plants via tobacco mosaic virus (TMV). Although this virus can not be called a harmless passenger virus, it is readily available to us for these first pilot studies, and other more appropriate viruses and mRNA's can be tried later.

Brown agouti rabbits of about 2-3 kg were injected daily by the subcutaneous route with 1.0 ml of filtered 2.5 % w/v phenylhydrazine hydrochloride for 4-5 days or until

their reticulocyte count was 70-100%. They were then exsanguinated from the dorsal aorta, the blood was heparinized and cooled, washed with 0.9 % buffered saline (pH 7) with 0.01 M Tris, and the reticulocytes were collected by centrifugation. They were lysed in 2 volumes of 0.01 M Tris (pH 7.6), the unbroken red and white cells and the stroma were pelleted, and the supernatant fluid was treated twice with SDS-phenol. The aqueous phase containing the RNA was made 0.1 M in acetate buffer (pH 5) containing 3 % NaCl. The RNA was precipitated with 2 volumes of cold ethanol, centrifuged, and the precipitate was dissolved in 0.1 M NaCl. The RNA's were separated by zonal centrifugation in the B XV rotor using a 5-20 % sucrose gradient at 33,000 rpm for 20 hr.

A slight peak at 10-15S proved upon recentrifugation to contain only two peaks of RNA, one at 9S and the other at 14S. The 9S and 14S RNA were separated and further purified by zonal centrifugation in the S.W. 50.1 rotor to give a homogenous peak of each. The use of the B XV rotor permits the workup of relatively large amounts of material in one centrifuge run and usually gives a clean separation of the 9S and 14S peaks from 4S, 5S, 18S, and 28S RNA. This mRNA will then be used to modify RNA viruses by covalent attachment to them, in the hope of producing an infective virus with the ability to produce rabbit hemoglobin in a non-rabbit host.

Antibody against rabbit hemoglobin has been prepared, and a purification scheme for the isolation of a radioactive antibody-antigen precipitate has been tested.

LACTATE DEHYDROGENASE ACTIVITY LOSS AND AGING IN THE MOUSE

Robert J. Oliveira and Peter Pfuderer

Aging animals are characterized by a gradual loss of function and ability to respond to stress. At the molecular level, one would hope to find a corresponding loss of enzyme function in aging animals. If this loss could be traced back to the molecular factors causing it, a great deal would be learned about the mechanism of aging.

We decided to investigate one enzyme in depth in the mouse as a function of age. We chose lactate dehydrogenase (LDH) as an ideal enzyme for our purpose, since it is found in every cell and has a convenient assay.

BC3F₁ mice were kindly furnished by Dr. T. Makinodan from the colony of old mice maintained here. The tissues were extracted, homogenized, and assayed for LDH activity using standard methods.

Although there were various changes between 0 and 30 weeks of age, and male and female animals showed different LDH profiles, in general a large loss of LDH activity was observed in every tissue from 30 weeks to our oldest points. In particular, a 40 % drop in LDH activity was observed in muscle tissue, an 80 % drop in heart tissue, and a 50 % drop in kidney and brain tissue between the ages of 30 and 140 weeks.

LDH in the mouse is a tetrameric enzyme made up of two different types of subunits, heart type and muscle type.

When the composition of each sample was analyzed by gel electrophoresis and resolved into its component parts, both heart- and muscle-type subunits were found to decrease with age. Therefore, two different gene products were found to decrease with age.

One popular theory of aging states that an accumulation of missynthesized enzymes accompanies senescence and thus accounts for the enzyme activity loss. One test of this hypothesis would be to measure the molecular activity of a given enzyme as a function of age. An inherent limitation in such a test, however, is that a molecular species could preferentially be obtained, masking the possible presence of a multiplicity of the species. An alternative approach, which would overcome the pitfall described, would be to study the enzyme using two independent molecular markers as a function of age.

LDH activity was used as one of the molecular markers, and another monitor of LDH, a monospecific antibody to an LDH isozyme, was prepared. With the monospecific antibody, qualitative and quantitative experiments were conducted to test for missynthesized LDH with age. Immunoelectrophoresis of muscle from mice of various ages, a qualitative measure, indicated the apparent absence of missynthesized LDH with age. The method of radial immunodiffusion provided the second molecular monitor of LDH with age, the quantitation of the amount of LDH antigen. A strong parallelism in the two monitors of LDH, the activity and antigen titration as functions of mouse age, suggested that the decrease in LDH activity in mouse muscle with senescence is due to a decrease in the total number of fully active LDH molecules, with the apparent absence of missynthesized, partially active molecules. Tests for the presence of LDH inhibitors were also negative.

An inquiry into the nature of the decline in LDH activity in aging mice on the cellular level is presently being conducted by histochemical techniques in collaboration with others. We hope to learn whether the decline is due to a complete loss of cell function in some cells or a partial loss of activity in all cells.

In summary, LDH activity was found to decrease in all nondividing tissues examined. This decrease involves both gene products of LDH, the heart- and muscle-type subunits. The parallel between the loss in LDH activity and the loss in LDH antigen suggests that this large decrease is not due to the production of missynthesized enzyme.

LACTATE DEHYDROGENASE ACTIVITY IN THE CARP AND IN THE ANNUAL KILLIFISH AS A FUNCTION OF AGE

Peter Pfuderer, Andrew Francis, and J. Edward Brown

Enzyme activities have been shown to decrease with increasing age in several organisms and for several enzymes. It is probably true that in most organisms a period of senescence starts approximately at the time when active growth ceases, and from this time on many measurable vital parameters decrease with time.

Some aquatic vertebrates, the carp in particular, are capable of almost indeterminate growth, and may have an

indeterminate life-span under ideal conditions. If the carp does not undergo senescence, since it does not stop growing, we would not expect to find any change in enzyme activities, specifically lactate dehydrogenase (LDH) activity, with age. The annual killifish, genus Cynolebias, an egg-laying toothed carp, is programmed to die in about a year. Its population exhibits the survival curves typical of senescence after its growth rate stops at about 10 months of age. These two fish, the carp and the annual killifish, should form a good basis for a comparative study on senescence.

We have examined LDH activities in carp collected from several local lakes. Some of these fish were generously given to us by the Division of Forestry, Fisheries, and Wildlife Development of TVA. The LDH activities in carp muscle, heart, and kidney tissue did not change with age in fish from 1 to 8 years of age, which is the oldest fish caught so far. There were two exceptions to this; two fish collected in the winter had extremely high LDH levels in all three tissues. This was assumed to be a seasonal variation to offset tissue temperature changes. The LDH activity in the brain was found to decrease 3-fold from 1 to 3 years, then remained relatively constant from 3 to 8 years. Over this time period, the brain, heart, and kidney tissue were all still growing in proportion with the rest of the animal. The carp had an overall growth rate which was rapid up to 1 year, then maintained a relatively constant rate from 1 to 8 years. Over this time span, then, there is no evidence for senescence in the carp, from either LDH levels or growth rates.

The colony of annual fish has been established, but not enough old fish are available as yet to do aging studies. Two species of Cynolebias have been started, Cynolebias bellottii, a large fish which has proved difficult to breed in an aquarium, and Cynolebias whitei, which is smaller and easier to breed.

The electrophoresis patterns of the LDH isozyme from these three teleosts are very different. The carp, which is a tetraploid species, shows a very complex isozyme pattern indicative of at least four different subunits in the tetrameric enzymes. The two killifish, although different from one another, show a simpler pattern, indicative of two or three subunits in a tetrameric enzyme. Thus, a changing LDH level in these fish could involve a change in far more than one gene product.

CHANGES IN tRNA IN SUPPRESSOR MUTANTS OF DROSOPHILA MELANOGASTER

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K. Bruce Jacobson, and J. B. Murphy

Suppression in procaryotic organisms is commonly accomplished by altering a tRNA at the anticodon site so that it will react with an abnormal codon in mRNA and thus "correct" a missense or nonsense mutation. In eucaryotic organisms, the mechanism of suppression is not as clearly defined. For yeast it appears likely that the correction of missense and nonsense mutations does occur as outlined above, but in higher organisms there is little known about the mechanism of suppression. For Drosophila melanogaster several

suppressor mutants are known. We are examining the tRNA of these mutants to determine what alterations exist in the mutants and how such alterations may cause the suppression of mutant characteristics.

The vermilion mutant of *Drosophila* is deficient in tryptophan pyrrolase; but in the double mutant, vermilion-suppressor [v, su(s)²], the enzyme activity is restored to 30% of the wild type. We have examined some tRNA's of the double mutant and studied their effects on the activity of tryptophan pyrrolase of the vermilion mutant.

Previously we reported that an alteration in tRNA^{Tyr} exists in a suppressor mutant, su(s)², of *Drosophila*, in that the second of two major isoacceptors is absent. The absence of a tRNA species is not compatible with the now classical mechanism of suppression in procaryotes that involves an alteration of the anticodon of that tRNA. Therefore we postulated that the missing tRNA^{Tyr-2} directly inhibits tryptophan pyrrolase of the vermilion mutant. This was borne out by the observations that (1) tryptophan pyrrolase of the vermilion mutant is activated by RNase, (2) the activated enzyme is inhibited by wild-type tRNA, and (3) the inhibition by tRNA is due exclusively to tRNA^{Tyr-2}, the species that is missing in the suppressor mutant. From these observations we hypothesize that wild-type tryptophan pyrrolase normally has tRNA^{Tyr-2} bound to it and that the enzyme produced by the vermilion mutant is altered in such a way that the bound tRNA^{Tyr-2} becomes severely inhibitory, perhaps by blocking the coenzyme site of the enzyme. Removal of the tRNA by RNase or by preventing its synthesis (suppressor mutant) allows the enzyme to function.

Thus, a mechanism for suppression is demonstrated for a eucaryotic organism that is different from that for procaryotes.

Cochromatography of precharged tRNA's from wild-type (Samarkand) *D. melanogaster* flies and vermilion (v; 1-33.0) larvae on 1.2 X 3.5 cm columns of hydroxylapatite demonstrate the presence of two main isoacceptor tRNA^{Tyr}'s in the third instar larvae of the vermilion mutant, which elute at the same position as those of the adults of the wild-type strain. These results are consistent with the finding that the vermilion tryptophan pyrrolase is inhibited by tRNA₂^{Tyr}.

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MUTATION OF THE HEMOGLOBIN LOCUS BY X-RAYS

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Many mutations of mice are known that have been induced by X-irradiation. None of these mutants have been characterized biochemically and, therefore, may have been caused either by gross damage to the chromosome or by modification of the purine and pyrimidine bases in the DNA. The latter possibility has never been demonstrated directly in the mouse.

To identify a "point mutation," the chemical composition of the gene or of the primary gene product must be obtained. Since hemoglobin consists of two kinds of polypeptide chains, it may be considered the primary product of two cistrons. If either cistron is altered by a point mutation, the amino acid sequence in one of the polypeptide chains may be altered. Abnormal forms of hemoglobin may be detected by their electrophoretic and solubility properties in cases where there is a substitution that alters the electrostatic charge or the hydrophilic properties of the protein.

If altered forms of hemoglobin are found as a result of X-ray-induced mutation, the mutants will be characterized by genetic analysis and by determining the amino acid sequence of the α - and β -chain polypeptides of the abnormal hemoglobin.

Electrophoretic and solubility techniques for examining mouse hemoglobin have been established. We are about to begin examining offspring of irradiated mice.

RECESSIVE LETHAL NONSENSE SUPPRESSION IN *SALMONELLA TYPHIMURIUM*

Jeremy Bruenn and K. Bruce Jacobson

Recently, a recessive lethal amber suppressor has been obtained in a partially diploid (merodiploid) strain of *Salmonella typhimurium*. The recessive-lethal character of this nonsense suppressor is thought to be the result of the existence of a nondispensible species of tRNA which, in the suppressor strain, is altered so that it will translate the nonsense codon UAG but will no longer translate the codon corresponding to the amino acid with which it is aminoacylated. The absence of any tRNA capable of reading this latter codon is lethal. The heterozygous merodiploid is viable, owing to the presence of one normal gene copy for the nondispensible tRNA.

The *in vitro* synthesis of T4 lysozyme was used as an assay for amber suppression. Synthesis is dependent on the addition of RNA from *E. coli* su⁻ cells infected with T4 bacteriophage. RNA from cells infected with phage strains carrying amber mutations in the *e* gene (the structural gene for lysozyme) does not cause the synthesis of active lysozyme *in vitro*, except in the presence of 30,000 X g supernatants or tRNA from amber suppressor strains of *E. coli* (1). Similarly, 30,000 X g supernatants or unfractionated tRNA from the recessive-lethal amber suppressor strain of *S. typhimurium* were found to cause *in vitro* suppression. This is the first example of recessive-lethal nonsense suppression shown to be mediated by tRNA.

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DEMONSTRATION OF ALTERED tRNA IN SUPPRESSOR MUTANTS OF YEAST

Jeremy Bruenn and K. Bruce Jacobson

Suppression is the phenotypic reversal of the effects of one mutation by the presence of another. The most ubiquitous types of suppression in procaryotic organisms are those effected by the presence of abnormal species of tRNA, which correct "errors" at the translational level of information transfer. Such species of tRNA generally continue to be aminoacylated by the "correct" aminoacyl synthetases but recognize different mRNA codons. If one of the chain-terminating codons (UAA, UAG, UGA) is recognized, the suppression is termed nonsense suppression. Yeast is the only eucaryotic organism in which there exists good evidence for nonsense suppression. Hawthorne and Mortimer (1) isolated a number of "supersuppressors," which by a variety of genetic criteria appear to be nonsense suppressors. Gilmore et al. (2) have shown that eight supersuppressors with similar allele specificity all insert tyrosine at a site in iso-1-cytochrome *c*, whose mRNA codon is known to be UAA.

Transfer RNA was prepared (1) from a yeast strain carrying one of these eight ochre (UAA) suppressors, (2) from a strain carrying an allelic amber (UAG) suppressor, and (3) from the wild type by phenol extraction and DEAE-cellulose chromatography. The tRNA's were aminoacylated with either ¹⁴C- or ³H-tyrosine by a tRNA-free crude synthetase preparation from the wild-type strain. Aminoacylated tRNA was purified on DEAE-cellulose and chromatographed on one of the reversed-phase chromatographic systems of Pearson et al. (3). By cochromatography of differentially labeled tyrosyl-tRNA from the amber suppressor strain and from the wild type, a new species of tRNA^{Tyr} was demonstrated in the amber suppressor strain. The wild type has only a single resolvable species of tRNA^{Tyr}. These observations are currently being extended to the ochre suppressor strain. This appears to be the first demonstration that suppressor mutants of yeast have altered forms of tRNA.

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2. R. A. Gilmore, J. W. Stewart, and F. Sherman, *Biochim. Biophys. Acta* **161**, 270 (1968).
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STUDIES ON PHENYLALANYL-tRNA SYNTHETASE OF NEUROSPORA CRASSA

J. E. Strickland and K. Bruce Jacobson

We have been following up the observation of Kull and Jacobson (1) that phenylalanyl-tRNA synthetase from *Neurospora crassa* cytoplasm occurs as three peaks of activity when it is chromatographed on hydroxylapatite columns. The two major forms appeared to be of similar molecular weight, and did not interconvert when they were rechromatographed.

We found mycelial growth on glucose consistently greater than on glycerol, and more than twice as much synthetase (Syn^{Phe}) per gram of mycelia was produced when glucose was used as the carbon source. Behavior of Syn^{Phe} on hydroxylapatite was independent of carbon source and appeared to be related to the size or growth stage of the culture. In smaller cultures the enzyme chromatographed as a single late peak, which was partially converted to earlier peaks when homogenates were incubated overnight at 4° C or when broken cells were frozen and thawed. In larger cultures the enzyme chromatographed as a single early peak, or both peaks were present. When a single peak was present, incubation produced no change; but when two peaks were present, incubation resulted in further conversion to the early-eluting form. In more recent experiments, however, all preparations have given single peaks of Syn^{Phe} activity that do not change upon incubation. The reason for this discrepancy is not clear, but it may be due to the use of a new lot of hydroxylapatite with different properties.

Polyacrylamide-SDS gel electrophoresis of Syn^{Phe} purified to homogeneity indicated subunit structure for the enzyme.

1. F. J. Kull and K. Bruce Jacobson, *Biol. Div. Ann. Progr. Rept.*, Dec. 31, 1969, ORNL-4535, p. 43.

AN ALTERNATIVE COUPLING AGENT FOR THE ESTIMATION OF L-KYNURENINE BY DIAZOTIZATION

J. F. Calvino* and K. Bruce Jacobson

L-Kynurenine, a degradation product of L-tryptophan, is usually estimated by diazotization, coupling to 1-naphthyl-ethylenediamine, and reading of the absorbance at 560 nm (1,2). The assay, however, is characterized by high blank values of L-tryptophan. Comparison of the infrared spectra of recrystallized and nonrecrystallized L-tryptophan, L-kynurenine, and mixtures of these compounds indicates that L-kynurenine, although present in trace amounts in the nonrecrystallized L-tryptophan, does not account for the high blank values observed. The visible spectra of the final L-tryptophan and L-kynurenine products in the assay are identical. Several other coupling agents were tested in an effort to find a coupler which would give a clear separation of the colors obtained.

Of 15 potential couplers tested, only 1-naphthol proved satisfactory. At concentrations of L-tryptophan and L-kynurenine usually encountered in the assay for tryptophan pyrrolase activity (2), the absorbance maxima of the tryptophan and kynurenine products were 480 and 510 nm, respectively. Approximate molar absorbance coefficients ($M^{-1}cm^{-1}$) at this wavelength are as follows: 430 for L-tryptophan and 15,900 for L-kynurenine at 480 nm; 350 for L-tryptophan and 19,900 for L-kynurenine at 510 nm. The concentration dependence of the L-kynurenine product absorbance at 510 nm is linear in the range of concentration studied (3.3-33 μM). The concentration dependence of the L-tryptophan product has not been determined yet.

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2. G. A. Marzluf, Z. Vererbungsl. 97, 10 (1965).

EFFECT OF 2-MERCAPTOETHANOL ON THE ESTIMATION OF L-KYNURENINE BY THE BRATTON-MARSHALL PROCEDURE

J. F. Calvino* and K. Bruce Jacobson

Marzluf (1) substituted ascorbate by 2-mercaptoethanol in the tryptophan pyrrolase assay of Kaufman (2). The assay depends upon the estimation of kynurenine by the diazotization procedure of Bratton and Marshall (3). Marzluf observed an initial activation of the enzyme by 2-mercaptoethanol (up to 0.75 mM) and a subsequent inhibition, with practically no activity remaining at a concentration of 2-mercaptoethanol of 10 mM. We have studied the effect of this compound on the kynurenine assay. Increasing amounts of 2-mercaptoethanol, added just before the nitrite, result in a corresponding decrease in the absorbance at 560 nm; at 10 mM the absorbance is reduced by 80% in the case of kynurenine and by 99% with tryptophan. These results suggest that the apparent inhibition of tryptophan pyrrolase at high concentrations of 2-mercaptoethanol may be due to an effect on the diazotization assay, possibly through a depletion of the nitrous acid needed to form the diazonium salt of L-kynurenine.

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3. A. C. Bratton and E. E. Marshall, J. Biol. Chem. 128, 537 (1939).

REACTION OF CACODYLIC ACID WITH SULFHYDRYL COMPOUNDS OF BIOLOGICAL IMPORTANCE

K. Bruce Jacobson, B. Das Sarma,* and J. B. Murphy

Cacodylic acid is commonly used as a buffer in biochemical research; furthermore, it is used as an herbicide in commercial application. The reaction of arsenous compounds with sulfhydryl reagents is well known, but little application of this knowledge seems to have been made to the use of cacodylic acid in these two instances. We are exploring the reaction of cacodylic acid and other arsenous compounds with various sulfur-containing compounds of biological interest.

The reaction of cacodylic acid with mercaptoethanol, cysteine, and glutathione and the properties of the products were reported previously (1). We further find that some thiopurines also react with cacodylic acid, but 4-thiouracil does not. Also, no change in the 4-thiouracil spectrum of tRNA occurred in the presence of cacodylic acid. We have not determined whether cacodylic acid reacts with thiopurines in tRNA.

To understand the reaction better we are attempting to isolate and identify the products of reaction between arsenous compound and sulfhydryl compounds. The infrared spectra of such products confirm the disappearance of free SH reported before (1).

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1. K. B. Jacobson and J. B. Murphy, Biol. Div. Ann. Progr. Rept., Dec. 31, 1969, ORNL-4535, p. 44.

FUNCTION OF METHYL GROUPS IN tRNA

M. P. Stulberg, L. R. Shugart, K. R. Isham, and Barbara H. Chastain

We are continuing our investigation of the functions of methylated bases in tRNA (1). Our previous studies indicated that undermethylated tRNA from a relaxed strain of E. coli, starved for methionine, was less active in amino acid activation. We have now confirmed these results and have produced further data on differences in its function and activity.

We have conclusively demonstrated that this tRNA^{Phe} differs from the fully methylated species in the initial velocity of the aminoacylation reaction, although the K_m is the same (2.5×10^{-7}). The extent of aminoacylation in any preparation, whether normal or undermethylated, is proportional only to the purity of the preparation. In a ribosome-protein synthesis system stimulated by poly(U), undermethylated tRNA^{Phe} is threefold less active in promoting polyphenylalanine synthesis. These data support our hypothesis that such tRNA can be a critical factor in controlling the rate of protein biosynthesis.

The conformation of undermethylated tRNA^{Phe} is to a small degree significantly different from its methylated counterpart when measured by optical rotary dispersion and circular dichroism spectra and especially by A_{260} melting profiles.

The final goal in this project is to purify this tRNA and the individual methylating enzymes sufficiently to allow for a stepwise examination of the effects of methylation on activity and structure.

1. L. R. Shugart, B. H. Chastain, K. R. Isham, E. C. Hise III, and M. P. Stulberg, Biol. Div. Ann. Progr. Rept., Dec. 31, 1969, ORNL-4535, p. 41.

THE tRNA-METHYLATING ENZYMES

L. R. Shugart, M. P. Stulberg, K. R. Isham, Barbara H. Chastain, and J. G. Farrelly

In conjunction with the preceding study, we are isolating and purifying the individual methylases that are necessary for the formation of 5-methyluridine, 7-methylguanosine, and the 2-methylthio group of isopentenyladenosine in tRNA^{Phe} of E. coli.

Thus far we have concentrated on the isolation of the 5-methyluridine methylase that contributes approximately 90% of the methylation of our preparations of undermethylated tRNA. As a group, the tRNA-methylating enzymes are extremely labile, and the 7-methyl guanosine methylase is perhaps the most labile, accounting partly for low guanosine methylation. The methylase is protected against heat inactivation by undermethylated tRNA alone among all substrates tested. It is activated by Na^+ and inhibited by K^+ . Upon exposure to anion-exchange column chromatography, it appears that a bound nucleic acid component is separated from the enzyme, producing extreme lability. Readdition of this component, tRNA, or undermethylated tRNA does not restabilize the enzyme. At this time we have separated the 5-methyluridine methylase from other methylases and RNases and purified it sufficiently to initiate stepwise methylation of undermethylated tRNA^{Phe}.

In the future we intend to examine methylases from mammalian sources. In mammals, these enzymes are subject to the influences of natural inhibitors, hormones, senescence, carcinogenesis, and differentiation. These correlations can be elucidated by a detailed examination of the enzymology and biology involved.

A STUDY OF tRNA IN WHEAT AND ITS ROLE IN CELLULAR REGULATION

L. R. Shugart and Barbara H. Chastain

Senescence has been hypothesized to be the consequence of the loss of certain translational capacities of the cell. This investigation was undertaken to determine whether or not the protein-synthesizing apparatus of senescing tissue contains competent tRNA populations. The wheat leaf was chosen for study because it is a senescing plant (1). The apical portion of the leaf is physiologically an older tissue than the basal growing portion. Attention was directed specifically toward the phenylalanine tRNA of the wheat leaf, because published data concerning its biochemical and physical properties suggest a possible regulatory role for the molecule in protein synthesis.

Phenylalanine tRNA isolated from senescing tissue of 8-day-old wheat shoots demonstrates a greatly reduced capacity to participate in poly(U)-directed polyphenylalanine synthesis compared to phenylalanine tRNA obtained from growing tissue of the same shoot. This difference is correlated with an apparent modification to the Y base of the phenylalanine tRNA, as shown by fluorescent emission spectra, chemical excision studies, and reversed-phase column chromatography.

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THE ISOLATION AND CHARACTERIZATION OF AMINOACYL-tRNA SYNTHETASE:tRNA COMPLEXES

J. G. Farrelly, J. W. Longworth, and M. P. Stulberg

Among the important problems in protein biosynthesis is the structural basis for the high degree of specificity between aminoacyl-tRNA synthetases and their cognate tRNA species.

In an effort to elucidate the basis for this specificity in a well-defined system, we have been continuing our efforts (1) toward the isolation of stable enzyme-tRNA complexes.

We have been studying two systems, the phenylalanine system from *E. coli* B and the arginine system from *E. coli* K-12. The latter system of activation is reportedly influenced by tRNA in its $\text{PP} \leftrightarrow \text{ATP}$ exchange activity and is thus a good candidate for observing conformational changes during complex formation. Also, the molecular weight of the arginine enzyme is approximately half that of the phenylalanine enzyme.

We have isolated the complex between phenylalanyl-tRNA synthetase and tRNA^{Phe} in sucrose gradients. The complex dissociates at pH 6.5 and above. The stoichiometry of the complex is one mole of tRNA per mole of enzyme and is specific for tRNA^{Phe}. At pH 5.0, the complex has an association constant of $1 \times 10^7 \text{ M}^{-1}$.

The technique of quenching of fluorescence of the enzyme tryptophan was used to follow the formation of complex. Upon the addition of tRNA^{Phe}, the fluorescence of the enzyme was markedly quenched. The quenching was specific for tRNA^{Phe}, but it was found that at pH 5.0 a concentration of tRNA tenfold in excess of that needed to produce maximum complex formation on sucrose gradients was required to produce maximum fluorescence quenching.

The complex between arginyl-tRNA synthetase and tRNA^{Arg} has also been isolated on sucrose gradients. The interaction was studied by fluorescence quenching and Sephadex G-100 chromatography. At pH 6.25 the stoichiometry as measured by sucrose gradient centrifugation and fluorescence quenching is one mole of tRNA per mole of enzyme.

In sucrose gradient centrifugation, the sedimentation velocity is dependent upon pH, suggesting an active enzyme multimer formation. Preliminary results suggest that there may be a change in the circular dichroism spectra of the enzyme upon complex formation.

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SEQUENTIAL DEGRADATION OF NUCLEIC ACIDS

Mayo Uziel and A. Jeannine Bandy

The automated nucleic acid sequenator described in the last report (1) has been modified so that it can perform all the required operations. In preliminary tests the instrument has taken a sample of tRNA through six cycles with an average yield per cycle of 98%. To achieve this modest performance, a number of modifications from the original design were incorporated. The filtering properties of the insoluble enzyme were greatly improved by ion-exchange binding to Plaskon (powdered Kel F) coated with an insoluble quaternary ammonium compound (Adogen 464). A number of other configurations of insoluble enzyme proved unworkable, including covalent binding to glass beads and adsorption to a variety of surfaces through the enzyme's anion exchanger potential. Optimum conditions were determined for binding the native alkaline phosphatase to the insoluble succinic anhydride copolymer, so that specific activity and stability were maximal.

Because of numerous technical problems in the use and handling of cetyltrimethylammonium ion as the RNA precipitating agent, we examined several other mechanisms to perform the separation step. Adsorption and elution from insoluble ion exchangers was limited by the low total capacity and low extraction coefficients. Rapid thin-film dialysis was found to be the most efficient means of separating the residual RNA from the reaction products. The half-life for removal of low-molecular-weight components is about 2 min. The diagram (Fig. 1) outlines the relationships of the various parts of the reactor. To improve yield and establish minimum times, we have investigated the kinetics of base release and phosphate elimination as a function of amine, pH, ionic strength, and periodate concentration, using tRNA as a substrate. The observed kinetics are quite similar to those of the mononucleotides undergoing the same reaction. At neutral pH, phosphate ester elimination is the rate-limiting step.

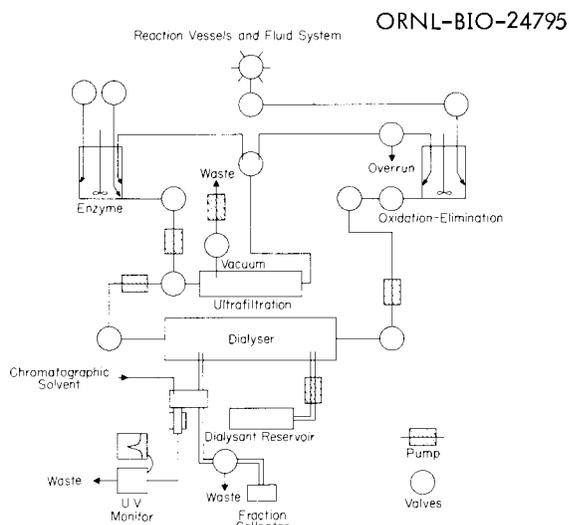


Fig. 1. Diagram of the automated nucleic acid sequencer.

The successful operation through six cycles was carried out using cyclohexylamine and N,N,N',N' -tetramethylethylenediamine catalysis at pH 9.5. This pH is not ideally suited for continued sequential degradation due to spurious hydrolysis of the RNA. We are examining the reaction kinetics as a function of pH, type and concentration of amine, and periodate concentration to decide what are the optimal conditions for the chemical degradation of RNA.

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MODIFICATION OF *E. COLI* tRNA^{Glu} WITH BISULFITE

Ram P. Singhal

Bisulfite (HSO_3^-) reacts with cytosine compounds at pH 6 to form pyrimidine 6-sulfonates, the 4-NH₂ group of cytosine being lost in the process. At pH 9, the added SO₃ groups are lost, leaving only uracil residues in place of the original cytosines, but this does not occur if the pH

is maintained at 6. I have used this reaction to determine which cytidine residues in glutamate tRNA (*E. coli*) are "exposed" to sulfite modification and what effect the modification has upon the biological activity of this tRNA^{Glu}.

In 2 M HSO_3^- (labeled with ³⁵S) at pH 6 and 20°C, three of the 27 cytidines are modified within 3 hr and three more in another 21 hr, after which no further modification takes place (up to 4 days). The sixth residue is altered slowly, requiring about 12 hr. The modified tRNA^{Glu} was hydrolyzed with T₁ RNase (at pH 6 for the reason given) and the resultant oligonucleotides were separated by DEAE-cellulose chromatography (again at pH 6). Comparison of the map thus obtained with the one given by unmodified tRNA^{Glu} showed that certain oligonucleotides of the latter had shifted in the direction of increased acidity (each SO₃⁻ added contributes one negative charge) and that each such modified oligonucleotide had radioactivity (³⁵S) corresponding to the modification of either one or three cytidine residues; this was confirmed by subsequent conversion of the modified cytidines (now uridine sulfonates) to uridines by raising the pH. The fact that the ³⁵S was so discretely distributed indicated that the modification was not random but was specific for certain selected residues, and that each of these had been totally modified.

Identification of the labeled oligonucleotides and comparison of these with the proposed (1) primary structure of tRNA^{Glu} of *E. coli* (see Fig. 1) indicated that the six reactive cytidines are all nonhydrogen-bonded in the standard "cloverleaf" structure, five being in "loop" rather than "stem" regions with one in the -C-C-A terminus. However, the three cytidines located in the so-called dihydrouridine loop were not modified, nor was the one (also nonhydrogen-bonded) at the junction of the stem of this loop with the acceptor stem. This indicates that these parts of the tRNA are not as "exposed" as the two-dimensional cloverleaf representation indicates; rather, they are probably buried (at least in 2 M NaHSO₃ at

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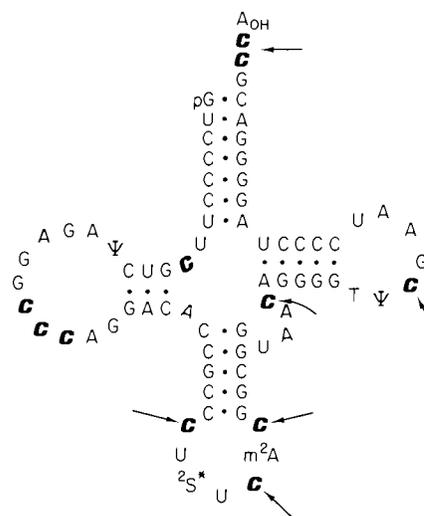


Fig. 1. Cytidine reaction sites (arrows) in the proposed cloverleaf model of tRNA^{Glu} (*E. coli* B).

Nonhydrogen-bonded cytidines are in boldface. 2S* is 5-methylaminomethyl-2-thiouridine.

20° C) so as to be inaccessible to the reagent. The reactivity of only one of the two cytidines in the -C-C-A terminus may point to some similar inhibition of the reactivity of one of these.

Thus modified (and converted to uridines at pH 9), the tRNA^{Glu} with six cytidines has lower $\frac{T}{m}$'s than originally, a higher extinction coefficient, and a decreased retention volume on gel columns, all pointing to a degree of unfolding (2). It had no glutamate acceptance activity under standard conditions. Whether this is related to the change at the acceptor end alone or to conformational changes is presently unknown. However, the results suggest that one or more of the altered residues are part of the specific recognition site(s) for glutamyl-tRNA synthetase. Alternatively, the conformational changes result either in masking the ligase recognition site(s) or destroying the specific tRNA configuration necessary for recognition. It thus appears that the reactive cytosines in this tRNA are involved in maintaining the molecular conformation or in recognition of the ligase binding or both.

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ANALYTICAL SEPARATION OF NUCLEOSIDES BY ANION-EXCHANGE CHROMATOGRAPHY

Ram P. Singhal and Waldo E. Cohn

Investigations of the structure of nucleic acids containing minor nucleosides (e.g. tRNA's) require analytical systems for the identification and assay of the primary building blocks, the base-containing components. Cation exchangers generally have greater exchange capacities than do anion exchangers, which is usually an advantage. Nevertheless, the separations of uridine from its minor congeners (ribothymidine and pseudouridine) and of uridine from guanosine are poor with cation-exchangers, particularly when, as in tRNA, the minor components are present in much smaller amounts than the major ones and thus may be obscured by overlap. It was this gap in analytical chemistry that led us to explore single-solvent anion-exchange systems for the separation of nucleoside mixtures as a comparison technique to the cation-exchange system established earlier (1).

The effects of pH, alcohols, temperature, ionic strength, and flow rate on the anion-exchange separation of nucleosides on Dowex-1-X8 have been studied with respect to the basic parameters of distribution coefficient (D), peak width (σ), and height-equivalent of a theoretical plate.

An appropriate choice of pH and solvent enables one to assay the members of the uridine group even when they are present in widely disparate amounts and to move them away from adenosine. Alcohols influence those substances that are more "organic" in nature. Temperature influences both σ and D and thus shortens the analysis time. While higher molarities reduce the analysis time, the decrease in resolution in the uridine group becomes critical. Flow rate has a negative influence, higher elution rates producing poorer resolution, especially with substances that are not ionically bound.

The optimum condition for the assay of the major nucleosides is around pH 9; when m^1m^7 Guo, iAdo, rThu, Ψ rd, m^2 Guo, and m^2 Guo are present along with the major nucleosides, it is pH 9.7 in the presence of 5% 2-propanol or 7% 1-butanol, in 0.3 M acetate at 50° C.

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MODIFICATION OF *E. COLI* tRNA^{Phe} WITH PARACHLOROMERCURIBENZOATE

B. C. Pal, K. R. Isham, L. R. Shugart, and M. P. Stulberg

The modification of 4-thiouridine moieties in *E. coli* tRNA^{Phe} with parachloromercuribenzoate has been investigated with a view to preparing tRNA labeled with a heavy metal at a specific site for X-ray crystallography and to the elucidation of the biochemical function of 4-thiouridine. Evidence for 1:1 stoichiometry in the reaction of parachloromercuribenzoate with 4-thiouridine has been obtained by carrying out the reaction with the methyl analog of 4-thiouridine and isolating and characterizing the product, 1-methyl-4-thiouracilyl paramercuribenzoic acid [m.p., 236° C (dec.); ϵ_{310} (pH 7), 18,000]. This compound has also been investigated by X-ray crystallography, which showed that the sulfur is covalently linked to the mercury (1). The *E. coli* tRNA^{Phe} was treated with ¹⁴C-parachloromercuribenzoate and subsequently dialyzed against water to remove the excess reagent. A part of the mercurated tRNA was then hydrolyzed to nucleosides using a mixture of venom phosphodiesterase and alkaline phosphatase, and the modified 4-thiouridine was then isolated by chromatography on DEAE-cellulose and identified spectrophotometrically. All the radioactivity was associated with the peak whose position corresponds to that of modified 4-thiouridine. This confirms that only the 4-thiouridine moiety in *E. coli* tRNA^{Phe} reacts with parachloromercuribenzoate. The amount of radioactivity in ¹⁴C-labeled mercurated tRNA^{Phe} indicates incorporation of one mole of the reagent per mole of tRNA^{Phe}. We attempted to assay the mercurated tRNA^{Phe} for amino acid acceptance. Unfortunately, the minimum amount of Mg⁺⁺ (0.0025 M) necessary for supporting enzyme activity was enough to dissociate the paramercuribenzoate moiety from the mercurated tRNA^{Phe} as indicated by the loss of ¹⁴C upon dialysis against water. The extent of aminoacylation of the tRNA that was dissociated from the mercurial was found to be the same as untreated tRNA^{Phe}. Attempts to replace Mg⁺⁺ with other ions such as Mn⁺⁺ were unsuccessful.

In conclusion, the modification of *E. coli* tRNA^{Phe} with parachloromercuribenzoate is specific and quantitative, and its use in X-ray crystallography appears promising. However, the modification is rather labile in the presence of Mg⁺⁺, and its effect on the amino acid acceptance of tRNA^{Phe} could not be studied for this reason.

1. S. W. Hawkinson, B. C. Pal, and J. R. Einstein, *Abstract of the Winter Meeting of the American Crystallography Association, March 2-5, 1970, New Orleans, Louisiana*

AN IMPROVED PROCEDURE FOR THE SYNTHESIS OF NUCLEOSIDE 5'-DIPHOSPHATES

B. C. Pal and V. Diane Grob

Nucleoside 5'-diphosphates are substrates for polynucleotide phosphorylase in the synthesis of polynucleotides. We have investigated a possible extension of the recent direct synthesis (1) of nucleoside 5'-monophosphates to the nucleoside 5'-diphosphates. The unprotected ribonucleosides were treated with POCl_3 in triethyl phosphate, and the intermediates, without isolation, were treated with tri-*n*-butylammonium phosphate. The nucleoside diphosphates were isolated from the reaction mixture by chromatography on DEAE-Sephadex, with a linear gradient of triethylammonium bicarbonate. In this manner we have been able to prepare UDP, CDP, GDP, ADP, and 5-BrUDP in about 30% yield, which is comparable with that obtainable by the laborious multistep procedure of Moffatt and Khorana (2). The procedure is simple and does not require dry-box techniques or isolation of the intermediate. Small amounts of unreacted nucleosides and nucleoside 5'-monophosphate (10-45%) are obtained as by-products. The method is applicable to the synthesis of the analogs of nucleoside 5'-diphosphates on a microscale and readily adaptable to the preparation of α - ^{32}P -labeled nucleoside diphosphates using radioactive POCl_3 .

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SYNTHESIS OF 2-THIOURIDINE AND 2-THIOISOURIDINE

B. C. Pal

Derivatives of 2-thiouridine have been discovered in mixed tRNA (1). Two of them, 5-methylaminomethyl-2-thiouridine (2) and 2-thiouridine-5-acetic acid methyl ester (3), are members of the anticodon triplet in tRNA^{Glu} from *E. coli* and from yeast, respectively. In order to study the properties of 2-thiouridine, I synthesized the compound by the mercuri procedure. Contrary to an earlier report (4), $(2\text{-thiouracil})_2\text{Hg}$ (I) can be obtained from 2-thiouracil and HgCl_2 . On treatment with 2,3,5-tri-*O*-benzoyl-*D*-ribofuranosyl chloride, it formed one disubstituted (II) and two monosubstituted derivatives (III and IV).

These compounds, on debenzoylation with sodium methoxide, formed 2-thiouridine (V) and 2-thioisouridine (VI), respectively (Fig. 1). Both V and VI are converted into uridine and isouridine, respectively, by cyanogen bromide. Both V and VI react with parachloromercuribenzoate, as is evident from difference spectra. The structures of both 2-thiouridine and 2-thioisouridine have been determined by X-ray crystallography (S. Hawkinson, J. R. Einstein).

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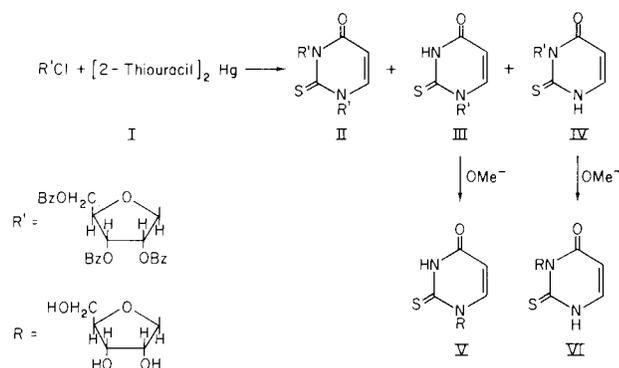


Fig. 1. Synthesis of 2-thiouridine and 2-thioisouridine.

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THE UPTAKE OF HUMAN LYMPHOBLASTOID RNA BY HUMAN SKIN CELLS IN TISSUE CULTURE

Elliot Volkin, M. Helen Jones, J. D. Regan, and W. H. Lee

Pulse-labeled RNA in human lymphoblastoid cell lines has been characterized in the following ways: (1) It specifically hybridizes to human DNA with about 10 times greater efficiency than uniformly labeled RNA. (2) A disproportionately large fraction of the RNA binds avidly to methylated albumin-kieselguhr columns and must be removed by anionic detergents. (3) The sedimentation behavior is unlike the bulk of cellular RNA. (4) The composition of the pulse-labeled RNA is similar to the composition of human DNA. We are studying the incorporation of this RNA into human skin cell fibroblasts. A fairly high level of nucleic acid uptake can be demonstrated.

In this system, incorporation proceeds at the polynucleotide level — it cannot be competed for by low molecular weight precursors. Our results show that either selected species of RNA are incorporated as such, or the RNA incorporated is broken down inside the cell and reutilized for the synthesis of selected species of RNA. Various lines of evidence indicate the selection mechanism to be the case. The kinds of RNA taken up by the skin cells seem to be mRNA and/or nuclear-DNA-like RNA. Thus, this system could prove useful in exploiting the possibility of inserting gene products from one cell line into a different one.

PROCEDURE FOR FRACTIONATION OF GUANOSINE OLIGOMERS

Ann C. Olson and Elliot Volkin

Many different series of oligonucleotides have been successfully separated on DEAE columns with an NaCl gradient in 7 M urea (1). Lipsett (2) recommended it for separation of guanosine oligomers. When a partial alkaline hydrolysate of poly(G) is run on this column, eight peaks are obtained, the largest being the third, fourth, and fifth. The rest of the material, which varies in amount according to the hydrolysis time, is not resolved but can be eluted batchwise with 1 M NaCl. If the partial hydrolysate is incubated in 0.002 M EDTA-0.01 M Tris (pH 7.4)-7 M urea (ETU) at 47° C overnight and run on DEAE-Sephadex (A25) with a gradient of guanidinium chloride (GCl) in ETU at 37° C, the profile contains, in addition to the first eight peaks, material eluting in the region of 9-12 mers and ≥ 20 mers, but there is a marked absence of material in the region in between. Except for this valley, the profile is similar to that given by cytidine oligomers with both NaCl and GCl gradients. This procedure has been used to study the distribution of guanosine oligomers resulting from partial T₁ RNase digestion of poly(G) and partial synthesis of poly(G) with polynucleotide phosphorylase, as well as from partial alkaline hydrolysis.

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IDENTIFICATION AND ORIGIN OF CERTAIN COMPONENTS OF ORGANELLES

D. R. Joseph and W. E. Barnett

One of the distinguishing features of cellular organelles is that they contain a unique (and genetically functioning) DNA as well as a unique translational apparatus for the synthesis of protein. We have demonstrated that both mitochondria and chloroplasts contain an apparently complete and unique tRNA and synthetase complement. This statement is based on several observations: (1) The isolated organelles contain a complete complement of chromatographically distinct tRNA's, which, in the case of Euglena chloroplasts, are light inducible and are absent in mutants that contain no chloroplast DNA or chloroplast structure. (2) The isolated organelles contain complete and unique sets of chromatographically distinct synthetases. In most cases the organelle synthetases have very different acylation specificities, thus providing further evidence for the unique organelle system.

We have also found that, in contrast to the chloroplast tRNA's, the chloroplast aminoacyl-RNA synthetases are present in the bleached mutant strains of Euglena, which contain no chloroplast DNA or chloroplast structure. Thus, the synthetases are synthesized in the cytoplasm and coded

by nuclear genes, and the presence of a unique set of synthetases in the chloroplast indicates a case of intracellular compartmentalization rather than autonomy in the sense of synthesis and retention within the organelle.

We are presently involved in isolating and characterizing the mitochondrial synthetases of Euglena in order to ask the following question: In an organism with two very different kinds of organelles, mitochondria and chloroplasts, is there a single organelle translational apparatus, or are there two different organelle systems plus the cytoplasmic apparatus? Our data at the present time indicate that the two organelles share a common set of synthetases.

ON THE SIMILARITY BETWEEN ORGANELLE AND PROCARYOTIC tRNA'S

S. A. Fairfield, W. E. Barnett, and Diane J. Goins

The origin of cellular organelles has long been an issue of discussion among biologists and biochemists. The notion that mitochondria and chloroplasts evolved from endosymbiotic procaryotic cells has received much attention, largely as a result of their apparent autonomous replication and their unique DNA content. In the past few years, this concept has gained considerable impetus from studies detailing translation within these organelles. Briefly stated, organelle protein synthesis is mechanistically quite analogous to that of bacterial systems (e.g. drug sensitivity, chain initiation) and in this regard is strikingly different from cytoplasmic protein synthesis. In addition, the organelle tRNA's, aminoacyl-RNA synthetases, and ribosomes have been found to be distinct from their cytoplasmic counterparts.

We have found an additional parallel between the translational apparatus of organelles and procaryotic cells in that neither contains a fluorescent base "Y." Base "Y" is of particular interest because it resides adjacent to the 3'-adenosine of the anticodon of eucaryotic tRNA^{Phe}'s and is necessary for normal codon recognition. It has not, however, been found in procaryotic tRNA's. Specifically, we have shown that neither Euglena chloroplast tRNA nor Neurospora mitochondrial tRNA (both of which participate in protein synthesis) contains a fluorescent base "Y," whereas cytoplasmic tRNA's from both organisms do.

Thus, while fluorescent base "Y" is restricted to the tRNA's of eucaryotes, a much more stringent restriction exists, in that base "Y" is present only in eucaryotic cytoplasmic tRNA's. The absence of base "Y" in organelle tRNA's indicates that codon-anticodon interaction in mitochondria and chloroplasts is, at least in this regard, completely analogous to that of procaryotes.

INDUCIBLE CHLOROPLAST tRNA'S IN A CYTOPLASMIC CONSTITUTIVE MUTANT

Diane J. Goins and W. E. Barnett

The chloroplast-specific tRNA's of Euglena which have been identified are all inducible; i.e., they are not detectable in cells grown in the dark. As a beginning to understanding the control of this induction phenomenon, we have

surveyed a series of heat-, X-ray-, UV-, and streptomycin-induced mutants of *Euglena* for the light induction of tRNA's. This effort has been made in collaboration with Dr. Jerome Schiff (Brandeis University), who generously provided a spectrum of *Euglena* mutants ranging from those which are completely aplastic to those with chloroplasts differing from normal in the absence of a single enzyme.

Our results to date indicate that aplastic cells do not show the light induction of chloroplast tRNA's and that the level of induction reflects the mutant's ability to make chloroplasts of normal appearance (even though they are incapable of photosynthetic growth).

More interestingly, however, we have found one mutant, presumably cytoplasmic, which has lost the light-dependent control of tRNA induction: dark-grown cells exhibit ~50% of the maximal light-induced level of chloroplast tRNA's. Thus, we have the bacterial equivalent of a constitutive mutant for the operon controlling the synthesis of organelle tRNA's.

THE ENZYMIC MECHANISM OF COMPLEMENTARY SEQUENCE RECOGNITION

C. G. Mead

An assay has been developed that is capable of detecting an enzymatic activity responsible for the recognition of complementary sequences in DNA. The assay depends on the observation that the 5'-terminal phosphates of phage λ DNA become insensitive to bacterial alkaline phosphatase when the DNA is in the circular form with its complementary ends hydrogen bonded.

The substrate molecules are prepared by removing the 5'-terminal phosphates of phage λ DNA with alkaline phosphatase and then labeling these 5'-termini with ^{32}P , using polynucleotide kinase and γ -labeled ^{32}P -ATP. An enzymatic activity that catalyzes the recognition of complementary sequences can then be measured by the appearance of alkaline-phosphatase-insensitive $^{32}\text{P}\text{O}_4$.

Protein extracts from mammalian meiotic tissues are being examined for the presence of such an activity. The involvement of membrane attachment sites is also being considered.

ANALYSIS OF DNA SYNTHESIS UNDER NONPERMISSIVE CONDITIONS FOR DNA POLYMERASE

R. K. Fujimura

DNA synthesis was analyzed in bacteria infected with a mutant of bacteriophage T5 that has a temperature-sensitive DNA polymerase. Under nonpermissive conditions in a deoxybromouridine (dBrUrd)-containing medium, the DNA synthesized does not have a heavier buoyant density. The ^{32}P -labeled nucleotides incorporated into T5 DNA under these conditions have no ^{32}P -dBrUMP. The amount of ^{32}P -nucleotide incorporated was about 1-5% of the parental DNA, with not too much dependence on the length of incubation of the infected cells. When *E. coli* DNA or parental T5 DNA was labeled with dBrUrd, there was no incorporation of dBrUrd from degradation of these DNA's. The DNA synthesis under nonpermissive conditions was also studied in ^{15}N - ^2H

medium. Under these conditions a density shift of newly synthesized DNA was observed, but not as much as for normally replicated DNA. DNA synthesis under nonpermissive conditions is inhibited by chloramphenicol added at the time of infection. Thus, it depends on a phage gene product. It is concluded that DNA synthesis under nonpermissive conditions is distinctly different from normal replication, and it is very likely of the repair type.

Currently we are trying to determine why dBrUrd is not utilized as a precursor for DNA synthesis under the nonpermissive temperature in cells infected with this T5 mutant. It is not due to a mutation in thymidine kinase or T5-induced deoxynucleotide kinase. It is not a natural property of a normal T5 polymerase. The presence of dBrUrd in the medium inhibits the incorporation of thymidine without incorporation of dBrUrd but does not have an effect on the incorporation of [^3H]-deoxyuridine as deoxycytidylic acid.

THE ANNEALING PROPERTIES OF OLIGORIBONUCLEOTIDES DERIVED FROM T5 DNA-DIRECTED RNA COMPARED TO THOSE FROM T2 AND T7 DNA-DIRECTED RNA'S

Salil K. Niyogi

RNA made *in vitro* on T5 phage DNA template was subjected to a limited digestion with ribonuclease T₁ and fractionated according to chain length. The oligonucleotides of various chain lengths were tested for their annealing at different temperatures to denatured DNA's immobilized on membrane filters. As observed before for the T-even and T-odd phage systems (1), a chain length of ten or more ribonucleotides is required to form a ribonuclease-resistant complex with denatured T5 DNA in the absence of Mg^{++} . The shortest oligonucleotides capable of forming a complex with denatured T5 DNA display a high degree of specificity for species: these oligonucleotides do not anneal to either T2 or T7 DNA. For the same chain length, T5-produced oligonucleotides having a (G + C) content intermediate between those of T2- and T7-produced oligonucleotides form complexes having stabilities intermediate between those of the latter. Indeed, one can derive linear relationships between the melting temperature and (G + C) content for oligoribonucleotides of defined chain lengths. This makes it possible to characterize oligoribonucleotide-denatured DNA complexes as a function of both the chain length and the base composition of the oligoribonucleotide.

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THE EFFECT OF SIGMA FACTOR AND OLIGORIBONUCLEOTIDES ON THE TRANSCRIPTION OF WELL-DEFINED TEMPLATES WITH THE RNA POLYMERASE OF *E. COLI*

Salil K. Niyogi

The sigma factor of *E. coli* RNA polymerase has a substantial stimulatory effect on the transcription of certain well-defined templates, especially single-stranded polymers. This stimulation, dependent on both the base composition (and/or sequence) and the secondary structure of the template, is

maximal at low ribonucleoside triphosphate concentrations, presumably because chain initiation is rate limiting. Among the polyribonucleotide templates, poly(U) shows the greatest dependence on sigma, whereas poly(C) is completely independent of sigma. The transcription of uridine residues in mixed ribopolymers, however, shows a reduced dependence on sigma. Among the polydeoxynucleotide templates, poly(dA)- and poly(dT)-directed reactions are substantially stimulated by sigma; here again, the dependence on sigma is reduced in mixed deoxypolymers. The dependence on the sigma factor for transcription is removed by oligoribonucleotides complementary to the template. Kinetic studies and experiments with γ - ^{32}P -labeled ribonucleoside triphosphates indicate that oligoribonucleotides are, presumably, bypassing the sigma-dependent initiation step.

ISOLATION AND CHARACTERIZATION OF RIBONUCLEOTIDE REDUCTASE FROM EUGLENA GRACILIS

Franklin D. Hamilton

Progress has been made in the purification of the enzyme ribonucleotide reductase from cultured Euglena cells. Enzymic activity is obtained in the 0-65% ammonium sulfate fraction of the crude extract. This fraction can be stored at -50°C for up to 3 months without loss of activity. Preliminary experiments have indicated that a ribonucleoside triphosphate is the substrate for the enzyme reaction. CTP reduction can be stimulated by low concentrations of dATP and ATP, but high concentrations of ATP inhibit the reduction. Efforts are being made to purify the enzyme to homogeneity through the use of column chromatography.

ROLE OF RNA SYNTHESIS IN M13 PHAGE MULTIPLICATION

Sankar Mitra

M13, a small, filamentous phage that is specific for male strains of E. coli, multiplies in the host with the simultaneous multiplication of the latter. The progeny phage leak out of intact cells. The half-life of M13 RNA was found to be at least 18 min (1). In the present investigations, it has been observed that both actinomycin D and rifampicin block phage synthesis as soon as they are added, even late after infection, suggesting that continuous RNA synthesis is necessary for phage production. In a rifampicin-resistant strain with altered RNA polymerase (2), addition of RNA polymerase does not block phage production, unlike in another strain isolated in this laboratory. Further experiments are in progress to evaluate the role of RNA synthesis in M13 multiplication.

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DISPROPORTIONATE INCORPORATION OF PURINE AND RIBOSE FROM PURINE NUCLEOSIDES INTO ATP OF ARTEMIA SALINA

F. J. Finamore and Rose P. Feldman

The brine shrimp, Artemia salina, is incapable of synthesizing purines de novo and, as a consequence, converts guanine-containing nucleotides, principally P^1, P^4 -diguanosine tetraphosphate, to adenine derivatives. In our studies of the mechanism by which this conversion occurs, we observed that the purine and ribose moieties of newly synthesized adenine nucleotides are derived from different sources. For instance, we exposed nauplii to exogenous sources of uniformly labeled ^{14}C -guanosine and uniformly labeled ^{14}C -adenosine (each with purine:ribose = 1:1). After short periods of incubation, the acid soluble nucleotides were isolated and hydrolyzed to determine the distribution of ^{14}C in the purines and ribose of the purified nucleotides. We observed that when ^{14}C -guanosine was used, the purine:ribose ratio of newly synthesized GMP, GDP, and GTP was 1:1, as would be expected if guanosine were being phosphorylated directly. Newly synthesized ATP, on the other hand, was found to have a purine:ribose ratio of only 0.2, indicating that in the synthesis of ATP from guanine-containing precursors, the source of purine is different from the source of ribose and that ribose is labeled much more heavily than adenine.

When nauplii were exposed to ^{14}C -adenosine under the same conditions, a completely different picture emerged. Here the purine:ribose ratios of GMP, GDP, and GTP all were 1:1, whereas the ratio in ATP was found to be about 5:1. Thus, in the synthesis of ATP from uniformly labeled ^{14}C -adenosine, once again the source of purine is different from the source of ribose, but now adenine is much more heavily labeled than ribose.

Therefore, these results indicate that when nauplii are exposed to an exogenous supply of guanosine, it is primarily the ribose moiety that is used for ATP synthesis, but when the organism is supplied with adenosine, it is primarily the purine that is incorporated into newly synthesized ATP.

ASCORBIC SULFATE — A NATURALLY OCCURRING ANTAGONIST OF VITAMIN C?

A. L. Golub and F. J. Finamore

We have recently undertaken experiments to determine whether ascorbic sulfate exhibits antiscorbutic activity when it is presented as a dietary supplement to scorbutic guinea pigs. These animals, like primates, require an exogenous supply of vitamin C, since they are unable to convert L-gulonolactone to ascorbic acid.

For these studies, male guinea pigs weighing 300-350 g were fed a commercial diet free of vitamin C for 14 days. Control animals were maintained on a complete guinea pig ration. Both groups were given ascorbic sulfate, ascorbic acid, or a combination of the two compounds for a period of 14-21 additional days while continuing on their respective

diets. Depending on experimental design, ascorbic acid, ascorbic sulfate, or water was administered either intraperitoneally or orally. The effect of each treatment was assayed by the body weight change, measured daily, and by the serum alkaline phosphatase level, measured every third day.

Further assessment of the effect of each treatment was obtained by postmortem examination of the animals and histological preparation of selected tissues.

Both scorbutic and control animals showed a parallel linear increase in weight until the 21st day of their specific diets. Thereafter, the weight of animals fed a scorbutigenic diet began to fall sharply, culminating in death some 10-15 days later.

The level of serum alkaline phosphatase in guinea pigs appears to be a specific measure of scurvy and is not affected by other pathological conditions. In all these studies, the onset of scurvy was heralded by a 50-100% increase in the basal alkaline phosphatase level. As the disease progressed, the alkaline phosphatase level declined until it reached a value approximately 10% that of normal. The alkaline phosphatase levels of animals receiving ascorbic acid remained constant throughout the experimental period.

Ascorbic sulfate, given either orally or intraperitoneally in doses ranging from 1 to 18 mg per animal per day, in no instance prevented the development of scurvy in animals fed a scorbutigenic diet. In several cases, however, the administration of ascorbic sulfate clearly modified the time course of the onset and progress of scurvy. For example, a short stationary period in the onset of scurvy appeared in animals receiving ascorbic sulfate. During this period, body weights remained relatively constant for several days, while alkaline phosphatase levels decreased significantly but more slowly than in scorbutic animals receiving sham water injections only.

Of considerable significance, however, is the fact that scurvy also developed in animals receiving ascorbic sulfate while being maintained on a normal ascorbic-acid-containing diet. This induction of scurvy suggests that ascorbic sulfate might actually interfere with the normal metabolic activity of ascorbic acid itself.

Thus, although ascorbic sulfate cannot substitute for ascorbic acid in preventing scurvy in guinea pigs, it may actually compete with ascorbic acid in some reactions in which the latter compound is required. If this proves to be

the case, then we have at our disposal a compound that will help us elucidate at least some of the biochemical functions of ascorbic acid.

PURINE INTERCONVERSION IN ARTEMIA SALINA

E. A. Hiss

Although brine shrimp cannot synthesize purines *de novo*, they can interconvert purines that are furnished exogenously. Therefore, a study was undertaken of the pathway(s) by which guanine and related purine derivatives are converted to adenine.

Nauplii were incubated for 4 hr in sterile sea water containing the appropriate ^{14}C -labeled precursors, beginning 1-1/2 days after hatching. After incubation, before any incorporation into RNA or DNA was observed, the nauplii were harvested, washed thoroughly in acid, and the acid-soluble nucleotides were prepared as described previously (1). The total nucleotide fraction was hydrolyzed in acid, and the resulting free purines were separated by column chromatography on Dow 50-H⁺. The purine fractions were pooled, and the total radioactivity in each fraction was determined.

Our preliminary data indicate that when ^{14}C -xanthine is supplied to nauplii it cannot serve as a precursor for adenine or guanine. When ^{14}C -adenine is used, it is more readily converted to xanthine than to guanine. However, ^{14}C -hypoxanthine is rapidly and preferentially converted to adenine by the nauplii. In addition, we observed that 2,6-diaminopurine is converted primarily to guanine rather than adenine, suggesting the presence of an active 6-aminopurine oxidative deaminase.

In another series of experiments, we determined that both adenylosuccinate synthetase and adenylosuccinate lyase activities are present in nauplii and, as a consequence, exogenously supplied IMP is rapidly converted to AMP.

In view of these results, we believe that hypoxanthine and/or its derivatives are probably transitory intermediates in the conversion of guanine-containing nucleotides to adenine-containing nucleotides during the development of Artemia salina.

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BIOPHYSICS AND CELL PHYSIOLOGY SECTION

R. B. Setlow

Cell Physiology

Peter Mazur

S. P. Leibo

Chromosome Chemistry

D. E. Olins

Ada L. Olins^bGrowth and Regeneration

Dorothy M. Skinner

P. C. Kao^aVirginia S. Mayo^aMolecular Biology

W. D. Fisher

D. P. Allison

L. O. Ingram^a

F. W. Shull, Jr.

Radiation Biophysics

P. A. Swenson

W. L. Carrier

J. S. Cook

J. E. Donnellan, Jr.

R. D. Ley^a

R. B. Setlow

G. L. Vaughan^aR. J. Wilkins^aX-Ray Diffraction

J. R. Einstein

F. C. Hartman

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Excited-State Biophysics

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Plant Physiology

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RELATION BETWEEN PERMEATION BY GLYCEROL AND THE RESPONSES OF RED BLOOD CELLS TO FREEZING AND THAWING

Peter Mazur, R. H. Miller, and S. P. Leibo

One of the central tenets of cryobiology is that in order for a protective solute to prevent freezing injury it must permeate the cell. We have been testing this view with respect to the protection of ox red blood cells by glycerol. Our approach has been to hold cells in 1–3 M glycerol at 0, 10, 15, or 20°C for 1–145 min and then freeze them and determine the percent hemolysis after thawing.

We estimated the amount of glycerol that had permeated the cells prior to freezing and the osmolal ratio of glycerol to salts in the cell as follows: Measurements were made of the times required for 50% hemolysis of cells in 1, 2, and 3 M solutions of glycerol that were hypotonic with respect to salt. Standard fragility measurements were used to estimate the relative volume of cell water at hemolysis. The equations describing the inflow of glycerol and the volume of cell water were then solved iteratively on a computer, the value of the permeability constant being adjusted to make the calculated volume of water in the cell reach the observed critical hemolytic volume at the observed time. The equations are as follows:

$$V(t) = \frac{M_i + 1000 s(t)}{M_e + M_s}, \quad (1)$$

where $V(t)$ is the relative volume of water in the erythrocyte at time t , M_i is the osmolal concentration of nonpermeating solute in an isotonic cell, $S(t)$ is the number of osmoles of glycerol that have permeated the cell at time t , and M_e and M_s are the osmolal concentrations of nonpermeating solute (i.e. salts) and glycerol in the extracellular medium;

$$\frac{ds}{dt} = PA[M_s/1000 - S/V(t)]; \quad (2)$$

and its integrated form,

$$S(t+x) = \frac{V(t)M_s}{1000} - V(t) \left[\frac{M_s}{1000} - \frac{s(t)}{V(t)} \right] e^{-PAX/V(t)}, \quad (3)$$

where P is the permeability constant for glycerol, X a small interval of time, and A the cell surface area.

A second way of estimating permeation of glycerol was to determine the time in glycerol required to produce osmotic hemolysis after rapid dilution. The two types of measurements were performed at 0, 10, 15, and 20°C. Both methods yielded similar values for P , the permeability constant for glycerol, and both showed an activation energy for permeation of about 21 kcal/mole — i.e., the rate was halved for every 5°C drop in temperature (Fig. 1).

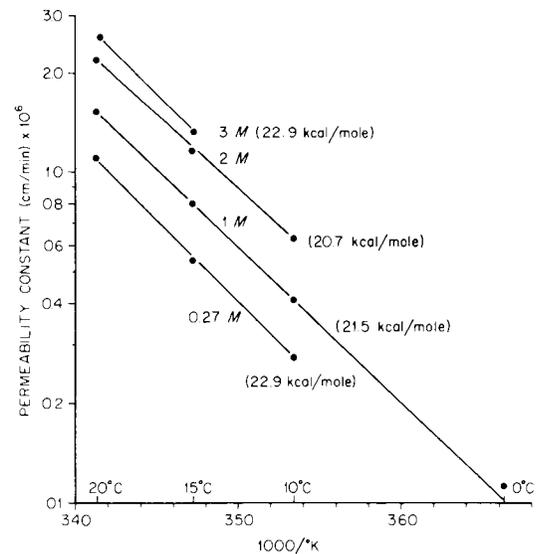


Fig. 1. Arrhenius plot of the permeability constant (P) for glycerol in ox red cells as a function of the reciprocal of the absolute temperature.

P was estimated from determination of the time in glycerol + hypotonic salts required to produce 50% hemolysis.

In the freezing experiments, the survival of the cells frozen after 1 min in 2 M glycerol was as high as after 30 min, regardless of whether the preefreezing contact with glycerol occurred at 0, 15, or 20°C, and in spite of the fact that the amount of glycerol that had permeated the cell differed widely in the several cases (Table I). The same percentage of cells also survived when exposed for 1–30 min

TABLE I. Relation between the ability of ox red cells to survive freezing at 50°C min⁻¹ and the amount of glycerol that has permeated the cells prior to freezing

Time in glycerol prior to freezing	Temp. (°C)	[Glycerol]/[Salts] in cell (osmolal/osmolal)	Survival (%)
1	0	0.05	57
30	0	0.75	55
1	20	0.3	60
30	20	3.5	53

and then frozen in 1.4 M sucrose, a solution of the same osmolality as 2 M glycerol. Since sucrose does not permeate the bovine red cell, and since permeability calculations demonstrate that the intracellular glycerol concentration is too low during the first minute or two for it to reduce the intracellular salt concentration substantially at any subzero temperature, we conclude that protection does not require permeation. We have, however, observed a transitory but dramatic decrease in survival of cells exposed for several minutes to glycerol prior to freezing. This decrease is temperature and concentration dependent, the time at which it

occurs increasing with increasing glycerol concentration and decreasing temperature. It is not due to "thermal shock," but its cause is unknown. The observation that additives do not have to permeate to confer protection, if found to be generally true, will have important theoretical and practical implications. One implication is that the chief site of damage is the cell membrane.

THE DEATH OF CHINESE HAMSTER TISSUE-CULTURE CELLS FROZEN SLOWLY IN VARIOUS SOLUTIONS

S. P. Leibo, Peter Mazur, and E. H. Y. Chu

When Chinese hamster tissue-culture cells are suspended in one of a variety of media and frozen to -196°C , they are killed at both low and high cooling rates. Their survival is maximum at intermediate, optimum rates. Microorganisms and red blood cells exhibit similar behavior. A mathematical analysis, coupled with microscopic examination, has demonstrated that the death of red cells frozen at rates greater than the optimum is due to the formation of intracellular ice during freezing and its recrystallization during thawing. By analogy with these cells, the death of rapidly cooled hamster cells has been attributed to the same cause, and this interpretation is currently being tested by electron microscopic examination of freeze-etched cells.

A variety of data suggest that the death of all these cells cooled at rates less than the optimum is due to changes in the chemical and physical properties of the suspending solutions during slow freezing. The nature and effect of such changes on cell survival are being investigated in both bovine erythrocytes and Chinese hamster cells.

The approach adopted for hamster cells has been to determine the cells' sensitivity to slow freezing as a function of the chemical composition of the suspending media. The method used has been to suspend cells in Hanks' balanced salt solution (HBSS) alone or in HBSS containing 0.004 M polyvinylpyrrolidone (PVP), 0.4, 0.7, or 1.0 M glycerol, 0.4, 0.7, or 1.0 M dimethyl sulfoxide (DMSO), or in 0.4 or 0.7 M sucrose, cool the suspensions at about $1.5^{\circ}\text{C min}^{-1}$ to various subzero temperatures, warm them rapidly to 0°C , and then measure survival by standard plating procedures.

The results in Fig. 1 demonstrate that the addition of any of the compounds listed above to HBSS lowers the subzero temperature at which hamster cells are killed during slow cooling. But these data also contradict predictions based on the widely held view that low-molecular-weight compounds protect simply by acting as a "salt buffer." If that view were correct, then equal molarities of glycerol, sucrose, and DMSO should protect equally well, which obviously is not true. Moreover, increasing the concentration of the additive from 0.4 to 0.7 M confers no more protection with sucrose, only slightly more protection with DMSO, but some significant protection with glycerol; and increasing the concentration from 0.7 to 1.0 M provides no additional protection with glycerol, but substantially more protection with DMSO. In other words, solute concentration is not the explanation of the ability of these various compounds to protect against freezing injury. In fact, a very low concentration (0.004 M) of PVP, a high-molecular-weight, nonpermeating addi-

tive, protects better than sucrose and almost as well as glycerol at high subzero temperatures and protects almost as well as DMSO at temperatures of -80°C and below.

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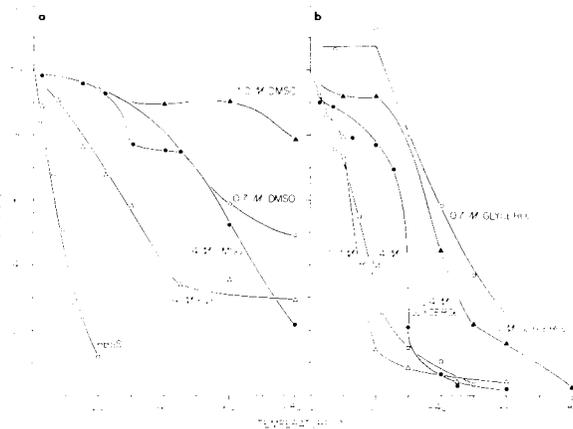


Fig. 1. The survival of Chinese hamster tissue-culture cells.

The cells were suspended in HBSS or in HBSS containing each of the indicated concentrations of solute, cooled at about $1.5^{\circ}\text{C min}^{-1}$ to various temperatures, and warmed rapidly. Survival was measured by standard plating procedures.

The survival curves for hamster cells in various compounds as a function of the minimum temperature of exposure suggest that the mode of protection varies in these different solutions. This, in turn, raises the possibility that a mixture of two compounds might provide more protection than each compound acting singly.

This possibility has been confirmed, since we have now found that a combination of PVP and DMSO will provide almost 100% protection to hamster cells cooled to -196°C . Experiments are underway to determine whether such additive effects of two compounds will protect other types of cells against freezing injury.

Preliminary observations have also demonstrated that the electrolyte composition of the suspending medium, as well as the nonionic component, plays a role in the survival of frozen-thawed hamster cells. That is, virtually 100% of the cells are killed when cooled to -30°C or below in solutions of PVP prepared in isotonic NaCl. The data in Fig. 1 demonstrate that PVP prepared in HBSS provides substantial protection to cells cooled to -45°C and that there is no further deleterious effect of cooling below -45°C . However, this difference between HBSS and NaCl is not true for solutions containing DMSO and glycerol. The survival curves of cells in each of these latter compounds prepared in NaCl are approximately the same as those shown in Fig. 1.

The data already available for hamster cells and erythrocytes (1) indicate that the increase in intracellular electrolytes produced during the freezing of cell suspensions in complex solutions is not responsible for cell death. But the data do not yet suggest what changes in the physical and chemical properties of such solutions are responsible. The explanation of this question is still being pursued experimentally.

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RELATION BETWEEN ULTRASTRUCTURE AND VIABILITY OF FROZEN-THAWED CHINESE HAMSTER TISSUE-CULTURE CELLS

ORNL-BIO-25656

Harvey Bank* and Peter Mazur

The precise mechanisms responsible for the decrease in cell viability during a freeze-thaw cycle remain obscure. Recent studies have correlated the survival of nucleated mammalian cells with cooling and/or warming velocities both in the presence and in the absence of protective agents (1, 2). For each type and concentration of protective agent tested, maximum survival of bone marrow and tissue-culture cells has been found to occur at a specific rate of cooling. Furthermore, cells cooled faster than this optimum rate are highly sensitive to the rate of rewarming, whereas cells cooled at rates less than the optimum are relatively insensitive to the rate of rewarming. These observations suggest that two or more factors affect survival — namely, solution effects (e.g. high solute concentrations), which kill slowly cooled cells, and intracellular freezing, which kills rapidly cooled cells. Thus, the optimum rate for survival is that "which minimizes both the time of exposure to concentrated solutes and the probability or extent of intracellular freezing (1)."

The purposes of the present study on Chinese hamster tissue-culture cells are to determine (1) whether ultrastructural changes occur after freezing; (2) whether there is a correlation between viability and morphology — specifically, whether the differences in the survival responses of slowly and rapidly cooled cells are reflected in their morphology; and (3) whether a protective additive, dimethyl sulfoxide, affects morphology and influences the relation between morphology and viability.

Cells suspended in Hanks' balanced salt solution, cooled to -196°C at rates exceeding the survival optimum, and then thawed and fixed exhibit different patterns and extents of ultrastructural alteration from those of cells cooled or warmed at rates lower than the optimum (Fig. 1). Even though the addition of 0.4 M dimethyl sulfoxide confers some protection in terms of survival, it does not prevent structural alteration. The types of alterations, however, differ from those found in cells frozen in Hanks' alone. Freeze-thaw treatments that produced similar percentages of cell survival do not necessarily cause similar structural alterations, nor is there a simple correlation between structural alteration and survival.

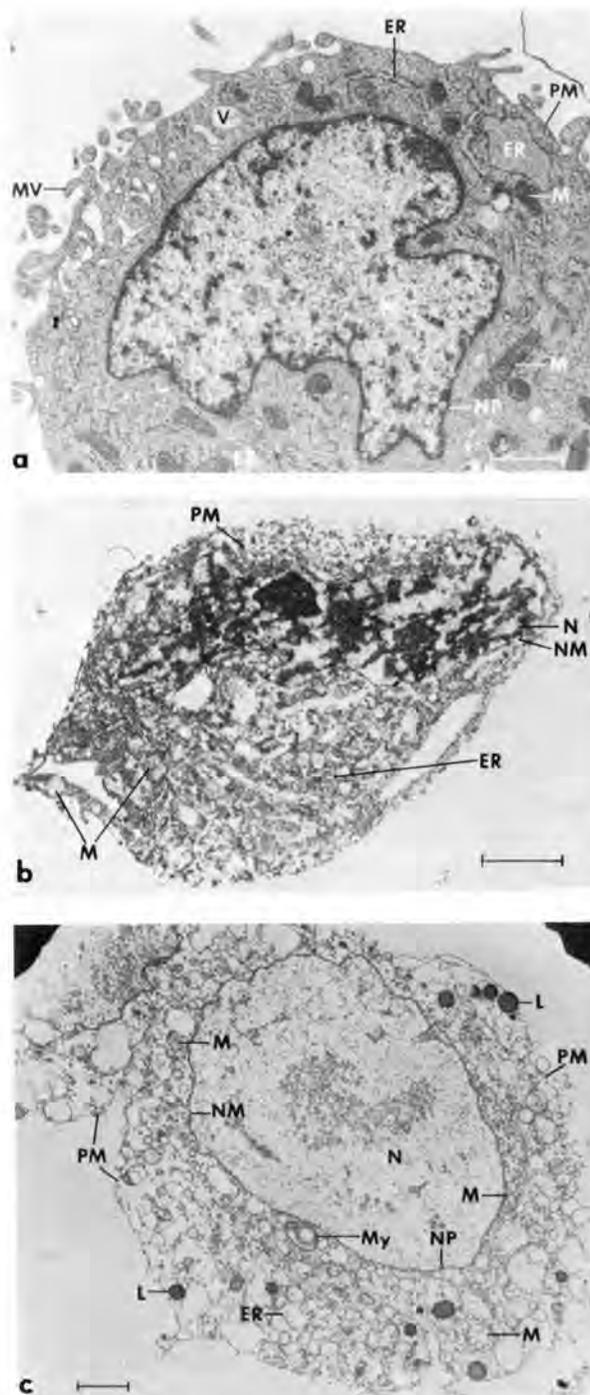


Fig. 1. Hamster cells in Hanks' balanced salt solution.

(a) Unfrozen control cell. (b) Cooled at $500^{\circ}\text{C min}^{-1}$ to -196°C and warmed slowly at $1^{\circ}\text{C min}^{-1}$. (c) Cooled at $2^{\circ}\text{C min}^{-1}$ to -196°C and warmed slowly at $1^{\circ}\text{C min}^{-1}$. NM, nuclear membrane; N, nucleus; ER, endoplasmic reticulum; M, mitochondria; V, vacuole; PM, plasma membrane; MV, microvillus.

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CONSERVATION OF NUCLEOTIDE SEQUENCES IN d(G+C)-RICH SATELLITE DNA'S OF CRUSTACEA

Dale E. Graham* and Dorothy M. Skinner

Three percent of the total DNA of the Bermuda land crab, *Gecarcinus lateralis*, is a d(G+C)-rich satellite DNA (67% G+C), which has been isolated by a modification of thermal chromatography on hydroxylapatite (1). Subcellular fractionation has shown the satellite to be nuclear and, in common with other nuclear satellites, repetitious. However, unlike the d(G+C)-rich satellite DNA's of some metazoans, this satellite does not code for ribosomal RNA (2). To begin to define the function of this satellite DNA, we have determined the extent of homology, under conditions promoting high specificity, between it and the total DNA's of other crustaceans, some of whose DNA's also contain d(G+C)-rich satellites (56-67% G+C). Radioactively labeled RNA synthesized from *Gecarcinus* satellite DNA by *E. coli* DNA-dependent RNA polymerase was used as the tester nuclei acid. The degree of homology was determined at various taxonomic levels: at the section level (the lobster, *Homarus americanus*), at the superfamily level (the spider crab, *Libinia dubia*), and at the family level (various marine crabs). Nonspecific binding in this system is less than 0.1%, as shown by the amount of homology between the d(G+C)-rich satellite DNA sequences of *Gecarcinus* and the DNA of *Micrococcus luteus* (72% G+C). In marked contrast, up to 20% homology exists between the *Gecarcinus* satellite and the other crustacean DNA's studied, under conditions of limiting RNA concentrations. Two factors are to be considered in the interpretation of the degree of conservation of nucleotide sequences in these satellite DNA's. (1) It can be interpreted as a measure of taxonomic relatedness. (2) Because of selection pressure on the rate of nucleotide substitution, it can also indicate the preservation of some function, analogous to the conservation of the sequences coding for ribosomal RNA.

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STIMULATION OF PRECOCIOUS MOLTS BY LIMB LOSS IN CRUSTACEA

Dale E. Graham,* Wanda G. Beattie, and Dorothy M. Skinner

Previously, we reported that the removal of six or more pereiopods (walking legs) from the land crab, *Gecarcinus lateralis*, triggered molting preparations (1). We have now determined the minimum number of limbs effective in eliciting this response. The loss of six or more legs elicits the response at almost a 100% level, whereas the loss of five legs is only 50% effective. Presumably, the reaction is mediated by blocking, at some step, of the activity of the molt-inhibitory hormone, which is located in the eyestalks. Some factor other than loss of a critical mass of tissue must be involved, because the loss of two chelipeds, weighing considerably more than six walking legs is only sporadically effective. The effective factor may be related to the number of

nerve endings of the autotomic appendages that are cut. The size of the regenerative load affects the size of the appendages regenerated. In brief, regeneration of one to six limbs produces new limbs a third smaller than those lost; and replacement of eight limbs decreases the sizes of the regenerates to half those of normal limbs.

These studies have been extended to marine species. The fiddler crabs, *Uca pugnax* and *U. pugilator*, and the portunid crab, *Callinectes sapidus*, respond as does *Gecarcinus*; that is, a precocious molt is initiated. The spider crab, *Libinia emarginata*, does not; nor does another portunid, the green crab *Carcinus maenas*.

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MAINTENANCE OF CRUSTACEAN TISSUES IN ORGAN CULTURE

Dorothy M. Skinner and Wanda G. Beattie

Crustacea, like other metazoa, synthesize macromolecules at low rates *in vivo*. We have, therefore, established conditions for maintaining crustacean tissue *in vitro* in order to carry out studies on the control of growth and differentiation. We have successfully maintained epidermis, midgut gland (hepatopancreas), and regenerating limbs of the land crab, *Gecarcinus lateralis*. Our principal studies to date have been with regenerating limbs. We use Eagle's minimal essential medium with specific inorganic electrolysis adjusted to concentrations characteristic of *Gecarcinus* hemolymph (1) and supplemented with either 10% fetal calf or *Gecarcinus* serum. Under these conditions tissues remain synthetically active for a week. The characteristics of the nucleic acids synthesized *in vitro* are those of crab tissues and not of bacterial contaminants. ³H-thymidine is incorporated into the poly d(A-T) satellite typical of *Gecarcinus* as well as main-band DNA. ³H-uridine is incorporated into ribosomal RNA with electrophoretic properties characteristic of metazoa, including *Gecarcinus*. During the molting period, two distinct phases are seen in the rates of synthesis of ribosomal RNA and DNA in regenerating limbs. In the early premolt period (D₀), when their growth is most rapid, there is a 10-fold greater rate of incorporation of uridine into RNA than in later stages (D₂, late to D₃; 5 days before ecdysis), when limb regeneration is essentially complete and the animal is occupied with other premolt preparations. With ³H-thymidine and ³H-adenosine (at 8 μCi/ml each in the culture medium for 16 hr), main-band DNA and poly d(A-T) of specific activities 1-2 X 10³ cpm/μg can be recovered from D₀ tissues; in the later stages, there is no detectable ³H-thymidine incorporation. The rates of synthesis are not determined by differences either in permeability or pool size; radioactive substrates are present in cell water at almost identical levels, and chromatographic data indicate that pool sizes at early and late premolt stages are similar. Studies on the effects of the addition of exogenous ecdysterone as well as sera from different premolt stages are in progress.

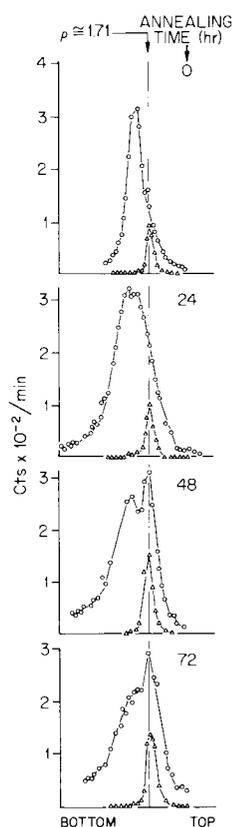
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A POSSIBLE METHOD FOR ISOLATING SPECIFIC REGIONS OF THE BACTERIAL CHROMOSOME. HYBRIDIZATION STUDIES OF CONJUGALLY TRANSFERRED DNA

J. A. Fralick* and W. D. Fisher

Minicells derived from an F^- strain of *E. coli* (P678-54) can act as recipients in bacterial conjugation; the DNA isolated from minicells after mating to a variety of Hfr strains is predominantly single stranded. Since minicells derived from cells without plasmids contain little or no DNA and can be separated from donor cells by centrifugation, it is relatively simple to isolate conjugally acquired DNA. This method has been used to confirm the unidirectional transfer of DNA during conjugation by hybridization studies on DNA strands isolated from minicells mated to Hfr strains that transfer the *proC* region of the bacterial chromosome near the origin but in opposite directions.

The DNA's transferred during separate mating were mixed together and hybridized to determine whether they contained complementary nucleotide sequences (Fig. 1).



ORNL-BIO-24077
 Fig. 1. Neutral CsCl density-gradient centrifugation patterns of cross-annealed Hfr OR56 + Hfr OR56 conjugally acquired DNA.

Aliquots of ^3H -labeled Hfr OR56 and Hfr OR7 DNA ($\sim 4 \mu\text{g ml}^{-1}$) were isolated from minicells after separate matings, mixed together, and annealed in 2X SSC buffer at 65°C . After various annealing times, the mixtures were centrifuged on neutral CsCl gradients (O—O). Native ^{14}C -labeled *E. coli* DNA ($\Delta\text{—}\Delta$) was used as a density marker.

There is a progressive shift in effective buoyant density toward that of native DNA with increasing annealing time. Since these same DNA molecules do not exhibit self-annealing, the observed hybridization indicates the existence of a complementary strand. The observed annealing is not due to an included portion of F DNA at the origin of transfer, since annealing did not occur between either Hfr OR56 or Hfr OR7 transferred DNA and the DNA transferred by Hfr

OR49. The latter strain has an origin far enough removed from the *proC* region to exclude the transfer of any complementary chromosomal regions, but it would contain F DNA if the origin of transfer were to reside within the F DNA, as postulated by some investigators.

These data support the unidirectional transfer of single-stranded DNA during conjugation. The procedure could be used to isolate specific regions of the *E. coli* chromosome. By using selected Hfr or F' strains, one could isolate, from mated minicells, DNA strands which would be complementary only at specific overlapping genetic regions. By hybridization and subsequent enzymatic digestion of the non-complementary and nonannealed single-stranded regions, it should be possible to isolate specific double-stranded regions of the bacterial chromosome.

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STUDIES ON A CELLULAR FACTOR THAT PROMOTES CELL DIVISION IN RADIATION-INDUCED BACTERIAL FILAMENTS

W. D. Fisher and F. W. Shull, Jr.

Radiation-induced filaments of *E. coli* strain 1899NM, which grow and synthesize macromolecular constituents at about the normal rate, are unable to form cross-septa and do not form macrocolonies when plated on nutrient agar. However, these filaments can be induced to divide and form macrocolonies by certain chemical or physical agents and by bacterial cell extracts (1). The division-promoting factor in the cellular extracts has been partially purified, and some of its characteristics have been described. The active material is associated with a particulate fraction (200S), is heat labile, resistant to nucleases and proteases, but is destroyed by ionic detergents and lipases (2). Its density ($\rho \approx 1.2 \text{ g cm}^{-3}$) and sensitivity to detergents and lipases suggest that it may be a membrane fraction or membrane associated.

Although we have been able to produce extracts with division-promoting activity by earlier procedures, we were not able to make extracts of uniform and consistently high activity from the same bacterial cultures (2). Besides complicating routine extract preparation, this variation in activity precluded a meaningful comparison of the biological activity of extracts from different sources, such as dividing or nondividing cells and filaments. The preparative procedure has been improved by pretreatment of donor cells with sucrose-EDTA and lysozyme to yield extracts of high uniform activity. Such extracts are 5–10 times as active, based on the mass of donor cells, as our best previous preparations.

We have compared the heat sensitivity of extracts prepared from B/r cells to extracts made from a temperature-sensitive (T_s) mutant. This T_s mutant (BUG-6) is unable to form cross-septa at the nonpermissive temperature (42°C) but does undergo nuclear division, and the heat-induced filaments divide into normal-sized cells when shifted to the permissive temperature, 30°C (3). Extracts from non- T_s mutants are stable for at least 2 hr at 45°C , while extracts of T_s

mutants show substantial inactivation after 30 min incubation. Extracts prepared from T₅ mutants held at the nonpermissive temperature are also less active (per gm of donor cells) than comparable extracts of normal cells. This suggests that radiation-induced filament formation in 1899NM and heat-induced filament formation in BUG-6 may be related. Study and comparison of extracts of the two types of filaments may yield new information on the mode of action of the extracts.

In our earlier studies (1, 2), we were not able to demonstrate a consistent response of irradiated 1899NM to the extracts in liquid culture, and our assays involved plating cells in agar in the presence of the extracts. This made biochemical studies of the action of the extracts technically difficult or impossible. We have now developed a liquid assay.

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STUDIES ON CONJUGALLY TRANSFERRED DEOXYRIBONUCLEIC ACID IN *E. COLI* MINICELLS. MEMBRANE ASSOCIATION

F. W. Shull, Jr. and W. D. Fisher

A mutant strain of *E. coli* K-12 produces large numbers of anucleate cells (minicells), approximately one-tenth the size (volume) of the parent cell, as a result of aberrant cell division near the ends of the parent cells (1). These minicells contain no detectable DNA. Minicells derived from F⁻ parents will participate as recipients in bacterial conjugation (2). The mated minicells contain, exclusively, labeled DNA acquired during conjugation and, because of their small size, are easily separated from the much larger donor cells.

We have examined DNA transferred to minicells from F⁺ and Hfr donors to determine the extent of its membrane attachment (3). The donor DNA was labeled with ³H-thymidine prior to mating with unlabeled F⁻ minicells. Mated minicells were isolated from donor cells by two cycles of rate, zone centrifugation on 10–30% linear sucrose gradients, which reduced the donor cell contamination to one donor cell per 10⁵–10⁷ minicells.

The conjugally acquired DNA in minicells treated in this manner is found to be 80–100% membrane associated when assayed on Mg–Sarkosinate gradients. A variety of controls have been run to ascertain the specificity of the Sarkosyl technique as applied to this system. No more than 10–15% of the exogenously added DNA under a variety of conditions is recovered in the M band.

Evidence from X-irradiation studies *in vivo* shows that there is no decrease in DNA-membrane association under conditions that reduce the DNA to one-sixth its original size and suggests the possibility of multiple DNA-membrane association sites. Preliminary enzymatic studies indicate the involvement of protein, DNA, and lipids in the membrane association of the DNA.

Recently, several reports have appeared showing that DNA can be introduced into minicells by the segregation of extrachromosomal elements. We have looked at the membrane association of segregated plasmid DNA in minicells containing either R factor, Colicin, or F factor and find such DNA to be largely (78–93%) membrane associated.

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PAPER STRIP METHOD FOR ASSAYING GRADIENT FRACTIONS CONTAINING RADIOACTIVE MACROMOLECULES

W. L. Carrier and R. B. Setlow

The filter-paper disc technique has been a big time-saver in the collection and detection of radioactive macromolecules sedimenting in centrifugal fields.

A simple modification of the paper disc method that we have used for sucrose and cesium chloride gradients reduces costs and eliminates the tediousness associated with writing numbers on the discs, putting the discs onto pins, and sorting the discs after they are washed. Drops from a centrifuge tube are collected onto cellulose paper strips marked off into sections by pencil lines. Each section is the equivalent of a paper disc, and the radioactive molecules in a given section are confined to that section by the liquid samples on either side of it. After the liquid soaks into the paper, the strips are gently washed and dried using the same solvents as for filter paper discs. The strips may be scanned in a strip scanner or they may be cut into sections, put into scintillation vials, and the radioactivity determined by scintillation counting. The strips we used (Whatman No. 17, 1.0 X 57 cm in 1.8-cm sections) are sufficient to collect a 4-ml gradient. Larger volumes may be collected by the use of wider or longer strips. There is no loss of resolution in using the strip instead of discs.

STRAND-REJOINING REPAIR, PHOTOREACTIVATION, AND EXCISION REPAIR IN *E. COLI* MINICELLS HARBORING R FACTOR DNA

M. C. Paterson* and K. J. Roosen*

Chromosomeless minicells are formed by misplaced cell fissions near the polar extremities of an *E. coli* K-12 mutant strain. Resistance-factor (R) DNA is introduced into minicells by segregation from an R⁺ (R64-11) derivative of the original mutant. This R⁺ minicell-producing strain is more resistant than is a wild-type strain toward the lethal effects produced by UV and γ radiations. Thus, it is pos-

sible to measure the DNA repair capabilities of minicells per se.

Sedimentation in alkaline sucrose indicated that about 25% of R DNA obtained from purified minicells consists of covalently closed circular DNA. The closed circular DNA species sedimented 3–4 times faster in alkaline sucrose than did linear DNA (that is, R DNA containing a strand break). The kinetics of γ -induced single-strand breaks in R DNA from either minicells or normal-sized cells were first order as indicated by an exponential decline in the surviving fraction of closed circular DNA as a function of γ -ray dose. Alkaline sedimentation studies revealed that minicells and normal-sized cells are equally competent at rejoining γ -induced single-strand breaks in R DNA.

The criterion for photoreactivation and excision repair was the selective disappearance of pyrimidine dimers from trichloroacetic acid-insoluble material during post-UV incubation in the presence or absence of photoreactivating light. The photorepair of dimers in R DNA of minicells and in chromosomal DNA of parental cells followed exponential kinetics; however, dimers were eliminated twice as quickly in parent cells as in minicells. The finding that R⁺ minicells perform enzymatic photoreactivation contrasts with an earlier unpublished observation of J. Setlow and A. Cohen (1), in which extracts of DNA-less minicells derived from photoreactivable R⁻ parents were reported to lack detectable photoreactivating enzyme activity. Thus, it may be inferred that photoreactivating enzyme activity is concentrated in the vicinity of R DNA and, therefore, tends to segregate with the plasmid into minicells.

In sharp contrast to the efficient excision mechanism operative in normal cells, minicells appeared defective in the excision process, since no net dimer removal was detected during post-UV incubation. In an excision-proficient strain, as expected, single-strand breaks in closed circular DNA of normal cells, manifested in sucrose sedimentation profiles as a decrease in the relative percentage of total radioactivity in the circular DNA, appeared and subsequently disappeared during post-UV excision repair. In UV-irradiated minicells, however, single-strand breaks did not appear in R DNA at any time during the incubation period. A simple interpretation of this observation is that minicells do not possess an endonuclease responsible for mediating the initial incision step necessary for dimer removal.

Assuming that the DNA repair properties of R⁺ minicells reflect the concentration of repair enzymes located in the plasmid-containing polar caps of entire *E. coli* cells, we are led to the following conclusions: (1) Strand-rejoining repair enzymes are distributed throughout the cell. (2) Photoreactivating enzyme molecules tend to be concentrated near bacterial and, to a lesser extent, plasmid DNA. (3) The activity of the excision-repair UV-specific endonuclease appears to be confined to the central region, not the polar extremities of the cell.

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ULTRAVIOLET- AND X-RAY-INDUCED RESPONSES OF A DNA POLYMERASE-DEFICIENT MUTANT OF *E. COLI*

M. C. Paterson,* J. M. Boyle, and R. B. Setlow

E. coli polA1⁻ (previously designated as P3478²) is a K-12 mutant strain which, although able to grow normally, (1) yields cell-free extracts possessing much reduced levels of Kornberg DNA polymerase activity (1) and (2) is sensitive to inactivation by UV light (1) and by X-rays (2).

An earlier study (2) revealed that polA1⁻ cells excise UV-induced thymine dimers normally but degrade their damaged DNA extensively after UV irradiation. In an attempt to clarify further the reason for the increased radiosensitivity of the polA1⁻ strain, we have compared the mutant and its parent (W3110 thy⁻) on the basis of a number of responses to UV- and X-irradiation.

For both types of radiation, the mutant strain is more sensitive by approximately the same factor, as measured by reduction in colony formation, depression of DNA synthesis, and enhancement of DNA degradation. PolA1⁻ cells possess only a limited ability to repair single-strand scissions introduced into DNA, whether these gaps are caused directly by X-irradiation or by events subsequent to UV irradiation. These facts indicate that the polA mutation causes a defective step in the repair of DNA lesions produced by UV or X-rays, as a result of which the changes in DNA are indistinguishable after a certain postirradiation incubation period.

Alkaline sucrose gradient analysis showed that the average molecular weight of DNA isolated from UV-irradiated mutant cells is always considerably larger than expected if one single-strand scission per dimer is produced during post-UV incubation. This finding indicates that the solubilization of damaged DNA in these mutant cells arises from extensive exonuclease activity and not from the action of a rampant endonuclease.

Although polA1⁻ is able to perform a considerable amount of host cell reactivation, UV-irradiated polA1⁻ cells are almost totally deficient in the reactivation of either un-irradiated or UV-irradiated λ phage. Thus, it appears that the participation of a functional product of the polA gene is most critical for host cell reactivation but not necessarily for the other two repair processes.

In conclusion, all evidence reported here and elsewhere (2) indicates that the lack of DNA polymerase activity in UV- or X-irradiated polA1⁻ cells leads to the rampant action of an exonuclease which, by causing extensive DNA degradation, prevents the detectable patching of gaps in the DNA and the sealing of breaks by the strand-rejoining enzyme, polynucleotide ligase. Although the identity of the enzyme responsible for the observed DNA degradation remains obscure, our data are consistent with the notion that the presence of the polA1⁻ mutation leads to a defective DNA polymerase possessing a reduced capability to perform repair synthesis while retaining normal exonuclease activity.

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ROLE OF PYRIDINE NUCLEOTIDES IN 5-FLUOROURACIL-MEDIATED REACTIVATION IN ULTRAVIOLET RADIATION DAMAGE

J. M. Boyle, R. L. Schenley, and P. A. Swenson

Ultraviolet irradiation (520 erg mm^{-2} at 254 nm) causes the respiration of *E. coli* B/r cells to cease after about 90 min of postirradiation incubation in a minimal medium containing glycerol as the sole source of carbon. The cessation of respiration is associated with loss of pyridine nucleotides. Agents that interfere with postirradiation transcription and translation prevent cessation of respiration. We have studied the effects of one of these agents, 5-fluorouracil (FU), on respiration, pyridine nucleotide levels, viability, capacity to support phage growth, and the repair of irradiated DNA.

Addition of FU to cells immediately following irradiation results in the continuation of respiration at a linear rate and the maintenance of high levels of pyridine nucleotides. Cellular viability increases dramatically during the first 60 min of postirradiation incubation in the presence of FU. The ability of irradiated cells to support the growth of phage T4 is also greatly increased. FU treatment has no effect on the kinetics of pyrimidine dimer excision or the degradation of DNA. However, treated cells repair single-strand breaks resulting from early steps in excision repair slightly more efficiently than do untreated cells.

The results support the hypothesis that one of the causes of death in these irradiated cells is the disappearance of pyridine nucleotides, coenzymes of certain respiratory dehydrogenases, and, in the case of the nicotinamide adenine dinucleotide for polynucleotide ligase, the enzyme activity responsible for the final step in the repair of DNA.

VIABILITY AND DIVISION OF ULTRAVIOLET-IRRADIATED *E. COLI* fil^+ AND fil^- CELLS. EFFECTS OF 5-FLUOROURACIL AND PANTOYL LACTONE

P. A. Swenson, R. L. Schenley, and J. M. Boyle

E. coli B Hill (fil^+) normally forms filaments after UV irradiation, but strain B/r (fil^-) does not. Each strain, when grown on minimal medium containing glycerol, turns off its respiration after UV irradiation (520 erg mm^{-2}) for a period of several hours. Using this system, treatment of cells with 5-fluorouracil (FU) resulted in two effects on cell viability: (1) Preirradiation treatment with FU enhances survival of *E. coli* B Hill (fil^+) but not that of B/r (fil^-) when survival is measured immediately after irradiation. (2) Treatment of either B Hill or B/r with FU either before or after irradiation results in a large and rapid increase in cell viability that is observed during the first hour of postirradiation incubation. Pantoyl lactone (PL) also favors an increase in viability upon incubation, but maintenance of respiration and growth is favored only by FU. Regardless of postirradiation conditions in liquid media, cell viability of B Hill is higher when irradiated cells are plated on agar containing PL than without PL. Irradiated B Hill cells under our experimental conditions do not form filaments unless FU is present. If filaments are al-

lowed to form and FU is replaced by PL, the filaments break up and viability increases. Thus FU and PL produce increased viability during postirradiation incubation by different means; PL by stimulation of cell division, and FU by maintenance of respiration.

INTERFERENCE WITH RESPIRATORY CONTROL BY IONIZING RADIATIONS IN *E. COLI* B/r

P. A. Swenson, R. L. Schenley, and J. M. Boyle

Irradiation of log-phase *E. coli* B/r cells with ^{60}Co γ -rays (30 krad) causes respiration to cease about 40 min after irradiation. When glycerol is the carbon source, cessation is complete for several hours. Addition of caseamino acids or substitution of glucose for glycerol favors early recovery. Respiration does not turn off when 5-fluorouracil, rifampin, or chloroamphenicol is added immediately after irradiation. In all these situations, the behavior of γ -irradiated cells resembles that of cells irradiated with UV light, and we conclude that in both cases RNA synthesis and protein synthesis are necessary for respiration to be turned off. Unlike UV-irradiated cells, in which an early increase in viability is favored by maintenance of respiration with 5-fluorouracil treatment, addition of this analog to γ -irradiated cells completely prevents even the normal exponential increase in viability, which begins about 60 min after irradiation. We conclude that RNA synthesis is necessary for a step in the repair of DNA or in the subsequent recovery process.

PRODUCTION OF CYCLOBUTANE DIMERS IN BACTERIAL SPORES

J. E. Donnellan, Jr. and R. S. Stafford

Bacterial spores, irradiated in aqueous suspension with UV light, are more resistant and exhibit a different photochemistry than the corresponding vegetative cells. Whereas the thymine photoproduct formed in vegetative cells is chiefly the cyclobutane dimer, the photoproduct observed in spores is mainly 5'-thyminy-5-(5,6-dihydrothymine) (the spore photoproduct). The resistance of spores can be correlated with the presence of the spore photoproduct, which is some ten times less effective in killing spores than are cyclobutane dimers in killing vegetative cells. The degree of hydration of bacterial spores has been shown to affect the survival of spores after UV and X-irradiation (1). We have attempted to correlate these changes in survival with changes in the photochemistry of the bacterial spore.

Spores were irradiated either moist on membrane filters, on filters *in vacuo*, or under various conditions after evacuation. Qualitatively, the photoproducts formed before evacuation and after exposure to room air following evacuation were the same as those formed in aqueous suspensions. The products formed *in vacuo*, however, consisted of approximately equal amounts of spore photoproducts and a product that chromatographed in the region of cyclobutane dimers. The same mixture of these two photoproducts was

obtained in spores irradiated in dry air or dry nitrogen following evacuation. The new photoproduct appears to be cyclobutane dimer, since a second irradiation of the photoproducts eluted from the chromatogram causes the dimer to revert to thymine and leaves the spore product intact. The absorption of moisture by the previously evacuated spore sample appears to be very rapid. Exposure to room air of 25% humidity causes a complete reversion of the spore state within 5 min.

Attempts to increase the relative proportion of cyclobutane dimers have been unsuccessful. This might suggest that half the spores are altered and half are not. This possibility is eliminated, however, by the further observation that irradiation under vacuum yields a survival several orders of magnitude below that of spores at the same dose.

Thus, the region of the spore that contains DNA, long thought to be dry even in aqueous suspension, can be drastically altered by the presence or absence of moisture in the surrounding atmosphere. Further study of this phenomenon may help to elucidate the unique structure of the bacterial spore and thus the cause of its resistance to environmental insults.

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STUDIES ON PHOTOREACTIVATING ENZYMES WITH ACETOPHENONE-TREATED DNA AS SUBSTRATE

J. S. Cook, T. E. Worthy, and W. R. Proctor*

For studies on photoreactivating enzymes, we have developed a new assay that utilizes a substrate ^3H -thymine-labeled *E. coli* DNA treated with the photosensitizer acetophenone and light of 310 nm. Up to 35% of the thymine residues in the substrate are in the form of cyclobutyl thymine-thymine dimers, with less than 2% of the radioactivity in all other photoproducts (1). The DNA itself is double stranded and has a molecular weight of about 5×10^5 . For our assay, we measure by a simple chromatographic procedure the rate at which the dimers are converted to monomeric thymine in the presence of enzyme and light. We have utilized the assay under three sets of conditions.

First, under intense illumination (Blacklight at $100 \text{ erg mm}^{-2} \text{ sec}^{-1}$), the kinetics of monomerization are pseudo-first-order for the first 80% of the reaction, with a rate constant that is linearly proportional to enzyme concentration. Knowing the absolute enzyme concentration in a crude preparation from Baker's yeast (see below) we calculate this rate constant to be $10^8 \text{ liter mol}^{-1} \text{ sec}^{-1}$ at 36°C and at optimum ionic strength. As shown previously (2), the activity is optimum at ionic strength of 0.17–0.19 and falls off steeply on both sides of this value. In a series of uni-univalent salts, the peak enzyme activity showed the same concentration dependence. The cationic or anionic species of the salt used was irrelevant. Under optimum conditions the enzyme is strongly inhibited by the addition of unirradiated denatured calf-thymus DNA; undenatured DNA is about 25 times less

effective as an inhibitor. Conversely, the rate constant for the repair of dimers in irradiated DNA is about three times greater for undenatured than denatured substrate.

The molecular weight of photoreactivating activity in crude yeast preparations was estimated by gel-filtration chromatography and by neutral sucrose-gradient centrifugation; both techniques yielded a mean molecular weight of approximately 67,000.

The ionic strength dependence of photoreactivating activity from a number of aquatic animals was examined; the animals came from habitats ranging in salinity from fresh water ponds to the saturated Great Salt Lake. Regardless of habitat, all showed the same ionic strength dependence, which was the same as that for yeast enzyme. However, all these animal species regulate the ionic content of their cells such that the intracellular environment is compatible with a reasonably high activity of the enzyme.

Second, enzyme activity was observed in flash photolysis studies. Since the enzyme binds tightly to pyrimidine dimers in the dark, Rupert *et al.* (summarized in Ref. 2) were able to show that the absolute number of enzyme molecules could be measured by the number of lesions repaired by a single brief light flash of sufficient intensity. We have used this technique with acetophenone-treated DNA in the determination of the absolute rate constant given above. We have also used this method to show that the enzyme binds with unirradiated DNA in the dark with a tenfold greater affinity for denatured than for undenatured DNA.

Third, at sufficiently low light intensity and high enzyme concentration, the reaction rate is more dependent on substrate concentration than enzyme concentration. Under these conditions, the reaction is very sensitive to competitive inhibition and can be used to measure concentrations of UV lesions in competing unlabeled DNA. We have used this technique to show that the highly repair-competent *Micrococcus luteus*, following a UV dose of 1000 erg mm^{-2} at 254 nm, repairs half its competing lesions (pyrimidine dimers) in 7 min at 33°C .

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PHOTOENZYMATIC REPAIR OF MITOCHONDRIAL DNA IN *XENOPUS LAEVIS*

J. S. Cook and T. E. Worthy

We have compared the photoenzymatic repair in DNA's from nuclei and mitochondria isolated from cultured cells of *Xenopus* (cell line A8W2, cloned by J. D. Regan from a line established by K. A. Rafferty, Jr.). The criterion for repair was the light-dependent loss of UV-induced pyrimidine dimers from trichloroacetic acid-insoluble material. Cells were grown and labeled with ^3H -thymidine at 25°C , UV-irradiated with monochromatic light at 254 nm, and subsequently

incubated in the dark or photoreactivated under black light. Following gentle disruption of the cells, nuclei-rich and mitochondria-rich fractions were prepared by differential centrifugation, and the mitochondria were treated with DNase and washed. All fractions were extracted with isoamyl alcohol-chloroform and loaded onto small hydroxylapatite columns in 0.12 M sodium phosphate buffer at room temperature. After extensive washing, the temperature of the column was raised. The midpoint of the mitochondrial DNA elution curve from such columns is 77°C, and that of the nuclear DNA is 87°C. At 80°C, 75% of the mitochondrial DNA and less than 1% of the nuclear DNA in any fraction is eluted. ³H-Thymine-dimer analysis of DNA's separated by these thermal elution properties shows that dimers are induced in both DNA species to approximately the same extent by UV and are photoreactivated in both types of DNA with similar kinetics.

If mitochondria are prepared from unlabeled, UV-irradiated cells and then sonicated, such preparations contain photoreactivating enzyme activity, as assayed by their capacity to monomerize pyrimidine dimers *in vitro* in acetophenone-treated *E. coli* DNA.

ULTRAVIOLET SENSITIVITY OF DNA CONTAINING BROMODEOXYURIDINE

R. B. Setlow, W. L. Carrier, and R. D. Ley

DNA's that contain bromodeoxyuridine (BrdUrd) are more sensitive to UV irradiation than unsubstituted DNA's, in terms of their effects on both biological activity and the induction of chain scissions, as detected in alkali. The high sensitivity has been utilized for the selective photolysis of repair-replicated regions in DNA. We have measured chain scission as a function of wavelength (248–334 nm) and as a function of the percentage of BrdUrd substitution (14–100). We wished to determine the most advantageous wavelength to use in the selective photolysis technique.

The action spectra for the production of chain scissions in substituted DNA's of *E. coli* *in vivo* and *in vitro* and for the DNA in λ phage are similar to those for the biological inactivation of substituted DNA's. (There are a negligible number of scissions in unsubstituted DNA's.) The ratio of the cross section at 313 nm to that at 265 nm is $\sim 10^{-2}$. (The ratio for unsubstituted DNA's is $\sim 10^{-4}$.) At 313 nm, the cross section for chain scission is approximately proportional to the percentage of substitution, but at 265 nm it is not. At the latter wavelength there is probably energy transfer from the normal bases of the DNA to the BrdUrd residues, since at this wavelength all the bases absorb about equally. At 313 nm, however, only BrdUrd residues are the important absorbers, so there is little net energy transfer from other bases to these residues.

We conclude that 313 nm is the best wavelength to use for the quantitative analysis of repair replication by BrdUrd photolysis, because the probability of a chain break is proportional to the percentage of substitution and because of the high signal-to-noise ratio at this wavelength.

THE MAGNITUDE OF REPAIR REPLICATION IN *E. COLI* AS MEASURED BY THE PHOTOLYSIS OF BROMODEOXYURIDINE

R. D. Ley and R. B. Setlow

Repair replication in UV-irradiated *E. coli* can be measured by the incorporation of bromodeoxyuridine (BrdUrd) into light parental DNA. Presumably, the analog is incorporated into the gaps resulting from the excision of pyrimidine dimers. Because of the high sensitivity at 313 nm of BrdUrd-containing as compared to unsubstituted DNA, it is possible selectively to photolyze some of the repaired regions and convert them to single-strand breaks, as detected by sedimentation in alkaline sucrose. Cells were labeled with ³H-thymidine, irradiated with 300 erg mm⁻² of 254-nm radiation, and incubated in the presence of nonradioactive BrdUrd. After excision of 45 dimers per 10⁸ daltons in a K-12 (uvr⁺) strain, the cells were exposed to 10⁶ erg mm⁻² of 313 nm, lysed, and sedimented. The 313-nm treatment resulted in seven single-strand breaks per 10⁸ daltons. The excision of 40 dimers per 10⁸ daltons in strain 15 TAU-bar cells in the presence of BrdUrd resulted in 5.5 single-strand breaks per 10⁸ daltons following exposure to 313 nm. Treatment of an excisionless K-12 mutant (uvrA-6) under the same conditions led to only 1.5 single-strand breaks per 10⁸ daltons. Therefore, excision in the presence of BrdUrd results in 5.5 single-strand breaks per 10⁸ daltons (K-12) and four single-strand breaks per 10⁸ daltons (15 TAU-bar) following exposure to 313-nm radiation. Since irradiation with the same dose of 313 nm of DNA fully substituted with BrdUrd results in one single-strand break per 30 BrdUrd residues in *E. coli* K-12 and one single-strand break per 55 BrdUrd residues in *E. coli* 15 TAU-bar, we can calculate that the repaired regions in *E. coli* contain an average of 4–6 BrdUrd residues. Photoreactivation experiments indicate that in *E. coli* the excision of pyrimidine dimers in the presence of BrdUrd is the major source of repaired regions selectively photolyzed by 313-nm radiation.

INCREASED SENSITIVITY OF THE BROMODEOXYURIDINE-PHOTOLYSIS METHOD OF MEASURING REPAIR REPLICATION. APPLICATION TO GAMMA-RAY DAMAGE TO HUMAN CELLS

R. B. Setlow and James D. Regan

The photolysis of bromodeoxyuridine (BrdUrd) residues in polynucleotide strands by 313-nm radiation results in strand scissions that are detected by sedimentation of radioactive DNA's in alkaline gradients. The kinetics of strand breakage as a function of exposure have been used to estimate the sizes of repaired regions in UV-irradiated DNA's (1, 2). If the repaired regions are few or small in size, the very high fluences of 313-nm radiation needed to photolyze the BrdUrd residues may result in sufficient numbers of chain breaks in the nonrepaired DNA to obscure the breakage of repaired regions. We have improved the sensitivity of the original technique (1) almost tenfold by use of double (and triple) radioactive labels.

Table I shows the scheme of a typical experiment used to measure small numbers of breaks in repaired regions that contain BrdUrd. Three separate cultures of human fibroblasts

TABLE I. Experiment to measure breaks in repaired regions of DNA

Labeled DNA	Treatment	Incubation medium	
$^3\text{H-dT}$	10 krad	BrdUrd	Irradiation (313 nm) → Sedimentation
$^{32}\text{PO}_4$	10 krad	dT	
$^{33}\text{PO}_4$	None	BrdUrd	

are treated as indicated. After incubation in the separate media the cells are mixed, irradiated together, and sedimented together in alkaline sucrose. The acid-insoluble radioactivity in the different fractions of the gradients is counted in a scintillation counter set for two- (or three-) channel operation. Any radioactive phosphorus in RNA, protein, or phospholipid can be ignored, because it is either acid-soluble as a result of alkaline hydrolysis or in small molecules that do not sediment appreciable distances. The use of this technique means that the differences in the molecular weights of the various DNA's are approximately independent of variations in dosimetry, sedimentation procedure, and gradient collection procedure. For example, even after large 313-nm exposures of untreated DNA's, the differences among the three labels amount approximately to one break in 2×10^9 daltons.

Human fibroblasts were irradiated with 10 krad, incubated for 90 min, in either BrdUrd or dT, and exposed to 313 nm. There were more breaks in the DNA of cells incubated in BrdUrd than in those incubated in dT. The difference was approximately one break per 2×10^8 daltons (tenfold higher than the noise level in these double-label experiments). We assume that BrdUrd residues are incorporated at the sites of chain breaks during their rapid repair. From the known sensitivity of DNA's fully substituted with BrdUrd and the known numbers of chain breaks introduced by 10 krad, we are able to calculate that there are 0.5 BrdUrd residues inserted per repaired chain break. Thus, the process of chain-break repair after ionizing radiation involves little excision of nucleotides compared to that following repair of UV lesions (~ 20 BrdUrd residues inserted per repaired region).

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LEVEL OF EXCISION REPAIR IN SEVERAL MAMMALIAN CELL LINES

R. B. Setlow, James D. Regan, and W. L. Carrier

Excision repair of UV damage involves the removal of UV-induced lesions (mainly pyrimidine dimers) and the replacement of the excised and possibly enlarged region by new

nucleotides. If repair takes place in the presence of bromodeoxyuridine (BrdUrd), the repaired regions may be selectively photolyzed by 313-nm irradiation. The photolyzed regions appear as chain breaks in alkaline sucrose (1). The sensitivity of the technique is greatly enhanced by comparing directly the effects of 313-nm radiation on cells incubated in BrdUrd and cells incubated in dT (2). We have used these techniques to investigate the magnitude of repair in normal human fibroblasts, Chinese hamster cells, mouse 3T3 cells, and fibroblasts of an individual affected with xeroderma pigmentosum (XP).

Figure 1 shows changes in the reciprocal of the molecular weight (a measure of the number of strand scissions) as a function of 313-nm exposure for these different cells incubated for several different times after UV irradiation. It is apparent that there are big differences in sensitivity to 313-nm exposure among the various cell lines and that the technique is capable of measuring the changes that are manifest after small UV exposures. For example, 50 erg mm^{-2} makes one pyrimidine dimer per 10^7 daltons. If normal human fibroblasts are taken as having a level of excision repair equal to 1, then these data indicate that hamster is approximately 0.5, mouse 0.2, and XP 0.03. The sizes of the repaired regions are all approximately the same — molecular weight about 30,000. Their numbers, however, differ drastically.

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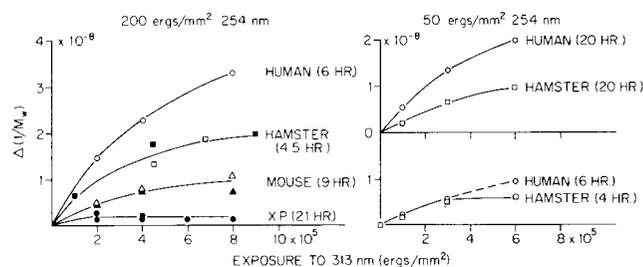


Fig. 1. The change in the reciprocal of the weight-average molecular weight ($1/M_w$) with exposure to 313 nm for cells treated as indicated.

The ordinates represent the differences between cells incubated in BrdUrd and in thymidine after 254-nm irradiation.

The measurements of excision repair by photolysis of cells incubated in BrdUrd after UV irradiation correlate well with the direct measurements of excision of pyrimidine dimers. Thus, following 50 erg mm^{-2} of 254-nm radiation, in 20 hr we measured excision of $\sim 80\%$ for human, 40% for hamster, 10% for mouse, and not detectable for XP.

The high sensitivity of the BrdUrd photolysis technique is indicated by the fact that earlier experiments did not detect such excision in hamster cells.

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POSTREPLICATION REPAIR OF DNA IN
ULTRAVIOLET-IRRADIATED
MAMMALIAN CELLS

A. R. Lehmann

L5178Y mouse lymphoma cells do not excise pyrimidine dimers from their DNA after UV irradiation but can nevertheless survive doses producing about 10^5 dimers in their DNA. The replication machinery in these cells, as in *E. coli* (1), has a mechanism for bypassing the dimers.

DNA synthesized in UV-irradiated L5178Y cells is smaller than that made in unirradiated cells, as shown by pulse-labeling with tritiated thymidine, followed by sedimentation of the DNA in alkaline sucrose gradients. Hence, as in *E. coli* (1), DNA synthesized on UV-irradiated DNA templates contains discontinuities. The estimated distance between these discontinuities is comparable to the average spacing between pyrimidine dimers in the parental strands, suggesting that the replication machinery leaves gaps opposite the dimers. On subsequent incubation, these discontinuities are filled in by a process that is inhibited by hydroxyurea.

Rupp and Howard-Flanders (1) postulated that in *E. coli* these gaps are filled in by a recombinational mechanism involving exchange with the parental strand of the sister duplex, and they have recently produced evidence in support of this hypothesis (2). In order to determine whether the gaps formed in L5178Y cells are filled in by a recombinational mechanism or by *de novo* synthesis, irradiated cells were pulse-labeled with radioactive thymidine, followed by incubation in bromodeoxyuridine and exposure to light of wavelength 313 nm. Light of this wavelength selectively introduces breaks into bromouracil-containing sections of DNA (3). Thus, if the gaps in the radioactively labeled daughter strands are filled in by exchange with thymidine-containing DNA from the parental strands (recombinational exchange), they will not contain bromouracil and will not be reformed by light at 313 nm. Hence, the molecular-weight distributions of the radioactive DNA from initially irradiated and unirradiated cells will be similar after exposure to 313-nm light. On the other hand, if the gaps are filled in by bromouracil-substituted DNA (arising from *de novo* synthesis) they will be reopened by exposure at 313 nm, and the small pieces of radioactive DNA synthesized in the initially irradiated cells during the pulse-labeling will be reformed. In this case, the molecular-weight distribution from the irradiated cells will have a lower average molecular weight than that from similarly treated unirradiated cells. The results are in accordance with the second alternative, indicating that the gaps in the daughter strands opposite the pyrimidine dimers are filled in with newly synthesized DNA. This excludes many of the recombinational models that have been put forward to account for similar gap-filling phenomena observed in bacteria (2).

The efficiency of breakage of bromouracil-substituted DNA was determined in a separate experiment. From this value and from the exposure at which the gaps were reintroduced into the DNA by light at 313 nm, I estimated that the average size of the filled-in piece in each gap is about 800 nucleotides. This is comparable to the size of the gap in irradiated *E. coli*, determined by an independent method (4).

In contrast to the DNA synthesized immediately after irradiation, which is relatively small, the newly synthesized

DNA made a few hours after irradiation (i.e. within a time period considerably less than the generation time of the cells) has a molecular weight similar to that from unirradiated cells. The reason for this unexpected observation is being investigated at the present time.

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2. P. Howard-Flanders, W. D. Rupp, C. Wilde, and D. Reno, *Proceedings of the Tenth International Congress on Microbiology* (1971).
3. J. D. Regan, R. B. Setlow, and R. D. Ley, *Proc. Nat. Acad. Sci. U.S.A.* **68**, 708 (1971).
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EFFECTS OF CAFFEINE ON DNA SYNTHESIS IN
MAMMALIAN CELLS

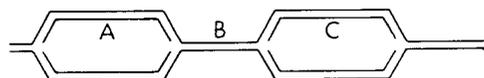
A. R. Lehmann

Caffeine sensitizes mammalian cells to UV light, and it is thought to exert its sensitizing activity during DNA synthesis following UV irradiation (1). It was hoped that it might be possible to elucidate its mode of action by examining its effect on postreplication repair; however, it was found that caffeine had a considerable effect on DNA synthesis even in unirradiated cells. This effect has been investigated in detail.

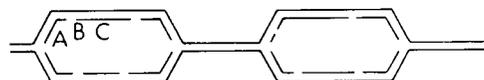
Alkaline sucrose gradient experiments were performed on L5178Y cells pulse-labeled with ^3H -thymidine in the presence and in the absence of caffeine. The DNA synthesized in the presence of 2×10^{-3} M caffeine had a lower average molecular weight than that synthesized in the absence of caffeine. Since caffeine does not introduce breaks into preformed DNA, there are two possible explanations for this observation (Fig. 1). (1) The DNA synthesized in the

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a. UNTREATED CELL



b. GAPS



c. SHORTER UNITS

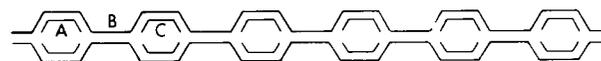


Fig. 1. Models for possible modes of action of DNA.

The inside (growing) strands become labeled in a pulse-labeling experiment.

presence of caffeine contains gaps (Fig. 1b), similar to those seen in the DNA made in UV-irradiated cells. (2) Some kind of aberrant synthesis takes place in the presence of caffeine, so that the DNA is synthesized in shorter units (e.g., see Fig. 1c). Arguments in favor of the latter alternative are (1) that the overall rate of DNA synthesis is the same in the presence and absence of caffeine, unlike the situation after UV irradiation, which causes a substantial decrease in the rate of synthesis; and (2) using the method of bromodeoxyuridine chase and selective photolysis with 313-nm irradiation, it was found that the spacing between separate synthesis sections (i.e. the size of the space B between pieces A and C in Fig. 1) was of the same order of magnitude as that in untreated cells. If it were merely a gap, it would be expected to be several orders of magnitude smaller.

Various other effects of caffeine have been observed: (1) When cells are pulse-labeled and then chased in cold thymidine in the presence of caffeine, the increase in molecular weight of the radioactive DNA, corresponding to the joining-up of adjacent replicating units, is delayed. (2) All effects appear to be reversible on removing the caffeine. Cells pulse-labeled in its presence and chased in its absence produce DNA sedimentation profiles similar to those from untreated cells. (3) Caffeine neither produces breaks in previously synthesized DNA nor prevents the rejoining of breaks induced by γ -rays.

It can be concluded that caffeine affects DNA synthesis in such a way that the DNA is synthesized in somewhat smaller pieces than those made in untreated cells.

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DNA-MEMBRANE COMPLEXES OF MAMMALIAN CELLS

A. R. Lehmann and D. P. Allison

If mouse lymphoma cells (L5178Y) are lysed without shearing in the detergent sarkosyl, on top of sucrose gradients containing cesium chloride, and the gradients are subsequently centrifuged at 40,000 rpm for 16–20 hr, the DNA bands at a density of about 1.5 g cm^{-3} . This suggests that it is complexed with some lighter cell components. If breaks are introduced into the DNA by X-irradiation of the cells prior to lysis, on subsequent centrifugation the DNA bands at the density of free DNA, 1.70 g cm^{-3} (1). Electron microscopic examination of the low-density material from unshered lysates has revealed the presence of extremely long DNA strands attached to membrane-like material. This adds further weight to the hypothesis (1) that DNA might be attached to the nuclear membrane inside mammalian cells.

1. M. G. Ormerod and A. R. Lehmann, Biochim. Biophys. Acta 228, 331 (1971)

ISOLATION AND CHARACTERIZATION OF TRIOSE PHOSPHATE ISOMERASE FROM RABBIT MUSCLE AND YEAST

F. C. Hartman, I. Lucille Norton, C. D. Stringer, and P. Pfuderer

Studies on the active site of rabbit muscle triose phosphate isomerase require large amounts of the enzyme. The difficulty in obtaining sufficient quantities by a previously published procedure, the heterogeneity of commercial material, and conflicting data in the literature concerning the chemical and physical properties of the enzyme prompted the development of an isolation procedure and an investigation of the purified enzyme's properties. The isolation procedure devised—acetone fractionation, heat treatment, Sephadex G-100 chromatography, $(\text{NH}_4)_2\text{SO}_4$ fractionation, and SE-Sephadex chromatography—gives about 100 mg of crystalline enzyme from 1 pound of muscle. This enzyme is homogeneous, as judged by analytical ultracentrifugation and disc gel electrophoresis. The enzyme has a molecular weight of 52,900 by sedimentation equilibrium, 56,000 by amino acid composition, and 53,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Data from carboxypeptidase digestion of the enzyme are consistent with two identical subunits having a C-terminal sequence of $-(\text{Val}, \text{Asp}, \text{Phe}, \text{Ile})-\text{Asn}-\text{Ile}-\text{Ala}-\text{Lys}-\text{Gln}$. Electrofocusing of triose phosphate isomerase reveals the presence of two major and two minor species with similar specific activities.

Recently, the corresponding enzyme from yeast has been isolated, and comparative studies have been initiated.

CHARACTERIZATION OF THE ACTIVE SITE OF RABBIT MUSCLE TRIOSE PHOSPHATE ISOMERASE

F. C. Hartman

I have recently reported the synthesis of 3-haloacetol phosphates, reactive analogs of the substrate dihydroxyacetone phosphate and, thus, potential active-site-specific reagents for triose phosphate isomerase, aldolase, glycerophosphate dehydrogenase, and glycerol kinase. These reagents inactivate triose phosphate isomerase by a selective modification of the active site, as demonstrated by the following: (1) Loss of enzymic activity is pseudo-first order. (2) Competitive inhibitors protect against inactivation. (3) One mole of reagent is covalently incorporated per mole of catalytic subunit inactivated. (4) Autoradiograms of peptide maps for enzyme inactivated with ^{32}P -haloacetol phosphates show most of the radioactivity in a single peptide. (5) Chloroacetol phosphate inactivates triose phosphate isomerase from all species that have been tested—rabbit muscle, mouse liver, human whole blood, yeast, *E. coli*, and spinach.

A pentadecapeptide containing the incorporated reagent has been isolated from a tryptic digest of rabbit muscle triose

phosphate isomerase inactivated by chloroacetyl phosphate. Quantitative ester assays with hydroxylamine and subsequent conversion of the resulting hydroxamate into an amine identify glutamic acid as the essential residue esterified by chloroacetyl phosphate. The sequence of the active-site peptide is Trp-Val-Leu-Ala-Tyr-Glu-Pro-Val-Trp-Ala-Ile-Gly-Thr-Gly-Lys.

Current efforts are directed toward comparative studies of the active sites of triose phosphate isomerase from a variety of species, so that the degree of homology in the primary sequences adjacent to the essential glutamate residue can be determined.

CHLOROACETOL PHOSPHATE AS AN ACTIVE-SITE REAGENT FOR FRUCTOSE DIPHOSPHATE ALDOLASE FROM RABBIT MUSCLE

M. C. Paterson* and F. C. Hartman

Rabbit muscle aldolase, a tetramer believed to possess only three substrate binding sites, catalyzes the condensation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate to give fructose-1,6-diphosphate (FDP). 1-Hydroxy-3-chloro-2-propanone phosphate (chloroacetyl phosphate, CAP), a compound structurally indistinguishable from DHAP except for a chlorine atom in place of a hydroxyl group at C-3, has been used to label residues at the active site of FDP aldolase. Under mild conditions, CAP irreversibly inactivates the enzyme concomitant with the modification of 6-8 of the 28-32 free sulfhydryl groups in aldolase. The amount of reagent incorporated is equivalent to the loss of sulfhydryl groups; thus, other amino acid side chains in aldolase are not modified by CAP. FDP and inorganic phosphate protect the enzyme from inactivation by CAP. Our data best fit the hypothesis that complete inactivation of aldolase involves the covalent modification of two essential sulfhydryl groups at each of the three proposed substrate binding sites. Autoradiograms of peptide maps of aldolase after modification by ^{32}P -CAP reveal one major radioactive peptide, a finding entirely consistent with this hypothesis.

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AFFINITY LABELING OF YEAST ALDOLASE BY HALOACETOL PHOSPHATES

F. C. Hartman, I. Lucille Norton, Y. Lin,* and R. D. Kobes*

Class I (Schiff-base-forming) and class II (metal-requiring) fructose diphosphate aldolases have a common mechanism, involving the C-3 carbanion of dihydroxyacetone phosphate, and they may also be structurally related, in that their amino acid compositions and subunit sizes are quite similar. The active site of rabbit muscle aldolase (the prototype of class I enzymes) has been partially characterized, whereas the active site of yeast aldolase (the prototype of class II enzymes) has not been characterized except for the finding that enzyme-

bound Zn^{++} is catalytically essential. As a further probe of the active sites of fructose diphosphate aldolases, "affinity labeling" with haloacetyl phosphates is being attempted.

Under mild conditions, chloro- and bromoacetyl phosphate, reactive analogs of dihydroxyacetone phosphate, inactivate yeast fructose diphosphate aldolase by alkylation of about one SH group per molecule of catalytic subunit. Protein side chains other than SH are not modified, since the amount of reagent incorporated is equivalent to the loss of SH groups. The observed stoichiometry, the kinetics of inactivation, and substrate protection data suggest that the SH group alkylated by these reagents is in the active-site region of this class II aldolase.

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ISOLATION AND CHARACTERIZATION OF ARGINYL tRNA SYNTHETASE FROM *E. COLI*

J. G. Farrelly, I. Lucille Norton, C. D. Stringer, and F. C. Hartman

As a preliminary step to long-range studies of the physical, chemical, and physiological properties of aminoacyl-tRNA synthetases, we have developed a convenient purification procedure for arginyl-tRNA synthetase from *E. coli*. The enzyme (obtained in about 30% recovery from the initial cellular extract) is at least 90% pure, based on analytical disc gel electrophoresis, and has a specific activity four-fold greater than previously described preparations. Another indication that our material is substantially purer than earlier preparations is that our enzyme's amino acid composition, which is highly reproducible, bears little resemblance to a previously published composition. The molecular weight of arginyl-tRNA synthetase is about 84,000, as determined from amino acid analysis and acrylamide electrophoresis in the presence of sodium dodecyl sulfate. We are currently attempting to design active-site-specific reagents for this enzyme.

TRIOSE PHOSPHATE ISOMERASE FROM YEAST . CRYSTALLIZATION AND PRELIMINARY X-RAY DATA

S. W. Hawkinson, C. H. Wei, F. C. Hartman, and J. R. Einstein

Triose phosphate isomerase from yeast has been crystallized previously (1) as tiny needles unsuitable for X-ray work. We have crystallized the enzyme in two ways. In microdiffusion cells (2), in which the ammonium sulfate concentration was raised from 40% to 65 or 75% saturation during a period of about a month, crystals appeared after several weeks as prisms of diamond-shaped cross section, some with a length greater than 0.5 mm. Crystals were obtained at pH 8.5 but not at pH 6 or 7. Alternatively, solid ammonium sulfate or buffered ammonium sulfate solutions were added

directly to enzyme solutions. Crystals appeared in 5 days, some with a length of 0.25 mm.

X-ray precession photographs, obtained with an Elliott rotating-anode generator and a copper target, have shown that the diffraction pattern extends beyond 3-Å spacings. The crystals are orthorhombic, with unit-cell dimensions approximately $160 \times 63 \times 47 \text{ \AA}$ and a unit-cell volume of $474,000 \text{ \AA}^3$. The space group is either $P222$, $P2_122$, $P2_12_12$, or $P2_12_12_1$; the assignment awaits further X-ray work at high resolution. In any case, there are four asymmetric units per unit cell. Calculations of the solvent content indicate that the asymmetric unit contains one molecule of triose phosphate isomerase (mol. wt. 56,000). There are, then, four molecules per unit cell, and the solvent content is about 40% by volume. Efforts are planned to improve the crystallization and to find isomorphous heavy-atom derivatives.

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THE CRYSTAL STRUCTURE OF 2-THIOURIDINE

S. W. Hawkinson and B. C. Pal

Derivatives of 2-thiouridine have been isolated from several tRNA species. Recently, the anticodon sequence of GAA-specific glutamic acid tRNA from yeast was determined (1) and shown to have a 2-thiouridine derivative in the first letter of that anticodon.

The compound crystallizes from water in the monoclinic space group $P2_1$, with cell parameters $a = 5.049(2)$, $b = 7.526(2)$, $c = 14.050(3) \text{ \AA}$, $\beta = 90.17(2)^\circ$, and $d(\text{calc}) = 1.619 \text{ g cm}^{-3}$ for $Z = 2$, as compared to $d(\text{obs}) = 1.62 \text{ g cm}^{-3}$. The intensities of 1334 reflections were measured with an Oak Ridge Computer-Controlled X-Ray Diffractometer to a limit of $\sin \theta/\lambda = 0.65 \text{ \AA}^{-1}$ using Nb-filtered $\text{MoK}\alpha$ radiation. The structure was solved directly from a sharpened Patterson function, calculated with $(E^2 - 1)$ coefficients. After four cycles of least-squares refinement, all the hydrogen atoms were located in a difference Fourier. Subsequent refinement, without absorption or extinction corrections, has yielded $R(F) = 0.024$ for all data, and positional e.s.d.'s of 0.002 \AA .

The bond lengths and angles in the structure agree well with those found in studies of other pyrimidine nucleosides. The conformation of the sugar ring relative to the base is *anti*, with a torsion angle of -17° between the $\text{O1}'\text{-C1}'$ bond and the N1-C6 bond (see Fig. 1). The sugar exists in the 3'*endo* conformation. The $\text{O5}'\text{-C5}'$ bond is *gauche* to $\text{C4}'\text{-O1}$ and *trans* to $\text{C4}'\text{-C3}'$ (torsion angles of 74° and -169° , respectively).

The most striking feature of the crystal structure is the base-linked hydrogen bonding between N3-H and O4 ($\text{N3}\dots\text{O4}$ distance = 2.89 \AA), which forms an infinite ribbon parallel to b , as in the structure of 5-chlorouridine (2).

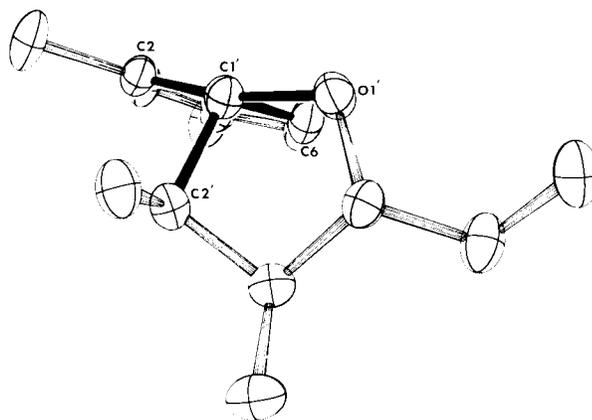


Fig. 1. The structure of 2-thiouridine viewed down glycosidic bond $\text{C1}'\text{-N1}$, showing the relative orientation of the sugar (foreground) to the base.

The ribbons are held together by a hydrogen-bonding system, $\text{O2}'\text{-H}\dots\text{O3}'\text{-H}\dots\text{O5}'\text{-H}\dots\text{S}$, involving three molecules. The lengths of these bonds are: $\text{O2}'\text{-O3}' = 2.69$, $\text{O3}'\text{-O5}' = 2.78$, and $\text{O5}'\text{-S} = 3.35 \text{ \AA}$.

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THE CRYSTAL STRUCTURE OF 1-METHYL-4-THIOURACIL

S. W. Hawkinson and B. C. Pal

The structure of 1-methyl-4-thiouracil, a minor base from *E. coli* tRNA, has been determined by three-dimensional X-ray analysis in order to provide accurate dimensions for the 4-thiouracil moiety and to give some additional data on the $\text{N-H}\dots\text{S}$ hydrogen-bonding system (1).

The compound crystallizes from water as pale yellow monoclinic prisms, in the space group $P2_1/c$ with cell dimensions $a = 6.757$, $b = 13.618$, $c = 7.010 \text{ \AA}$, and $\beta = 99^\circ 22'$. The density calculated for four molecules per cell is 1.481 , as compared to the observed density of 1.484 g cm^{-3} . The structure was solved directly from a sharpened Patterson function, using data obtained by standard diffractometer techniques. The final refined structure, including hydrogen atoms, yielded an R -value of 0.031 for the 920 significant data.

The molecular parameters determined are fairly standard for pyrimidine bases. The bond length from C4 to S is 1.67 \AA . The molecules are hydrogen-bonded in pairs about centers of symmetry with N to S distances of 3.33 \AA for the $\text{N3-H}\dots\text{S}$ hydrogen bond. This arrangement is analogous to the hydrogen-bonding scheme in 1-methylthymine (2) with sulfur replacing oxygen and with the bond lengthened from the

more normal 2.83 Å. This lengthened hydrogen bond may have important consequences in the roles played by minor sulfur-containing nucleosides in tRNA.

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THE CRYSTAL AND MOLECULAR STRUCTURE OF 2-THIOISOURIDINE

Lois M. Jacob,* B. C. Pal, and J. R. Einstein

2-Thio-(3-β-D-ribofuranosyl)-uracil, commonly called 2-thioisouridine, is a nucleoside that has not yet been found in nature. However, the structure has unusual features, which enlarge our knowledge of nucleoside stereochemistry.

Crystals of 2-thioisouridine, obtained by evaporation of solutions in absolute ethanol, have the space group $P2_12_12_1$ with unit-cell dimensions $a = 19.919(4)$, $b = 7.610(2)$, $c = 7.467(1)$ Å, and four molecules per unit cell. Counter data were recorded using Nb-filtered $MoK\alpha$ radiation for 1528 reflections of minimum spacing 0.68 Å. The structure was solved by the heavy-atom method and refined by full-matrix least squares to an R factor of 0.046. The bond lengths (average estimated standard deviation = 0.004 Å) and angles (average e.s.d. = 0.3°) for the C, N, O, and S atoms agree quite well with those of other nucleosides.

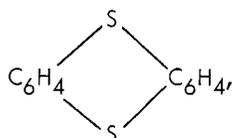
Three unusual conformational features were found. (1) The torsion angle X around the glycosidic bond is 74°, a value differing by 30 or 40° from a typical anti conformation. (2) The pyrimidine ring is definitely nonplanar, with out-of-plane deviations of 0.1 Å for C(4) and 0.2 Å for O(4). (3) The sugar ring is C(4') *exo*, a conformation never previously found for a β nucleoside, in which the sugar conformation is normally C(2') or C(3') *endo*. All of these anomalies have been attributed to the crowding of the sugar ring by the two exocyclic atoms adjacent to the glycosidic bond, S and O(4). It is interesting to speculate whether internucleoside crowding in a molecule such as tRNA might result in any similar effects.

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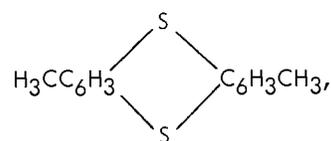
THE CRYSTAL STRUCTURE OF 2,7-DIMETHYLTHIANTHRENE, AN ANTIPARASITIC AGENT

C. H. Wei

The crystal and molecular structure of thianthrene,



has been determined previously by X-ray structure analysis (1). Because of the tendency for the sulfur atoms to retain their tetrahedral valency angles, thianthrene possesses a configuration folded along the S—S line. Structural analysis of similar 2,7-dimethylthianthrene,



known to be an effective scabies-treating medicine, by the single-crystal X-ray diffraction method was carried out to provide more accurate stereochemical information (including those of all the hydrogen atoms) on the nature of this heterocyclic system, and to provide a comparison of its molecular architecture, S—C_{sp2} distances in particular, with those of closely related compounds.

The compound crystallizes with four molecules in an orthorhombic unit cell of symmetry $P2_12_12_1$ and parameters $a = 8.175(2)$, $b = 11.351(2)$, and $c = 13.197(2)$ Å. The structure was solved by the heavy-atom technique and was refined by the full-matrix least-squares method to $R(F) = 3.1\%$ based on 1282 independent intensity data. The molecule possesses an idealized C_2 symmetry, with all the non-hydrogen atoms approximately in two planes that intersect along the S—S line to form a folded configuration with a dihedral angle of 131.1°. By comparing the average S—C_{sp2} distance of 1.764(2) Å with those of other related compounds, it seems safe to conclude that the S—C_{sp2} distance is lengthened slightly by the order of 0.01 to 0.02 Å as the coordination number of the sulfur atom is increased from 2 to 3 in this group of heterocyclic compounds. The maximum individual standard deviations for bond distances and bond angles involving carbon atoms are 0.005 Å and 0.3°, respectively.

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THERMALLY INDUCED CHAIN BREAKAGE IN MERCURATED DNA

R. O. Rahn and Jay Hirsh*

Mercuric ions interact with the bases of DNA and form cross-links between the strands. These cross-links prevent strand separation, as measured optically, even upon heating for more than 15 min at 100°C. However, exposure of DNA to elevated temperatures for extended periods of time results in a loss of transforming activity and a decrease in the intrinsic viscosity. To explain these results, we measured the number of chain breaks formed in mercurated and nonmercurated DNA following heating and tried to correlate these changes with the decrease in viscosity.

In mercurated, denatured DNA, seven breaks per 10^6 daltons were formed in 60 min at 100°C, as determined by centrifugation in an alkaline sucrose gradient. In nonmercurated, denatured DNA, only one break per 10^6 daltons was formed upon similar heat treatment. Hence, Hg(II) greatly accelerates the breakdown of denatured DNA. In native DNA containing mercuric ions, the number of breaks upon heating was about half that for denatured mercurated DNA.

Of these breaks, about 25% were due to double-strand chain breaks, as determined by centrifugation in a neutral gradient. We feel that these double-strand breaks are mainly responsible for the observed decrease in viscosity and either are caused by mechanical shear or else are due to a single-strand hydrolysis mechanism in which the formation of a single-strand break on one strand aids in the formation of a break on the opposite strand. Such a mechanism might have as its first step the loss of a purine from one strand, and this could be aided by complexing with Hg(II).

*Student trainee.

CHAIN BREAKAGE DURING PHOTOSENSITIZATION OF DNA

R. O. Rahn, W. L. Carrier, L. C. Landry, and Claude Helene*

The relative efficiency of thymine dimer production by UV irradiation of DNA at 313 nm in the presence of acetone, acetophenone, or benzophenone was compared with the number of chain breaks produced. At a concentration of sensitizer giving an absorbance at 313 nm of 0.2, an exposure of 1×10^4 erg mm⁻² converted 0.6% of the thymine to thymine-thymine dimers, regardless of the sensitizer used. The number-average molecular weight of the DNA strands after this photosensitization procedure was determined by sedimentation of the DNA in alkaline sucrose gradients. The decrease in molecular weight was the same whether the DNA was analyzed in an alkaline gradient or first denatured with formamide and then analyzed in a neutral gradient. Hence, the chain breaks observed are not dependent upon the alkaline conditions. We observed one chain break for every six dimers with benzophenone as the sensitizer, whereas for both acetone and acetophenone there was one break for every 60 dimers. Oxygen had no large effect on chain breaking for either benzophenone or acetophenone. Sensitivity to chain breaking was an order of magnitude greater in denatured DNA than in native DNA. The mechanism for chain breaking is thought to involve an attack by an OH radical at the deoxyribose moieties. Such a radical is produced when the triplet state of benzophenone abstracts a hydrogen from water, forming a ketyl radical ($\text{O}_2\text{-}\dot{\text{C}}\text{OH}$) and a hydroxyl radical (OH•). Presumably, the deoxyribose moieties are much more susceptible to attack by OH• in denatured DNA than in native DNA.

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ULTRAVIOLET MICROSCOPY OF CHROMOSOMES STAINED WITH MERCURIC IONS

R. O. Rahn, R. F. Kimball, Stella W. Perdue, and J. G. Brewen

When mercuric ions, Hg(II), bind to DNA, the absorbance maximum is shifted from 260 to 280 nm. We used a UV microscope to examine marsupial chromosomes fixed to a quartz slide and then soaked in HgCl₂. The UV character-

istics of these chromosomes reveal that binding of Hg(II) to the DNA has occurred. In some cases, preferential binding of Hg(II) seems to have taken place at the ends of certain chromosomes. We are currently examining easily recognizable chromosomes from Chinese hamster cells treated with Hg(II), to see whether there are well-defined changes in the UV absorbance profile and whether these changes offer an opportunity to resolve structural features of the chromosomes.

EXCITED-STATE PROPERTIES OF HEAVY METAL-NUCLEIC ACID COMPLEXES

R. O. Rahn and L. C. Landry

There is currently considerable interest in the mode of interaction of heavy metals such as lead, mercury, and cadmium with cellular constituents. Ions of these metals, along with those of silver and copper, interact quite strongly with the bases of nucleic acids. We have examined a number of physical properties of nucleic acid-heavy metal complexes in order to learn more about the nature of the interaction and also to examine the usefulness of these heavy metals as tools for varying the photochemistry and excited-state properties of the nucleic acids. We have demonstrated previously (1) that mercuric ions, when bound to the bases of DNA, have a very large effect on the luminescence and photochemistry of DNA. More recently, we have carried out similar experiments with silver ions, Ag(I), which also bind to the bases.

We summarize our results as follows: (1) When silver ions bind to the nucleic acids, there is complete quenching of fluorescence and a large enhancement of phosphorescence. These results are due to the well-known "internal heavy-atom effect" in emission spectroscopy. (2) Silver ions enhance the rate of dimerization in DNA by as much as 28 times, depending upon the DNA used. (3) Silver ions drastically reduce the rate of photohydration in poly(U) and poly(dU) but do not enhance the rate of dimerization. (4) The binding of Ag(I) to DNA appears, from the luminescence and photochemical data, to be preferentially at the thymine-rich regions, as with Hg(II). (5) Spectrophotometric titrations, in contrast, suggest that (G-C)-rich regions bind Hg(II) the strongest. These results suggest that binding to (G-C)-rich regions in DNA sensitizes triplet states of thymine, which can then phosphoresce or undergo photochemistry.

In summary, Ag(I) has been shown to have a tremendous effect on the luminescence and photochemistry of nucleic acids and should prove to be a very useful tool in investigating the excited-state properties of these systems.

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INFLUENCE OF Ag(I) ON THE ULTRAVIOLET SENSITIVITY OF TRANSFORMING DNA AND VIRUSES FROM HAEMOPHILUS INFLUENZAE

R. O. Rahn and Jane K. Setlow

The UV inactivation of *Haemophilus influenzae* transforming DNA is strongly correlated with the formation of

pyrimidine dimers. Since silver ions were found to enhance the rate of thymine dimerization in DNA 30-fold, it was of interest to see what effect Ag^+ would have on the UV inactivation of transforming DNA. We have previously shown that mercuration decreases the sensitivity of transforming DNA to UV inactivation. No loss of transforming ability occurs following treatment of the DNA with Ag^+ . Irradiation of transforming DNA in the presence of Ag^+ results in an increase in the UV sensitivity by a factor of 30. Enzymatic photoreactivation of irradiated DNA indicates that Ag^+ enhances both photoreactivable and nonphotoreactivable biological damage to the same extent.

H. influenzae virus HPI₁C1 was also irradiated with Ag^+ present, and its UV sensitivity was found to be greatly enhanced by the heavy metal. This virus was found to be sensitive to inactivation by Hg(II), in contrast to T4B virus, which is not inactivated by Hg(II), so the complementary experiments with Hg(II) were not performed with the HPI₁C1 virus.

We conclude that heavy metal ions such as Ag(I) and Hg(II) can serve as useful tools for varying the UV response of viruses and transforming DNA.

MECHANISM OF INACTIVATION OF TRANSFORMING DNA BY SONIC RADIATION

M. L. Randolph and Jane K. Setlow

As a complement to previous X-ray and UV studies (1, 2) on the mechanism of inactivation of Haemophilus influenzae transforming DNA, we have studied the mechanism of inactivation by sonic irradiation. Our ultimate goal in such studies is to understand the molecular mechanisms by which transforming DNA works, which we can infer indirectly from the mechanisms of inactivation.

Using wild-type Haemophilus cells and transforming DNA that had been subjected to sonic irradiation, we measured the reductions in genetic transformation of the cells to drug resistance, cellular uptake of the transforming DNA, incorporation of the transforming DNA into the genome, and double- and single-strand lengths of the irradiated DNA. The bacterial strains, DNA preparations, and techniques employed were essentially as described previously (2), except for the sonication. For these experiments, radioactively labeled transforming DNA at about 4°C was subjected to 10-kHz irradiations of 3 sec to 18 min duration in a Raytheon Model D F-101 sonicator. Sonicated materials were frozen for storage.

We find, as we found previously for X-irradiation (2), that radiation sensitivities for transformation, integration, and double-strand breaking are roughly comparable and much greater than for uptake. This implies that the mechanism of sonic inactivation of transforming DNA is the production of broken double strands which, although taken into the cell, are not integrated into the DNA of the recipient cell and hence cannot transform the cell.

Using the model of Guild et al. (3), fits of our data for transformation and weight-average double-strand molecular weights suggest 0.2–0.25 switches per million daltons and a

minimum mass of transforming DNA of about 5×10^5 daltons. Guild et al. (3) reported corresponding values of 0.2–0.3 switches per million and 400 ± 100 nucleotides or about 2.7×10^5 daltons, respectively.

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QUALITATIVE TESTS OF A GENERALIZED TARGET THEORY APPLICABLE TO INDIRECT EFFECTS

M. L. Randolph

Although indirect effects of ionizing radiations are at least as likely as direct effects, there has been no generally recognized theory for indirect effects comparable to target theory for direct effects. To fill this void and, initially, to provide a common basis for understanding the differences in radiobiological effects of different ionizing radiations, a generalized target theory applicable to indirect effect is proposed, and qualitative predictions from it are compared with experimental results. This is an extension of previous work (1).

A biological effect is assumed to occur whenever more than a characteristic minimum energy is deposited within a crucial (spherical) mass surrounding the actual biological target. Microdosimetric concepts are inherent in the formulation. Survival, S , of the tested function is

$$S = \int_0^{\infty} P[Z(d)] dZ(d),$$

where $Z(d)$ is the energy per unit mass deposited in a (microscopic) sphere of diameter d , and $P(Z)$ is the probability that a sphere will receive energy density $Z(d)$.

Qualitatively, application of the theory can be used to predict such observable biological results as bell-shaped curves of relative biological effectiveness (RBE) versus linear energy transfer (LET); decreased effectiveness of low-LET and, to a lesser extent, high-LET radiations at low dose rates; minimum radiosensitivities at low doses of various radiations regardless of dose rate; lesser effects of protective compounds and smaller oxygen effects for high-LET radiations; decreasing RBE with increasing dose; and reasons to expect complex dose-effect curves in certain circumstances, which are more likely for low- than high-LET radiations. Physical and radiobiological data still seem too fragmentary for rigorous quantitative analyses by this approach.

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QUANTITATIVE CONSIDERATIONS IN ELECTRON SPIN RESONANCE STUDIES OF BIOLOGICAL MATERIALS

M. L. Randolph

Absolute measurements of electron spin resonance (ESR) concentrations are needed for many studies, such as determinations of photochemical quantum yields, determinations of radiochemical G values, and studies of free-radical kinetics. We have analyzed the significance of instrumental and experimental variables used in current ESR spectroscopy to determine spin concentrations.

In doing such work one must first decide what accuracy is required. When samples with identical shape, saturation characteristics, and geometry are compared in a dual cavity, accurate relative values may be obtained from measurements of derivative peak heights. If, however, the samples compared have dissimilar geometry, saturation characteristics, or line shape, or if absolute values are needed, more sophisticated calculations must be made, and more attention must be given to such factors as scan width, microwave power, modulation, instrumental drifts, and seemingly small measurement errors. Equations and typical calculations of how these factors affect absolute quantitative measurements have been set down.

Spin concentrations can be estimated by second-integral, first-moment, or corrected second-integral calculations from the usually presented derivative output of an ESR spectrometer. The uncertainty of the results depends in different ways on possible systematic and random signal errors, with the corrected second integral being the most reliable method.

The best compromises in instrument settings differ for quantitative work and maximum sensitivity or determination of line shape. For quantitative work, scan width must generally be greater than for other work, but restrictions of low power and modulation can be relaxed. Scan rate and dispersion are relatively unimportant. Although dual cavities reduce many instrumental problems, they do not eliminate all that one might expect.

The ideal quantitative ESR standard is a stable material with a well-known number of spins, having line shape, width, and power saturation characteristics similar to those of the test sample. However, although a variety of standards have been proposed, a single universal standard seems impractical.

General understanding of these problems should enable workers to appraise realistically the importance of various parameters of quantitative measurements and hence to obtain more accurate concentration values.

INTRAMOLECULAR TRIPLET-TRIPLET ENERGY TRANSFER IN POLYADENYLIC ACIDS. THE INFLUENCE OF TRIPLET ENERGY TRAPPING ON THE DECAY KINETICS OF DELAYED FLUORESCENCE PRODUCED BY A TRIPLET-TRIPLET ANNIHILATION

Claude Helene* and J. W. Longworth

A delayed fluorescence is observed from both poly-riboadenylate and polydeoxyriboadenylate helices, but not

from a random conformer of poly(rA). The delayed fluorescence depends approximately on the square of the triplet concentration and is believed to originate from a triplet-triplet annihilation occurring within the polymer helices. The decay of the delayed fluorescence is nonexponential, with a short-lived component of 70-msec decay and a longer-lived component of 670 msec. Increasing temperature from 77°K decreases the fractional contribution of the short component. The failure of phosphorescence intensity to increase linearly with exciting-light intensity and its having a shorter rise time to steady-state levels than decay indicate that the emission occurs from an exciting-light-saturable site. As there was no evidence for a depletion of the ground state by optical pumping to the triplet level at light intensities that gave appreciable reduction in rate of approach to steady state, all the phosphorescence emissions must originate from an intrinsic lower-energy trap site. Previous studies of the Stokes' shift of phosphorescence from the polymers have found an unexplained addition to the Stokes' shift of 15 cm^{-1} , which is not present in oligomer helices. These observations were consistent with a simple trapping model for the production of delayed fluorescence. The triplet excitons become rapidly trapped by these lower-energy sites, and delayed fluorescence is formed by thermal activation to the polymer host level, followed by a diffusive migration and annihilation with a trapped triplet exciton. This model is fully consistent with the quenching of phosphorescence by transition metals, where, although extensive reduction in phosphorescence yield occurred, no alteration in lifetime was found.

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MEASUREMENT OF NANOSECOND FLUORESCENCE LIFETIME BY SINGLE-PHOTOELECTRON TIMING BY A DELAYED COINCIDENCE

J. W. Longworth, R. M. Pearlstein, and S. S. Stevens

We are interested in two disparate spectral regions: the ultraviolet, where proteins and nucleic acids emit, and the visible red, where photosynthetic organelles fluoresce. Each brings special problems. The UV region requires a subnanosecond light flash where there is an intense quartz UV output, and the red region requires a high-speed photomultiplier with a high sensitivity to red light. These demands can now largely be accommodated.

The research is directed primarily to timing processes with relaxations between 0.1 and 10 nsec, a previously unexplored region of operation for photomultipliers detecting single photons. Yet this is the time interval imposed by the known lifetimes of proteins, nucleic acids, and photosynthetic organelles. The research described in the subsequent reports demonstrates that, although there are serious difficulties to operating in this time region, from our understanding of the nature of the performance of photomultipliers it is feasible to determine relaxations with decay constants less than 10 nsec.

Our prime interest is in decomposing nonexponential fluorescence decays into constituent parts and identifying

each component with the individual source of emission, e.g. a specific tryptophan of a protein or the reaction center of a photosynthetic unit. The nonexponential decays arise from multiple emission sites, which are the rule with biological macromolecules and organelles. A second phenomenon that shows nonexponential relaxation in this time domain is the time dependence of fluorescence anisotropy. Analysis of the time dependence provides direct information (previously unavailable) on the dynamics of electronic energy transfer within macromolecules. This phenomenon is of direct concern to nucleic acid photochemistry, photosynthesis, and the visual response, and it greatly complicates any application of fluorescence procedures in enzymatic function studies. Another application is to determine macromolecular rotatory diffusive relaxation rates, and a common development is to investigate macromolecular associations and interactions with such a procedure; e.g., immunoglobulins and nucleic acid-enzyme associations are now being studied with these procedures, although observations are limited to events longer than 10 nsec.

A procedure has been developed to measure nanosecond luminescent decays with standard commercial nuclear instrumentation modules. We have developed instrumentation to utilize this method for measuring fluorescence delays with decay constants less than 10 nsec. The method detects the time when a single photon is emitted by a system previously excited by a flash of light lasting less than 1 nsec. Repetition of the brief light flash builds up within a computer memory a time histogram of the probability density function for the illumination process. Statistical considerations show that this is equal to the intensity decay of the fluorescence excited by the light flash only when single photons are detected.

Crucial elements in the system are (1) producing a subnanosecond light flash, (2) detecting a single photon with a sufficiently rapid responding photodetector (a high-speed photomultiplier is the only choice available), and (3) deriving a precise timing pulse from the photomultiplier anode output pulse with a timing discriminator. These features are described in the following four individual reports.

Our demonstration of a bimodal time response for the single-electron response of a high-speed photomultiplier greatly modifies previous studies in the region of 1-10 nsec. A clear example is provided by previous photosynthetic organelle lifetime studies that failed to account adequately for the presence of a late pulse. ORTEC and Bendix Corporations have obtained preliminary indication that single-channel secondary multiplication does not produce late pulse outputs. We will continue to consult with ORTEC and encourage the development of a photomultiplier by Bendix that will have a stack of channels — a channel-plate secondary multiplier. Such a tube will have a 100 psec response, comparable efficiency to photomultipliers now available, and an excellent pulse-height response. As a result of this superior performance, widespread application to all scintillation counting — timing, pulse height, and pulse shape — is to be expected. The considerable need for a photomultiplier with these features has become clear from our investigation of the speed of response of the photomultipliers currently available. The procedures we have devised make it possible to analyze the performance of a photomultiplier at times less than 10 nsec.

CONSTANT FRACTION OF PULSE-HEIGHT DISCRIMINATION OF ANODE PULSES FORMED BY AN RTC XP 1023 PHOTOMULTIPLIER

S. S. Stevens and J. W. Longworth

The single-electron pulses formed at the anode of an RTC XP 1023 photomultiplier have a pulse-height variation of 10:1 because of the variance in the gain of secondary multiplication. To operate fast timing electronic circuits, it is necessary to derive from these variable-height pulses a precisely time-correlated standard-height pulse. Discriminators are used to perform this function, but clearly the normal integral discriminator that functions with a fixed level will be subject to severe timing walk from the variation in pulse height. A method that significantly improves the situation depends upon obtaining a level that is a fixed fraction of pulse height and so is no longer dependent on the pulse height. This is readily achieved by summing the pulse with an inverted and attenuated version of the same pulse, where the delays are adjusted to allow the formation of a bipolar pulse at the leading edge of the unattenuated pulse. A defined zero crossing occurs at the same point, independent of pulse-height variation. Commercial constant-fraction discriminators are unsatisfactory, because they have poorly shielded power inputs and cannot operate in the neighborhood of our spark discharge. We have constructed a constant-fraction bipolar pulse by mixing dynode and anode pulses; the positive dynode pulse precedes the anode pulse, which is sevenfold greater in amplitude. To detect the zero crossing we use a differential discriminator in the so-called low-level timing mode. The low-level discriminator functions as a fast zero crossing discriminator, and the high-level discriminator gates its output so that only single-electron pulses produce outputs. Significant improvement in timing is obtained for the XP 1023.

BIMODAL TIME DISTRIBUTION OF THE SINGLE-ELECTRON RESPONSE OF HIGH-SPEED PHOTOMULTIPLIERS

J. W. Longworth and S. S. Stevens

Two generic photomultipliers produced by RTC, a 56 DUVP and an XP 1023, were investigated together with two generic tubes manufactured by RCA, an 8575 and an 8850. The single-electron response, the impulse response for these photomultipliers, was determined by the delayed coincidence timing method, using a spark discharge in air for the illumination impulse. A bimodal time distribution of anode output pulses was observed. The majority of pulses formed at the anode of the RTC tubes appear in a distribution with a full width half maximum (FWHM) of 1.6-1.8 nsec, but a fraction (1-5%) appear later (9-12 nsec) with an equivalent FWHM for their distribution. A fraction of the anode outputs are formed 9-12 nsec later than the majority, and we term these pulses "late pulses." The frequency of late pulsing is not dependent on photon energy between 298 nm and 680 nm. Any alteration of the electrooptic focusing lens

for the secondary multiplier entrance from the recommended potential values greatly enhances the probability of late pulsing.

The RCA 8575 and 8850 tubes also late pulse, but the phenomenon is distinct from that observed with RTC tubes. The probability of late pulsing is less (< 1%), and the pulses are formed later (21–25 nsec) and appear in a far broader distribution of times than their majority pulses. The single-electron response of the RCA 8850 was 1.5–1.7 nsec, and the FWHM of the late pulse distribution was 6–10 nsec.

The mechanism for and the origin of the late pulses are not known.

A NANOSECOND DIGITAL AUTOCORRELATION OF SINGLE-ELECTRON PULSES FORMED RANDOMLY BY PHOTOMULTIPLIERS

J. W. Longworth and S. S. Stevens

Late pulsing by high-speed photomultipliers leads to a bimodal time distribution for the single-electron response. We wished to demonstrate the presence of late pulses by a method that does not utilize an impulse illumination, so we chose to perform an autocorrelation of the single-electron height background pulses formed within photomultipliers, largely by thermionic emission from the photocathode. This required the implementation of a digital autocorrelator with nanosecond resolution, and this was achieved through a gated fast coincidence. An individual anode pulse was used to trigger a gate pulse that has a fast transition (1 nsec). The gate pulse, 50 nsec to 1 μ sec, is applied to a fourfold fast coincidence (limiter type) together with the anode pulses. Fixed delays were set to place the anode pulse that triggered the gate to be in anticoincidence to the gate output but triggered all subsequent pulses formed by the photomultiplier to be in coincidence. The coincidence output was discriminated, and the fast logic pulse output was used to stop a time-to-amplitude converter that had been previously started by the discriminator fast logic output that generated the gate pulse. A pulse-height analyzer accepted conversion from the time-to-amplitude converter. An autocorrelation builds up in the memory, where the initial 5 nsec is not resolved. Correlations are found at 9–12, 28, 300, and 600 nsec. The principal distribution is exponential, with the slope set by the mean anode pulse rate. Single-channel analysis showed that the 9- to 12- and 28-nsec pulses are single height, whereas the 300- and 600-nsec correlations are multiple height. These large pulses are due to positive-ion feedback to the photocathode from the secondary multiplier entrance, where the entrance electrooptical lens functions as a time-of-flight analyzer. The 28-nsec correlation is caused by an anode scintillation feedback optically to the photocathode and is characterized by being equal in decay to the transit time of the phototube. The late pulses clearly account for the 9- to 12-nsec correlations.

This digital autocorrelation is a very powerful method to analyze noise statistics from photomultipliers and clearly will become a widely used method for investigation of correlations in phototube anode pulses in the 5 nsec to 80 μ sec

range covered by the time-to-amplitude converter analog sweep. A particularly obvious application is to determine macromolecular rotatory relaxation from depolarized Rayleigh-scattered laser light, utilizing a clipped single-photon autocorrelation procedure.

PULSE-HEIGHT DISTRIBUTION OF PROMPT AND LATE PULSES FORMED IN FAST PHOTOMULTIPLIERS

S. S. Stevens and J. W. Longworth

We compared the pulse-height distribution of prompt and late pulses formed at the anode of an RTC XP 1023 photomultiplier. A nanoflash was used to form a single-electron pulse at a defined time, and a trigger derived from the spark discharge was used to initiate a fast-transition gate pulse. The gate pulse was delayed to be in coincidence with the anode pulse at a fast coincidence. Coincidence outputs were discriminated and subjected to ratio scaling with nanoflashes (gate trigger fast logic outputs). The gain of the secondary multiplier was varied by altering the dynode potential, and an integral bias curve was collected. Identical integral bias curves were obtained when the coincidence delays were set to scale prompt and late pulses. The late pulses have a single-electron pulse-height distribution.

ILLUMINATION IMPULSES OF AN 11pF CAPACITOR SPARK DISCHARGE IN AIR

S. S. Stevens and J. W. Longworth

A subnanosecond flash of light is required to excite proteins and nucleic acids, to allow measurement of their fluorescence decay behavior. We charged a coaxial 11pF capacitor to 5–15 kV and investigated its spark discharge in air at 50 pascal pressure. A sampling oscilloscope was inductively coupled to the potential collapse and indicated that only a single current pulse accompanies the gap breakdown, with a full width half maximum (FWHM) of approximately 1 nsec. The illumination impulse was determined with the single-photoelectron method of delayed coincidence timing. Light from the spark was dispersed through a monochromator and scattered by colloidal quartz onto the faceplate of an end-window fast photomultiplier (RTC XP 1023 or RCA 8850). The spark discharge light is predominantly from prominent band heads and their rotational components from the $C^3\pi_u \rightarrow B^3\pi_g$ transition of the second positive system of nitrogen located at 316, 337, and 358 nm and an $O\ III\ 4p^5P_2 \rightarrow 3d^3D_2$ transition of oxygen at 298 nm. A weak plasma continuum occurs, peaking below 290 nm. The FWHM of the illumination impulse was 1.7 nsec for the prominent lines of N_2 and O_2 , but the quartz UV continuum decayed at 15 nsec.

Positron-electron annihilation gammas produced after a positron decay of ^{22}Na were used as a source of coincident excitation. The single-electron response of the stop photomultiplier, an RCT XP 1023, was measured. A Naton 111 scintillator coupled to an RCA 8575 was used to obtain the starts; stop photoelectrons were derived from Cerenkov light

generated within the faceplate of the tube from energetic secondaries formed in Compton scatters of the 511-keV annihilation gamma. The single-electron response had an FWHM of 1.7 nsec. Hence, we concluded that the FWHM of the illumination impulse for the nanoflash discharge was less than 1 nsec.

THE FLUORESCENCE LIFETIME OF CHLORELLA

J. W. Longworth, S. S. Stevens, W. A. Arnold,
J. R. Azzi, R. M. Pearlstein, and G. R. Welch*

The lifetime of Chlorella fluorescence has previously been investigated with single-photoelectron detection methods. Two relaxation paths were suggested to account for the observed data. But previous workers were not aware that the fast phototubes of RTC give late pulse outputs, so we were prompted to reinvestigate the phenomenon using high-resolution phototubes and circuits. No difference could be observed between the single-electron response of the red-sensitive RCA 8850 tube we used to detect chlorophyll fluorescence from Chlorella and the single-electron response obtained from the nanoflasher. We conclude that the fluorescence decay occurs at a rate faster than we can measure readily; moreover, the previous study is incorrect. Recourse will be taken to determine the centroid shift rather than attempt to analyze the relaxation profile. To eliminate a centroid shift associated with photon energy, single-electron responses were obtained from the nanoflasher between 298 and 435 nm, and at 680 nm from fluorescein quenched to 0.01 of its radiative lifetime by iodide (45 psec lifetime). With careful calibration of the time-to-amplitude converter linearity with the Tennelec correlated clock system and a cross correlation of clock-noise pulses, an adequate resolution of the centroid shift is expected.

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PULSE PILEUP REJECTOR CIRCUIT

S. S. Stevens and J. W. Longworth

For protein fluorescence lifetime studies, single-photoelectron timing procedures can be significantly improved in efficiency if multiphoton cycles can be distinguished and rejected (not stored into the pulse-height analyzer memory). We applied a fast-transition gate derived by the first stop pulse detected and applied the gate to a fast coincidence together with the stop tube anode pulses. Any subsequent stop pulses produce a coincidence output, which initiates a delay-gate generator to control the slow-logic anticoincidence gate input of the pulse-height analyzer analog-to-digital converter. Time-to-pulse-height conversions in anticoincidence with the delay-gate outputs are stored; multiple-photon events are rejected. A pulse pair resolution of 5 nsec is reached, set by the gate rise time and anode pulse width. As protein fluorescence lifetimes are of the order of 5 nsec, the pile-up rejector is of no value but performs adequately with longer-

lived fluorescence decays. Implementing a differential coincidence will significantly improve the rejector performance; or, alternatively, utilizing an integrating single-channel analysis of the output of a UV-sensitive phototube with negative-affinity III-IV semiconductive dynodes will permit the use of rejector circuits for these faster times.

THE ISOLATION OF A PHOTOCHEMICALLY ACTIVE MACROMOLECULAR COMPLEX FROM CHLOROPSEUDOMONAS ETHYLICA

C. F. Fowler, N. A. Nugent, and R. C. Fuller

Green photosynthetic bacteria, unlike purple bacteria, contain a photochemical apparatus apparently independent of the bacterial cytoplasmic membrane and with unique physical and chemical properties. From these "vesicles" it has been possible to isolate a photochemically active electron-transport complex. This complex is apparently devoid of light-harvesting "Chlorobium chlorophyll" and contains bacteriochlorophyll a , a carotenoid, cytochromes, and P840. Cytochromes and P840 were found to undergo reversible light-induced absorbancy changes similar to those obtained with whole cells. The measurement of the absorbancy change associated with P840 as a function of redox environment indicates that P840 has a midpoint potential near 0.25 V at pH 7.5. A measurement of the cytochromes suggests that C553 has a midpoint potential near 0.16 V at pH 7.5. The absorbancy change associated with the light-induced oxidation of C560 could not be separated from C553 oxidation unless the redox potential was below 0.0 V. These measurements suggest that the primary donor P840 and its associated cytochrome operate at lower redox potentials in Chloropseudomonas ethylica than P700 in green plants or P890 in purple bacteria (Fig. 1).

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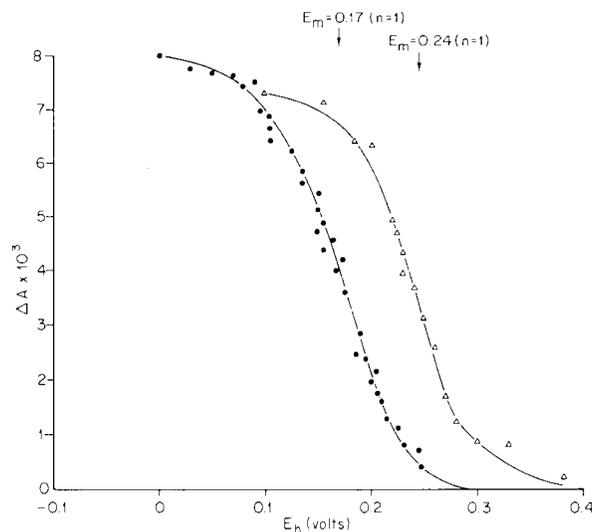


Fig. 1. Redox titration of light-induced oxidation of P840 (Δ—Δ) and its associated cytochrome (●—●) from C. ethylica.

ISOLATION, IDENTIFICATION, AND LOCALIZATION OF THE COMPONENTS OF THE PHOTOSYNTHETIC SYSTEM OF GREEN BACTERIA

N. A. Nugent, C. F. Fowler, N. Rigopoulos, R. C. Fuller, and P. M. Heath*

Isolation of the photosynthetic apparatus of the green bacterium *Chloropseudomonas ethylica* has been accomplished. A photograph of the isolated units negatively stained in phosphotungstic acid (Fig. 1) shows the uniqueness of these

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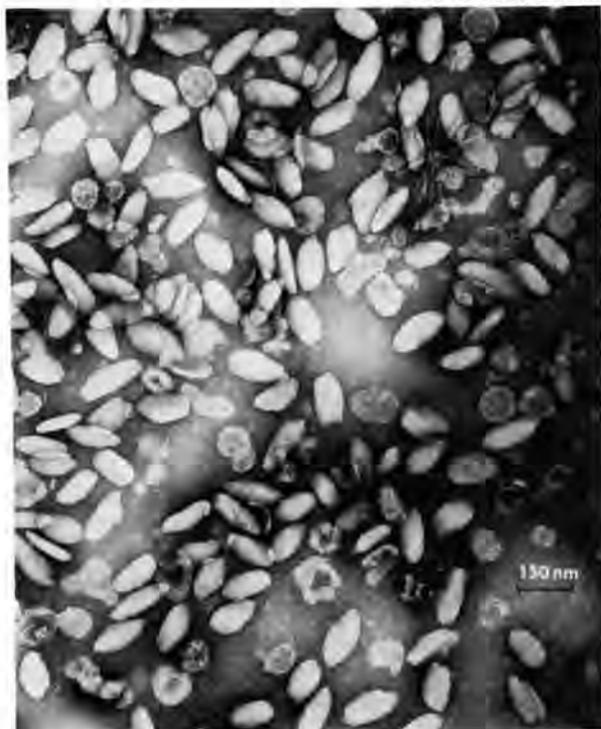


Fig. 1. Isolated photosynthetic apparatus negatively stained in phosphotungstic acid.

structures. In sections, they do not appear to be constructed of bilayer membranes, as is the case in all other classes of photosynthetic organisms. These structures are common to all green bacteria (chlorobacteriaceae) and have been termed "Chlorobium vesicles."

Chemical analysis of these units has shown the presence of bacteriochlorophyll *a* (BChl_a), Chlorobium chlorophyll 660 (Chl 660), γ -carotene and related compounds, cytochrome (C553), ferredoxin, rubredoxin, pteridine, naphthoquinone, and galactosyl glycerides.

The photosynthetic vesicles have been fractionated, yielding a photochemically active macromolecular complex that contains BChl_a, carotenoid, C553, pteridine, and the reaction center P840. The subunit size appears to be $1.5-2.0 \times 10^6$ daltons. The absorption spectrum of this active particle (reaction-center complex) is compared to the spectrum of the inactive particle (light-harvesting complex) also derived from the intact vesicle.

Several pieces of evidence indicate that the photochemically active protein complex is located on the surface of the vesicle. The best evidence was obtained from a study of the isoelectric points of the intact vesicle, as compared to the isolated protein. Both isoelectric points occur at pH 4.5.

Positive proof of the location of the photochemically active complex is currently being sought through the use of an immunological procedure. A key protein-containing bacteriochlorophyll (BchlP) was isolated and purified from the intact system. This protein and the isolated active complex have been injected into rabbits for the purpose of producing antibodies to these components. The antibodies will then be tagged with fluorescein isothiocyanate and cross-reacted with intact vesicles. Ferritin-tagged antibody will be used in conjunction with electron microscopy of thin sections to visualize the location and distribution of the photochemically active centers. To date, antisera to BchlP have been prepared, and the fluorescein tagging of the purified antibody has been accomplished.

The results described have contributed significantly toward a final understanding of the organization and photochemical mechanisms of an evolutionarily unique class of photosynthetic organisms.

*Student trainee.

PHOTOSYNTHESIS

W. A. Arnold and J. R. Azzi

In trying to understand photosynthesis — that is, how green plants reduce carbon dioxide to carbohydrates — we need a detailed picture of what happens between the absorption of a light quantum by chlorophyll and the appearance of oxidizing and reducing power. To develop this picture, we are studying delayed light.

In 1951, a communication from this laboratory described a light emission (delayed light) lasting for some seconds after illumination. We have studied this emission over a time range from milliseconds to hours. In 1970, we found that an electric field across a suspension of chloroplasts will stimulate the emission of delayed light. This stimulation is large, for a field of a few hundred volts per cm can make the delayed light 50 times brighter. The phenomenon is fast; the change in the light emission follows the change in voltage within tenths of milliseconds. This electrical effect gives us a new tool in studying the storage of energy in green plants.

We have three papers in press describing the effect of electric fields on delayed light.

EXCITED-STATE ENERGY TRANSFER. GENERAL THEORY AND APPLICATIONS TO A MODEL SYSTEM

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

Energy transfer in photosynthetic systems, in excited nucleic acids, and in excited proteins is usually described in terms of mobile excited states, or excitons. Work applicable

to specific biological systems is covered in separate reports below. Our previous work (1) on the general theory of exciton kinetics in condensed molecular systems has been extended in several ways. First, using standard mathematical techniques involving Green's functions, we have shown explicitly the precise relation between the master equation and random-walk descriptions of a variety of problems involving incoherent excitons on quenched linear chains. In so doing, we have generalized the usual description of these problems to include arbitrary quenching probability constant, free-end as well as periodic boundary conditions, the treatment of higher moments of the chain excitation function, and finite excitation lifetime in the absence of quenching (the parameter τ). Each of these generalizations is of interest in experimental applications to linear polymers.

In addition, we have applied the Green's function formalism to obtain the spectrum of decay rates for short-range, nondisruptive quenching of rings and of open chains with arbitrary quencher location. These spectral results are applied in turn to the analytical solution of a finite- τ problem and investigation of the excitation function of a chain excited and quenched at opposite ends.

We have shown that the coherent potential approximation originally used to find densities of states is, if anything, even more useful in random-walk applications. Using this approximation, we have solved explicitly for the first passage time (zereth moment at infinite τ of the excitation function) on an infinite chain with a finite concentration of randomly placed nondisruptive quenchers.

Work is in progress to extend many of these one-dimensional results to two- and three-dimensional systems. We are particularly interested in a class of organic mixed crystals, the doped polyacenes (e.g. tetracene-doped anthracene), whose energy transfer properties should be quite similar to those of photosynthetic units. Unlike that of the chlorophyll units, the structure of the polyacenes is well known. Hence, theory is more severely tested with the latter than the former. The fact that the experimentally determined exciton quenching kinetics in the doped polyacenes is anomalous heightens interest in this problem. At the present time, we believe that this anomalous kinetics can be explained by a competition for the excited-state energy between the irreversibly quenching dopant and partially reversible host traps, both of which processes can be described by our theory. This may be not unlike the competition for excitation energy between Systems I and II of green plants.

The other difficulty concerns the lack of sufficient time resolution in existing experimental results to obtain from them higher moments of the excitation function. These higher moments are necessary if the hopping nature of the quenched excitons is to be demonstrated by comparison with theory. A method especially sensitive at short times, such as that of single-photon counting, is also necessary to extend previous work to higher quencher concentrations. Higher time resolution also would be helpful in settling the first difficulty. Since the necessary instrumentation is being developed (2), a decision on the nature of triplet excitons in polyriboadenylic acid can be expected in the near future.

Our general work on the quenching of coherent, as contrasted with incoherent or hopping, excitons in linear polymers has been substantially completed. The most interesting new result concerns the predicted behavior of nondisruptive quenchers that are not near chain ends. For such quenchers, the optimum quenching strength (1) for band-edge excitons is much smaller (by a factor on the order of the quencher concentration) than that for end quenchers. Although the optimum strength for end quenchers is probably too large to be readily attained, that for a moderate concentration of "non-end" quenchers should be experimentally accessible.

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1. R. M. Pearlstein and R. P. Hemenger, *Biol. Div. Ann. Progr. Rept.*, Dec. 31, 1969, ORNL-4535, p. 78.
2. J. W. Longworth, R. M. Pearlstein, and S. S. Stevens, this report, p. 41.

EXCITON KINETICS IN POLYRIBOADENYLIC ACID AND OTHER LINEAR POLYMERS

R. M. Pearlstein and R. P. Hemenger

We are interested in the nature of excitons in biopolymers. If, as some suspect, metal-ion quenching of the phosphorescence of polyriboadenylic acid [poly(rA)] involves the hopping of triplet excitons, the quenching kinetics can be described by our theory. If necessary, higher moments of the excitation function can be calculated, even for finite τ , although the expressions are then much more complicated. Corrections to the coherent potential approximation used in the theory are also calculable.

Quenched poly(rA) seems an almost ideal system to test this theory. Available polymer is long enough for end effects to be negligible. For triplet excitons, interactions other than those between nearest neighbors are also negligible, as is excimer formation (unlike the case of singlet excitons in nucleic acids). There is no evidence that the quenching process alters the triplet-triplet interactions between the immediately quenched base and its nearest neighbors. Hence, regardless of the precise nature of the quenching mechanism, the quenching must be considered nondisruptive.

There are two difficulties yet to be resolved. One of these is the apparent existence of defect sites, which trap some of the triplets or otherwise isolate them from quenching sites. However, it seems likely that in future experiments these defects either can be annealed out, or else their effects can be calculated by suitable control experiments. Recent experiments involving the delayed fluorescence produced by the annihilation of pairs of triplet excitons in poly(rA), one member of each pair being trapped, certainly provide one method of calculating the effects of the trapping defects.

SPILLOVER AMONG PHOTOSYNTHETIC UNITS OF GREEN PLANTS

R. M. Pearlstein

Energy transfer in chlorophyll units is usually described in terms of incoherent or hopping excitons. New theoretical results show that if the excitons are coherent over small regions of the chlorophyll array, it is possible for the excitons to provide an efficient, dynamic mechanism to control spillover. (Spillover in green plants refers to use by System I of excess energy absorbed by System II.) The mechanism would work as follows. For definiteness, imagine that the chlorophyll arrays are planar with $\sim 1\%$ of the reaction centers placed periodically. If a reaction center is also a "center of coherence" for an exciton, the exciton would form a standing wave, peaked at the reaction center and minimal at the perimeter of the coherence region. Once the reaction center is bleached — i.e., the excitation energy is used — a "hole" would be formed in the array as far as the next exciton is concerned. This exciton would form a standing wave that is minimal at the "hole," or bleached reaction center. Hence, excitation energy would readily escape only from those regions of the chlorophyll array that contain bleached reaction centers, i.e. where the energy is excessive, and would be trapped in those regions that are short of excitation. Preliminary calculations show that the relative spillover efficiency would be increased by at least a factor of 2 with coherent, as compared to incoherent, excitons.

FORMATION OF THE INITIAL PROTRUSION OF A LEAF PRIMORDIUM WITHOUT CONCURRENT, PERICLINAL CELL DIVISIONS

D. E. Foard and Rhonda F. Irwin

The view that periclinal cell divisions cause the initial protrusion of a leaf primordium may be tested by using ionizing radiation to prevent cell division without preventing growth. After receiving 800 krad of γ -rays, wheat grains containing embryos with three leaf primordia produce seedlings in which a fourth protrusion of the shoot apex forms, unaccompanied by cell divisions. This protrusion without periclinal divisions occurs in the same phyllotactic position as that of the fourth leaf primordium, in which periclinal divisions occur. In addition to proper phyllotactic position, the protrusion without cell divisions is formed by the outermost cell layer, as is the initial protrusion of a typical leaf primordium of wheat; moreover, the initial number of cells involved is the same in both kinds of protrusions. Therefore, the fourth protrusion in seedlings from irradiated grain is interpreted as the initial protrusion of a leaf primordium that formed without periclinal cell divisions. Measured along the axis of greatest extension, the protrusions without cell divisions represent about a 4- to 8-fold increase over the anticlinal dimension of the surface cell layer in the embryo. These protrusions do not develop further. The absence of cell divisions limits the extent of primordial growth, but does not prevent its inception. Periclinal cell divisions do not cause the initial protrusion of a leaf primordium.

PHYSIOLOGICALLY NORMAL SENESCENCE IN SEEDLINGS GROWN WITHOUT CELL DIVISION AFTER MASSIVE GAMMA IRRADIATION OF SEEDS

D. E. Foard, A. H. Haber, and Rhonda F. Irwin

Senescence was studied both in gamma-plantlets and in unirradiated control wheat seedlings. The slow chlorophyll loss in the gamma-plantlet leaf resembles that in the unirradiated control with respect to time course and chemical regulation. In both gamma-plantlet and normal roots, protoplasmic streaming occurs in all growing root hairs, and synthesis of insoluble RNA is detected in all cells before they stop growing. In root hairs of both gamma-plantlets and unirradiated controls, protoplasmic streaming stops within a day after cessation of growth. In a given region of roots, detectable synthesis of insoluble RNA stops within a day of cessation of growth in that region, except that in gamma-plantlets this RNA synthesis persists for up to $2\frac{1}{2}$ weeks in an extreme apical portion. The lack of gross acceleration of senescence in gamma-plantlets indicates that the doses of 0.5 Mrad or more given to dry wheat grains are not lethal in the physiological sense, despite their lethality in the genetic and proliferative senses.

A METHOD FOR TESTING THE SPECIFICITY OF INHIBITORS OF DNA SYNTHESIS IN STUDIES OF GROWTH

A. H. Haber, O. J. Schwarz, and Lee W. Evans*

Inhibitors of DNA synthesis are often used in plant growth experiments to study the role of DNA synthesis in a given aspect of growth regulation. The usefulness of such an inhibitor depends upon the extent to which a given concentration fulfills two requirements: (1) effectiveness — the inhibitor should truly prevent subsequent DNA synthesis — and (2) specificity — essentially all the growth inhibition should be a result of inhibition of DNA synthesis; i.e., there should be an absence of "nonspecific growth inhibition" (here defined as a reduction in growth that is not caused directly or indirectly by inhibition of DNA synthesis).

The requirement of effectiveness is generally appreciated and often tested experimentally by using various methods of checking for absence of DNA synthesis. By contrast, the requirement of specificity is seldom, if ever, checked in growth studies, probably owing to the lack of a suitable test method. We describe here a method for testing the specificity of inhibitors of DNA synthesis in growth studies. We also show how this test of specificity can be coupled with an independent test of effectiveness, to permit conclusions concerning the suitability or unsuitability of a possible specific inhibitor of DNA synthesis for growth studies.

We test the specificity by examining the actions of inhibitors on the germination of heavily irradiated lettuce seeds, which occurs without detectable DNA synthesis (1), and the growth of wheat gamma-plantlet seedlings, which does not require DNA synthesis (2). Despite the heavy seed irradiation

and because of the absence of many of the typical cytogenetic effects of ionizing irradiations (2), the germination and growth of such seeds and seedlings are remarkably normal in many respects, including normal responses to a wide variety of growth-regulating chemicals (1, 2). Since in gamma-plantlets there is no DNA synthesis to inhibit, any growth inhibition that is caused by an inhibitor of DNA synthesis must therefore be non-specific. The inhibitors used were 5-fluorodeoxyuridine (FdUrd), hydroxyurea, and phenethyl alcohol.

The capacity of the inhibitors to produce nonspecific growth inhibition can be seen as a decreased growth resulting from their application to gamma-plantlets, in which there is no DNA synthesis to inhibit, owing to prior γ -irradiation (500 krad) of the dry seeds. The inhibitors seem more effective in producing nonspecific growth inhibition when they are applied from the beginning of inhibition than when they are applied a few days later, during early germination.

For a substance to be useful as a specific inhibitor of DNA synthesis, the ranges of concentrations for which the requirements of specificity and effectiveness are met must overlap. Using unirradiated seedlings, in which there is DNA synthesis to inhibit, we determined DNA content per seedling so as to determine the concentration range necessary to fulfill the requirement of effectiveness for each inhibitor. The concentrations of inhibitors necessary to prevent detectable increases in DNA content, when applied to unirradiated seedlings, are represented in Fig. 1 by the solid bars. We believe that the high concentrations necessary for apparent inhibition of DNA synthesis are partly a reflection of the method of application of the inhibitors to the whole seedlings and partly a reflection of our requirement that the inhibition of increase in DNA per seedling be complete rather than partial. Absence of a significant increase in DNA content would not preclude DNA turnover or undetectably small amounts of synthesis. Consequently, the true range of concentrations for fulfilling completely the requirement of effectiveness might be somewhat higher than represented by the solid bars in Fig. 1.

For comparison, we also plot as stippled bars in Fig. 1 the range of concentrations for which we could not find any nonspecific growth inhibition, i.e., for which gamma-plantlet seedling growth was not significantly affected by the inhibitors. Thus, the stippled bars represent the range of concentrations for which the requirement of specificity seems to be met. If we consider the possibility that there may be subtle, nonspecific effects of the inhibitors that might not show up on growth in the gamma-plantlet test system, the true range of concentrations for absolute biochemical specificity might be somewhat lower than the range indicated in Fig. 1 by the stippled bars, which pertain only to growth. Consequently, the concentration range for which FdUrd is both effective and specific may actually be smaller than indicated in Fig. 1. Also, for hydroxyurea and phenethyl alcohol the concentration range for which there is neither effectiveness nor specificity may be greater than indicated in Fig. 1. We conclude, therefore, that for early wheat seedling growth neither hydroxyurea nor phenethyl alcohol concentrations exist that

satisfy both requirements. By contrast, a wide range of concentrations of FdUrd seem to satisfy the requirements for a specific inhibitor of DNA synthesis for studies of growth in young wheat seedlings. The ineffectiveness of FdUrd on growth of gamma-plantlet seedlings relative to unirradiated seedlings cannot be attributed to differences in uptake of the inhibitor, because the uptake of $2\text{-}^{14}\text{C}$ -FdUrd was the same into gamma-plantlet and unirradiated control seedlings of comparable size.

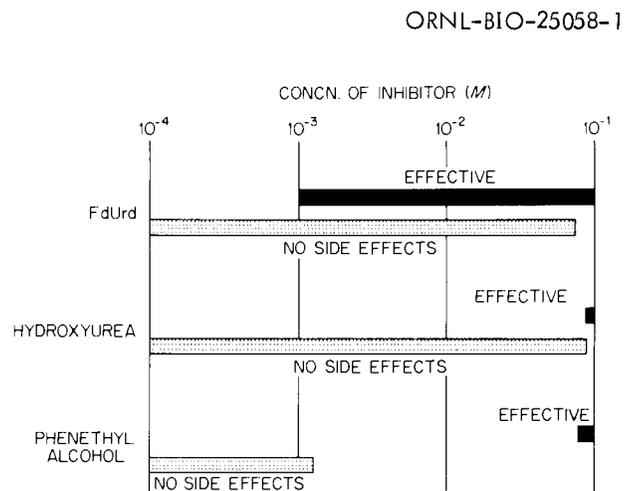


Fig. 1. Comparison of the range of concentrations for which the requirements of effectiveness (solid bars) and specificity (stippled bars) are met after treatment of germinating wheat seedlings with inhibitors of DNA synthesis.

Experiments with lettuce gave results similar in three respects to those with wheat. (1) Treatment of seedlings produced smaller nonspecific growth inhibition than did treatment of dry seeds. (2) On a molar concentration basis, the order of effectiveness in producing nonspecific growth inhibitions was the same for the three inhibitors tested. (3) Concentrations of FdUrd were the only ones found that satisfied both requirements.

These findings suggest a general approach that might be used or adapted in other plant systems for testing the specificity of these and other inhibitors of DNA synthesis.

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COMPARISON OF CERTAIN DEVELOPMENTAL PARAMETERS IN GAMMA-PLANTLETS AND UNIRRADIATED CONTROLS AND THEIR IMPLICATIONS FOR AGING RESEARCH

O. J. Schwarz and A. H. Haber

We studied various developmental parameters as a function of time during germination, growth, and senescence of gamma-plantlets (seedlings growing without cell division after 500 krad γ -irradiation of dry seeds). During the first 96 hr after germination, we compared gamma-plantlets and unirradiated controls with respect to the following: dry weight; DNA, RNA, and soluble protein contents; and time of onset and level of activity of uridine, cytidine, deoxyuridine, deoxycytidine, and thymidine kinases. In gamma-plantlets, except for the absence of increase in DNA, the developmental sequence of events reflected in these parameters seems to be essentially unaltered. The results with the kinases, which are initially undetectable, indicate that the prevention of DNA synthesis in the gamma-plantlet cannot be attributed to inactivation or repression of pyrimidine nucleoside and deoxynucleoside kinases. These results also illustrate the physiological normalcy of gamma-plantlets and their suitability for developmental studies.

When the pyrimidine nucleoside and deoxynucleoside kinase activities were followed throughout the gamma-plantlet life-span, they were found to increase to maximal levels at around 8–10 days, after which they decline. These age-dependent changes may be useful biochemical indices of senescence in this developmentally simple, completely determinate system.

DEVELOPMENT OF THYMIDINE KINASE ACTIVITY IN WHEAT SEEDLINGS GROWING WITHOUT DNA SYNTHESIS AND CELL DIVISION

O. J. Schwarz and A. H. Haber

Thymidine kinase activity is usually low or undetectable in cells that are not undergoing DNA synthesis and cell division. Conversely, thymidine kinase activity is usually high in systems, like regenerating rat liver and cell cultures growing logarithmically, that have rapid DNA synthesis and cell division (1). To test how closely coupled the kinase activity is to DNA synthesis and cell division, we carried out a study of its activity in relation to DNA synthesis during germination and growth of normal wheat seedlings and in gamma-plantlets (2). Control of the time of appearance and/or activity levels of the kinase have been found to be sensitive to physiological insults, including ionizing irradiation (1, 3). Therefore, a study of the developmental profiles of thymidine kinase in gamma-plantlets and unirradiated controls might also give information concerning possible radiation injury to developmental control mechanisms, in gamma-plantlets.

Thymidine kinase activity (4) and DNA content were determined in gamma-plantlet and unirradiated control seedlings from 0 to 96 hr after sowing. Thymidine kinase activity

was not measurable at 12 hr in either gamma-plantlets or unirradiated controls. By 24 hr both systems had activity, which continued to increase throughout the period studied. The DNA levels of the controls in the period from 0 to 96 hr parallel the rise in thymidine kinase activity. The DNA content of the gamma-plantlets remained constant throughout the time interval. Despite the absence of DNA synthesis (as shown by both autoradiographic and chemical determinations) and the absence of cell division, the time of appearance and subsequent increase of thymidine kinase activity were not essentially altered from the unirradiated controls. This system represents a complete uncoupling, from DNA synthesis and cell division, of the biochemical regulation of onset of and subsequent increase in activity of thymidine kinase. In contrast to the irradiated systems studied by others (1, 3), gamma-plantlets showed no significant alteration in the developmental activity profile of thymidine kinase. This is consistent with the absence in gamma-plantlets of many other typical radiation effects (2).

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ENDOSPERM PERMEABILITY AND THE STORAGE OF LETTUCE SEEDS UNDER SOLVENTS

L. L. Triplett and A. H. Haber

The effects of various chemicals on germination are almost always studied by applying the chemicals in aqueous solutions. Since imbibition of water and concomitant uptake of the test chemical take time, the chemical is not present from zero time in the sequence of events in metabolic activation during germination. The usefulness of certain solvents, principally dichloromethane and to a lesser extent acetone, in introducing test chemicals into lettuce seeds has been reported (1). The method reported involves putting seeds into solutions of the test chemicals dissolved in the solvent. After such application, the solvent is evaporated off *in vacuo*, presumably leaving the test chemical behind in the embryo. The seeds are then sown in water, using normal procedures, so that normal germination proceeds, presumably with the test chemical present from zero time in the sequence of events in metabolic activation during germination (1).

We have confirmed the findings that lettuce seeds can be stored under dichloromethane, acetone, or ethanol for extended periods of at least several days without loss of viability, as shown by germination tests after evaporation of the solvents *in vacuo*. We also find that appreciable amounts of moisture (e.g. 8% water impurity in ethanol) in the solvent rapidly causes loss of viability. We conclude, however, that the survival of the lettuce seeds under these pure solvents is not caused by any capacity of the embryo to withstand the solvents.

On the contrary, the survival is caused by the inability of the solvents to reach the embryo, owing to the impermeability of the endosperm. Our conclusion is based on the following findings: (1) Seeds cut so as to remove the endosperm as a barrier between the external solution and the embryo lost viability when immersed in the solvents. (2) Control seeds similarly cut, but not exposed to the solvents, retained viability. (3) A variety of organic dyes dissolved in the pure solvents, which caused no loss of viability of intact seeds, failed to penetrate the endosperm of intact seeds. (4) The organic dyes were seen to penetrate to the embryo of intact seeds when the solvents had sufficient amounts of water to cause loss of viability. (5) The organic dyes dissolved in the pure solvents did reach the embryo of cut seeds, which showed loss of viability.

Our conclusions fail to confirm the possibility of supplying test chemicals to embryos within lettuce seeds by using organic solvents. Nevertheless, the finding that lettuce seeds can be stored without loss of viability for extended periods (e.g. at least 6 months under ethanol with essentially complete germination capacity) opens up new approaches to radiobiological and other studies of germination (2).

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RADIOBIOLOGICAL STUDIES USING LETTUCE SEEDS STORED UNDER ETHANOL

A. H. Haber, L. L. Triplett, and M. L. Randolph

Because of the impermeability of the endosperm, intact lettuce seeds will survive prolonged storage under pure ethanol. When vacuum-dried seeds (with about 7.6% water content, as determined by conventional oven-drying methods) were put into pure ethanol, they lost additional water, about 2% of their total weight, as independently determined by Karl Fischer titrations or by gas chromatographic analysis. The ethanol treatment can, therefore, be useful for radiobiological studies, because it produces dryer viable seeds than have been produced by other known methods. Gamma-irradiated seeds stored *in vacuo* before sowing show the typical "postirradiation storage phenomenon" (i.e. apparent increase in radiation damage with increasing duration of seed storage). Irradiated seeds stored in ethanol show a more pronounced postirradiation storage phenomenon than do the irradiated seeds stored *in vacuo*. The long-lived electron spin resonance produced by irradiation shows similar decay kinetics whether the irradiated seeds are stored after irradiation *in vacuo* or under ethanol. These results indicate that the differences in water content between irradiated seeds stored *in vacuo* and under pure ethanol are accompanied by differences in the postirradiation storage phenomenon that are unrelated to any significant alteration in decay of the radiation-induced electron-spin-resonance signal.

ATTEMPTS TO DEFINE THE LOCUS OF PERCEPTION FOR LIGHT-INDUCED RETARDATION OF SENESCENCE IN WHEAT LEAF TISSUE

Paula J. Thompson,* A. H. Haber, and J. R. Azzi

In the presence of aminotriazole, which prevents chloroplast formation, light retards chlorophyll loss (concomitant with chloroplast disintegration) in detached wheat leaf tissue. Dichlorophenyl dimethylurea (DCMU), which prevents photosynthetic carbon fixation, does not inhibit the retardation of senescence (1).

We have found that the light effect is a general effect on leaf senescence, in that light retards protein loss, as well as chloroplast disintegration, in the wheat leaf tissue. We have asked whether this light effect is solely or primarily on the chloroplast or on the entire plant cell. We isolated chloroplasts according to the method of Ridley and Leech (2) and measured chlorophyll loss after incubation in the light and dark. Chlorophyll decayed faster in the light than in the dark. Ridley and Leech (3) reported similar results for bean chloroplasts kept in the light. Radiobiological studies established such bleaching, at light intensities that normally do not cause bleaching or chlorophyll in the living tissue, as a criterion of lethality (4). Thus, our isolated chloroplasts, like those of Ridley and Leech (3), may not show the effect because they are dead, not because they are potentially incapable. We obtained aplastidic plants by germinating and growing wheat in aminotriazole. Preliminary results showed that light did not retard protein loss in detached aplastidic wheat leaf tissue as it did in green tissue.

These results suggest that the locus of perception for the light effect is the chloroplast in the plant cell. The retardation of protein loss may be solely on chloroplast proteins, or it may include other proteins secondarily. A study of the loss of activity of organelle-specific enzymes might answer this question.

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ISOLATION AND PURIFICATION OF EYESPOT GRANULES FROM *EUGLENA GRACILIS* BY COMBINED RATE ZONAL AND ISOPYCNIC CENTRIFUGATION

Carol J. Bartlett,* Patricia L. Walne,* O. J. Schwarz, and D. H. Brown†

Large volumes of eyespot granules were isolated from homogenates of *Euglena gracilis* var. *bacillaris* by centrifugation in a B-XV zonal rotor in a sucrose density gradient.

The granules were further purified by centrifugation through a sucrose density gradient in a swinging bucket rotor and banded at the interface of the sample and the buffer overlay. Examination with the electron microscope showed the eyespot granules to be free from other cellular material.

Absorption spectra of extracts (hexane or petroleum ether) of isolated eyespot granules were obtained after initial isolation in the zonal rotor, after purification, and after freezing. Neither freezing nor the addition of cysteine to the fractions had any significant effect on the absorption properties of eyespots between 350 and 600 nm. A small peak at 675–700 nm was observed in the extracts of both crude and purified eyespot preparations. The significance of this peak is under investigation.

The procedures developed here make possible the isolation, with reasonable purity, of large quantities of eyespots of euglenoid flagellates. With this capability, these organelles can now be characterized biochemically in various phases of growth, in senescence, and after various kinds of experimental treatment that should yield information relating to their structure and function.

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GENETICS AND DEVELOPMENTAL BIOLOGY SECTION

W. E. Barnett

<u>Mutagenesis and Cytochemistry</u>	<u>Mammalian Cytology and Cell Genetics</u>	<u>Cell Growth and Differentiation</u>
R. F. Kimball ^d	E. H. Y. Chu	Tuneo Yamada
	Chia-cheng Chang ^a	T. G. Connelly ^a
<u>Microbial Genetics and Radiation Microbiology</u>	B. Nai-Chau Sun	J. N. Dumont
Roy Curtiss III		J. J. Eppig, Jr. ^a
H. I. Adler	<u>Mammalian Biochemical Genetics</u>	Aida Goldstein ^a
Anne C. Frazer ^a	R. A. Popp	V. P. Idoyaga-Vargas ^a
Louis Glatzer ^a	G. P. Hirsch ^a	
G. G. Khachatourians ^a	Diana M. Popp	<u>Chromosome Ultrastructure</u>
		O. L. Miller, Jr.
<u>Mammalian Cytogenetics</u>	<u>Fungal Genetics</u>	Aimée H. Bakken ^a
J. G. Brewen	F. J. de Serres	Barbara A. Hamkalo
R. J. Preston	C. R. Fisher ^d	
	H. V. Malling	<u>Vitellogenesis</u>
<u>Human Genetic Biochemical Defects Analysis</u>	B. E. Matter ^b	R. A. Wallace
<u>Genetics and Cell Culture of Human Variants and DNA Repair</u>	M. M. Nawar ^b	D. W. Jared
J. D. Regan	Tong-man Ong ^a	
W. B. Roess ^e	M. E. Schupbach ^a	<u>Molecular Basis of Recombination and Repair of DNA</u>
	Elizabeth S. Von Halle ^c	Jane K. Setlow
<u>Biochemical Analysis of Human Variants</u>	<u>Drosophila Biochemical Genetics</u>	K. L. Beattie
J. L. Epler	E. H. Grell	M. E. Boling
J. X. Khyrn	C. E. Nix ^a	N. K. Notani ^b
	<u>Drosophila Chromosomal Behavior</u>	
<u>Enzymology of Human Genetic Genetic Defects</u>	Rhoda F. Grell	<u>Hymenopteran Genetics and Genetic Methods for Insect Control</u>
W. E. Barnett	<u>Structure and Function of Multienzyme Complexes</u>	R. H. Smith
C. G. Mead	F. H. Gaertner	Anna R. Whiting ^c
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THE MUTAGENIC AND LETHAL EFFECTS OF MONO-FUNCTIONAL METHYLATING AGENTS IN STRAINS OF HAEMOPHILUS INFLUENZAE DEFECTIVE IN REPAIR PROCESSES

R. F. Kimball, Jane K. Setlow, and Mini Liu*

Four mutant strains of *Haemophilus influenzae* were tested for their response to the monofunctional methylating agents *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and methyl methanesulfonate (MMS). Two of these strains (DB112 and DB116) are unable to excise pyrimidine dimers, one (DB115) is slow in rejoining breaks produced by excision, and one (DB117) is unable to support recombination or rejoin the single-strand breaks produced in DNA by X-rays (1). DB112, DB115, and DB116 are like wild type in their sensitivity to killing by the two agents; DB117 is more sensitive to both agents by a factor of 3-4. MMS does not induce a detectable number of mutations to cathomycin resistance at doses that give equal survival from mutagenic doses of MNNG and approximately equal numbers of induced single-strand breaks in the DNA. It is suggested that the mutation induced by MNNG is due to a specific alteration—perhaps alkylation of guanine on the oxygen at the 6 position—produced by this agent but not by MMS. DB115, DB116, and DB117 give approximately the same mutation yield as wild type with MNNG. DB112 and a derivative obtained by transforming wild type with DB112 DNA give a distinctly lower yield. DB112 has been shown earlier to lack the endonuclease that makes the initial incision for the excision repair process in DNA that contains pyrimidine dimers (2). It is possible that this endonuclease can also act on the MNNG-specific mutagenic alteration to enhance the probability that it will cause mutation.

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MUTATION INDUCTION IN HAEMOPHILUS INFLUENZAE BY TRANSFORMATION WITH NITROSOGUANIDINE-TREATED DNA

R. F. Kimball and Jane K. Setlow

Mutations were induced by treatment of *Haemophilus influenzae* DNA *in vitro* with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and subsequent transformation of the bacteria with the treated DNA. The treated DNA was derived from streptomycin-resistant strains, and transformants were selected by selecting for streptomycin resistance. Mutations were

detected among the transformants in appreciable frequency in two loci (cathomycin and kanamycin resistance) that are closely linked to the streptomycin resistance locus but were detected in a very low frequency at most in two loci (erythromycin and viomycin resistance) that are not closely linked. The frequency of mutation in the linked loci is at least an order of magnitude lower than can be obtained by treating the bacteria directly. A still lower frequency, approaching zero, was found when bacteria were treated, lysed immediately, and the lysate used for transformation. However, the frequency increased to about the level for direct treatment when the treated bacteria were incubated at 37° C for 30 min before lysing. The presence of a sulfhydryl compound, glutathione, during the treatment of the DNA was without effect. This compound has been reported to facilitate some of the reactions of nitrosoguanidine with DNA *in vitro*.

A UV-sensitive strain that gives a low mutation yield when treated directly with MNNG also gives a low yield, approaching zero, when used as a recipient for MNNG-treated DNA. Attempts to obtain a higher yield by treatment *in vitro* with a UV endonuclease, which seems to be missing in this strain, were unsuccessful.

STUDIES ON THE CELL CYCLE AND CELL SIZE DURING GROWTH AND DECLINE OF CHINESE HAMSTER CELL CULTURES

R. F. Kimball, Stella W. Perdue, E. H. Y. Chu, and J. R. Ortiz*

A combination of microphotometric determinations of the DNA and protein content and dry mass of single cells and autoradiography was used to follow the changes in cell cycle parameters and cell growth in cultures of Chinese hamster cells as they passed from the initial stages of growth, through the exponential phase, into culture decline. Cell counts and total protein determinations were used to follow the overall culture growth. In the lag phase and early part of the exponential growth phase, cell size and the relative duration of the combined periods S through M increased. In the latter half of the exponential phase, cell size at mitosis and the duration of S through M decreased, while the length of the cell cycle and the size of the cells at the initiation of DNA synthesis remained essentially constant. As the cultures went into the decline phase resulting from cell death, the cell cycle increased greatly, primarily because of an increase in G₁. At the same time, cell size at mitosis and at the initiation of DNA synthesis decreased, because the cells continued to go through the cycle with very little net increase in protein content. There was evidence that at least 40% of the cells remained in the cycle well into the decline period, and the simplest interpretation was that all cells remained in the cycle. A description of the events can be given in terms of the rates of three processes: protein synthesis, initiation of DNA synthesis, and initiation of mitosis. There are indications that the rate of initiation of mitosis is less closely associated with the rate of protein synthesis than is the rate of initiation of DNA synthesis.

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THE MOLECULAR MECHANISM OF RECOMBINATION
FOLLOWING CONJUGAL CHROMOSOME TRANSFER

R. J. Sheehy* and Roy Curtiss III

The purpose of this study is to investigate the molecular mechanism of recombination following conjugal chromosome transfer. One of the main factors which has hindered an exact description of molecular events leading to recombination has been the operational problem of isolating and describing the physical form of the interacting parental DNA molecules arising as a consequence of recombination. A mutant strain of *E. coli* produces minicells, which are formed as a result of abnormal cell division. Minicells, which are approximately one-tenth the size of normal cells, can be conveniently separated from larger cells on 5–20% sucrose gradients. When the minicell-producing mutant is infected with a bacterial plasmid, e.g. R, Col, or F factors, these factors segregate into the minicells at high frequency. Since most bacterial plasmids exist as covalently closed circles, they can be isolated from minicells on alkaline sucrose gradients and identified by appropriate radioactive labeling techniques. Conjugal transfer of DNA has also been shown to occur between normal-sized cells and minicells. Thus, the advantage of this system is that labeled DNA can be transferred from donors of normal size to plasmid-containing minicells, and the ensuing recombinant structure can be isolated as a closed circular molecule on alkaline sucrose gradients. The elimination of donor DNA and a reduction in the amount of DNA not directly involved in recombination make this system appealing for recombinational analysis.

Methods have been developed for the efficient conjugal transfer of plasmid DNA from donor cells to minicells harboring plasmids of the same type. The system is being further improved by the development of methods to completely eliminate the problem of surface exclusion that is observed in donor-donor matings.

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ANALYSIS OF THE EFFECTS OF MUTATIONS THAT
DISRUPT DNA SYNTHESIS ON CONJUGATION
IN *E. COLI* K-12

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and Roy Curtiss III

Our continuing interest in conjugation has prompted us to examine the effects of several mutations on this process. We have paid particular attention to mutations that affect DNA metabolism in one way or another, and we have taken advantage of the fact that minicells of *E. coli* K-12 can act as either recipient (1) or donor (2) in a mating and then be rapidly separated from the other parent by sedimentation in sucrose gradients.

The DNA-ts mutation described by Bonhoeffer (3) inhibits replication immediately when the mutant is placed at the restrictive temperature of 43°C. When it is in the donor cell, this mutation is reported to have no effect on

conjugal transfer; but we have found that donor cells at the restrictive temperature and in the presence of minicells incorporate four times more radioactive thymidine than the same cells in a nonmating situation. In addition, when the donor cells contain a transmissible plasmid, such as an R factor, up to 30% of the thymidine is incorporated into covalently closed circular DNA molecules. In comparison with other data we have collected on the amount of plasmid DNA that can be isolated as closed circles, this indicates that most of the synthesis involves plasmid DNA. The amount of isotope in the recipient minicells increases during the mating, and the fact that labeled DNA is transferred to the recipients indicates that the plasmids have initiated multiple rounds of transfer. Thus, the original conclusion drawn from work with this mutation (3) — that DNA synthesis is not necessary in the donor for transfer — is not valid, because the act of conjugation itself stimulates replication of the DNA being transferred.

The results are not as straightforward when the DNA-ts mutation is in the recipient cells, where it is known to affect haploid recombinant formation but not transfer (4), and plasmid-containing minicells are used as donors. We can detect the transfer of prelabeled F⁺lac from minicells to cells at the restrictive temperature, but our inability to isolate this plasmid as a closed circular molecule from the strains used in these studies has made it difficult to interpret the effects of the mutation. R factors, which are readily isolable as closed circles, are transferred at such low frequencies from minicells that their transfer is impossible to follow.

We have also examined the effect of mutant forms of the Kornberg DNA polymerase (*polA*⁻) on conjugal transfer. We have done this by making a minicell-producing strain *polA*⁻ and using the minicells from the strain as recipients in matings. Hfr donors transfer fragments of DNA with molecular weights of about 25 X 10⁶ to *polA*⁻ minicells. After a 60-min mating, only about half as much DNA is transferred to *polA*⁻ minicells, and the fragments are also smaller. The apparently lower level of transfer may be due to less transfer, transfer of smaller pieces of DNA, or more rapid degradation during mating in the *polA*⁻ minicells. Actually, the rate of degradation of transferred DNA is the same in both types of minicells examined after 60 min of mating. Thus, *polA*⁻-stimulated degradation may not be involved in the lower level of transfer. With *polA*⁻ recipient cells, however, we could detect no depression of recombinant frequencies in Hfr matings, so this mutation may have a greater effect in the minicell than in the normal system. In matings involving transfer of the F sex factor, again, only about 50% as much DNA can be isolated from *polA*⁻ minicells. However, since we can detect no circularization of transferred plasmid DNA in *polA*⁺ minicells, it is not possible to examine the effect of the mutation on this process.

These results lend support to the idea that DNA synthesis is required in the donor for conjugal transfer and, furthermore, have demonstrated that the minicell mating system has a wide range of uses for studies on conjugation.

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PLASMID-CONTAINING MINICELLS

K. J. Roozen and R. G. Fenwick, Jr.

Several years ago, during studies on the genetic control of cell division and radiation sensitivity, a mutant F^- strain of *E. coli* K-12 was discovered that produced DNA-deficient minicells easily separable from their larger parental cells. In our search for a method to introduce specific DNA molecules into these minicells in order to study the replication, transcription, etc. of this DNA in the absence of chromosomally determined events, we have found that when a minicell-producing strain is infected with a suitable plasmid (i. e. F , F' , R , Col , λdv), DNA is recovered from purified minicells. Since plasmid DNA usually exists as a covalently closed circular molecule with sedimentation properties different from those of chromosomal DNA, we have used a variety of physical techniques to demonstrate that the DNA recovered from R^+ , Col^+ , and λdv^+ minicells is predominantly plasmid DNA. The ability of minicells produced by F' -containing strains to transfer episomal but not chromosomal markers is taken as evidence that the DNA located in minicells produced by these strains is plasmid DNA. Studies to determine the amount of DNA segregated into minicells produced by a variety of strains indicate that different plasmid types are segregated into minicells with different efficiencies. This system offers a unique method for isolating plasmid DNA molecules.

SYNTHETIC CAPABILITIES OF PLASMID-CONTAINING MINICELLS

K. J. Roozen and R. G. Fenwick, Jr.

After it was established that plasmid DNA can be segregated into minicells, these minicells were examined for their ability to carry out various cellular processes. Using primarily R^+ and Col^+ minicells, we found that they are capable of RNA and protein synthesis, as well as a limited amount of DNA replication. RNA synthesis was assayed by the incorporation of labeled RNA precursors into trichloroacetic acid-insoluble material. Polyacrylamide gel electrophoresis of RNA synthesized in minicells reveals that the bulk of material synthesized is between 6 and 14S. The 16 and 23S ribosomal RNA subunits are not synthesized in plasmid-containing minicells, indicating the absence of chromosomal genes. The significance of low-molecular-weight (3-5S) material prepared from minicells is currently under investigation. Protein products specified by plasmids have not yet been extensively examined. The ability of plasmid-containing minicells to produce viable T4 bacteriophage, however, is taken as evidence for the biological relevance of the synthetic capacities of these minicells. Since plasmids

can be examined in the absence of the usual masking of the chromosomal events, this system offers an excellent opportunity to study plasmid synthetic events and has potential for the isolation of plasmid-specified mRNA and protein molecules.

REPAIR IN PLASMID-CONTAINING MINICELLS

K. J. Roozen and M. C. Paterson*

Plasmid-containing minicells have been examined for their ability to carry out a variety of repair processes, in order to assess their potential as a tool for studying repair and recombination processes. Since these minicells contain a large percentage of covalently closed circular molecules, which are converted to open circles by a single-strand break, the number of single-strand breaks induced by irradiation can be easily measured by assaying the ratio of closed to non-closed circular molecules. Similarly, the repair of single-strand breaks in plasmid DNA can be assessed by assaying for an increase in closed circular molecules during postirradiation incubation. R^+ minicells are capable of repairing 70-80% of the single-strand breaks in plasmid DNA. Another easily assayed repair process is the photoreactivation of UV-induced pyrimidine dimers. R^+ minicells appear to be capable of photoreactivation, although at a slower rate than their parental cells exposed to the same UV dose, which results in the same percentage of thymine dimers. Pyrimidine dimers may also be repaired by the light-independent excision repair process. Unlike their parents, R^+ minicells are unable to remove pyrimidine dimers from DNA. Further investigation has revealed that R^+ minicells are unable to perform the initial UV-endonuclease attack that results in a single-strand break, detected as in the strand-rejoining repair experiments.

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THE CAPACITY FOR SYNTHESIS OF FUNCTIONAL GENE PRODUCTS BY PLASMID-CONTAINING MINICELLS

Anne Cornish Frazer and Roy Curtiss III

Previously, this laboratory and others have demonstrated the following transcriptional and translational synthetic capabilities of plasmid-containing minicells: (1) RNA and protein precursors are incorporated into trichloroacetic acid-precipitable material. (2) Characteristic patterns are revealed upon electrophoresis of these RNA and protein species. (3) A normal lytic cycle of T4 bacteriophage is supported. These observations suggest that plasmid-containing minicells constitute a useful tool for isolating and exploring the function and regulation of plasmid-specific gene products; plasmids of particular interest include those that carry essential chromosomal genes.

In order to analyze this potentially important system and to compare and contrast the regulation of transcription and translation in minicells and normal-sized cells, we are attempting to examine the kinetics of induction and derepression of the well-characterized lactose and tryptophan operons in minicells carrying F'_{lac} and $col-trp$ plasmids, respectively. Efforts to determine the differential rate of β -galactosidase

synthesis in F⁺lac-containing minicells of χ 797 have been unsuccessful. However, in two experiments with col-trp-containing minicells of χ 1041, derepression of anthranilate synthetase is indicated. Two operational difficulties have particularly hindered these studies: (1) sufficient cell contamination in minicell suspensions to obscure the responses of minicells upon induction, and (2) physiological maintenance of minicells during the minicell purification procedure. We have attacked the first problem by constructing a diamino-pimelic-acid-requiring minicell producer, by the use of which cell contamination should be decreased to a very low level. We are approaching the second problem by continuing to explore alternate isolation procedures, including milipore filtration and the use of renografin and ficol gradients.

TIME-LAPSE CINEMATOGRAPHIC STUDIES OF TEMPERATURE-SENSITIVE CELL DIVISION MUTANTS OF E. COLI

Alice A. Hardigree and Howard I. Adler

Several temperature-sensitive cell division mutants of E. coli AB1157 have been isolated by D. J. Clark and co-workers (1). We have studied the growth and division of one of these mutants on nutrient-agar-covered microscope slides by means of time-lapse cinematography.

At the permissive temperature of 30° C, cells of this strain, BUG-6, undergo normal division, producing short, rod-shaped cells. Growth at 42° C, however, resulted in the production of long, nonseptate filaments. After a shift to 42° C, cells which had already begun a normal division at 30° C and were almost separated appeared to fuse before continuing to elongate. However, these filaments did retain a slight indentation at the original division point, even after extensive elongation. The filament appeared to be less dense in this area, as if the cell contents had been stretched out or diluted. After a 2-hr period of growth at 42° C and a shift to 30° C, we observed the rapid division of filaments into normal-length cells in 10-20 min. Indentations occurred nearly simultaneously at many points throughout the length of the filament, and further normal divisions followed.

Strain χ 1260, a minicell-producing derivative of BUG-6, is another temperature-sensitive mutant, which we obtained from J. N. Reeve. At 30° C these cells divide normally, with the production of minicells from the ends of many of the cells. A shift to 42° C causes filament formation, as with BUG-6, but minicell production ceases. As in the case of BUG-6, growth at 42° C followed by a shift to 30° C results in the rapid division of many of the filaments into normal-length cells. Other filaments do not divide normally but produce minicells at a very rapid rate, usually from the ends of cells which had originally been producing minicells before the shift to 42° C. These results support previous observations (2) that minicell production occurs only under conditions that permit normal cell division.

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2. H. I. Adler and A. A. Hardigree, Biol. Div. Ann. Progr. Rept., Dec. 31, 1967, ORNL-4240, p. 135.

STUDIES ON THE STRUCTURE AND INTEGRATION OF THE F EPISOME IN E. COLI

Louis Glatzer and Roy Curtiss III

In an attempt to test whether a small portion of the integrated F episome is transferred to a recipient on the lead region of the donor chromosome during Hfr-mediated conjugation, we have isolated several hundred isogenic strains from a single 2-hr Hfr X F⁻ mating. When selection for prototrophy at a terminal marker (A⁻, Z⁺) was carried out, only 5% of the strains demonstrated any kind of fertility, as opposed to 85% when selection was carried out for both proximal and terminal markers (A⁺, Z⁺). No fertility was observed in strains selected at a proximal site only (A⁺, Z⁻) after short-term matings. Two A⁻, Z⁺ strains have been obtained that have reduced fertility relative to the Hfr parent strain, as measured by recombination frequency. One of these strains has been used as a "phenocopy" recipient to select for A⁺, Z⁺ derivatives in either 15- or 120-min matings. Analysis was made of 150 A⁺, Z⁺ strains selected after 15 min of mating, and none were restored to the normal fertility of the parental Hfr strain. Of those strains selected as A⁺, Z⁺ after 120 min of mating, several were restored to an equal or better fertility than the original Hfr parent, and several showed decreases in fertility. Thus, a two-step complementation by proximal and terminal portions of episomal DNA from an Hfr strain has not been substantiated as originally postulated by Wollman and Jacob (1). Additional work on other Hfr strains is necessary to test whether the postulated "complementation" is unique to certain Hfr strains or perhaps just an exceedingly rare event.

We are now initiating a molecular approach to the problem of whether F DNA is present at the lead region of the chromosome during Hfr-mediated conjugation. F⁻ minicells (2) have been used to isolate fragments of radioactively labeled DNA transferred to them by an Hfr parent in Hfr X F⁻ minicell matings. Following reisolation and lysis of the minicells, the DNA has been analyzed by physical techniques. The DNA appears to be transferred as a single strand, substantiating the findings of Cohen et al. (3). Contrary to the original findings of Fralick (4), the DNA fragments appear to be somewhat heterogeneous in nature. Thus, as much as 50% of the DNA transferred may be small, single-stranded fragments of 2 X 10⁶ daltons or less. There is a size range of greater than 25 X 10⁶ daltons to less than 2 X 10⁶ daltons. This finding has been substantiated by examination of the DNA under the electron microscope in collaboration with Dr. Philip Sharp and Dr. Norman Davidson at the California Institute of Technology. We intend to extend this collaboration during the coming months. Dr. Glatzer has been awarded a fellowship to visit Dr. Davidson's laboratory to learn several techniques useful in our work. Dr. Davidson and his co-workers have been able to isolate F DNA as closed covalent circles on a preparative scale (5). F DNA can be "duplexed" *in vitro* with single-stranded DNA of related strains, and regions of homologous heteroduplexed DNA can be identified under the electron microscope (6). We expect to learn these techniques to test, first, whether a portion of F is transferred with the lead region. DNA transferred to minicells by Hfr strains will be annealed with purified F DNA. Examination of the DNA for regions of heteroduplexed strands will answer the question

directly. If this question is answered affirmatively, we shall examine a series of isogenic Hfr strains to determine whether the portion of F at the "origin" during transfer is variable or constant. This information will help define the precision with which a single-stranded scission is introduced in the donor chromosome during Hfr transfer, as well as the specificity of the integration site during F integration into the host chromosome. Standard DNA-DNA duplex formations will then be carried out on filter discs to substantiate the findings by electron microscopy.

1. E. L. Wollman, and F. Jacob, C. R. Acad. Sci. 246, 536 (1958).
2. H. I. Adler, W. D. Fisher, A. Cohen, and A. Hardigree, Proc. Nat. Acad. Sci. U.S.A. 57, 321 (1967).
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6. P. A. Sharp, M. T. Hsu, and N. Davidson, Biophys. Soc. Abstr. 11, 265 (1971).

CYTOGENETIC STUDIES FOLLOWING AN ACCIDENTAL WHOLE-BODY EXPOSURE TO ^{60}Co X-RAYS

J. G. Brewen, R. J. Preston, and L. G. Littlefield*

In February, 1971, an employee at the UT-AEC Agricultural Research Laboratory was accidentally irradiated with a significant dose of ^{60}Co X-rays. The distribution of this radiation over the anterior surface of the body was relatively homogeneous. Mock-up physical measurements were made by members of the staff at the Medical Division, Oak Ridge Associated Universities, using an Alderson Phantom and LiF termoluminescent dosimeters. Their measurements indicated that the patient received a mean midline dose of 127r.

Blood samples were obtained as early as 6 hr after exposure and periodically thereafter for 140 days, at this writing. Short-term peripheral leukocyte cultures were made, and suitable metaphase spreads were analyzed for chromosome damage. The data are summarized in Fig. 1. Concurrent with these studies, an acute ^{60}Co X-ray dose-response curve was plotted, using normal human blood irradiated in vitro. The yields of chromosome damage at various doses were fitted to the quadratic model, $Y = bD + cD^2$, by least-squares regression. The resulting estimates of the one- and two-track coefficients were $b = (0.393 \pm 0.106) \times 10^{-3}$ rings + dicentrics/cell/r and $c = (8.16 \pm 0.110) \times 10^{-6}$ rings + dicentrics/cell/r².

The mean aberration yield obtained over the first 32 days after exposure was 0.226 rings + dicentrics/cell. When this value is substituted for Y in the quadratic expression above, it provides an estimate for D of ~~144r~~. This value is in remarkably good agreement with the physically derived estimate.

John G. Brewen

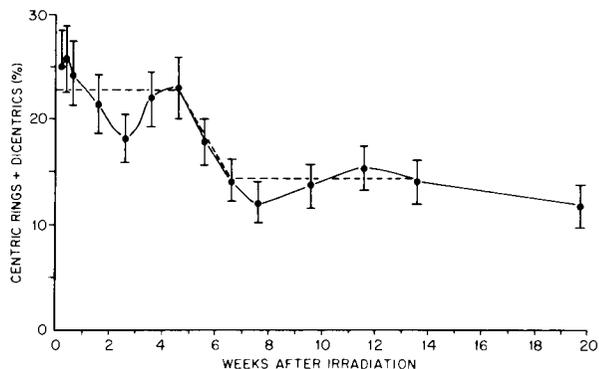


Fig. 1. The yield of asymmetrical exchange aberrations in human peripheral leukocytes at various times after an accidental whole-body exposure to ^{60}Co X-rays.

The broken line represents the mean frequency for the periods indicated.

This accidental exposure is unique in several respects. It is the first instance in which an individual with no prior history of exposure to significant levels of radiation or chemical mutagens received a high dose of homogeneously distributed radiation of a single quality, and the first in which blood samples were obtained so quickly after the exposure. The excellent agreement between the physically and cytogenetically derived dose estimates reaffirms the conviction that in vitro studies are a true reflection of the in vivo situation.

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RADIATION-INDUCED HUMAN CHROMOSOME ABERRATIONS. HUMAN IN VITRO IRRADIATION COMPARED TO IN VITRO AND IN VIVO IRRADIATION OF MARMOSSET LEUKOCYTES

J. G. Brewen and N. Gengozian*

One of the pressing problems in human radiation genetics is the question whether studies on the response of human cells treated in tissue culture are a true reflection of the response in vivo. Since there is a great wealth of data on the cytogenetic effects of ionizing radiation on human leukocytes treated in vitro, we attempted to compare the cytogenetic in vitro and in vivo responses of a suitable laboratory animal. The animal we chose was the new world primate S. F. illigeri, because it has a karyotype that is very similar to man's (Fig. 1).

Acute 250 kV X-ray dose-response curves were plotted by irradiating freshly drawn blood samples from both the marmoset and man. The doses, yields, and kinetics of aberration production are summarized in Fig. 2. The dose kinetics and aberration yields for the two species are very similar, as would be expected on the basis of the similarities in the karyotypes.

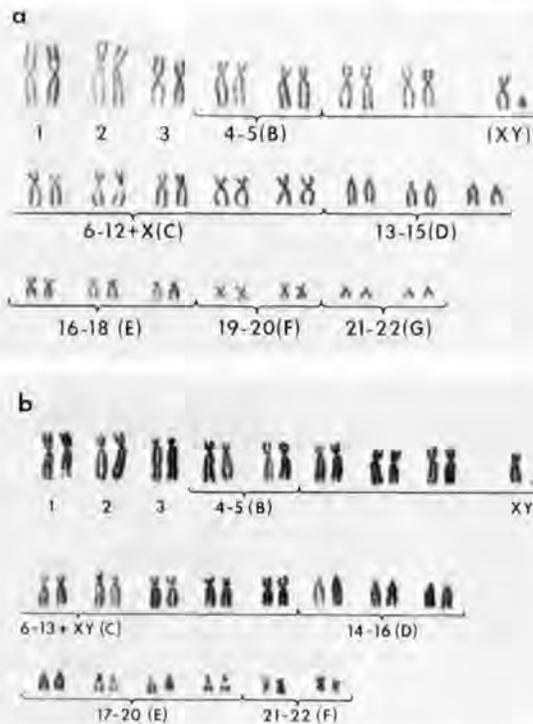


Fig. 1. Karyotypes of typical male human (a) and marmoset (b) metaphase cells.

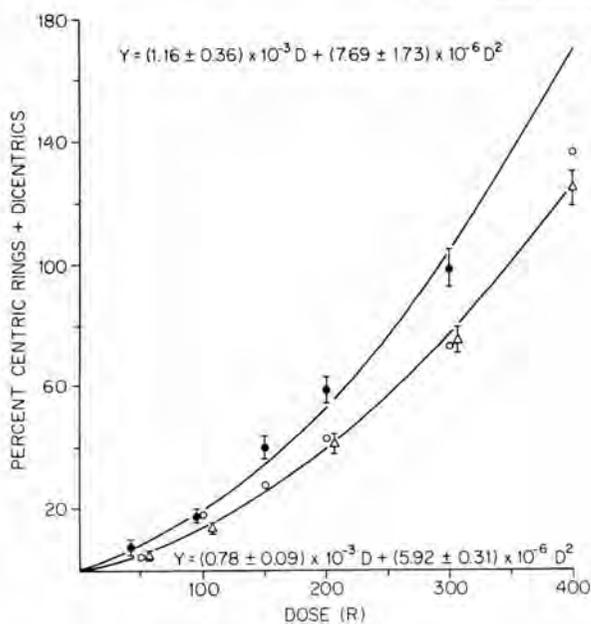


Fig. 2. The actual observed yields of centric rings plus dicentric chromosomes obtained when whole blood cultures of human (Δ - Δ) and marmoset (\bullet - \bullet) were irradiated, plotted against the "best-fit" regression curves.

The earlier data from irradiation of human whole blood samples are also given (O). These points are offset from the actual dose levels for the purpose of clarity.

In vivo and *in vitro* comparisons were then made by giving whole-body irradiation to several marmosets and drawing blood samples immediately and 24 hr after the irradiation. Concurrent to these studies, freshly drawn whole blood was irradiated with the same quality radiation at an identical dose rate. The radiation in these latter studies was ^{60}Co X-rays delivered at 3.7 r min^{-1} .

The doses used and the aberration yields observed are summarized in Tables I and II.

TABLE I. Chromosome aberration yields observed in marmoset leukocytes after whole-body ^{60}Co X-irradiation

Dose (R)	Animal number	Number of cells	Rings + dicentric (% \pm S. E.)
100	1	600	14.2 \pm 1.5
100	2	400	13.0 \pm 1.8
200	4	600	41.2 \pm 2.6
200	5	350	38.6 \pm 3.3
300	6	300	70.7 \pm 4.9
400	8	250	94.4 \pm 6.1

TABLE II. Chromosome aberration yields observed in marmoset leukocytes after *in vitro* ^{60}Co X-irradiation

Dose (R)	Number of cells	Rings + dicentric (% \pm S. E.)
0	300	0.0
100	300	13.3 \pm 2.1
200	300	33.3 \pm 3.3
300	300	68.7 \pm 4.8
400	300	98.3 \pm 5.7

Since in the *in vivo* studies the aberration yields in the blood samples drawn immediately and 24 hr after exposure were identical, the data are pooled in Table I.

It is obvious that there is no difference in the yield of chromosome aberrations induced in the *in vivo* and *in vitro* irradiations. These results suggest that the data obtained from *in vitro* irradiation of human leukocytes are in fact a direct measure of the damage expected from an *in vivo* exposure and add credibility to the assertion that *in vitro* studies on the effect of ionizing radiations can be applied directly to estimating man's hazard.

*Medical Division, Oak Ridge Associated Universities.

RADIATION-INDUCED HUMAN CHROMOSOME
ABERRATIONS. CHRONIC AND ACUTE
DOSE RATE STUDIES

J. G. Brewen and H. E. Luippold

One of the basic arguments that has developed over the past several years is whether the dose-response kinetics for the production of human chromosome aberrations are predominantly linear or dose square (1, 2). If the kinetics are essentially linear, alterations in dose rate will have little effect on the yield produced per unit dose. If the kinetics are dose square, however, fairly small alterations in dose rate will dramatically affect the yield per unit dose, with chronic dose rates producing relatively negligible yields. This issue assumes reasonable importance when it is considered that the great majority of ionizing radiations to which the general public is exposed are of a chronic nature. Hence, one argument states that this chronic exposure would be almost as effective as an equal acute dose, but the other states that it would be orders of magnitude less effective.

In order to clarify this issue somewhat, we performed experiments in which freshly drawn human peripheral blood was irradiated with either acute (25-100 r min⁻¹) 250 kV X-rays or chronic (0.07-0.5 r min⁻¹) ¹³⁷Cs γ -rays. Various doses were given over a constant time interval in each case, and the resulting yields of chromosome aberrations were fitted to various dose-response models. The models were $Y = bD$ (linear), $Y = cD^2$ (dose square), and $Y = bD + cD^2$ (linear + dose square). In both instances the best fit was to the model $Y = bD + cD^2$, indicating the presence of a significant linear as well as a significant dose-square component. The actual data and fits are summarized in Fig. 1.

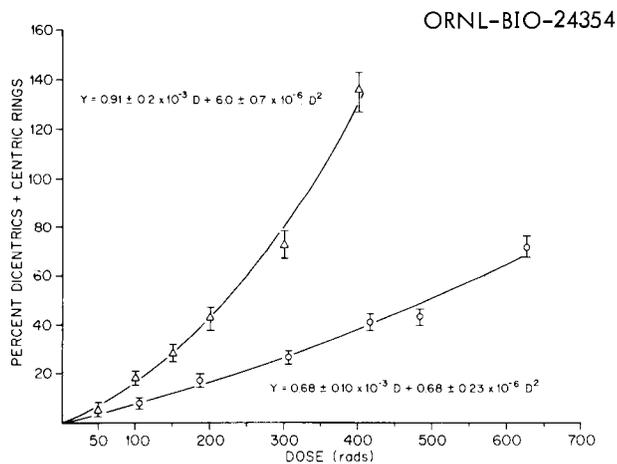


Fig. 1. Plot of the actual data for centric rings plus dicentrics against the best-fit estimates derived by least-squares regression analysis for the acute (Δ) and chronic (O) dose-response curves.

Standard errors are indicated at each point.

The linear component in both the acute and chronic dose-response curves is essentially the same if relative biological effectiveness is considered. The dose-square component, however, is reduced one order of magnitude, as would be expected on the basis of years of research in the area of radiation cytogenetics. These results imply that there is a reasonable genetic hazard from chronic radiation exposures, but that the hazards are certainly far less than the data of Evans (1) suggest.

Further investigations into the responses to chronic exposures are desirable. Hopefully, experiments can be done where the exposure times can be increased to weeks, so that a much more accurate estimation of the linear component can be made.

1. H. J. Evans, Annex C, Report of the United Nations Scientific Committee on the Effects of Atomic Radiation, p. 98. General Assembly, 24th Session, United Nations, New York (1967).
2. M. A. Bender, in *Advances in Radiation Biology* (L. G. Augenstein, R. Mason, and M. Zelle, eds.), Vol. 3, p. 215. Academic Press, New York (1969).

A COMPARISON OF THE FREQUENCIES OF CHROMOSOME ABERRATIONS IN CHINESE HAMSTER LEUKOCYTES FOLLOWING IN VIVO AND IN VITRO IRRADIATION

R. J. Preston, J. G. Brewen, and K. P. Jones

The induction of chromosome aberrations in human peripheral leukocytes following in vitro irradiation has been extensively studied. In order to extrapolate this information to the expected in vivo response, and subsequently to determine a relationship between this somatic cell damage and the amount of transmissible genetic damage, it is first of all necessary to determine for a laboratory animal whether the yield of chromosome aberrations is similar for in vivo and in vitro irradiations. Such a comparison has been made for the Chinese hamster, since it is a karyotypically suitable animal.

Blood was withdrawn by heart puncture either prior to or immediately after X-irradiation. Buffy-coat cultures were prepared and placed in small diffusion chambers as described by Brewen et al. (1). For the in vitro experiments, the leukocyte cultures were irradiated within the chambers. The chambers were then inserted into the body cavities of mice and remained there for 56-60 hr. At this time they were removed and cut open, and the contents were transferred to tissue-culture medium containing colchicine (2×10^{-7} M) and allowed to incubate at 37° C for 2 hr. Dissociation of the cells, fixation, and slide preparation were carried out as previously described (1).

X-ray (250 kV) doses of 50, 100, 200, 300, and 400 r were given in vitro, and 50, 200, and 400 r were given in vivo. The dose-response curve for dicentric and ring aberrations in both cases gave a good fit to the quadratic equation, and there was no significant difference between the in vivo and in vitro irradiations (Fig. 1).

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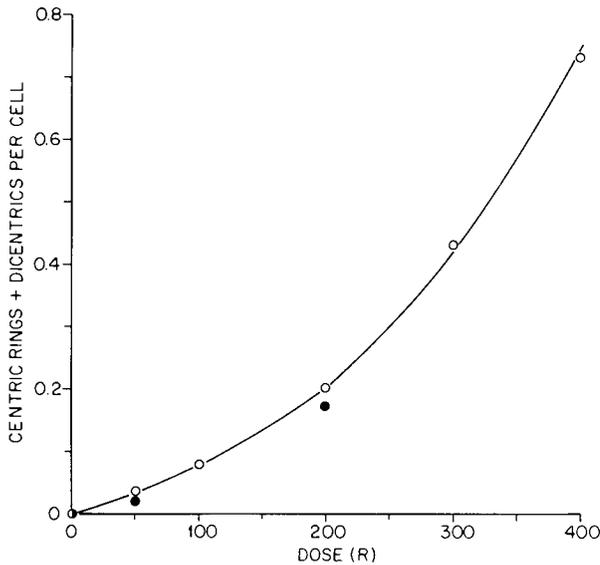


Fig. 1. X-ray dose-response curve for Chinese hamster cells irradiated *in vivo* (●) and *in vitro* (○).

Using chromosome aberration data obtained in this and other laboratories for leukocytes of several species, we have determined that for any X-ray dose there is a linear relationship between chromosome arm number and the yield of dicentric and ring aberrations. The yields of exchange aberrations for Chinese hamster leukocytes following *in vivo* and *in vitro* treatment were as predicted on the basis of the chromosome arm number; i. e., the Chinese hamster is only half as sensitive as the human.

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A COMPARISON OF THE RADIOSENSITIVITY OF BONE MARROW AND SPERMATOGONIAL CELLS OF THE CHINESE HAMSTER

J. G. Brewen and R. J. Preston

It was reported by Brooks and Lengemann (1) that there was a much higher yield of chromatid aberrations in spermatogonia than in bone marrow cells of the Chinese hamster at 6, 9, and 12 hr after X-irradiation, although there was little if any difference for cells sampled 3 hr after irradiation. This seemed a rather surprising result, and on analyzing the data we felt that it could possibly be explained by the fact that the cells from the two tissues sampled 6 hr or more after irradiation were in different stages of the cell cycle at the time of irradiation. This would be an important consideration, because it is well known that cells in G_2 are considerably more sensitive to chromatid aberration production than those in S .

Therefore, we conducted a series of experiments to test this hypothesis. Male hamsters were injected with ^3H -thymidine ($15.9 \text{ Ci mmole}^{-1}$, $225 \mu\text{Ci ml}^{-1}$) and then irradiated with an X-ray dose of 25, 50, 100, or 150 r. Colchicine was injected into the animals 3 hr prior to sampling. At 3, 6, 9, and 12 hr after irradiation, animals were killed, the testes removed, and spermatogonial cell preparations made. At the same time, bone marrow was obtained from both femurs, and chromosome preparations were made. The slides were then dipped in Kodak NTB nuclear emulsion and developed after a 2-week exposure. Unirradiated control animals were sampled at the same times following injection with ^3H -thymidine and were subsequently treated in the same way as the irradiated animals.

No labeled metaphase cells were seen in the spermatogonial cells of the control animals at 3 and 6 hr after injection, whereas some 40% of the bone marrow metaphase cells were labeled at 6 hr. Both spermatogonia and bone marrow metaphases were labeled at 9 and 12 hr after treatment. Following irradiation at the lowest dose, there were no labeled spermatogonial metaphases even at 12 hr after irradiation, whereas some 40% of the bone marrow metaphases were still labeled at 6 hr after irradiation. This indicated that only G_2 cells reached metaphase in irradiated spermatogonia, and that the S cells were either subject to a very long division delay or they were killed; but bone marrow cells that were in S at the time of irradiation reached mitosis 6 hr after irradiation.

The metaphase cells in samples taken 3 hr after irradiation in both spermatogonia and bone marrow were in G_2 (unlabeled) at the time of irradiation, and the yields of chromatid aberrations were not significantly different. However, spermatogonial metaphases sampled 6 hr after irradiation were those in G_2 at the time of irradiation, whereas the bone marrow metaphases sampled at this time would have been in S and G_2 at the time of irradiation. A comparison of unlabeled (G_2) cells showed only that the yield of chromatid aberrations is still the same in both bone marrow and spermatogonial cells 6 hr after irradiation, but that the yield in labeled (S) bone marrow cells is only about 25% of that in unlabeled (G_2) cells. This finding is in agreement with the observation that G_2 cells are much more sensitive to aberration induction than S cells. We can also conclude that if no account is taken of cell cycle stage, then it could appear that the spermatogonial cells are more sensitive than the bone marrow. However, when cells in the same stage of the cycle are compared, it is clear that there is no difference in sensitivity between bone marrow (somatic) and spermatogonial (germ) cells.

This finding is of particular importance in the studies being conducted in this laboratory to estimate the transmissible genetic damage in man from observations on chromosomal damage in somatic cells. The estimation will be facilitated by the finding that somatic and germ cells have the same sensitivity to chromosome aberration production.

1. A. L. Brooks and F. W. Lengemann, *Radiat. Res.* 32, 587 (1967).

NORMAL AND DEFECTIVE REPAIR OF DAMAGED DNA IN HUMAN CELLS. A SENSITIVE ASSAY UTILIZING THE PHOTOLYSIS OF BROMODEOXYURIDINE

James D. Regan, R. B. Setlow, and R. D. Ley

A new technique has been developed for studying the extent of repair of UV radiation damage to DNA in human cells. It is easy to use, has excellent sensitivity, and provides rapid quantitative estimates of repair. UV-irradiated cells whose DNA has been previously labeled with a radioisotope are grown after irradiation in nonradioactive bromodeoxyuridine, which is incorporated at the breaks induced by repair enzymes. After a period of growth in the thymidine analog, the cells are exposed to a large flux of 313-nm radiation and then lysed on top of an alkaline sucrose gradient. Bromodeoxyuridine-containing sections of the DNA are thus selectively photolysed (Fig. 1).

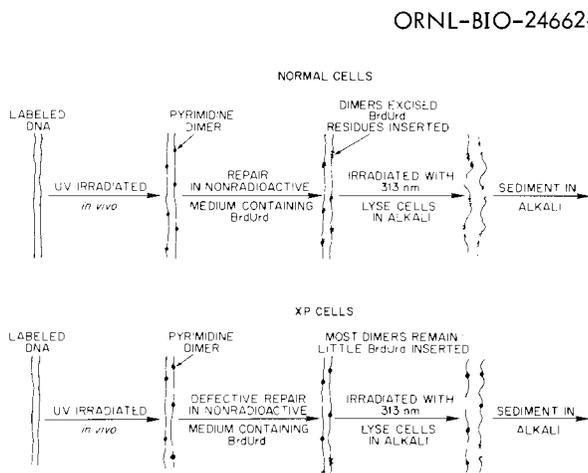


Fig. 1. Scheme for the detection of normal or defective repair in normal or xeroderma pigmentosum (XP) cells.

For control purposes, both cell types are incubated in nonradioactive medium containing dT rather than BrdUrd.

Sedimentation in the alkaline gradient reveals the average molecular weight of disrupted segments and gives a measure of the number of breaks induced by repair enzymes over the whole period allowed for repair. The large change in average molecular weight observed upon exposure of normal repairing cells to 313-nm radiation is not observed in the repair-deficient cells from patients with xeroderma pigmentosum (Fig. 2). The quantitative aspects of this assay for repair and its sensitivity should make it applicable to the study of repair induced by agents other than UV radiation. Utilization of this technique in a number of repair studies, employing a variety of DNA-damaging agents such as γ -rays and chemical mutagens and carcinogens, is described elsewhere in this annual report.

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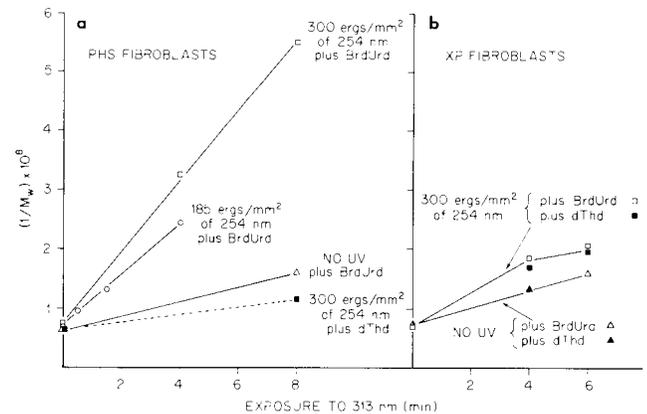


Fig. 2. Relationship between the reciprocal of the weight-average molecular weight ($1/M_w$) of DNA from (a) normal and (b) XP cells and exposure to 313-nm radiation.

XERODERMA PIGMENTOSUM. A RAPID, SENSITIVE METHOD FOR PRENATAL DIAGNOSIS

James D. Regan, R. B. Setlow, Michael M. Kaback,* R. Rodney Howell,* Edmund Klein,[†] and Gordon Burgess[†]

A method for prenatal diagnosis of a genetic defect should be unequivocal and reasonably rapid, so that therapeutic abortion, if indicated, can be performed as soon as possible. In the case of the rare autosomal recessive human disease xeroderma pigmentosum (XP), no prenatal diagnostic test with these characteristics has previously been described.

When normal human cells, capable of repairing UV-induced lesions in their DNA, are incubated in the thymidine analog 5-bromodeoxyuridine (BrdUrd) after UV irradiation, the analog is incorporated into the repaired regions. When such BrdUrd-repaired cells are subsequently irradiated with 313-nm radiation and placed in alkali, breaks appear in the DNA at sites of BrdUrd incorporation, inducing a dramatic downward shift in the sedimentation constant of the DNA. Cells from patients with XP, which involves high sensitivity to UV, are incapable or only minimally capable of repair; such cells incorporate little BrdUrd into their DNA under these conditions and, upon 313-nm irradiation and sedimentation in alkali, exhibit only minor shifts in DNA sedimentation constants. When these experimental conditions are applied to fibroblasts developed from normal and XP skin biopsies, as well as cells cultured from midtrimester amniotic fluid samples in a prenatal diagnostic procedure, one can demonstrate unequivocal differences between normal and XP cells. XP heterozygotes are also clearly distinguishable from homozygous mutants, and results can be made available within a few hours after the initial radioactive labeling of the cultures.

Fig. 1 shows typical results obtained with this method, using normal skin cells, heterozygote skin cells, XP skin cells, and normal amniocentetic cells drawn prenatally. The XP cells differ from the other three cell types in that the 313-nm radiation does not induce the marked decrease in molecular weight seen in the other cells, indicating only minimal BrdUrd insertion after UV damage.

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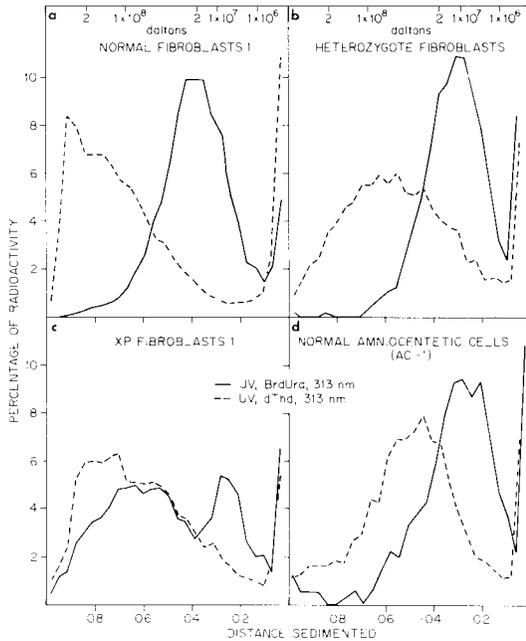


Fig. 1. Sedimentation patterns in alkaline sucrose of the radioactivity from ^3H -Thd-labeled cells irradiated with 200 erg mm^{-2} of 254-nm radiation, incubated for 16-20 hr in BrdUrd or thymidine (dThd), and then exposed to 313-nm radiation before being lysed on top of the gradients.

The molecular-weight scale for single-stranded DNA, by reference to separately sedimented phage T4 DNA, λ DNA, and ϕX174 DNA, is shown at the top of the figure. (a, c, d) $4.5 \times 10^6 \text{ erg mm}^{-2}$, (b) $7 \times 10^5 \text{ erg mm}^{-2}$ of 313 nm.

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BIOCHEMICAL ANALYSIS OF HUMAN GENETIC DEFECTS

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The study of mutation has long been a primary project in the Biology Division. Recently, a new approach to the study of human mutation has been initiated. The latest methodology in column chromatography is being used to analyze the body fluids of individuals who have a high probability of genetically

controlled metabolic disorders. Part of the instrumentation, a high resolution UV analyzer, was developed in other divisions at ORNL. The project combines the genetic, biochemical, and tissue-culture abilities of the biologist with advanced chemical technology and instrumentation.

Body fluids from large numbers of "high genetic risk" individuals are screened for unusual compounds. The protocol of the experiments has been set up so that examples of previously described genetic disorders can be quickly excluded. Thus, the research focuses on new "inborn errors of metabolism" that are reflected by the appearance of grossly different or increased amounts of metabolites in body fluids. After examples of potentially new mutations have been identified, cell cultures are derived from the individuals involved. Eventually, the specific biochemical pathways that are blocked or altered will be uncovered.

At the present stage, a number of suspect cases have been detected and are being investigated in depth. Studies are under way to rule out dietary and drug effects. A number of clinical and pediatric contacts are channeling selected samples into the study. A recent result is the finding of two siblings institutionalized at Murdoch Center, N. C., who show an elevated urinary urocanic acid (a histidine metabolite) by UV chromatography. Control studies with parents and normal siblings are underway. Levels of other metabolites in the pathway are being assayed both in urine and in serum.

In a number of other cases, grossly elevated levels of resolvable UV-absorbing or ninhydrin-positive compounds have been detected but not identified. In order to follow up on these potential mutations, we have initiated work on the identification of the unknown accumulants, using multiple-analytical techniques such as gas chromatography and mass spectroscopy. Much of this preliminary work has been carried out with the cooperation of the Body Fluids Analysis Program at ORNL.

The initial results indicate that increasing amounts of subsequent activities will also be devoted to cell culture and enzymology of presumptive human mutations. The end result of this work, a natural extension of the Division's existing research in biochemical genetics of microorganisms, should be an increased insight into human genetic defects.

CHARACTERIZATION OF X-RAY-INDUCED FORWARD MUTATIONS IN CHINESE HAMSTER CELL CULTURES*

E. H. Y. Chu and Patricia A. Brimer

An aneuploid cell line (V79) from the lung of a male Chinese hamster was used to determine the spontaneous and X-ray-induced mutations that are responsible for the acquisition of resistance to a purine analog, 8-azaguanine. The cells were sensitive to the analog at concentrations greater than $1 \mu\text{g ml}^{-1}$. Resistance arises from a loss or reduction in activity of hypoxanthine guanine phosphoribosyl transferase. In the Lesch-Nyhan syndrome in man, the control of the same enzyme activity is X-linked.

Our previous work (1, 2) demonstrated in hamster cells both spontaneous and chemically induced forward and reverse mutations at this locus. The rate of spontaneous forward

mutation to azaguanine resistance was 1.5×10^{-8} per cell per generation.

In the present study, cells were exposed to X-ray doses of 0-1200 r (250 kVp, 30 mA, with 3-mm Al filter, 100 r min^{-1}). The X-ray survival of these cells approximately fits a multi-target relation. The frequency of spontaneous mutation was 10^{-7} . Within the X-ray dose range tested, the rate of induced forward mutations (expressed as function of survivors) increases from 4.2×10^{-7} per locus per r with 200 r to 1.8×10^{-6} per locus per r with 1200 r. Several factors, including cell density, markedly influence the mutational yield. Reversion tests using specific chemical mutagens on 72 randomly isolated, azaguanine-resistant mutants suggest that both point mutations and chromosome deletions may have occurred in the hamster cells after exposure to ionizing radiation (3).

*Research jointly sponsored by the National Cancer Institute and by the U. S. Atomic Energy Commission under contract with the Union Carbide Corporation.

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EFFECTS OF CAFFEINE ON THE INDUCTION OF MUTATIONS IN CHINESE HAMSTER CELLS BY ULTRAVIOLET LIGHT

J. E. Trosko* and E. H. Y. Chu

Caffeine, theophylline, and theobromine have been shown to increase the frequencies of mutations and of killing in UV-treated bacteria. The mechanism of caffeine sensitization of UV damage in bacteria has been attributed to inhibition of the excision of pyrimidine dimers. In mouse L cells and Chinese hamster cells irradiated with UV light, caffeine decreases colony-forming ability. Caffeine does not inhibit excision of UV-induced pyrimidine dimers in human cells.

Although it induces chromosome aberrations in mammalian cells, caffeine by itself appears to be ineffective in the induction of auxotrophic mutants. Using the quantitative method for the detection of forward mutations to 8-azaguanine resistance, we have found that a nontoxic concentration of caffeine ($1 \times 10^{-3} \text{ M}$) lowers the frequency of mutations in UV-irradiated, as well as in nonirradiated, cultures. The data also confirm reported observations that caffeine sensitizes the irradiated cells in terms of colony-forming ability. It has been inferred from our results that in Chinese hamster cells caffeine inhibits a recombination repair mechanism (1) that is known to exist in *E. coli*.

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ISOLATION AND CHARACTERIZATION OF TEMPERATURE-SENSITIVE MUTANTS IN CHINESE HAMSTER CELLS IN VITRO

Diana B. Smith* and E. H. Y. Chu

A Chinese hamster cell line (V79) normally grown at 37°C was plated at 41°C , and a surviving clone was isolated and maintained at this elevated temperature. The thermo-tolerant cells were treated with ethyl methanesulfonate, plated at 37°C as single-cell clones in plastic microdepression plates, and subcultured by replica plating. The duplicate plates were exposed to both temperatures, and temperature-sensitive (ts) clones were isolated by this nonselective technique. Most of these ts mutant lines exhibited a density-dependent survival at 41°C . Several clear-cut ts mutants that died within 24 hr at 41°C regardless of cell density were chosen for further study. The average generation time for the parental as well as the mutant cell lines is 12 hr at 37°C .

Exponentially growing populations of mutant cells were transferred from 37 to 41°C , and their uptake of radioactively labeled thymidine, uridine, and leucine in different cell samples was periodically determined. The rates of macromolecular synthesis measured in this way were affected by cell density. The present study not only demonstrates the feasibility of isolating conditional lethal mutants in a mammalian cell line but also indicates the prospects of studying the genetic control of cellular functions in animal cells.

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RESISTANCE TO CYTOSINE ARABINOSIDE IN CHINESE HAMSTER CELLS

Diana B. Smith* and E. H. Y. Chu

Cytosine arabinoside (ara-C) is an inhibitor of DNA synthesis in mammalian cells and has been shown to break chromosomes *in vivo* and *in vitro*. It has a cytotoxic effect on cells in tissue culture, as measured by decreased plating efficiency. A current hypothesis for the effect of ara-C on sensitive cells is that ara-CTP competitively inhibits DNA polymerase. The genetic nature of resistance to ara-C in Chinese hamster cells is now being studied. Ara-C-resistant clones have been isolated from two Chinese hamster mutant cell lines, one resistant to 8-azaguanine and the other to 5-bromodeoxyuridine. Various ara-C-resistant clones from the two different parental backgrounds were hybridized with the aid of inactivated Sendai virus and selected on a medium supplemented with thymidine, hypoxanthine, aminopterin, and glycine. Neither parental line can grow alone on this medium. The hybrid clones isolated were then tested for their relative plating efficiencies on standard medium and on medium supplemented with ara-C. The putative hybrid clones have been confirmed cytologically. If ara-C resistance is a recessive characteristic, then hybrids between ara-C-resistant and wild-type cells will not survive on medium supplemented with ara-C. This is what has been found for the eight such hybrids so far studied. When ara-C-resistant clones are combined in hybrids, the

complementation pattern should indicate whether there is one locus or more controlling the resistance characteristic. Our results obtained to date suggest the existence of one locus, as well as intragenic complementation in some hybrids.

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AN IMPROVED METHOD FOR CHROMOSOME PREPARATION FROM MOUSE LIVER

N. C. Sun and E. H. Y. Chu

A simple method for preparing mitotic chromosomes from adult mouse liver is described. The procedure involves the accumulation of metaphases in regenerating liver resulting from CCl_4 treatment, followed by trypsin perfusion, hypotonic pretreatment and fixation of cells, and flame-drying of slides to spread chromosomes. Approximately 2×10^6 intact liver cells can be obtained from a single mouse liver — enough to prepare 50 slides. A peak mitotic activity, with more than 1% of cells in mitosis, was observed 72 hr after subcutaneous injection of 0.1 ml of 45% CCl_4 per animal. The distribution of diploid, tetraploid, and octaploid cells in mitosis was about 86, 11, and 3%, respectively. The abundant number of analyzable metaphases in such preparations makes this method valuable for cytogenetic analysis of normally nonproliferating tissue from adult laboratory mammals. This method is being used in studies on the cumulative cytogenetic effects in mouse liver of aging and experimental treatment with radiation and chemicals, which are now in progress.

EARLY MORTALITY INDUCED BY THE INJECTION OF SOLUBILIZED PLASMA MEMBRANE INTO MICE

R. A. Popp, M. W. Francis, and G. E. Cosgrove

Efforts are being continued to procure soluble murine transplantation antigens, to test their potential for inducing tolerance to skin and organ grafts. In general, soluble antigens are better toleragens than particulate antigens. Most preparations of solubilized transplantation antigens obtained from tissue extracts also contain other membrane components; therefore, we undertook to investigate whether the injection of solubilized erythrocyte membrane would have any effect on the physiology and pathology of mice.

The biphasic butanol-water system was used to solubilize erythrocyte stroma. Multiple injections, i.e. six injections of 1 mg of protein each time at weekly intervals, of the water-soluble phase of C57BL/6 red cell stroma into C57BL/10.D2 mice induced a delayed debilitation that grossly resembles the wasting disease of radiation-induced, hematopoietic chimeras. Mice that died 4–12 months after treatment had severe pyelonephritis and a generalized amyloidosis in their liver, spleen, and kidneys. The results suggest that caution should be taken in the use of crude preparations of solubilized transplantation antigens as toleragens in potential human organ transplantation recipients.

DUPLICATE HEMOGLOBIN BETA-CHAIN GENES IN MICE

R. A. Popp and E. G. Bailiff

The beta-chain polypeptides of hemoglobin from BALB/c and C3H mice are polymorphic, in contrast to the single type of beta-chain polypeptide of hemoglobin from C57BL mice. The genetic and biochemical bases for hemoglobin beta-chain polymorphism in mice have not been defined. The amino acid sequences of the multiple forms of beta chains in BALB/c mice are being determined to learn whether the polymorphism results from duplicate beta-chain genes, translational variation, or other possibilities.

The ratio of the major and minor components of the beta-chain polypeptides is approximately 85/15. Four residues showing amino acid duplexity have been found in the first 60 residues, beginning at the amino-terminal end. Residue 16 contains 0.83 units of glycine and 0.17 units of alanine. Quantitation at the other positions is more difficult to determine as accurately, but the major and minor amino acids at positions 9, 20, and 58 are alanine and serine, serine and proline, and alanine and proline, respectively. Data suggest that the major and minor hemoglobin beta-chain polypeptides from BALB/c mice are controlled by duplicate genes at the beta-chain locus. The amino acid duplexities within the first 60 residues do not explain the electrophoretic mobility differences between the major and minor polypeptides. The remainder of the residues are being examined to determine the total number of amino acid differences between these polypeptides and what amino acid differences account for their differential electrophoretic mobilities.

CHEMICAL MODIFICATION OF MEMBRANE-BOUND HISTOCOMPATIBILITY ANTIGENS IN MICE

R. A. Popp and Diana M. Popp

The effects of chemical alterations of specific amino acids on the immunological activities of antigens and antibodies have been used to yield some information about the chemical properties of these molecules. We have used similar chemical methods to learn what amino acids are involved in the antigenic sites of murine histocompatibility antigens. Mouse red cell stroma has been used as an immunoadsorbant for anti-H-2 and anti-H-14 antibodies. The effects of chemical treatment of the stroma are determined by analyzing the absorption and subsequent elution of isoantibody by normal and chemically treated stroma.

Maleic anhydride blocks the antigenic sites of H-2 specificities 3 and 11, but not 4, of strain A/Sn erythrocyte stroma. Maleylation also blocks H-2.2 and H-14^b specificities of C57BL erythrocyte stroma; it does not block H-2.8 and H-2.9, but it does block H-14^a of RFM erythrocyte stroma. That maleylation affects both H-14^a specificities indicates that lysine is located in or near the antigenic site; however, it also suggests that lysine is not the amino acid that imparts the specificity distinguishing the allelic forms of the antigens controlled by the H-14 locus. Similar studies show that N -acetylimidazole, which acetylates exposed tyrosyl residues, and neuraminidase, which releases neuraminic acid from oligosaccharides, have no effect on several antigenic sites tested. Treatment of strain A/Sn erythrocyte

stroma with water-soluble carbodiimide, which complexes with exposed carboxyl residues, causes a 50% reduction in the absorption of antibodies to H-2 specificities 3, 4, and 11. Studies on the chemical modification of specific amino acid residues in transplantation antigens do not define the primary or tertiary structures of these antigenic sites, but they do provide some information on the amino acids involved in these specific antigen-antibody reactions.

IN VIVO LYMPHOCYTE TRANSFORMATION INDUCED BY H-2 AND H-14 MOUSE ISOANTIGENS

Diana M. Popp and M. L. Davis

Mammalian small lymphocytes undergo blast-cell transformation upon contact with foreign antigens. *In vitro* transformation can be demonstrated following the injection of allogeneic or xenogeneic lymphocytes into irradiated recipients. The patterns of lymphocyte transformation caused by major (H-2) and minor (non-H-2) isoantigen incompatibilities differ markedly, but the detailed histology of *in vivo* lymphocyte transformation caused by minor isoantigens has never been reported. *In vivo* lymphocyte transformation caused by H-14 isoantigen was compared with a similar response to H-2 isoantigen, to determine whether the patterns of lymphocyte transformation correlate with the dual origin of the thymus-dependent and thymus-independent lymphocytes in the lymphatic follicle. Donor lymph node cells were infused into lethally irradiated recipients, and the histology of the splenic follicles was followed at regular intervals. Congenic mice differing at H-14 (C57/Ha and C57.F) and H-2 (C57BL/10 and B10.D2) were used. *In vivo* lymphocyte transformation induced by H-2 antigenic stimulation begins at 24 hr, while that induced by H-14 antigenic stimulation begins at 96 hr. In strains of mice that differ at H-2, lymphocyte transformation was prominent in the thymus-dependent regions; however, in mice that differ at H-14, lymphocyte transformation was restricted to the germinal center regions of lymphatic follicles. The differential response to H-2 and H-14 isoantigens seems to correlate with the dual origin of lymphocytes.

MUTABILITY OF ULTRAVIOLET-SENSITIVE STRAINS OF NEUROSPORA CRASSA

F. J. de Serres

The effects of seven different mutations that enhance the UV-induced inactivation of conidia from haploid strains of *Neurospora crassa* have been studied on spontaneous and UV-induced forward mutation frequencies of the *ad-3A* and *ad-3B* loci. The UV-sensitive strains were made homozygous with wild-type OR74A; they carry the genetic markers *al-2*, *cot*, and *pan-2*. Present data suggest that strains carrying *uvs-1* or *uvs-4* may give a lower spontaneous mutation frequency than wild type; *uvs-2*, *uvs-5*, *nuc-2*, and *upr-1* strains have the same spontaneous mutability as wild type ($\sim 0.3 \times 10^{-6}$); and *uvs-3* is about 10 times more mutable. After UV irradiation and incubation (in the dark), a peak mutation frequency of about 250×10^{-6} survivors was obtained with wild-type

strains after a UV dose of 1200 erg mm^{-2} ; at higher doses, the dose-effect curve for mutation induction saturates, and lower mutation frequencies are found. Present data indicate that *uvs-1*, *upr-1* and *uvs-5* strains are slightly less mutable after UV irradiation than wild type or show dose-effect curves that saturate at lower UV doses. The *uvs-2* strain is 5-10 times more sensitive to mutation induction with UV than wild-type strains. Two of the strains, *uvs-3* and *uvs-4*, showed markedly reduced UV-induced mutability; no UV-induced *ad-3* mutants were found with doses giving survival levels ranging from 100 to 1%. The marked differences observed between the dose-effect curves for mutation induction in *uvs* and wild-type strains demonstrates the need for a wide range of UV doses (rather than a single dose) for comparison of the mutability of *uvs* strains. Samples of spontaneous and UV-induced *ad-3* mutants obtained in these experiments have been reserved for genetic characterization, to determine whether there are qualitative differences in the spectra of UV-induced *ad-3* mutations as well as quantitative.

INACTIVATION OF *ad-3* MUTATIONS IN NEUROSPORA CRASSA DURING INCUBATION IN MICE AND RATS

H. V. Malling

In the host-mediated assay for the detection of mutagenic activity in animals, an indicator organism is injected into the peritoneal cavity of an animal, which then is treated with a mutagen or premutagen. When *Neurospora* was used as the indicator organism and kept for more than 24 hr in the peritoneal cavity of untreated mice, the *ad-3* mutation frequency among the surviving conidia increased sharply, and more so in rats than in mice. This increase in the *ad-3* mutation frequency could be due to cellular contact, macromolecules, or smaller molecules already present in untreated animals. Conidia enclosed in dialysis tubing or in diffusion chambers surgically placed into the peritoneal cavity had a much smaller frequency of *ad-3* mutations than conidia injected as a suspension into the peritoneal cavity, indicating that most of the mutations are mediated through cellular contact or interaction with small molecules. To determine whether the dialysis bags were permeable to mutagens, the mutation frequencies among conidia in dialysis bags and free in the cavity were determined after treatment with methyl methanesulfonate (MMS). Eight hours after the conidia were placed into the animals, 100 mg kg^{-1} MMS was injected in the tail vein. The conidia were recovered 10 hr after the injection of the mutagen. The MMS-induced mutation frequency was the same among both groups of conidia.

Autopsies of animals injected 24 days previously with conidia suspension showed that they conidia were still present, even though essentially 100% of them had been inactivated by 96 hr after injection. The inactivation of these fungi spores may result from an enzymatic degradation of the fungal DNA, mediated by the host. Halting this process prematurely may result in induction of recessive-lethal mutations. Thus, the induction of mutations in infectious organisms may be a part of the defense mechanism of higher animals.

THE AUTOMATED RETRIEVAL OF MUTAGENIC DATA

H. V. Malling, J. S. Wassom, and E. S. von Halle

The Environmental Mutagen Information Center (EMIC) is a specialized information facility that began collecting data on the mutagenic action of chemicals and adapting computer programs for the automated retrieval of this information in October, 1969. In the past, these data were never easily accessible and could be obtained only after a laborious manual search. There are a few automated multidiscipline facilities to which the researcher could turn for assistance; but, as a result of broad subject coverages, the output from these centers usually contains a great amount of unusable material, and coverage of the specific search area usually is incomplete. To overcome these handicaps in an era when publications are doubling annually, EMIC began with the cooperation of Union Carbide's Computer Technology Center to devise new and/or adapt existing machine programs to handle our output requirements.

To date, we have assembled and organized a unique collection from the world literature reporting on the genetic and allied effects of chemical compounds. The number of documents that have been indexed and stored in our data banks number 3600. The majority of these documents are those published after 1968. EMIC searches these data cells to provide information to requisitioners concerning the mutagenicity of chemicals. Requests for searches arrive at the Center at a rate of 2-3 per week. Special indexed literature surveys are also compiled and published on various subjects. Some of our more recent compilations are collections on the mutagenicity of psychotropic drugs, chromosome-breaking agents, chemical mu-

tagens in mammals, genetic effects of caffeine, and the mutagenicity of nitroso compounds. We also compiled a survey of the literature on chemical mutagenesis during 1969 and published it as a supplement to the Environmental Mutagen Society's Newsletter No. 3. To date, 920 copies of this document have been distributed.

Presently, our data are retrievable only by limited indices (e.g. by author, keywords from titles, article source, year of publication, and EMIC accession and/or identification code). We have several programs under study that will make our information more flexible, thereby increasing its value. Initial results from these tentative programs have been most encouraging.

For example, a program of tabular abstracting would be of great value. Use of this abstracting scheme makes it possible to segregate essential data under different column designators. All items under these headings will be searchable. Table I shows an example of this technique as it will appear when printed by the computer. To supplement the information entered under our data tabulation format, it will be necessary to create a chemical dictionary with synonyms. Also, in order to bring about an even better understanding of chemical-biological interactions, implementation of a substructural search program would be extremely helpful. This would enable the user to draw correlations between chemically active groups and mutagenic activity, a capability that might have some prognostic value for predicting the mutagenic activity of newly created chemicals. In connection with these data, a program capable of relating the metabolic fate of chemical compounds in different organisms would be highly desirable.

Through EMIC's present and experimental programs designed for the automated retrieval of mutagenic data, the literature reporting the testing of chemicals for mutagenicity will be rendered more useful and more accessible.

TABLE I.

Agent	Test organism	Assay system	Dose		Observed and/or reported biological effect	EMIC accession number
			Range	Minimum effective dose		
Ethylene oxide	<u>Neurospora crassa</u>	Conidia	0.14 M		Point mutations and reverse mutations	34
Ethylene oxide	Maize	Plant cells	1 part ethylene oxide to 20 parts air		Chromosome breaks	25
Ethylmercury chloride	<u>Triticum</u>	Root tips	0.5 to 1 %		Mitotic aberrations	357
Ethylmercury	<u>Secale cereale</u>	Root tips	0.5 to 1 %		Mitotic aberrations	357
Lindane	<u>Allium cepa</u>	Root tips	.0006 to 2 %	.00125 %	Induced C-mitosis	571

THE EFFECT OF A CHANGE IN THE FREQUENCY OF
RECOMBINATION BETWEEN NONALLELIC MARKERS
ON THE FREQUENCIES OF RECIPROCAL AND
NONRECIPROCAL RECOMBINATION

F. J. de Serres

Studies on intercrosses of ad-3A and ad-3B mutants of Neurospora crassa induced in the same wild-type strain and in different wild-type strains have shown significant differences in recombination frequency, as determined by the frequency of adenine-independent progeny. By using closely linked genetic markers both to the left and to the right of the ad-3 region, it has been possible to determine the effect of a change in the frequency of recombination on the frequencies of reciprocal and nonreciprocal recombination. Tetrad analyses by others (1) have shown that the majority of asci showing reciprocal recombination were of the genotype expected from a single crossover, whereas recombinants resulting from nonreciprocal recombination were of all four genotypes with reference to outside markers. This finding makes it possible to estimate the frequency of reciprocal and nonreciprocal recombination in ad-3A X ad-3B crosses by determining the relative frequencies of adenine-independent progeny of genotypes expected to arise from a single crossover and from multiple crossovers, respectively. The data from the analysis of ad-3A X ad-3B crosses of mutants induced in the same wild-type strain versus the intercrosses between mutants induced in different wild-type strains has shown that the higher recombination frequencies observed in the latter crosses result from an increase in the frequency of reciprocal recombination alone. The frequencies of nonreciprocal recombination are not significantly different in the two groups of crosses.

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DUPLICATIONS OF THE 5S RNA LOCUS IN
DROSOPHILA MELANOGASTER

Carroll Nix

In Drosophila, the genes for 18 and 28S RNA are clustered at the nucleolus organizer region on the X and Y chromosome (1). Since ribosomes, from whatever source, contain one molecule of 5S RNA in addition to one molecule each of 16-18S and 23-28S RNA, one might expect all three loci to be tightly linked. This seems to be the case in Bacillus subtilis, but Tartof and Perry (2) have excluded the sex chromosomes as the site of 5S RNA in Drosophila melanogaster. More recently, Wimber and Steffensen (3) have presented evidence, using in situ hybridization and autoradiography, that most of the 5S RNA genes are located on 2R at 56 E-F of the salivary map.

In order to confirm and extend the results of the above investigators we have produced several duplications of the 56 E-F region. These duplications were produced in two different ways. The first was made by obtaining a recombinant between two different inversions, In(2R)Cy and In(2R)-C129. By selecting for the appropriate markers, a recom-

binant was obtained which contained the left end of In(2R)Cy (42A2-3;58A4-B1) and the right end of In(2R)C129 (43F;56E). Thus the recombinant, designated as In(2R)CY^LC129^R, was duplicated for 56E;58A4-B1 and deficient for 42A2-3;43F. [The reciprocal recombinant In(2R)C129^LCy^R was not recovered.]

In order to obtain chromosomes duplicated for 56 E-F, but without a corresponding deficiency, bw/bw females were irradiated with 4500 r and mated to M(2)173/SM5, al² Cy lit^y sp² males. Since most Minutes are deficiencies of various sizes, it was expected that most Minute suppressors would be duplications. Thus, non-Cy progeny were scored for the presence or absence of the Minute phenotype. Stocks were established from non-Minute progeny, and the salivary chromosomes were examined for the presence of duplications. Five suppressors of M(2)173 were recovered out of 5053 progeny examined. Upon analysis, two proved to be rather large tandem duplications with the following tentative break points: (1) Su^{II}-56C;59D and (2) Su^{IV}-56F3-58A-B. Thus far, I have been unable to identify cytologically the duplications, if any, present in the other three suppressors.

Although it has been relatively easy to produce duplications for the 56 E-F region, all attempts to produce deficiencies have failed.

DNA-RNA hybridizations will be done with each suppressor in order to determine the number of 5S RNA genes.

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APPROXIMATE LOCATION OF A GLUTAMIC-OXALO-
ACETIC TRANSAMINASE GENE IN DROSOPHILA
MELANOGASTER

E. H. Grell

The genetics of a number of enzymes have been studied in Drosophila melanogaster. In previous work, initial progress has depended on the finding of at least two naturally occurring alleles of the gene that specifies the primary structure of the enzyme. Most often, the two alleles were associated with electrophoretically different forms of the enzyme. Several enzymes do not display the required natural electrophoretic variants, and one of these is glutamic-oxaloacetic transaminase (GOT). There is, however, an electrophoretic difference between GOT of D. melanogaster and that of a closely related species, D. simulans. These two species may be crossed to produce certain kinds of F₁ hybrids, but the F₁'s of both sexes are sterile, and therefore segregation of marker genes can not be used to assign a chromosomal location to genetic differences in the usual way. The method of X-ray-induced partial hybrids devised by Muller and Pontecorvo circumvents the problem to some extent. The partial hybrids are the result of the fertilization of a D. melanogaster egg containing an extra chromosome by a sperm that is effectively

deficient in that chromosome as a result of X-ray treatment.

The partial hybrids were made, and it was found that flies that were homozygous for *D. melanogaster* second chromosomes contained only the *D. melanogaster* GOT, regardless of the heterozygosity of other chromosomes. Therefore, the GOT gene must be located on the second chromosome. Further refinement of the technique indicates that the locus is on the left arm of the second chromosome. This rough location of the gene opens the way to the induction of variants of GOT in *D. melanogaster* and proves that the method is practical for several other enzymes.

THE BEHAVIOR OF METACENTRIC CHROMOSOMES AT DISTRIBUTIVE PAIRING

Charleen M. Moore* and Rhoda F. Grell

Distributive pairing, a postexchange process, has been shown to be an essential feature of regular chromosome segregation during oogenesis in *D. melanogaster*. There is evidence to suggest that it may be operative in human gametogenesis as well (1). Certain rules governing the process have been clarified, but a number of questions remain. For example, it has been established that participation is limited to noncrossover chromosomes, that recognition for pairing is size dependent and homology independent, that compound chromosomes always participate regardless of exchange between their homologous arms, and that disjunction from trivalents is characterized by the tendency of the intermediate chromosome to migrate to one pole and the small and large chromosomes to the other. On the other hand, analyses of size dependency and disjunctive properties, which were accomplished with the use of one-armed (acrocentric) chromosomes, have revealed little concerning the role of chromosome configuration in the process. It was not known whether, for a two-armed (metacentric) chromosome, size recognition is dependent on arm length or on total length, or whether disjunction is altered by the presence of a second arm. A set of experiments designed to answer these questions has been carried out.

The present tests utilized a compound metacentric fourth chromosome ($\overline{44}$), constructed by attaching two acrocentric 4's to a single centromere, so that its arm length is identical to that of a normal 4 but its total length is twice that of a 4. Recognition was judged by the frequency with which the $\overline{44}$ segregated from a series of different-sized X-duplications (\overline{Dp}) ranging from ≤ 0.3 to > 4 times the size of a single 4. $\overline{Dp}, \overline{44}$ segregation was measured in the absence and in the presence of a competitor.

A comparison of the $\overline{Dp}, \overline{44}$ recognition curves with those previously obtained for the same X-duplications and a single 4, showed, for both competitive and noncompetitive situations, that the curves are very similar in shape; but in each case the $\overline{Dp}, \overline{44}$ curve is displaced one unit to the right, corresponding precisely to the difference in size between the 4 and $\overline{44}$. This means that total length and not arm length confers the characteristic recognition property to the $\overline{44}$.

While rules governing chromosome recognition derived from acrocentrics appear to be applicable to metacentrics,

the disjunctive behavior of a metacentric was found to differ. The second arm permits the $\overline{44}$ to act as the intermediate of a trivalent when the size criterion shows it to be the smallest element. The capacity to assume intermediacy is itself size dependent, since the property is lost when the size ratio of the small metacentric to the intermediate acrocentric becomes $\sim 5/9$ or less.

The enhanced ability of a metacentric to act as the intermediate when it is the smallest chromosome of the trivalent prompted an investigation to determine whether a metacentric possesses a similar ability when it is the largest chromosome of the trivalent. Structurally modified X chromosomes that differed in size through the addition or deletion of heterochromatin and, in some instances, differed in configuration through the addition of a second heterochromatic arm were used for the study. Heterozygous inversions insured the participation of the X's in the distributive process with a high frequency. In the competitive studies, the third chromosome was the small $\overline{44}$.

When the two X's were both single-armed, the smaller X directed the larger \overline{X} and the very small $\overline{44}$ to the same pole; when the larger X carried a second arm, it assumed the directing role; when the size ratio of the smaller acrocentric X to the larger metacentric X became less than $\sim 5/9$, the smaller X again directed the other two. A metacentric, whether the smallest or largest member of a trivalent, possesses the ability, then, within restricted size limits, to act as the directing element — a property reserved solely for the intermediate chromosome when only acrocentrics are involved.

Configuration does not alter the recognition properties of chromosomes, but it does modify their disjunctive behavior.

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1. R. F. Grell, *Ann. Genet.* (in press).

HEAT-INDUCED ALTERATIONS IN INTERFERENCE PATTERNS

Rhoda F. Grell

Whether DNA replication and recombination are coupled or independent processes is unknown. Elevated temperature increases the frequency of recombination in certain chromosomal regions and has been widely used as a tool in eucaryotic systems to mark exchange chronologically. Genetic studies with a variety of organisms have shown that increases in exchange are induced when temperature treatment is given at premeiotic interphase, coinciding at least partially with DNA replication.

An independent approach to the localization of the exchange process involves an analysis of the effect of heat on chromosome interference. Positive interference is defined as the tendency of one crossover to suppress other crossovers in its vicinity. Interference increases as loci become closer and decreases as they become more distant. In *Drosophila*,

interference is considered to be complete within 12 cross-over units, partial between 12 and 40 units, and to disappear beyond 40 units. If, as is generally assumed, interference arises through the configurations that homologs assume when they pair for exchange, changes in interference patterns should reflect changes in the process of exchange pairing.

The ability of an increase of 10° C for 12 hr to alter interference patterns in the X chromosome of *Drosophila* has been examined. Temperature treatment was given to different groups of females at sequential time periods, so that progressively older germ cells were exposed. The results show that heat decreased interference between some regions and increased interference between others so as to drastically alter the normal pattern. The greatest decreases occurred between nearest neighbors, so that interference was sometimes abolished between regions where it is normally complete. Increases occurred between distant regions, where interference is normally absent.

The effect of heat on interference showed no correlation with its effect on exchange. Maximum effects on interference occurred with treatment given immediately preceding or coinciding with oocyte formation; lesser but pronounced effects occurred during premeiotic interphase.

THE BEHAVIOR OF A RING CHROMOSOME AT DISTRIBUTIVE PAIRING

Charleen M. Moore* and Rhoda F. Grell

The properties of chromosomes at distributive pairing have been shown to be size dependent. The experiments that provided this information utilized a series of rod-shaped chromosomes, for which increase in length corresponds to increase in mass. To determine whether size dependency is essentially a reflection of mass dependency, the segregation pattern of a ring-X and a rod-X of identical mass were examined in the presence and absence of a small competitor, a compound-4 (44). When the 44 was absent, recognition between the X's was not affected by the difference in their form. The addition of the 44 induced a 100-fold increase in X-nondisjunction, and trivalent formation occurred frequently. When trivalents were formed, the ring-X acted as a chromosome of intermediate size, directing the rod-X and 44 to the same pole while it migrated to the other. Recovery of the 44 with the rod-X occurred three times as frequently as with the ring-X. The premise that mass alone determines distributive behavior was rejected, since altered configuration confers altered disjunctive properties.

This study provided an opportunity to examine the requirement for a telomere in the segregational process. The ability of the ring-X to participate in trivalent formation, as well as its ability to direct disjunction from the trivalent, indicates that a telomere is not essential for these aspects of chromosome behavior.

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NONHOMOLOGOUS PAIRING IN THE SOMATIC CELLS OF *DROSOPHILA*

Charleen M. Moore*

A cytological study of oogonial cells of *Drosophila melanogaster* has revealed that in females of the proper genotype nonhomologous chromosomes undergo a size-dependent pairing (1). To determine whether such pairing is limited to the female sex and to the germ-cell line, ganglia taken from male and female larvae as well as oogonia cells were examined. Three different genotypes were studied. In each case a compound-4 (44) comprised one of the nonhomologs and one of three different-sized X-duplications the other. Pairing was determined both by inspection and by precise measurements for a sample of the cells, and the results agreed in virtually every case. The X-duplication whose length was the same as the 44 showed the highest frequency of pairing in each of the three tissues (53%, oogonia; 63%, ♀ ganglia; 53%, ♂ ganglia); the duplication with a length twice that of the 44 showed intermediate frequencies (39%; 45% and; 43%, respectively); and the duplication 1/7 the size of the 44 showed the lowest frequencies (26%; 30%; 23%, respectively). Homologous pairing of the autosomes and X's ranged from 89-97%, but the X and Y in ganglia cells of male larvae resembled nonhomologs by pairing < 50%.

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1. R. F. Grell and J. W. Day, *Chromosoma* 31, 434 (1970)

HEAT-INDUCED STERILITY OF *DROSOPHILA* MALES

Rhoda F. Grell, R. Wilkerson, and W. Whitmer*

Effects of thermal pollution on the reproductive capacity of animals have not been well characterized. A study has begun to evaluate the ability of elevated temperature to alter the normal course of gametogenesis in the *Drosophila* male. Treatment consists of an increase of 7° C (or 10° C) for 24 hr to different groups of males on successive days of development. Mating of the treated males following their eclosion permits detection of those heat-induced alterations in the germ cells during the maturation process that lead to sterility. The day of treatment roughly identifies the stage the germ cell has reached at the time of response. The study includes a comparison of the responses of strains originating from different geographic locations to reveal whether the genetically specified temperature range for normal sperm development is correlated with the native climate.

Two strains have been studied thus far. Heat treatment (7° C increase) during the latter half of male development induces complete sterility in one strain and partial sterility in the other. An additional increment of 3° C increases sterility in the latter case, suggesting a strain-specific threshold. The sensitive stages include the mature sperm, spermiogenesis, and part if not all of spermatogenesis. Brooding of the treated males indicates sterility is temporary, although

normal fertility is not attained. Cytological examination discloses nonmotile sperm with tail irregularities.

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CATALYTIC FACILITATION BY ENZYME COMPLEXES. ANALYSIS OF THE POSSIBLE INFLUENCE OF MULTIENZYME COMPLEXES ON TRANSITION TIME

F. H. Gaertner

Compared to unaggregated multienzyme systems, multienzyme complexes may possess unique catalytic properties. Among these are (1) compartmentalization or channeling of a sequence of intermediate substrates, (2) reduction of transition times or increase in catalytic efficiency by elimination of rate-limiting diffusion steps between sequential enzymes, (3) inhibition or activation of a multienzyme system through the conformational change or allosteric regulation of a complex, and (4) reduction of transition times or a decrease in the time required for a multienzyme system to proceed from one steady state to another. Each of these properties may be examined *in vitro* with isolated multienzyme complexes, but as yet such studies are limited, and investigations of transition times have not been made.

In general, providing steady state can be adequately approximated, the transition time for an irreversible monoliner chain ($A \rightarrow B \rightarrow C$) is given by $\Delta t_x = \frac{x(B_f - B_0)}{(v_1 - v_2)} - \ln \frac{(1-x)v_2 K_2 / (v_1 - v_2)^2}{(v_1 - v_2)^2}$, where Δt_x is the time for a fractional rise (x) to steady state, B_0 and B_f are the initial and final concentrations of B , v_2 is the v_{max} of reaction 2, K_2 is the K_m of 2, and v_1 is the velocity of the first reaction. Hence, the time required to achieve steady state ($x = 1$) is theoretically infinite, but Δt can be calculated for $x < 1$. Although the above expression is adequate for a two-step sequence (e.g. the anthranilate synthetase complex *Neurospora crassa*), the transition times for each step of a five-step process (e.g. the aromatic complex) will not be simply additive. In general, it is found for a sequence $A \rightarrow B \rightarrow C \rightarrow D \rightarrow E \rightarrow F$ that $\Delta t_{0.8} = 0.8(B_f - B_0) / (v_1 - v_5) + 1.61v_2 K_2 / (v_1 - v_5) + 0.8(C_f - C_0) / (v_1 - v_5) + \dots + 1.61v_5 K_5 / (v_1 - v_5)^2$, where $v_1 - v_5 = \frac{dC + dD + dE}{dt}$. Thus, the lag time of the overall reaction may be measured *in vitro* and compared to calculated values. Significant decrease from the transition time calculated for individual steps is theoretically a unique possibility for an aggregated multienzyme system.

EVIDENCE FOR DISTINCT KYNURENINASE AND HYDROXYKYNURENINASE ACTIVITIES IN NEUROSPORA CRASSA

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

Previous studies have indicated that a single enzyme, "kynureninase," catalyzes the reactions of L -kynurenine to anthranilate and L -3-hydroxykynurenine to 3-hydroxyanthranilate in *Neurospora crassa* and in other organisms.

Recently, we have demonstrated separate enzymes that catalyze these reactions in *N. crassa*. The first, a kynureninase, preferentially catalyzes kynurenine to anthranilate and is induced over 250-fold by tryptophan or a catabolite of tryptophan. The second, a hydroxykynureninase, is constitutive or noninducible by tryptophan and preferentially catalyzes L -3-hydroxykynurenine to 3-hydroxyanthranilate. The physiological significance of these enzymes can be inferred from the facts that (1) the noninducible enzyme, hydroxykynureninase, appears to be the only enzyme present in uninduced cells that is capable of catalyzing L -3-hydroxykynurenine to 3-hydroxyanthranilate for the indispensable synthesis of NAD, and (2) the inducible enzyme, kynureninase, is induced by tryptophan to concentrations far in excess of that needed to meet the cell's requirements for NAD, resulting in the excretion of anthranilate into the medium. Studies on an enzyme isolated from mouse liver suggest that the liver enzyme closely resembles the constitutive hydroxykynureninase from *N. crassa*.

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CHORISMATE SYNTHETASE. PROPERTIES OF THE AROMATIC BRANCH-POINT ENZYME FROM NEUROSPORA CRASSA

F. H. Gaertner and K. W. Cole

Preparations of anthranilate synthetase purified more than 800-fold from crude extracts of *Neurospora crassa* catalyze the overall conversion of shikimate to anthranilate. Samples of anthranilate synthetase obtained after 2000-fold purification by preparative-gel electrophoresis no longer catalyze this overall reaction. However, the shikimate-to-anthranilate activity can be restored when the appropriate fractions are combined. Thereby, the overall activity has been shown to consist of three separable catalytic activities: shikimate to 3-enolpyruvylshikimate 5-phosphate (EPSP), EPSP to chorismate, and chorismate to anthranilate. The chorismate-to-anthranilate activity is catalyzed by anthranilate synthetase, which has been characterized as one of three enzymes of the anthranilate synthetase multienzyme complex. The shikimate-to-EPSP activity is carried out by shikimate kinase and EPSP synthetase, which are known to be two of five enzymes contained in a second enzyme aggregate, the aromatic multienzyme complex. The remaining enzyme catalyzing EPSP to chorismate, known as chorismate synthetase, has been demonstrated in *E. coli*. Although the EPSP-to-chorismate activity involves the major branch-point of the aromatic pathway, it has not been studied extensively and has not been studied previously in *N. crassa*.

We have studied the properties of the enzyme catalyzing EPSP chorismate in *N. crassa* and have found that multiple molecular forms on DEAE-cellulose, activation by the substrate EPSP, absolute requirement for TPNH and no requirement for reduced flavin mononucleotide or anaerobic atmosphere are features which distinguish chorismate synthetase from *N. crassa* from the *E. coli* enzyme. As in *E. coli*, diaphorase provides a greater than 10-fold stimulation of the reaction.

ULTRASTRUCTURAL STUDIES OF CELLULAR DEDIFFERENTIATION

James N. Dumont and Tuneo Yamada

Although the differentiated state of animal tissue cells is generally stable, there are exceptional cases where cellular differentiation is reversible. Reversion of not only overt but also covert differentiation is called dedifferentiation. Since carcinogenesis can be defined as pathological dedifferentiation, a basic understanding of dedifferentiation is a prerequisite in the cellular approach to the cancer problem. The iris epithelial cells of adult newts in lens regeneration represent almost the only system in which occurrence of dedifferentiation has been sufficiently documented and generally conceded. However, cellular and molecular mechanisms by which dedifferentiation is brought about have not been elucidated even in this system. We propose to concentrate our efforts on the problem of cellular dedifferentiation.

As the first approach, ultrastructural studies of iris epithelial cells in the process of dedifferentiation have been carried out. The terminally differentiated iris epithelial cells are characterized by a large number of melanosomes filling their cytoplasm. These melanosomes disappear, during the first 10–12 days after lentiectomy, from those iris epithelial cells that later transform into lens cells. Disappearance of melanosomes is accompanied by profound morphological changes in the cell surface. The normal iris epithelial cells have cuboidal or discoidal shapes and a smooth cell surface. Around day 4, a small number of large protrusions are produced from the cell surface. In the subsequent 3–5 days, the protrusions are gradually extended into slender processes. The tips of these processes, filled up with melanosomes, become fragmented. Macrophages that invade the iris epithelium contact iris cell processes with their villi and take up melanosomes either individually or in clusters. Fragments of iris cell cytoplasm and cell surface often accompany the melanosomes and are incorporated into the macrophages. There are indications that some of the cytoplasmic fragments degenerate extracellularly. When the iris epithelial cells have lost most of their melanosomes, the amount of cytoplasm is greatly reduced, and the large number of desmosomes that characterize the normal iris epithelium have disappeared. Our earlier autoradiographic studies demonstrate that at this stage incorporation of labeled leucine in the cytoplasm is enhanced. Lens-specific antigens appear only after these changes in the cytoplasm. Taken as a whole, these data seem to suggest the possibility that dedifferentiation is effected by removal of the whole population of melanosomes and a part of cytoplasm and cell surface, and that in the normal iris epithelial cells, those removed structures have repressive control over the nuclear synthesis. This will be our working hypothesis for subsequent research.

CELLULAR DEDIFFERENTIATION IN VITRO

J. R. Ortiz* and Tuneo Yamada

In an attempt to develop a method to study cellular dedifferentiation during Wolffian lens regeneration, cells from the dorsal iris epithelium from the adult newt,

Notophthalmus viridescens, were cultured in vitro for up to 12 days.

With aseptic procedures used throughout, the dorsal iris epithelium from the adult newt was removed and placed over a glass coverslip in a plastic culture dish containing amphibian-adjusted Leibovitz culture medium and 15 % fetal calf serum. The cultures were kept at room temperature and were observed daily. Pictures of the cultures were taken at different time intervals. Cultures were fixed in Bouin's 4, 6, 8, and 12 days after being cultured in vitro, stained with haemalum, eosin, and picronigrosin, and mounted on a glass slide.

When the dorsal iris epithelium explants are placed in culture, they attach to the coverslip after 1–3 days in vitro. Epithelial cells, partially pigmented as well as totally pigmented, can be observed migrating out of the explant by the fourth day. The migration of these cells continues throughout the entire culture period. Free melanosomes can be observed in the culture medium from the third day. A gradual decrease in pigmentation of these cells occurs during the culture period. The epithelial cells show different shapes, ranging from elongated to stellate. Cell processes are abundant, and melanosomes can be seen moving into them. Many of the cells have nuclei with more than one nucleolus, and some have more than one nucleus. Mitotic figures were never observed in vitro, although a few were found in the fixed cultures. Besides the presence of melanosomes in the medium, cells were observed shedding pieces of cytoplasm containing melanosomes. In the fixed preparations, pieces of cytoplasm containing melanosomes were also observed. Thus, the pigmented cells from the dorsal iris epithelium have the capacity to shed a part of their cytoplasm with cell surface as well as melanosomes in the cell culture condition. These observations support the working hypothesis discussed in the preceding article. Experiments are being conducted to test the effects of various specific inhibitors on cytoplasmic shedding of cultured iris epithelial cells.

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WOLFFIAN LENS REGENERATION IN VITRO

Tuneo Yamada

It has been speculated that transformation of the newt iris lens after lentiectomy is dependent upon a factor coming from the retina, referred to as the retinal factor. Isolation and characterization of this factor is necessary for understanding the mechanism of transformation. Before this can be achieved, a good assay system employing tissue-culture methods must be worked out. Our earlier studies show that isolated normal dorsal iris from the adult newt cultured alone under various conditions does not produce lens. Addition of fetal calf serum in the medium does not help. However, when the same tissue was put in contact with the neural retina of a lentiectomized eyeball from Rana pipiens larvae and cultured in vitro with medium 199 adjusted to amphibian tonicity and supplemented with 8% fetal calf serum, ~90% of the irises showed lens formation. Sixteen to twenty days

of culture were required for lens formation. The lens fibers thus formed indicated positive reaction for gamma-crystallins. The difference in size and stainability of nuclei between newt and frog made it easy to distinguish cells coming from the newt iris. When the donor of the retina started metamorphosis, the frequency of lens formation dropped to a level of 60%. When the normal ventral iris, which does not produce lens after lentectomy, was combined with the retina from a young frog larva, no lens formation occurred. The normal dorsal irises cultured with tissues such as lung, thymus, spleen, and liver failed to produce lens under the same culture conditions. So far, the only tissue other than the neural retina that has the capacity to support lens formation from the dorsal iris in tissue culture is the pituitary (1). The present work indicates that a factor from the neural retina is essential for the transformation of dorsal iris epithelial cells into lens cells and opens up the possibility for isolation and characterization of that factor.

1. T. G. Connelly and J. R. Ortiz, this page.

EFFECT OF PITUITARY ON TRANSFORMATION OF IRIS INTO LENS

T. G. Connelly and J. R. Ortiz*

In view of the fact that removal of the pituitary affects the progress of Wolffian lens regeneration, the effect of pituitary on dorsal iris cultured *in vitro* was tested. Dorsal irises were removed from normal adult eyes and cultured with pituitaries in several media containing 8% fetal calf serum. Control consisted of iris alone, with pieces of muscle or with kidney and adrenal. Irises cultured alone or on kidney and adrenal showed no signs of depigmented cells. In addition, 20-30% of the cases showed lens formation by depigmented cells, and 20% of the cases went through depigmentation without forming lens. According to Dr. David S. McDevitt, the lens fiber cells formed in the pituitary series are positive for immunofluorescence that is specific for gamma-crystallins. When the whole iris along with the cornea was cultured on pituitaries, 60% of the cases showed lens formation. When the pituitaries were replaced by ethanol-fixed or frozen pituitaries, transformation of iris did not proceed beyond the early lens vesicle stage. Thus, the live pituitary has the capacity to promote the transformation of iris cells into lens fiber cells *in vitro*.

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ACTIVATION OF RNA SYNTHESIS WITHOUT LENS REMOVAL IN THE WOLFFIAN LENS REGENERATING SYSTEM

Fritz Jauker and Tuneo Yamada

The process of Wolffian lens regeneration in the newt is initiated by removal of the lens. The removal needs to be complete for regeneration of a functional lens to be obtained. In this study, surgical injury was applied to the

cornea alone without touching the lens, a manipulation that does not lead to any regenerative process, and the response of the dorsal iris in RNA synthesis was studied. First, it was found that cutting the cornea in the same way as for lentectomy is sufficient to increase the incorporation of ^3H -uridine into RNA of the dorsal iris by a factor of 2 to 3 in 4 days after treatment. The increase is comparable to the dorsal iris 4 days after lentectomy. The enhancement is even stronger when the cornea is punctured. When the sedimentation pattern of labeled RNA of the dorsal iris 4 days after lentectomy is compared with that of dorsal iris 4 days after puncturing cornea, the patterns are basically similar. However, a clear difference is noticed in the presence of a prominent peak in the second series close to the bottom of the gradient. It is the highest peak in the RNA profiles of this series and is definitely heavier than ribosomal precursor RNA, which is estimated to be 40S. Studies of comparable patterns of *in vitro* cultured dorsal iris indicate that the same fraction is present in this case. The peak becomes prominent later than 4 days of culture, when the tissue starts to show signs of degeneration. (No lens is formed in this culture.) The fraction was found to be relatively insensitive to actinomycin treatment and was not labeled with radioactive methionine. Thus, it does not appear to be related to ribosomal RNA. One possibility from the above results may be that this fraction is synthesized in activated iris but is quickly processed when the iris is engaged in regeneration. In the absence of regeneration, or when regeneration is disturbed, the fraction becomes accumulated. If this is the case, the fraction should have an important role in the progress of regeneration.

IN VITRO ACTIVATION OF THE EARLY WOLFFIAN LENS REGENERATION RESPONSE IN THE NEWT IRIS

D. H. Reese, J. R. Ortiz,* and M. V. Cone

We have previously shown that normal dorsal iris, when isolated in culture, begins to synthesize ribosomal RNA (rRNA) at increased rates within 1 day of explantation (1). This observation is comparable to the *in vivo* condition, where the iris is allowed to remain in the *lenticotomized* eye. We now report other parameters of *in vitro* activation, as well as some additional parameters of rRNA activation.

Short-term pulse labelings of tissues with ^3H -uridine reveal that the rate of precursor rRNA synthesis (pre-rRNA) in 4-day, *in vitro* activated irises is on the order of 3.2 times greater than the rate observed in normal irises. The rate of pre-rRNA synthesis in 4-day, *in vivo* activated irises is nearly identical to the observed rate in 4-day *in vivo* activated tissue. Labeling of tissues with ^3H -methyl methionine also reveals that 4-day, *in vitro* activated tissues synthesize pre-rRNA at 3.2 times the rate of normal irises. The ratio of ^3H -methyl methionine labeling in the 4S regions of the two tissues, however, is considerably less. (The 4S ratio of 4-day to normal tissue is 1.3.) This suggests that both tissues have equal access to labeled methionine, so the observed ratio of 3.2 is not due to unequal pools.

The time course of ^3H -uridine incorporation into trichloroacetic acid-soluble and insoluble material in normal and 4-day, *in vitro* activated irises has been followed. The data show that the soluble and insoluble 4-day-to-normal ratios

are the same and are all on the order of 3. This indicates that the rate of ^3H -uridine incorporation into RNA is controlled by the rate of ^3H -uridine uptake into the tissues. Chromatography of the acid-soluble pools of ^3H -uridine-labeled normal and 4-day, *in vitro* activated tissues indicates that both tissues take up uridine at nearly the same rate and in excess, but that 4-day, *in vitro* activated tissues phosphorylate uridine at a higher rate. These data suggest that uridine and uridylylate kinase activity is increased in 4-day irises.

Another parameter of *in vivo* lens regeneration is the onset of DNA replication, which begins abruptly between 3 and 4 days after lens removal. To determine whether this occurs in *in vitro* activated irises, dorsal irises were cultured continuously in medium containing ^3H -methyl thymidine and were removed and fixed for autoradiography at 24-hr intervals. The criterion for labeled nuclei was arbitrarily set at a minimum of five grains per nucleus. Labeled nuclei were not detected until the fourth day after explantation, at which time 20.5% of the explant nuclei were labeled.

The above data, plus the data presented previously, show that the iris is activated *in vitro*, and the degree of activation is quantitatively and qualitatively equivalent to that which occurs *in vivo*. This system thus provides a means under controlled conditions for elucidating the various factors responsible for initiation of the early events associated with Wolffian lens regeneration.

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1. D. H. Reese, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1969*, ORNL-4535, p. 107.

PROTEIN UPTAKE AND MICROTUBULES IN *XENOPUS LAEVIS* OOCYTES

James N. Dumont

Previous reports have indicated that yolk precursor protein (vitellogenin) is incorporated from the serum into the oocyte by the process of micropinocytosis. Classically, micropinocytosis involves two steps: (1) the formation of small invaginations or pits on the surface membrane of the cell and (2) the subsequent closure of the pits to form vesicles in the cytoplasm. In *Xenopus* oocytes, the vesicles, which are thought to contain yolk precursor protein, move deeper into the cytoplasm, fuse, and contribute to the formation of yolk platelets. Experiments using electron-opaque and fluorescent tracers confirm that material contained within the vesicles reaches the yolk platelets.

Recent electron microscope studies, using the tracer peroxidase and unusually thick (3,000 Å) sections (normally, sections are about 600 Å thick), suggest that what was previously interpreted as micropinocytotic vesicles may actually be an extensive anastomosing system of membranous tubules, reaching from the oocyte surface to the forming yolk platelets. This new finding suggests that material is transported not by vesicles, as was previously thought, but through this tubular network. The data suggest that the network is a stable

system; that is, it is not continuously broken down and formed and therefore does not require continual synthesis of new cellular membranes. The classical view of micropinocytosis, on the other hand, implies extensive new membrane synthesis and renewal, since surface membrane would be lost when vesicles are formed. Further examination of this tubular system is planned, using thicker sections (1 μ), the 650 kV electron microscope, and stereo electron microscopy.

The factor(s) that maintain and support such an elaborate intracellular tubular transport system are also being investigated. It has been shown by others (1) that microtubules play an important role in maintaining cell shape. By exposing oocytes to agents such as vinblastine and cold (0°C), both of which selectively destroy microtubules, Wallace *et al.* (2) have shown that *in vitro* uptake of yolk is dramatically reduced. Examination of vinblastine-treated oocytes reveals that the elaborate infolded surface normally present on oocytes is lost. The surface is essentially flat, and only microvilli remain protruding from it. Furthermore, the number of micropinocytotic pits on the surface is reduced. These findings, coupled with reduced uptake of proteins, implicate the function of microtubules in the acquisition of yolks.

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1. L. G. Tilney, in *Origin and Continuity of Cell Organelles* (J. Reinard and H. Ursprung, eds.), p. 222. Springer-Verlag, New York (1971).
 2. R. A. Wallace, D. W. Jared, and T. Ho, *J. Exp. Zool.* (submitted).

DIFFERENTIATION IN PIGMENT-PRODUCING CELLS. AMPHIBIAN OOCYTES AND EMBRYONIC EYES

John J. Eppig, Jr. and James N. Dumont

Cells that produce melanin are well suited for the study of cell growth and differentiation. The compound dihydroxyphenylalanine (DOPA) is a specific precursor of melanin and can be readily employed in cytochemical and precursor-uptake studies. Also, measurement of the melanogenic enzyme tyrosinase gives an indication of the status of differentiation and can be used as an indicator in studies on the control of pigment production and hence cellular development.

Early stages in the development of the pigmented epithelium have been examined with the electron microscope. The earliest presumptive pigmented epithelial cells contain large amounts of glycogen, yolk platelets, and a few melanosomes, all of these presumably formed during the development of the oocyte. The pigmented epithelial cells then commence synthesis of endoplasmic reticulum and Golgi complexes. At this time, the cells also begin to engulf many oocyte melanosomes, which are released into the space (primary cavity) between the prospective neural retina and pigmented epithelium by the prospective neural retinal cells. These melanosomes are engulfed singly or in groups containing several melanosomes. Many of these oocyte melanosomes become enclosed in newly synthesized melanin to form "complex" melanosomes, which have been described previously.

Tyrosinase, which is involved in the initial steps of melanin synthesis, has been localized cytochemically by incubating

fixed tissue in a medium containing DOPA. The reaction product, probably melanin, can be identified by its electron density. In pigmented epithelial cells, reaction product is found in the active face of the Golgi apparatus and in a continuous system of smooth-surfaced channels that communicate with both typical premelanosomes and developing complex melanosomes. In addition, reaction product is found in an extensive system of tubules and cisternae connecting with the premelanosomes in several places and interconnecting neighboring premelanosomes. Experiments utilizing the melanogenesis inhibitor phenylthiourea suggest that tyrosinase is active in the DOPA-positive channels as well as in intermediate and late premelanosomes. This suggests that melanogenesis is a dynamic process involving tyrosinase enzyme condensation in the Golgi apparatus, with the possibility that melanin synthesis begins in the channels, with further melanin synthesis and deposition in premelanosomes. This intricate system may function to facilitate the development of numerous premelanosomes at this stage of differentiation in the pigmented epithelium.

Tyrosinase has also been localized cytochemically by the DOPA reaction in oocytes in various stages of development from frogs induced to ovulate with human chorionic gonadotropin (HCG). The reaction product is present only in oocytes of Stages II, III, and IV. No reaction product is present in smaller Stage I or larger Stage V oocytes of HCG-stimulated animals or in the oocytes of unstimulated frogs. The reaction product is localized in the active face of the Golgi apparatus and an anastomosing smooth membrane system connected to the Golgi apparatus. This system is similar to but less extensive than that of pigmented epithelial cells. There is no DOPA-positive material in early premelanosomes, even in oocytes where the Golgi complexes are DOPA-positive. The DOPA-positive Golgi complexes of the smaller oocytes are about 13 μ from the cell periphery, where premelanosomes are formed in the endoplasmic reticulum. During later stages of oocyte development, when melanization of premelanosomes occurs, the Golgi complexes are much closer to the oocyte periphery (5-9 μ) and hence to the developing premelanosomes. This apposition may also facilitate the transfer of tyrosinase to the premelanosomes. Occasionally, the premelanosomes are connected directly to the Golgi complex via smooth-surfaced channels. Some DOPA-positive Golgi complexes are also involved in the synthesis of cortical granules, which suggests a segregation of function within a single Golgi complex. This may not be a complete segregation, however, since some of the cortical granules also appear to contain some DOPA reaction product. Since mature cortical granules do not contain this reaction product, the tyrosinase may become inhibited in them.

Tyrosinase activity has also been assayed biochemically by incubating oocyte homogenates in ^{14}C -tyrosine and measuring trichloroacetic acid-insoluble radioactivity. Such assays were made on isolated oocytes of various developmental stages from HCG-stimulated and unstimulated frogs. The oocytes from HCG-stimulated animals show that tyrosinase activity is greatly increased in Stage III and IV oocytes, only slightly in Stage II oocytes, and not at all in Stage V oocytes. Once melanization is complete (Stage V), elevated tyrosinase activity cannot be induced. Oocytes

of *Xenopus* are therefore competent to be stimulated to synthesize pigment only during specific stages of their development. The degree of stimulation is related to the stage to which the oocyte has developed within the competent period.

ACID PHOSPHATASE ACTIVITY IN THE DEVELOPING OOCYTE OF *XENOPUS LAEVIS*

Anthony Vitto, Jr.,* James N. Dumont, and John J. Eppig, Jr.

Acid phosphatase, a phosphomonoesterase optimally active at acidic pH, has been shown biochemically to exist in the oocytes of *Xenopus laevis*. Oocytes at various stages of development were homogenized at pH 5.0, treated with 1% triton X-100 for 1 hr, and incubated with *p*-nitrophenylphosphate for 0.5 hr. The *p*-nitrophenolate ions released enzymatically are read colorimetrically. Enzyme activity per oocyte increases with increasing oocyte development. The enzyme levels appear to be the same in oocytes of unstimulated animals as in those of animals stimulated with human chorionic gonadotropin (HCG). This contrasts with recent studies on the enzyme tyrosinase, which is inducible by stimulation with HCG (1).

Preliminary cytochemical studies using the electron microscope suggest that acid phosphatase activity is localized around yolk platelets (perhaps in the superficial layer), in lysosomal-type bodies existing in early oocyte stages (Stages I and II), and in pinocytotic vesicles on the oocyte surface. This hydrolytic enzyme may play a role in pinocytotic uptake of yolk protein from the blood and/or subsequent breakdown and formation of yolk platelets. On the other hand, it may simply be stored in an inactive state in the oocyte until embryogenesis, when it may function in the breakdown and utilization of yolk. These possibilities are being investigated further.

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CHROMOSOME STRUCTURE AND FUNCTION DURING MAMMALIAN OOGENESIS

Aimée H. Bakken and O. L. Miller, Jr.

During the past year, a program was initiated within the Chromosome Ultrastructure Group with the immediate goal of determining the feasibility of isolating and mapping sites of active RNA synthesis on predicyate chromosomes of mammalian oocytes. If mapping procedures can be developed, an obvious long-range project would be to determine whether early embryonic defects can be correlated with abnormal activity or absence of normal activity at specific chromosomal loci.

A primary obstacle in this study is the lack of dependable techniques for isolating and dispersing the contents of

relatively small nuclei for electron microscopy. Consequently, preliminary efforts have been focused on experiments to derive a chemical means of breaking down the mechanical barrier of the nuclear envelope without concomitant degradation of structural relationships within the nucleus. The effects of various detergents, enzymes, and pH conditions are being studied, using amphibian oocyte nuclei as an assay system. The advantage of the latter cell is that the structure of nuclear organelles isolated following chemical treatments can be quickly compared with their structure following isolation by manual means.

Ionic detergents, such as deoxycholate and sodium dodecylsulfate, readily solubilize nuclear envelopes; however, all of the ionic detergents so far examined have adverse effects on chromosome structure. For example, deoxycholate denatures RNA polymerases, causing gene products to dissociate from active sites, essentially mimicking the action of RNase. Sodium dodecylsulfate, on the other hand, entirely dissociates all nucleoprotein complexes of the chromosomes. When used alone, nonionic detergents, such as the Tweens, Triton X-100, and Brij-58, show little short-term effect on nuclear envelope stability, even in concentrations 10-fold higher than required to solubilize plasma membranes. So far, the most promising development is the finding that nuclear envelopes from both mammalian and amphibian oocytes can be more or less disintegrated by brief exposure to pH 11.5. The structural appearance of isolated amphibian chromosomes is unaltered by this treatment. Possible synergistic effects of high pH, nonionic detergents, and lipid-degrading enzymes are being investigated. If chemical means can be found to solubilize nuclear envelopes without adversely affecting nuclear contents, electron microscopic visualization of genetically active chromosomes should be possible for numerous cell types with small nuclei, in addition to mammalian oocytes.

POSSIBLE FUNCTION OF SPACER DNA IN AMPHIBIAN NUCLEOLI

O. L. Miller, Jr. and Christie A. Holland*

The nucleolus organizer locus of amphibian consists of a repetitive sequence of several hundred genes coding for ribosomal RNA (rRNA) precursor molecules that give rise to the 18S and 28S rRNA's of cytoplasmic ribosomes. Each gene within an organizer is separated from its neighbors by short "spacer" segments of DNA that appear to be transcriptionally inactive (1). Using hybridization criteria, Brown et al. (2) have shown that the rRNA genes of the closely related species *Xenopus laevis* and *X. mullerii* are indistinguishable. On the other hand, although spacer lengths are similar, buoyant density differences indicate that the base content of spacer segments has diverged enough between the two species to allow separation of their respective nucleolar genes in density gradients. Obviously, spacer function does not require stringent conservation of base composition, but it does appear to require maintenance of a constant length.

Techniques have been developed previously that allow electron microscopic visualization of nucleolar genes and

associated gene products isolated from amphibian oocytes (3). Following routine isolations, spacer segments appear devoid of any attached matrix, although granules similar in size and electron opacity to the RNA polymerases on the rRNA genes often are present. Recently, however, a fibrogranular material has been found associated with spacer segments in *Triturus viridescens* under two conditions: in preparations from oocytes of animals collected prior to ovulation and stored at 12° C for several months and in preparations following treatment of oocytes with actinomycin D. In both cases, the attached material is similar in structure to the granular cortex component of amphibian nucleoli.

This relationship suggests that spacers may be involved in processing of mature rRNA precursor molecules. For example, one could postulate the following events. RNA polymerases cease RNA synthesis at the termination signal of a nucleolar gene but move onto the adjacent spacer segment with mature rRNA precursor molecules still attached. The driving force for movement of the polymerases on the spacers conceivably could be provided by rotation of the DNA. This mechanism would result in confinement of precursor molecules to a specific site for the first cleavage event involved in ribosome subunit formation. If this is the case, the absence of precursors from spacers isolated from freshly collected oocytes indicates a much more labile association of precursors with nontranscribing than with transcribing polymerases.

Cycloheximide, a protein synthesis inhibitor, is known to lengthen considerably the time for processing mature rRNA precursor molecules in HeLa cells (4). The effects of this inhibitor on the structural aspects of rRNA precursor processing in amphibians should be of interest.

*Student trainee.

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ELECTRON MICROSCOPIC VISUALIZATION OF BACTERIAL TRANSCRIPTION

Barbara A. Hamkalo and O. L. Miller, Jr.

Using preparative techniques previously described (1), we have extended electron microscopic studies of the genetic activity of both *E. coli* and *Salmonella typhimurium* chromosomes. Biochemical evidence has shown that bacterial 16S and 23S ribosomal RNA (rRNA) cistrons are contiguous (2, 3), and that there are about six 16S-23S doublets per chromosome equivalent (3, 4). Under maximal growth conditions, it has been estimated that about 80 closely packed RNA polymerase molecules simultaneously transcribe each rRNA locus. From these data and the molecular weights of the two rRNA molecules, we can identify regions (Fig. 1) that almost certainly are ribosomal RNA genes.

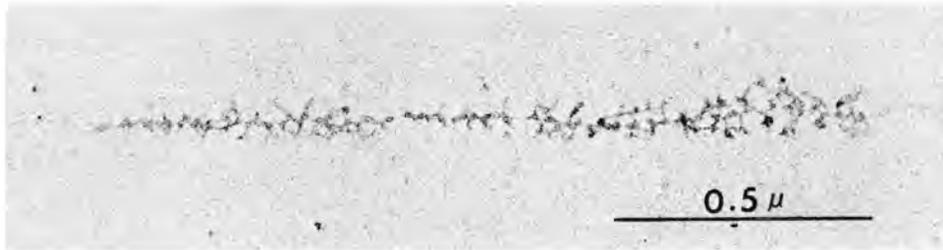


Fig. 1. An rRNA locus in *E. coli* chromosome showing activity of 16S (short gradient) and 23S (long gradient) cistrons under optimal growth conditions.

Each region is about 1.3μ long, slightly shorter than the length of DNA predicted from the combined molecular weights of the rRNA's. However, extensive local denaturation to permit transcription at closely adjacent sites on the DNA probably would foreshorten such regions. The ribonucleoprotein (RNP) fibrils comprising each matrix are made up of nascent rRNA chains and associated ribosomal proteins. The RNP fibrils are arranged in two gradients of unequal length. The relative lengths of the gradients correspond to those predicted from the molecular weights of the 16S and 23S rRNA's.

Since there are two gradients, the two bacterial rRNA's must be synthesized as separate molecules, rather than as

part of a single large precursor molecule, as is the case in eukaryotic cells. From this structural arrangement, however, one cannot distinguish whether there are one or two RNA polymerase binding sites per rRNA region. In order to choose between these alternatives, the structure of rRNA regions was studied after the addition of rifampin, a drug that inhibits the initiation of transcription by RNA polymerase but does not affect chain elongation. Fig. 2a shows that 40 sec after drug addition to a growing culture, the entire 16S gradient is removed but the 23S gradient remains intact; at a later time (Fig. 2b), the 23S regions begin to disappear. These experiments corroborate biochemical data (5, 6) indicating that a single initiation site at the proximal end of the 16S gradient exists for both cistrons.

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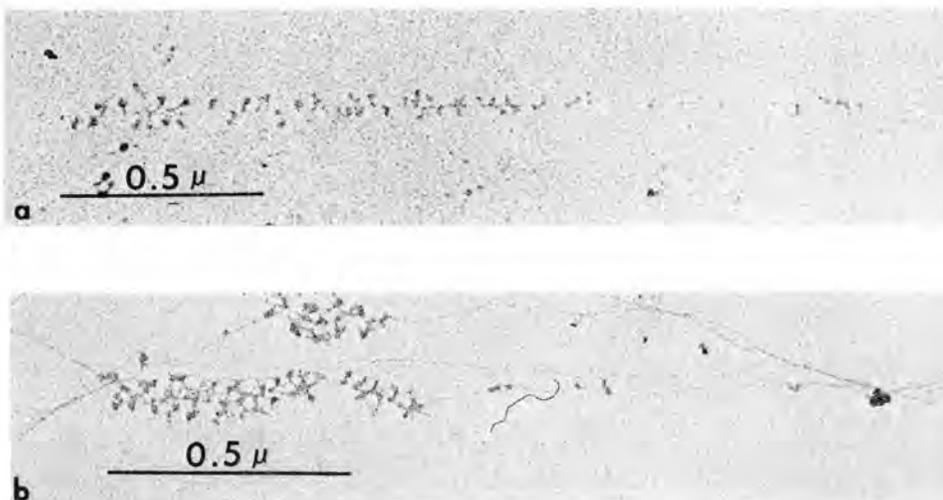


Fig. 2. (a) An *E. coli* rRNA locus 40 sec after rifampin treatment. The 16S cistron is essentially cleared whereas the 23S cistron shows a normal complement of fibrils. (b) An rRNA locus 80 sec after rifampin treatment. Approximately one-third of the 23S gradient has now been cleared of fibrils.

When cell generation time is increased, the number of ribosomes and thus the rRNA synthesized per unit time are reduced. Bacterial cells apparently accomplish this by reducing the frequency of initiation of transcription, rather than by reducing the rate of transcription. Fig. 3 shows an rRNA locus from a slowly growing culture. Each matrix unit consists of 16-20 irregularly spaced RNP fibrils. Under step-up growth conditions, the number of fibrils per locus (= frequency of initiation) increases.

Although some genetic and biochemical data (4, 7) suggest that rRNA genes are clustered on bacterial chromosomes, we have never found closely adjacent rRNA loci in our preparations. Fig. 4 shows a single rRNA region bracketed by structural gene activity (DNA-RNA polymerase-polyribosome complexes). Such regions, showing transcription of both structural and rRNA genes, have been observed to extend over 10 μ of the genome.

These studies are being extended to cells infected with virulent and lysogenic bacteriophages. T7, a small (26 X 10⁶ daltons of DNA), virulent virus, is currently being in-

vestigated. Early after infection, host RNA polymerase is used to synthesize the messengers for a few phage proteins, including a T7-specific RNA polymerase, that is insensitive to rifampin inhibition. Under conditions that result in cell lysis at 12 min, host transcription and translation are gradually turned off, until inhibition (i.e. the absence of rifampin-sensitive genetic activity) is complete at about 5 min. At this time, most of the host genome remains intact and appears to have many RNA polymerases attached. These observations suggest that the phage does, in fact, exert inhibitory control over host polymerase activity, as suggested by Summers (8). At 5 min, polyribosomes appear to be associated primarily with membrane fragments. T7 mRNA is thought to be stable ($t_{1/2}$ = 20 min) (8). If this is true, translation of these messengers in membrane-associated polyribosomes may play a role in such stabilization.

T7 RNA and DNA polymerase mutants currently are being used in attempts to study both early transcription by *E. coli* RNA polymerase and subsequent T7 RNA polymerase activity without the handicap of large quantities of replicating T7 DNA.

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Fig. 3. Activity of an *E. coli* rRNA locus from bacterial culture grown under suboptimal conditions.

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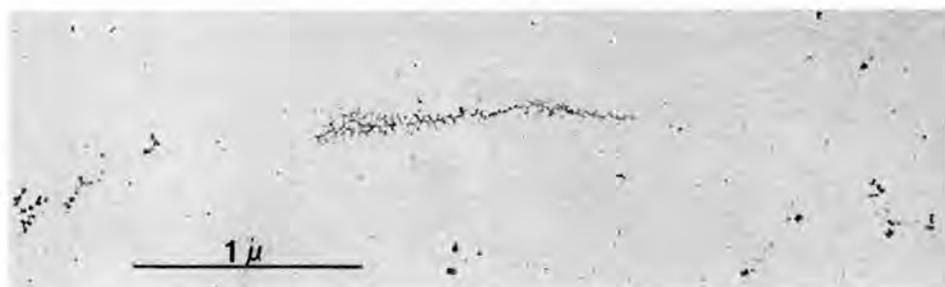


Fig. 4. An *E. coli* rRNA locus bracketed by polyribosomes attached to active structural genes.

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VISUALIZATION OF EUGLENA GRACILIS CHLOROPLAST GENOMES

O. L. Miller, Jr., B. A. Hamkalo, W. E. Barnett, and Diane J. Gains

Chloroplasts synthesize organelle-specific ribosomal RNA's (rRNA's), tRNA's, and presumably many of the enzymes involved in organelle DNA, RNA, and protein synthesis (1). There is conflicting evidence as to whether or not the processes of transcription and translation are intimately coupled in chloroplasts as they are in bacteria (2).

Chloroplasts were isolated from log-phase, autotrophically grown *Euglena gracilis* cultures by French pressing after rapid cooling of cells to ice temperature. The chloroplasts then were osmotically shocked and processed for electron microscopy by techniques similar to those successfully used to observe genes of bacteria and amphibian oocyte nuclei (3, 4).

Preliminary results (Fig. 1) indicate that genetically active portions of chloroplast genomes can be visualized and have attached structures resembling bacterial polyribosomes. As yet, no indication of rRNA gene action has been observed.

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STUDIES ON VITELLOGENIN IN VIVO AND IN VITRO

R. A. Wallace

Vitellogenin, a sex-limited serum phosphoprotein normally found in *Xenopus* females, can be produced in males by injection of estradiol-17 β (1). In males, however, vitellogenin is not removed from the bloodstream, and in data from several hundred male animals a clear correlation has been found between protein phosphorus (P) and both total serum protein (p) and total serum calcium (Ca) according to the relationships $p = 67.6P + 47.4 \text{ mg ml}^{-1}$ and $\text{Ca} = 1.30P + 0.078 \text{ mg ml}^{-1}$. These results suggest that if vitellogenin is the only component released into the bloodstream after estrogen administration, then (1) it has a protein phosphorus content of 1.48% and (2) the atomic ratio of serum calcium to vitellogenin-bound phosphorus is 1.0. Gel filtration and chromatographic studies on serum from estrogen-treated males and females have verified that vitellogenin is the only new component; further, it was found that calcium does not appear to be strongly bound to vitellogenin but that the latter can strongly bind circulating iron.

Vitellogenin can be isolated from serum by chromatography on TEAE-cellulose, and chemical and physical measurements have been performed on the isolated product. Sedimentation velocity and equilibrium runs in the ultracentrifuge indicate a homogeneous material with $S_{20,w}^0 = 13.6S$ (derived both from phosphate- and NaCl-EDTA-buffered systems) and with $d \log f/dr^2 = 0.661$ at 7923 rpm. From these and other measurements, vitellogenin was found to be a lipophosphoprotein with a molecular weight of 460,000 and to have a lipid content and protein phosphorus content of 12 and 1.4%, respectively. Considering the available evidence (2, 3), a hypothesis has been constructed whereby vitellogenin is considered to be (1) a covalently bound complex of one lipovitellin dimer and two phosvitin molecules and (2) the immediate precursor of these yolk proteins during yolk-platelet assembly *in vitro*.

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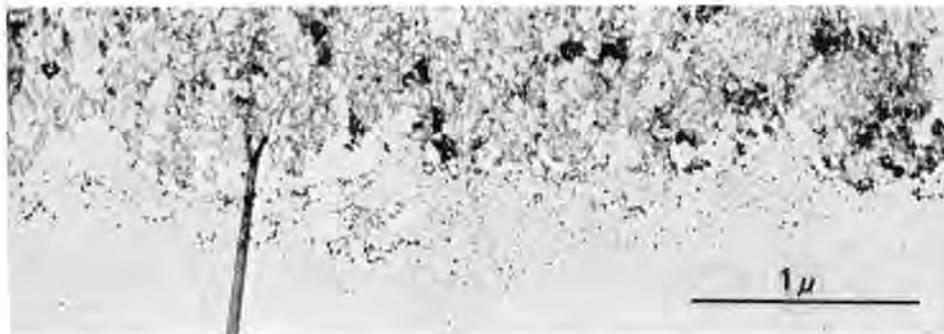


Fig. 1. A portion of an osmotically shocked chloroplast, showing the extruded genome with associated structures resembling polyribosomes (*Euglena gracilis*).

PROTEIN INCORPORATION BY ISOLATED AMPHIBIAN OOCYTES

R. A. Wallace and D. W. Jared

In the intact female amphibian, vitellogenin is rapidly incorporated by ovarian tissue and specifically by developing oocytes (1). During this process, it must enter the capillary network of the theca and from there pass through the connective tissue and follicular epithelium to the oocyte surface, where it is sequestered by a pinocytotic mechanism. The factors involved during these steps are difficult to study using the intact animal. We have therefore developed a culture procedure for oocytes that employs 50% labeled serum protein equilibrated against a simple saline solution (2); the labeled serum is also prepared in such a way that at least 98% of the labeled protein incorporated by the isolated oocytes is represented by vitellogenin.

In our initial studies, we have found that developing *Xenopus* oocytes divested of their outermost cellular layers (theca and surface epithelium) but with their innermost cellular layer (follicular epithelium) intact will incorporate protein continuously for a period of at least 6 days when they are cultured *in vitro*. Protein uptake is negligible when oocytes are incubated either with all their cellular layers intact or with all their cellular layers removed (by EDTA or pronase treatment). These results imply that the outer surface epithelium is relatively impermeable to protein and that the integrity of the investing follicular epithelium is essential for vigorous protein incorporation by the oocyte.

In further experiments, we found that the rate of protein uptake by isolated oocytes depends upon: (1) the extent to which the donor has been stimulated by gonadotropin, with multiple injections producing an additive effect; (2) the length of time since the donor was last stimulated with gonadotropin, with $t_{1/2} = 9$ days for the decline in the rate of protein-incorporating activity; (3) the size of the oocyte, since only oocytes in a size range of 0.6-1.2 mm sequester protein to any marked extent (the rate of incorporation also seems to be proportional to the surface area of the oocyte throughout most of this size range); and (4) the protein composition of the medium, since a mechanism exists to sequester selected protein and vitellogenin in particular. With these variables thus defined, we should now be able to proceed with an investigation into the regulatory mechanisms for protein incorporation and oocyte growth.

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THE RELATIVE ROLES OF AUTOSYNTHETIC AND HETERO-SYNTHETIC PROCESSES DURING YOLK-PROTEIN ASSEMBLY BY ISOLATED OOCYTES

R. A. Wallace and Ti Ho

A long-standing controversy in reproductive biology has been centered around the question of whether yolk material in developing oocytes is synthesized within the oocyte ("autosynthetic") or, rather, is elaborated by some external tissue and subsequently transferred to the oocyte

intact ("heterosynthetic"). In order to evaluate this question, two basic experiments were performed.

In the first experiment, isolated developing oocytes were incubated in the presence of vitellogenin doubly labeled with ^3H -leucine and ^{32}P -phosphate for 1 hr and subsequently transferred to unlabeled medium for various lengths of time. Protein was then extracted from the oocytes and chromatographed on TEAE-cellulose, and the pattern of labeling was observed. After the 1-hr "pulse," label was exclusively associated with vitellogenin; but after an additional 1-hr "chase," a very heterogeneous labeling pattern was present. After a 4-hr "chase," essentially all the ^3H -leucine was associated with lipovitellin, and most of the ^{32}P -phosphate was associated with phosvitin. Lipovitellin and phosvitin are the only two proteins present in the crystallin yolk-platelet, and they contain 98% and 80% of the total platelet protein leucine and protein phosphorus, respectively. During the period in culture, the low-molecular-weight pools remain unlabeled.

In the second experiment, oocytes were incubated for 1 hr in medium containing free ^3H -leucine and ^{32}P -phosphate and also subsequently transferred to unlabeled medium for various lengths of time. In this case, however, the low-molecular-weight pools rapidly became labeled, while no incorporation into lipovitellin or phosvitin was observed, even after 18 hr.

Autoradiographs of oocytes were made concurrently during the incorporation and transformation process, and the most important finding was that after the 1-hr "pulse" with labeled vitellogenin, radioactive material was found within the cortex of the oocyte rather than at the surface of the oocyte or in the follicle cells. Thus, (1) vitellogenin, which normally is made by the liver (1), is taken up directly by the oocyte, (2) once incorporated it is transformed within the oocyte into lipovitellin and phosvitin, (3) this transformation apparently involves a macromolecular restructuring rather than breakdown and resynthesis, and (4) an autosynthetic contribution to yolk-protein assembly appears to be nonexistent.

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A COMPLEX OF RECOMBINATION AND REPAIR GENES IN HAEMOPHILUS INFLUENZAE

Jane K. Setlow, Maxon E. Boling, and Kenneth L. Beattie

Two transformation-defective strains of *Haemophilus influenzae*, one of which was obtained by transforming wild-type cells with DNA from the other, UV-sensitive strain, have been studied with respect to their transforming ability, their repair of DNA damage induced by UV, X-rays, and methyl methanesulfonate (MMS), their ability to allow recombination of temperature-sensitive mutants of the *H. influenzae* phage HP1c1, and their inducibility following lysogenization with wild-type phage. These strains have been crossed by transformation with wild type and with each other, and repair and recombination in the transformants have been investigated.

The results indicate that both strains contain a defect in their recombination mechanisms, since phage recombination is not measurable in either of them. The recombination defects in the two strains are different by the following criteria. (1) The residual transformation frequencies in the strains are different, about 10^{-6} and 10^{-7} that of the wild type. (2) The recombination defect in the strain with the higher transformation frequency, containing a mutation we have called rec_1^- , is inseparable from UV sensitivity, X-ray and MMS sensitivity, and lack of inducibility following lysogenization. The other strain, which is rec_1^+ (UV resistant and inducible), has an intermediate sensitivity to MMS and X-rays. (3) rec_1^+ can be transformed into the rec_1^- strain, either with wild-type DNA or with DNA from the rec_1^+ recombination-defective mutant; rec_1^- can also be transformed into the wild type. We have been unable to transform the lower-frequency transformation defect into the wild type, nor have we succeeded in transforming the rec_1^+ recombination-defective strain to normal recombination, either with wild-type DNA or with DNA from the rec_1^- mutant.

A hypothetical scheme has been proposed to account for the anomalous result that two different recombination defects appear to have been present in a single strain, but when one of these defects is transformed into wild-type, cells, DNA from the resulting new recombination-defective strain can transform the original strain to rec^+ . It is postulated that the original rec^- strain contains two rec mutations, rec_1^- and rec_3^- , and that the derived strain is rec_1^+ and rec_3^- , the rec_3^- mutation conferring intermediate sensitivity to MMS and X-rays. It is further postulated that the derived rec^- strain contains a third altered gene, rec_2^- , which was obtained through the selection procedure used to isolate the strain from the wild-type culture exposed to $rec_1^- rec_2^+ rec_3^-$ DNA, and that the combination $rec_2^- rec_3^-$ causes the rec^- phenotype. The selection procedure involved repeated exposure of the transformed culture to the DNA from a related microorganism, *H. parainfluenzae*, a treatment that is lethal for rec^+ strains able to integrate transforming DNA but does not affect rec^- strains. The rec_2^- gene is considered to have come from the *H. parainfluenzae* DNA.

THE FREQUENCY OF TRANSFORMATION OF A RECOMBINATION GENE IN *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow, Kenneth L. Beattie, and Maxon E. Boling

Since the transformation frequency of the recombination-deficient (rec^-) mutants of *Haemophilus influenzae* isolated in this laboratory is around 10^{-8} , it is not possible to detect rec^+ transformants of rec^- cells with DNA from the wild-type strain by examination of clones from individual cells isolated from the mixture of competent cells and DNA. However, such rec^+ transformants can be obtained if selection is first made for an antibiotic marker closely linked to the rec^+ gene in the transforming DNA.

The rec^- cells were made highly competent, exposed to rec^+ DNA, allowed to express any transformed markers, and then incubated in growth medium containing the antibiotic. When the transformed culture was grown, samples

were plated and single colonies were isolated. These were individually made competent and tested for their ability to be transformed to resistance to some other antibiotic. When the selected antibiotic marker was streptomycin resistance, 124 out of 231 isolates, or about 50%, were rec^+ . When the selected marker was cathomycin resistance, at a locus linked to streptomycin resistance, only three out of 212 isolates were rec^+ . Selection for erythromycin resistance, a marker that is relatively far away from the other two antibiotic markers, yielded no rec^+ transformants among 180 isolates tested. We conclude that the rec gene is tightly linked to the streptomycin gene, loosely linked to the cathomycin gene, and unlinked to the erythromycin gene.

When the recipient cells were rec^+ and the donor DNA was rec^- , the tight linkage to the streptomycin gene was not observed, since only nine out of 258 streptomycin-resistant isolates (4%) were rec^- . Without any selection, no rec^- transformants in 119 isolates were found, although the competence of the rec^+ culture exposed to rec^- DNA was very high, with 3% of the cells transformed to antibiotic resistance. These data suggest that when rec^- DNA enters the rec^+ cell and pairs with the recipient DNA, it interferes with its own integration, such as by making a gene product unfavorable for transformation. The hypothesis is strengthened by two observations. (1) The frequency of streptomycin transformation in the rec^+ cell is about seven times lower with rec^- DNA than with rec^+ DNA. (2) The apparent linkage between streptomycin and cathomycin resistance is about three times higher in rec^+ cells transformed with rec^- DNA than with rec^+ DNA. This is to be expected if almost all the streptomycin transformations with rec^- DNA are caused by DNA molecules not carrying the rec^- gene, which therefore would be more likely to contain the gene for cathomycin resistance as well, on the side away from the rec^- gene.

EXPRESSION OF A RECOMBINATION GENE ON TRANSFORMING DNA IN A RECOMBINATION-DEFECTIVE *HAEMOPHILUS INFLUENZAE* RECIPIENT CELL

Jane K. Setlow, Kenneth L. Beattie, and Maxon E. Boling

The rec_1^- mutation not only prevents transformation and phage recombination but also is responsible for the UV sensitivity of cells carrying it. To determine whether rec_1^- cells can show evidence of expression of a rec_1^+ gene that enters the competent cell, the mutant competent cells were exposed to transforming DNA containing either the rec_1^+ gene or the rec_1^- gene and then irradiated with various doses of UV. As a control, the same competent cells were irradiated without preexposure to DNA. The cells exposed to rec_1^+ DNA and irradiated with doses greater than 50 erg mm^{-2} were more resistant than cells exposed to rec_1^- DNA. The slope of the rec_1^+ DNA curve beyond this dose was identical to the slope of the dose-effect curve for competent wild-type cells. Without any DNA exposure, the cells were the most sensitive. The slight increase in resistance due to the rec^- DNA could be duplicated by exposing the cells to nonhomologous DNA from *E. coli*, and is considered to result from the protective effect of these DNA's in competing for nucleases of the rec_1^- cell that can degrade the irradiated cell genome.

The fraction of the cell population that acquired wild-type resistance could be estimated from the survival curve as about 0.5%. Assuming that the probability that a rec_1^+ gene on a piece of transforming DNA will be present in a competent rec^- cell is about twice the probability of maximum transformation in rec^+ cells or about 10%, then approximately one out of 100 rec_1^+ genes present in the rec^- cells can confer UV resistance. This is orders of magnitude greater than the probability of integration of a rec_1^+ gene into a rec_1^- cell genome; therefore, the rec_1^+ gene must be transcribed without being integrated about 1% of the time.

DNA SYNTHESIZED AFTER ULTRAVIOLET IRRADIATION IN RECOMBINATION-DEFECTIVE AND NORMALLY RECOMBINING STRAINS OF HAEMOPHILUS INFLUENZAE

J. Eugene LeClerc and Jane K. Setlow

DNA synthesized after UV irradiation in a UV-sensitive, recombination-defective strain of Haemophilus influenzae is broken down much more rapidly than in wild-type cells or than DNA made in the mutant before irradiation. In wild-type and recombination-defective strains, there is a dose-dependent decrease in single-strand molecular weight of DNA made after irradiation, as judged by alkaline sucrose sedimentation of the DNA from cells irradiated and then pulse-labeled with tritiated thymidine. When the cells are incubated in cold medium following the pulse label, the single-strand molecular weight returns to the control value in the wild type and in an excision-defective, UV-sensitive mutant with normal recombination. However, in the UV-sensitive, recombination-defective strain, the single strands of DNA remain short. In another recombination-defective but UV-resistant strain, the sedimentation patterns are identical to those of the wild type.

It is concluded that the inability to rejoin gaps in DNA synthesized after irradiation is lethal for some irradiated cells. However, the rejoining of such gaps does not necessarily require a functional recombination system, since it takes place in a strain with no measurable phage recombination and a transformation level seven orders of magnitude lower than that of the wild type.

EVIDENCE THAT HOST CELL RESTRICTION GENES IN HAEMOPHILUS INFLUENZAE ARE ON AN EPISOME

Jane K. Setlow and Nazima Khan*

A host cell restriction system for Haemophilus influenzae phage was discovered previously in this laboratory. H. influenzae restriction mutants break down the DNA of infecting phage, so that the efficiency of infection decreases about two orders of magnitude. At least two genes are involved with this cell property.

Treatment of cells with acridine orange is known to eliminate episomes. Acridine orange readily alters the restriction properties of H. influenzae cells, changing the

nonrestricting strain into a restricting one and vice versa. The data indicate that at least some of the restriction genes are located on an episome. There is a high spontaneous rate of reversion of the acridine-orange-induced mutants, suggesting that cells "cured" of the episome can be reinfected by DNA carrying restriction genes, which could reestablish the the episome as a replicating unit in the cell.

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DIFFERENCES BETWEEN INFECTION OF HAEMOPHILUS INFLUENZAE BY WHOLE PHAGE, PHAGE DNA, AND DNA FROM LYSOGENIC CELLS

Maxon E. Boling, Jane K. Setlow, and David P. Allison

A recombination-defective strain of Haemophilus influenzae is infected with purified DNA from phage HP1c1 much less efficiently than is the wild type, although DNA uptake and whole phage infection are equally efficient in the two strains. Following whole phage infection of both strains, lysogenization readily occurs, but we have detected no lysogenization after transfection with either phage DNA or DNA from lysogenic cells. When the cells have been made competent in rich organic medium, the efficiency of transfection of wild-type cells by phage DNA, but not by DNA from lysogenic cells, increases with increasing concentration of DNA. However, in the rec^- strain, or in wild-type cells made competent in a defined medium, the transfection efficiency is independent of concentration.

Measurement of lengths of phage DNA molecules from electron micrographs shows that linear and circular dimers and trimers as well as monomer forms exist in our purified DNA preparations. There is good agreement between these measurements and the values calculated from the sedimentation velocity of phage DNA labeled with tritiated thymidine. Conditions that maximize circularization of the DNA drastically decrease transfection, probably because circular molecules cannot enter the competent cell. Linear dimer forms of phage DNA separated by sedimentation velocity transfect the wild-type cell made competent in the organic medium more efficiently than monomer DNA but do not transfect the rec^- strain or wild-type cells made competent in the defined medium.

It is concluded that recombination is required for most wild-type transfections in the rich medium, either dimer molecules recombining with themselves, or monomer molecules combining with each other, to convert damaged molecules to single intact molecules with two cohesive ends, capable of replication. The dependence of transfection on DNA concentration observed in wild-type cells is considered to be caused partly by a concentration-dependent equilibrium between monomer and multiple forms of phage DNA in solution. These conclusions are in accord with the observation that recombination of temperature-sensitive phage in wild-type cells made competent in rich medium is enhanced about 50-fold for infection by phage DNA, as compared to infection by whole phage.

VARIATION IN REPAIR OF ULTRAVIOLET-IRRADIATED PHAGE DNA IN HAEMOPHILUS INFLUENZAE WITH THE TYPE OF INFECTION

Jane K. Setlow and Maxon E. Boling

It was previously found that Haemophilus influenzae that have the ability to excise UV-induced pyrimidine dimers from their DNA, but not excision-defective mutants, are able to repair UV-damaged phage DNA from phage irradiated before infection. The amount of such repair varies markedly, depending on whether the UV-irradiated phage DNA enters the cells by injection or as pure DNA or as part of DNA from lysogenic cells. These differences reflect the different events necessary for successful infection by the three methods.

Transfection by irradiated DNA from lysogenic cells is inactivated to the same extent when assayed in excising and in excision-defective strains, apparently because UV lesions within the prophage part of the transforming DNA prevent the emergence of the prophage from the transforming DNA, a process that is necessary for infection. Since only prophage that escapes UV damage can transfect, no repair of such DNA is observed in any host, and the UV sensitivity assayed in all hosts is the same as that of whole phage assayed on excisionless hosts. Transfecting DNA extracted from phage is more UV-sensitive than whole phage assayed in wild-type hosts, apparently because transfection, which is an inefficient process, must be assayed at a high multiplicity of infection, and there is competitive inhibition for repair of UV damage. The UV sensitivity of such DNA assayed in strains that are excision-defective but able to recombine phage DNA is twice as great as whole phage sensitivity in these strains, since most successful transfection involves a recombination between two units of phage DNA, so the irradiation "target" becomes twice as large.

THE CAPACITY OF ULTRAVIOLET-IRRADIATED HAEMOPHILUS INFLUENZAE TO PRODUCE VIABLE PHAGE

Jane K. Setlow and Maxon E. Boling

Ultraviolet-irradiated cells have a decreased capacity to propagate unirradiated phage. The UV sensitivity of this capacity depends on the repair capability of the host cell and on the method of infection. When infection is by whole phage, the most resistant strains are those that lack an excision mechanism, probably because these strains do not turn off respiration following UV irradiation. A recombination defect in the host cell does not alter the sensitivity to loss of capacity upon infection with whole phage. However, in a rec^- mutant infected with phage DNA, capacity is much less sensitive than in the wild type, suggesting that the recombination required for most transfection in the wild type is depressed by competition for enzymes needed for both recombination and repair of cell DNA. Capacity for transfection by phage DNA in the wild type is considerably more UV sensitive than capacity following infection by whole phage, while capacity for transfection by DNA from lysogenic cells is intermediate in sensitivity. Transfection

capacity damaged by UV can be repaired by the excision mechanism, as judged by the greater sensitivity of capacity for transfection in excision-defective mutants.

CHARACTERIZATION OF TEMPERATURE-SENSITIVE MUTANTS OF HAEMOPHILUS INFLUENZAE BACTERIOPHAGE

Maxon E. Boling, David P. Allison, and Jane K. Setlow

Temperature-sensitive mutants of Haemophilus influenzae phage isolated in this laboratory grow normally at 34°C but fail to form viable virus at 40°C. The mutants can recombine with each other to produce wild-type phage that grow at 40°C, provided the host cell is not defective in recombination.

Upon velocity sedimentation in a CsCl gradient, lysates of the temperature-sensitive mutants grown at 34°C banded in the same position as wild-type phage grown at 34, 37, or 40°C. The sedimentation pattern of two of the mutants grown at 37°C, intermediate between the permissive and restrictive temperatures, showed two bands, one in the same position as the normal band and another below it. After growth of the phage at 40°C, only the lower band was seen. Electron micrographs of material from these bands showed that the phage of the upper band were morphologically normal and that the lower band contained only phage heads without tails. A third temperature-sensitive mutant was found to make normal tails but abnormal heads at the restrictive temperature.

Since we have found that the phage head and tail mutations map close to one another, it is concluded that head and tail genes in the H. influenzae phage are clustered, as has been found to be the case for phage λ .

REPAIR OF CARCINOGEN DAMAGE TO TRANSFORMING DNA FROM HAEMOPHILUS INFLUENZAE

Jane K. Setlow and Paul O. P. Ts'o*

3,4-Benzpyrene can be covalently linked to DNA by incubating a mixture of the DNA and the carcinogen with 10^{-4} M iodine at pH 6.8. This procedure causes no measurable DNA degradation, and one molecule of the carcinogen is linked to about every 200 bases of the DNA. Such treatment inactivates the transforming ability of the DNA, to an extent which varies with the genetic marker. When it is assayed in mutants of Haemophilus influenzae that lack the ability to excise pyrimidine dimers, the transforming DNA appears considerably more sensitive than when it is assayed on wild-type cells.

The data indicate that DNA damage from 3,4-benzpyrene is repairable by the excision mechanism, suggesting that excision repair may affect the carcinogenic properties of this compound.

* Johns Hopkins University.

REPAIR OF ULTRAVIOLET DAMAGE IN YEAST

ORNL-BIO-25287

Michael A. Resnick and Jane K. Setlow

Two types of repair mechanism have been under investigation in the yeast *Saccharomyces cerevisiae* — excision repair and photoreactivation. Previous work had demonstrated that a photoreactivationless mutant, *phr1*, does lack the photoreactivating (PR) enzyme. We have found gene dosage effects of the *phr1* gene in tetraploid strains in terms of survival and amount of PR enzyme, as determined *in vitro*.

To measure dark repair of UV-induced pyrimidine dimers, it has been necessary to use an assay that does not rely on specifically labeling the DNA, since yeast lack a thymidine kinase. We have shown that pyrimidine dimers induced in the DNA of yeast cells compete with UV-irradiated transforming DNA for PR enzyme when the yeast DNA is present in crude cell extracts. Based on a calibration of the competition with UV-irradiated calf thymus DNA, an incident dose of $1,500 \text{ erg mm}^{-2}$ is shown to produce $2.5\text{--}5 \times 10^4$ dimers per cell. Wild-type cells irradiated in the exponential phase of growth remove more than 85% of the dimers within 3 hr 40 min after irradiation, while exponentially growing or stationary-phase UV-sensitive cells (*rad2-17*) do not exhibit any detectable alteration of dimers. Dimers induced in the stationary-phase wild-type cells appear to remain in the DNA; however, the dimers or dimer regions are altered in such a way as to make them less photoreactivable *in vivo* although photoreactivable *in vitro*. We propose that the wild-type cells, unlike the UV-sensitive cells, contain an endonuclease that is specific for UV-induced pyrimidine dimers.

SELECTION FOR MUTANTS OF YEAST WITH ENHANCED MUTATION RATES

C. M. Steinberg,* Dorna J. C. Gottlieb,[†] Katherine T. Cain, and R. C. von Borstel[‡]

We have developed a method for inducing and selecting mutants that have enhanced spontaneous mutation rates. This is done by treating a strain of *Saccharomyces cerevisiae* with ethyl methanesulfonate and selecting for papillating colonies (see Fig. 1 for a diagram of the complete procedure). The strain contains the supersuppressible markers *ade2-1*, *lys1-1*, *his5-2*, *trp5-48*, and *arg4-17*. The papillations are mostly from mutations in genes encoding enzymes that catalyze steps earlier in the adenine pathway than the *ade2*, or from supersuppressor mutations: these have a slightly faster growth rate than the original strain. The papillating colonies must be picked and taken through a second step of screening. Each papillating colony is placed in buffer and spread at high titre on complete medium with lysine as the growth-limiting factor. Under this condition each inoculum will grow, but eventually growth is stopped, except for that of the revertants to lysine independence. Those strains with a greatly increased reversion frequency over that of controls are then restreaked and retested on limiting lysine as well as on parafluorophenylalanine. If the limiting lysine plate shows a high frequency of revertants, if there are few revertants on the plates that contain no lysine, and if the frequency of parafluorophenylalanine-resistant mutants is high, then these strains are tested for their spontaneous mutation rates.

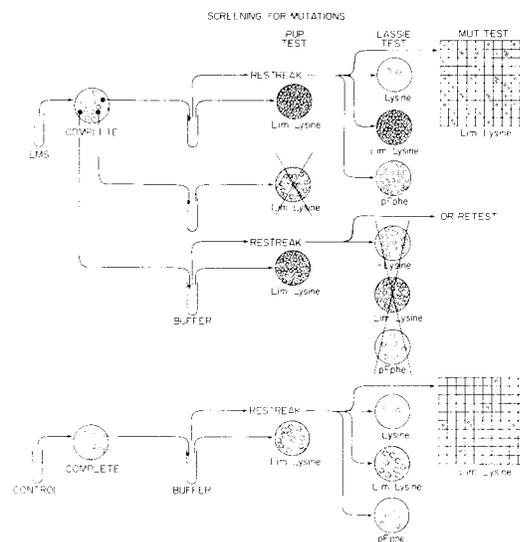


Fig. 1. Diagram of steps followed in the procedure for inducing and selecting mutants that enhance the spontaneous mutation rate.

Lim. Lysine, insufficient lysine for complete growth; -Lysine, no lysine in the medium; pFphe, parafluorophenylalanine; EMS, ethyl methanesulfonate.

The first mutagenization resulted in over 100 different mutants, which were designated as presumptive mutators after the three steps of selection. Of the first 38 tested, 35 are mutators indeed. Of the first 25 tested, not one is sensitive to UV radiation. The frequency of the induced presumptive mutators is about one-tenth that of the frequency of induced auxotrophic mutants recovered after the same mutagen treatment. Some of the data are shown in Table I.

TABLE I. Spontaneous mutation rates for reversion from lysine dependence to independence among a random sampling of "presumptive mutator" (VA) strains

Strains	Mutations/cell/ generation ($\times 10^{-8}$)	Strains	Mutations/cell/ generation ($\times 10^{-8}$)
Wild-type	3.4	VA-16	20.2
VA-1	27.5	VA-17	6.7
VA-3	71.0	VA-19	33.8
VA-4	19.5	VA-20	17.9
VA-5	13.8	VA-21	9.5
VA-6	17.5	VA-22	14.8
VA-7	19.1	VA-44	15.2
VA-13	13.1	VA-45	16.2
VA-14	38.9	VA-105	145.7
VA-15	16.9		

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PRELIMINARY CHARACTERIZATION OF TWO MUTATOR STRAINS IN YEAST

Dorma J. C. Gottlieb* and R. C. von Borstel†

Recently, it has been the purpose of our experiments to induce and select mutator genes in order to investigate further the spontaneous mutation processes in yeast. The procedure and the results of such a search are reported elsewhere (1). Efforts to characterize these mutators are now underway.

The two strains, provisionally designated VA-3 and VA-105, exhibit the highest spontaneous mutation rates of any mutants obtained thus far. The measured mutation rates were 64.9×10^{-8} and 145.7×10^{-8} , respectively, compared to a control value of 4.3×10^{-8} . These values were obtained using the rate of reversion from lysine dependence to lysine independence (2).

Testing for sensitivity to X-rays and UV radiation in these two strains revealed no significant differences from the controls. When mated to a "nonmutator" strain, the resulting diploids sporulated normally, although the frequency of mating with VA-3 appeared abnormally low.

The most striking observation was that all the mutations in these strains were mutants of the supersuppressor type and not reversions at the lysine locus. The supersuppressor mutations were almost exclusively Class I suppressors, which have been demonstrated to be ochre-reading mutants (3) of genes coding for tyrosine tRNA's (4). This specific enhancement in supersuppressor mutations has also been observed in meiosis (5) and in strains carrying the radiation-sensitive genes uxs1-1 and xrs3-1 (Ref. 2).

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ELECTROPHORETIC VARIANTS FOR ESTERASE IN HABROBRACON SERINOPAE

Roger H. Smith and Richard H. Reindollar*

Reciprocal crosses were made between two strains of Habrobracon serinopae — Hs(p), with plum eye color, and DEL, a wild strain from India — to study inheritance of two major isozymes of esterase. Parental strains, F₁ hybrids, and the F₂ haploid male progeny from virgin hybrid females were homogenized individually in 50% sucrose and 0.075 M Tris-sulfate, pH 9. All samples were centrifuged at 1040 X g for 20 min, and the supernatant was frozen at -20°C. The samples were run with the ORTEC 4100 pulsed constant power supply and the ORTEC 4200 electrophoresis system. The bridge buffer was Tris-borate, 6.5×10^{-2} M, pH 9.0. Gradient gels of three layers were cast with Tris-sulfate; the lower layer 8% polyacrylamide, the middle 6%, and the upper 4.5%. Each run was made at 300 V and 300 pulses per sec for 55 min.

The nonspecific esterases were stained by first equilibrating the gel in 0.04 M Tris-chloride for 5 min at 37°C followed by 15 min in a solution of Fast Blue B R, 0.04 Tris-chloride, pH 6.6, and a 1% substrate in acetone. The reaction was terminated in 20% ethyl alcohol in 10% acetic acid. After 15 min, the gels were placed in 10% acetic acid for storage.

Both major esterases showed isozyme differences between the strains. The two different loci were named est A and est B, est A being the closer to the origin and est B more distant. Alleles of each locus of DEL were designated according to their relative distance from their respective standard locus, the plum mutation. In this case, the alleles were designated est A^{1.00} and est B^{1.00} as the standard, and in Hs were assigned est A^{0.94} and est B^{0.95}. The superscripts refer to the relative mobility of each isozyme to the isozymes assigned as the standards. This type of designation is used for Drosophila (1).

Hybrid bands were not found for either est A or est B in the F₁ females. This indicates that these enzymes are probably monomer proteins. The est A and est B, as well as est A and plum, were found to segregate independently of one another in the analysis of the F₂ haploid offspring. Alleles of plum eye color and est B were found to be associated with their parental types. Table I shows that plum and est B are linked with a crossover frequency of about 5%. This is the first report of an electrophoretic variant located in the genome of Habrobracon.

*Student trainee.

1. J. C. Hubby and R. C. Lewontin, Genetics 54, 577 (1966).

TABLE I. F₂ progeny from the F₁ female Habrobracon [Hs(p) X DEL]

Alleles	Number in F ₂ progeny	Gene combinations	Recombinant types (No.)	Frequency
p- <u>est</u> A ^{1.00} - <u>est</u> B ^{1.00}	10			
+ <u>est</u> A ^{0.94} - <u>est</u> B ^{0.95}	15			
+ <u>est</u> A ^{1.00} - <u>est</u> B ^{1.00}	0	p - <u>est</u> A	29	0.52
p- <u>est</u> A ^{0.94} - <u>est</u> B ^{0.95}	1			
p- <u>est</u> A ^{0.94} - <u>est</u> B ^{1.00}	16	p - <u>est</u> B	3	0.05
+ <u>est</u> A ^{1.00} - <u>est</u> B ^{0.95}	12			
p- <u>est</u> A ^{1.00} - <u>est</u> B ^{0.95}	1	<u>est</u> A - <u>est</u> B	30	0.54
+ <u>est</u> A ^{0.94} - <u>est</u> B ^{1.00}	1			
	56			

MUTATIONAL RESPONSE OF HABROBRACON MALES TO X-RADIATION AND ETHYL METHANESULFONATE

Roger H. Smith

A comparison was made between X-rays and the alkylating agent ethyl methanesulfonate (EMS) for a spectrum of mutations induced in sperm of Habrobracon.

One-day-old males were exposed to 2000r of X-rays at 575 r min⁻¹. Another group of males were fed 0.005 M and 0.01 M EMS in 50% sucrose, while a control group was fed only sucrose. All males were then mated to virgin females, and these females were set for oviposition. The routine procedure for mutagenic analysis was followed (1).

The results of this study are presented in Table I. Feeding EMS produced a higher frequency of recessive-lethal mutations and a higher proportion of temperature-sensitive

lethal mutations than X-rays, when the two mutagens were compared on the basis of dominant- to recessive-lethal mutations. X-rays, however, were more effective in inducing translocations when compared on the same basis. These results probably reflect different mechanisms in the production of mutational events by the two mutagens.

The results in the F₂ analysis were identical for the group of males fed the two different concentrations of EMS. This suggests that the sperm were saturated by mutational events that lead to recessive-lethal mutations.

1. R. H. Smith and R. C. von Borstel, in Chemical Mutagens, vol. II, p. 445. Plenum Press, New York (1971).

TABLE I. Mutation analysis of sperm from Habrobracon males exposed to X-rays and to EMS

Exposure	Eggs (No.)	Dominant lethality	Stage 1 deaths	Frequency		
				F ₂ analysis		
				Recessive lethals	Temperature sensitive	Translocations
Sucrose	1475	0.02	~ 0	~ 0	~ 0	0
2000r X-rays	806	0.56	0.48	0.16	0.17	0.10
0.005 M EMS	3099	0.05	0.04	0.63	0.32	0.02
0.01 M EMS	3610	0.35	0.32	0.63	0.32	0.03

MAMMALIAN GENETICS SECTION

W. L. Russell

Genetic Effects of Radiation in Mice

W. L. Russell

Azucena L. Carpena^a

Elizabeth M. Kelly

Mammalian Cytogenetics and Development

Liane B. Russell

Mammalian Cytochemistry and Mutagenesis

R. B. Cumming

G. A. Sega^aEffects of Radiation on Mammalian Gametogenesis

E. F. Oakberg

Mammalian Comparative Mutagenesis

W. L. Russell

R. B. Cumming

W. M. Generoso

^aPostdoctoral investigator^bVisiting investigator from abroad

TRITIUM MUTAGENESIS IN THE MOUSE

W. L. Russell, R. B. Cumming, Elizabeth M. Kelly,
and Marva F. Walton

Relatively little is known about the genetic effects of tritium in any organism. This condition persists, in spite of the increasing awareness and concern about the possible biological impact of man-made environmental tritium and in spite of the obvious scientific interest in a comparison of the genetic effects of low-energy β particles with those of higher-energy external radiation sources. With regard to intact mammals, the lack of information is even more striking. Prior to experiments in progress in our laboratory, there were no mammalian specific-locus mutation data or other mammalian gene mutation information available for tritium in water, in organic molecules, or in any other form; and there were no data on any kind of genetic effect of tritiated water on mammalian germ cells. There have been limited experiments in mice on dominant-lethal effects of tritiated thymidine (1), but these are not extensive enough to allow a quantitative comparison to other, genetically better known forms of radiation.

It is convenient to divide the consideration of the genetic effects of tritium into two separate topics — effects due to tritium specifically incorporated into organic molecules of biological significance and effects due to tritiated water. The latter category is far more important from the standpoint of assessing the hazard of environmental tritium and is in many ways more comparable to externally applied radiation.

Dosimetry of β radiation from tritium decay within specific cells of a mammal is more complicated than one would at first suspect. The problems are quite different for tritiated DNA precursors and tritiated water. In the case of compounds like tritiated thymidine, the majority of the activity is metabolized to tritiated water, and this then becomes subject to all the dose considerations for that material. The remainder of the radioactivity is incorporated directly into the DNA molecules of dividing cells. The amount incorporated and the biological half-life are different for each cell type and depend upon a multitude of biological factors, including the pharmacology of thymidine in the target tissue and the cell kinetics of the particular cell. Dosimetry of radiation from ^3H decay from tritiated thymidine in mammalian male germ cells is not a trivial problem, but the understanding of the kinetics of stem cell renewal in seminiferous epithelium recently achieved by Oakberg (2, 3) for the mouse and by Huckins (4) for the rat makes a solution to this problem possible.

We have started, on a moderate scale, a specific-locus experiment on male mice, using a single injection of tritiated water as the radiation source. A study on the dosimetry of injected tritiated water is also in progress. Male offspring from the specific-locus experiment will be tested for translocation heterozygosity, and a dominant-lethal mutation experiment is planned, also with injected tritiated water.

Other projects may develop in the future, such as tritiated thymidine mutagenesis, the effects of tritiated drinking water, and the genetic effects of tritium on female mice.

An isolator system for treated mice that prevents tritium release has been worked out and operates well. To date, five groups of 18 male mice each have been treated and mated in the specific-locus experiment. Two dose levels have been used in these preliminary experiments. The first two groups received 0.75 mCi per gram of body weight, and the remaining three groups received 0.50 mCi g^{-1} . Fertility prior to the expected sterile period lasted only 2 weeks at the higher dose level, with greatly reduced litter sizes, particularly in the second week. The sterile period in the higher dose groups was about as long as would be expected from an acute dose of 1000 r of X-rays. In the groups that received the higher dose, one presumed specific-locus mutation was observed in 515 offspring from cells irradiated in postspermatogonial stages. In the three groups that received 0.50 mCi g^{-1} , litter size and fertility were depressed but not to the same extent as with the higher dose level. In these groups we will have about 1000 offspring from irradiated postspermatogonial stages, and we have thus far detected two presumed specific-locus mutations and another animal which appears to be mottled.

These experiments will be continued. It appears that we have a workable — though laborious — system for using the specific-locus test to detect mutations induced by tritiated water. The frequencies are high enough to allow us to obtain adequate data with an experiment of modest size.

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2. E. F. Oakberg, *Anat. Rec.* 169, 515 (1971).
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FURTHER EVIDENCE ON THE EFFECT OF RADIATION DOSE RATE ON THE INDUCTION OF $\underline{\text{X}}$ -CHROMOSOME LOSS IN FEMALE MICE

W. L. Russell, Elizabeth M. Kelly, Patricia R. Hunsicker,
Carolyn M. Vaughan, Georgia M. Guinn, and
Elizabeth B. Edwards

As was pointed out in an earlier report (1), the purpose of this experiment is to test whether radiation dose rate, which has a marked effect on the frequency of mutations induced at specific loci, will prove to have a similar effect on a different type of genetic damage — namely, $\underline{\text{X}}$ -chromosome loss.

The preliminary results presented in the earlier report already showed that the frequency of $\underline{\text{X}}$ -chromosome losses in the offspring of females exposed to a low dose rate (approximately 0.6 r min^{-1}) was significantly below the frequency obtained in the progeny of females exposed to the same dose (400 r) at a higher dose rate (approximately 80 r min^{-1}). The experimental details are given in the earlier report (1).

The additional data now obtained support the earlier finding of a dose-rate effect and provide a more reliable estimate of the ratio of effects at the two dose rates. In a

total of 13,359 female offspring in the 80 r min^{-1} experiment, there were 85-102 cases of \underline{X} -chromosome loss. The upper figure includes 17 in which the tests are not yet finished or in which the animals died before testing was completed. In the 0.6 r min^{-1} data, the corresponding figures were 41-46 in a total of 10,525.

The difference between the frequencies at the two dose rates is not quite as large as in the preliminary data, but it remains statistically highly significant ($P = 0.0013$ for a one-tailed test), when the maximum frequencies of 85 and 46 are compared. If the statistically much more conservative comparison between the minimum frequency of 85 in the high-dose-rate experiment and the maximum figure of 46 in the low-dose-rate data is made, a statistically significant difference ($P = 0.025$) still is obtained. Subtracting the spontaneous frequency (7 \underline{X} -chromosome losses in a total of 10,491 offspring) increases the degree of statistical significance in both comparisons.

The ratio of the induced frequencies of \underline{X} -chromosome loss at low and high dose rates, based on the maximum values at each, now stands at 0.53. The possibility of a further reduction in frequency at lower dose rates is now being explored.

-
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NONLINEARITY OF THE DOSE-FREQUENCY CURVE FOR RADIATION INDUCTION OF \underline{X} -CHROMOSOME LOSS IN FEMALE MICE

W. L. Russell, Elizabeth M. Kelly, Patricia R. Hunsicker, Carolyn M. Vaughan, Georgia M. Guinn, and Elizabeth B. Edwards

In an earlier report (1), in which the frequencies of \underline{X} -chromosome loss in female mice exposed to doses of 50 and 400 r of X-rays at approximately 80 r min^{-1} were compared, the observed frequencies at the two doses were so close to those expected on the basis of a straight-line relation between frequency and dose that no departure from linearity was suggested. With the collection of nearly eight times as much data, there is now evidence that the effect at 50 r is less than would be expected on a linear basis.

It is now clear that, in the earlier report, the frequencies at both doses were erroneously high. Further testing has revealed that some of the \underline{X} -chromosome losses observed were not new occurrences but were inherited from $\underline{X}/0$ mothers already existing in the mouse strain used.

The experimental procedure is described in earlier reports (1, 2). The present data give a frequency of 12-17 \underline{X} -chromosome losses in 21,895 female offspring of females exposed to 50 r. The upper figure includes five in which tests were not completed. In the 400-r data, the frequency was 85-102 \underline{X} -chromosome losses in 10,525.

The number of \underline{X} -chromosome losses expected at 50 r, on the basis of linear interpolation between the control frequency (7 in 10,491) and the frequency at 400 r, is 30.2 if we take the lower value in the 400-r data and 33.7 if we take the upper value. Thus, the observed frequency at 50 r, only 12-17, falls far short of the figures expected on the basis of linearity.

Tests of statistical significance are complicated by the appreciable spontaneous frequency; but, assuming that deviations from the expected frequency at 50 r are normally distributed, the observed frequency at 50 r can be shown to be significantly lower ($P < 0.01$) than expected (3).

It is concluded that, as with specific-locus mutations in the mouse, this entirely different type of genetic hazard also shows less risk of occurrence per roentgen at low doses than at high ones.

The number of spontaneous \underline{X} -chromosome losses expected in the 21,895 female offspring in the 50-r experiment is 14.6. This lies between the two values, 12 and 17, observed for the experimental frequency of spontaneous and induced occurrences combined. Even the upper value is not significantly different from the spontaneous frequency. If there is, in fact, any effect of 50 r, the present data are inadequate to show a significant increase above the control level.

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EFFECT OF DOSE FRACTIONATION ON THE X-RAY INDUCTION OF \underline{X} -CHROMOSOME LOSS IN FEMALE MICE

A. L. Carpena and W. L. Russell

The finding of W. L. Russell *et al.* (1) that the X-ray-induced frequency of \underline{X} -chromosome loss in female mice is affected by dose rate suggests that dose fractionation may have an effect also on this type of genetic damage. Our experiment was carried out to test this possibility and to see how the effect, if any, of a certain type of dose fractionation on \underline{X} -chromosome loss compares with the observed effect on specific-locus mutation.

Eight- to eleven-week-old female mice from the cross (101 X C3H) or its reciprocal were exposed to a total dose of 400 r of X-rays at $65-70 \text{ r min}^{-1}$, given either all at one time (single dose) or in eight fractions of 50 r each with a 75-min interval between fractions (fractionated dose). The irradiated females were mated to males carrying the sex-linked gene Greasy (Gs) at least 24 hr after the last exposure. The offspring were checked 8-10 days after birth for the presence of Gs females, which presumably are $\underline{X}/0$'s (Gs/0's).

Cytological and breeding tests were done on each G_s daughter to determine whether she lacked an X chromosome. Chromosome counts were also made on the mothers of $G_s/0$ females, so that data from parents that were already $X/0$'s could be excluded. In the cytological test, chromosome preparations were made from bone marrow aspirated from a femur of the animal and incubated with a small amount of medium 199 and colcemide for 2-3 hr at 37° C. For the breeding test, males carrying another sex-linked gene, sparsfur, were used as testers.

The single-dose experiment yielded 26 $G_s/0$ daughters out of 2921 female offspring ($G_s/0$ plus $G_s/+$) or 31 $G_s/0$'s out of 2938 daughters if litters from mothers that died before their chromosomes could be counted were included. The fractionated dose gave 14 $G_s/0$'s out of 3473 daughters or 17 out of 3486 if litters of untested mothers were also considered. Two $G_s/0$ females were obtained from the control group (unirradiated) of 2820 daughters.

The frequency at the fractionated dose is significantly lower than that at the single dose ($0.05 > P > 0.01$) for all comparisons, e.g. maximum frequency at single dose (31/2938) versus maximum frequency at fractionated dose (17/3486). The minimum induced frequency (14/3473) shows a highly significant ($P < 0.01$) increase over that of the control (2/2820). It appears, therefore, that dose fractionation does have an effect on the frequency of X-chromosome loss in female mice.

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SEX-CHROMOSOME LOSS INDUCED IN MOUSE SPERMATOGONIA BY SINGLE AND FRACTIONATED DOSES OF X-RAYS

Liane B. Russell and Clyde S. Montgomery

Sex-chromosome losses have been widely used by us (1,2) as an index of induced chromosomal damage, since such losses result in viable animals that can be readily detected by phenotype when appropriate markers are used. At the time of the last annual report, we had just begun an experiment designed to fill a big gap in the series of sex-chromosome-loss experiments — namely, the induction of such losses in spermatogonia, a stage of high importance in the evaluation of genetic hazards. In addition, the experiment was designed to test whether 24-hr fractionation, which had earlier been found to increase greatly the frequency of specific-locus mutations (3), would also augment this type of chromosomal damage.

(101 X C3H) F_1 males were irradiated with acute X-ray doses, either 600 r or 100 r + 500 r (24-hr interval), and mated to G_s/G_s females to obtain spermatozoal (4) and spermatogonial data. Exceptional progeny were tested genetically and cytologically.

With 26 cases of paternal sex-chromosome loss in over 13,000 offspring classified to date, the rate in irradiated spermatogonia (0.205 % for the two groups combined) is not significantly higher than in contemporary controls (0.193 %). The adjusted induced frequency is $0.02 r^{-1} \times 10^5$ — about two orders of magnitude lower than in spermatozoa. No augmentation due to dose fractionation could be detected: the rate in the group that received 100 r + 500 r is actually somewhat below that for 600 r. There was only one case of presumed paternal nondisjunction ($G_s/+/Y$), and that occurred in controls.

An earlier, smaller experiment (5) had yielded no cases of paternal sex-chromosome loss in either irradiated or control groups (a total of 2855 mice scored). While this result indicates a control rate considerably below ours, it supports our finding on lack of recoverable induced losses.

Since it is known from translocation findings that chromosome breakage can be induced in mouse spermatogonia (6), it is unlikely that the absence of recoverable induced sex-chromosome losses indicates an absence of initial damage. It appears more probable that selection is operating. Whether this occurs during spermatogonial stages (against 0/Y or X/0 gonia) or during meiotic stages (e.g. because of deficient sex vesicles) is not known at this time. Successive tabulations in both irradiated and control series show a slight decline in frequency of recovered sex-chromosome loss with age of the males.

In the course of the cytological verification of exceptional progeny in this experiment, we discovered a submetacentric chromosome, resulting from an autosomal translocation with one break presumably in one of the almost imperceptible "short arms" of the complement (i.e. not a Robertsonian type of translocation). This will be very useful in cytogenetic studies.

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INTERPRETATION OF THE NATURE OF RADIATION-INDUCED SPECIFIC-LOCUS MUTATIONS IN THE MOUSE ON THE BASIS OF FINE-STRUCTURE MAPPING

Liane B. Russell

Mutation-rate experiments in the mouse have, over the past 20 years, yielded a large number of genetic changes involving specific loci. The nature of these specific-locus

mutations has been a question of some interest, especially since it was known from the use of two closely linked markers that certain types of treatments could induce deficiencies. For the interpretation of radiation-genetic data in general, it was of interest to determine whether the mutations are the result of single-hit or single-track events, on the one hand, or of two independent events, on the other.

Complementation mapping of a small chromosomal region has now been used to give more accurate definition to the nature of specific-locus mutations in that region. Mutations used for this purpose were detected by their visible phenotype in combination with the test stock's linkage-group-2 markers, *d* and *se* (recombination frequency 0.16%). A total of 235 mutants of this type have been recovered, and 108 of these were used in complementation tests involving over 900 combinations and about 40,000 progeny (1).

While the original screening for mutants employed only two markers, the subsequent analysis has, so far, revealed 18 complementation groups, spanning nine or ten functional units. Involvement in a mutation of two or more functional units is taken to indicate chromosomal aberration. All mutations are consistent with a linear map, except for two, which appear to "skip" units but have been shown not to result from recombinationally separable changes. These two mutations, one of which gives "conversion"-like results in recombination tests, are interpreted as small rearrangements. Neither of them appears to decrease recombination between two close outside markers (tests still in progress). A third mutation was apparently the result of two independent events separable by recombination. The remaining multi-unit mutations are, for the time being, taken to be deficiencies. Although there is evidence that homozygous deficiency of either one of the marked loci gives a visible effect, this by itself is no proof that the single-unit mutations are not "point" mutations (perhaps single base-pair changes).

A strong effect of irradiated cell stage can be demonstrated, both on the locus spectrum (i. e., relative frequencies of events involving *d*, *se*, or both) and on frequency of mutations that are interpreted as aberrations. The latter ranges from 13.5% in most X- or γ -irradiated spermatogonia, through 42.3% in postgonial stages, to 65.6% in oocytes. Within spermatogonia, neutron irradiation and 24-hr fractionation of X-rays shifts the distribution in the direction of postgonial results. The rest of the irradiated spermatogonia closely resemble the control distribution.

The recombinational length of most of the aberrations is very small, 75-86% of them spanning less than two crossover units. Even in those groups that have a high total frequency of aberrations (postgonial stages and oocytes), no more than 23% of all mutations exceed this length; and the figure is 0% for X- or γ -irradiated spermatogonia (excluding 24-hr fractionation data). This finding supports W. L. Russell's conclusion (2) that his specific-locus method measures mostly single-track events, even in irradiated oocytes. Results from X- and γ -irradiated spermatogonia indicate a clearly restricted nature of the genetic damage.

Heterozygous effects on viability were detected in *se* lethals that include two or more functional lethals but not in those that include only one. In the case of *d*-locus lethals, the relationship between heterozygous effect and number of lethal units involved is apparently not a simple one.

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ACTIVITY AND BEHAVIOR OF THE MOUSE X CHROMOSOME

Liane B. Russell, Gary R. Dunn,* and Nestor L. A. Cacheiro

Work on activity and behavior of the mouse X chromosome has continued along several avenues. Three of these will be summarized here. (Considerable additional work that has continued on X-autosome translocations will be reported in the future.)

(1) We have attempted to demonstrate different inactivating states of normal mouse X chromosomes. The results from our work with reciprocal $\bar{T}(X;A)$'s several years ago suggested the existence of an inactivation center in the X chromosome (1). More recent work by Cattanach (2) and others indicates that the X-controlling element, *X_{ce}*, which may correspond to the inactivation center, exists in alternative states.

We are attempting to check for different states of *X_{ce}* in normal X chromosomes. X chromosomes derived from different inbred strains are tested with respect to their effect on the expression of *Bent-tail* (*B_n*) carried on another X of near-inbred derivation. Males from the stocks whose X is being tested (BALB/c, CFW, BL10, 101, and MWA) are crossed to uniform *Blo/+* females from a C3H-*Blo* stock, and the *Blo/+** progeny (+* representing the X being tested) are crossed to *B_n/Y* males from the *B_{ncp}* stock (near inbred). Tail kinking is scored in *Blo/B_n* and *+*/B_n* daughters of the second series of crosses. To date, 752 daughters of this cross have been scored.

If tail-kinking frequency is under control of only the X chromosome, it should be identical in the *Blo/B_n* segregants of all groups, since (except for rare *Blo-X_{ce}* crossovers) each X involved comes from a uniform source. However, the frequency shows significant variations, from 42% to 76%. In each strain-derived group, the frequency of kinks was significantly lower in females carrying *+Blo* on the X opposite *X_{B_n}* than in those carrying *Blo*. One possible explanation is a relatively greater developmental inviability of cells in which *X_{Blo}* is active. Tail-kinking frequency in *+*/B_n* varied from 18% in *+BL10/B_n* to 50% in *+BALB/c/B_n*. The data indicate at least two alternate forms of *+**, but the interpretation is somewhat obscured by the demonstrated effect of general genetic background on the expression of kinking.

(2) Arguments recently raised by Grüneberg against the single-active-X hypothesis have been tested (3). Grüneberg (4) had proposed the "complemental X" hypothesis, according to which the two X's of a female interact intracellularly, the total activity being equivalent to a dosage of one, but the relative contributions of the two X's to this total varying from cell to cell.

Grüneberg claims that the phenotype of mice heterozygous for X-linked genes cannot be interpreted on the basis of separate cellular "heredities." To test this claim, fusion chimeras were made between *G_s/G_s* (or *G_s/Y*) and *+/+* (or *+/Y*) embryos, and the phenotypes of the resultant mosaic

mice were compared with those of $G_s/+$ heterozygotes. Detailed comparison of several hair parameters indicates that the $G_s/+$ hair phenotype is not merely a summation of the G_s and $+$ phenotypes, and it is closely paralleled by the phenotype of the fusion chimeras. Therefore, it could be interpreted on the basis of separate cellular heredities. Both in heterozygotes and fusion chimeras, there appears to be selection of $+$ -bearing lineages with increasing age.

(3) Cytological investigations in X -autosome translocations have concentrated on meiotic chromosomes and, more recently, on detailed mitotic studies made possible through the use of the new fluorescence methods. Meiotic studies, most of which must be done in females (due to stoppage of spermatogenesis in pachytene) reveal a high frequency of quadrivalents (81-96%) in all six of the reciprocal $T(X;A)$'s analyzed, in contrast to only 30-50% quadrivalents in the case of the flecked insertion. The relative frequencies of chain and ring quadrivalents differed for the different translocations (5). The use of fluorescence methods has led to the cytological identification of linkage groups 1 and 8. It further indicates that the long chromosome has an X centromere in the case of one $T(X;8)$ and two $T(X;1)$'s but an autosomal centromere in the case of our other X -autosome translocations. This corroborates tentative conclusions drawn on the basis of the meiotic evidence. Perfection in our laboratory of the fluorescence techniques will be highly useful in the study of numerous chromosome aberrations.

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THE X-RAY INDUCTION OF SPECIFIC-LOCUS MUTATIONS IN MALE MICE AT VARIOUS AGES FROM NEWBORN TO YOUNG ADULT

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The specific-locus mutation frequency resulting from 300 r of X -irradiation at 80 r min^{-1} is being determined for the reproductive cells present in male mice at 0, 2, 4, 6, 8, 10, 14, 21, 28, and 35 days of age. All irradiated mice were F_1 hybrids from a cross of strains 101 and C3Hf. Each mouse was irradiated at one of the above ages, and then after growing up it was mated with a female of our T stock, which is homozygous for seven recessive marker genes. The offspring produced during the entire reproductive life of the irradiated males are being examined for mutations at the seven genetically marked loci.

The intent of this research is to obtain a fairly accurate measurement of the mutation rate of the reproductive cells present in day-0 mice. All mice irradiated on day 0 were

actually irradiated within 9 hr after birth. This project is also designed to screen the mutation rates of the reproductive cells present at the other nine ages in such a way that any extremely high rate would be detected. It is important to know whether the reproductive cells present at these ages show the same sensitivity to mutation induction as the cells of the adult. At 35 days of age, the testis is histologically similar to an adult testis (1); however, the mutation frequency for the reproductive cells present in it has never been determined.

To date, the point estimate of the specific-locus mutation rate for male mice irradiated on day 0 is $13.7 \times 10^{-8} \text{ r}^{-1} \text{ locus}^{-1}$, based on 13 (plus one questionable) presumed mutations out of a total of 48,675 offspring. In contrast, the point estimate of the mutation frequency of similarly irradiated adult males is $29.0 \times 10^{-8} \text{ r}^{-1} \text{ locus}^{-1}$ (2). The rate for day-0 males is statistically significantly lower ($P = 0.015$) than the rate for similarly irradiated adults. There are strong indications that the distribution of mutations among the loci is different. There is some indication that the mutation rate resulting from the irradiation of the reproductive cells in day-0 males may decrease after these males are about 400 days old.

More clusters of mutations occur in male mice irradiated on day 0 than in similarly irradiated adults. This difference is significant ($P < 0.02$) and presumably results from there being relatively fewer surviving reproductive cells following irradiation of the day-0 testis.

Taken together, the remaining nine groups of males have thus far yielded 38 presumed mutations in 67,765 offspring. This compares to 40 mutations in 65,548 offspring in similarly irradiated adult males (2). Although one of the nine groups (day 8) has a point estimate 1.6 times that for adults, none has a mutation rate statistically significantly higher than the adult rate. To date, five of the point estimates are lower than the adult rate and four are higher.

The data contain some indications of a possible trend, supporting the idea that the transition from day-0 sensitivity for mutation induction to adult sensitivity may occur prior to day 8. Day 8 had the highest rate of all the groups, and of the five point estimates lower than that for adults, three were for the groups irradiated on days 2, 4, and 6. Furthermore, the point estimates for the data when arbitrarily grouped are as shown in Table I.

TABLE I. Point estimates of mutation rates of male mice irradiated at various ages

Age at irradiation (days)	Mutation rate [$\times 10^{-8}$] ($\text{r}^{-1} \text{ locus}^{-1}$)
0	13.7
2-6 combined	18
8-35 combined	30.5
Adult	29.0

It would be of great interest to know whether or not the cellular basis for this change in sensitivity, which could be abrupt or gradual, is the transformation of gonocytes into A_s

spermatogonia. This transformation has been thought to occur on or near day 0 in the mouse, but it has never been precisely timed in the hybrid mice used in this experiment.

In summary, it appears highly unlikely that the mouse testis passes through any stages of development between birth and adulthood during which the reproductive cells in it are very much more sensitive to the induction of specific-locus mutations than those of the adult. These stages may not, in fact, be any more sensitive. The mutation rate resulting from the irradiation of day-0 male mice is half as great as that resulting from the irradiation of adult male mice.

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X-RAY INDUCTION OF SPECIFIC-LOCUS MUTATIONS IN NEWBORN FEMALE MICE

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There has been a gap in our knowledge of the mutational sensitivity of newborn female mice. The purpose of this experiment was to fill that gap. Recently, there has been additional interest in this area, because cytological observations give some reason to believe that some of the oocyte stages present in late fetal and newborn female mice may be more similar than those in female mice of other ages to the bulk of the oocytes in human females. However, it should be kept firmly in mind that similarities in chromosome configuration do not necessarily mean that there would be similarities in mutational sensitivity.

The specific-locus mutation frequency resulting from 300 r of X-irradiation at 80 r min^{-1} has been determined for the reproductive cells present in female mice within 9 hr after birth. All irradiated females were F_1 hybrids from a cross of strains 101 and C3Hf, and they were all irradiated some time during the 9 hr after they were born. After growing up, each female was mated with a male of our T stock, which is homozygous for seven recessive marker genes. The offspring produced during the entire reproductive life of each female were examined for mutations at the seven genetically marked loci.

Three mutants were found out of a total of 14,259 offspring. This gives a point estimate of the mutation rate of $10.0 \times 10^{-8} \text{ r}^{-1} \text{ locus}^{-1}$. Russell (1) found that the point estimate for adult females that received 400 r of X-irradiation at 90 r min^{-1} is $55.3 \times 10^{-8} \text{ r}^{-1} \text{ locus}^{-1}$. The mutation rate for the newborn females is highly statistically significantly lower ($P = 0.0009$).

The interpretation of this result is complicated by many factors, however. One major factor is that female mice irradiated with 200 or 400 r as adults become sterile within just a few weeks, whereas most females irradiated as newborns continue to be fertile for many months. When adults are exposed to an X-ray dose low enough to permit extended fertility, namely 50 r, the mutation frequency drops to zero

or near-zero levels in offspring conceived more than 6 weeks after irradiation. In contrast, the only mutations scored in the present experiment were in offspring conceived more than 20 weeks after irradiation. However, when the mutation frequency per roentgen for offspring conceived over the whole reproductive life-span in the present experiment is compared with the same figure from the 50-r experiment on adults, no significant difference is seen. And since we can expect that newborns, like adults, may give a lower per-roentgen mutation rate with 50 than with 300 r, the present data, as a whole, do not indicate a higher mutation rate in newborns than that reported for adults.

Within the data of the present experiment, however, there is one odd finding that suggests another possible complication in interpretation. All three mutants in this experiment on day-0 oocytes were conceived within the narrow interval of time 21-23 weeks after the time of irradiation. The probability of this occurring due to chance is very small; however, that does not rule out the fact that chance alone might have caused it. If it did have a biological basis, though, one way of explaining it would invoke a precise stage of development of the oocyte (probably within either pachytene or diplotene) with a fairly high mutation rate. If strict regimentation of the oocytes occurs throughout development, most or all mutant offspring would be born within a narrow interval of time. If this postulated precise stage of oocyte development happened to correspond to the "resting stage" of the human oocyte, then the genetic hazard from irradiation of women could be greater than that which would be estimated from the average mutation rate in newborn mice.

In summary, the point estimate of the mutation rate in oocytes of female mice irradiated within 9 hr after birth is less than 1/5 of the point estimate of the mutation rate in the oocytes of adult female mice that received a comparable dose. More work is needed, though, before this result can be applied meaningfully to the estimation of human hazards.

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SPERMATOGONIAL STEM CELL RENEWAL IN THE MOUSE

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The stem cells of the seminiferous epithelium have been identified as isolated type A spermatogonia with oval, dark-staining, uniformly granular nuclei and high resistance to radiation. The designation A_s (A_{stem}) has been given to these cells because they have the property of replacing themselves by mitotic division, with the daughter cells becoming widely separated, and also to form pairs of cells that remain connected by cytoplasmic bridges.

The spermatogonial pairs divide, forming branching chains of cells with nuclear morphology identical to that of A_s spermatogonia. At stage VII of the cycle, the aligned cells then transform, without division, into type A_1 spermatogonia. Division of A_1 gives rise to A_2 and, by successive division, A_3 , A_4 , In, and B spermatogonia. Tubule whole

mounts indicate that cytoplasmic bridges persist throughout this developmental sequence. It appears, therefore, that the formation of a pair of cells connected by a cytoplasmic bridge is the initial visible step in the developmental sequence.

Heavily labeled A_5 spermatogonia persist for as long as 10 days, but by 15 days only lightly labeled cells could be found. This suggests that some A_5 cells have a very long cycle, but that they divide at least once each cycle of the seminiferous epithelium (207 hr). The long persistence of heavily labeled cells also suggests that these cells may have a long G_2 . Further evidence for this is obtained from curves of labeled mitoses, where some A_5 cells reach metaphase as soon as A aligned spermatogonia, but the curve then flattens out. Elucidation of stem cell kinetics is difficult, owing to the extreme variability in duration of the cell cycle.

RADIATION RESPONSE OF A_5 SPERMATOGONIA

E. F. Oakberg, Claire Huckins,* and Patricia D. Tyrrell

The A_5 spermatogonia are the most resistant of the type A population and are the only survivors after doses of 150r or more. Aligned cells degenerate as they begin division at stage IX, but before this process is completed, formation of new pairs by surviving A_5 cells has begun; it is only at stage V that their maximum depletion of A_5 gonidia can be estimated. At doses above 150r, pair formation occurs later and has returned to normal frequency by 207 hr.

As the testis regenerates, the normal developmental sequence is repeated. The newly formed pairs divide to form a chain of four, which divides to form a chain of eight, etc. Finally, these chains of cells transform into spermatogonia A_1 , and the entire sequence of differentiating cells is reestablished. It is very clear that A_4 spermatogonia give rise only to the intermediate type.

It was observed that the aligned spermatogonia degenerate in groups; i.e., all members of a chain either degenerate or survive. After 20r (about the LD_{40}), chains of dead cells could be observed intermingled with chains that appeared normal. Since the probability of killing all members of a chain with 20r is low, it must mean that inactivation of one (or a few) cells kills the whole chain. It frequently was observed that degeneration appeared to spread along the chain in a wave-like fashion; normal cells could be observed at one end of the chain, frankly necrotic cells at the other, and an ordered sequence of degenerative steps in between. After nuclear degeneration reached a certain stage, the cytoplasmic bridges broke, and the late necrotic stages appeared as chains of separate dead cells. The response of aligned spermatogonia as groups, therefore, must be a significant factor in the high radiation sensitivity of the A_1 - A_4 spermatogonia. Since the chain is the unit of death, inactivation of one cell is amplified.

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EFFECT OF DOSE IN THE INDUCTION OF CHROMOSOME ABERRATIONS BY ETHYL METHANESULFONATE IN THE MOUSE

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The exposure of the human population to chemicals occurs predominantly at chronic levels. Certain drugs are obvious exceptions. Before hazards of chemicals to humans can be properly evaluated, it is necessary to study whether there is a mechanism in the human body that protects partially or completely against low doses before genetic damage can occur or be inherited. In other words, what is the shape of the dose-effect curve, and is there a dose threshold? Data on this problem are lacking. The present report and the following one are attempts to answer this question in the mouse.

Twelve-week-old (101 X C3H) F_1 male mice were injected intraperitoneally with 50, 100, 150, 200, or 250 mg kg^{-1} of ethyl methanesulfonate (EMS), and the frequencies of dominant-lethal mutations and heritable translocations were measured in germ cells treated in the early spermatozoa stage. In the dominant-lethals experiment, males were mated, 4-1/2 to 7-1/2 days after injection, with (101 X C3H) F_1 virgin females, which were killed during pregnancy for uterine analysis. In the translocation experiment, males were mated with normal females 6-1/2 to 7-1/2 days after injection, and F_1 male offspring were tested for translocation heterozygosity. F_1 males were tested for sterility or semisterility by caging each one with a (SEC X C57BL) F_1 female and allowing it to produce four litters. F_1 males that were either sterile or exhibited a slight indication of semisterility were further tested by mating each one to six more (C3H X C57BL) F_1 females, which were killed during pregnancy for uterine analysis.

No significant increase in dominant-lethal mutations was detected after the dose of 100 mg kg^{-1} . At 150 mg kg^{-1} , an 18.6% increase in detectable dominant lethals was observed, and the frequency increased very sharply with dose, with 52.0 and 92.1% at 200 and 250 mg kg^{-1} , respectively. In the translocation experiment, however, increase in the frequency was already detectable at 50 mg kg^{-1} , with six presumed translocations in 853 F_1 males tested ($P = 0.022$). In the control, one presumed translocation was found in 1218 F_1 males tested. Eighteen presumed translocations out of 1013 F_1 males were found at 100 mg kg^{-1} , 46 presumed translocations out of 621 at 150 mg kg^{-1} , and 79 presumed translocations out of 246 at 200 mg kg^{-1} . All F_1 males that were confirmed sterile or semisterile are presently being examined cytologically.

It is clear that genetic damage can be induced at doses as low as 50 mg kg^{-1} , which is the lowest dose used in this experiment. The threshold for genetic effects of EMS, if there is any, must be at a lower dose. It is interesting to point out that, in contrast to induced translocations, dominant lethals induced by EMS were convincingly detected only at 150 mg kg^{-1} and above. It is likely that dominant lethals had also been induced at 50 and 100 mg kg^{-1} but were not detected, owing to the relative insensitivity of the dominant-lethals procedure. (It should be emphasized that this comparative study involved the germ cell stage that is known to be

most sensitive to dominant-lethal induction with EMS but not necessarily to translocation induction.) Thus, for the detection of low levels of chromosome breakage, translocations are a much more reliable endpoint than dominant-lethal mutations.

The dominant-lethal and translocation curves (Fig. 1) may indicate that there is a point in dose of EMS at which the protective mechanism in the mouse body is saturated, and beyond which additional chemical results in a very sharp increase in mutation rates. This dose appears to be between 100 and 150 mg kg⁻¹. The protective mechanisms that operate at lower doses may manifest themselves before the chemical reaches the target cell, or within the target cell before damage to the chromosomes, or after damage to the chromosomes has been done (repair). There is, in fact, strong evidence that an effective protective mechanism operates after the chemical has reached the target cell. In a parallel study, R. B. Cumming (1) found effective ethylation of sperm heads at 50 mg kg⁻¹, and this dose yielded about 10 times fewer ethylations than 200 mg kg⁻¹. In the present genetic results, on the other hand, 50 mg kg⁻¹ yielded 46 times fewer translocations than 200 mg kg⁻¹. Thus, there was a much more pronounced drop with dose in the yield of genetic damage than would be expected from ethylation of the target cells.

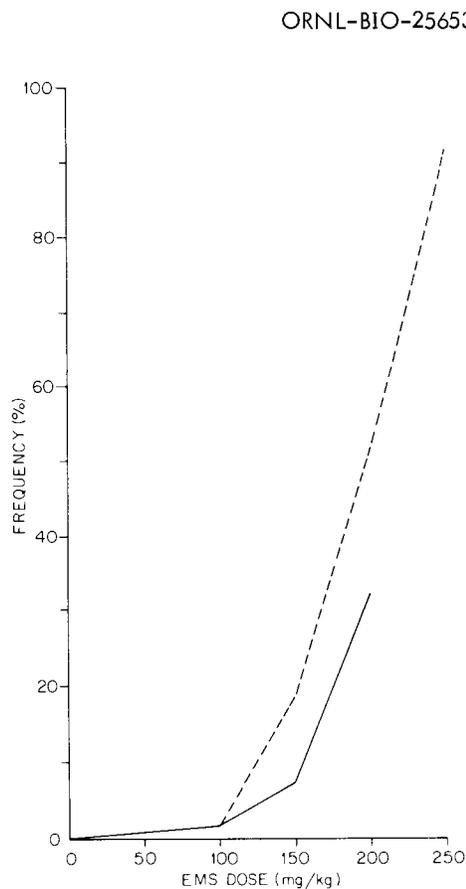


Fig. 1. Frequencies of translocations (solid line) and dominant-lethal mutations (broken line) induced in mice by various doses of EMS.

The nonlinearity of the EMS dose-response curves is in sharp contrast to that obtained for triethylenemelamine (2). The results for the two mutagens indicate that *in vivo* there is a very complex interaction between chemical and animal response, and that the type of interaction may vary from chemical to chemical. Therefore, it is necessary to study the dose-response curves for a wide variety of chemical mutagens.

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DOSE-RESPONSE STUDIES ON THE INDUCTION OF DOMINANT-LETHAL MUTATIONS IN POST-SPERMATOGONIAL STAGES OF MICE TREATED WITH TRIETHYLENEMELAMINE

B. E. Matter* and W. M. Generoso

Experiments on the dose-response relationships between dominant-lethal mutations and translocations induced by ethyl methanesulfonate (EMS) suggest that low doses of EMS are relatively ineffective, possibly because some physiological mechanism greatly reduces the degree of damage (1). Certainly it cannot be expected that the threshold problem and the shape of the curve will be the same for all chemicals. For a proper evaluation of the hazards to man, it is necessary, therefore, to study the dose effects of a wide variety of compounds. The present experiment was designed to study the effect of dose on the induction of dominant-lethal mutations in different postspermatogonial stages by triethylenemelamine (TEM). TEM was selected because, in many respects, its effects in the mouse are similar to those of radiation. Furthermore, TEM has been shown to induce dominant-lethal mutations and heritable translocations at very low doses, relative to the approximate lethal dose (2, 3), in postspermatogonial stages. This indicates that there might be a marked difference between TEM and EMS in the ratio of genetically effective dose to lethal dose.

Twelve-week-old (101 X C3H)F₁ male mice were injected intraperitoneally with various concentrations of TEM. The frequencies of dominant-lethal mutations were measured in germ cells treated in the early spermatozoal and mid-spermatid stages (4-1/2 to 7-1/2 and 11-1/2 to 15-1/2 days after treatment, respectively). These posttreatment intervals were selected on the basis of results of preliminary work with a dose of 0.2 mg kg⁻¹. The mid-spermatid stage was found to be most sensitive for dominant-lethal induction with TEM. Treated males were mated to B1H virgin females, 7-14 weeks old. Between 40 and 60 females per dose were killed for uterine analysis. The frequencies of dominant-lethal mutations were calculated as a reciprocal of the ratios of living implants in experimental and control animals.

The results (Fig. 1) indicate that TEM is extremely effective in the induction of dominant-lethal mutations in both stages studied. The mutagenic effect was already clearly detectable at 0.05 mg kg⁻¹. For early spermatozoa, the frequency increased with dose, being 1.2, 9.7, 20.8, 55.9, 80.7, and 91.9% for TEM doses of 0.035, 0.05, 0.1, 0.2, 0.3, and 0.4 mg kg⁻¹, respectively. For spermatids, the

frequencies were found to be slightly higher at corresponding doses — namely, 4.5, 15.1, 39.5, 73.2, 92.9, and 96.8%. At the dose of 0.035 mg kg^{-1} , there were slight increases over controls in the frequencies of dead implantations, both in the spermatozoa and spermatid groups — namely, 9.4 versus 4.9% in controls and 11.1 versus 5.5% in controls, respectively. However, it is questionable whether these increases represent dominant-lethal effects, since at these levels the inherent variability in the natural level of dead implantations prohibits accurate measurement of dominant-lethal effects. In contrast to EMS results, the TEM dose-response curve approaches linearity.

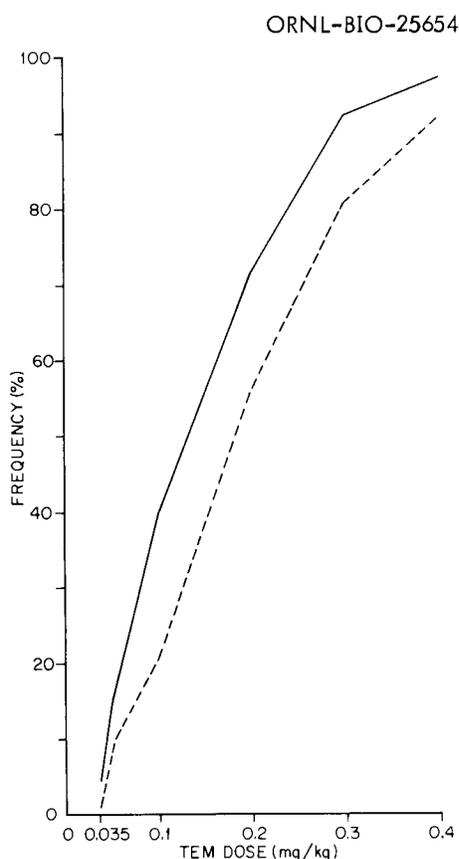


Fig. 1. Frequencies of translocations (solid line) and dominant-lethal mutations (broken line) induced in mice by various doses of TEM.

One interesting difference between TEM and EMS that has importance from a practical point of view is revealed when comparison is based on the ratio between the genetically effective dose and the lethal dose. For EMS, the lowest dose that kills 100% of the animals is approximately 525 mg kg^{-1} ; for TEM this dose is 5 mg kg^{-1} . The lowest genetically effective doses (as measured by dominant lethals) for EMS and TEM are 150 and 0.05 mg kg^{-1} , respectively, which yielded comparable frequencies of dominant lethals. Thus, the ratio of genetically effective dose to lethal dose for TEM is 1/100, while for EMS it is only 1/3.5. TEM, therefore, in contrast to EMS, is mutagenic far below the toxic level, and the protective mechanism that is apparent for EMS does not seem to exist for TEM. Obviously, these results have important implications for the practical problems

of evaluating mutagenic effects of chemicals at chronic levels. An experiment is now in progress to study effects of TEM dose in the induction of heritable translocations.

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GENETIC EFFECTS OF TRIETHYLENEMELAMINE IN MOUSE SPERMATOGONIA

B. E. Matter* and W. M. Generoso

It is generally known that certain alkylating agents are very effective in the induction of chromosomal breakage in late spermatogenic stages of the mouse. Very little is known, however, about this type of effect in the spermatogonial stage, which, in terms of hazards, is more important. It has been shown that doses of certain alkylating agents that produce high frequencies of chromosome breaks in postspermatogonial stages induce very low levels of specific-locus mutations in spermatogonia (1). For a comprehensive evaluation of genetic hazards, therefore, it is necessary to study the chemical induction of chromosome aberrations at the spermatogonial stage. Such an experiment with triethylenemelamine (TEM) has been initiated and will be reported in the future, TEM having been selected because of the similarity of many of its effects to those of X-rays. This compound has earlier been shown to induce cell killing and specific-locus mutations in spermatogonia (2). Preparatory to the chromosome aberration experiment, however, it has been necessary to obtain some measure of the action of different dose levels of TEM on spermatogonial cell killing and repopulation. In the present study, this has been accomplished indirectly by observing the induction of sterility and the length of time required for the recovery of fertility.

Twelve-week-old (101 X C3H) F_1 male mice were injected intraperitoneally with various concentrations of TEM, ranging from 0.1 to 2.0 mg kg^{-1} . Each male was caged with three T-stock females during the period 34 to 41 days after treatment and with a new batch of three T-stock females from 42 days after treatment until at least one female of the three was visibly pregnant. The first period gives information about differentiating spermatogonia, the second about spermatogonial stem cells. Fertility effects are shown in Tables I and II. The time required for recovery of fertility was scored on the basis of average delay in the appearance of the first litter.

Up to 0.80 mg kg^{-1} , TEM has no measurable effect on the fertility of males during either the first or the second period. At higher doses, clear effects were observed. During the mating period from days 34 to 41, the number of sterile males increased, while the number of pregnant females and average litter size decreased with increasing dose. For matings that started 42 days after treatment, sterile periods were ob-

served. These were of 7.0, 9.3, and 11.9 days in duration for doses of 1.2, 1.6, and 2.0 mg kg⁻¹, respectively. One presumed specific-locus mutant (s locus) was found among 3759 progeny. This mutant was recovered in the 1.2 mg kg⁻¹ dose group in a mating made following the sterile period.

TABLE I. Effects of TEM on fertility of males mated 34-41 days after treatment (differentiating spermatogonia)

Dose (mg/kg)	Males treated (No.)	Sterile males (No.)	Pregnant females (No.)	Avg. litter size per pregnant female
Control (HBSS)	20	0	55	6.5
0.10	19	0	54	7.2
0.20	20	0	49	6.4
0.40	20	0	51	6.9
0.80	20	1	45	5.2
1.20	20	2	32	6.1
1.60	20	4	25	3.8
2.00	17	10	11	3.6

TABLE II. Effects of TEM on fertility of males mated beginning 42 days after treatment (spermatogonial stem cells)

Dose (mg/kg)	Males treated (No.)	Pregnant females (No.)	Time after mating that first litter appeared (Avg. no. of days)	Length of sterile period (Avg., in days)	Avg. litter size per pregnant female
Control (HBSS)	19	54	19.8	—	6.2
0.10	19	56	19.8	—	6.7
0.20	20	60	19.7	—	6.6
0.40	20	57	20.3	—	6.6
0.80	20	59	21.6	1.8	6.2
1.20	20	56	26.8	7.0	6.5
1.60	20	56	29.1	9.3	6.6
2.00	17	49	31.7	11.9	7.4

The magnitude of the spermatogonial cell-killing effect of TEM, as measured by induced sterility, is in marked contrast to that found with X-rays. A TEM dose of 0.2 mg kg⁻¹, which induced about 50% dominant-lethal mutations in post-spermatogonial stages (3), showed no effect on fertility. On the other hand, 600r of X-rays, which also induced about 50% dominant lethals in postspermatogonial stages (4), resulted in a sterile period of at least 32 days (5). To illustrate the difference further, it should be noted that the dose of 2.0 mg kg⁻¹ induced only about 12 days of sterility. Experiments are now in progress to determine the length of the sterile period after doses of 3.0 and 4.0 mg kg⁻¹. In collaboration with Dr. Julian Preston of the Mammalian Cytogenetics group, treated spermatogonial stem cells are being analyzed cytologically at the spermatocyte stage for translocations. Results of the fertility and cytological studies will form the basis for the selection of the TEM dose to be used in studying induction of heritable translocations.

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A SEQUENTIAL PROCEDURE FOR THE DETECTION OF TRANSLOCATION HETEROZYGOTES IN MICE

W. M. Generoso and D. G. Gosslee*

The study of chemical induction of chromosomal aberrations in the mouse is important in view of the many human genetic diseases that result from chromosome anomalies of one type or another. So far, evaluation of chromosomal aberrations as genetic hazards of chemicals has been made primarily by using results of the dominant-lethal test in mice and rats and results of cytogenetic studies of somatic cells. These two test systems do not measure transmissible genetic effects, which are the most important mutagenic effects. Thus, there is a need for obtaining information on heritable translocations.

The conventional procedure for detecting translocation heterozygotes is to test F₁ male progeny of treated parents for sterility or semisterility by mating each one to three or more different females. Each female is opened at midpregnancy, and the numbers of living and dead implants are counted. Detection of sterile translocations is straightforward, but because of (1) the natural variation in the fertility of background females and (2) chance variations in the type of sperm used in fertilization, detection of semisterile males is more difficult. From the fertility data on three or more females, a semisterile male is expected to sire less than half as many living embryos, on the average, as a normal male, with an accompanying increase in the number of dead implants. This procedure for

detecting translocation males is obviously expensive and requires a good deal of animal handling and record keeping. The procedure described below reduces these problems; hence it is more suitable for wide-scale screening and experimentation.

Each F_1 male to be tested is caged with a female of the (SEC X C57BL) F_1 strain. The females are used beginning when they are about 12 weeks old up to the time when the twelfth litter is produced. If the first litter is large enough, the male is declared fertile and discarded immediately after the litter is scored. If the first litter is not large enough, a second litter is allowed to be produced. If the second litter size is large enough, the male is declared fertile and immediately discarded; otherwise, the male is suspect and is tested further by being mated to three virgin females, which are killed during pregnancy. In either case, another male is placed with the original female 1 week after the litter is born, and the same procedure is followed until the last male is added no later than after the tenth litter. Since the female is normally mated shortly after parturition, a maximum of three litters are used up per male — two litters if a male is declared fertile after siring his first litter and three litters if the decision is made after the second litter.

From the ethyl methanesulfonate translocation experiment (1), it was found that out of 462 litters sired by 98 confirmed semisterile males, only nine litters, which were sired by independent males, contained ten or more offspring, and only three contained eleven or more. The probability that a semisterile male will sire a litter of ten or more is then calculated as 0.0195, and for a litter of eleven or more it is 0.0087.

The key to this screening procedure is the exceptional fertility of (SEC X C57BL) F_1 females. Data on the fertility of these females mated with fully fertile males are shown in Table I. Analysis of the fertility data revealed that if a

litter of ten or more sired by a male in his first or second litter is used as the indicator of full fertility, an average of 5.27 males can be tested per female.

This average was calculated by considering all possible configurations of two and three litters required for males for each female. Since each configuration is not equally likely, the average is a weighted average. For each configuration, the number of males that can be tested is weighted by the chance that it will occur. There are 21 combinations of two- and three-litter periods that can test four, five, or six males. For example, six males can be tested in six periods of two litters, five males can be tested in one period of three litters followed by four periods of two litters, and four males can be tested in four periods of three litters. The chances of the three configurations are 0.293, 0.264, and 0.002, respectively. The chances are computed using the last column in Table I, which gives the probability that a fertile male will be declared fertile and thus require only two litters. The three configurations above can be combined with the other 18 and grouped according to the number of males that can be tested. The estimates of the proportions for four, five, and six males that can be tested are 0.0225, 0.6845, and 0.2930, respectively. The weighted average of four, five, and six, using the above proportions, is 5.27.

Thus, a large percentage of the males can be declared fertile by the simple procedure of counting the number of live births in at most two litters, with a small risk of declaring a semisterile male as fertile. Since (SEC X C57BL) F_1 females normally produce twelve good litters, it is now possible to test several F_1 males per female instead of killing three or more females per F_1 male as in the old procedure. A translocation induction experiment that uses the procedure described above and in which litter size of ten or more is used to detect full fertility is now in progress.

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1. W. M. Generoso, Sandra W. Huff, and Sandra K. Stout, this report, p. 93.

TABLE I. Reproductive performance of (SEC X C57BL) F_1 females mated with normal males

Litter generation	Mean size	Total litters	Number < 10	Number > 9	Proportion with > 9
1	9.15	970	488	482	.50
2	11.21	970	120	850	.88
3	11.42	970	111	859	.89
4	11.35	970	143	827	.85
5	11.92	71	2	69	.97
6	11.56	71	8	63	.89
7	11.73	71	6	65	.92
8	11.86	71	8	63	.89
9	11.31	71	11	60	.84
10	10.86	71	15	56	.79
11	10.36	69	19	50	.72
12	9.29	68	31	37	.54

DOSE IN MAMMALIAN CHEMICAL MUTAGENESIS

R. B. Cumming and Marva F. Walton

Adequate measurement of dose is essential both to an understanding of the relationship of dose to effect and to a comparison of the relative sensitivities of different test systems to a particular mutagen. That is to say, there is no real study of mutagenesis in the absence of a consideration of dose. In addition, the comparative study of radiation-induced mutations with those induced by other agents requires meaningful dosimetry both of the radiation and of whatever other agent may be involved. The dosimetry of chemical agents in mutagenesis is an important and neglected subject. We have approached this problem by attempting to measure the dose of alkylating agents in the mouse in terms of the amount of alkylation of biological targets at the molecular level with various administered doses.

The first studies of molecular dosimetry are in progress with ethyl methanesulfonate (EMS), an ethylating agent. The results to date allow the dose to be converted from mg kg^{-1} injected intraperitoneally to ethylations per cell or ethylations per nucleotide in specific germ cell types. We are thus able to express the relationship between the yield of dominant-lethal mutations, heritable translocations or specific-locus mutations and ethylations per nucleotide in the germ cells involved. These data — genetic events per ethylation per nucleotide in the target DNA — can be compared directly with similar data that are becoming available for other organisms (1, 2).

To do these experiments, EMS labeled with ^{14}C in the ethyl group was injected into male (C3H X 101) F_1 mice at doses ranging from 50 to 250 mg kg^{-1} . The animals were killed at various times after injection, and spermatozoa were isolated from the *vas deferentia* by centrifuging them through Ficoll solutions. Some of the EMS-treated animals had been given tritiated thymidine about 1 month before treatment with the mutagen, so that the treated spermatozoa were ^3H -labeled in the DNA. The resulting double-labeled sperm cells were divided into two aliquots. In the first, whole sperm heads were solubilized and counted to give dpm of each isotope per cell. From a second, larger aliquot, DNA oligonucleotides were extracted and counted to give the amount of ^{14}C label covalently bound to the DNA. The technique is similar to that used by Segal and Lee (1, 2) for *Drosophila*.

Ethylation of mouse sperm DNA proceeds for at least 4 hr after intraperitoneal injection of EMS. The number of ethylations resulting to mature sperm DNA in the mouse from such injection is about ten times as high from an administered dose of 200 mg kg^{-1} as from a dose of 50 mg kg^{-1} . The dose-action curve is not linear, and it requires very many ethylations ($\sim 1 \times 10^5$ per cell) to produce measurable genetic damage.

The measurement of ethylations per nucleotide produced by EMS in mature sperm of the mouse allows a direct comparison of the relative sensitivity of this germ cell stage in the mouse and in *Drosophila*. The *Drosophila* data come from the laboratory of W. R. Lee of Louisiana State University. It turns out that *Drosophila* fed EMS by the method of Lewis receive a dose to mature sperm that is about 1000 times higher than that received by the mouse injected with 200 mg kg^{-1} , when both doses are translated to ethylations per nucleotide. The mouse is clearly much more sensitive to genetic damage by EMS ethylation when this genetic damage is expressed in terms of equal molecular dose.

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GENETIC EFFECTS OF CYCLOPHOSPHAMIDE IN THE GERM CELLS OF MALE MICE

R. B. Cumming

Cyclophosphamide (Cytoxan, Endoxan) is a nitrogen mustard derivative developed by Arnold and his colleagues (1) in 1958 and introduced by them in that year as a new type of cancer chemotherapeutic agent. This compound is entirely inactive *in vitro*, but it is metabolized in higher organisms, particularly by the liver microsomes of mammals, to an active alkylating intermediate which is cytotoxic, breaks chromosomes, and is mutagenic. The alkylating intermediate is selective in its attack, in such a way that this drug is very effective against some types of tumors but shows relatively low toxicity to the host organism. Cyclophosphamide is widely used to combat some types of neoplasia in humans and also for certain other medical procedures.

This compound is generally nonmutagenic in microorganisms and fungi, but it has been shown to cause chromosome aberrations in higher plants (2). It also breaks somatic chromosomes in the mouse *in vivo* (3). Röhrborn (4) showed that it produces sex-linked recessive-lethal mutations in *Drosophila*, and Brittinger (5) presented evidence that it produces dominant-lethal mutations in all stages of spermatogenesis in the mouse. In this latter organism, we have in progress a small specific-locus experiment and a translocation induction experiment. We are also examining the effect of butylated hydroxytoluene (BHT) pretreatment on the mutagenicity of this compound in mice.

The specific-locus mutation experiment and the translocation experiment involve F_1 offspring of hybrid males that were treated with cyclophosphamide at 350 mg kg^{-1} . In the 3642 offspring obtained from germ cells treated in post-spermatogonial stages, three specific-locus mutations were observed. To date, no specific-locus mutations have been obtained in the 1768 offspring that resulted from treated spermatogonia. Thus, it is clear that cyclophosphamide does induce specific-locus mutations in post-spermatogonial germ cell stages, but the evidence is inconclusive for spermatogonia.

Table I shows results to date on translocations induced by cyclophosphamide. Of the 507 F_1 males tested from treated post-spermatogonial cell stages, 97 are presumed translocations. The process has been started of verifying the presumed translocations cytologically, and all of those examined to date have been confirmed. A higher overall percentage of translocations occurs in the animals pretreated with BHT, but a day-by-day comparison indicates that BHT potentiates

TABLE I. Offspring tested for cyclophosphamide-induced translocations

Cell stage	Pretreatment	No. of offspring tested				Presumed translocations (%)
		Fertile	Partially sterile	Sterile	Total	
Postspematogonial	None	241	33	8	282	14.5
	BHT 0.75 %	169	41	15	225	24.9
Spermatogonial	None	70	1	3	74	5.4

translocation induction by this compound in sperm but protects against translocation induction in spermatids. Only a small number of offspring derived from treated spermatogonia have been tested, but it is evident that some translocations are induced in this cell stage that are transmitted. Thus, the compound does have some effect on the germ cell stage that is the most critical from the standpoint of human hazard.

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PATHOLOGY AND IMMUNOLOGY SECTION

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Mammalian Radiation Recovery

C. C. Congdon

Radiation Recovery of Hemopoietic Cells

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Experimental Hematology

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Radiation Immunology and Senescence

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Hematopoietic Regulation

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Cellular Immunology

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Histology and Autoradiography

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Mammalian Radiobiology

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IMMUNE STATUS IN THYMECTOMIZED
 RADIATION CHIMERAS

C. C. Congdon, Helen S. Payne, and N. Gengozian*

 SECONDARY DISEASE (HETEROLOGOUS).
 DESIGN VIII

C. C. Congdon, T. J. Mitchell,* and D. A. Gardiner*

A series of experiments has been designed in an attempt to determine conditions that minimize mortality due to secondary disease in mice lethally irradiated and then injected with rat bone marrow. Preliminary analysis of the 90-day mortality data has been made on the main part of the design. This covers four age levels of donor marrow (15, 30, 45, and 60 days) and six levels of cell dose (25.5, 32, 40, 62.5, 78.1, and 97.7×10^6 cells). The experiment was restricted to males kept in an unlimited filter-top environment, and the time of injection was held at 1 day after irradiation. Previous experiments in this series had indicated that lowest mortality could be achieved under these conditions.

The design was constructed to permit a precise fit of a quadratic response function in the variables "age of donor" and "log cell dose." The statistical analysis indicated, however, that a model representing the 90-day mortality as a simple linear function of log dose was suitable over the range of the two factors considered. Under this model, the 90-day mortality decreases with increasing cell dose, implying that it will be necessary to extend the range of dose still further before we are able to identify an optimum.

Linear and quadratic terms in age of donor were statistically significant in one replicate of the experiment but not in the other, nor were they significant when the two replicates were considered together. The age factor remains extremely difficult to pin down. Unfortunately, each marrow pool is necessarily associated with only one age of donor, and the variation in response from one pool of donor marrow to another is quite large.

The possibility of a change in the dose effect from one age to another has also been investigated. The statistical significance was borderline (significant at the 0.10 level) and appeared to result from the difference between a very weak dose effect at the two intermediate donor ages (30 and 45 days) and a strong dose effect at ages of 15 and 60 days.

Although there are still some unresolved questions, one of the goals of this study, which was to achieve a relatively low mortality, has been attained. At the two highest levels of cell dose, for example, only 22.5% (18 out of 80) of the mice were dead at 90 days. This compares favorably with 90-day mortalities of 65-90% in earlier work with rat marrow transplants.

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The cellular and humoral immune states and the anatomical condition of lymphatic tissue of thymectomized radiation chimeras were evaluated at monthly intervals. These animals serve as controls for thymectomized radiation chimeras injected with *Mycobacterium leprae*. Three-month-old adult B6C3F₁ mice were thymectomized. Ten days later, they were exposed to 950 r total-body X-radiation and injected intravenously with 5×10^6 syngeneic bone marrow cells. Allogeneic tail skin grafts were used to measure cellular immunity, and rat erythrocytes were used as antigens to measure humoral immunity. The anatomical condition of the lymph nodes and spleen was examined for gross and cellular changes. Normal mice were used as controls for this study.

Body, lymph node, and spleen weights were substantially lower in thymectomized radiation chimeras than in normal control mice throughout the 6-month period of study. The thymectomy lesion involved loss of lymphocytes from the thymus-dependent region of the spleen white pulp and from lymph-node cortex throughout the 6-month interval. Delayed rejection or persistence of skin allografts and a defective primary humoral antibody response to rat erythrocyte antigens were observed throughout the 6-month interval in chimeras, as compared with normal mice. Occasionally, experimental animals developed an extreme wasting disorder, with death occurring when the body weight reached 13-15 g. It is concluded that for the 6-month period after thymectomy, irradiation, and injection of syngeneic bone marrow cells, the immunological responsiveness is substantially reduced.

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 THE INFLUENCE OF DONOR AGE ON BONE MARROW
 TRANSPLANTATION IN MICE

R. E. Toya, M. L. Davis,* and C. C. Congdon

The functional changes in the hemopoietic organs of donor mice with age have received little attention until recently. The immune status of animals has been shown to deteriorate with age, and experiments have been designed to explore the effect of age on the induction of secondary disease and the ability of aged marrow to provide active hemopoiesis.

Lethally irradiated mice were injected with allogeneic bone marrow. Secondary disease was assayed by 90-day mortality and histologic evaluation of the recipient spleen, and hemopoiesis was studied by gross and microscopic spleen colony assay.

Graft-versus-host disease decreases as a function of age, unless the allogeneic donors are pretreated with recipient antigens. Pretreatment of the donors 21 days prior to

use eliminated the age-associated decrease in secondary disease. Gross and microscopic colonies of hemopoiesis were not observed in the recipient spleen 6 days after transplantation of allogeneic marrow.

Lethally irradiated mice receiving syngeneic marrow from donors of various ages were used to assay the donor marrow for its hemopoietic potential. The gross or macroscopic spleen colony-forming unit (CFU) increased to a peak at about 12 months of age and then steadily declined through the remainder of the life-span of the donor animals. The total microscopic CFU's followed the same pattern until late in life, when an increase in CFU's was observed. Microscopic evaluation of the colonies did not reveal a shift in the differentials. The number of nucleated cells per femur in the donor animals was found to increase with age, nearly doubling between 4 and 35 months of donor age. The data indicate that with increasing mouse age, the number of nucleated cells in the femoral marrow increases; however, the capacity for this marrow to provide active hemopoiesis and graft-versus-host reactive cells is depressed.

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ALTERED LIPID AND SERINE METABOLISM IN GRAFT-VERSUS-HOST DISEASE

R. E. Toya

Alterations in lipid and serine metabolism in the liver may be associated with graft-versus-host disease. The test system chosen to explore this association was the parent-to-F₁ foreign spleen reaction.

The total lipids in the liver were extracted and separated by thin-layer chromatography. Respiration pattern analysis of L-3-¹⁴C-serine and unlabeled formate was made. Total lipids in the liver increased to maximum values 6 days after treatment. Thin-layer chromatography, at this time, revealed an increase in triglycerides and a decrease in phosphatidyl serine. Six days after treatment with parent spleen cells, the respiration pattern of L-3-¹⁴C-serine showed an increase in serine metabolism. The respiration pattern of labeled serine could not be altered by the simultaneous injection of unlabeled formate.

These data indicate that accumulation of lipids in the liver of mice undergoing an acute graft-versus-host disease is probably the result of an increase in serine metabolism, which in turn causes a decrease in phosphatidyl serine. The lack of effect of unlabeled formate on the rate of transformation of labeled serine into ¹⁴CO₂ implies a defect in l-C metabolism, possibly because of alteration in the function of tetrahydrofolic acid.

THE EFFECT OF HYDROXYUREA AND VINBLASTINE ON PROLIFERATING CELLS IN THE SPLEEN

A. L. Kretchmar* and W. R. Conover†

To investigate mechanisms controlling the rate of proliferation among hemopoietic precursor cells, normal bone

marrow cells were injected intravenously into lethally irradiated, isologous C3H mice, and the sensitivity of proliferating cells in the spleen to hydroxyurea and vinblastine was measured by retransplantation. Various drug doses were investigated at several times after transplantation. Four hours after transplantation, hydroxyurea killed 41% of the spleen colony-forming potential, whereas at 4 days, 60% was killed. This suggested a change in mean generation time among the hemopoietic precursor cells. At 4 days, the sensitivity to various levels of hydroxyurea showed a characteristic relation to dose; no effect on colony-forming potential was noted until a dose of 20 mg kg⁻¹ was reached, and no further loss beyond 60% was found at doses higher than 100 mg kg⁻¹. The results are consistent with evidence from other laboratories that hydroxyurea kills cells in the S phase of the generation cycle and prevents G₁ cells from entering S.

The sensitivity of colony-forming potential to various levels of vinblastine shows a different relation to dose. The percentage of colony-forming potential killed shows a progressive increase to more than 90% as the dose is increased from 5 to 60 µg. The recovering endogenous spleen colony-forming potential in sublethally irradiated mice not given bone marrow cells also undergoes a change in sensitivity between 1 and 4 days. One aspect of the control of the rate of proliferation among hemopoietic precursor cells appears to be a variable generation time.

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DISTRIBUTION OF COLONIES IN SPLEENS OF LETHALLY IRRADIATED MICE GIVEN ISOLOGOUS BONE MARROW CELLS

K. L. Mossman,* W. R. Conover,† and A. L. Kretchmar‡

In order to investigate the underlying mechanisms involved in the spleen colony assay for hemopoietic stem cells, we investigated the statistical distribution of colonies in assay mice. It was found that the number of cells which transplant and form colonies does not follow a Poisson distribution. This appears to be true for spleen cell suspensions as well as bone marrow suspensions. The data are more closely distributed about the mean colony count than is predicted by Poisson statistics. The explanation for this deviation from expectation is being investigated in current experiments.

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RADIOPROTECTION BY PHENYLHYDRAZINE

L. H. Smith and T. W. McKinley, Jr.

Phenylhydrazine belongs to a class of biostimulant radioprotectors that differ from chemical protectors such as mercaptoethylguanidine and cysteine, in that the latter agents must be present at the time of irradiation to be effective. Biostimulant protectors administered one to several days before irradiation appear to confer protection by altering cell populations. In the case of phenylhydrazine, the two most likely cell populations involved are those of the hemopoietic and reticuloendothelial systems, since both are stimulated by the drug. Our primary goal has been to determine the basis for radioprotection by phenylhydrazine and, more specifically, to relate changes in hemopoiesis resulting from its administration to changes in whole-body radiosensitivity of mice. Some of the studies also relate to the hypothesis that radioprotection by this drug is due to an increase in the number of hemopoietic stem cells at the time of irradiation.

Phenylhydrazine is radioprotective only when injected prior to irradiation; and when it is given 7, 8, or 9 days before exposure, the dose reduction factor, based on the LD₅₀/30 is about 1.2. When it is given 3 days before exposure, the LD₅₀/30 is considerably reduced, indicating a radiosensitization. Our data show that sensitization results from persistent, induced anemia, because the LD₅₀/30 can be restored to normal by transfusion of isogeneic erythrocytes.

Dose studies show that (1) the individual dose providing the best protection (3.0 mg, or about 100 mg kg⁻¹) is not within the lethal range for the drug; (2) radioprotection is linear with log dose over the range 0.5 to 3.0 mg; and (3) multiple injections are protective, but no more so than a single injection.

Life-span data for phenylhydrazine-protected mice indicate that radioprotection against acute hemopoietic death is not temporary, although animals die according to the radiation-induced life-shortening processes that would be anticipated to occur in proportion to the X-ray exposure. Thus, the drug probably does not protect against late effects of irradiation, such as tumor induction and nephrosclerosis.

Hemopoietic recovery in irradiated (850 r) mice given phenylhydrazine 7 days before exposure begins about 2 weeks after exposure. Indices measured were peripheral erythrocyte and spleen ⁵⁹Fe uptake and endogenous spleen colony formation. Other than the fact that regeneration was somewhat slow, these results were expected, since hemopoietic recovery must occur in order for mice to survive.

The hemopoietic status of animals at the time of irradiation could be an important factor in determining radiosensitivity, and since phenylhydrazine alters this status, we studied its effect on several parameters of hemopoiesis in normal mice. An important observation was an increase in the total number of spleen colony-forming cells in the spleen but not the bone marrow throughout the period of radioprotection. However, the greatest increase in colony-forming cells from the spleen occurred on day 5, not on days 7, 8, or 9, when protection was maximum. Thus, either colony-forming cells and hemopoietic stem cells are synonymous, but radioprotection by phenylhydrazine is not the result of an

increased number of these cells at the time of irradiation, or the stem-cell radioprotection hypothesis is correct, but colony-forming cells and hemopoietic stem cells are not identical under all conditions.

THE COMBINED ACTION OF LACTIC DEHYDROGENASE VIRUS AND RAUSCHER LEUKEMIA VIRUS ON MOUSE LYMPHATIC TISSUE

M. R. Proffitt*

The lactic dehydrogenase virus (LDV) is known to be an unwanted contaminant in many transplantable tumors, as well as some pools of other animal viruses, including the much-studied Rauscher leukemia virus (RLV). Although LDV has shown conclusive evidence of being oncogenic or even pathogenic, it does induce a rather dramatic hyperplasia of lymphatic tissue germinal centers. These facts, coupled with evidence that germinal center cells may be targets for C-type viruses such as RLV, prompted us to determine whether and how the presence of LDV might influence the expression of the diseases induced by contaminated virus pools.

Mice inoculated with animal-passaged RLV contaminated with LDV showed early gross and histological changes in their lymphatic tissues, comparable to those in mice inoculated with LDV alone. The changes closely resembled those that have been ascribed to stress, which are mediated by corticosteroid hormones. Except for early erythroblast activation, mice inoculated with a pool of animal-passaged RLV derived from cell culture and not contaminated with LDV did not undergo these changes. Most notable were the absence of early lymphocyte destruction in thymus-dependent areas responsible for cell-mediated immunity and the lack of significant immunoblast activation and proliferation in lymphoid germinal centers (humoral immune compartments).

The presence of LDV in RLV pools or its addition to the uncontaminated pool resulted in an enhancement of the "erythroblastic reaction" associated with Rauscher disease, as indicated by an increase in spleen weight. There was also prominent diffuse and persisting hyperplasia of germinal center cells (immunoblasts and immunocytes) after LDV was added to the uncontaminated RLV pool. Furthermore, the presence of the LDV enhanced the immunosuppressive properties of RLV.

Two possibilities were considered to account for the enhancement of Rauscher disease by LDV. First, by stimulating germinal center hyperplasia, the LDV could supply greater numbers of target cells for RLV replication. Second, the depression of the cell-mediated immune response by LDV could prevent the inactivation of RLV particles or tumor cells by lymphocytes.

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EFFECT OF TOLERANT THYMOCYTES ON LONG-TERM SURVIVAL OF PARENT-TO-F₁ RADIATION CHIMERAS

Joan Wright Goodman and Nancy L. Basford

Although parental (P) thymocytes given intravenously to irradiated hybrid (F₁) recipients of P bone marrow (i.e. P → F₁ chimeras) considerably augment early growth of the marrow, 90-day survival of such chimeras is very poor (1). In the present study, we have investigated the effect of tolerant thymocytes — incapable of graft-versus-host (GVH) or graft-versus-graft (GVG) activity — on survival of P → F₁ chimeras.

B6D2F₁ hybrids were exposed to 900 rads of whole-body X-radiation. After 22–24 hr, they were given intravenously 10⁶ B6 bone marrow cells and 10⁸ thymocytes either from normal B6 or D2 mice or from chimeric mice of one of the following constitutions: B6 → B6, B6 → B6C3F₁, B6 → B6D2F₁, or D2 → B6D2F₁. Thymocytes from these chimeras were serotyped and shown in every case to be of marrow donor origin (i.e. parental). Simonsen assays (2) of portions of thymus cell suspensions revealed that chimeric thymocytes were specifically tolerant to the host but immunologically reactive against other alloantigens.

Long-term (90-day) survival results of four individual experiments are shown in Fig. 1. The curves in Fig. 1a show

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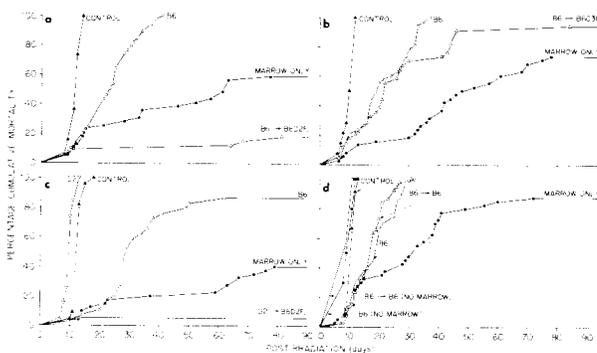


Fig. 1. Cumulative mortality data from irradiated B6D2F₁ mice given 10⁶ B6 marrow cells and/or 10⁸ thymocytes from untreated normal donors or from the following chimeras: (a) B6 → B6D2F₁ (specifically tolerant of host), (b) B6 → B6C3F₁ (not tolerant of host), (c) D2 → B6D2F₁ (specifically tolerant of donor and host), (d) B6 → B6 (not tolerant of host).

that when B6 thymocytes are incapable of GVH and of GVG, they improve long-term survival of B6 → B6D2F₁ chimeras. Thymus cells from normal (untreated) B6 donors, on the other hand, increase the mortality rate relative to that of marrow-only recipients. The curves in Fig. 1c and d show that B6 thymocytes from B6 → B6 or from B6 → B6C3F₁ chimeras, which are capable of GVH activity (i.e. not specifically tolerant of B6D2F₁ host), behave like B6 cells from normal donors in accelerating death from secondary disease. D2 thymocytes from normal donors, capable of both GVH and GVG, produce a mortality curve similar to that of radiation controls (Fig. 1c). Cells from D2 → B6D2F₁ chimeras, on the other hand, specifically tolerant of both host and marrow donor, considerably improve survival.

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AUGMENTATION OF HEMOPOIESIS IN MARROW-PLUS-THYMOCYTE CHIMERAS. DEPENDENCE ON AGE OF THYMUS DONOR

Joan Wright Goodman and Sarah G. Shinpock

Because of the changes in size and immunological importance of the thymus with age, we thought it of interest to study the effect of age of donor on the ability of thymocytes to augment hemopoiesis in radiation chimeras. Heavily irradiated, 3- to 4-month-old B6D2F₁ males were given intravenously 10⁶ B6 bone marrow cells plus 10⁸ B6 thymocytes from 12-week-old donors. Male and female donors were pooled in most cases from more than a single birth date, as indicated by horizontal bars along the abscissa of Fig. 1.

A week after transplantation, 0.5 μCi ⁵⁹Fe was injected intravenously into each chimera, and 24-hr uptake into circulating erythrocytes was determined. The average 24-hr ⁵⁹Fe uptake value of recipients of marrow only was arbitrarily set at 1.0, and the other data — those of recipients of marrow plus thymocytes — are expressed in values relative to that unit.

The data from a single experiment are shown in Fig. 1.

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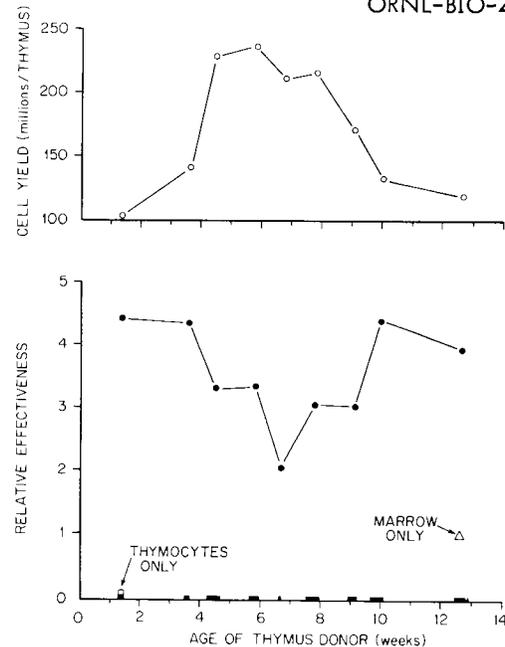


Fig. 1. Effect of age of donor on ability of 10⁸ B6 thymocytes to augment erythropoiesis in irradiated B6D2F₁ females given 10⁶ B6 marrow cells. Top values give thymus yields, bottom values relative effectiveness of thymocytes. Each bottom value represents 17–24 mice; each top value represents duplicate counts on a single suspension made from 20 or more thymuses.

There is a definite decrease in effectiveness of thymocytes from 5- to 8-week-old donors, as compared to other donor age groups. These findings confirm two earlier experiments, each of which tested mice of four different ages between 1 and 13 weeks, and which showed a similar decrease in effectiveness of 5- to 8-week-old thymocytes. All three experiments showed 12- to 13-week-old thymocytes to be slightly less effective than those from donors 9-10 weeks old. The ability of thymus cells to augment erythropoiesis was inversely related to the cellular yield, as Fig. 1 shows. Corresponding relationships were found in the other two experiments not plotted here.

Several thymuses from B6 donors of different ages were taken for histologic examination. No obvious differences in cellular populations were seen among 5-, 10-, and 15-week-old organs. This confirms Metcalf's (1) finding of a fairly constant cortex-to-medulla ratio (9:1) that declines only in relatively old mice.

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MARROW PLATING EFFICIENCY IN VIVO. AUGMENTATION BY PARENTAL THYMOCYTES

Linda L. Pritchard* and Joan Wright Goodman

The growth of C57BL bone marrow (measured by ^{59}Fe uptake by red blood cells and spleen) in lethally irradiated (C57BL X C3H) F_1 mice can be improved by concomitant transplantation of thymus cells. This thymus effect is specific: C3H thymus does not augment marrow growth, while F_1 thymus gives only marginal augmentation (1). Increased hemopoiesis in the presence of thymus may occur in one of at least two different ways. First, thymus may increase the "plating efficiency" of colony-forming units (CFU) in the marrow transplant, allowing a greater number of precursor cells to proliferate into hemopoietic clones. Second, thymus may increase the growth rate of extant hemopoietic clones, resulting in a greater number of progeny cells per clone at the time of assay.

Till and McCulloch's (2) gross nodule technique provided the means to test the first possibility directly. (C57BL X C3H) F_1 female mice, 12-16 weeks old, were exposed to 900 r X-irradiation. After exposure, some mice received both bone marrow and thymus cell transplants from C57BL females, while others received just bone marrow, just thymus, or just phosphate-buffered saline (PBS). Cells were transplanted by intravenous injection of a known number of live (determined by eosin exclusion) nucleated cells suspended in PBS. Bone marrow cell doses ranged from 0.4 to 1.0×10^6 , and thymus doses were 20-40 times the marrow dose. Six

to ten days after marrow transplantation, animals were killed by cervical dislocation, and their spleens were removed and fixed in Tellyesniczky's solution for at least 24 hr. Grossly observable splenic nodules were counted under a dissecting microscope. Spleens from animals receiving both marrow and thymus had roughly twice as many nodules as spleens from animals receiving only marrow. When no marrow was given, nodules almost never appeared in the spleen. These results indicate clearly that thymus does in fact improve the CFU "plating efficiency" of C57BL marrow in F_1 recipients. Whether the growth rate also increases in the presence of thymus remains to be seen.

This increase in CFU "plating efficiency" in the spleen probably does not occur at the expense of hemopoiesis at other sites. Iron uptake studies show that while splenic hemopoiesis is increasing by several hundred percent, hemopoiesis in bone marrow may increase by as much as 50%.

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RELATIVE EFFECTIVENESS OF THYMOCYTES FROM IRRADIATED DONORS ON HEMOPOIESIS IN PARENT-TO- F_1 CHIMERAS

F. A. Salinas* and Joan Wright Goodman

The ability of thymocytes to augment hemopoiesis in P \rightarrow F_1 chimeras (heavily irradiated B6D2 F_1 recipients of B6 bone marrow) has been shown to depend on the age of thymus donors, within the limits of 1 week to 3 months of age (1). Although a preliminary histologic examination of thymuses taken from 5-, 10-, and 15-week-old mice revealed no differences in cortex-to-medulla ratio, it was reasoned that the age-dependent changes in augmentative capacity reflected alterations in concentration of the effective cell type in the organ during development.

We have investigated the effectiveness of thymocytes from mice 72 hr after whole-body exposure to 200 r X-radiation, at which time the cellular population of the thymus has been dramatically changed by the irradiation. From such studies we hope to gain more information about which cell type is responsible for the augmentative effect.

To assay the effectiveness of thymocytes from irradiated and normal donors, the spleen colony technique of Till and McCulloch (2) was used. B6D2 F_1 mice were heavily irradiated (900 r) and given 5×10^5 B6 marrow cells plus thymocytes intravenously. Eight days later, spleens were removed and fixed in Tellyesniczky's fluid. The following day, macroscopic colonies were counted.

Results of three experiments are shown in Table I. It is clear that in all three experiments and at every thymocyte dose, cells from irradiated donors were more effective than those from normal (untreated mice). The difference was almost twofold in two cases (experiments A and C) and was smaller in the third (B). The control level — i.e. the number of nodules produced by 5×10^5 marrow cells alone — for females (C) was around half that for males (A and B), a finding that confirms data of McCulloch and Till (3), who used a different parent- F_1 hybrid combination. In all three experiments, when thymocytes were taken from irradiated donors, at least twofold augmentation of hemopoiesis was produced at a ratio (thymocyte to marrow cells) of 8:1. This is a much smaller value than the lowest ratio (40:1) at which augmentation can be measured by the ^{59}Fe -uptake method.

TABLE I. Splenic colonies in heavily irradiated B6D2F₁ mice given 5×10^5 B6 marrow cells plus B6 thymocytes

Expt. (sex of host & donor)	Thymocyte cell dose (millions)	Thymocyte donor	
		Normal (mean no. colonies \pm S. E.)	Irradiated*
A (σ)	0	9.7 \pm 1.13	
	4	15.5 \pm 0.87	27.9 \pm 1.43
	8	24.5 \pm 1.39	31.5 \pm 1.51
	16	24.9 \pm 0.56	41.8 \pm 1.61
B (σ)	0	9.1 \pm 1.06	
	4	11.3 \pm 1.47	17.1 \pm 1.72
	8	22.3 \pm 1.20	23.2 \pm 1.15
	16	25.6 \pm 2.67	32.4 \pm 0.64
C (♀)	0	3.7 \pm 0.67	
	4	6.9 \pm 0.95	12.8 \pm 1.04
	8	13.4 \pm 1.26	22.2 \pm 1.08
	16	17.0 \pm 1.01	31.5 \pm 0.75

*Exposed to 200 rads whole-body X-irradiation 72 hr before thymocytes taken.

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1. J. S. Goodman and S. G. Shinpock, this report, p. 104.
2. J. E. Till and E. A. McCulloch, *Radiat. Res.* 14, 213 (1961).
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CELL SIZE DISTRIBUTION IN THE THYMUS

F. A. Salinas,* L. H. Smith, and Joan Wright Goodman

Different approaches to the relationship between thymocytes and the immune response or erythropoiesis have been reported. Comparatively little is known, however, about morphophysiological aspects of thymocytes. The purpose of this study was to examine possible changes during development and aging in the cell size distribution of C57BL/6 mouse thymocytes, using a Model B Coulter Counter attached to a Model J plotter.

Cell size distribution analysis was made of cell suspensions from thymus donors in the age range of a few hours to 84 weeks. The results show that adult (10- to 12-week-old) thymus cells have two major components. The first population has an average mean cell size of about $110 \mu^3$ (5.9μ diameter), and the second, which is the predominant population, around $290 \mu^3$ (8.0μ diameter). The pattern appears to be dependent on the age of the thymus donor. In the earliest stages after birth there is a predominance of the smaller cell size, suggesting that major histologic changes occur between birth and early adulthood.

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DIAGNOSTIC LABORATORY PROGRAM

P. C. Estes, C. B. Richter, J. A. Franklin, and F. S. Shults

The testing laboratory and diagnostic facility have continued to monitor the health status and the environment of the experimental animals in the Biology Division. Approximately 1500 routine tests are performed to fulfill various diagnostic demands within the Division. Special emphasis has been given to disease problems encountered by individual investigators in their experimental animal populations.

Latent and acute infections in laboratory animals from commercial sources continue to be a problem during experiments. Continuous monitoring of in-house production and retired breeders from commercial sources is essential in order to recognize and control such infections. Our efforts to eliminate *Pseudomonas* from our experimental animals has resulted in almost complete elimination of early deaths due to this cause in radiation experiments. Some specific problems encountered during the past year were *Salmonella*-contaminated feed, Tyzzer's disease outbreak in experimental rabbits, staphylococcus infections in SPF mice, demodectic mange in hamsters, and various outbreaks of respiratory infections in mice, rats, and hamsters, caused by latent and acute contagious infections with murine respiratory viruses and mycoplasma.

A method for microorganism decontamination of large areas such as animal quarters and laboratories has been adopted and successfully tested. The method utilizes paraformaldehyde vapors and is available for use when the need arises.

Initial steps were taken to reduce the ectoparasite load in our common animal facility through the use of dichlorvos (Vapona) in animal rooms. This program offers promise for reducing the total parasite load on experimental animals. Methods for mass internal deparasitization are now under consideration. LD_{50/30} radiation experiments are now being used as endpoints in studies on cage population size, to determine more economical methods of animal care without jeopardizing current high standards.

CONTINUING STUDIES ON ACUTE SPONTANEOUS LEUKEMIA OF RATS

C. B. Richter, P. C. Estes, and R. L. Hendren

Continuing studies on acute leukemias of young rats further strengthen the concept that this may be an important model for acute human leukemias. Previously considered as lymphocytic, these are now regarded as poorly differentiated stem cell leukemias (1), a common type in children. Serologic examination indicates that rats bearing long-term solid forms of the tumor possess none of the group-specific antibodies commonly seen in mouse leukemias. Further studies under electron microscopy show that no overt viruses are associated with the rat leukemias. These findings also parallel those for humans.

Sinusoidal histiocytosis in regional lymph nodes has been seen in some long-term tumor bearers, which suggests that further studies are necessary to determine whether this type of reaction plays a role in ultimate tumor rejection, as is the case with human breast carcinoma. Some long-term tumor bearers eventually succumb, while others reject the tumor completely. Experiments to correlate this behavior with lymph node sinusoidal histiocytosis are now in progress.

Basic biochemical properties are currently under investigation by several Biology Division staff members.

1. C. B. Richter, P. C. Estes, and R. W. Tennant, in preparation.

INTRINSIC CELLULAR DEFICIENCIES OF IMMUNITY IN AGING

G. B. Price* and T. Makinodan

The purpose of this investigation was to characterize an immune deficiency that occurs in old mice, which is manifested as a 20- to 50-fold reduction in generation of IgM-specific antibody-forming cells in response to antigen stimulation. Intrinsic deficiencies, deficiencies intimately associated with the principal cell populations participating in the events leading to production of IgM-specific antibody-forming cells, were analyzed by limiting-dilution and dose-response analyses. These studies were conducted in (C57BL/Cum ♀ X C3H/Anf Cum ♂)F₁ mice, which have a mean life-span of about 30 months. Immunological activities of cells from young adult mice (3-6 months old) and old mice (30-35 months old) were compared. Limiting-dilution analysis indi-

cates that there is a twofold increase in the frequency of thymus-derived cells and nearly a fourfold decrease in the frequency of bone-marrow-derived cells in spleen cell suspensions from old, as compared to young mice. The frequency of the functional immunocompetent unit comprised of both cell types is decreased by fivefold in old mice.

Antigen-mediated events required ten times as much antigen in old as in young mice to stimulate maximally generation of antibody-forming cells. Dose-response analysis indicates that both thymus-derived and bone-marrow-derived cells from old mice are about ten times less capable of growth and proliferation in response to maximally stimulating doses of antigen. Thus, the intrinsically deficient state of the antibody-forming cell response to antigen was found to include a reduction in functional capacities of thymus-derived and bone-marrow-derived cells, manifested in growth and proliferation and a reduction of sensitivity to antigen.

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EXTRINSIC DEFICIENCIES OF IMMUNITY IN AGING

G. B. Price* and T. Makinodan

Extrinsic deficiencies, i.e. environmental deficiencies, causing reduced immune capacity in old (30-35 months) as compared to young (3-6 months) BC3F₁ mice were analyzed. Extrinsic deficiencies, as determined by assessment of 19S IgM antibody-forming cells after transfer of young spleen cells and antigen into X-irradiated old and young syngeneic environments, showed about a twofold reduction in the peak level of production of antibody-forming cells and a 3-day delay in attainment of peak production if cells were transferred to an old environment. The factor(s) responsible for reduced activity of young spleen cells was effective across the cell-impermeable membranes of diffusion chambers with young cells implanted into old environments and therefore is a noncellular factor(s).

Long-term recovery of old spleen cells in young, irradiated, syngeneic environments did not change the immune capacity of those cells but instead converted the young recipient into an immunologically senescent mouse. The existence of programmed low homeostatic levels in spleen cells derived from old mice is one reasonable interpretation of such data.

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ABILITY OF OLD MICE TO BE SENSITIZED TO UNDERGO A SECONDARY ANTIBODY RESPONSE

T. Makinodan

The following observations prompted this investigation: (1) Antigens localize in the follicles of the spleen and lymph nodes in a highly efficient manner shortly after injection into

immunologically competent young adults. In association with follicular antigen localization, germinal centers are formed de novo, and preexisting germinal centers (1) display increased proliferative activity. (2) These cellular events are intimately associated with the formation of mercaptoethanol-resistant 7S IgG antibody, the predominant immunoglobulin class synthesized in a secondary response (2). It has been speculated that antigen-stimulated proliferative activity of germinal centers reflects growth in the number of IgG antibody-forming cells and their precursors, the "memory" cells. (3) In old mice, antigen localizes poorly in the follicles (3), and associated with this deficiency is the apparent absence of germinal centers before and even after antigen stimulation.

These observations suggest that if germinal centers are the sites of growth in the number of memory cells and IgG antibody-forming cells, then the ability of old mice to be sensitized to undergo a secondary response should be sharply curtailed, especially among those with diseases of the follicles. Experiments were therefore carried out to resolve this problem.

The results established that old (30–32 months) BC3F₁ mice can be sensitized with sheep red blood cells to undergo a rather vigorous secondary response, measured in terms of numbers of IgG antibody-forming cells in the spleen, the major site of antibody formation. This is especially true for old mice whose splenic follicles are not sarcomatous or atrophic. Their secondary response, in fact, is almost comparable to that of young mice. Even those with sarcomatous and atrophic splenic follicles respond by generating ten times as many IgG antibody-forming cells as in the primary response. At this age range, 30 months or older, germinal centers cannot be detected even in the disease-free follicles of the spleen. Thus, these findings support the view that follicular antigen localization followed by the appearance of mitotically active germinal centers, as seen in antigen-stimulated young adults, is not obligatory for the proliferating growth of memory cells and IgG antibody-forming cells in old mice.

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SYNERGISM BETWEEN SPLEEN CELLS OF IMMUNOLOGICALLY ACTIVE YOUNG AND INACTIVE AGED MICE

C. P. Peter and T. Makinodan

Previous studies showed that there is a decline in humoral immune potential as an animal ages (1). It has been suggested that this reduction is due to deficiencies both intrinsic and extrinsic to the immunocompetent cell population. In order to gain insight into the nature of the intrinsic deficiency, we have assayed the primary immune potential of spleen cells from young adult and aged mice, separately and in combination, in lethally irradiated adult recipients.

Spleen cell suspensions were prepared from young adult (3- to 4-month-old) and aged (30-month-old) mice with and without reticulum cell sarcoma.

Primary antibody-forming potential of aged spleen, as determined from the peak number of direct (19S) or indirect (7S) plaque-forming cells, was markedly lower than that of the young adult cells. This was in agreement with previous findings. When aged cells from nontumorous spleens were injected with young adult cells, both direct and indirect plaque-forming cell responses were significantly higher than expected—particularly, the indirect. (The response was expected to be equal to the sum of the responses of old and young cells injected separately.) In contrast, when young cells were injected with aged tumorous cells, the response was significantly lower than expected.

Histological examination revealed that spleens from mice that had received young adult cells 7 days previously had well-developed lymphoid follicles with various numbers of germinal centers. There were moderate numbers of large pyroninophilic cells around the central arteriole. The lymphoid follicles of spleens from mice that had received aged cells were less cellular, especially in those that had received aged tumorous cells. There was no apparent germinal center development in the lymphoid follicles of these spleens. However, the spleens of mice that had received a mixture of young and aged nontumorous cells revealed large lymphoid follicles with many germinal centers. There were also large numbers of large pyroninophilic cells around the central arteriole. In contrast, when mice were injected with a mixture of young and aged tumorous cells, the lymphoid follicles of spleen in most cases consisted primarily of reticular cells. However, there were occasional normal lymphoid follicles with germinal centers.

These results suggest that the spleen cells of young adult mice enhance the activity of aged nontumorous cells. We can only speculate about the nature of this synergism. It is possible that the young cells replace or supplement a deficient component of old spleen cells. The results also suggest that tumorous spleen cells can suppress the immune potential of young spleen cells; these findings concur with the *in vitro* observations made by Jaroslow and his co-workers (2).

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AUTOIMMUNE MANIFESTATION IN AGED MICE WITH MEDIUM AND LONG LIFE-SPANS

W. J. Peterson and T. Makinodan

Walford's (1) immunologic theory of aging proposes that aging is due to weak histoincompatibility reactions induced by somatic variation in the immune competent tissue and/or other endogenous tissues. In certain cases, histoincompatibility reactions are reflected by the appearance of autoantibodies. Autoantibodies appear with advancing age, and sometimes they are found associated with diseases commonly referred to as autoimmune diseases. Previous studies (2) have shown an inverse relationship between the activity of the immune system and the appearance of autoantibodies in mice with a short life-span.

Studies were undertaken using mice with medium and long life-spans to determine whether the occurrence of autoantibodies in disease and in aging is due to a common underlying cause or to separate mechanisms. Results were obtained with the use of untreated and trypsinized syngeneic red blood cells (RBC) to detect anti-RBC autoantibodies in the plasma of aged mice. The occurrence of autoantibodies in mice appears to be independent of the mouse strain, the length of their life-span, and the conditions under which they are reared. The frequency of mice with these autoantibodies increases with advancing age and tends to be higher in females. The appearance of these autoantibodies in mice free of obvious autoimmune diseases suggests that they are not associated with disease. They are distinct from natural heteroantibodies but reactive against RBC from other mouse strains. Their activity is temperature sensitive to the extent that they are more reactive at 22 than at 37°C. The striking affinity of these autoantibodies against trypsinized RBC suggests that their activity is against inaccessible non-proteinaceous haptens, most likely carbohydrate haptens. They are primarily IgM immunoglobulins, and in some cases they can be IgA immunoglobulins. RBC from young and from old mice show the same immunogenic potential; thus, changes in the RBC due to somatic variations is an unlikely cause for their origin. The origin and significance of these autoantibodies is unknown. One possibility is that they may have a role in homeostasis. That is, it is conceivable that the spontaneous appearance of what has been referred to as autoantibodies may be a reflection of the normal physiological adjustment in a decaying biological system. These autoantibodies may serve as a protective coating of RBC *in situ*. In this respect, Najjar *et al.* (3) have shown that the binding of normal gamma globulin to the RBC membrane serves to protect them against osmotic and mechanical stress.

In any event, it can be concluded that the presence of these autoantibodies can be used as an index of aging in mice and possibly other species. The advantage of this index is in the technique. It is simple, sensitive, and requires only a small volume of blood, which means that individual mice can be sampled repeatedly.

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INCIDENCE OF DELAYED MORTALITY (SECONDARY DISEASE) IN ALLOGENEIC RADIATION CHIMERAS RECEIVING DONOR BONE MARROW FROM AGED MICE

M. G. Chen, G. B. Price,* and T. Makinodan

Because the number of cells immunocompetent for antibody formation decreases with age in the mouse (1), we investigated possible similar alterations in the capacity of cells in the bone marrow of aged donor mice to cause secondary disease and death in young, lethally irradiated, allogeneic recipient mice. The concentration of hematopoietic stem cells (CFU) decreases with age at a slower rate than the

antibody-forming cells, so the possibility that a dose of old bone marrow might contain adequate CFU for restoration of the lethally irradiated host, but insufficient numbers of cells reactive for secondary disease was tested. Also, the postulated direct or indirect influence of thymus-derived cells and/or the thymus on reactions of this type was investigated.

Briefly, BC3F₁ (H-2^{b,k}) mice of both sexes were used as bone marrow donors; old mice were ≥ 25 months old, young mice 3–6 months old. Recipient CD2F₁ (H-2^{d,d}) mice of both sexes, 2–5 months old, received 850 r whole-body radiation and were injected with donor bone marrow cells within 3 hr of irradiation. The number of bone marrow cells infused was adjusted to contain approximately equal numbers of CFU in old and young inocula, as determined by parallel assessment of donor suspensions for CFU content in BC3F₁ recipients. Thymectomies were performed on groups of old and young BC3F₁ donors and CD2F₁ recipients at 8–12 weeks of age. Deaths were tabulated daily; postmortem examinations and histological studies were made on a random sample of mice dying in each group.

The data demonstrate that aging does not result in a loss in the ability of bone marrow cells at this dose level to initiate secondary disease; in fact, mortality occurs at a slightly faster rate in the mice receiving the old cells. The characteristic lesions of secondary disease were observed in all the mice necropsied. Attempts to reduce the number of thymus-derived cells in the donor marrow by adult thymectomy with or without a dose of 400 r whole-body X-radiation 3 weeks before cell transfer accelerated the death rate. Thymectomy of the CD2F₁ recipients significantly reduced mortality during the first 2 months after transplantation, but by 3–4 months mortality was not significantly different from the nonthymectomized controls. Further studies into the nature of the cell(s) mediating secondary disease and the factors involved in their differentiation from earlier precursors are in progress.

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EFFECTS OF VARIOUS INSULTS ON PATHOLOGY OF BC3F₁ MICE

C. P. Peter

Studies have been initiated to determine the long-term effects of various insults (surgical, physical, and chemical) on the pathology and life-span of long-lived hybrid BC3F₁ mice. Experimental data are available at this time only on those subjected to splenectomy and thymectomy at various ages.

BC3F₁ mice have been shown to have a high incidence (60%) of reticular neoplasm (1). This is one of the most common causes of death in this strain of mice. It has been suggested that the neoplasm arises from the spleen (2). Thus, removal of the spleen during early life should reduce the incidence of reticulum cell sarcoma and possibly increase the

life-span. The thymus plays an important part in the development of the immune system. It has been shown that neonatal thymectomy reduces the capacity to respond immunologically to several different kinds of stimuli (3), and it was thought that thymectomy would increase susceptibility to infectious agents and thus shorten life-span.

Conventional mice were thymectomized or splenectomized at various ages between 2 and 24 months. Controls were sham operated. Of 42 splenectomized animals examined at autopsy, there was no marked difference in the incidence of various pathologic processes associated with mortality, except for a slight decrease in the incidence of reticulum cell sarcoma (43% as opposed to 53%). When the individual age groups are examined separately, it can be seen that this difference is due to the decreased incidence of reticulum cell sarcoma in animals that were splenectomized as young adults. The sample size for pathology studies from each age group is too small to generalize at this time on the possible role of the spleen in the development of reticulum cell sarcoma.

Among mice thymectomized at various ages (between 2 and 26 months) there is a higher incidence of infectious processes than in the sham-operated controls. These processes include bronchopneumonia and widespread septicemia, with abscess formation in various organs such as heart, kidney, and liver.

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A LONGITUDINAL STUDY OF IMMUNOLOGIC AND CYTOGENETIC ACTIVITIES IN MAN

T. Makinodan, J. G. Brewen, T. A. Lincoln,*
W. J. Peterson, and Lucia H. Cacheiro

Studies on aging that have been conducted in the Biology Division have used primarily small animals and cytological and serological techniques. Some of the principles established in these studies now need to be evaluated systematically in a representative human population. The employees of ORNL can serve as an important source of human biological data.

The purposes of this study are (1) to define in humans age-related changes in various immunologic and cytogenic indices, (2) to relate these indices to one another and to age, sex, and low-level radiation exposure, and (3) to identify individuals with "unusual" indices so that their medical histories can be examined for possible factors that may not have been considered.

The reason for undertaking the immunologic study is that although the immune system has been implicated as a factor in many old-age-associated degenerative diseases, including neoplasia, systematic age-related immunologic studies of non-hospitalized individuals are at best very meager. The reason for undertaking the cytogenetic study, which offers a direct

measure of the damage to the genetic material, is to determine whether spontaneously occurring chromosome aberrations are accumulated in somatic cells and consequently are observed in higher frequencies in the aged.

It would seem that past difficulties in obtaining predictive information can be overcome by the use of modern methods to monitor immunologic and cytogenetic indices from blood samples of employees at this laboratory during their periodic complete physical examinations. Needless to say, information derived from such a longitudinal study should benefit not only basic, clinical, and social scientists but also those who are concerned with the long-term effects of exposure to low levels of ionizing radiations.

Our laboratory offers several unique features for such a comprehensive longitudinal study in man. One is that because all employees of the Oak Ridge National Laboratory, numbering about 4700 with a mean age of 43 years, are offered complete physical examinations periodically, there will be no difficulty in monitoring individuals throughout their company service. The population is remarkably stable, with the average employee having 14.4 years of company service. Many of our employees remain in this area after retirement and, with suitable inducement such as periodic physical examination, would be available for continuing studies after retirement. Current longitudinal studies of this nature supported by the NIH include fewer than 500 individuals.

Because of the health and safety program and the varied research and service activities, there is no one occupational stress that predominates. Although exposure to radiation is our single most important problem, there are large numbers of people who have no occupational radiation exposure other than background. Good records of all types of exposures are available.

In view of these considerations, preliminary cross-sectional studies have been initiated; i.e., various immunologic and cytogenetic indices are being assessed from 2-ml blood samples from individuals of various age groups. The indices that are being evaluated are (1) the agglutinating and absorbing capacity of red blood cells, (2) plant mitogen- and antigen-stimulated blastogenic indices of lymphocytes, (3) chromosome anomalies of lymphocytes, (4) phagocytic index of white blood cells, (5) frequency of immunocompetent cells and hematopoietic stem cells in the blood, and (6) serum levels of immunoglobulin concentration, complement, opsonins, bacterial activity, isoagglutinins, heteroagglutinins, and auto-antibodies against DNA.

Preliminary results obtained with blood samples from male donors show, with few exceptions, a lack of age-related change in the indices tested to date. These results are in contrast to those obtained from inbred mice, supporting the view that predictive age-related immunologic and cytogenetic indices from a noninbred population of individuals, such as man, can best be obtained through a longitudinal study.

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PHAGOCYTOTIC ACTIVITY OF AGED MICE

E. H. Perkins and Charlene A. Seibert

As a part of a study documenting the prominent decline of the immune system with advancing age, the phagocytic activity of aged mice is being assessed. Based on *in vitro* cellular engulfing and degradative indices and *in vivo* blood clearance and organ deposition studies, we have found no evidence to indicate that phagocytic activity decreases with advancing age. Thus, the uptake of opsonized sheep erythrocytes at fixed erythrocyte-to-macrophage ratios, which measures the engulfing ability of peritoneal macrophages, was greater in macrophages harvested from gelatin-stimulated, aged mice than in those from young adult mice. Similarly, no impairment of subsequent degradative activity was seen; the half-times of engulfed sheep erythrocytes were 2.27 and 3.33 hr in macrophages of aged and young adult mice, respectively. Furthermore, the total number of peritoneal macrophages harvested from aged mice was significantly greater than that from young adult mice. Mean blood clearance rates of ^{51}Cr -labeled sheep erythrocytes in aged mice were comparable or significantly accelerated, as compared to young adult mice. No correlation between blood clearance rates and limited, selected organ pathology was noted among aged mice. Moreover, the 4-hr deposition of labeled erythrocytes was greater in aged than in young adult mice. Finally, although the peak plaque-forming cell response was markedly depressed in aged mice, no correlation existed in either young adult or aged mice between antibody response and blood clearance or organ deposition of the labeled antigen. Hence, by these indices, the age-related decrease in antibody response does not appear to result from impaired phagocytic activity.

AGE-ASSOCIATED AND X-RAY-INDUCED CHANGES IN DNA OF MOUSE BRAIN CELL NUCLEI

G. B. Price, * S. P. Modak, [†] and T. Makinodan

An age-associated accumulation of damage to DNA of brain cell nuclei has been reported previously (1). Exogenous DNA polymerase-catalyzed incorporation of deoxyribonucleotide monophosphates in nuclei of young brain cells fixed after 10,000 r X-irradiation was compared with that of unirradiated young and old brain cells (2). The change in template activity of nuclear DNA in young brain cells after 10,000 r was comparable to that seen as a function of age. We conclude that increased DNA template activity seen in brain cells of 35-month-old, as compared to 3-month-old mice, is analogous to that caused by DNA strand scission induced by 10,000 r X-irradiation. Therefore, certain aging phenomena may be consequences of damage to nuclear DNA of certain cells, not unlike strand scission induced by X-rays.

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CHANGES IN THE CLONABILITY OF HEMATOPOIETIC STEM CELLS IN SPLEENS OF AGED MICE

M. G. Chen

We have recently reported age-related changes in the concentration of hematopoietic spleen colony-forming cells (CFU) in the femoral bone marrow and spleen of the long-lived BC3F₁ hybrid mouse (1); these assays were performed in syngeneic young (3-month-old) recipient mice. Because the *in vivo* assay system (2) involves the cloning of CFU in the spleens of lethally irradiated recipient mice and the subsequent proliferation of these cells to form a macroscopic nodule 9 days after transplantation, it was necessary to determine the clonability of CFU as a function of the age of the donor in order to establish that the changes in CFU numbers with senescence were due to actual differences in numbers of CFU in the donor inoculum and not to fewer stem cells cloning in the spleen. In addition, determination of cloning efficiency or "f" factor in old and young recipients allows a limited estimate of the receptivity of the host's splenic environment to stem cell colonization.

The data demonstrate that the cloning efficiencies for young and old cells in young recipients are not significantly different. However, a decrease of more than 50% in the number of young (3 months) and old (23 months) CFU resulted when the assay was made in old instead of young recipients; this is partly attributable to a decrease in the "f" factor in the old recipient, as determined by the method of Siminovitch et al. (3). Histological classification of spleen colonies obtained in all combinations of heterochronic transplantations did not show differences in the percentages of differentiated colony types, suggesting no changes in the overall pattern of CFU differentiation with senescence. Further cell transfer studies into the functional capacities of CFU and other dependent stem cell populations of aging mice are in progress.

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IMPAIRED ELKIND RECOVERY IN HEMATOPOIETIC
SPLEEN COLONY-FORMING CELLS OF
AGED MICE

M. G. Chen

Although small variations in the radiosensitivity of hematopoietic spleen colony-forming cells (CFU) occur with senescence (1), a more striking finding is the decrease in extrapolation number (loss of shoulder) on dose-survival curves for CFU from irradiated aging mice. Because the loss of shoulders on radiation survival curves is correlated with lack of repair of sublethal radiation damage (Elkind recovery), studies were done on the recovery of CFU from young (3 months) and old (27 months) bone marrow donors after fractionated sublethal doses of X-rays *in vivo* in young syngeneic recipients, using Till and McCulloch's adaptation (2) of the technique of Luria and Latarjet (3).

Our results show a significant impairment of Elkind recovery in CFU after two equal (200 r) fractions of X-ray exposure *in vivo*. At the peak of recovery (5 hr between doses), the survival ratio—the ratio of the percentage of surviving CFU after two doses separated by a time interval, t , to the percentage survival for the same total dose given as a single exposure—is 3.1 ± 0.2 for the young and 1.5 ± 0.1 for the old CFU. Dose-survival curves at 0, 5, and 10 hr after transplantation for both young and old cells do not exhibit different slopes within each age group that could account for the differences in survival ratio observed. These findings suggest changes with senescence in some physiological parameter of the stem cell itself (e.g. proportion of CFU in active cell cycle). The possible relationship of these findings to radiation recovery in the intact old animal is under investigation.

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IMPROVED CONDITIONS FOR HEMOLYTIC PLAQUE-FORMING CELL ASSESSMENT IN DIFFUSION CHAMBERS

Sarah A. Goodman* and M. G. Chen

Studies of various parameters of the Millipore diffusion chamber technique were made in an attempt to improve this system for the culture of immunocompetent cells. The technique is valuable because of its wide applicability as an *in vivo*, yet closed system for cytokinetic studies. Modifications of this technique now generate a peak primary immune response of mouse spleen cells to sheep red blood cells of 10^4 direct plaque-forming cells (DPFC) per 10^6 nucleated cells harvested and a burst size of nearly 5000, a response which is superior to those obtained *in situ*, *in vitro*, in cell transfer,

and in the previously used chamber system (Table I). Background levels of DPFC are decreased fivefold or more in comparison to previous data obtained in this laboratory; as in the past, however, significant numbers of indirect plaque-forming cells were not detected during the period of study. The improved DPFC response was obtained by the use of larger chambers, chamber detoxification, culture medium containing horse serum, and lethal irradiation of recipient mice.

TABLE I. Comparison of the DPFC response to sheep red blood cells assessed by different methods*

Culture method	DPFC per 10^6 cells recovered	Burst size	Reference
<i>In situ</i>	2000	250	Perkins <i>et al.</i> , 1969 (1)
Cell transfer (Primary response)	200	1100	Bosma <i>et al.</i> , 1968 (2)
<i>In vitro</i>	2000	—	Mishell & Dutton, 1967 (3)
Chamber Previously	5000	750	Groves <i>et al.</i> , 1970 (4)
Presently	10000	4800	

*Values are approximate.

The usefulness of the technique was extended by the demonstration that cyclophosphamide, a radiomimetic drug, could be used in place of irradiation for the pretreatment of chamber hosts, and that a DPFC response equivalent to that in irradiated chamber hosts could be obtained. Cyclophosphamide can therefore be used by investigators at research institutions that have no available X-ray source. Also, immunocompetent cells were cultured successfully in allogeneic and xenogeneic hosts, which broadens the potential applicability of the technique to studies in the species.

Studies were carried out to determine the mechanism of the improved response. The results indicate that the improved response can be attributed to the larger burst size and the two-fold increase in culturing efficiency. The improved response was shown not to be due to an increased efficiency of interaction of cells initiating the primary immune response. The improvements and wider applicability of this technique make it a valuable system with which to study immunocompetent cells.

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KINETICS OF THE DIRECT PLAQUE-FORMING CELL RESPONSE IN STUDIES USING DIFFUSION CHAMBERS

C. F. Gottlieb and E. H. Perkins

In our continuing study of the growth kinetics of plaque-forming cells (PFC), we are asking the following questions: (1) What are the growth patterns of immunocompetent cell populations cultured in this closed system, where recruitment from other lymphoid organs is not possible? (2) Is there both 19S and 7S antibody production in the closed chambers during initiation of the primary antibody response, and if so, are the growth patterns of the cells producing these two classes of antibody the same or different? (3) What factors are involved in the regulation of the response; does antibody cause end-product inhibition, or are there other regulatory factors involved?

Normal (nonprimed) mouse spleen cells were cultured together with sheep erythrocyte antigen in cell-impermeable diffusion chambers implanted in irradiated (650 or 900 r) syngeneic recipients. The time profile of the direct PFC response was similar to that of intact animals, being characterized by an exponential rise ($t_{1/2}$ about 6.3 hr) beginning after 3 days in culture and continuing until the peak response was reached at 6 days. A rapid, exponential decline ($t_{1/2}$ about 19 hr) followed, reaching background levels by 15 days.

Close-interval sampling (2-hr intervals) during the ascending limb of the response curve demonstrated a staircase growth pattern like that seen previously *in situ* (1) and with primed cells in chambers (2). Cell doubling time was about 3.9 hr. Approximately seven steps were required to reach peak response, with an average amplification at each step of 3.9-fold (range 2.8–7.9) and an average shelf time of 8 hr (range 7–9). We interpret these findings as indicating multiple recruitment from a population of synchronously dividing cells. Culture of small numbers of spleen cells produced no clear-cut staircase growth pattern, indicating that all or most of the immunocompetent cells were initially triggered, leaving few for later recruitment.

Of particular significance was the absence of indirect PFC during the 20 days of culture. Therefore, the rapid decline of direct PFC ($t_{1/2}$ about 19 hr) cannot be attributed to feedback regulation by 7S antibody. This idea is further supported by the observation that when normal cells with antigen are cultured in one side of a double-compartmented chamber (two compartments separated by a single, cell-impermeable membrane) and primed spleen cells with antigen in the other side, the kinetics of the normal cells are identical to cells cultured in the absence of the primed cells. These findings indicate that there may be other regulatory factors unrelated to 7S antibody, or that direct PFC have a definite lifetime and die normally.

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THE EFFECT OF PRESENSITIZATION OF PARENTAL DONORS ON GRAFT-VERSUS-HOST DISEASE IN IRRADIATED F₁ HYBRID MICE

G. E. Cosgrove and M. L. Davis*

The graft-versus-host (GVH) disease induced in 1C3F₁ hybrid mice after irradiation and injection of parental spleen or bone marrow cells was noted previously to be mild compared to that occurring in some other strain combinations. However, presensitization of the parental donor by spleen cells of the F₁ or of the reciprocal parent results in a severe GVH reaction under the same conditions (1, 2).

Mice were exposed to 500 r whole-body X-radiation, and some mice were given intraperitoneal injections of approximately 15×10^6 C3H spleen cells. Donors were presensitized by intraperitoneal injections of 101 spleen cells at 1–7 days, 28 days, or 7–24 months prior to use as donors. Four groups of appropriate controls were used (Table I): (1) non-sensitized donors, (2) syngeneic donors, (3) nonirradiated, spleen injected, and (4) radiation alone.

TABLE I. Mortality and average survival of 1C3F₁ mice receiving unsensitized parental or isogenic spleen cells after 500 r X-ray exposure

Exposure (r)	Donor	Donor age	Mortality at day 60 (%)
500	—	—	16
500	C3H	12–16 wk	19
500	C3H	12–23 mo	23
500	1C3F ₁	12–16 wk	3
500	1C3F ₁	12–17 mo	0
0	C3H*	12–16 wk	20

*Donors were sensitized 28 days prior to use by injection of reciprocal parental spleen cells.

Donor presensitization seems to be effective only after at least 3 days, is near maximum effectiveness at 7 days, and only gradually falls off through the life of the donor (Table II). Sensitization is still active 24 months after treatment. This implies that the transplanted cells responsible for GVH disease either have a long life and long immunologic memory or give rise to generations of offspring capable of reacting in the same way.

TABLE II. The effect of time of donor presensitization on mortality and average survival in $1C3F_1$ mice receiving C3H spleen cells after 500r X-ray exposure

Elapsed time since donor sensitization	Mortality at day 60 (%)
1-3 days	6
4 days	33
5 days	56
6 days	80
7 days	84
28 days	100
28 days*	100
7-11 months	92
12-24 months	79

*Donors were 12-17 months old at time of sensitization.

In the experiments reported here, presensitization of parental donors at various times produced a rapidly heightening effectiveness of GVH-inducing capability in the early days after sensitization, with a prolonged persistence of this capability.

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INFLUENCE OF DOSE FRACTIONATION AND PROTRACTION ON SOME LATE EFFECTS OF FISSION NEUTRONS AND GAMMA RAYS IN MICE

E. B. Darden, Jr., J. M. Yuhas, M. C. Jernigan, and J. B. Storer

The rate of delivery of ionizing radiations has an important influence on their relative biological effectiveness (RBE). For many effects, densely ionizing or high-LET radiations such as fast neutrons show less dose-rate dependence than low-LET radiations, so the RBE of the high-LET radiations

is increased when exposure is protracted. Public controversy about acceptable radiation levels has created a greater concern than usual among radiobiologists as to dose-rate effects in mammals. Less widely recognized, perhaps, is the changing nature of the radiation hazards of concern. The growing use of small transuranium fission sources, particularly Californium-252, in medicine, industry, and other fields raises for the first time the possibility that members of the general public may be exposed to low-level, high-LET radiation, perhaps for relatively long intervals. Definitive information on effects of protracted neutron exposure is scanty, however, even for mammals other than man. The evidence for an increase in RBE at low dose rates comes from theory or from experiments with fractionated rather than protracted exposures.

Prompted by these considerations, we are trying to evaluate the influence of dose rate on late radiation effects in mice, principally changes in longevity, induction of neoplasia, and lens opacification. Replication groups of BALB/c mice will receive up to 200 rads of fission neutrons in single or fractionated doses (25 rads weekly) at a dose rate of 25 rad min^{-1} in the Health Physics Research Reactor (HPRR). Other groups will receive corresponding amounts of protracted fast-neutron radiation (1 rad daily) in a 0.5-Ci ^{252}Cf point source facility recently installed in the Biology Division. In the exposure position, the ^{252}Cf capsule is surrounded by a thick spherical housing of depleted uranium. The uranium serves to moderate the slightly more energetic ^{252}Cf fission neutrons in order to simulate more nearly the fission spectrum of the HPRR and is thick enough to absorb most of the ^{252}Cf gamma emission, which otherwise would amount to about a third of the total neutron-gamma absorbed dose. For the RBE determinations, the neutron-irradiated groups will be compared with groups that have received corresponding exposures to gamma radiation (except for a scaling factor of about 2), in the dose-rate studies conducted by Dr. Yuhas.

Exploratory studies using the HPRR are under way, and neutron irradiation for the main experiment was begun in mid-summer 1971.

EFFECTS OF FAST NEUTRONS AND X-RAYS ON THE MOUSE EMBRYO IN THE EARLY PRENATAL PERIOD

E. B. Darden, Jr., W. Friedberg,* C. D. Hanneman,* and D. N. Faulkner*

This report summarizes progress in a study initiated earlier (1) on the effects of densely ionizing radiation on the mammal in the prenatal period, especially prior to implantation of the zygote. The principal endpoint to date has been relative embryo survival, based on assay of the uterine contents shortly before term in pregnant mice irradiated with single doses of fission neutrons (Health Physics Research Reactor) or X-rays a few hours after mating. With both fast neutrons and X-rays, survival appears to decrease exponentially with increasing dose over the range studied (down to about 30% survival), the D_0 values being approximately 16 rads and 76 rads, respectively. The relative biological effectiveness (RBE) over this range lies between 4 and 5. Preliminary data from recently started dose-rate and dose-fractionation studies show that with neutrons the survival values are about the same whether the dose (12 rads) is delivered in 6 min or spread over 5.5 hr (two 2-hr exposures separated by a 1.5-hr interval).

The results are thus consistent with the interpretation that embryo death after irradiation of the zygote with neutrons is the consequence of a single, irreparable lethal injury.

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1. E. B. Darden, Jr., W. Friedberg, C. D. Hanneman, and D. N. Faulkner, *Biol. Div. Ann. Progr. Rept.* Dec. 31, 1969, ORNL-4535, p. 148.

EFFECTS OF RADIATION ON THE THROMBOCYTOPOIETIC SYSTEM OF MICE

T. T. Odell, Jr. and C. W. Jackson

Three-month-old male RFM/Unf mice were exposed to 650 r X-rays, approximately an LD_{50/30}. Their hemopoietic tissue and peripheral blood were studied at intervals, with particular reference to the megakaryocyte-platelet system. We propose that the early increase we observed in the number of megakaryocytes in femoral marrow and the early decline in platelet count result from a temporary inhibition of platelet production by mature megakaryocytes. After 2-3 days the megakaryocytes and platelets began to decline, reaching a nadir at about 6 and 8 days, respectively. In relation to recent information about the timing of megakaryocytopoiesis, the time characteristics of the decline of the megakaryocyte population after radiation indicate that megakaryocytes are rather insensitive to radiation, beginning with committed diploid precursor cells. These cells continue to undergo maturation and to produce blood platelets. The dividing precursors, on the other hand, are radiosensitive. Recovery of megakaryocytes and platelets was not yet complete by 39 days, when the investigation was terminated.

EFFECTS OF THROMBOCYTOPOIESIS ON MEGAKARYOCYTOPOIESIS

T. T. Odell, Jr., C. W. Jackson, and J. R. Murphy

Additional experiments on the effects of reduction of the peripheral platelet count on megakaryocytopoiesis are being carried out to define further the physiological mechanisms that maintain the normal circulating platelet count. Population samples of marrow megakaryocytes and peripheral blood platelets taken at frequent intervals for 5 days after a single injection of platelet-specific antiserum sufficient to reduce the peripheral platelet count to a small percentage of normal are being assessed for number, size, and morphologic characteristics, as well as the DNA content and mitotic index of the megakaryocytes. Megakaryocyte number showed a rapid increase, being well above normal by 18 hr and reaching a peak of about 150% of normal between 42 and 60 hr. Thereafter, the megakaryocyte count declined rapidly, although the size of megakaryocytes continued to be greater than normal. Concomitantly, the mitotic index of megakaryocytes rose rapidly to a peak about five times the control level at 36 hr; it then declined approximately to normal by 72 hr. Apparently, therefore, there was an essentially immediate

response of the unrecognized megakaryocyte precursors, which supply the recognized population, to the reduced circulating platelet mass. (Two cell generation cycles of approximately 9 hr each are required to progress from a diploid precursor to a recognized 8N megakaryocyte.) Moreover, the increases in mitotic index and megakaryocyte number ceased before the peripheral platelet count had reached control levels, showing that it is not necessary to have a large platelet mass to terminate the stimulus for increased megakaryocytopoiesis. It was also noted that platelet size was greater for 42 hr after inducing thrombocytopenia, supporting earlier observations that newly formed platelets are larger than the average size in control populations.

CHOLINESTERASE AS A POSSIBLE MARKER FOR UNRECOGNIZED CELLS OF THE MEGAKARYOCYTIC SERIES

C. W. Jackson, T. T. Odell, Jr., and J. R. Murphy

The study of megakaryocytopoiesis presents special problems since, unlike the other hemopoietic cells, megakaryocytes do not differentiate morphologically at 2N. Consequently, recognition of immature megakaryocytes is based primarily on size; few are recognized before they reach 8N. In this study, we investigated the use of cholinesterase (ChE) activity as a marker for the unrecognizable cells of the megakaryocytic series in rats. In 1954, Zajicek (1) reported that among the differentiated hemopoietic cells in rat bone marrow only megakaryocytes showed high cholinesterase activity. In our studies, megakaryocytes showed intense ChE activity, while in the erythrocytic, lymphocytic, and granulocytic series only basophils occasionally showed weak staining. Some small, undifferentiated cells, however, were highly positive for ChE. They averaged about 16% of the total ChE-staining cell population in untreated rats. When platelet counts were reduced to less than 5% by injection of platelet-specific antiserum, the relative number of small ChE-positive cells was increased about twofold during the first few hours, with the earliest increase seen at 1 hr. The small ChE-positive cells increased in size with time, and the relative number remained elevated for at least 10 hr. The increase in small ChE-positive cells is followed by an increase in megakaryocyte number, beginning about 18 hr after injection. These data suggest that ChE activity can be used as a marker for cells in the megakaryocyte series that are unrecognizable with conventional staining methods.

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1. J. Zajicek, *Acta Haematol.* 12, 238 (1954).

LOW (MEDIUM) LEVEL EXPERIMENT

L. J. Serrano, J. M. Yuhas, E. B. Darden, Jr., G. E. Cosgrove, and J. B. Storer

The objective of this experiment is to determine the relation of small doses of gamma radiation to shortening of life-span, induction of neoplasms, and other late-occurring degenerative diseases and to determine whether the rate of irradiation changes these dose-response relationships. At present it is

difficult to estimate with confidence the shape of the dose-response curve for somatic effects at doses below about 100 rads. Yet, the chance of individuals being exposed to low doses appears to be increasing with the increased use of radioactive sources in power production, science, industry, and medicine. Thus, information such as can be gained from the experiment is necessary if we are to estimate with confidence the effects of irradiation in the dose range approaching present maximum permissible levels.

The experiment was divided into two phases. The first phase was concerned with identifying specific late effects induced by exposure to gamma radiation. The second phase was concerned with quantifying the changes in these predictable effects when the same dose was given at a much lower rate.

Previous progress reports have described the experiment in detail. To summarize: In phase one, groups taken each week from a total of 120 10-week-old RFM mice were exposed to a single whole-body dose of 10-300 rad of gamma radiation at a rate of 40 rad min⁻¹. They were then maintained under rigorously controlled environmental conditions to protect them from disease and to reduce the influence of other variables, since the difference in results for control and experimental mice was expected to be small. Most of the 16,000 mice were allowed to live until death occurred naturally. Some of the mice were killed at weekly intervals from 12 to 120 weeks of age (serial sacrifice group). All mice that died or were killed were examined by pathologists. In phase two, four groups taken each week from a total of 56 RFM mice were exposed for 20 hr each day to a whole-body dose of 50-400 rad at a rate of 0.007 rad min⁻¹. Three other groups taken from 24 RFM mice were exposed as in phase one and served as controls. Phase two of the experiment was replicated concurrently with another strain (BALB/c) of mice.

About 1500 mice in phase one are still alive, and the last one may not die for another year. The input of mice to phase two is about 70% complete. Preparations for analyzing the data are being completed. Some of the data emerging from this experiment have been analyzed for other purposes by Yuhas and Clapp (1).

1. J. M. Yuhas and N. K. Clapp, this report, p. 122.

VARIABILITY DUE TO ORDER OF BIRTH OF EXPERIMENTAL ANIMALS

L. J. Serrano

Analysis of preliminary data from a large experiment suggested that longevity and incidence of diseases were related to the order of birth of the litter in which the mice were born. Similar observations suggesting that parity or age of mothers influences their progeny's adaptability to environmental or experimental stresses have been made by others. For example, Strong (1) analyzed data from mice born in the first to twelfth litters and found that litter order was related to latent period for induction of tumors and to survival time. Others have found maternal influences on incidence of spontaneous tumors and leukemias, immunity, longevity, and anom-

alies in offspring. Because of such findings, it was reasonable to expect that responses to the stress of irradiation could also be modified by order of birth.

Our system of data collection for the large experiment was designed to allow analysis of the influence of litter order on longevity, incidences of late-occurring diseases and other responses. But the total data from the large experiment would not be available for a year or more, and it seemed desirable to test the hypothesis that order of birth influenced the responses of experimental animals to irradiation.

Mice born in first through sixth litters were exposed to single whole-body doses of 300 or 675 rad or to continuous irradiation until death. Preliminary results suggest that survival 30 days after 675 rad (a dose slightly less than an LD_{50/30}) decreases with litter order; i.e., mice from earlier litters live longer. Other results are not yet available.

1. L. C. Strong, *J. Gerontol.* 6, 339 (1951).

QUATERNARY AMMONIUM DISINFECTANT CAUSES LOSS OF EXPERIMENTAL MICE

L. J. Serrano

A sudden outbreak of a disease in mice was characterized by the following signs: discoloration or soiling of the hair between the scapulae and the base of the skull; depilation, sloughing of a dermal pellet (0.5-1.5 cm in diameter) or ulceration in the same region; and injection, inflammation, or necrosis of the ears. Morbidity in mice with advanced signs was high, but only a few died. Only mice held in one of the 20 mouse rooms and only one or two of the eight mice in each cage showed these signs. Examination of historical and laboratory data and methods of handling the mice suggested that the signs were caused by a quaternary ammonium compound used as a disinfectant. The results of an experiment to test this hypothesis confirmed that the accidental use of the concentrated form of the disinfectant had produced the disease.

ROLE OF THE MICROBIAL ENVIRONMENT IN RADIATION-INDUCED PATHOLOGY AND ITS RELATION TO HOST DEFENSE SYSTEMS

H. E. Walburg, Jr.

Early studies on the competence of the host defense systems of germfree (GF) animals suggested a defect in both humoral and cellular responses. More recent data, both from this laboratory and elsewhere, have documented that rather than being defective, the capacity to respond to foreign antigens and cells is slightly but consistently greater in GF than in conventional (CONV) animals. In general, GF animals reflect their understimulated state by decreased numbers of functional cells and their products (e.g. antibodies). On the other hand, these animals demonstrate a slightly increased capacity to respond to specific stimuli, probably as a result of lack of competing stimuli normally present in the microbial

flora. Thus, if immunologic competence plays an important role in the development of radiation-induced diseases, GF animals might be expected to respond similarly to CONV animals, perhaps with a reduced incidence of those diseases inhibited by immunological reactions, e.g. malignant neoplasms(?), and an increased incidence of those diseases that are the result of such reactions, e.g. glomerulosclerosis(?).

Studies on the influence of the microbial environment on late somatic effects of radiation currently in progress involve determination of survival time and causes of death in GF and CONV unirradiated mice and mice exposed to 300 r whole-body X-radiation. Of the studies undertaken, two have been completed, those with the noninbred ICR and the inbred RFMf/Wg. In these strains, the principal radiation-induced diseases are neoplasms of the ovary, pituitary, breast, Harderian gland, and hemopoietic system, including thymic lymphoma and myeloid leukemia as well as glomerulosclerosis, a nonneoplastic disease. Absence of the microbial environment does not appear to affect the development of radiation-induced tumors of the ovary, pituitary, or breast in female mice or tumors of the Harderian gland in both sexes but does significantly alter the incidence of myeloid leukemia and thymic lymphoma. Whole-body exposure of male RFM mice to 300 r X-radiation in a CONV environment results in development of myeloid leukemia in moderate incidence (15–50%), but this disease is not induced under comparable conditions in mice reared in a GF environment. On the other hand, the incidence of thymic lymphoma is elevated in irradiated GF mice, as compared to their CONV counterparts. While the lower incidence of myeloid leukemia might be ascribed to the greater immunologic competence of GF mice, such an explanation does not take into account the increased incidence in GF mice of the related neoplasm, thymic lymphoma. Perhaps a better explanation involves the understimulation of the granulocytopenic tissue (1), resulting in a reduced number of stem cells at risk to neoplastic transformation. Glomerulosclerosis, a complex disease with multiple pathogeneses, is affected by the microbial environment in unirradiated animals, and the incidence is increased by whole-body irradiation. However, the component of glomerulosclerosis that is radiation-induced does not seem to be dependent on the microbial flora, GF mice demonstrating a radiation effect similar to that observed in CONV mice. Thus, most radiation-induced diseases studied to date are not seriously affected by the microbial flora, suggesting that if immunologic mechanisms are significant for development of those diseases, immunologic competence of GF animals must be equivalent to that for CONV animals. This conclusion agrees with the data from immunologic studies. In the case of hemopoietic neoplasms, where differences between GF and CONV animals have been observed, an explanation based on a difference in immunologic competence does not appear to be consistent with the data.

1. V. K. Jenkins, H. E. Walburg, Jr., A. C. Upton, and L. C. Satterfield, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1968*, ORNL-4412, p. 150.

CAUSES OF MAMMALIAN RADIATION LETHALITY

H. E. Walburg, Jr.

Survival following whole-body exposure to radiation is clearly related to perturbation of the cellular kinetics of rapidly proliferating tissue. This is particularly true in exposure ranges of 500–1000 r, where failure of the hemopoietic tissues results in anemia, hemorrhage, and infection, all contributing to death. It has also been suggested that physiological functions not related to cellular kinetics are affected by radiation in this exposure range, leading to fluid loss, electrolyte imbalance, anorexia, etc., which may also contribute to death. Attempts to isolate these multiple pathogeneses are confounded by the inability to control postirradiation infection, which consistently leads to death following irradiation in the higher (800–1000 r) exposure range. Germfree animals free of the complication of postirradiation infection provide a useful tool to study the relative importance of noninfectious causes of radiation lethality. As previously discussed (1), repopulation of hemopoietic tissues is comparable in germfree and conventional mice, with the exception of the lymphopoietic system. To study the role of anemia in mammalian radiation lethality, germfree male and female C3H mice were exposed to lethal amounts of radiation and treated with various schedules of washed isogenic erythrocytes and/or platelets.

Preliminary experiments (Table I, Expts. 1–9) demonstrated that a single intravenous injection of erythrocytes or platelets at 11 days after exposure to 700 r X-radiation was effective in preventing lethality, but the same treatment administered 6 days after exposure was ineffective. However, single or multiple doses at 8 and 11 days after exposure to 900 r X-radiation were not protective. All mice dying after exposure to radiation, whether they were treated with erythrocytes and platelets or not, showed evidence of massive hemorrhage, particularly in the lung, intestine, and brain, as well as fatty degeneration of the liver, suggesting hypoxia. Peripheral blood counts verified the loss of circulating erythrocytes, leukocytes, and platelets, the packed cell volume dropping from about 40% to as low as 2% before death. The repopulation rate of hemopoietic organs was dependent on exposure level, and the greater time required for repopulation following exposure to 900 r explains the failure of early treatment with erythrocytes or platelets at this exposure level.

Additional experiments (Table I, Expts. 10–12) were carried out with intravenous injections of $3-4 \times 10^9$ erythrocytes and $0.3-4.0 \times 10^8$ platelets twice weekly throughout a 30-day period following exposure to 900 r X-radiation. About 60% survival was obtained in the first of these experiments, with all but a few of the mice dying between 23 and 26 days after exposure. These deaths occurred because of a change to single weekly injections during the third week, when hemopoietic repopulation was not sufficiently advanced. In the last two experiments, where two injections weekly were continued through the 27th day after irradiation, greater than 90% survival was obtained, with the few deaths occurring

TABLE I. Effects of isogenic bone marrow and/or erythrocyte injections at various times after irradiation with 700 or 900r X-rays

Expt. no.	Treatment	Treatment days*	Survival	
			No. alive/ no. at risk	Percent
1	700r	D ₀	3/60	5
2	700r + 10 ⁶ IBM†	D ₀	82/82	100
3	700r + 3 X 10 ⁹ MRBC‡	D ₆	0/9	0
4	700r + 2 X 10 ⁸ platelets	D ₆	1/9	9
5	700r + 3 X 10 ⁹ MRBC	D ₁₁	6/7	88
6	700r + 5 X 10 ⁸ platelets	D ₁₁	7/8	89
7	900r	D ₀	0/19	0
8	900r + 4 X 10 ⁹ MRBC + 6 X 10 ⁸ platelets	D ₈ , D ₁₁	1/13	8
9	900r + 4 X 10 ⁹ MRBC + 1 X 10 ⁸ platelets	D ₈	0/50	0
10	900r + 4 X 10 ⁹ MRBC + 0.8-4 X 10 ⁸ platelets	D ₅ , D ₈ , D ₁₂ , D ₁₅ , D ₁₉ , D ₂₆	21/41	57
11	900r + 4 X 10 ⁹ MRBC + 0.3-3 X 10 ⁸ platelets	D ₆ , D ₉ , D ₁₂ , D ₁₅ , D ₁₉ , D ₂₂ , D ₂₇	32/36	92
12	900r + 4 X 10 ⁹ MRBC + 1-3 X 10 ⁸ platelets	D ₅ , D ₈ , D ₁₂ , D ₁₅ , D ₁₉ , D ₂₂ , D ₂₇	59/62	95

*Irradiation always given on D₀.

†Isogenic bone marrow.

‡Isogenic mouse erythrocytes.

between 30 and 45 days. These data demonstrate the importance of anemia resulting from hemorrhage and failure of erythropoiesis in radiation lethality and cast doubt on the importance of radiation perturbation of physiological functions not related to cellular kinetics of the hemopoietic system.

1. H. E. Walburg, Jr. and E. I. Mynatt, *Biol. Div. Ann. Progr. Rept.* Dec. 31, 1968, ORNL-4412, p. 154.

STATISTICAL ANALYSIS OF SURVIVAL EXPERIMENTS

H. E. Walburg, Jr. and D. G. Hoel*

Animal survival experiments are among the most difficult to design, conduct, and analyze, due not only to the duration of such experiments and their inherent biological variability but also to the numerous and competing causes of death, which are often independently affected by the treatment being studied. The final incidences of late-occurring diseases are seriously affected by mortality rates preceding their onset. Serial killing can be used to determine the incidence of a disease free of such errors, but these experiments give no information about the effect of a treatment on survival or on causes of death. Meaningful analysis of survival experiments can be obtained by determining the estimates of the net probability of death due to the cause of interest. This net probability is the probability of death if the specific risk is the only risk acting on the population.

Assessment of mortality caused by a specific disease assumes that the cause of death can be determined accurately. This is admittedly a difficult and sometimes speculative decision. However, where considerable attention is paid to the individual experimental animal and adequate tissue from all vital organs is available for histological examination, the causes of death in inbred strains of mice can be determined in most cases.

Several methods are used for correcting for competing lethal diseases. One general method of analysis, the interval method, involves dividing time into a series of equal contiguous intervals and reducing the age-at-death data to the numbers of animals dying in each age interval. Then, for each interval, estimates of the probability of death if the risk of interest is the only risk in effect on the population are calculated. Also it is possible to develop corrected cumulative mortality curves due to a specific disease if it is the only risk operating and to develop a standard error for each point on the curve.

Another method is the Kaplan-Meier representation (1) of the cumulative mortality function, which uses basic age-at-death data. This method can be used for describing the cumulative mortality with respect to a specific risk. This technique has the advantage of avoiding an assumption necessary to the interval technique, that there is a constant force of mortality across the interval. In addition, the Kaplan-Meier technique gives more information about the statistic $P(t)$, i.e. probability that an animal will die before a given time if the disease of interest is the only disease causing death. It is possible to test such cumulative mortality curves for the presence of a significant difference between different popula-

tions with a nonparametric test for randomly censored data, given by Breslow (2). This avoids the error inherent in selecting a point where a difference is noted after the data have been examined.

It must be understood that the techniques described above can only be used for lethal diseases, since changes in the mortality patterns for competing lethal diseases will seriously affect the "net probabilities" for nonlethal diseases. In this case, the usual quantity studied is the proportion of animals with the disease present. Such a quantity can be determined easily using an interval method, e.g. the number of animals dying in an interval with a nonlethal disease present divided by the total number of animals dying in that interval. A non-interval method similar to the Kaplan-Meier technique for lethal diseases can also be developed.

Thus, it is possible to correct data obtained from survival experiments so that the confusing effect of competing risks (diseases) does not occur and so that a clear interpretation of the effect of a treatment on disease induction or acceleration can be made. This can be accomplished with only a few, biologically reasonable assumptions.

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1. E. Kaplan and P. Meier, J. Amer. Statist. Ass. 53, 457 (1958).
2. N. Breslow, Biometrika 57, 579 (1970).

LOCALIZATION OF GALLIUM-67 CITRATE DURING EMBRYOGENESIS

J. A. Otten, R. L. Tyndall, and P. C. Estes

Much evidence has accumulated recently concerning the relationship between embryonic and leukemic murine tissues. Biochemical, serologic, and histologic evidence suggests a variety of similarities between such cells. Several recent studies have also demonstrated the localization of ^{67}Ga in nonosseous tumors of humans and animals, including leukemic tissues in mice (1, 2). The reason(s) for this localization is unknown. The analogies between embryonic and leukemic mouse tissues and the observed localization of ^{67}Ga in leukemic tissues prompted an investigation into the degree and specificity of ^{67}Ga localization during embryogenesis in mice.

^{67}Ga uptake in embryos was determined 24 hr after intravenous injection of pregnant BALB/c mice with 1 μCi of ^{67}Ga . Autoradiograms were prepared from embryos and related placental tissue of mice inoculated intravenously with 2 mCi of either ^{67}Ga or ^3H -thymidine. Total ^{67}Ga counts in 4- to 7-day embryos were comparable to those previously seen in normal adult mouse tissues, whereas uptake of ^{67}Ga in 8- to 10-day embryos was equivalent to or surpassed that noted in leukemic tissues of mice. By day 12, ^{67}Ga counts were diminished. Autoradiograms revealed that ^{67}Ga localized in fetal membranes, limb buds, and car-

tiginous precursors of skeletal structures of the embryos and in the decidua. Conversely, embryos and decidua labeled with ^3H -thymidine indicated no such selectivity. Therefore, incorporation of ^{67}Ga is neither selected for nor a reflection of cell turnover as judged by ^3H -thymidine incorporation.

Thus, in addition to other analogies between embryonic and leukemic tissues in mice, selective localization of ^{67}Ga is also associated with both tissues. These studies were undertaken in the hope of providing a better understanding of the basis for ^{67}Ga uptake as it is related to carcinogenesis. Localization of ^{67}Ga during embryogenesis could be useful as a model system for studying ^{67}Ga uptake, because of the well delineated sequence of events during fetal development. Conversely, the isotope may also prove to be a useful tool for the study of embryogenesis.

1. C. L. Edwards and R. L. Hayes, J. Amer. Med. Ass. 212, 1182 (1970).
2. D. C. Swartzendruber, B. L. Bird, R. L. Hayes, B. Nelson, and R. L. Tyndall, J. Nat. Cancer Inst. 44, 695 (1970).

ONCOGENIC EFFECTS OF REPEATED INOCULATIONS OF SHEEP ERYTHROCYTES IN BALB/c MICE

R. L. Tyndall, J. A. Otten, P. C. Estes, and Carole S. King

Preliminary studies indicated either direct oncogenic effects in mice of repeated inoculations of sheep erythrocytes (SRBC) or suggested an increased incidence of reticular cell sarcomas (RCS) in newborn mice injected with spleen cells from mice undergoing SRBC inoculations (1). In attempts to confirm either or both of these observations, additional BALB/c mice were inoculated twice weekly with SRBC, while control mice were similarly injected with phosphate-buffered saline. At various intervals during the inoculation regimen, random mice both from control and from SRBC groups were killed, and their spleen cells (1×10^6) were injected into newborn recipients. Both treated mice and recipients of spleen cells from treated mice were observed for 16-18 months for development of RCS. Mice treated directly with SRBC for periods of 10-20 weeks showed no evidence of RCS at 18 months of age, whereas RCS developed in 29% of newborn BALB/c mice within 14-18 months after the mice were injected with histologically normal spleen cells from mice inoculated repeatedly with SRBC (Table I). Control mice, injected with spleen cells of mice inoculated repeatedly with phosphate-buffered saline, showed no evidence of malignancy (Table I). The sarcomas arising in mice inoculated with spleen cells from SRBC-treated donor animals were readily transplantable and contained demonstrable murine leukemia virus antigens. Electron microscopic analysis of transplantable tumors revealed intracisternal, budding A-type viruses. Mature C-type particles were localized in the enfoldings of the reticular cells. Few budding C-type viruses were observed.

TABLE I. Effect on newborn mice of spleen cells from mice treated with sheep erythrocytes (SRBC) or phosphate-buffered saline (PBS)

Expt. no.	Treatment of donor mice	Duration of treatment prior to transfer of spleen cells to recipient mice (days)	No. of recipient mice injected*	Observation period of recipient mice† (months)	No. RCS/no. injected	Latent period from injection to onset of RCS (days)
1	SRBC	83	7	18	3/7	129-354
	PBS	107	11	18	0/11	—
	SRBC	107	8	18	8/8	270-489
2	SRBC	131	14	16	5/14	335-350
	SRBC	315	15	16	1/15	279
	SRBC	315	11	16	6/11	251-454
	PBS	315	8	16	0/8	—
	PBS	315	12	16	0/12	—
	PBS	131	10	16	0/10	—
3	SRBC	72	9	14	1/9	328
	SRBC	72	14	14	1/14	405
	PBS	131	17	14	0/17	—
	SRBC	131	9	14	0/9	—

*1- to 5-day-old BALB/c mice. †From date of birth.

Although no RCS developed in the SRBC-treated mice *per se*, the present results, as in the original study, confirm and extend the previous observation that inoculation of spleen cells from SRBC-treated mice results in an increased incidence of RCS in the recipient mice. The lack of neoplasia in the donor mice, except for the original experiments, suggests a suppressive mechanism(s) preventing the direct oncogenic expression of SRBC treatment. The role of endogenous murine leukemia viruses in the oncogenic effect of SRBC treatment is currently under study, as is the characterization of the transplantation antigens associated with the resultant RCS.

1. R. L. Tyndall and J. A. Otten, *Proc. Amer. Ass. Cancer Res.* 10, 94 (1969).

INTERACTIONS OF PARVOVIRUSES AND CELLS

R. W. Tennant and R. E. Hand, Jr.

The paroviruses comprise a group of at least six distinct viruses and related serotypes and are the smallest DNA-containing animal viruses. In addition to their minute size and small, single-stranded DNA genome, some of these viruses induce a variety of birth defects. Work in our laboratory has shown that Kilham rat virus (KRV), the prototype parvovirus, preferentially infects actively dividing cells in culture and

that the virus is dependent on one or more radiosensitive (X-ray or UV) cellular functions, not required by other DNA animal viruses.

Subsequently, we have found that H-1 and MVM parvoviruses likewise require radiosensitive cell functions, but that the requirement(s) of MVM may be somewhat different from that of the other two viruses. The adenoassociated viruses are defective parvoviruses and require coinfection with an adenovirus to complete the replication cycle. However, they do not require the same type of cell function required by the other parvoviruses, and their requirements are presumably fulfilled by the coreplicating adenovirus. Limited attempts to "rescue" the radiosensitive cell function by coinfection with adenovirus type 12 or polyoma virus have been unsuccessful in the case of KRV.

We have found also that KRV has a specific inhibiting effect on DNA synthesis and mitosis in cell cultures. The effect on DNA synthesis occurred much earlier than any effects on protein or RNA synthesis. Experiments suggest that KRV may compete for some cellular function associated with DNA synthesis.

These results suggest that the requirement for specific cell function(s) is a parvovirus subgroup characteristic and that this property is directly related to the interaction of the viruses with cells and the pathogenesis of the diseases induced by parvoviruses.

ULTRAVIOLET PHOTSENSITIVITY OF A SINGLE-STRANDED DNA ANIMAL VIRUS

W. R. Proctor,* J. S. Cook, and R. W. Tennant

The effects of UV radiation on Kilham rat virus (KRV) were studied to determine the relative sensitivity of a single-stranded DNA animal virus. Inactivation of KRV was assayed in rat embryo cells, using the fluorescent antibody technique to enumerate cells synthesizing virus in the first cycle of replication, a method comparable to the plaque assay technique. KRV showed a D₃₇ (UV survival dose) of 130 erg mm⁻² at 254 nm. This D₃₇ value is comparable to that of phage ϕ -174, which has been reported to be 65–130 erg mm⁻² but is about 20-fold less than the D₃₇ doses reported for double-stranded DNA animal viruses.

The DNA of KRV develops UV-induced lesions at the same rate as phage ϕ X-174. Analysis of the UV-induced products gave a linear dose-response curve and a value of 0.30% thymine-containing dimers per 1000 erg mm⁻²; at the D₃₇ dose, 0.66 pyrimidine dimers per virus were produced, which is about twice that of phage systems, which were reported at values of 0.33 dimers per virus at their D₃₇ dose of 65 erg mm⁻².

These results show that the single-stranded DNA animal viruses may be similar to the single-stranded DNA-containing bacterial viruses in UV sensitivity and thus may be valuable for exploration of UV photobiological problems in mammalian cell systems.

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THE CARCINOGENIC EFFECTS OF GAMMA RAYS DELIVERED THROUGHOUT THE PRECONCEPTION AND FOETAL PERIODS

J. M. Yuhas and W. C. Klima

Retrospective studies in human populations have reported what appears to be an elevated risk of leukemia and other neoplasms in children whose parents received radiation exposure prior to their conception, or who received diagnostic exposure during the foetal period. A prospective study in humans exposed at the time of the Hiroshima and Nagasaki detonations fails to confirm these observations, but cannot disprove their validity.

Since the data obtained suggest that the foetus may be extremely sensitive, a resolution to this controversy should be sought in the laboratory. Unfortunately, all of the existing data are concerned with relatively high total exposures delivered acutely at some point during gestation. This introduces the complications of artificial selection, as well as the possibility that the foetal stage irradiated is not the one that is sensitive to cancer induction. In order to avoid these complications and to provide an estimate of the risk of cancer

as a function of the radiation dose received by the parents before conception, we are exposing mating pairs of BALB/c mice to 8 rads a day. As of the time a litter is born, it has received a total of 168–176 rads, and the genetic material from which it was derived received a variable but known exposure. Each mouse is specified, therefore, by the amount of exposure its parents received prior to its conception, as well as a standard exposure during the foetal period. By comparison of the cancer risk in these mice (200 males and 200 females) to that of their similar but unirradiated counterparts, we hope to determine the effects of chronic irradiation during the foetal period. Similarly, by comparing the elevated cancer risk in these mice to that in similarly exposed adults, we should be able to determine whether the foetus is indeed extremely sensitive. Lastly, an analysis of cancer risk within the foetal irradiation group as a function of the exposure the parents received should allow the estimation of the effects of preconception exposure.

The irradiations for the experiment involving chronic exposure to 8 rads per day are now complete, and as expected we encountered both an increased incidence of sterile matings and a decreased litter size in the irradiated groups. As part of our studies on the effects of total dose and dose rate on the late effects of radiation, we intend to repeat this experiment using a daily dose of 1 rad.

AGING, IMMUNE COMPETENCE, AND THE ONSET OF DISEASE IN CONTROL AND CHRONICALLY IRRADIATED POPULATIONS

J. M. Yuhas

The concept of immunologic surveillance proposes that neoplasms are antigenically different from the host in which they reside, and therefore the immune system will keep these growths in a repressed state unless age, trauma, or disease reduces its ability to function. If valid, this concept is of prime importance to the study of age-dependent diseases and the late effects of radiation. As an example, if we assume that the number of malignant transformations induced by radiation is independent of the dose rate at which the exposure is received, as is often done, we would still expect to obtain fewer clinical cancers following a low dose-rate exposure, because the immune system is much more resistant to exposures at low dose rates. In other words, the reduced efficiency of low dose-rate exposures may involve intercellular interactions, in addition to any intracellular phenomena that may be operative.

We are currently studying the carcinogenic effects of four different total doses of γ -rays given at dose rates of 1 r per day through 1 r per sec. As a corollary to these studies, we are studying the status of the immune system as a function of age in the various groups in the hopes of testing the hypothesis that reduced immune competence will be associated with an elevated risk of cancer.

Results to date have established the baseline values for immune competence as a function of age in two strains of mice, BALB/c and RFM. These data will provide an adequate test of the proposal that diseases which affect immune competence increase the cancer risk, and more importantly that the depression of the immune function precedes the development of neoplasms. The RFM mice have been included since we have available extensive information on the incidence of neoplastic and nonneoplastic diseases as a function of age in this strain for both spontaneously dying mice and mice killed at predetermined intervals.

The shape of the curve relating immune competence to age in these two strains is essentially the same as that observed by others for the BC3F₁ mouse (1) but with a contracted time scale, owing to the fact that the BALB/c and RFM mice have a much shorter life-span. We are currently completing the histological analysis of the mice, in the hope of relating immune competence to disease status for individual records.

1. T. Makinodan, this report, p. 107.

RADIOSENSITIVITY AND RECOVERY POTENTIAL OF HUMAN BLOOD-FORMING TISSUES

J. M. Yuhas, Mildred G. Hayes, Judith O. Proctor, and C. C. Lushbaugh*

In spite of all the studies that have been conducted on irradiated human populations, we do not yet have available a quantitative means of predicting the acute response of normal man to ionizing radiation exposure, nor do we have a rational means of predicting the best combination of total exposure, number of fractions, and total time of protraction for the treatment of disseminated neoplasms. In the hope of being able to provide some information on these two points, Dr. Lushbaugh has gathered the clinical records of more than 2000 humans who were exposed to various regimens of low-LET total-body irradiation. This report presents an analysis of their peripheral blood cell responses.

The objective of our first study was to determine the radiosensitivity and recovery potential of the blood-forming tissues in four disease categories: chronic myelogenous leukemia (CML), chronic lymphatic leukemia (CLL), lymphosarcoma (LSAR), and diseases without direct effects on the blood-forming tissues (NORMALS). The endpoint studied was the white blood cell count at the nadir or lowest point following exposure, expressed as a percentage of the pre-irradiation levels. Based on the degree of clinical follow-up and the accuracy of the dosimetry, we excluded all but 518 records, which were distributed among the four disease categories as shown in Table I. Since each record is characterized by its own combination of total exposure (E) and time over which it was given (T), we employed a multiple regression analysis, through which it is possible to determine the effects of each, independent of the other. For the present, we have ignored the number of fractions into which the exposure was divided. The protraction time, T, is equal to the number of days over which the exposure was given, with single acute exposures having T = 1.

TABLE I. Results of the multiple regression analysis on the clinical records of 518 patients given total-body irradiation

	CML	CLL	LSAR	Normals
Number of patients	131	200	66	121
Slopes:				
WBC on exposure	-0.94	-0.83	-1.06	-1.03
WBC on time	0.39	0.22	0.23	0.63
Correlation coefficient	0.61	0.59	0.68	0.55
All correlations are statistically significant to at least the 0.01 level.				

Each set of data was best fit by a power function of the form

$$\% S = \frac{K (100) (T)^\beta}{(E)^\alpha}$$

Table I includes the results of these analyses and shows that the correlation coefficients for curves are extremely good. The slope of % S on exposure does not differ among the four categories, indicating that the diseases themselves do not affect the inherent radiosensitivity of the cell population that gives rise to the peripheral blood. On the other hand, the slopes of % S at the nadir (at any exposure) as a function of the time of protraction do show significant differences. The slope is highest for the NORMALS group, indicating that this group is able to recover from the exposure most efficiently as the exposure is protracted in time. The CML group has a lower slope, but it does not differ significantly from the NORMALS. Both diseases that affect the lymphatic tissues, CLL and LSAR, are characterized by a recovery slope three times smaller than that of NORMALS.

We conclude, therefore, that the major factor controlling differences in radiation resistance among the four groups is not differences in inherent radiosensitivity but rather differences in the ability to recover when the exposure is given over a period of time.

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CORRELATED DISEASE OCCURRENCE IN AGING POPULATIONS OF MICE. NEOPLASTIC AND NONNEOPLASTIC DISEASES

J. M. Yuhas and N. K. Clapp

Many of the currently tenable theories of aging and carcinogenesis carry with them predictions of correlated disease occurrence. For example, if the theory of immunologic surveillance is valid, then diseases that reduce immune competence should increase the risk of cancer. A promising method of testing these theories, then, should be to determine whether the correlated occurrences they predict are indeed observed.

In order to test the possibility that autoimmune diseases lead to leukemia and that leukemias which invade the immunocompetent organs lead to neoplasms of other organs, we have conducted a study of correlated disease occurrence in a population of 311 RF mice that died spontaneously and were then examined extensively by one pathologist. Results obtained to date have shown that a presumed autoimmune disease, glomerulosclerosis, increases the risk of two other presumed autoimmune diseases, polyarteritis and arterial hyalinization, by factors of 4 and 8, respectively. By themselves, these observations would appear to indicate that autoimmune diseases occur in different sites as the result of a common etiology, but a further correlation makes this unlikely. The presence of glomerulosclerosis reduces the risk of leukemia by a factor of 2, and in combination with polyarteritis or arterial hyalinization or both, glomerulosclerosis can reduce the risk of leukemia by a factor of up to 3. Based on the known pathogenesis of leukemia and the basis of the three others, we interpret these correlations as indicating that glomerulosclerosis, polyarteritis, and arterial hyalinization are all manifestations of an immunologic response to a virus, and that this virus is leukemogenic. The presence of an adequate response to this virus, as indicated by the presence of what were thought to be autoimmune diseases, would then reduce the risk of leukemia. This interpretation is further supported by the fact that these three diseases show no correlation with other types of neoplasms, which are thought to be independent of the action of the leukemia virus. We are testing this interpretation directly.

Our attempts to determine a correlated occurrence of leukemias that affect immunocompetent organs and neoplasms of other sites have thus far been negative (i.e., they apparently occur independent of one another). This does not refute the possibility that immunologic surveillance is of importance in this mouse strain but does indicate that if it is operative it is not of sufficient importance to allow its demonstration by a simple disease analysis.

IMPROVED RADIOTHERAPY OF MURINE LUNG ADENOMAS THROUGH THE USE OF A RADIOPROTECTIVE DRUG (WR-2721)

J. M. Yuhas and W. D. Gude

In order for a radiotherapeutic treatment to be considered successful, one must be able to eradicate or at least control the tumor without inducing adverse side effects in the normal tissues. In practice this is difficult to achieve, since the deficient blood supply of the tumor renders it anoxic, and the tumor is therefore more resistant to radiation than are the surrounding normal tissues. A variety of methods have been tried in an attempt to decrease tumor resistance, but to date none have proven to be of practical value. We have taken the opposite approach, in that we are attempting to increase the resistance of the normal tissues through pre-irradiation injection of a radioprotective drug, S-2-(3-aminopropylamino)ethylphosphorothioic acid or WR-2721.

The rationale for these studies is that the deficient blood supply of the tumor should inhibit the drug from entering it, while the adequate vasculature of the normal tissues should allow its rapid absorption. Excellent increases in

resistance have been obtained with this drug for a variety of normal tissue injury modes: hematopoietic death, gastrointestinal death, skin ulceration, epilation, immune responsiveness, residual radiation injury, and death from localized exposure of the lungs. In each instance, the resistance has been increased by a factor of 2-3. Earlier data on a transplantable mammary tumor failed to demonstrate any protection of the tumor, and we therefore expanded our studies to include a tumor that more closely simulated the problems encountered in radiotherapy, namely lung cancer in the mouse.

A model system has been developed that allows the enumeration and sizing of lung adenomas in the mouse. Mice are injected with urethane and maintained for 6 months, at which time each mouse possesses 8-10 lung adenomas. The mice are then given localized exposures to the lungs (1500-7500 r), with and without prior injection of 500 mg kg⁻¹ of WR-2721. At intervals of 15-160 days later, these mice along with unirradiated controls are killed, and the numbers and sizes of the tumors are determined. Our data are complete through the 60-day postirradiation period and indicate that at equal levels of normal tissue injury, the lung tumor volume per mouse is more than four times smaller in drug-treated mice than in mice that received no drug prior to exposure. This is attributed to the fact that the drug-treated animals require larger radiation doses to induce injury in normal tissues, while injury to the tumor is apparently independent of the drug injection.

Since the murine lung adenoma is not of the same histologic type as that observed in humans, we are expanding our studies to a squamous cell carcinoma of the lung, which has been isolated by Dr. Paul Nettesheim. These experiments should provide as definitive a test of our approach as can be expected from studies in the mouse.

RADIATION CARCINOGENESIS AS A FUNCTION OF BOTH TOTAL DOSE AND DOSE RATE

J. M. Yuhas

In experimental animals, it has been established that the same total dose of γ -rays will induce less cancer if it is given at a low rate than if it is given at a very rapid rate. This statement adequately summarizes our knowledge of the effects of dose rate on carcinogenesis. The basis of this effect can be accounted for theoretically, but few experimental data exist to substantiate these interpretations. Similarly, we are unable to describe the rate at which carcinogenic effectiveness declines as the dose rate is lowered. If we are to understand the mechanisms of radiation carcinogenesis and predict the hazards of low dose-rate, low-level exposures, these voids in our knowledge must be resolved.

In order to meet this need, we are currently studying the carcinogenic effectiveness of ¹³⁷Cs γ -rays given at dose rates of 1 r per sec down to 1 r per day over the accumulated dose range of 49-392 r. From these studies we hope to describe the carcinogenic and other effects of γ -rays as a function of both total dose and dose rate, and to provide a rational means of extrapolating beyond the observed range of doses and rates.

As a corollary to these studies, we are investigating the possible mechanisms that have been proposed as theoretical bases of the dose-rate effect. These include age-dependent changes in resistance, intracellular recovery, dose-size dependence for effects, and the role of immunologic factors. In conjunction with other studies of a similar nature conducted with neutrons, we hope to be able to elucidate the mechanism of radiation carcinogenesis through the analysis of relative biological effectiveness.

The performance of this study required the construction of a new laboratory, which has been completed during the past year. We anticipate completion of the irradiations within the coming year. The studies related to the mechanisms of the dose-rate effect have been initiated, but results are too preliminary to warrant discussion.

RADIOPROTECTIVE AND ACCELERATING EFFECTS OF S-2-(3-AMINOPROPYLAMINO)ETHYLPHOSPHOROTHIOIC ACID ON THE PRIMARY IMMUNE RESPONSE

J. M. Yuhas

The radioprotective drug S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721) is currently being studied as a possible adjunct to radiation therapy of cancer. From our studies in a variety of systems, it appears that this drug offers excellent protection to normal tissues but little if any protection to tumor tissue. As part of our analysis of the ability of this drug to protect normal tissues, we have initiated studies on its ability to protect the primary and secondary immune responses. Our first studies on the primary immune response

are now complete and have demonstrated not only the radioprotective capabilities of the drug but also its ability to accelerate the maturation of the primary immune response.

If the maximum tolerated dose (500 mg kg^{-1}) is injected into BALB/c mice 16 hr before an injection of sheep red blood cells, the direct plaque-forming cells (DPFC) appear earlier but reach a lower maximum level than in untreated mice given the same antigen. The exact nature of this effect is presently under study. Our data thus far indicate that the effect is not due to a persistent metabolite of the drug, but rather that a specific component of the primary response is affected. By altering the time of drug injection relative to the time of antigen injection we have been able to show that the suppression of this component persists for 4 days, after which time it recovers linearly toward normal, reaching essentially control values by 10 days after injection, and that the ability of drug injections given after the antigen to suppress the response decreases as the time at which the drug is injected increases. No other drugs in the same class exhibit this accelerating behavior.

Due to the complicating effects of the drug itself on immune responsiveness, we determined the radioprotective effect at 90 days after exposure. Mice were exposed to graded sublethal doses of γ -rays, with and without prior injection of 500 mg kg^{-1} of WR-2721. Ninety days later, we assayed the primary immune response. From the slopes of DPFC on radiation dose we obtained an estimate of protection for WR-2721. This drug is able to increase the radioresistance of the primary response by a factor of 3.4 and therefore protects the tissues sufficiently from the standpoint of possible application to radiotherapy.

We are continuing these studies and attempting to determine whether WR-2721 is able to protect the secondary immune response, and we are studying the nature of its accelerating effect.

CARCINOGENESIS PROGRAM

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Respiratory Carcinogenesis

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 T. H. Corbett
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Mechanisms of Chemical Carcinogenesis

William Lijinsky

Mutagenicity of Carcinogens

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Somatic Cell Genetics

E. H. Y. Chu

Repair Mechanisms in Carcinogenesis

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 J. S. Cook
 J. D. Regan

Immunology of Carcinogenesis

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RNA Tumor Virus-Cell Biology

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Enzymology of Carcinogenesis

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Regulation of Gene Expression

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Biochemistry of Viral Carcinogenesis

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RNA Tumor Virus-Developmental Processes

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LUNG TUMOR INCIDENCE AND HISTOPATHOLOGICAL FINDINGS IN MICE CHRONICALLY EXPOSED TO CALCIUM CHROMATE DUST

P. Nettesheim, M. G. Hanna, Jr., D. G. Doherty, R. F. Newell, and A. Hellman

A number of epidemiological studies on men working in chromium plants have indicated that high occupational exposures to chromium compounds increase the risk of bronchogenic carcinoma. However, the specific agent responsible for this increased risk has not been identified to date, partly because most experimental attempts to produce respiratory-tract tumors with identified chromium compounds and mixtures have met with little success.

A total of 1090 specific-pathogen-free C57BL/6 mice were exposed in two inhalation chambers to calcium chromate dust 5 hr per day, 5 days per week, throughout their lifetime. Half of the animals in each of the two inhalation chambers were irradiated 4 weeks before the start of the inhalation exposure with 100 r whole-body X-radiation. All the mice in one of the chambers were infected with influenza virus 2 weeks before CaCrO₄ exposure was begun. The same pretreatment (100 r whole-body X-irradiation, PR8 virus infection) was given to 1090 control animals, which then were exposed to filtered air.

Chronic inhalation exposure to calcium chromate dust resulted in a fourfold increase in lung tumor incidence (adenomas and adenocarcinomas). Acute exposure to 100 r whole-body X-radiation caused a similar increase in tumor incidence. The effects of the two tumorigenic insults were not additive (Table I). PR8 influenza virus infection suppressed spontaneous as well as X-ray- and CaCrO₄-induced

TABLE I. Effects of X-radiation and CaCrO₄ exposures on lung tumor incidence*

	X-ray	No X-ray
CaCrO ₄	24	21
Air	19	5

*Numbers indicate number of animals with lung tumors in each exposure group. For each of the 4 exposure combinations, 545 mice were under test.

lung tumor development (similar suppression was observed in a previous experiment using gasoline fume exposure). CaCrO₄ also induced other pathological changes in the respiratory tract, such as epithelial necrosis and regenerative hyperplasia in the conducting airways, alveolar epithelialization, alveolar proteinosis, and emphysema-like changes.

EFFECT OF CHRONIC EXPOSURE TO ARTIFICIAL SMOG AND FERRIC OXIDE ON DEVELOPMENT OF DIETHYLNITROSAMINE-INDUCED LUNG TUMORS IN HAMSTERS

P. Nettesheim and D. A. Creasia

This inhalation program is a continuation of the study to determine the role of air pollutants on the induction of lung tumors. The major purpose of the study is to determine whether inhalation of gaseous and particulate air contaminants, such as synthetic smog and ferric oxide dust, would affect the respiratory tract tumor response of hamsters induced by diethylnitrosamine. Synthetic smog had previously been shown to increase the lung tumor incidence in mice, and ferric oxide dust, when injected intratracheally, was found to increase the lung tumor response of hamsters to diethylnitrosamine. The design of the experiment and the exposure and mortality data are summarized in Table I.

TABLE I. Hamsters. Chamber operations through June 30, 1971

Chamber	Date started (1969)	Co-treatment	Chemical	Exposure (weeks)	Animals (no.)	Total dead
29*	3/26	None	None	119	135	123
30*	4/9	DEN†	None	114	135	135
31	5/7	None	Ozone, 1 ppm; gasoline, 108 mg/m ³	113	135	126
32	5/21	DEN†	Ozone, 1 ppm; gasoline, 108 mg/m ³	111	135	125
33	10/1	None	Ozone, 1 ppm; gasoline, 108 mg/m ³ ; Fe ₂ O ₃ , 40 mg/m ³	92	135	93
34	10/15	DEN†	Ozone, 1 ppm; gasoline, 108 mg/m ³ ; Fe ₂ O ₃ , 40 mg/m ³	90	135	111
35	6/11	None	Fe ₂ O ₃ , 40 mg/m ³	108	135	113
36	7/2	DEN†	Fe ₂ O ₃ , 40 mg/m ³	105	135	126
37	10/29	None	Filtered air	88	135	92
38	11/13	DEN†	Filtered air	86	135	89
Total (all chambers)					1348	1133

*Nonchamber controls, kept in wire cages.

†3 mg total, given in 12 weekly subcutaneous injections of 0.25 mg each starting at 6 weeks of age.

The hamster inhalation program has been in operation for more than 2 years. One group of nonchamber control animals has already been terminated, and four more exposure groups are down to the last few animals. To date, approximately 70% of the original 1348 hamsters have died. Histological data from all animals that have died or been killed are currently being evaluated. Data on tumor incidence as related to the various exposures are expected to become available in the next 6 months.

Lung contents of ferric oxide have been determined after exposures of various durations. There is a direct correlation between length of exposure and amount of ferric oxide retained in the lungs, which ranges as high as 2700 μg for a 3-month exposure. Ferric oxide contents from longer exposure times are yet to be determined.

CHRONIC EXPOSURE OF HAMSTERS AND RATS TO PROTOTYPE AIR POLLUTANTS

D. A. Creasia and P. Nettesheim

The three major gaseous pollutants present in ambient air of large urban areas are SO_2 , NO_2 , and ozone. Much attention has been given to these pollutants as causative agents in the development of chronic respiratory disease. Recently it was shown that at least one of these pollutants, SO_2 , may also enhance the carcinogenic effect of certain inhaled carcinogens. Systematic studies are needed to confirm and extend this finding: Do other reactive gases have a similar enhancement effect on the carcinogenic process, and by what factor is the tumor yield increased? Because of the differences in tumor response and susceptibility to respiratory infection and other diseases, the selection of animal species for such studies is of utmost importance. Therefore, we conducted subchronic toxicity studies in rats and hamsters.

Three exposure systems were set up. One system exposed animals to SO_2 alone, one exposed them to SO_2 and an NaCl aerosol, and the other system exposed the animals to NO_2 alone. SO_2 is an upper respiratory irritant and NO_2 a lower respiratory irritant. SO_2 was used with an NaCl aerosol because the aerosol is inert by itself but potentiates the effect of SO_2 . It has been rather well established that SO_2 alone, in concentrations usually found in polluted atmosphere, does not cause much respiratory distress.

Because of the rat's known susceptibility to respiratory infection and its possible role in tumor induction, bacteriology and virology tests were performed on all animals at autopsy. It appears that chronic exposure to these pollutants increases the incidence of respiratory infection, as indicated by serum antibodies that developed against several viral agents. The animals were killed serially after exposures of various lengths, and the entire respiratory tract was prepared for histology. So far, no morphological evidence of respiratory tract infection has been observed (in spite of the serological findings). The response of the tracheobronchial epithelium to the toxic inhalants is still being studied. Both rats and hamsters seem to be well suited for chronic cocarcinogenesis experiments. In both species, respiratory tract tumors can be induced with topical and systemic carcinogens.

INDUCTION OF SQUAMOUS CELL CARCINOMA IN THE RESPIRATORY TRACT OF MICE

P. Nettesheim and Anna S. Hammons

In respiratory carcinogenesis studies, rats and hamsters have by and large replaced the mouse as the experimental animals of choice. The reason is that most investigators seem to believe that the so-called pulmonary adenoma is the only tumor readily inducible in the mouse respiratory tract.

We injected BC3F₁ and DBA/2 mice with 4-6 weekly intratracheal injections of 3-methylcholanthrene. Out of the 36 BC3F₁ mice surviving the carcinogen application, 31 developed invasive, metastasizing squamous and adeno-squamous carcinomas within 4-24 weeks after the last injection. The tumor incidence in DBA/2 mice was less. Some of the tumors were transplanted for 4-7 generations and have retained their squamous cell characteristics. Two tumor lines are still being maintained. The exact origin of the tumor has not yet been established. Involvement of large and small bronchi was demonstrated in several instances.

Our findings suggest that in spite of earlier discouraging reports, the mouse may yet be a useful experimental animal in respiratory carcinogenesis studies.

EARLY RESPONSE OF RESPIRATORY TRACT TISSUES OF RATS AND HAMSTERS TO POLYCYCLIC AROMATIC HYDROCARBONS

Hans Schreiber, D. H. Martin, and P. Nettesheim

Some of the newly developed lung cancer models are based on the observation that mixtures of finely dispersed polycyclic hydrocarbons and inert carrier dusts, when introduced into the respiratory tract of rats or hamsters, induce a high incidence of respiratory tract tumors. To refine the existing experimental models further, several problems still need to be resolved. (1) What is the early response of hamster and rat lung tissue to the most commonly used carcinogenic polycyclic hydrocarbons, methylcholanthrene and benzpyrene? How do these two animal species differ in their response? How do the two carcinogens differ in the response they evoke? (2) What is the site of origin of the primary carcinogen-induced lesions, and how do they progress to neoplastic lesions? (3) What is the role of the carrier dust in the experimental lung cancer models?

Our experiments, which are still in progress, are beginning to answer some of these questions. In rats, high doses of benzpyrene and methylcholanthrene induce an early, rapidly expanding metaplastic response, which originates in the terminal and respiratory bronchioles rather than in the bronchi. The resulting lesions may occupy as much as half of the entire lung parenchyma. Addition of carrier dust (ferric oxide) to the carcinogen does not noticeably affect this metaplastic response.

The response of hamster lungs to an identical treatment is quite different. MCA and BP alone induce only microscopic granulomas in the parenchyma (i. e. within 2 to 3 months after administration of the carcinogen). Few metaplastic changes in the trachea and the bronchi are found.

The addition of ferric oxide to the carcinogen markedly enhances the response in hamsters, as is evident from the early appearance of premalignant lesions in the tracheobronchial tree.

MORPHOGENESIS OF ALVEOLAR BRONCHIOLIZATION

P. Nettesheim and A. K. Szakal

So-called bronchiolization of alveoli can be observed after a variety of insults, such as respiratory infection, exposure to chemical irritants and carcinogens, and even circulatory disturbances involving the lung. It is uncertain whether the cuboidal and often columnar cells lining the alveoli resemble in all instances typical bronchiolar epithelium, and whether the histogenesis of the alveolar "epithelialization" is the same in the various pathological conditions.

We observed alveolar bronchiolization in mice exposed to ozonized gasoline fumes or CaCrO_4 dust. The epithelial cells lining the affected alveoli were identified by electron microscopy as typical bronchiolar cells. Basement membrane pores were often found in the bronchiolar walls of exposed mice, laterally connecting the bronchioles with adjacent alveoli [Fig. 1 (below)]. In animals exposed to gasoline fumes, these openings were always occupied by cells resembling bronchiolar epithelium. In CaCrO_4 -exposed animals with bronchiolar epithelial hyperplasia, the pores appeared to have developed into small bronchiolar-alveolar channels with a patent lumen and a length of 2-3 cell diameters. These bronchiolar-alveolar pores have hitherto not been described. Bronchiolar epithelialization was seen only occasionally in age-matched control animals; openings in the basement membrane have not yet been identified in controls. We propose that colonization of alveoli with bronchiolar cells via bronchiolar-alveolar pores is one morphogenetic mechanism of alveolar bronchiolization.

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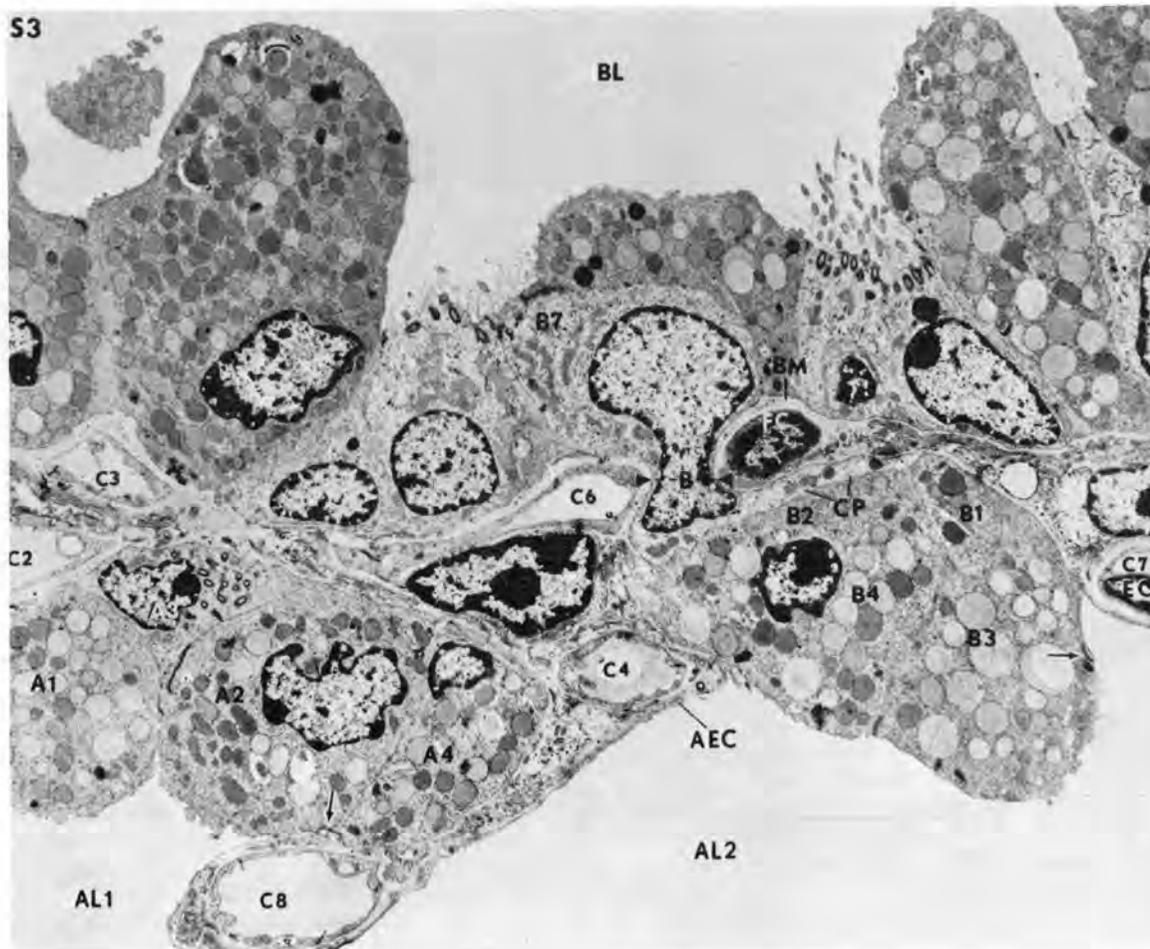


Fig. 1. Electron micrograph showing fenestration of bronchiolar-alveolar basement membrane with bronchiolar cell "migrating" into alveolus.

BL = bronchiolar lumen; AL2 = alveolar lumen; the arrows (B) point to bronchiolar-alveolar pore with "migrating" bronchiolar cell. Magnification $\sim 6000\times$.

DEVELOPMENT OF A BIOASSAY FOR INHALED CARCINOGENS

D. A. Creasia, F. D. Harvey, and P. Nettesheim

A major difficulty in any inhalation program using small laboratory animals is getting a large quantity of the test substance past the nasal labyrinth to the lung. These animals are usually obligatory nose breathers and cannot be maintained for extended periods of time with occluded nares. We have recently developed a technique for intratracheal cannulation of hamsters that effectively bypasses the nasal labyrinth without causing any major alterations in the respiratory pattern. The animals are not kept under anesthesia, and the nose bypass device has been kept in place for up to 5 hr.

The animal is first lightly anesthetized. The cannula is inserted into the trachea via the oral cavity and through the glottis. The cannula has an attached bite piece, and the jaws are clamped over it and held in place by a flexible but strong rubber head band. This also prevents the animal from expelling the cannula. Direct measurement of respiratory rate and tidal volume every 10 min for up to 5 hr has shown no significant change before and after cannulation. The animal is kept from physically removing the cannula by a loose-fitting, rigid, tubular body holder, through which only the neck and head protrude.

The essential aspects of this methodology for short-term exposure is complete. A comparison between cannulated and noncannulated animals exposed for 4 hr to ferric oxide particulates (mass concentration 40 mg m^{-3} and average size $< 1.0 \mu$) shows that lung deposition is increased by 200% for the cannulated animals.

Present studies are directed mainly toward adapting the technique for cigarette smoke exposure. The increased deposition results in rapid onset of nicotine and carbon monoxide toxicity. Therapeutic techniques are being evaluated for combating the toxicity while maintaining the increased deposition of smoke. Also under investigation is the feasibility of daily cannulation over many weeks, optimum cannula diameter, and different cannula material and design. Casts have been made of the hamster's oral cavity for optimizing the design of the cannula and bite piece. Deposition studies with radioactively labeled cigarette smoke are in progress, in collaboration with M. R. Guerin and W. L. Wayne of the Analytical Chemistry Division, ORNL.

DEVELOPMENT OF A PULMONARY CYTOLOGY TEST FOR USE IN EXPERIMENTAL RESPIRATORY CARCINOGENESIS STUDIES

Hans Schreiber, D. H. Martin, and P. Nettesheim

In recent years, several experimental models have been developed to study the morphogenesis and pathogenesis of bronchogenic carcinoma. To make full use of these animal models it is necessary to develop a sensitive technique by which early changes in respiratory-tract epithelium caused

by noncarcinogenic and carcinogenic substances can be spotted and which permits detection of the sequential cellular changes following the application of such substances. Therefore, we developed a method to obtain repeated exfoliative cytology specimens from rats and hamsters without causing damage to the respiratory tract. The principle of the equipment used is illustrated in Fig. 1. We have shown that adequate samples can be obtained from normal and carcinogen-treated animals. Preliminary attempts to correlate the cytological with the histopathological findings (specific tumor type) found in carcinogen-treated animals seem promising. Cells suggestive of malignancy were detected several months before the animals died from respiratory tract carcinomas. We believe that this newly developed technique might also assist the interpretation of some of the findings made by clinical cytologists who are concerned with the diagnosis of lung cancer in man.

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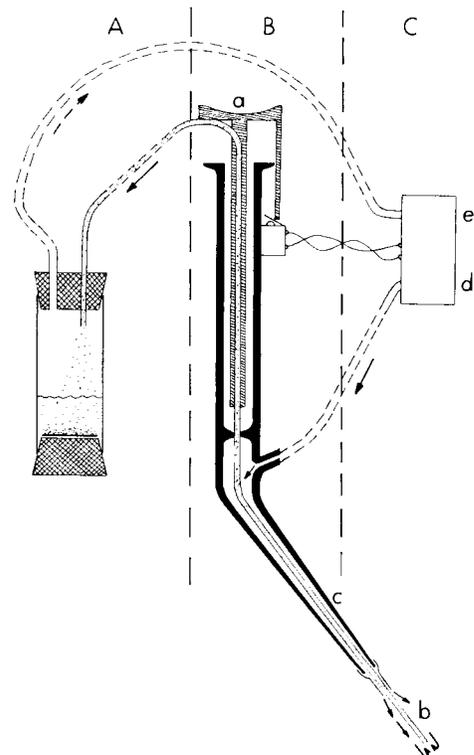


Fig. 1. Diagram of the intratracheal sampling device.

A, collecting system; B, sampling catheter; C, regulating unit. The distal end of the catheter shaft is inserted into the larynx. Then the plunger (a) is pressed down; this pushes the collecting tube (b) out of the shaft (c) into the trachea and at the same time activates the injection pump (d) and the vacuum (e). The released washing fluid rinses the tracheal walls and is reaspirated by vacuum into the collecting tubing (b) and delivered into the collection vessel (A).

THE METABOLISM OF CARCINOGENIC HYDROCARBONS

T. H. Corbett

Certain polycyclic, aromatic hydrocarbons are among the most potent carcinogens. The metabolism of these compounds, which leads to their covalent binding to tissue constituents (the event probably responsible for their carcinogenic activity) is not understood. We are attempting to obtain information concerning this metabolism for the potent carcinogenic hydrocarbons 9,10-dimethyl-1,2-benzanthracene and 3-methylcholanthrene. We have been able to cleave several bound forms of these hydrocarbons (two and three forms, respectively from cellular proteins by treatment with Raney nickel. One can conclude that the compounds are bound through the sulfur of cysteine (Raney nickel selectively cleaves carbon-sulfur covalent bonds).

Strain and species variations in metabolism are being studied. BALB/c, BC3F₁, and DBA/2 mice carry out essentially the same hydrocarbon metabolism in both lung and skin, although the DBA/2 mouse is much less efficient in this regard. In all cases, the skin tissue is about tenfold more efficient than the lung in metabolizing the hydrocarbons to sulfur-bound forms. Rat and hamster tissues are also being studied. It appears that there are marked species differences in hydrocarbon metabolism.

Studies are also in progress concerning the metabolism of these carcinogens in various parts of the respiratory tract—i.e. trachea, lung parenchyma, and alveolar macrophages.

DEVELOPMENT OF COMBINED IN VITRO AND IN VIVO SYSTEMS FOR CHEMICAL CARCINOGENESIS STUDIES WITH RESPIRATORY TRACT TISSUES

T. H. Corbett

Attempts are being made to develop cell cultures from adult mice, rats, and hamsters. We have found that stable cell cultures from tracheas but not from lungs can be easily established. Cultures were treated for periods of a few hours up to several days with various amounts of one of the following carcinogens: dimethylbenzanthracene, methylcholanthrene, benzpyrene, propane sulfone, or ethylnitrosourea. After carcinogen exposure, the cells were subcutaneously injected into syngeneic hosts, which were kept for several months to observe possible tumor development.

Organ cultures of adult mouse and rat tracheas were also established and were successfully kept *in vitro* for up to 6 weeks. Short-term cultures were exposed for several hours or days to the same carcinogens listed above and were then implanted into syngeneic hosts. Similar studies were also carried out with fetal lung tissue. The outcome of these experiments is presently still uncertain, because of the short observation period.

EFFECT OF INFLUENZA VIRUS INFECTION ON THE PULMONARY CLEARANCE OF ⁵¹Cr₂O₃ PARTICLES IN MICE

D. A. Creasia and P. Nettesheim

The respiratory tract is the prime route of entry for airborne carcinogens into the body. The factors that control the residence time of inhaled particulates is therefore important in evaluating the exposure to inhaled carcinogens. Experiments in which influenza-infected mice were injected intratracheally with ⁵¹Cr₂O₃ show that pulmonary clearance is greatly reduced in infected animals.

Mice were injected with ⁵¹Cr₂O₃ on days -2, 0, and +2 and weeks 1, 3, 5, 7, and 9 after infection. All animals showed a positive serum titer to the virus. Fig. 1 is typical of the data produced at all time points of intratracheal injection. Animal number 12.4 in Fig. 1 did not show any gross pathology at autopsy, despite a positive serum titer. All animals with less than 10% lung scarring cleared ⁵¹Cr₂O₃ particles similarly to noninfected animals.

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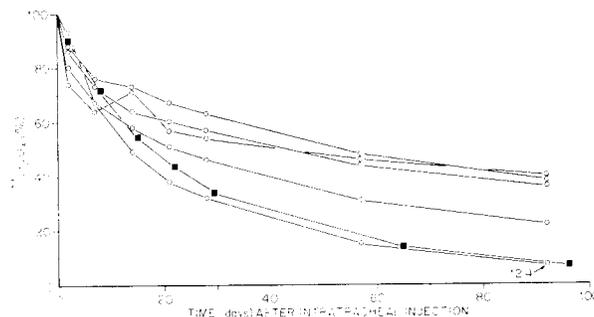


Fig. 1. Respiratory clearance of ⁵¹Cr₂O₃ by influenza-infected mice 3 weeks postinjection.

■—■, controls; ○—○, infected mice.

Preliminary interpretation of the data suggests that the deep lung clearance mechanism (phagocytosis) *per se* is not impaired, but that the ⁵¹Cr₂O₃ becomes inaccessible, possibly due to incorporation into inflammatory or scar tissue. For 10 days after intratracheal injection, the clearance pattern of infected animals is similar to that of noninfected animals. This indicates that the main respiratory clearance mechanism is not impaired. After 10 days, however, the rate of clearance in the infected animals becomes very slow. Particulates that have been "sequestered" by pathologically altered lung tissue may not be available to phagocytosis and may have to be cleared, if at all, via the lymphatic system.

EFFECT OF ANTIGEN INJECTION ON URETHANE-INDUCED LUNG TUMOR INCIDENCE IN MICE

P. Nettesheim and Anna S. Hammons

Evidence is accumulating in our laboratory indicating that the lung tumor response to chemical carcinogens may be enhanced by intercurrent respiratory tract infection. The mechanism for this enhancement effect of infection is unknown. In addition to perturbation of the normal cellular proliferation and differentiation by the respiratory infection, failure of the local immune surveillance mechanism due to preoccupation with the microbial antigens may be a contributing factor. To test this hypothesis, BALB/c mice were injected with a dose of urethane sufficient to induce several pulmonary adenomas per mouse within 3-4 months. Four weeks after urethane administration, the animals were subjected to massive antigenic stimulation by intratracheal and intraperitoneal injection of four different nonproliferating antigens. Control mice received either urethane plus isologous red blood cells or antigens only. The mean tumor count per animal (20 animals per group) 4 months after urethane treatment (or 3 months after antigen treatment) showed 0.05 tumors in the antigen-only controls, 3.3 tumors in the urethane and isologous red blood cell group, and 7.9 tumors per animal in the urethane plus antigen group. These results indicate that massive antigenic stimulation markedly affects the urethane-induced tumor response of the lung.

EFFECT OF MICROBIAL FLORA, RESPIRATORY INFECTION, AND VITAMIN A ON N-NITROSOHEPTAMETHYLENIMINE-INDUCED LUNG TUMORS IN RATS

Hans Schreiber, P. Nettesheim, C. B. Richter, H. E. Walburg, Jr., and W. Lijinsky*

This study was initiated to determine whether the general microbial flora, respiratory tract infection, and A hypervitaminosis affect tumor induction or the type of lung tumor induced in Sprague-Dawley rats by N-nitrosoheptamethyleneimine. The following groups of rats received the carcinogen in drinking water (22 weeks, total dose 140 mg): (1) germ-free rats, (2) specific-pathogen-free (SPF) rats, (3) conventional rats with clinical symptoms of spontaneous respiratory tract infection, (4) SPF rats intranasally infected with *Mycoplasma pneumoniae* and Sendai virus, and (5) SPF rats treated with high levels of vitamin A. The vitamin A treatment was chosen because there is evidence in the literature suggesting that this vitamin may inhibit tumor development in mucous membranes.

Two weeks after the cessation of the treatment the animals were killed. At the present time the SPF group and the group with spontaneous respiratory tract infection have been terminated and macroscopically evaluated, while the other experimental groups are still in program. In the SPF group, 43% of the males and 86% of the females showed tumors of 2 mm or larger diameter; the tumor incidence in the infected rats was 83% for the males and 100% for the females. These preliminary data suggest that pulmonary infection "promotes" the development of lung tumors in rats.

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INITIATION OF THE HUMORAL ANTIBODY RESPONSE VIA THE RESPIRATORY TRACT

P. Nettesheim and Mary L. Williams

Little is known about the capability of the lung and the associated lymphatic tissues to handle antigens and mount an immune response against them. This lack of basic information is surprising in view of the facts that the respiratory tract, next to the digestive tract, is the organ system most frequently exposed to living and nonliving antigens and that disease due to contact with such antigens is very common. To develop more basic information regarding immunocompetence residing in the respiratory system is also highly desirable for the development of more effective immunotherapy of lung cancer.

Therefore, we initiated a series of studies designed to investigate the immune response following intratracheal antigenic stimulation of normal mice and mice preexposed to *Bordetella pertussis*. We found that in normal mice, antigenic stimulation via the respiratory tract is very ineffective compared with the intravenous or intraperitoneal route (Fig. 1); 10^9 sheep red blood cells were required intratracheally to obtain an antibody response comparable to that following intravenous injection of 10^6 cells. However, when antigenic stimulation was preceded by intratracheal *Bordetella pertussis* injection (1-9 days prior to antigen), the response to intratracheally administered sheep red blood cells was greatly increased.

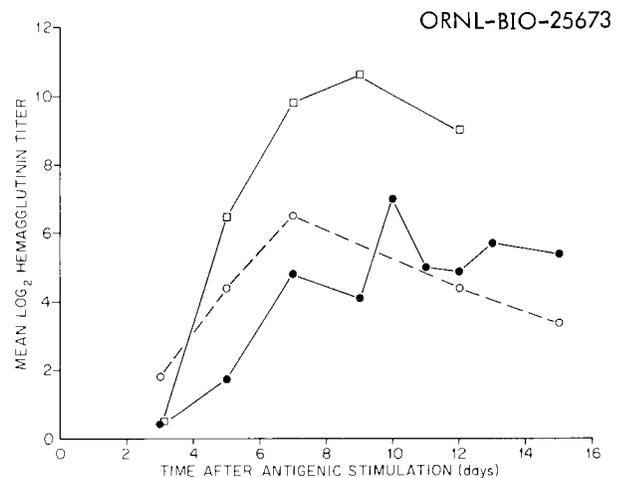


Fig. 1. Serum hemagglutinin response of BC3F₁ mice injected with 10^6 sheep RBC intravenously ○—○; 10^9 sheep RBC intratracheally ●—●; and 10^9 sheep RBC intratracheally 6 days after intratracheal *Bordetella pertussis* administration □—□. Five to 10 animals per point.

Extensive studies were performed to determine the site of humoral antibody formation, using the hemolytic plaque technique. These studies showed that the vast majority of the antibody-forming cells both in the normal and in the *B. pertussis*-stimulated animals reside in the spleen. The number of antibody-forming cells found in the tracheobronchial lymph nodes and in the lung was 1% or less of the number detached in the spleen.

Further studies are under way to determine the fate of intratracheally administered antigen, using soluble and particulate (aggregated) labeled serum proteins. We are also developing a lung tumor assay to study tumor immunity in the respiratory tract.

RECOVERY OF THE ANTIGEN RETENTION MECHANISM AFTER X-IRRADIATION. EFFECT OF TRANSFER OF CELLS DERIVED FROM VARIOUS SOURCES

P. Nettesheim and Anna S. Hammons

The effects of spleen, thymus, bone marrow, and peritoneal exudate cells on the recovery of X-ray-damaged antigen localization in spleen follicles was determined. Infusion of spleen or peritoneal exudate cells leads to immediate, partial restoration of the antigen-trapping system and progressive recovery. No significant recovery was observed on day 1 or 8 after 600 r in animals receiving either thymus cells, bone marrow cells, or no cells. The data suggest that spleen and peritoneal exudate cell suspensions contain cells (or their precursors) involved in follicular localization. Further studies are needed to determine the nature of the cells involved and precisely what role they play in the enhancement of recovery of antigen localization after X-irradiation.

ROLE OF ACUTE RESPIRATORY INFECTION IN LUNG CANCER

C. B. Richter, Carole S. King, and J. A. Franklin

A previous report was concerned primarily with the study of chronic respiratory infection and its possible role in lung cancer, using the mouse as a model. Those studies are long-term and not yet completed. During the course of experimental pathology studies with acute parainfluenza virus (Sendai) infections in mice, considerable damage was found in the bronchial epithelium, particularly in the secondary bronchi and smaller bronchioles. The virus is lytic to infected epithelial cells and results in cell death and rapid replacement by division of surviving cells. Replacement cells are frequently anaplastic and disoriented during the period immediately following rapid growth. For these reasons, it was decided to study the cell kinetics of mouse parainfluenza virus infection to determine whether single events during the peak period of cell replication could significantly alter the incidence of bronchogenic carcinomas.

Light microscopic autoradiograms of control DBA/2 mice have shown a bronchial epithelium labeling index of about 1:375, using ^3H -thymidine as labeling material. This suggests that one of every 375 bronchial epithelial cells is dividing at any given time in this strain of mouse. During peak cell division in obviously infected bronchi after experimental virus inoculation, the ratio changes to 1:5 and in some bronchi is as high as 1:2. This indicates that very high percentages of epithelial cells are synthesizing DNA during this period, and thus large populations of cells are presumably available for carcinogenic action during a critical period.

Studies currently in progress will employ single- and multiple-hit carcinogen exposures during the peak period

versus single- and multiple-hit exposures in uninfected control animals. Further details of the experimental pathology in the bronchi and alveoli remain to be completed.

MUTAGENIC ACTIVATION OF DIMETHYLNITROSAMINE BY MOUSE LIVER MICROSOMES

H. V. Malling, E. H. Y. Chu, and D. Wild*

Dimethylnitrosamine (DMN) is a potent carcinogen in several rodents and is thought to be metabolized before becoming tumorigenic. This probably results in the intracellular formation of a methylating agent. These metabolic processes can be carried out by the microsomal fraction of liver. A direct study of the mutagenicity of the metabolic breakdown products of DMN can be made by incubating DMN with a suspension of liver microsomes in the presence of an indicator organism for mutagenicity. The livers from 4-6 mice [(C57BL/6 X C3H) F_1], 10-14 weeks of age, were used in each experiment.

Four different, histidine-requiring strains of *Salmonella typhimurium* were used as indicator organisms for mutagenic activity. The induction of mutations was scored by observing histidine revertants in any of these four strains. Two of the strains (TA1530 and G46) contain the same mutant allele, which can be reverted by a reverse mutation or by a variety of extragenic suppressor mutations. Strain TA1530 contains in addition a deletion of the gal-bio-uvr B region of the chromosome. Strain C207 reverts of single base-pair addition. Strain C3076 reverts by single base-pair deletion.

The data indicate that only the strains which revert by base-pair substitution revert after treatment with DMN and liver enzymes. Strain TA1530 was then selected for further study.

The increase in the frequency of histidine revertants in TA1530 after incubation for various lengths of time in a reaction mixture containing DMN, a suspension of liver microsomes, and required cofactors is exponential with the power of 3.3 of the treatment time. Since the reaction mixture is complex, the importance of the different components in the reaction mixture was studied. The results indicate that the complete system is necessary for maximum activity. Only in the case where magnesium is deleted from the reaction mixture is any residual activity found. This could be due to traces of magnesium present in the microsome fraction.

Preliminary studies with Chinese hamster cells in tissue culture have shown that a combined treatment with DMN and microsomes gives a significant increase in the frequency of azaguanine-resistant mutants over treatments with the components alone, which did not show any difference from the untreated control. One of the serious problems for test systems for mutagenicity is the degree of relevance to man. A system utilizing human liver fractions and a microbial or mammalian indicator system for mutagenic activity similar to the one described in this paper is a direct approach to this problem.

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THE MUTAGENICITY OF CHEMICAL CARCINOGENS IN *NEUROSPORA CRASSA*

Tong-man Ong and F. J. de Serres

The mutagenicity of aflatoxins (B_1 , B_2 , G_1 , and G_2), ethyleneimine (EI), 1,2,3,4-diepoxy cyclohexane (DECH), 1,2,4,5-diepoxy pentane (DEP), 1,2,7,8-diepoxy octane (DEO), 1-phenyl-3,3-dimethyl triazene (PDMT), and 1-phenyl-3-monomethyl triazene (PMMT) was tested in the *ad-3* system of *Neurospora crassa* in an attempt to determine whether there is a correlation between carcinogenicity and mutagenicity of chemical carcinogens. Aflatoxins B_1 and G_1 are carcinogenic in rats, whereas aflatoxin B_2 is not. At a much higher dosage, aflatoxin B_2 is carcinogenic in rainbow trout but not aflatoxin G_2 . The carcinogenicity of aflatoxin B_1 both in rats and in rainbow trout is much higher than that of aflatoxin G_1 . Both EI and triethylenemelamine (TEM) are carcinogenic in rats, but the carcinogenicity of the polyfunctional compound TEM is higher than that of the monofunctional compound EI. DEP and DEO are carcinogenic in mice, whereas the related compound DECH is not. Both PMMT and PDMT are carcinogenic in rats; however, PDMT is less carcinogenic than PMMT.

Treatment of a genetically marked two-component heterokaryon of *N. crassa* with the agents described above was carried out by one of the following methods: (1) conidia were treated with the chemicals in 0.067 M sodium-phosphate buffer solution for 2 hr at 25° C, or (2) vegetative cultures were grown on a medium containing the chemicals. The treated conidia or conidia from the cultures grown on the medium were used for analyzing for the presence of *ad-3* mutants by the modified direct method (1). The results show that aflatoxin B_1 , aflatoxin G_1 , EI, DEP, DEO, PDMT, and PMMT are mutagenic, whereas aflatoxin B_2 , aflatoxin G_2 , and DECH are not mutagenic in *N. crassa*. The mutagenicity of aflatoxin B_1 is higher than that of aflatoxin G_1 . EI has lower mutagenicity than TEM (2), and PMMT was found to be a more effective mutagen than PDMT. Hence, there is a positive correlation between carcinogenicity and mutagenicity of the compounds tested (Table I).

TABLE I. Comparison between carcinogenicity and mutagenicity of several chemical agents

Group	Compound	Mutagenicity	Carcinogenicity
1	Aflatoxin B_1	++	++
	Aflatoxin B_2	-	±
	Aflatoxin G_1	+	+
	Aflatoxin G_2	-	-
2	DEP	+	+
	DEO	+	+
	DECH	-	-
3	EI	+	+
	TEM	++	++
4	PDMT	+	+
	PMMT	++	++

The number of plus signs (+) reflect qualitative differences in the activity of compounds within the same group.

The isolated *ad-3* mutants are being characterized by a series of genetic tests, which in turn will determine whether there is a relationship between carcinogenicity and the type of genetic alterations caused by chemical carcinogens.

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2. H. V. Malling, personal communication.

INDUCTION OF RECESSIVE-LETHAL MUTATIONS IN *NEUROSPORA CRASSA* BY 2-NAPHTHYLAMINE

Tong-man Ong

2-Naphthylamine (2-AmNap) is a known chemical carcinogen that causes bladder tumors in dogs and man and induces hepatoma in mice. A closely related compound, 1-naphthylamine (1-AmNap), is a very weak bladder carcinogen compared to 2-AmNap, and 1-AmNap does not induce hepatoma in mice. Both 2- and 1-AmNap have recently been isolated from cigarette smoke.

Studies on the mutagenicity of 1- and 2-AmNap have shown that both compounds failed to induce point mutations in *Drosophila melanogaster* and *E. coli*. The mutagenicity of both agents has now been tested in *Neurospora crassa*. The results show that both 1- and 2-AmNap are not mutagenic when tested under conditions where resting conidia were treated with 1- or 2-AmNap (0.8 mM) in sodium-phosphate buffer (pH 7) at 25° C for 2 hr. However, 2-AmNap is mutagenic in metabolically active, vegetative cultures of *N. crassa*. When the vegetative cultures were treated with 2-AmNap (0.4 mM) the *ad-3* mutation frequency increased to 27×10^{-6} survivors, which is a 65-fold increase over the spontaneous mutation frequency. The mutation frequency was not significantly increased over the spontaneous mutation frequency when vegetative cultures were treated with 1-AmNap (0.4 mM). Hence, there is a positive correlation between the carcinogenicity and mutagenicity of both compounds.

It has been suggested that metabolic conversion might be necessary for the carcinogenicity of 1- and 2-AmNap. It seems that metabolic conversion might also be necessary for the mutagenicity of 2-AmNap (probably also 1-AmNap), and that this conversion could occur in the enzymatically active vegetative cultures of *N. crassa*. Therefore, tests for the mutagenicity of chemical carcinogens in vegetative cultures of *N. crassa* might be an ideal method for those compounds requiring metabolic conversion for their carcinogenic activities.

EFFECT OF HEAT ON THE MUTAGENICITY OF AFLATOXINS B_1 AND G_1

Tong-man Ong

Aflatoxins, the metabolites of certain strains of *Aspergillus flavus*, are carcinogenic in rats and rainbow trout, teratogenic in hamsters, mutagenic in *Neurospora crassa* and *Drosophila melanogaster*, and toxic in all animal species tested. Unfortunately, aflatoxins have been found as natural contaminants in various foodstuffs, and many of these foodstuffs were found to contain a biologically significant level of these compounds. Due to the direct and indirect hazards of aflatoxins to human health, attempts have been made to detoxify aflatoxins by heat, chemical, and biological treatments.

The effect of heat on the mutagenicity of aflatoxins B₁ and G₁ has been tested in *N. crassa* by growing vegetative cultures of *Neurospora* in 10 ml of Fries' basal medium (supplemented with 1% sucrose and 1.5% agar) containing 400 µg of aflatoxin B₁ or G₁. Aflatoxin was added either before or after the medium was autoclaved for 15 or 30 min. Conidia from these vegetative cultures were harvested and assayed for the presence of ad-3 mutants by the modified direct method. The results show that the ad-3 mutation frequencies induced by aflatoxin B₁ or G₁ autoclaved with medium at 15 lb in⁻² (122° C) for 15 or 30 min are not significantly different from the mutation frequencies induced by unautoclaved aflatoxin B₁ or G₁. This study indicates that the mutagenicity and possibly also the carcinogenicity of aflatoxins B₁ and G₁ cannot be destroyed by ordinary cooking or autoclaving.

MUTAGENIC ACTIVITY OF 4-NITROQUINOLINE 1-OXIDE AND 4-HYDROXYAMINOQUINOLINE 1-OXIDE IN *NEUROSPORA CRASSA*

B. E. Matter* and Tong-man Ong

4-Nitroquinoline 1-oxide (4NQO) and 4-hydroxyaminoquinoline 1-oxide (4HAQO) are very potent carcinogens in mice and rats. 4HAQO is a metabolic intermediate of 4NQO and is suspected to be the active compound in regard to 4NQO carcinogenesis (1).

The mutagenicity of both compounds was tested in the ad-3 system of *Neurospora crassa*. Conidia from a two-component heterokaryon were treated for 2 hr with various concentrations of 4NQO and 4HAQO, ranging from 2 to 16 X 10⁻⁸ M and 7.5 to 60 X 10⁻⁸ M, respectively. The ad-3 mutants were selected by the direct method (2) and can result from either point mutations or chromosomal deletions. The results indicate that both compounds are mutagenic in *N. crassa*, 4HAQO to a somewhat lesser extent than 4NQO. Studies are in progress to characterize the ad-3 mutants and to compare the spectra of 4NQO- and 4HAQO-induced ad-3 mutations at similar forward-mutation frequencies.

*Visiting investigator from abroad. Permanent address: Pharmaceutical Division, SANDOZ Ltd., Basel, Switzerland.

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MUTAGENESIS AND MUTAGEN SPECIFICITY OF 2-ACETYLAMINOFLUORENE AND ITS METABOLITES

H. V. Mallng and F. J. de Serres

The role of the induction of mutations in the process of carcinogenesis is still unclear. One way to understand it is to study the mutagenicity of potent carcinogens. Many potent carcinogens do not react as such with the biological macromolecules in cells but are metabolized into very

reactive compounds (the ultimate carcinogens), some of which are mutagenic. 2-Acetylaminofluorene (2-AAF) is a strong carcinogen. One of its metabolic breakdown products is N-hydroxy-acetylaminofluorene (N-OH-AAF). This compound can be esterified at the N-OH position; some of these esters are probably the ultimate carcinogens. The sulfate and phosphate esters formed *in vivo* are very unstable compounds. The acetate ester is more stable; it has not been isolated in the *in vivo* systems but is known to react with biological macromolecules in the same way as the sulfate ester. The esters of N-OH-AAF are the only compounds known to alkylate the 8-carbon position of guanine in DNA.

The genetic effects of these compounds were studied on mutants obtained at specific loci in the ad-3 region of a dikaryon of *Neurospora crassa*. The ad-3 mutants are in the process of being characterized by means of tests for genotype and allelic complementation, as well as dikaryon and trikaryon tests to distinguish point mutations from multilocus deletions.

Even after very extensive attempts and varied experimental conditions, 2-AAF did not show any mutagenic activity. N-OH-AAF is a weak mutagen, whereas acetyl-N-OH-AAF is a very strong mutagen. Preliminary analysis of the acetyl-N-OH-AAF-induced ad-3 mutations has shown that most mutants resulted from point mutations and that few chromosome breaks were induced.

MUTAGENICITY OF CHEMICAL CARCINOGENS IN MAMMALIAN CELLS

E. H. Y. Chu, E. G. Bailiff, and H. V. Mallng

With the availability of genetic markers and the demonstration of chemical mutagenesis in tissue-cultured mammalian cells, it has become feasible to test in the same system both carcinogenicity and mutagenicity of a particular agent. We have studied the cytotoxicity and photodynamic effect of several carcinogenic and noncarcinogenic polycyclic hydrocarbons (1). This report summarizes some preliminary results on mutation induction in Chinese hamster cells with these compounds. The mutagenic change studied was from sensitivity to 8-azaguanine (wild type) to resistance. The compounds were first dissolved in dimethyl formamide and then added, at appropriate concentrations, to the cell cultures. The solvent at a final concentration of 1% in the medium reduced cell survival to about 70% but was not itself mutagenic. Cells were exposed to various polycyclic hydrocarbons for 24 hr in the dark, at concentrations that would permit normal or slightly reduced survival. Our results to date clearly show that 9,10-dimethyl-1,2-benzanthracene was highly mutagenic; 1,2,3,4-dibenzanthracene was nonmutagenic, whereas 1,2,5,6-dibenzanthracene caused slight but significant increases in the forward-mutation frequency. On the other hand, neither 1,2-benzpyrene nor 3,4-benzpyrene was shown to be mutagenic. These results are similar to a parallel study in *Neurospora*, with the exception that in hamster cells 1,2,5,6-dibenzanthracene exhibited mutagenic activity. The work has been extended to include other carcinogens, such as aromatic amines.

Our aim is to test the possibility that metabolic derivatives rather than the test compound *per se* were responsible for the final effect. It is hoped that studies along these lines may throw some light on the somatic mutation theory of carcinogenesis.

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IN VIVO INDUCTION OF CHROMOSOME ABERRATIONS WITH ALKYLATING AGENTS IN EHRlich ASCITES TUMOR CELLS AND RAT BONE MARROW CELLS

E. H. Y. Chu

Tumor-bearing C3H mice, 2–3 months of age, were treated intravenously with single, sublethal doses of ethyl methanesulfonate (EMS), methyl methanesulfonate (MMS), or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Tumor cells, without prior colchicine treatment, were repeatedly sampled from the same animal and fixed at successive 12-hr periods following injection of the chemical. In connection with a host-mediated study of mutations in *Neurospora*, cesarean-derived Charles River rats (Sprague-Dawley strain) were similarly treated with alkylating agents. After 1 hr of exposure to colchicine, the rat bone marrow cells were removed for chromosome preparations.

There was an exceedingly low frequency of spontaneous chromosome aberrations in control animals treated with saline alone. In both systems, and at the maximum sublethal doses used, neither EMS or MNNG induced chromosome aberrations. On the other hand, MMS effectively induced achromatic lesions (gaps), chromatid breaks, and exchanges in both types of cells. The frequency of aberrations increased with the amount of MMS administered and with time. Chromatid-type aberrations continued to appear in cell populations as late as 60 hr after treatment. No chromosome-type aberrations were found.

The delayed and continued appearance of chromatid-type aberrations in MMS-treated cells and the absence of chromosome-type aberrations cannot be explained entirely by mitotic inhibition. The possibilities of differential cell-stage sensitivity to MMS and of molecular action of MMS at sub-chromosomal levels have been considered and tested.

INDUCTION OF MUTATIONS IN MAMMALIAN CELLS IMPLANTED IN HETEROLOGOUS HOSTS

E. H. Y. Chu and H. V. Malling

Gabridge and Legator (1) have developed an animal-mediated microbial assay for a test of mutagenicity of chemicals. The indicator microorganisms used are *Salmonella typhimurium* and *Neurospora crassa* (2). We have used an aneuploid cell line (V79) of Chinese hamster for the same purpose. The cells are suspended in balanced saline, enclosed in dialysis bags, and inserted surgically into the peritoneal cavities of untreated rats. After various periods of time, the bags are removed and the recovered cells are plated *in vitro* to determine cell survival and the frequency of forward mutations to 8-azaguanine resistance.

Six, 24, and 48 hr after implantation, 20–40% of the original inoculum appeared viable by a dye exclusion test. The plating efficiencies of the recovered cells were 100, 28, and 5%, respectively, at these periods. In five independent experiments, the frequency of spontaneous mutations at this locus increased significantly after 18 hr and drastically after 24 or more hr of incubation in the rat. The spontaneous mutation frequency in different cell populations *in vitro* at the time of implantation experiments varied from 0.3 to 3.8×10^{-5} . In rats there was no difference in mutation frequency among cell survivors after 6 or 12 hr. After 24 hr, however, the mutation frequency increased to as high as 10^{-3} among survivors. These results confirm and extend a similar observation in *Neurospora* (3). The increase in the spontaneous mutation frequency in hamster cells observed in our experiments might have been caused by substance(s) that penetrated the dialysis bag.

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2. H. V. Malling and G. E. Cosgrove, in *Chemical Mutagenesis in Mammals and Man* (F. Vogel and G. Röhrborn, eds.), p. 271. Springer-Verlag, New York (1970).
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REPAIR OF CHEMICAL DAMAGE TO THE DNA OF HUMAN CELLS

R. B. Setlow and James D. Regan

If chemical damage to DNA *in vivo* is repaired by an excision-type mechanism in the presence of bromodeoxyuridine (BrdUrd), the characteristics of the repaired regions can be investigated by photolysis with 313-nm radiation followed by sedimentation in alkaline sucrose (1). Radioactively labeled cells were treated with chemicals, incubated in BrdUrd (^3H -labeled DNA) or dT (^{32}P -labeled DNA), and irradiated with 313 nm. The difference between the molecular weights of the two DNA's was measured by cosedimentation in alkaline sucrose.

Methyl methanesulfonate at 5×10^{-5} M for 2 hr produced 3.2 alkaline-labile regions, or breaks, per 10^8 daltons of DNA. These alkaline-labile regions did not disappear with time. A high fluence of 313-nm radiation resulted in the additional production of 0.35 scissions per 10^8 daltons in the BrdUrd-incubated material. Thus, there is a low level of repair that tends to be masked by the high level of unrepaired damage.

Ethyl methanesulfonate treatment at 10^{-2} M for 2 hr resulted in 3.3 alkaline-labile regions, or breaks, in 10^8 daltons. These regions did not disappear in a 20-hr incubation. Large fluences of 313-nm radiation made one additional break in 10^8 daltons of the DNA that had been incubated in BrdUrd. Thus, the number of repaired regions was approximately one-third of the number of detectable lesions.

4-Nitroquinoline oxide (NQO) was tested by a 90-min treatment followed by incubation for 20 hr. The results were: (1) NQO results in chain breaks in the DNA. In alkali there were more observable chain breaks in fibroblasts of xeroderma pigmentosum (XP) patients than of normal individuals. (2) After 20 hr incubation, there were fewer detectable chain breaks. Either the breaks were repaired or their alkaline

lability had decayed. (The recovery of molecular weight was not as great in XP cells as in normal cells.) (3) Unsubstituted DNA incubated in the presence of NQO is sensitized to 313-nm radiation, presumably because of a photodynamic effect of the bound dye. When cells are incubated after removal of NQO, the sensitivity of the DNA to 313 nm returns to that of untreated cells. The decay of this NQO-enhanced sensitivity was more rapid for normal than for XP cells. (4) Incubation in BrdUrd following NQO treatment results in an increased sensitivity to 313 nm, compared to cells incubated in dT. This result indicates the existence of repair-replicated regions in both XP and normal fibroblasts.

We conclude that there are at least two types of repair of NQO damage (and therefore at least two types of damage). One type acts in both XP and normal cells, the other preferentially in normal cells.

1. J. D. Regan, R. B. Setlow, and R. D. Ley, this report, p. 61.

OZONE IN THE UPPER ATMOSPHERE AND SKIN CANCER

R. B. Setlow

Ozone absorbs the shorter wavelengths in sunlight. The absorption is especially strong for those wavelengths that affect the biological activity of nucleic acids. Any permanent decrease in the level of ozone in the upper atmosphere, such as might occur from a fleet of supersonic transports, could have drastic biological consequences. I used fundamental biological data to estimate the quantitative relation between a decrease in ozone and an increase in the incidence rate of skin cancer among the U.S. white population. The estimate indicates that a 1% decrease in the average ozone level would result in an increase of 5000 additional skin cancer cases per year. An outline of the analysis goes as follows:

1. Skin cancer is associated with exposure to sunlight. (The incidence rate increases exponentially with decrease in latitude.) From the facts that individuals defective in the repair of UV damage to DNA are supersensitive to cancer induction and that wavelengths greater than 320 nm are ineffective in inducing skin cancer in experimental animals, we conclude that UV damage to cellular DNA results ultimately in the induction of skin cancer.
2. (a) The intensity in sunlight as a function of wavelength decreases rapidly for wavelengths less than 300 nm.
(b) The quantitative biological effects of UV light on simple biological systems decreases rapidly for wavelengths greater than 300 nm. (The effect follows the absorption spectrum of DNA.)
(c) The point-by-point product of the two curves in (a) and (b) (corrected for absorption of light by the skin) has a maximum between 295 and 300 nm. The area under this product curve is proportional to the biological effect of sunlight on DNA.
3. From the known absorption coefficient of ozone as a function of wavelength and the data in (2) above, it is possible to show that a 1% decrease in ozone results in a 4% increase in biological effect for equal times of exposure to sunlight.

4. Since the increase in incidence rate of skin cancer goes exponentially with the decrease in latitude (and increase in the sun's UV flux), we may infer from (3) that a 1% decrease in ozone will give a 4% increase in the incidence rate (5,000 new cases per year).
5. It is of interest that if the estimate in (4) is correct, we can show that approximately one-fourth of the increase in incidence of skin cancer with decrease in latitude arises from an increase in the sun's intensity and that three-fourths of the effect arises from the longer time spent in the light at the lower latitudes.

EXCISION REPAIR OF ULTRAVIOLET-INDUCED PYRIMIDINE DIMERS IN HUMAN CELLS

James D. Regan, W. L. Carrier, and R. B. Setlow

In our original demonstration of excision repair of pyrimidine dimers in human cells (1) we used two established human cell lines (RA and HeLa) and one diploid cell strain (WI-38). Recently, two publications (2, 3) have suggested that pyrimidine dimers may not be biologically important, since repair of dimers has been demonstrated in only one cell line commonly in use (HeLa). Ignoring the non sequitur aspect of this statement, we now demonstrate its falsity by the data in Table I.

TABLE I. Excision repair of UV-induced pyrimidine dimers in human and other primate cells

Cell	Origin	UV dose (ergs/mm ²)	Total pyrimidine dimers excised in 24 hr (%)
Human Malignant Cells:			
D98	Human carcinoma	200	35
KB	Human carcinoma	200	40
Hep-2	Human carcinoma	200	32
P-4365	Human leukemia	200	21
PGLC-51E	Human infectious mononucleosis	200	60
HPA	Human carcinoma	200	37
Human Cells (not from malignancies):			
LN 284	Lesch-Nyhan syndrome (skin)	100	72
HS	Human skin (normal)	150	63
MS	Xeroderma heterozygote (skin)	150	56
DS	Xeroderma heterozygote (skin)	150	52
HSB	Human skin (normal)	150	60
HG-99	Fanconi's anemia (skin)	150	55
HG-46	Bloom's syndrome (skin)	150	45
HG-173	Bloom's syndrome (skin)	150	25
Human Cells from Xeroderma Pigmentosum Homozygotes:			
XP-1	Uncomplicated XP (skin)	100	2
XP-2	Uncomplicated XP (skin)	100	21
XP-3	Uncomplicated XP (skin)	100	7
XP-4	De Sanctis-Cacchione syndrome (skin)	100	8
XP-5	De Sanctis-Cacchione syndrome (skin)	100	15
Non-Human Primates:			
CV1	Green monkey (kidney)	150	60
RMH	Rhesus monkey (heart)	150	43

These data show that excision repair of UV-induced pyrimidine dimers is a general property of human and other primate cells. Only in repair-specific mutations such as xeroderma pigmentosum is there an absence or a severe reduction in the excising capacity of human cells.

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2. A. Han and W. Sinclair, *Biophys. J.* **9**, 1171 (1969).
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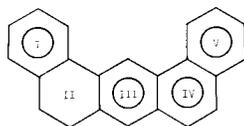
X-RAY STRUCTURAL ANALYSES OF TWO CARCINOGENS, 5,6-DIHYDRODIBENZ[*a,j*]ANTHRACENE AND 5,6-DIHYDRODIBENZ[*a,h*]ANTHRACENE

C. H. Wei and J. R. Einstein

Polycyclic aromatic hydrocarbons are known to display a wide variety of carcinogenic activities, and a number of these compounds and their derivatives have been tested for carcinogenicity in animals. Recently, a series of partially hydrogenated dibenzanthracene derivatives were prepared and further investigated for their tumorigenic activity on Swiss female mice (1). Among the compounds tested, 5,6-dihydrodibenz[*a,j*]anthracene has been found to be significantly carcinogenic, in contrast to the weak carcinogenicity of its parent, dibenz[*a,j*]anthracene. On the other hand, 5,6-dihydrodibenz[*a,h*]anthracene has been found to be weakly carcinogenic in the skin test, and apparently not carcinogenic when injected subcutaneously, in contrast to the potent carcinogenicity of its parent, dibenz[*a,h*]anthracene. The crystal and molecular structures of the title compounds, kindly furnished by Dr. W. Lijinsky of the Eppley Institute for Research in Cancer, have been determined from three-dimensional X-ray diffractometer data.

5,6-Dihydrodibenz[*a,j*]anthracene crystallizes with eight molecules in a monoclinic unit cell of symmetry $P2_1/c$ and α parameters $a = 12.1434(3)$, $b = 8.0864(4)$, $c = 30.6369(11)$ Å, and $\beta = 101.130(1)^\circ$ at 22°C . The structure was solved by direct methods and refined by the full-matrix least-squares method to $R(F) = 8.2\%$ and $R(F^2) = 7.2\%$ based on 3090 independent intensity data. One of the two independent molecules (A) is normal, whereas the successfully refined model for the other molecule (B) assumes its distribution in two disordered sites, with orientations approximately related to each other by a mirror plane through the central atoms of ring III:

ORNL-BIO-25665 (a)

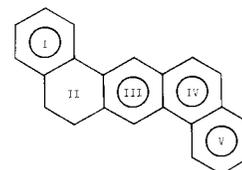


5,6-Dihydrodibenz[*a,j*]anthracene

The ratio of occupancy factors for the two disordered orientations is 7:3. Each aromatic ring in both independent molecules is almost perfectly planar, and the phenanthrene moieties are approximately planar, but with an observable distortion of the rings attributable to overcrowding of hydrogen atoms. The bond length in the electron-rich K region of the phenanthrene moiety is $1.334(6)$ Å for molecule A. The saturated carbon atoms are above and below the plane defined by the other carbon atoms of the ring II by about 0.35 Å, and the adjacent end aromatic ring I is twisted 21° from the plane of the center aromatic ring III.

5,6-Dihydrodibenz[*a,h*]anthracene crystallizes with four molecules in an orthorhombic unit cell of symmetry $P2_12_12_1$ and dimensions $a = 8.465(3)$ Å, $b = 15.082(4)$ Å, and $c = 11.616(3)$ Å at 22°C . The structure was solved by the application of the symbolic addition phase-determination procedure and refined by the full-matrix least-squares method to $R(F) = 7.5\%$ based on 854 observed diffractometer data. The solution of the structure was based on the assumption that the molecule occupies two disordered sites of partial occupancy, each centered near the origin of the unit cell, and related to each other by a pseudocenter of symmetry. The ratio of occupancy factors for the two molecular sites is approximately 3:2. Although the disorder prevents a precise determination of the conformation of an individual molecule, we have estimated the twist of ring I relative to ring III about the single bond joining them to be within a few degrees of 16.5° , as compared with 21° for 5,6-dihydrodibenz[*a,j*]anthracene:

ORNL-BIO-25665 (b)



5,6-Dihydrodibenz[*a,h*]anthracene

Furthermore, it appears that this twist in either molecule may vary considerably with the molecular environment. Therefore, it appears unlikely that the difference in carcinogenicity between these two molecules is due to a difference in the degree of twist.

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INCREASE IN SURFACE-MEMBRANE Na-K-ATPase ACTIVITY AND OUABAIN-BINDING SITES IN TRANSFORMED MAMMALIAN CELLS

G. L. Vaughan, R. W. Tennant, and J. S. Cook

Determination of the number of ouabain-binding sites on the surfaces of a number of mammalian cell types has led us to the generalization that transformed cells bind more ouabain and have a higher titer of glycoside-sensitive Na-K-ATPase activity than nontransformed cells. We have now further characterized these relationships in a single system—Swiss

mouse embryo 3T3 cells, Swiss 3T3 cells undergoing transformation by the Maloney isolate of murine sarcoma virus (MSV), and a nonproducer clone of MSV-transformed Swiss 3T3 cells (S^+L^-). The S^+L^- line shows a 2.5- to 3.5-fold increase in the number of ouabain-binding sites and a concomitant increase in the Na-K-ATPase activity, as compared with the parent line. No increase in either activity could be detected 4 days after infection of the parent line with MSV, but 21 days after infection increases could be seen in the mixed population of transformed and nontransformed cells. The change in the Na-K-ATPase titer, which is associated with the cells' electrolyte transport system, could be a response to increased membrane leakiness to alkali cations and thus indirectly related to viral activity rather than a direct expression of the MSV genome.

FURTHER STUDIES ON THE SERINE REQUIREMENT OF HUMAN BLOOD NEOPLASIAS

Kathryn P. Lore,* James D. Regan, F. M. Faulcon, and W. H. Lee

We have shown previously that serine is an essential requirement for the growth of human chronic granulocytic leukemia cells (1). We have extended these studies to include certain other blood neoplasias. Our results indicate that chronic lymphocytic leukemia leukocytes require serine but not asparagine, while Hodgkin's disease and lymphosarcoma leukocytes have a major requirement for serine and a minor requirement for asparagine. Asparagine is not required by chronic lymphocytic leukemia leukocytes, and this suggests that (1) asparagine nutritionally directed therapy would be unsuccessful in chronic lymphocytic leukemia, (2) serine analogs or serine-catabolic enzymes should be of therapeutic value in chronic lymphocytic leukemia, and (3) circulating lymphocytic leukocytes are possibly of different origin than the circulating granulocytic lymphosarcoma and Hodgkin's cells.

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1. J. D. Regan, H. Vodopick, S. Takeda, W. H. Lee, and F. M. Faulcon, *Science* **163**, 1452 (1969).

EFFECT OF AN ASPARAGINE ANALOG ON ASPARAGINE-REQUIRING RAT LEUKEMIA CELLS

James D. Regan, D. G. Doherty, C. E. Richter, and F. M. Faulcon

Certain types of human and rodent leukemias have an essential requirement for a supposedly "nonessential" amino acid, asparagine. Asparaginase has been an effective therapeutic agent in some cases. It is not, however, without certain undesirable side effects, many of which are apparently caused by the patient's immune reaction to the foreign protein.

We have synthesized an asparagine analog, diamino succinic acid, and have examined its ability to inhibit macromolecular synthesis in rat leukemia cells that have a severe requirement for asparagine. In medium containing 10^{-4} M asparagine, 2×10^{-4} M diaminosuccinic acid caused a 50% inhibition of DNA synthesis in these cells. Further work on effects on RNA and protein synthesis and separation of the DL forms of the analog for use in *in vitro* and *in vivo* testing is in progress.

THE INTERACTION OF RNA TUMOR VIRUSES AND THE IMMUNE SYSTEM. IMMUNE CAPACITY AND PATHOGENESIS

M. G. Hanna, Jr., R. W. Tennant, and Jane A. Treber

It has been assumed that immunologic inadequacy during leukemogenesis is a primary condition for neoplasia, and that immunologically competent lymphoid cells may serve as a source of target cells to the leukemogenic virus. One recent important observation related to the association of RNA tumor viruses and central immune components is the concentration of endogenous C-type virus in lymphatic tissue germinal centers of a variety of mouse strains. This finding is of extreme interest, since studies with other antigens have shown that antigens to which an animal is truly tolerant do not localize in these areas of peripheral lymphatic tissue; and it raises the possibility that rather than tolerance to endogenous RNA tumor virus, we have been seeing an expression of some level of immune reactivity to the virus. Further, histologic and ultrastructural studies in BALB/c mice injected with Rauscher leukemia virus (RLV) suggest that one of the earliest detectable tissue sites of C-type virus localization, as well as of replication in immunocompetent progenitor cells, is the nonthymus-dependent areas of spleen lymphatic nodules and lymph nodes, particularly in the germinal centers. We attribute the initial localization of the virus primarily to its antigenic quality.

It is not unreasonable that RNA viruses would be associated either normally or during early intervals of infection with parenchymal cells in germinal centers, when the high mitotic activity of this region is recognized and related to a general assumption that lesion or malignant transformation of cells by viruses depends on a specific interaction between the viral and host cell genomes. A replication requirement of the host cell for virus production would limit other cells, such as mature lymphocytes and reticular cells of the nodule, as target cells.

Because of this morphologic study, we concluded that a selective replication of the virus in proliferating cells of the germinal centers causes lymphoblastosis, an early and essential aspect of the splenomegaly associated with Rauscher disease. Further, by developing an enlarged population of proliferating immunocompetent cells in lymphatic nodules of germfree mice, we have been able to enhance RLV-induced splenomegaly. This provides a direct correlation between the size of the immunocompetent cell compartment and RLV-induced pathogenesis.

It may well be that what we are confronted with in RNA tumor virus-induced leukemogenesis in the mouse system is a balance between innate immunity to endogenous virus and a direct involvement of the immune system with the pathogenesis of the disease. Suppression of the immune system via aging, chemicals, antilymphocyte serum, and radiation has been shown to favor the disease processes associated with RNA tumor viruses. However, data relevant to innate immunity to endogenous tumor viruses are inadequate to support this speculation.

Recently we have obtained preliminary data from strain RFM mice, from which a correlation can be made to support this point. Our results show that while murine leukemia virus (MLV) antigen can be detected in the thymus at 1 week of age, it cannot be detected in the spleen until 4 weeks of age. A marked decrease in detectable MLV antigen in the spleen occurs between 6 and 20 weeks of age, which is reminiscent of an immune clearance pattern. In these mice, immune competence of the spleen, as measured by the ability to localize ^{125}I -human gamma globulin in germinal centers, as well as by *de novo* formation of germinal centers and serum immune elimination of the antigen, is first detectable at 4 weeks of age and peaks at 7 weeks of age. Thus, the time when the spleen is immunologically competent precedes the elimination of MLV in the same order.

A further correlation that needs to be made is the finding that glomerulosclerosis in the RFM mice is first detectable at 8 weeks of age and increases in both incidence and severity to about 12 months of age. Since it is proposed that the hyalinization of the glomeruli is a consequence of assimilation of antigen-antibody complexes, and since we have demonstrated serologically that MLV antigen is present in the kidney during this time interval, it is possible that this is a manifestation of the immune process. Our data strongly suggest but do not conclusively demonstrate that there is a chronic immunologic reactivity to endogenous RNA tumor viruses.

ULTRASTRUCTURAL LOCALIZATION AND T-CELL CYTOTOXICITY OF LACTIC DEHYDROGENASE VIRUS

M. J. Snodgrass and M. G. Hanna, Jr.

Although long-lasting and hyperplastic germinal centers appear in lymphatic tissue after infection with lactic dehydrogenase virus (LDV), this virus does not stimulate the production of a complete neutralizing antibody. Instead, it acts as an immunological adjuvant, having a synergistic effect with oncogenic viruses such as the Rauscher leukemia virus that affect blast cells of the humoral immune system. A more immediate result of LDV infection is a transient depression of the cell-mediated immune response, which seems to be due to its effect on both the reticuloendothelial system and lymphocytes of thymic origin (T cells). Accordingly, this study involves a systematic investigation of the cytological and histopathological effects of LDV infection in mice. The results indicate that infection results in a two-phase response, in which the virus is preferentially associated with phagocytic reticular cells (Fig. 1) in lymphatic tissue, and that this association is ultimately cytotoxic to T cells. It

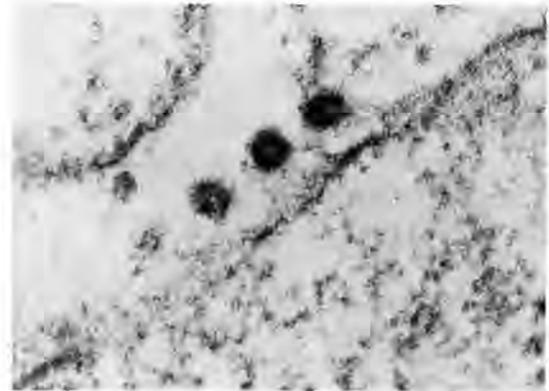


Fig. 1. Intercellular LDV particles that are attached to the plasmalemma of a phagocytic reticular cell; one of them appears to be in the process of budding. 210,000X

also involves studies to determine whether the lymphoid involution is due to a stress response with a resultant hypersecretion of adrenocorticosteroids, and whether the degenerating lymphocytes are a source of the elevated serum lactate dehydrogenase (LDH) level, which is characteristic of LDV infection.

Male BALB/c mice, 10 to 14 weeks old, were separated into six groups and treated with LDV as follows: (1) conventional animals injected intraperitoneally, (2) conventional animals injected intrathymically, (3) thymectomized animals exposed to 400 r whole-body radiation and injected intraperitoneally, (4) adrenalectomized animals injected intraperitoneally at 48 hr or 2 weeks after surgery, (5) animals given a second intraperitoneal injection after 25 days, the second injection consisting of virus either from the same animal or from the original virus pool, and (6) conventional animals treated in the same manner as the various groups above but not given LDV.

Spleen, mesenteric lymph node and thymus were removed at intervals from 6 hr through 30 days after virus infection, weighed, and prepared according to conventional methods for histological and ultrastructural examination. Serum was collected and assayed for LDH activity.

The serum LDH activity increased sevenfold by 4-7 days after intraperitoneal injection and declined to fivefold for the remainder of the experiment in all animals given virus. Spleen and mesenteric lymph nodes weights increased and then returned to normal, whereas the thymic weight decreased at the early intervals after injection. Thymic-dependent areas of the spleen and lymph node were depleted of lymphocytes from 1 to 4 days after injection in all animals, including the adrenalectomized ones, during which time LDV particles were found in close association with phagocytic reticular cells. However, this sequence did not recur after a second injection of the virus. By 4 days after intrathymic injection, the lymphocytes of the cortex of the thymus were totally depleted, and only a peripheral layer of lymphoblasts remained. By 7 days the cortex was repopulated, as were the thymic dependent areas of the other lymphatic organs.

Regardless of the route of injection, LDV particles were found in close association with phagocytic reticular cells of the lymphoid organs within the first 24 hr after injection. Shortly thereafter, T cells were destroyed and engulfed by macrophages. This was very prominent after intrathymic injection. However, the dying lymphocytes were not the source of the elevated serum enzyme activity, since this activity was equally elevated in thymectomized, irradiated animals. Thus, this two-phase response consists of virus infectivity and replication and T-cell cytotoxicity. LDV particles were not associated with the thymocytes, indicating that these cells are affected by some other factor. Our data also indicate that the factor is not an adrenal corticosteroid, since these events also occurred in the adrenalectomized animals.

SUPPRESSIVE EFFECT OF IMMUNIZATION WITH MOUSE FETAL ANTIGEN ON GROWTH OF CELLS INFECTED WITH MURINE LEUKEMIA VIRUS AND ON PLASMA CELL TUMORS

M. G. Hanna, Jr., R. W. Tennant, J. H. Coggin, Jr.,* and Jane A. Treber

Numerous reports have suggested that fetal cells have antigens that crossreact with specific transplantation antigens in tumor cells. In humans, three classes of embryonal antigens in tumor cells have been described. In rodents, it has been demonstrated that hamster, mouse, and human fetal cells contain antigen that crossreacts with tumor-specific transplantation antigen of hamster and mouse SV40 tumor cells. The reappearance of embryo-specific antigens in tumors induced by viruses, chemicals, and radiation, and in spontaneous murine tumors, has also recently been demonstrated using diffusion precipitin reactions.

The present studies were undertaken to examine the protective effects of fetal antigen immunization on the growth of spleen cells infected with Rauscher leukemia virus (RLV) and plasma cell tumors in BALB/c mice. BALB/c male mice were immunized at 3 weekly intervals with X-irradiated 14-day-old syngeneic embryo cells. The embryo cells were obtained from first litters. The results of these studies demonstrate that the recovery of spleen cells infected with RLV and grown in Millipore diffusion chambers, the development of RLV-induced splenomegaly, and the cumulative mortality from a transplanted ascites plasma cell tumor were all suppressed in young adult BALB/c mice previously primed with X-irradiated syngeneic embryo cells. RLV-induced splenomegaly was also suppressed by adoptive transfer of postpartal spleen cells, as well as spleen cells from animals primed with syngeneic embryo cells. Similar suppressions were not observed in mice primed with neonatal or normal syngeneic cells. Further, injection of fetal cells was not effective in suppressing the immune function of normal spleen cells, as measured by ability to elaborate a primary IgM response to heterologous erythrocyte antigen.

These data suggest that BALB/c embryos, at approximately 3/4 of their gestation process, possess components tentatively presumed to be antigen(s) common to both RLV-infected spleen cells and a plasma cell ascites tumor. Equally important is the finding that growth and pathogenesis of tumors in-

duced by RNA virus are suppressed in adult animals immunized with embryo-specific antigen. This agrees with recent findings on DNA virus-induced tumors. An important consideration in the suppression of virally induced splenomegaly in intact mice immunized with fetal antigen is our earlier finding that immunization with noncrossreactive antigen enhances splenomegaly, compared to that in normal RLV-infected mice. Thus, suppression of splenomegaly in RLV-infected mice immunized with fetal antigen carries particular immunologic significance. Furthermore, the tumor systems used in this report normally rendered the animal immunologically inadequate with respect to humoral antibody production. Thus, the protective effect by embryonic antigen immunization is most likely a consequence of residual antibody developed after the last fetal immunization.

A major consideration in our studies is the possibility that the immunity was provided by murine leukemia virus antigen in the fetal cell preparation rather than by a crossreactive embryonic antigen. Since the murine leukemia viruses isolated from BALB/c mice and other mouse strains are of the Gross or AKR virus serotype, we feel that any virus present in the embryo cell preparation in our study probably has no neutralizing or crossreacting activity with the RLV, which is of the FMR serotype. Our results, however, do not rule out the possibility that the immunity elicited by embryonal antigens is against a virus-induced transplantation antigen. If a nondetectable level of group-specific antigen were present in the embryo cell preparation, it is unlikely that it would be effective as a source of crossreactive antigen, since mice appear to be unresponsive to immunization with the group-specific antigen of murine leukemia virus. It is still possible, however, that the phenomenon is related to the covert behavior of the RNA tumor virus, such that other antigens of the fetal cells could be expressed concomitantly with group-specific antigen, and both types of antigen could be representative of functions directed by virogenes or oncogenes.

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DECREASING IMMUNE COMPETENCE AND DEVELOPMENT OF RETICULUM CELL SARCOMAS IN LYMPHATIC TISSUE OF AGED MICE

M. G. Hanna, Jr., P. Nettlesheim, and M. J. Snodgrass

It is well established that both humoral and cellular immune capacity increases with age. In mice, there seems to be a close temporal relationship between decrease in immune function and development of neoplasia in major lymphatic organs. At present, it is not clear to what degree decreasing immunocompetence is related to the malignant transformation of essential elements of the lymphatic tissues. We have previously reported a 46% incidence of reticulum cell sarcomas (RCS) in aged male and female B6C3F₁ mice. These RCS primarily involve the spleen. Studies of specific antibody-forming capacity in intact and splenectomized aged B3C3F₁ mice with no neoplasia have demonstrated that the spleen is the major source of immunocompetence. The decrease of

primary and secondary antibody-forming potential with age has been associated with the reduction of antibody-forming cells as well as with the failure of the extracellular antigen-trapping mechanism.

We studied the relation between the development of RCS and reduced immune capacity in aged BC3F₁ mice. 125I-human gamma globulin (HGG) was used to analyze the relative immune clearance in antigen-trapping capacity of spleen germinal centers in 1.5-year-old, 2.5-year-old and 13-week-old mice; the histopathologic development of reticuloendothelial neoplasia was determined with respect to site of origin within the lymphatic nodules of the spleen. The results of this study demonstrate that the early stages of RCS were confined to the spleen, with no evidence of RCS in other organs. In animals with the most progressed stage of RCS, tumor masses occasionally appeared in extrasplenic sites. Our impression was that the first alteration of the spleen lymphatic nodules associated with the development of RCS was a proliferation of large cells in the thymus-dependent periarteriolar region of the nodules. The next stage of development was the prominence of reticular cells in this region, with continued hyperplasia. Eventually, the periarteriolar region of the follicle contained anaplastic reticular cells that invaded the cortical regions of the lymphatic follicles and eventually the spleen red pulp. During the development of the disease, the germinal center reticular cells apparently were not involved in the early reticuloendothelial neoplasia. This finding is in contrast to the pathogenesis of Type A and mixed Type B RCS in the SJL mouse.

The decrease in immune capacity in these aged mice is clearly not a function of the development of lymphoreticular neoplasia. The general loss of immune capacity begins before any detectable neoplasia in the lymphatic tissue of the spleen. This is supported by the fact that in 18-month-old animals a significant decrease in immunological function (antigen localization in the spleen and immune elimination in the serum) occurs without any indication on the light microscopic level of neoplasia. It is tempting, however, to speculate that the impaired immune capacity may be related causally to the developing tumor.

There was an increase of approximately 15% in nonlymphatic neoplasms in 2.5-year-old animals with severe destruction of spleen lymphatic tissues as a result of RCS or marked degenerative changes, over normal aged animals or those categorized in the earliest stage of RCS. These differences, although not highly significant, warrant further investigation. It may be considered that a further decrease in immune capacity of the spleen resulting from neoplasia or degenerative changes might contribute to the increased frequency of nonlymphatic tissue tumors in these animals.

EVIDENCE OF FUNCTIONAL MICROENVIRONMENTS IN LYMPHATIC TISSUE RESPONSE TO ANTIGEN

M. G. Hanna, Jr., P. Nettesheim, and Leona C. Peters

A major difference between studies of immunocompetent cell interaction performed *in vitro* and by *in vivo* transfer is that the primary immune response *in vitro* requires (1) cells derived from bone marrow (B cells), (2) thymus-dependent

cells (T cells), and (3) a third cell type commonly referred to as "adherent cells" because of their ability to adhere to glass or plastic. Nonadherent cells alone give an excellent primary response when transferred with antigen into X-irradiated recipients. Thus, it is reasonable to assume that upon transfer of dissociated unprimed spleen cells and antigen, the reticular remnant of the spleen supplies a functional cell population that may correspond to adherent cells.

Although B and T cell compartments have been successfully manipulated in *in vivo* transfer studies, little consideration has been given to manipulating the reticular stroma. It seemed to us that the aging mouse affords a natural system in which there is an alteration of the reticular stroma. The reduced immune response in aged mice has been attributed to a decreased activity of the immunocompetent cell population, but recent studies have suggested in addition that a microenvironmental change prevents maximum expression of existing immunocompetent cells. It has been clearly shown that antigen localization on dendritic reticular cells is markedly reduced in mice more than 1.5 years old. Thus, the aged animal provides an excellent model for studying the interaction of the microenvironments provided by stromal reticular cells and immunocompetent progenitor cells.

Experiments were designed to evaluate these cellular interactions in young adult and aged mice using the *in vivo* transfer method. We first attempted to compare the immunocompetent cell compartments of 12-week-old and 2-year-old mice after transfer with sheep erythrocyte antigen into 12-week-old irradiated recipients. The results showed that the indirect plaque-forming cell (7S) response of aged spleen cells in young recipients was 3% of that obtained with an equal number of 12-week-old spleen cells. There did appear to be an equal compartment size of direct and indirect plaque-forming cells among aged spleen cells stimulated with antigen in the stromal environment in spleens of young adult mice. This is of particular interest, since previous studies in intact aged animals have shown clearly that the major suppression of humoral antibody-forming capacity is in the 7S antibody response.

We next transferred young adult spleen cells and sheep erythrocyte antigen into old recipients. Except for a slight decrease at day 6 (possibly representing an effect on growth rate) the peak direct plaque-forming cell response was not below the normal response of cells transferred into young adult recipients. The 7S cellular response, however, was markedly depressed, with a measured peak response that was approximately 15% of normal. These results clearly indicate that even though there is a normal indirect plaque-forming cell potential in a dissociated cell pool transferred into old recipients, a marked impairment of the response occurs as a result of some interacting factors with the aged mouse spleen. Thus, it is clear that associated with the reticular remnants of the spleen in irradiated recipients are factors that do not contribute to the maximum expression of the 7S antibody-forming precursors. As shown in our study, the immunocompetent cell compartment of aged spleen cells is diminished approximately 22-fold, whereas the same compartment, as expressed by young spleen cells transferred into old recipients, is depressed approximately sevenfold. Thus, from these data one would surmise that while ~75% of the 7S depression in old animals is directly attributable to a lack of progenitor cells, ~25% of this depression can be attributed to an inadequate microenvironment.

We next attempted with a limited number of aged animals to supplement the reticular cell compartment of the spleen by direct implantation of reticular stroma from young adult spleen. The results of two experiments, although quite limited, demonstrated that any degree of success will be with respect to enhancing the 7S response.

In general, the overall results of these experiments with aged mice clearly support the concept that the reticular stroma of the spleen, possibly interacting with other humoral factors, is essential for expression of immunocompetent precursors directed toward 7S antibody production. That this is perhaps somehow associated with the interaction of reticular cells and antigen is suggested by the fact that in aged mice localization of antigen on dendritic reticular cells in the spleen decreases simultaneously with the overall loss of immune capacity. Numerous reports support the concept that this form of antigen localization and the development of germinal centers with these cells correspond to and appear to be essential components for the development of primary 7S antibody-forming capacity. We would propose from the present studies that microenvironments are supportive, if not essential, for aspects of primary immune capacity, and that immune deficiencies should be considered not only from the point of view of immunocompetent progenitor cell compartment size but also from the aspect of essential interacting stromal cells and their ability to create with antigen a microenvironment for overall cellular functions.

HOST IMMUNE MECHANISM INVOLVEMENT IN PLASMA CELL TUMORIGENESIS IN BALB/c MICE

M. G. Hanna, Jr., Wen-Kuang Yang, and H. E. Walburg, Jr.

Plasma cell neoplasms of humans and mice have been used in the study of specific aspects of the immunoglobulins produced by these malignancies and also for comparison of tumor-secreted protein with products of the normal immune response. While the normal immune response leading to the production of circulating antibodies has been intensively studied, little attention has been paid to the pathogenesis of plasma cell tumors. In mice, plasma cell tumors are induced in the BALB/c strain by introduction of irritants such as plastics or mineral oil in the peritoneal cavity. The tumors appear months later, following chronic interaction between host tissue and the foreign material. BALB/c mice develop a high incidence, approximately 80%, of plasma cell tumors following the introduction of mineral oil in the peritoneal cavity. It has recently been shown that germfree mice, which have a less developed lymphoreticular system, including a smaller number of plasma cells, than their conventional counterparts, when injected intraperitoneally with mineral oil, fail to develop the expected incidence of plasma cell tumors. This particular experiment suggests the importance of the microbial flora and/or the status of the immune system in the differentiation of plasma cells that respond to the carcinogenic stimulant in the genetically susceptible host.

The purpose of our study was to stimulate germfree mice in such a manner as to induce germinal center activity in the Peyer's patches of the intestines and to follow the immune stimulation by mineral oil injection. Histologic and serologic results of alterations in the Peyer's patches as well as spleen and mesenteric lymph nodes in these animals were compared with normal germfree animals stimulated with mineral oil alone. At the present time, two germfree isolators have been established. Interestingly, it was found that the only means of stimulating Peyer's patch development in these germfree mice was by intrarectal injection of antigen. The antigen used in these studies was sheep erythrocytes. Following intrarectal injection of half of the germfree mice, all animals were injected intraperitoneally with mineral oil.

The experiment is now in its eighth month. Thus far in the sheep-erythrocyte-stimulated, mineral-oil-injected animals, those with gross evidence of ascites have been killed. From peritoneal cell differentials, four of the animals have shown suspected plasma cell tumors, one of which has been successfully transplanted and is now in its second passage. Three animals were autopsied and found to have fibrosarcomas. In the germfree mice that received mineral oil only, three animals have died, none of these having neoplasia. These preliminary results suggest that antigenic stimulation of the intestines, as well as the peripheral lymphoid components, contributes to plasma cell tumorigenesis. The interesting aspect of these studies will be to determine whether the plasma cell tumors induced in heterologous-erythrocyte-stimulated, mineral-oil-treated animals will continue to produce specific antibody.

EFFECT OF ANTIGEN DOSE ON THE REQUIREMENT FOR CONTINUOUS ANTIGENIC STIMULATION IN THE DEVELOPMENT AND DIFFERENTIATION OF ANTIBODY-FORMING CELLS

M. G. Hanna, Jr. and Leona C. Peters

The concept that antigen has a continuous role in the recruitment and differentiation of immune progenitor cells has not only a functional basis but, in our estimation, a structural one as well. This morphologic basis rests on the observation during the primary response of extracellular localization and persistence of antigen and/or antigen-antibody complexes on unique dendritic reticular cells of lymphatic tissue. A strong argument can be made for the interdependency between antigen localization and sustained proliferation of the immunocompetent cells in the follicular, thymus-independent regions of lymphatic tissue. In terms of the functional aspect of immunity, the strongest support for this concept is derived from recent studies of passive antibody-mediated immune suppression. Also, the immune cell maturation scheme, commonly referred to as the X-Y-Z model, is based on the requirement of continuous antigenic stimulation in the development and differentiation of antibody-producing cells. What is actually involved is an antigen-sensitive unit which, upon antigenic stimulation, results in an intermediate sensitized cell that is not an efficient

antibody-producing cell but acts as a sensitized progenitor cell that is qualitatively distinct from the antigen-sensitive cells of unprimed animals. Stimulation of the Y cell by antigen results in proliferation of these sensitized cells, which expand the cell compartment prior to irreversible maturation of Y cells to functional antibody-producing Z cells.

One characteristic we would expect to observe during the primary antibody response, in which high levels of immunity are available, is the rapid transformation of the sensitized progenitor cells to functional cells. This condition would favor a primary antibody response rather than the establishment of secondary antibody-forming potential. Conversely, low levels of antigen would favor preservation of the sensitized cell compartment rather than production of antibody-producing cells. There is experimental evidence supporting both possibilities.

The purpose of this study was to evaluate the effect of antigen dose on sensitized cell exhaustion during the primary immune response. Hemolytic plaque-forming cell capacity during both primary and secondary immune reactions was studied, both in the intact animal and with the spleen cell transfer technique.

The results clearly demonstrate a higher detectable secondary immune capacity in the suboptimum antigen dose group than in the optimum antigen dose group. This was demonstrated from both 19S and 7S cellular responses, as well as with humoral antibody levels measured in the spleen cell recipient mice. It can be interpreted that in the presence of a suboptimum dose of antigen, which rapidly diminishes during the early intervals of the primary response, there is adequate recruitment with subsequent preservation or rescue from antigen-mediated depletion of the sensitized cell compartment, at the expense of the detectable primary response. These results support our previous study of the effect of injecting isologous specific antibody on the primary and secondary response to sheep erythrocyte antigens.

PRELEUKEMIC CHANGES IN AKR MICE. GROSS LEUKEMIA VIRUS-INDUCED GERMINAL CENTERS IN THE THYMUS OF YOUNG ADULT MICE

R. A. Hogg* and M. G. Hanna, Jr.

There is strong evidence that the thymus is the site for the development of the first leukemic cells in AKR mice. These animals have a higher than 90% incidence of thymoma between 8 and 14 months of age. There is strong evidence that the disease is initiated by the Gross leukemia virus and has a remarkable uniformity in its histologic manifestation in the thymus.

A most significant change, as described by Metcalf (1), was the *de novo* formation of lymphoid germinal centers in the thymus of 8-month-old AKR mice. It was suggested that this histological alteration of the thymus preceding thymoma development (preleukemia change), although not necessarily a premalignant condition, may in some way be related to the pathogenesis of the disease.

The present studies were initiated to examine the ultrastructure of germinal centers in the thymus of AKR mice. Because antigen injected directly into the thymus does result in

the formation of thymic germinal centers and the appearance of plasma cells, attempts were made to induce thymic germinal centers in 3-month-old AKR mice, using a Gross leukemia virus preparation grown in rat embryo cell culture and comparing these induced centers with those arising spontaneously in aged AKR mice, preceding the formation of thymomas.

The thymus was removed from mice at 1, 3, 5, 7, and 8 months of age, fixed in Bouin's fluid, sectioned, and stained with hematoxylin and eosin for histological analysis. Three-month-old normal mice injected with Gross leukemia virus were killed at 1, 3, 5, 9, and 15 days after injection. The tissue was examined on the histological and ultrastructural levels.

At ages 1 and 3 months, no alteration of thymus structure was detected on the histological level. In 5-month-old mice, a marked increase in medullary concentration of lymphoid cells was observed. The lymphoid cell concentration in the medulla appeared as primary lymphoid follicles. In 7- and 8-month-old mice, secondary follicles, which had many of the characteristics of lymphoid germinal centers, could be detected in many of the primary follicles of the thymus medulla. These secondary follicles, however, were less organized and never demonstrated densely and thinly populated regions.

The thymus medulla of mice that received virus injections was infiltrated with mononuclear cells 3 days after injection, forming primary follicles, several of which contained secondary follicles. These induced follicles closely resembled lymphoid germinal centers, and they appeared highly organized.

On the ultrastructural level, the essential feature of these germinal centers was the observation of C-type virus particles located extracellularly along the plasma membrane infoldings of dendritic reticular cells, a prominent feature of lymphoid germinal centers.

Although the immunological activity of secondary follicles in both thymus and lymphoid tissues has been previously demonstrated with other antigens, no such reactivity could be determined in the AKR system. As a matter of fact, there was a decrease in the latent period for thymoma development in these animals, indicating that the increased follicle activity may be in some way actually contributing to the pathogenesis of the disease, presumably the consequence of an additional source of proliferating target cells (immunoblasts) for viral replication.

Present studies involve the injection of groups of animals intrathymically with both virus antigen and a virus vaccine preparation. The thymuses of these animals will be studied both histologically and electron microscopically in an effort to gather more insight into the immunological and pathological manifestations of the disease process in these animals.

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INDUCED REGRESSION OF SYNGENEIC HEPATOMA CELL TRANSPLANTS IN GUINEA PIGS

M. G. Hanna, Jr., Leona C. Peters, and B. Zbar*

A considerable amount of data has been obtained on the suppression of tumor growth at the site of infection of Calmette Guerin bacillus (BCG). It has been shown that BCG injection into established interdermal tumors can induce regression of the progressively growing transplanted tumor in guinea pigs. This system provides a unique model for the study of histopathologic and functional immunologic changes during BCG-mediated tumor regression. These studies were performed in collaboration with Drs. B. Zbar and H. Rapp of the National Cancer Institute.

The initial experiment, started in April of this year, was a serial histopathologic study of the site of tumor growth and essential and peripheral lymphoid tissue during regressive tumor reaction to BCG. Three groups of guinea pigs were used in the experiments. Group 1 was injected with BCG at day 7 after interdermal tumor implantation, group 2 was injected with saline at day 7 after tumor implantation, and group 3 had their tumor papules excised at day 7. Histologically, it was established that metastasis of tumors to the proximal draining lymph node had occurred at the time of treatment. Twenty-four hours after treatment (saline, BCG, or tumor incision) histopathology of the proximal and distal draining lymph nodes revealed tumor cells in the subcapsular sinuses of the proximal lymph node. A major difference was that in the BCG-treated animals, the number of tumor cells in the subcapsular sinuses was greatly increased over the saline or tumor excision treatment groups. At 4 days after treatment, evidence of granulomatous inflammation could be observed in the proximal draining lymph nodes of the BCG-treated animals. A major change in the architecture of the proximal draining lymph nodes in the BCG-treated guinea pigs was a massive histiocyte infiltration into the sinuses of the lymph node. This response was not observed in the saline or tumor excision groups. Eight days after BCG treatment, interaction of "epithelioid reticular cells" and tumor cells could be observed in the lymph node sinuses. Many metastasized tumor cells at this time were pyknotic and obviously undergoing cell destruction. Eleven days after treatment, a syncytial histiocytosis occurred in the paracortical and sinus areas of the proximal draining lymph node, and no further evidence of metastasis existed in these nodes; saline-injected and tumor-excised animals showed massive infiltrative growth of tumor cells in the proximal draining lymph nodes. By day 25, the BCG-treated animals had essentially undergone complete tumor regression, and no evidence of metastasis existed in either proximal or distal draining nodes. The saline-injected group had a well-established tumor, and the proximal node was totally infiltrated and destroyed by metastatic cells. An interesting observation in the tumor-excised group was the fact that metastasis was well established in the proximal node, and the major response of the lymphoid component was a hyperplastic lymphoid lymphatic tissue reaction with well-established germinal centers. Long-term survival studies in these animals demonstrated that BCG-treated mice are still alive 8-9 months after treatment, while saline-injected and tumor-excised animals have died from metastasis between 3 and 4 months after tumor implantation.

Thus, it is demonstrated by these histopathological studies that BCG-mediated tumor regression stimulates a histiocytic reaction that is also capable of eliminating metastasis in draining nodes. It is interesting to point out that the effect of histiocytic response observed during this reaction correlates with histopathology of lymph nodes draining the sites of primary mammary tumors in humans, which has been used as a favorable prognosis. Further studies are underway, using this model system, in an attempt to delineate the functional immunologic factors directed against success of tumors and metastasis. This information should provide insight into rational approaches aimed at focusing immunity against the tumor.

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IMMUNOLOGY AND HISTOPATHOLOGY OF SV40 TUMOR DEVELOPMENT

G. W. Fortner,* J. H. Coggin, Jr.,† and M. G. Hanna, Jr.

This study is designed to determine the histopathologic changes, both specific and nonspecific, occurring in animals prior to and following tumor induction. The systems employed are the SV40-hamster model and the SV40-transformed cell-hamster model.

In the virus-hamster experiments, neonatal and 14-day-old hamsters are injected with SV40 subcutaneously in the upper right quadrant. A third group receives passage control fluid. The hamsters are killed at regular intervals, and their histopathology is studied. After the fourth week, diffusion chambers are implanted, and 5 days later autopsies are performed according to the pattern of cytostatic antibody present as a measure of the developmental phase of the tumor. The changes in histopathology are studied. The SV40-transformed cell-hamster experiments are carried out in the same way. Immunologic tests are to be performed on the sera and lymphoid cells of these two groups of animals. These include complement fixation and colony inhibition tests. An attempt will be made to establish a correlation between the immune status of the animal and development of a skin test for delayed hypersensitivity.

A further study will be made to correlate the functional immunity of the host to the histopathologic changes occurring during and following tumor induction. Animals with growing tumors in various phases will be injected with labeled SV40 neoantigens and studied for tolerance by antigen localization and immune clearance from the serum and spleen. This study will incorporate histology and low- and high-resolution autoradiography.

Animals from each group of experiments above will be used to study immunotherapy from the standpoint of surgery and Calmette Guerin bacillus treatment.

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ASSAY FOR FETAL-LIKE ANTIGENS IN LEUKEMIA VIRUS-INFECTED CELL CULTURES

R. W. Tennant, M. G. Hanna, Jr., and Sally A. Thompson

It has been shown that cells infected or transformed by mouse leukemia virus or by sarcoma virus produce surface antigens that are able to promote immunity to tumor cell transplants. It is not clear, however, whether the antigens are viral or cellular, since infection and transformation both are accompanied by viral multiplication, in which the cell membrane is involved. On the basis of evidence obtained recently by J. H. Coggin and others in the Molecular Anatomy Program at ORNL, we have attempted to determine whether infection by mouse leukemia virus can result in expression of fetal-like cell membrane antigens.

It was found that spleen cells from male C3Hf mice "immunized" with isogenic fetal cells are cytotoxic against normal C3Hf cells in culture and, to a greater extent, against cultures infected with Moloney leukemia virus. Contact with virus-infected cells also preferentially stimulated the spleen cells to increase their rate of DNA synthesis. The evidence suggests that reactions to indigenous mouse leukemia viruses are not involved in these experiments. Thus, these preliminary results indicate that the problem of nonviral cell transplantation antigens induced by RNA tumor virus may be explored by testing the expression of normal fetal cell antigen(s) in virus-infected cell cultures.

INTERACTIONS BETWEEN RNA TUMOR VIRUSES, CELLS, AND CARCINOGENS

R. W. Tennant, Sally A. Thompson, and R. E. Hand, Jr.

While an etiological relationship has been clearly established for both tumorigenesis *in vivo* and cell transformation in cell cultures by RNA tumor viruses, the mechanism of the effect of the viruses is unknown. Effects may involve the action of either covert or exogenous virus or both, and virus expression appears to be modified by both cell synthetic functions and certain genetic specificities. Recently it has been reported that virus expression and cell transformation in cell cultures can be influenced by certain chemical carcinogens. Our laboratory has been engaged in studies to define the mechanisms of cell virus interaction, using cell fusion techniques and selected chemical inhibitors. These studies also involve tests of the effects of irradiation (UV and X-rays) and N-ethylnitrosourea on virus expression and cell transformation. Results, thus far, fail to demonstrate an effect of acute treatments with the above agents on virus expression or cell transformation in certain cell cultures. Preliminary results of the fusion of permissive and nonpermissive cultured cells suggest that nonpermissiveness is dominant; i.e., virus expression is inhibited in heterokaryons. These studies are being extended to define the nature of the initial events that govern the response of cells to virus infection and the role of radiation or chemical carcinogens in interaction of cellular and viral functions.

DEVELOPMENT OF TESTS AND REAGENTS FOR DETECTION OF RNA TUMOR VIRUS EXPRESSION

R. W. Tennant and R. L. Tyndall

It has been shown that infection and/or transformation of cells by RNA tumor virus may be accompanied by only limited expression of the virus genome. Huebner *et al.* (1) have found that the internal group-specific antigens of the murine leukemia virus are the most frequent virus-specified functions detectable. Our efforts have concentrated on developing methods of similar sensitivity for detection of group- and type-specific viral antigens, infectious virus, and virus-induced neocell antigens.

In addition to the complement fixation test developed by Huebner *et al.* (1), we have applied the fluorescent antibody test to direct quantitation of cells synthesizing viral antigens and the micro Ouchterlony gel double diffusion technique to define antigen specificities. We are also attempting to quantitate the gel diffusion technique by microdensitometry. Our laboratories have applied the mixed-cell cytopathic technique and the focus rescue technique of Bassin *et al.* (2) to quantitation of infectious virus. Tests for cell-related antigens that we have recently applied include *in vitro* cytotoxicity assays and characterization of tumor cell transplantation antigens *in vivo*.

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DNA POLYMERASE ACTIVITY FROM RAUSCHER LEUKEMIA VIRUS. I. USE OF SYNTHETIC RIBO- AND DEOXYRIBONUCLEOTIDE POLYMERS

L. C. Waters, Wen-Kuang Yang, and L. G. Hardin

Characteristic association of RNA-dependent DNA polymerase activity with the RNA oncogenic viruses poses a question of the role of this enzyme activity in viral oncogenesis. Suggestive answers to this question may be obtained by studying the effect of inhibitors of the enzyme on viral oncogenicity and also by investigating the host factors affecting its activity in cells either susceptible or resistant to viral transformation. Our approach to the study of host factors began with a detailed analysis of optimal reaction conditions for the polymerase function, using a preparation from Rauscher leukemia virus and synthetic ribo- and deoxyribonucleotide polymers. The purpose is twofold: to define a sensitive method of enzyme assay and to understand the kinetic mechanism of enzyme function.

Rauscher leukemia virus, isolated by sucrose density gradient centrifugation from infected mouse plasma, was ruptured in 0.2% Nonidet NP 40. Polymerase activity was measured at a protein concentration of 0.01 mg ml⁻¹ in the reaction

mixture. Protection of the enzyme activity was found by the addition of bovine serum albumin, which works optimally at 0.17 mg ml^{-1} . The optimal Mg^{++} and/or Mn^{++} requirement for the polymerase, using $d(\text{A-T})_n$, $r(\text{A}\cdot\text{U})_n$, $r(\text{I}\cdot\text{C})_n$, $r(\text{I}\cdot\text{C})_n$, and DNase I-"activated" calf thymus DNA, have been determined. These differ markedly, depending on the template and slightly on the dNTP used as substrate. Mg^{++} is the preferred divalent ion in the DNA-dependent system. Mn^{++} , being much less stimulatory than Mg^{++} for DNA-dependent activity, is absolutely required for RNA-dependent activity. Combination of Mn^{++} and Mg^{++} gives best stimulation of RNA-dependent DNA polymerase activity. Commercially obtained $r(\text{A}\cdot\text{U})_n$ with 0.7:1.0 ratio of rA:rU and $r(\text{I}\cdot\text{C})_n$ with a 1:1 ratio of rI:rC were found to give below optimal template activity for dTMP and dGMP incorporation; adjustment of their ratio to 2.5:1.0 of rA:rU and to 1.0:2.0 of rI:rC gives 8–10 times more incorporation.

Kinetic studies showed the following: (1) K_m of $r(\text{A}\cdot\text{U})_n$ for the enzyme is less than one-tenth that of $d(\text{A-T})_n$, based on rA and dA content, indicating that the Rauscher leukemia virus polymerase may have a higher affinity for RNA than for DNA. (2) With "activated" calf thymus DNA as template-primer, two moles of the enzyme can bind to one mole of this DNA. (3) K_m of dTTP for $r(\text{A}\cdot\text{U})_n$ -dependent activity is approximately 0.05 mM, whereas that for "activated" DNA- and $d(\text{A-T})_n$ -dependent activities is 0.03 mM. (4) A sigmoidal curve was obtained by plotting the rates of $r(\text{A}\cdot\text{U})_n$ -dependent dTMP incorporation versus the concentration of dTTP in the reaction mixture, suggesting a rate-limiting step at the initiation of polydeoxynucleotide synthesis in this system.

DNA POLYMERASE ACTIVITY OF RAUSCHER LEUKEMIA VIRUS. II. EFFECT OF OLIGODEOXYNUCLEOTIDES

Wen-Kuang Yang, L. C. Waters, and Chongkun Koh

Our previous study with $r(\text{A}\cdot\text{U})_n$ -dependent incorporation of dTMP by the Rauscher leukemia virus (RLV) polymerase showed that a rate-limiting step was evident at the initiation of the polydeoxynucleotide chain. This seemed to be similar to the results obtained by Niyogi and Stevens (1), working with DNA-dependent RNA polymerase of *E. coli*. Their studies with the oligoribonucleotides suggested that a similar study with oligodeoxyribonucleotides in the RLV polymerase system may help to understand the mechanism of this enzyme action. This is important, especially because of the recent popular use of synthetic RNA-DNA duplexes by many workers in the assay of oncogenic RNA virus polymerase. Our results indicate that by the use of rA-dT one may fail to detect a critical function of RLV polymerase that can be detected by the use of rA-rU polymer.

The main findings are summarized as follow: (1) The sigmoidal curve of the rate of dTMP incorporation directed by $r(\text{A}\cdot\text{U})_n$ as a function of dTTP concentration in the reaction mixture was converted to a normal Menten-Michaelis kinetic curve when oligo-dT was added to the reaction. With $r\text{A}_n\cdot\text{oligo-dT}$, normal Menten-Michaelis kinetics were also obtained. (2) The effect of oligodeoxyribonucleotides (oligo-dN) is strictly temperature dependent. For example, $d(\text{pT})_{10}$

works optimally at 35–38°C, $d(\text{pT})_8$ at 28–32°C, $d(\text{pT})_6$ at 22–25°C and $d(\text{pT})_4$ at around 15°C. Increase of the reaction temperature above the optimal temperature caused a precipitous decline of the effect, indicating that hybridization of oligo-dN to RNA is essential. (3) The effect of oligo-dN is specific. For example, oligo-dG stimulates only dGMP incorporation by $r(\text{I}\cdot\text{C})_n$ but not dCMP incorporation by $r(\text{I}\cdot\text{C})_n$ nor dTMP incorporation by $r(\text{A}\cdot\text{U})_n$. This indicates that the action of oligo-dN may not be through an allosteric effect on the enzyme. (4) K_m of dTTP for the polymerase is 0.05 mM in $r(\text{A}\cdot\text{U})_n$ system, 0.03 mM in either $r(\text{A}\cdot\text{U})_n + \text{oligo-dT}$ or $r\text{A}_n\cdot\text{oligo-dT}$ system, and 0.03 mM in "activated" calf thymus DNA and $d(\text{A-T})_n$ systems. (5) Divalent metal requirement for the oligo-dN + RNA system has individual characteristics, distinct from those of RNA and DNA. (6) Preliminary data showed that oligo-dN acts as primer and may itself be incorporated into the polymerized product.

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DNA POLYMERASE ACTIVITIES IN RATS AS A FUNCTION OF AGE

J. M. Frazer and Wen-Kuang Yang

Quantitative or qualitative alterations in DNA polymerase activities could result in insufficient cell renewal by blocking DNA synthesis or could result in increased rates of mutation by insufficient repair to DNA. Either process could affect proper cell renewal and could play an important role in aging.

Studies have been initiated to determine specific activities and functional parameters of DNA polymerase activities in different subcellular fractions of 3-, 13-, and 25-month-old rats. To date, no significant differences have been found for activities extracted from liver nuclei. The specific activities of DNA polymerase requiring all four deoxynucleotide triphosphates and the "terminal transferase" activity polymerizing a single deoxynucleotide triphosphate (dTTP), have been the same for all three age groups. Further studies on these activities and their subcellular distribution and properties and similar studies on spleen tissue will be carried out.

ANALYSIS OF POSSIBLE TRANSLATIONAL FACTORS IN AGING

J. M. Frazer and Wen-Kuang Yang

We have tested Orgel's hypothesis (1) of "error catastrophe," one of many biochemical theories of aging. Briefly, he suggested that a random error incorporated into a metabolic enzyme at the translational process would be diluted out and not amplified, whereas an error in an enzyme or component involved in the expression of genetic information would not only be passed on in time but also increase in magnitude by decreasing the fidelity of genetic expression. This process

of error amplification would result in what Orgel termed "error catastrophe," the point at which the cell could no longer function. Some possible points to look at in the translational step of genetic information expression include tRNA, aminoacyl tRNA synthetases, ribosomal functions, and others.

We have investigated a partial spectrum of isoaccepting tRNA's by RPC-2 column chromatography and the aminoacyl tRNA synthetases in the BC3F₁ mouse strain. These mice live as long as 3 years, equivalent to more than 100 years for man. The comparison of tRNA profiles and enzyme activities in liver and brain between old (28-30 months) and young (9 months) animals should subject Orgel's hypothesis to an experimental test. Results on rate and extent of aminoacylation and cochromatographic analysis of isoaccepting species reveal no differences between young and old animals for arginyl-, aspartyl-, glutamyl-, histidiny-, leucyl-, lysyl-, phenylalanyl-, seryl-, or tyrosyl-tRNA's from liver or for arginyl-, aspartyl-, leucyl-, lysyl-, and seryl-tRNA's from brain. These results were obtained using homologous synthetase preparations and maximal acylation conditions determined separately for each amino acid. These results indicate no changes in tRNA as a function of age, and, since many different enzymes are necessary for producing active tRNA (modification enzymes), the absence of changes in tRNA's argues for the correct functioning of all of these enzymes with their usual specificity. This indirect biological assay of the correct functioning of more than twenty enzymes argues against significant error generation with aging.

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ANALYTICAL USE OF THE RPC-5 SYSTEM FOR MAMMALIAN ISOACCEPTING TRANSFER RNA'S

Chongkun Koh, Wen-Kuang Yang, and G. David Novelli

For the analysis of isoaccepting tRNA's, one of the reversed-phase chromatographic systems, the RPC-2, generally shows satisfactory resolving power. An analytical method for small quantities of tRNA using the RPC-2 system has been described (1). However, separation of isoaccepting species of tyrosyl-, prolyl-, valyl-, glycyl-, arginyl-, leucyl-, and threonyl-tRNA's from mammalian sources is only fairly achieved on the RPC-2 column. In the present study, we have found that the isoaccepting species of these tRNA's can be separated and hence analyzed by the newly developed RPC-5 system, which employs the same tricaprylmethylammonium chloride as the RPC-2 but uses Plaskon powder (polychlorotrifluoroethylene) in the place of Chromosorb W and Freon 214 (2). Tyrosyl-tRNA's from mouse tumor tissues were resolved into six peaks; prolyl-tRNA's from L-M cells, into three peaks; valyl-tRNA's from C57BL/6 mouse reticulocytes, into about six peaks; glycyl-tRNA's from C57BL/6 mouse reticulocytes, into three peaks; leucyl-tRNA's from rat liver, into five peaks. In all cases except valyl-tRNA's, the resolution of the peaks was satisfactory enough for distinct numbering of the peaks to be obtained. The RPC-5 column has proved to be especially useful in the analysis of tyrosyl-tRNA's from tumor, fetal, and control normal tissues.

The technique for the operation of the RPC-5 is generally the same as that previously described (1), except as follows: (1) Size of the column was either 0.5 X 50 cm or 0.5 X 25 cm. This reflects both the increased capacity of the RPC-5 for tRNA and its different chromatographic properties. (2) Optimal NaCl gradients were chosen by first chromatographing aminoacyl-tRNA's from 0.4 to 0.8 M NaCl and subsequently selecting a narrower range. The optimal gradients were determined to be 0.475-0.55 M for tyrosyl-, prolyl-, and glycyl-tRNA's, 0.5-0.725 M for leucyl-tRNA's, and 0.425-0.50 M for valyl-tRNA's. (3) The column was operated at 50-100 psi. This was necessary, due to the fine Plaskon powder. (4) Flow rates range from 0.2 to 0.5 ml min⁻¹. No loss of resolution of tRNA's was found by increasing the flow rate. Thus, with a gradient size of 120 ml, 200 fractions would be obtained in 4 hr.

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INHIBITION OF THE DNA POLYMERASE OF RAUSCHER LEUKEMIA VIRUS BY SINGLE-STRANDED POLYRIBONUCLEOTIDES

F. W. Tuominen, L. E. Roberson, and F. T. Kenney

To begin analysis of cellular repression mechanisms regulating the expression of oncogenic viruses in mice, we chose to follow the newly discovered "reverse transcriptase" as a convenient and quantifiable marker of virus expression. Initial objectives were: (1) to devise a reliable assay for the virus-type DNA polymerase to distinguish it from DNA-dependent DNA polymerases in cells and (2) to devise an experimental system in which the level of the virus polymerase can be manipulated. In following the first of these objectives, we found that the viral polymerase is severely inhibited by single-stranded polyribonucleotides.

These polymers inhibit the virus polymerase, whether endogenous RNA, DNA, or synthetic polymers such as the alternating copolymer poly[d(A-T)] are used as template. Inhibition is strictly competitive with the template. DNA polymerases from mouse embryo cells (partially purified by DNA-cellulose chromatography) or *E. coli* are not inhibited at high concentrations of ribopolymers. Control experiments established that failure to inhibit the nonviral polymerases is not due to contaminants that destroy the polyribonucleotides; mixtures of, e.g., mouse embryo DNA polymerase and poly(U), were capable of inhibiting virus polymerase after prolonged incubation at 37°C.

The strength of the inhibition of virus polymerase depends on the particular homopolymer employed: poly(U) > poly(G) >> poly(A) > poly(C). The K_i for poly(U) was 0.08 $\mu\text{g ml}^{-1}$, which represents an apparent affinity six times greater than that for viral RNA.

These observations provide a simple criterion to distinguish the virus-type DNA polymerase from cellular DNA polymerases; the former is a polymerase that is competitively inhibited by polyribonucleotides. Experiments are under way to

determine whether the specific affinity of virus polymerase for polyribonucleotides may be useful in separation and purification of the enzyme from cellular extracts, e.g. by specific elution with poly(U) from DNA-cellulose columns.

Perhaps of greater importance, these results provide a means to probe the role of the "reverse transcriptase" in virus replication and in virus-induced leukemia. If RNA-directed synthesis of DNA is of significance in leukemia or other cancers, it is conceivable that specific polynucleotide inhibitors could be used therapeutically. These possibilities are being studied in collaboration with virologists R. W. Tennant and R. L. Tyndall and pathologist M. G. Hanna, Jr.

SPECIFIC STEROID RECEPTORS IN CULTURED HEPATOMA CELLS

Kai-Lin Lee, Janice C. Laney, and F. T. Kenney

Previous studies (1) on the structural requirements of steroids for induction of tyrosine transaminase indicated that the steroids must bind to an intracellular receptor to initiate induction. The macromolecular cellular constituents that bind ^3H -hydrocortisone were found to be present in both nucleus and cytoplasm. To test whether the receptors so identified are actually involved in induction, we tested the competition of various unlabeled steroids with ^3H -hydrocortisone, with respect to both induction and binding of the receptors.

Radioactive hydrocortisone elevated the tyrosine transaminase content of cells and also produced appreciable labeling of supernatant and nuclear receptors. Supplementation with either estradiol or tetrahydrohydrocortisone had no effect on the enzyme level or on the extent of hydrocortisone binding in either cellular fraction. 17α - and 11α -hydroxyprogesterone lowered ^3H -hydrocortisone binding appreciably and also reduced induction by hydrocortisone. Corticosterone and 11β -hydroxyprogesterone similarly competed with labeled hydrocortisone for receptor sites in both soluble and nuclear fractions. Based on these observations, it can be concluded that the competition we observed previously in induction studies does, indeed, reflect competition for intracellular receptor sites. These results also support our earlier conclusion that the binding of steroid to receptor is a prerequisite for the induction process. That the binding of radioactive steroid to cytoplasmic receptor was not temperature dependent, while binding to the nuclear receptor was temperature dependent, suggests that only the latter step is an energy-dependent process. Preliminary results indicate that the nuclear receptor is located in chromatin. Therefore, we examined the template activity of the cells treated with hydrocortisone (in collaboration with Dr. K. Barker, University of Nebraska). Chromatin isolated from hydrocortisone-treated cells had 10-15% higher template activity than that from untreated cells. Maximal increase in template efficiency was achieved 1 hr after the addition of hydrocortisone. These results can be interpreted to mean that increased template activity reflects increased formation of messenger RNA coding for the transaminase.

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REGULATION OF TYROSINE TRANSAMINASE BY $\underline{\underline{\text{L}}}$ -LEUCINE IN CULTURED HEPATOMA CELLS

Kai-Lin Lee, Janice C. Laney, and F. T. Kenney

The activity of tyrosine transaminase in cultured H-35 cells was markedly stimulated by increasing the concentration of amino acids in the medium. The active component was identified as $\underline{\underline{\text{L}}}$ -leucine. The optimal concentration of $\underline{\underline{\text{L}}}$ -leucine was 5 mM, which elicited an 8- to 10-fold increase in the transaminase level. The half-life of the transaminase was estimated by following the increase or decrease of enzyme activity after addition or withdrawal of the amino acid. These gave values of 6 and 2 hr, respectively, which were in agreement with the values obtained by direct measurement of the enzyme half-life, using an immunochemical-isotopic method. Pulse-labeling experiments indicated that $\underline{\underline{\text{L}}}$ -leucine also increased the rate of transaminase synthesis by a factor of 3 to 4. Thus, $\underline{\underline{\text{L}}}$ -leucine increased the level of the transaminase by a dual effect on both synthesis and degradation of the enzyme. That hydrocortisone or insulin effectively induced the transaminase in $\underline{\underline{\text{L}}}$ -leucine-free medium and that combinations of $\underline{\underline{\text{L}}}$ -leucine with hydrocortisone or with insulin gave additive responses indicate that induction by either of these hormones is not mediated by $\underline{\underline{\text{L}}}$ -leucine. These results also imply that the mechanism of the leucine effect on enzyme synthesis differs from that of hydrocortisone or of insulin.

INDUCTION OF ALANINE TRANSAMINASE BY ADRENAL STEROIDS IN CULTURED HEPATOMA CELLS

Kai-Lin Lee and F. T. Kenney

Alanine transaminase is known to be inducible by hydrocortisone in rat liver. The maximum increase in enzyme level is attained only after prolonged steroid treatment. This slow response has hitherto made it impossible to determine whether steroids act directly on hepatic cells to induce this enzyme. Thus, we examined the induction of alanine transaminase by steroids in cultured cells of the H-35 hepatoma. Induction of this transaminase by hydrocortisone was observed in H-35 cells and was shown to be due to enhanced synthesis of the enzyme. Based on these results, it can be concluded that the reported induction of alanine transaminase in rat liver after glucocorticoid treatment is due to direct steroid action on hepatic cells. The similarity of the response of tyrosine and alanine transaminases to various concentrations and to modifications of the steroid structure suggest that the same mechanism is involved in the induction of these two transaminases. However, the extent of response of these enzymes to the steroid is not the same: 24 hr after the addition of hydrocortisone, the synthesis of tyrosine transaminase is elevated 10- to 12-fold, whereas that of alanine transaminase is approximately doubled. These observations suggest that synthesis of these two enzymes is stimulated in a sequential rather than coordinate fashion after hydrocortisone treatment.

STUDIES ON THE REGULATION OF ENZYME DEGRADATION IN CULTURED CELLS

R. W. Johnson and F. T. Kenney

Several hypotheses have been proposed to explain differential rates of enzyme degradation. The degradation rate may be determined by (1) the physical properties of the enzyme molecule, (2) ligand interaction, (3) a specific degrading enzyme, (4) the turnover rate of an organelle where an enzyme is localized, (5) molecular size, or (6) a specific posttranslational modification.

To test the first hypothesis, that the physical properties of the enzyme determine its turnover rate, fluorotryptophan was incorporated into tyrosine aminotransferase (TAT) in place of tryptophan in Reuber H-35 hepatoma cells. TAT from cells induced with hydrocortisone in the presence of 0.1 mM 4-fluorotryptophan, 5-fluorotryptophan, or 6-fluorotryptophan was shown to denature 1.24-, 2.2-, and 4.7-fold, respectively, more rapidly at 64°C than TAT from control cells. The degree of change in the heat stability of TAT is dose dependent. Increasing concentrations of fluorotryptophan in the induction medium lead to increased heat denaturation rates of TAT. TAT from 5-methyltryptophan-supplemented cells or tryptophan-starved cells had a heat denaturation rate identical to that of enzyme from control cells.

The immunochemical properties of tyrosine aminotransferase are also altered by fluorotryptophan incorporation; 1.6 times as much TAT-specific antibody is required to precipitate an equivalent number of analog enzyme units. This is consistent with the interpretation that the analog enzyme possesses less catalytic activity per molecule. It is concluded from the altered heat stability of the analog enzyme, the dose-response relationship between analog concentration and change in heat stability, and the altered immunochemical properties, that fluorotryptophan is incorporated into tyrosine aminotransferase and significantly changes the physical properties of the enzyme.

The degradation rate of the analog enzyme *in vivo* was measured in a double-label experiment. Cells were first induced for 8 hr on tryptophan-free media. The native enzyme, which is induced on a tryptophan-free medium, was then labeled for 2 hr with ^{14}C -valine. This medium was replaced by one containing ^3H -valine and 6F-tryptophan for an additional 2 hr to label the analog enzyme. The cells were chased with a cold medium for 4 hr. The radioactivity in the enzymes, both native and analog, was determined hourly by precipitating with antibody. The analog enzyme was degraded at the same rate, with a 2-hr half-life, as the native enzyme. This rate is identical to the rate determined simultaneously in control cells, as well as to the reported value in the literature. It is concluded from this series of experiments that the enzyme conformation is not a critical determinant of the rate of enzyme degradation.

ENZYME TURNOVER AND ENZYME MULTIPLE MOLECULAR FORMS

R. W. Johnson and F. T. Kenney

Three multiple forms of tyrosine aminotransferase (TAT) have been purified from rat liver, using CM-Sephadex chromatography. These forms have the following characteristics. They are stable when purified; they can be interconverted *in vitro* in crude soluble liver extracts; they are not aggregates, as judged by gel filtration chromatography; they have identical heat denaturation rates; and they are immunologically identical, as judged by immunodiffusion. It is concluded from these findings that the multiple forms are not products of multiple genes.

The relative proportions of the three multiple forms of TAT during the 18-hr hydrocortisone induction cycle was studied. All animals were killed at about the same time of day, and the soluble liver extracts were applied to the column as rapidly as possible to prevent interconversion.

Early in the induction cycle, when enzyme-specific activity is increasing, form I predominates (70%). At the induced steady-state level and in uninduced liver, the proportions of the three forms approximate 40:20:40. During the degradative phase of the cycle, form III predominates (50%). These findings indicate that forms II and III represent a posttranslational modification of form I, that the three forms are reversibly interconverted, and that they may represent intermediates in enzyme turnover.

STUDIES ON THE POSSIBLE ROLE OF LYSOSOMES IN PROTEIN TURNOVER

C. D. Stiles* and F. T. Kenney

The molecular mechanisms responsible for intracellular protein degradation in mammals are not clearly understood. Recently, Auricchio and Figuori (1) presented data that, they claimed, strongly implicated lysosomes as being responsible for the extremely rapid turnover exhibited by tyrosine aminotransferase (TAT) in rat liver and in tissue-culture lines derived from rat hepatomas. We performed two quick tests of this hypothesis, using Reuber H-35 cells, a tissue-culture line derived from a minimal-deviation rat hepatoma.

In one experiment, TAT was preinduced in several culture flasks by overnight incubation with 5×10^{-7} M hydrocortisone in the growth medium. At time 0, beef hemoglobin was added to half the flasks to a final concentration of 1 mg ml^{-1} . Controls received no addition. Hemoglobin is a good substrate for lysosomal peptidases, so if lysosomes were involved in TAT degradation, one might expect that hemoglobin would be taken up by the cells, act as a competitive inhibitor for TAT, and

result in a "superinduction" of the hemoglobin treated cells. No such superinduction was observed.

In a second experiment, TAT was preinduced in several culture flasks by overnight incubation with hydrocortisone. At time 0, the inducing medium was washed away from all flasks. Control flasks received fresh growth medium, and the remainder of the flasks received fresh growth medium containing 0.01% trypan blue, which has been shown to be a specific inhibitor of lysosomal peptidases in rat liver (2). If lysosomes were involved in TAT degradation, then trypan blue should noncompetitively inhibit the turnover of the enzyme; this inhibition would be reflected in the decay curve of TAT activity following removal of the inducer. In fact, no differences were observed in the TAT decay curves of trypan-blue-treated and control cells.

The results of these experiments are inconsistent with the hypothesis that lysosomes are responsible for TAT turnover, although, clearly, the idea cannot be ruled out on the basis of such negative data.

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STUDIES OF STEROID REGULATORY MECHANISMS IN SYNCHRONIZED RAT HEPATOMA CELLS

C. D. Stiles* and F. T. Kenney

A major obstacle to the study of regulatory mechanisms in mammals is the lack of regulatory mutants to serve as experimental controls. As an alternative to mammalian regulatory mutants, it may be possible to obtain populations of cells which, though genetically unaltered from the parent stock, do not respond to the regulatory substance.

Nonsynchronized Reuber H-35 cells, a tissue-culture line derived from a minimal-deviation rat hepatoma, exhibit an 8- to 10-fold induction of the enzyme tyrosine aminotransferase (TAT) following addition of physiological levels of hydrocortisone to the tissue-culture medium. Employing the cell shake-off technique of Terasima and Tomach (1), coupled with the use of colecemid, we obtained small populations of Reuber cells that were 90% synchronized at mitosis. Hydrocortisone, a presumptive transcriptional inducer of TAT, failed to induce the enzyme in the mitotic cells. Similar findings were reported earlier for HTC cells, a tissue-culture line similar in many respects to the Reuber cells used in our laboratory (2). Additionally, we obtained evidence suggesting that insulin, a presumptive translational inducer of TAT, does induce mitotic cells.

We are currently developing methods for obtaining large populations of mitotically synchronized Reuber cells, using inhibitors of DNA synthesis. We plan to use these mitotically synchronized cells as controls in the study of the molecular mechanism of steroid hormone regulation.

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BIOCHEMISTRY OF VIRAL CARCINOGENESIS

Stanfield Rogers

This report summarizes research in two areas: (1) studies of wild-type Shope rabbit papilloma virus and its domestic-rabbit recoverable mutant, the tumors they induce, and the cancers arising therein; and (2) development of the use of viruses to transmit genetic information with the aim of specifically treating patients with monogenic deficiency diseases such as argininemia. These studies include modification of viral nucleic acid and reassembly of the virion.

Comparative studies of the physical and chemical characteristics of wild-type (WT) Shope virus and its Rec mutant are being continued. The Rec mutant moves somewhat slower in sucrose gradients than the WT, but the mutant's tendency to aggregate has as yet made precision sedimentation velocity studies impossible. Using virus purified on sucrose gradients, comparisons are being made of amino acids in the protein coats of both viruses. WT protein coat is very stable, and in electron microscope pictures it has the usual capsomers. Rec, on the other hand, has no detectable capsomers. The arginases induced by the two virus lines are separable, as previously reported. There are other physical and chemical differences between the arginases, such as specific activity and specific rotation in optical rotatory dispersion. In collaboration with Dr. Raymond Popp, sequence studies of the amino acids of these two enzymes are being made.

Efforts have been made to find out why wild cottontail rabbits in Kansas produce much more virus than similar cottontails inoculated in the laboratory. Both Kansas-raised food and Kansas river water from the area these rabbits inhabit have been tested without increasing the virus yield in laboratory rabbits. Tests are under way to find out whether an individual rabbit whose papillomas at the time of capture had a high virus yield will continue to do so after a period of months in the laboratory. Papillomas from the wild rabbits thus far examined show a much larger virus yield when plucked on arrival from Kansas than regrowths of the same tumors while in Oak Ridge, even though all the food fed both rabbits originates from western Kansas.

In both wild and domestic rabbits, cancers arise in the virus-induced papillomas after a period of several months. In contrast to the constancy of morphology of the papillomas, the cancers differ among themselves over a wide range. Two such cancers have been transplanted over a period of many years. They differ markedly from each other in morphology and growth rate. The Vx-7 cancer still has the virus and the virus-induced arginase after 116 transplant generations. The Vx-2 has lost both and is in its 270th transplant generation. Upon comparing the amino acid uptakes *in vitro* of these two cancers, in addition to the change relating to the loss of

arginase (lower uptakes of arginine and no ornithine production in the Vx-2), we found that the Vx-2 takes up several times more glycine and aspartate than the Vx-7. The Vx-7, on the other hand, uses remarkable amounts of leucine. We plan to take advantage of these metabolic differences through the use of metabolic antagonists specifically designed for the individual tumor and by enzymatic control of the blood levels of these amino acids, using dialysis.

Upon reintroduction of the virus into the Vx-2, the ratios of lysine to ornithine and arginine to ornithine resemble those of the Vx-7 more closely. When this is done in tissue-culture Vx-2 cells and the arginase activity is measured (by measuring the amount of ^{14}C -ornithine found following incubation with ^{14}C -arginine), the virus-exposed cells yield more labeled ornithine. We have no evidence as yet that any coat protein of the virus is being made. Rabbits carrying the Vx-2 tumor in which the virus has been reintroduced do not make detectable antibody against the virus. The Vx-7 rabbits do.

It is hoped that these studies of the cancers arising in papillomas will in time cast light on the nature of the carcinogenic event. The randomness of the findings thus far makes a model resembling mutation likely.

Specifically hybridized Shope papilloma virus message has been isolated from papillomas and separated into five fractions, using cesium chloride. Using the method of Sela and Kaesberg (1), an *in vitro* protein synthesizing system using tobacco chloroplast ribosomes, we plan to find out whether these messages and/or messages derived from the Vx-7 and Vx-2 tumors will show activity. Using this system and tobacco mosaic virus (TMV) RNA, one can readily make TMV protein coat subunits.

A joint effort is under way with three groups of European investigators to use the Shope virus to treat two children who have argininemia. Using cultured fibroblasts from one of the children (AW), inoculation with the Shope virus has resulted in increased radioactive ornithine, using both cell extracts and cultured cells in a synthetic medium containing ^{14}C -arginine. Fluorescent antibody studies, using antibody against purified virus-induced arginine, indicate that about half the cultured cells are infected. When corrected for the number of infected cells, the specific activity of arginase per mg of protein extracted is about the same as that of normal human fibroblasts. In testing for arginase in the argininemic cells without virus infection, only trace amounts of arginase activity have been found. The two children in Germany are currently being treated with the Shope virus. These studies are not yet complete. The tissue-culture studies are being made in collaboration with Dr. Ernest Chu.

Studies using both TMV RNA and the Shope virus DNA are being continued. One of the problems is reassembly of the virion following any changes made in the nucleic acid. We are currently studying the effects of polybasic amino acids upon the infectivity of these nucleic acids and on their resistance to nucleases. TMV RNA can be protected against ribonuclease with polyornithine or polylysine, but the infectivity is low. Shope virus DNA, normally infective in rab-

bits, is rendered noninfective by coating with these basic polymers. Bancroft has found with the cow pea viruses and Brome mosaic virus of barley that the protein coat subunits are interchangeable in virus reassembly and in relation to protection of the RNA against diesterase; the plant host range of the starting viral RNA is not modified by the protein coat it has. He further found that Brome mosaic virus subunits protect double-stranded nucleic acids against nucleases. We are propagating this virus to provide coat subunits for protection of the Shope virus DNA and perhaps DNA from other viruses. Such protection is necessary to protect against nucleases when DNA's are introduced into animals.

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SIMILARITIES IN ISOENZYME PROFILES OF A VARIETY OF MALIGNANT AND FETAL TISSUES

R. L. Tyndall, J. A. Otten, and Lois H. Jordan

Previous electrophoretic characterizations of extracts from normal, leukemic, and fetal mouse tissues indicated that infection with Rauscher leukemia virus (RLV) resulted in the subsequent emergence of cells having profiles similar to fetal thymus and spleen tissues (1). In order to determine whether such patterns were peculiar to RLV-induced malignancies, similar analyses were made on extracts of thymic lymphoma tissues from spontaneous and X-ray-induced malignancies of AKR and RFM mice, respectively. The corresponding fetal and normal thymus tissues were also characterized. In addition, extracts of normal, fetal, and neoplastic lung and stomach tissues of RFM mice were analyzed. The lung and stomach neoplasms were obtained following oral administration of diethylnitrosamine.

The results of these studies indicated that isoenzyme patterns of all neoplastic and corresponding fetal-newborn tissues show similar alterations, compared to normal adult tissues. Lactic dehydrogenase (LDH) activity in both fetal and neoplastic tissues was predominantly associated with the more slowly migrating bands, in contrast to normal adult tissues. Esterase activity associated with proteins migrating similar to serum haptoglobins and prealbumins was markedly reduced in the extracts of fetal and neoplastic tissues, compared to extracts of adult tissues. Thus, as with RLV-induced malignancies, extracts of spontaneous, X-ray-induced, and DEN-induced neoplasias showed isoenzyme profiles similar to those of the corresponding fetal tissues and altered from the normal adult pattern. In view of these similarities, studies were undertaken to determine whether mice bearing leukemic tissue react to their malignancies in a manner analogous to mice bearing fetal tissues during pregnancy.

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SIMILARITY IN RESPONSE OF MICE BEARING LEUKEMIC AND FETAL TISSUES

R. L. Tyndall, J. A. Otten, N. D. Bowles, and M. R. Proffitt*

A variety of similarities between leukemic and corresponding fetal tissues have been observed in recent studies (1, 2, 3). Consequently, it was of interest to determine whether mice bearing leukemic tissues reacted to such tissues in a manner similar to mice bearing fetal tissues during pregnancy. Spleen and serum were collected from female mice at various intervals following coitus. Similarly, samples were collected at like intervals following Rauscher leukemia virus (RLV) infection or inoculation of antigen (sheep erythrocytes). Splens were weighed and examined histologically, and extracts were subsequently examined for their esterase isoenzyme profiles by electrophoresis on acrylamide gel. Protein profiles of the various serum samples were also determined electrophoretically, as was serum from AKR and RFM mice with spontaneous or X-ray-induced leukemias, respectively. Sera from normal and pregnant AKR and RFM mice were also analyzed for comparative purposes. In addition, the effects of spleen cells from pregnant and from virgin mice on the attachment and growth of control uninfected (JLS V6) and RLV-infected (JLS V5) cell cultures were tested.

Neither serum nor spleen tissue from mice injected with sheep erythrocytes showed any significant alterations, compared to tissues from normal adult mice. Conversely, increases in weight, erythropoiesis, and esterase activity were seen in splens from both pregnant and RLV-infected mice. These effects reached a maximum in pregnant mice at 10–12 days following coitus and returned to normal values by day 20, whereas the effects increased progressively with time following RLV infection. Serum protein profiles of pregnant or leukemic BALB/c, RFM, and AKR mice all showed identical, highly reproducible alterations. The bimodal distribution of the second and third prealbumins was lost, and a fourth prealbumin protein became apparent in sera from pregnant and leukemic mice.

In addition, increased amounts of a protein migrating in juxtaposition to serum transferrin was also seen in sera from pregnant and leukemic mice but not in sera from normal virgin mice. The attachment and growth of RLV-infected cell cultures but not control uninfected cells were also impaired in the presence of spleen cells from pregnant, as compared to normal mice. These results indicate some similarities in the responses of mice to the presence of leukemic and fetal tissues. The similarities in fetal and leukemic tissues and in the responses of mice to such tissues suggest a hypothesis by which the pseudofetal nature of leukemic tissues elicits a response from the host not unlike that of pregnancy, and which may inadvertently insure the continuance of the neoplasia. Such a hypothesis might warrant consideration in devising new therapeutic approaches against such neoplasias.

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THE COMBINED ACTION OF RAUSCHER LEUKEMIA VIRUS AND LACTIC DEHYDROGENASE VIRUS ON MOUSE LYMPHATIC TISSUE

M. R. Proffitt,* C. C. Congdon, and R. L. Tyndall

The majority of transplantable tumors and stocks of murine leukemia viruses are contaminated with lactic dehydrogenase virus (LDV). Rauscher leukemia virus propagated in cell cultures (TCRLV), however, is free of LDV. While TCRLV is leukemogenic for newborn mice following an extended latent period, it does not produce rapid erythroid hyperplasia and early leukemia on inoculation of adult mice as do conventional stocks of Rauscher leukemia virus (RLV) contaminated with LDV. Consequently, it was of interest to determine the role, if any, of LDV in the total "Rauscher disease" syndrome. Adult BALB/c mice were inoculated with LDV, conventional RLV contaminated with LDV, animal-passaged TCRLV free of LDV, or a mixture of LDV and TCRLV. The weight and histologic appearance of spleen tissues at various intervals following virus infection were determined. In addition, the immunosuppressive effect of the different virus preparations on antirat erythrocyte antibody formation was studied by inoculating test mice with virus preparations 8 days prior to challenge with rat erythrocytes.

The results showed that mice inoculated with conventional RLV contaminated with LDV had early gross and histological changes in their lymphatic tissues almost identical to those seen in mice inoculated with LDV alone. Except for early erythroblast activation, mice inoculated with the pool of animal-passaged TCRLV, which was not contaminated with LDV, did not undergo these changes. Most notable were the lack of early lymphocyte destruction in "thymus-dependent" areas responsible for cell-mediated immunity and the failure of significant immunoblast activation and proliferation to occur in lymphoid germinal centers (humoral immune compartments).

The presence of LDV in the RLV pools or its addition to the uncontaminated pool resulted in an enhancement of the erythroblastic reaction associated with Rauscher disease, as measured by increase in spleen weight (Table I). There was also prominent diffuse and persisting hyperplasia of germinal center cells (immunoblasts and immunocytes) and enhanced immunosuppression (Table I) after LDV was added to the uncontaminated RLV pool.

TABLE I. Primary hemagglutinin response in BALB/c mice infected with LDV or different pools of RLV 8 days prior to immunization with rat erythrocytes (RRBC's)

Days after RBC	Mean log ₂ titers ± (S.E.)*					
	Viruses					
	RLV	TCRLV + LDV	TCRLV	LDV	Vehicle	None
6	1.7 ± (0.4)	1.2 ± (0.2)	5.0 ± (0.8)	7.6 ± (0.4)	5.7 ± (0.4)	7.3 ± (0.2)
12	2.1 ± (0.1)	2.0 ± (0.1)	4.5 ± (0.2)	5.7 ± (0.4)	5.2 ± (0.1)	6.0 ± (0.1)
	Mean day-12 spleen weight (mg)/Body weight (g) ± (S.E.)					
	13.6 ± (3.0)	11.0 ± (0.4)	6.5 ± (1.2)	5.8 ± (0.1)	4.8 ± (0.3)	5.5 ± (0.2)

*Standard error of the mean (5 mice per group) ± 2 S.E. = 95% confidence limits.

Two possibilities were considered to account for the enhancement of Rauscher disease by LDV. First, by stimulating germinal center hyperplasia, LDV could supply greater numbers of target cells for RLV replication. Second, the depression of the cell-mediated immune response by LDV could prevent RLV or tumor cell inactivation by lymphocytes.

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DIFFERENCES IN TUMOR TYPES AND ORGAN SUSCEPTIBILITY IN BALB/c AND RF MICE FOLLOWING DIMETHYLNITROSAMINE AND DIETHYLNITROSAMINE

N. K. Clapp, R. L. Tyndall, and J. A. Otten

The differences in types and incidences of spontaneous malignancies among various mouse strains amply demonstrate the genetic influence on the expression of such malignancies. In light of such influence on spontaneous tumor development, the present study was made to evaluate tumorigenesis induced by dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) in male BALB/c mice, a strain with relatively low spontaneous incidences of tumors, and to compare the results with previous

studies in male RF mice, a strain in which there are high spontaneous incidences of lung tumors and reticulum cell sarcoma, a late-occurring leukemia.

Total doses of 300 mg kg⁻¹ of DMN and 515 and 1010 mg kg⁻¹ of DEN were given to adult BALB/c mice in their drinking water. The oncogenic results for this strain were compared with previously reported data on a noninbred sub-line of RF/Un mice handled under similar conditions (1, 2). DEN induced forestomach and esophageal squamous cell carcinomas in both strains but induced liver hemangiosarcoma in BALB/c mice and hepatomas in RF mice. In addition, DEN induced a high incidence of lung adenomas in the RF strain but was only slightly effective (3%) in BALB/c mice. In both strains, DMN induced lung adenomas and liver hemangiosarcomas. Differences in the liver sensitivity of the two strains to DEN suggest that different liver cells may metabolize DEN in the two strains, suggesting, in turn, an excellent tool for metabolic studies. No leukemogenic effect was observed following either DMN or DEN in either strain. Thus, just as with spontaneous tumor expression, the type and degree of oncogenic response of mice to DEN and DMN is a reflection, in part, of the mouse strain, but is not necessarily directly related to the spontaneous tumor incidence of the strain.

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