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BIOLOGY DIVISION
ANNUAL PROGRESS REPORT
For Period Ending June 30, 1972

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

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INTRODUCTION

The primary purpose of this document is to provide to the Oak Ridge National Laboratory a summary of the research activities in the Biology Division. Efforts have been made to keep each entry concise so that the large number of research projects in progress can all be represented in one volume of manageable size.

In addition to its primary purpose, we hope that this report is useful to the agencies that sponsor our work. Most of the activities in the Division are sponsored entirely by the Atomic Energy Commission. We have attempted by footnotes to call attention to activities which are the result of joint agreements between the AEC and other sponsoring organizations.

It is clear that concise summaries of the sort presented here cannot do justice to the work of the Division. I suggest that most readers who want a more detailed account of our activities request the annual bulletin and reprint selection list, or that they contact the individual investigators.

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Director, Biology Division

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CHEMICAL PROTECTION AND ENZYME CATALYSIS

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

We have been engaged in synthesizing a series of seven diamine dithiols and diamino monothiols with appropriate spacing of the charges for binding to nucleic acids. The preparation of these compounds has been complicated by the fact that most of the penultimate intermediates are unknown and, if they can be prepared, will exist as both optical and geometrical isomers. We have experienced considerable difficulty in the synthesis and separation of pure compounds and are exploring alternate routes that will hopefully yield the desired substances. In conjunction with J. D. Regan we have prepared eight possible serine antagonists, one of which seems promising, for testing in the human leukemia cell system.

CHARACTERIZATION OF THE GENOME TRANSCRIBED BY BOVINE LENS EPITHELIAL CELLS

B. C. Strnad and John Papaconstantinou

The vertebrate lens is composed of cells solely of ectodermal origin and is essentially the only tissue that consists of a pure population of cells that show distinct stages of cellular differentiation (1). In addition, the cells from this tissue synthesize large quantities of tissue-specific structural proteins. In the epithelial cell, for example, 60% of the total leucine incorporation is found in the α -crystallin subunits (2). This homogeneity of embryonic origin as well as the relative homogeneity of protein synthesis makes the lens an excellent tissue for studying the expression of specific genes in eucaryotic cells.

It has been shown in other laboratories that 40% of the bovine genome is composed of repeated DNA sequences and 60% of the genome is composed of single-copy sequences (3). In our studies, we have determined the percentage of RNA transcribed by the repeated and unique regions of the calf genome, using DNA-RNA hybridization. RNA of the epithelial cells was pulse-labeled with [³H]uridine for 1.5 hr. When the RNA was labeled under the same conditions with [¹⁴C]methionine, very little rRNA was synthesized.

Thus, the data obtained in these studies result from the hybridization of RNA species other than rRNA. To carry out the hybridizations, we mixed RNA with bovine thymus DNA that had been sheared to a molecular weight of 2.5×10^5 daltons. The input ratio of DNA to RNA was 32. The DNA-RNA mixture was dissolved in 50% formamide-5X SSC, heated to 100° C, and then quickly cooled to 37° C. The kinetics of DNA reassociation were followed optically, and the kinetics of DNA-RNA hybrid formation were followed by measurement of RNase-resistant, TCA-precipitable radioactivity. Only 20% of the input RNA hybridized by the time the DNA had undergone 90% reassociation ($C_0t = 2 \times 10^5$). Of the RNA that hybridized, 25% had done so by a C_0t of 1×10^3 , indicating that it was transcribed from the repeated sequences of the genome. The remaining 75% hybridized between a C_0t of 1×10^3 and 2×10^5 , indicating that it was transcribed from the unique sequences of the genome. The hybrids were melted to determine the specificity of the base pairing. None of the hybrids melted below 70° C. The T_m of the hybrids was 81° C, which compares favorably with the T_m of calf thymus DNA, 86° C. An increase of the DNA/RNA input ratio to 300 failed to increase the percentage of RNA hybridized. This might be explained by the existence of populations of molecules with different specific activities. The population whose hybrids are detected is probably the one with the higher specific activity. We conclude from these studies that the majority of the nonribosomal RNA in the lens is transcribed by the unique sequences. There is also a small but significant amount of RNA transcribed by the repeated sequences of the genome, which reassociate at C_0t between 10^2 and 10^3 . Attempts are now being made to define the various species of RNA that hybridize to the repeated and unique sequences.

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AN ATTEMPT TO ISOLATE AND CHARACTERIZE THE GLOBIN CISTRONS OF THE RABBIT GENOME

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

One of the major problems of developmental biology is the molecular mechanism by which eucaryotic cells control the expression of their genome. In our approach

to this problem, we have chosen first to devise procedures for the isolation of specific DNA sequences that code for the synthesis of tissue-specific proteins, such as hemoglobin, and to use these procedures to determine the degree of gene redundancy and the arrangement of these genes in the chromosome.

To study the reiteration frequency of globin cistrons in the rabbit genome, 9S globin mRNA was isolated and made radioactive by ultraviolet photoreduction in the presence of [^3H]sodium borohydride (1). The photoreduction had no effect on the sedimentation properties of the RNA. Small amounts of this high-specific-activity mRNA (10^4 cpm/ μg) were hybridized in liquid to DNA in a reaction where the ratio of DNA to RNA was $>10^3$ and incubation was to a C_{0t} of 10^4 . The DNA-RNA hybrids were isolated after RNase digestion and Sephadex and hydroxylapatite chromatography. Under these conditions, 70–80% of the DNA was reassociated and 5–8 ng of RNA hybridized per mg DNA. No hybridization occurs in reactions where the DNA C_{0t} is 10^3 or lower. Unlabeled rRNA under conditions in which the ratio of rRNA to mRNA is 500 does not significantly reduce the amount of hybridization of the 9S mRNA. The quality of the hybrids was verified by T_m determinations. Hybrid molecules melt with a T_m that is 5–10° C lower than the T_m for the total reassociated DNA. This indicates, at the most, a 15% mismatching in the base pairing of the hybrids.

From the amount of mRNA hybridized, we have calculated that the genes complementary to the 9S RNA (mol. wt. 2.2×10^5) are reiterated at least 50 times per haploid genome. This value compares favorably with estimates made in the duck reticulocyte system by Bishop *et al.* (2) and by Scherrer (3), who calculate that there are 5–10 and 50 genes, respectively, that hybridize to duck 10S RNA. Our data are in conflict with estimations made by Williamson *et al.* (4) in the mouse and Sanchez de Jimenez *et al.* (5) in the chicken, who find 5×10^4 and 1×10^5 genes per nucleus, respectively. However, it should be emphasized that these latter authors employed filter hybridizations, and their criteria for hybridizations are much less stringent than ours.

Recently we have attempted to fractionate the globin cistrons on $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradients where the ratio of Ag^+ to DNA-P is 0.35 (6). When ^3H -labeled 9S mRNA is hybridized to the fractionated DNA, it is bound preferentially to the DNA in the less dense region of the gradient. When it is recycled, this DNA has a decidedly lower buoyant density in $\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ than does the main-band DNA, but, paradoxically, its T_m is 5° C higher than that of the main band. Through the use of

$\text{Ag}^+ - \text{Cs}_2\text{SO}_4$ gradients and the recycling of the fractions that preferentially bind 9S RNA, we hope to attain a high degree of purification of the globin cistrons, which may then be used for further studies on the arrangement of genes in the genome and the differential expression of these genes.

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TRANSLATIONAL REGULATION OF PROTEIN SYNTHESIS IN EUKARYOTIC CELLS

B. E. Ledford and John Papaconstantinou

Two basic mechanisms exist for the translational regulation of protein synthesis -- regulation of the translational rate and regulation of the number of translational sites. Alterations, either in the rate of generalized protein synthesis or in the synthesis of a specific protein, must involve a change in one or both of these synthetic parameters. One of us (BEL) has developed a theoretical-experimental approach for the determination of the translational rate and the number of translational sites in cultured cells. The method was first applied to the synthesis of the α and β chains of hemoglobin in rabbit reticulocytes. The translational rates of the α and β chains are 60 and 75 amino acid residues per min, respectively, at 25° C. The numbers of translational sites involved in the synthesis of the α and β chains are 1.2×10^7 and 0.90×10^7 per reticulocyte, respectively, at 25° C. Thus, coordination of hemoglobin synthesis is achieved by compensating for a difference in the translational rate with a difference in the number of translational sites.

This approach is presently being applied to the synthesis of specific proteins associated with the structural and functional differentiation of cultured neuroblastoma cells. When transferred from suspension culture to monolayer culture, these cells extend axons that reach several millimeters in length, the specific activity of acetylcholine esterase increases 30-fold, and there is a pronounced increase in the cytoplasmic concentration of microtubular protein. The microtubular protein has been characterized by its colchicine-binding activity and its insolubility in the presence of 2×10^{-3} M

vinblastine sulfate. SDS-acrylamide gel electrophoresis resolves the microtubular protein into two components having molecular weights of 5×10^4 and 4.6×10^4 . Alterations in the synthesis of these two components during the morphological differentiation of neuroblastoma cells is presently being investigated.

AN ANALYSIS OF THE STRUCTURE OF THE POLYTENE CHROMOSOMES IN SALIVARY GLANDS OF *RHYNCHOSCIARA HOLLAENDERI*

E. T. Chin* and John Papaconstantinou

In the Sciarid dipteran, *Rhynchosciara hollaenderi*, polytene chromosomes are formed in many of the somatic tissues. The process of polytene chromosome formation begins during the first-instar stage of larval development, proceeds through the fourth-instar period, and results in the formation of giant chromosomes composed of lateral arrays of DNA strands. The salivary gland is a typical somatic tissue that forms polytene chromosomes, and we have used this tissue to determine whether the DNA of the polytene chromosome is assembled in discrete structural units. Furthermore, we have attempted to determine whether the DNA of the diploid chromosome is similarly assembled. To carry out these experiments, we use the gentle alkaline-lysis technique described by Lett *et al.* (1). It is our purpose in doing these experiments to measure the rate of release of high-molecular-weight DNA from the polytene and diploid chromosomes and to compare the size of the DNA released at various times. Salivary-gland DNA was labeled for 24 hr with [^3H]thymidine to ensure that most of the labeled DNA was high molecular weight. The salivary glands from 10 larvae were removed and lysed in alkaline buffer for 1, 4, and 6 hr. The lysate was then centrifuged in an alkaline sucrose gradient to determine the size of single-stranded DNA (ssDNA) released by this lysis (2, 3). The broadness of the peak, compared to that of phage T4 DNA under similar conditions of alkaline lysis and centrifugation, indicates a small degree of heterogeneity in the size of the DNA released. However, the data clearly show a rapid release of ssDNA, the majority of which peaked with an $S_{20,w}$ of 80S. Furthermore, no differences in the sedimentation properties of the ssDNA were detectable during the 6 hr of alkaline lysis. We conclude from these data that the DNA of the polytene chromosome is assembled in units of double-stranded DNA having a molecular weight of 168×10^6 (4).

A similar series of studies was done using a variety of diploid cells. In one set of experiments, *Drosophila* cells maintained in tissue culture clearly showed that after 4 hr of lysis ssDNA having an $S_{20,w}$ of 174–179S was released. This is equivalent to a double-stranded DNA with a molecular weight of $1240\text{--}1320 \times 10^6$. From 8 to 12 hr of lysis, the ssDNA released had a molecular weight of $3.3\text{--}3.6 \times 10^8$ ($660\text{--}720 \times 10^8$ for double-stranded DNA). In another series of experiments, the spermatocytes of midfourth-instar *Rhynchosciara* larvae were used as a source of diploid chromosomes. At this stage the spermatocytes are still dividing. In addition, there is a small percentage of interstitial cells in the testis that are forming polytene chromosomes. Analysis of a 4-hr lysate showed two distinct peaks, one with an $S_{20,w}$ of 172S and the other with an $S_{20,w}$ of 78S. Although this is a preliminary observation, we believe that the diploid chromosomes give rise to the 172S (ssDNA), whereas the polytene chromosomes give rise to the 78S (ssDNA).

We conclude from our studies that (1) DNA of the polytene chromosome is assembled in structural units having a molecular weight of approximately 168×10^6 ; (2) the DNA of the diploid chromosome is either a larger molecule or, if it exists as molecules similar in size to those seen in the polytene chromosome, more stable in alkali than the polytene chromosomal units; and (3) there is a basic difference between the assembly of the DNA of polytene and diploid chromosomes, which we propose to define more precisely in our future studies.

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CYTOCHEMICAL STUDIES OF POLYTENE CHROMOSOME STRUCTURE

Mary A. Handel, John Papaconstantinou, and R. O. Rahn

Because of the ease of cytological analysis, polytene chromosomes of *Rhynchosciara hollaenderi* have been chosen for an analysis of the DNA content of specific chromosomal regions. Several well-defined chromosomal loci can be described—the centromeric heterochromatin regions and loci that exhibit the phenomenon of DNA puffing. Our recent studies have focused

on centromeric heterochromatin. We have isolated an (A-T)-rich satellite DNA from *Rhynchosciara* salivary glands identical to that isolated from adult flies by Eckhardt and Gall (1) and localized by these workers to the centromeric heterochromatin of the salivary-gland chromosomes. In our studies, through the use of the quinacrine staining technique (2), we have demonstrated that only the chromosomal regions containing these (A-T)-rich satellite DNA sequences react specifically to enhance greatly the fluorescence of the quinacrine stain in cytological preparations.

Experiments are presently being done to determine the basis of the selective, intense quinacrine fluorescence, which may ultimately reveal some of the characteristics of the arrangement and assembly of the (A-T)-rich DNA sequences in the chromosome. Preliminary results indicate that the isolated (A-T)-rich satellite DNA enhances quinacrine fluorescence as measured by spectrophotofluorimetry, but not to the same extent as the fluorescence enhancement observed in cytological preparations. Cytological studies employing acid extraction of histones indicate that these chromosomal proteins do not affect quinacrine specificity. Alternatively, extraction of the acidic chromosomal proteins and pronase and urea treatment do affect the specificity of quinacrine fluorescence. These observations indicate that the acidic proteins and the close packing of the DNA within the chromosome do influence the nature of the specific interaction with quinacrine. It is hoped that such cytochemical studies correlated with biochemical studies will begin to define the relationship of DNA and protein association to chromosomal structure.

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SYNTHESIS OF MITOCHONDRIAL DNA DURING SPERMATOGENESIS IN *RHYNCHOSCIARA HOLLAENDERI*

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Emilia M. Julku, D. P. Allison, and E. T. Chin*

During the period between the last mitotic division and first meiotic division of the primary spermatocytes of *Rhynchosciara* larvae, the mitochondria undergo a significant enlargement, so that just prior to the first meiotic division the mitochondria occupy the entire cytoplasmic area. This increase in size is a unique characteristic of the spermatocyte mitochondria and appears to be due to the accumulation of a highly refractile proteinaceous inclusion. These mitochondria

ultimately fuse to form a single "nebenkern," which extends through the length of the tail of the mature sperm cell. The cytological and ultrastructural differentiation of mitochondria during spermatogenesis has been described for *Rhynchosciara* (1) and *Sciara* (2-4).

In view of the unique morphological change seen in the spermatocyte mitochondria, a series of studies was initiated to determine whether there is a specific DNA synthesis associated with the highly specific development of this organelle (5). These studies revealed the existence of an (A-T)-rich satellite DNA whose synthesis increases significantly during the period of morphological differentiation of the mitochondria. Analysis by ethidium bromide-CsCl gradients showed that a significant portion of this satellite DNA is circular. There is also an indication of the existence of a linear (A-T)-rich satellite, different from the circular DNA, which we believe is synthesized in the nucleus of the polytene interstitial cells of the testis. On ethidium bromide-CsCl gradients, nicked circular DNA and linear satellite DNA band at the same density. Electron microscopy of the DNA from this region of the gradient confirms that much of the satellite DNA is circular and that there is a linear component longer than the circular DNA.

In another series of experiments, attempts were made to determine whether the circular DNA is mitochondrial. A testis homogenate was prepared and centrifuged to obtain a pellet of mitochondria and broken membranes. The pellet was treated with DNase to digest all DNA except that remaining in the intact mitochondria. The DNA extracted from the DNase-treated pellet consisted only of circular and nicked circular species.

Some physical characteristics of the mitochondrial DNA have been determined. By electron microscopy the contour length was shown to be 9 μm . The absolute density was determined to be 1.681 g/cm^3 by analytical ultracentrifugation using *E. coli* DNA as a marker. By thermal gradient elution from hydroxylapatite, the T_m was determined to be 79.3° C (uncorrected). Alkaline CsCl gradient analysis using [³H] thymidine-labeled DNA resolves a major peak at 1.721 g/cm^3 with a shoulder at 1.713 g/cm^3 . The uneven labeling of these strands indicates that the heavy strand has a higher thymidine content than the light strand.

One of the most unique characteristics of this DNA is its relatively long contour length (9 μm). DNA from mitochondria of *Drosophila melanogaster* oocytes has a contour length of 5 μm (6). We are presently isolating mitochondrial DNA from *Rhynchosciara* oocytes for contour measurements to determine whether the mitochondrial DNA from *Rhynchosciara* spermatocytes is

unique, or whether all mitochondria from this insect contain a large circular DNA.

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ATTEMPTS TO ISOLATE POLYRIBOSOMES ENGAGED IN SYNTHESIS OF MICROTUBULAR PROTEIN FROM MOUSE NEUROBLASTOMA CELLS AND *RHYNCHOSCIARA* TESTIS

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and John Papaconstantinou

It has been shown in other laboratories that mouse neuroblastoma cells in tissue culture are an excellent source of microtubular protein (*1*). Electron microscope studies of spermatocyte development in both *Sciara* (*2–4*) and *Rhynchosciara* (*5*) show the formation of large arrays of microtubules in the tail of the mature sperm cell. These observations indicate that such sperm cells should be another excellent source of microtubular protein, and on the basis of these observations, we have assumed that these cells might be an excellent source of microtubular mRNA. The purpose of these studies is to identify and isolate the polyribosomes that synthesize microtubular proteins and to purify the mRNA from the polyribosomes for further use in studies of chromosome structure.

Mouse neuroblastoma cells, when attached to the surface of tissue-culture plates, differentiate and produce large processes that require microtubule formation, but when grown in a suspension culture they are free of such structures. We have succeeded in resolving the polyribosomes from attached and suspended neuroblastoma cells on sucrose density gradients. Analysis of gradient profiles of attached and suspended cell polyribosomes shows no detectable differences in the size distribution; however, the cells growing in suspension have a much greater amount of polyribosomal material per unit cell than do the attached cells. In other experiments, attached cells and suspended cells were briefly labeled with [³H]leucine and [¹⁴C]leucine, respectively, and were compared with respect to the amount of label incorporated into proteins in the polysomal regions. The sucrose gradients were adjusted so that the heavy polysomal region sedimented further

away from the bottom of the tube. There was very little radioactivity seen in the 60S and 40S subunit regions of the gradients, indicating that an insignificant amount of incorporation is due to ribosomal protein synthesis. By calculating the ratio of ³H to ¹⁴C incorporation, we hoped to identify a polyribosomal region whose ratio increased, indicating an increase in the microtubular polyribosomes. No significant change in the ratio was seen, but in both cases there were two peaks of high activity in the dense portion of the gradient. We believe that these peaks represent the microtubular protein polyribosomes.

We have also done sucrose gradient analysis of polyribosomes from *Rhynchosciara* testis, using tissue from a stage just after meiosis, when the microtubular assembly has occurred. It can be seen from this profile that there is excellent resolution of the small and intermediate-sized polyribosomes. Our efforts are now concentrated toward identifying the large polyribosomes involved in microtubular protein synthesis.

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STUDIES ON THE ALTERATION OF tRNA BY EXPOSURE OF *E. COLI* TO CHLORAMPHENICOL

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

We are continuing our studies on the tRNA's made by *E. coli* during inhibition of protein synthesis with chloramphenicol (CAP). As we mentioned last year, although the new tRNA's synthesized by cells during exposure to chloramphenicol give a different chromatographic profile from that of normal tRNA, they appear to have normal aminoacyl acceptance activity. Methylation appears to be normal. The 4-thiouracil content decreases markedly with time of CAP treatment, and other changes appear to have occurred. In order really to understand the nature of the changes that have occurred in these tRNA's it becomes important to isolate them in a pure state, obtain a "fingerprint" of the bases, and compare them to the "fingerprint" of the normal component.

During the past year we have been trying to perfect a new nucleoside fingerprinting technique developed by Randerath and Randerath (*1*). In essence, an oligonucle-

otide or polynucleotide such as tRNA is converted to nucleosides by digestion with pancreatic ribonuclease, snake venom phosphodiesterase, and alkaline phosphatase. The nucleosides are then converted to a dialdehyde derivative by oxidation with sodium metaperiodate and finally converted to the tritiated trialcohols with high-specific-activity tritiated borohydride. The tritiated nucleoside trialcohols are then separated by two-dimensional thin-layer chromatography on cellulose sheets. Low-temperature solid-scintillation fluorography with 2,5-diphenyloxazole as the scintillator is used to visualize the labeled compounds. The Randerath procedure can assay the four major bases and most minor bases in tRNA with the exception of certain thionucleosides and 2'-O-methylated nucleosides. Using purified bulk tRNA prepared from *E. coli* that had been exposed to CAP for various periods of time, we have obtained preliminary results indicating that there is a decrease in dihydrouridine content as a function of time after CAP treatment. Several other minor ribonucleosides as yet unidentified seem to decrease also. There is no appreciable change in ribothymidine and pseudouridine.

We have used this fingerprinting technique with several other *E. coli* tRNA's purified to a high degree of aminoacyl acceptance activity and have found good agreement with the results obtained by other methods. Bulk *E. coli* tRNA grown under conditions leading to undermethylation showed a marked decrease in content of the modified nucleosides pseudouridine, ribothymidine, dihydrouridine, 7-methylguanosine, and several other methylated nucleosides from that of tRNA grown under normal conditions. However, a mutant of *E. coli* relaxed for phenylalanine showed little change in the nucleoside composition of its bulk tRNA when grown in the presence or absence of phenylalanine. There was an alteration in the chromatographic profile for phenylalanine acceptance in the phenylalanine-deprived condition, which was comparable to that obtained from CAP-treated cells.

It is clear from our first experiments that some changes are too slight to affect the composition of bulk tRNA. We are trying to separate various tRNA's grown under CAP restriction and analyze them by the Randerath borohydride technique.

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SUPPRESSOR MUTATION AND THE POSSIBLE INVOLVEMENT OF tRNA IN *BACILLUS SUBTILIS*

Shigemi I. Simms and G. David Novelli

This is a collaborative program with Dr. Manley Mandel of the M. D. Anderson Hospital in Houston, Texas. The object of the study is to determine whether the introduction of suppressor genes into *Bacillus subtilis* will result in the synthesis of new species of tRNA that might be involved in the mechanism of suppression. It is already known from other studies that in some cases suppressor genes code for new tRNA species.

Dr. Mandel constructed the following strains of *B. subtilis*. He began with *B. subtilis* (Br151, which is Frank Young's strain) and then moved into this strain the necessary genetic markers to construct strain 129, which is the point of departure for subsequent experiments. For the purpose of this discussion, *B. subtilis* strain 129 can be considered the "standard." Phenotypically it is a normal spore-forming strain with a nutritional requirement for histidine and methionine. In addition, it is nonpermissive for the growth of two defective phages. To strain 129, Dr. Mandel added suppressor 1 (*Su1⁺*) to yield strain 135, and suppressor 3 (*Su3⁺*), which is strain 173. The tRNA profiles of strain 135 and 173 are to be compared with the tRNA profiles of strain 129 (*Su⁻*). At all other loci, these strains are genotypically identical. The cells were grown to late log phase in L-Broth with Ca^{2+} and glucose, and 50–100 g wet weight was harvested. Dr. Mandel purified the tRNA from each strain by a modification of the method of Von Ehrenstein and shipped it to Oak Ridge in absolute ethanol containing 1 mM 2-mercaptoethanol in dry ice. The tRNA's were aminoacylated, in separate runs, with ^{14}C - or ^3H -labeled phenylalanine, lysine, tryptophan, glycine, tyrosine, leucine, and serine, using a partially purified aminoacyl-tRNA synthetase prepared from an asporogenous mutant derived from wild-type *B. subtilis* 168, obtained from Dr. John Spizinen of the Scripps Clinic and Research Foundation.

The aminoacylated tRNA's from each mutant were cochromatographed on reversed-phase system 5 with the corresponding aminoacylated tRNA from strain 129 (*Su⁻*), carrying the same amino acid but with a different isotopic label.

The results to date, although preliminary, indicate the following features. For Lys-tRNA, Trp-tRNA, and Gly-tRNA there were no significant differences in profiles between the mutant and the *Su*⁻ strain. For Tyr-tRNA, Glu-tRNA, Leu-tRNA, and Ser-tRNA, multiple peaks of aminoacylated tRNA were observed with both mutants and *Su*⁻, but there were no peaks observed in the mutants that were not also present in the tRNA from the *Su*⁻ strain. However, with each of the aminoacylated tRNA's there were striking differences in the relative ratios of the peaks between the mutants and the *Su*⁻ strain. These are quantitative differences, and their significance is not known.

The most striking difference was observed with Phe-tRNA. In the *Su*⁻ strain as well as in strain 168, Phe-tRNA shows only a single peak of activity, eluting rather late in the chromatogram at 0.865 M NaCl. In strain 135 (*Sul*⁺), as well as in strain *B. subtilis* AT (*SpoA*), instead of a single peak of Phe-tRNA there are two peaks. One late-eluting peak is at the same position as the one in the *Su*⁻ strain that is quantitatively reduced in the two strains mentioned. In addition, there is an early-eluting peak in both the *Sul*⁺ and *SpoA* strains, which is absent in the *Su*⁻ strain and in strain 168. This new peak of Phe-tRNA elutes at 0.690 M NaCl and is not present in strain 173 (*Su3*⁺).

We are continuing to compare the aminoacyl-tRNA profiles of these strains with the amino acids not yet tested at this time. We also have plans to purify the Phe-tRNA's from the mutants with two chromatographic species and to compare their nucleotide "fingerprint" with the single species of Phe-tRNA from the *Su*⁻ strain in order to determine the differences in bases between the early-eluting and late-eluting species.

IN VITRO PROTEIN-SYNTHESIZING SYSTEM FOR THE ASSAY OF SPECIFIC mRNA

Helen G. Sellin

The *in vitro* protein-synthesizing system from *E. coli*, developed in previous years, will now synthesize lysozyme when directed by phage T3, T4, or T7 mRNA and SAMase (the enzyme that cleaves S-adenosyl methionine) when directed by T3 mRNA. Phage mRNA is really total RNA extracted from phage-infected *E. coli* strains. Lysozyme and SAMase activities, however, are present only after phage infection.

No system using such phage mRNA preparations can be really tRNA-dependent. Therefore we are continuing our investigation to develop a system that will use phage DNA as messenger (via RNA polymerase) for the *in vitro* synthesis of the same phage-specific enzymes.

In addition, D. Kelmers from the Chemical Technology Division, ORNL, is collaborating on the purification of mRNA. Devising a new flowsheet, he has worked out procedures reducing substantially the quantity of DNA and tRNA in the mRNA preparation. Work continues on removing rRNA and, eventually, separating various mRNA's.

Increased efficiency of *in vitro* enzyme synthesis would be desirable and might be achieved by increasing the concentration of a limiting component — e.g. RNA polymerase, initiation factors, elongation factors, or release factors. Addition of uninfected or T7 RNA polymerase has not improved T7-DNA-directed lysozyme synthesis. Therefore, we have turned to one of the other components. Currently I am isolating initiation factors and release factors, and A. N. Best of this division is isolating elongation factors, which we will test in the enzyme synthesis system, hoping to increase its efficiency.

ISOLATION AND PROPERTIES OF PRECURSOR tRNA FROM YEAST

J. Dijk*

Several investigators have demonstrated the existence of precursor tRNA in mammalian cells (HeLa cells and Krebs II ascites cells). A precursor of tyrosine tRNA has been isolated from phage-infected *E. coli*, and its sequence has been determined. The experiments described here were carried out with *Saccharomyces cerevisiae* (bakers' yeast), an eucaryotic organism that can easily be obtained in large amounts.

In these experiments precursor tRNA is characterized as a rapidly labeled RNA species (in pulse-label experiments) that has a molecular weight slightly smaller than that of 5S rRNA (expected size about 120 nucleotides) and can be converted *in vivo* (and perhaps also *in vitro*) into an RNA species with the same molecular weight as tRNA.

The presence of precursor tRNA in yeast cells can be demonstrated (as described below); however, in order to obtain a detectable amount of precursor molecule, the metabolism of the cells has to be slowed down just before the addition of the labeled nucleoside. The cells are treated with cycloheximide (actidione), which inhibits protein synthesis and rRNA synthesis (including 5S RNA). The expected effect is an accumulation of precursor tRNA, due to decreasing enzyme levels in the cell.

Rapidly growing yeast cells (in mid-log phase) are treated for 20 min with cycloheximide and then exposed to [³H]uridine for 30 min. Low-molecular-

weight RNA is extracted from the intact cells with cold phenol and fractionated by gel filtration. The precursor tRNA is eluted in nearly the same position as 5S RNA; its presence can be shown by the high specific activity (3–5 times higher than that of tRNA).

The precursor tRNA obtained is contaminated with a large amount of 5S RNA and a small amount of highly labeled tRNA. Several column chromatographic systems have been investigated (DEAE-Sephadex and the reversed-phase systems RPC-5, 6, and 7). RPC-5 turned out to be the most efficient and rapid system.

On RPC-5 columns the contaminating tRNA is eluted in the first 40% of the gradient and the 5S RNA at 50%. In addition two peaks with high specific activity are found, a sharp one (peak A) just in front of the 5S RNA peak and a broad one (peak B), eluting well behind the 5S RNA peak. Both peaks are absent in RNA preparations obtained from cells grown in the absence of cycloheximide and are decreased in size after a chase with cold uridine. The second peak (B) has a slightly higher molecular weight than peak A (shown by gel filtration). If necessary, these peaks can be purified on RPC-7 columns, on which they both elute in front of the 5S peak.

In order to show that these peaks really represent precursor tRNA, maturation experiments have been carried out *in vitro*. Either the mixture or the separated ³H-labeled peaks were exposed to the 30,000 × g supernatant of *S. cerevisiae* homogenate. The most successful experiment showed only about 20% "conversion" for both peaks; the "converted" material is eluted in the same position as tRNA from RPC-5 columns and has the same molecular weight. This partial conversion must be attributed, probably, to a very low level of maturation enzymes. Their very rigid cell walls make *S. cerevisiae* cells rather resistant to homogenization procedures. The experiments will be repeated with enzyme preparations from *S. fragilis*.

Attempts have been made to isolate unlabeled precursor tRNA in amounts high enough to detect it by its absorption at 260 nm. To accumulate large amounts of precursor molecules the cells were exposed for longer times (up to 3 hr) to cycloheximide. This also results in a sharp decrease of the amount of 5S RNA. The first experiments, carried out on 500 g of cells, were hindered by the presence of small peaks that eluted in nearly the same position as precursor tRNA on RPC-5 columns and seem to be associated with 5S RNA.

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TRANSFER RNA IN MOPC 31C TUMORS

J. M. Frazer and G. David Novelli

Yang *et al.* (1) have shown that L-M cells grown as a solid subcutaneous tumor have several new species of isoaccepting tRNA's (on reversed-phase chromatography) in addition to those tRNA species present when the cells are cultured *in vitro* in a serum-free medium. The tRNA's showing qualitative or strong quantitative differences are specific for aspartic acid, histidine, phenylalanine, and tyrosine. The new peaks arising in the tumor eluted chromatographically at the same salt concentration as the tRNA prepared from differentiated normal tissue, such as liver and reticulocytes. The cells in the tumor were approximately tenfold less tumorigenic than the cells grown *in vitro*, indicating a physiological change in the cells in addition to the tRNA changes. This type of "mixed" pattern of isoaccepting tRNA species has been shown for approximately 15 mouse and rat tumors of different origins. Recent results suggest that the tRNA peaks characteristic of L-M cells grown in serum-free medium lack a single nucleoside modification present in the peaks characteristic of differentiated cells.

The tRNA's showing a qualitative change for the two growth conditions (His, Asp, Tyr, but not Phe) all recognize codons ending in AU or AC. In *E. coli* these tRNA's apparently have a modified guanosine in the wobble position of the anticodon, and this base is sensitive to cyanogen bromide (CNBr). When tumor tRNA (having both tRNA "sets") is reacted with CNBr, aminoacylated with labeled amino acid, and chromatographed, only those isoacceptors characteristic of the cells grown *in vitro* retain their normal elution position; those isoacceptors characteristic of differentiated cells elute at a much higher salt concentration, while maintaining the same number of peaks and the same peak heights. This suggests that the tRNA isoacceptors (for Asp, His, and Tyr) characteristic of differentiated tissue contain a CNBr-sensitive nucleoside (presumably a modified guanosine as in *E. coli*) that is not present in the tRNA isoacceptors characteristic of the cells grown *in vitro* in a serum-free medium. Future experimental work will center on extending the chromatographic observations and analytical work to pin down the exact differences between the two apparent sets of tRNA's.

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NEW SMALL POLYPEPTIDES ASSOCIATED WITH DNA-DEPENDENT RNA POLYMERASE OF *E. COLI* AFTER INFECTION WITH PHAGE T4

Audrey L. Stevens and Diane D. Crowder

Upon infection of *E. coli* with T4 phage, a new program of transcription takes place. To understand better how the new program operates, we have continued investigation of the fate of DNA-dependent RNA polymerase of *E. coli* after infection. Four new small polypeptides are found associated with the enzyme after infection. Their molecular weights are as follows: polypeptide 1, 22×10^3 ; polypeptide 2, 14×10^3 ; polypeptide 3, $5-10 \times 10^3$; polypeptide 4, 12×10^3 . Two results link the new polypeptides to formation of late T4 mRNA, a process that starts about 10 min after infection and depends on concurrent DNA replication. The first is the finding that all four polypeptides are formed starting 6-8 min after infection at 30° C. T4 DNA synthesis starts about the same time. Second, two of the polypeptides are missing from the RNA polymerase when T4 amber mutants in the two maturation-defective genes are used for the infection process. Polypeptide 1 is missing when maturation-defective gene-55 mutants are used, and polypeptide 4 is missing when one maturation-defective gene-33 mutant is used. The maturation-defective amber mutants are characterized by having DNA replication but no late mRNA synthesis following infection of *E. coli* B.

The exact role of the new polypeptides in RNA formation is under investigation, using purified RNA polymerase from *E. coli* infected with appropriate T4 mutants. RNA polymerase obtained from *E. coli* infected with a Do amber mutant, which makes only early mRNA and has no DNA replication, has been compared with that from a maturation-defective amber mutant in gene 55. The first enzyme contains all four new polypeptides, whereas the second lacks polypeptide 1. The two enzymes appear identical in their content of all the other subunits, and they both have less ($1/2-1/3$) activity with T4 DNA as template than does normal *E. coli* enzyme. When purified by glycerol gradient centrifugation, both enzymes contain about 0.2 equivalent of the sigma subunit. The enzyme containing polypeptide 1 copies mature T4 DNA in a symmetric manner, and the enzyme lacking polypeptide 1 makes 70-80% early asymmetric T4 RNA. The results suggest that the new 22,000 mol. wt. polypeptide may alter the effect of the sigma subunit on the activity of RNA polymerase. Investigations of the function and synthesis of the new subunits are continuing.

ISOLATION OF A NEW HORMONE FROM THE WATER OF CROWDED GOLDFISH

Peter Pfuderer, A. A. Francis,
and Penny Williams*

When fish are in an overcrowded situation their growth and spawning slow up and finally stop, and their heart rate is depressed. If the overcrowding becomes more severe, they die, usually starting with the smaller fish. The cause of this phenomenon is a substance secreted into the water by the fish, inhibiting that species of fish.

The substance or substances causing these effects can be extracted from the water of overcrowded fish with lipid solvents. We have shown that crude solvent extracts of water from crowded goldfish can slow the growth of young goldfish fry, can slow their heart rate over 50%, and are lethal in higher doses. These effects all proved to be species specific, so a pheromone is involved and not just a toxic waste product.

We have used the heart rate depression as an assay to purify this pheromone from goldfish. It is a low-molecular-weight, nonpolar substance with a molecular weight of ~200 by Sephadex chromatography. It is very labile and is sensitive to UV, visible light, and heat, and it slowly loses activity in aqueous solutions. It has no amino acids detectable by acidic or basic hydrolysis, and it is ninhydrin negative. It is not a steroid or prostaglandin according to its chromatographic behavior. It has very few functional groups, but absorbs strongly in the UV range. In short, it seems to be a new biochemical compound.

The depression of heart rate is completely reversed by the addition of atropine, which indicates that the pheromone's effect is probably mediated through vagal control and implies that the brain is the most probable target tissue.

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AN AGE-RELATED DECREASE IN LACTIC DEHYDROGENASE IN BC3F₁ MICE

Peter Pfuderer, A. A. Francis,
and R. J. Oliveira*

We have observed a large decrease in lactic dehydrogenase (LDH) enzyme levels with increasing age in nondividing tissue (heart, kidney, brain, and muscle) of BC3F₁ mice. These decreases start at roughly 40 weeks of age and continue as far as our data and the life-span of the mouse permit. This drop in LDH activity is ~30-50% by 140 weeks and is a relatively smooth function once the onset of the decrease starts. The drop

apparently involves a decrease in both LDH₅ (muscle type) and LDH₁ (heart type) and therefore involves two different genetic loci, essentially two different enzymes, and was found in all four tissues. There were also pronounced differences observed between male and female mice, showing either hormonal or genetic influence.

Since LDH is an intracellular enzyme in these tissues, it is possible that we are assaying the number of functional cells in the tissues. A monospecific antibody to one LDH isozyme, LDH₅, was prepared. The amount of LDH₅ antigen as a function of age, as assayed by radial immunodiffusion, showed a decrease with age paralleling the decrease of LDH₅ activity with age in the same tissue. Therefore, the decrease in LDH activity is probably due to a decrease in the number of fully active LDH molecules and probably not to the presence of missynthesized, partially active molecules.

When frozen tissue sections of old and young animals were stained for LDH activity, the old tissues showed a wide variation in LDH activity, indicating the decline in LDH activity with age is not evenly distributed in the cells, but that some cells are declining faster than others in the same tissue.

FUNCTION OF METHYL GROUPS IN tRNA

K. R. Isham* and M. P. Stulberg

Our previous investigations of the function of methylated bases in tRNA (1, 2) have culminated in a high degree of purification of undermethylated tRNA and utilizable ⁵U-methyltransferase (3).

A protocol of cell extraction, DEAE-cellulose, RPC-5 and 7, and finally A-50 chromatography has produced a highly purified, undermethylated tRNA^{Phe}. This product demonstrates all the criteria of pure tRNA^{Phe} with little if any contamination from other tRNA's.

The undermethylated tRNA^{Phe} is now ready for stepwise methylation by the isolated methyltransferases. The effect of methylation will be studied by determining changes in structure and biological activity.

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PURIFICATION OF ⁵U-METHYLTRANSFERASE FROM *E. COLI*

L. R. Shugart and Barbara H. Chastain

The isolation and purification of ⁵U-methyltransferase, which methylates tRNA resulting in the formation of ribothymidine, is being continued. Although this enzyme represents about 90% of the total methylating activity found in our crude preparations, it is extremely labile, and purification of the enzyme from 100,000 × g supernatant extracts of the microorganism has proved difficult.

A preparation has been obtained after DEAE-cellulose column chromatography, however, that contains the ⁵U-methyltransferase activity almost exclusively. Further purification has been accomplished by Sephadex column chromatography. The enzyme at this step of purification has an apparent molecular weight of 50,000, is stimulated by NH₄⁺, and is free of contaminating RNase activities.

This preparation will be used to study the effect of stepwise methylation on an undermethylated tRNA molecule (1).

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THE EFFECT OF UNDERMETHYLATION OF tRNA ON OTHER BASE-MODIFYING PROCESSES

K. R. Isham, Audrey N. Best, and M. P. Stulberg

Very little is known about the effect of one type of base modification of tRNA on the large number of other modifications necessary for the maturation of the molecule. Are these modifications independent of each other or is there a system of interlocking controls leading to an orderly synthesis of a completely functional tRNA?

We have tested undermethylated tRNA^{Phe} for its content of modified bases. Initial experiments performed by the Randerath procedure (1) have tentatively indicated depletions in the known modified bases. We are now repeating these experiments with highly purified tRNA^{Phe} (2) in order to specify in detail the changes observed when the tRNA^{Phe} is lacking its normally methylated bases.

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AGE-RELATED ABNORMALITIES DETECTED BY UTILIZATION OF A SPECIFIC *IN VITRO* PROTEIN-SYNTHESIZING SYSTEM

M. P. Stulberg and K. R. Isham

A large amount of circumstantial evidence has accumulated concerning the relation between fluctuating methyltransferase activity and under- and overmethylated tRNA in systems affected by viral infection, neoplasia, hormonal fluctuation, and senescence. We have decided on a method of determining directly the effect of these lesions on protein biosynthesis and have chosen tissues of the aging mouse for our initial studies.

Our *in vitro* protein-synthesizing system will consist of components of ascites tumor using either EMC viral RNA or globin RNA as the messenger. Thus either viral capsid protein or globin will be measured as a specific end product. We will test the ability of tRNA or other protein-synthesis components extracted from aging tissue to support the synthesis of specific proteins in the *in vitro* ascites tumor system. If abnormalities are demonstrated we will then correlate them with specific components and thus be able to demonstrate a specific correlation with the age of the animal. We have obtained samples of EMC virus and Krebs II ascites tumor and have propagated the tumor in long-lived BC3F₁ mice.

We are in the process of stocking the tumor for further propagation and for use as a host for viral infection.

AGE-RELATED MODIFICATION OF "Y" BASE STRUCTURE IN PHENYLALANINE tRNA

B. L. Whitfield and L. R. Shugart

The lack of protein synthesis in senescing wheat leaves may be the result of an alteration in the "Y" base of phenylalanine tRNA. Earlier studies have demonstrated that tRNA^{Phe} from apical wheat tissue has a greatly reduced capacity to participate in poly(U)-directed polyphenylalanine synthesis compared to that from growing basal tissue. The Y base is apparently modified in some way, as revealed by reduced fluorescence and altered chromatographic behavior (1).

The present study is an attempt to obtain sufficient amounts of purified tRNA^{Phe} and Y base from apical and basal sections of 8-day-old wheat to permit physical and chemical determination of the Y base modification. A purification scheme for wheat leaf tRNA^{Phe} has been developed which involves separation on DEAE- and BD-cellulose and RPC-5 columns.

Phases of the earlier work have been confirmed and expanded. Phenylalanine tRNA from apical tissue is aminoacylated approximately 30% less than that from basal tissue at equal stages of purification. Fluorescence of Y base in the intact tRNA^{Phe} molecule is quantitatively the same in apical and basal tissue; however, the maximum fluorescence peak is shifted 10 nm toward the blue in apical tissue. Upon excision of Y base, the fluorescence greatly decreases if isolated from apical tissue and remains the same if isolated from basal tissue, indicating a possible break in the ring structure expressed only after excision. With thin-layer chromatography, the retardation factor of Y base from basal tRNA^{Phe} is 0.35 as detected by 260-nm absorption and 440-nm fluorescence. When Y base from apical tissue is chromatographed in the same system the 260-nm-absorbing material has a retardation factor of 0.35, but all remaining fluorescence migrates with the solvent front.

Phenylalanine tRNA from wheat grown in 3-amino-1,2,4-triazole and in the dark is being purified and examined for altered Y base. Final structural differences in the Y base will be determined by mass spectrometry.

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REVERSED-PHASE CHROMATOGRAPHY OF TYROSYL-tRNA FROM *DROSOPHILA MELANOGASTER*

J. F. Calvino,* K. B. Jacobson, and J. B. Murphy

The three plaskon-supported reversed-phase chromatography (RPC) systems introduced by Pearson *et al.* (1) have been screened for their usefulness in the separation of isoacceptor species of tyrosine-specific tRNA (tRNA^{Tyr}) from *Drosophila melanogaster* wild type (Samarkand) and selected mutant strains.

Of the three plaskon-supported RPC systems, only RPC-5 has been studied in detail. Runs on RPC-7 indicated this system is not appropriate for tyrosyl-tRNA from wild-type strains, since the nucleic acid will not bind to the quaternary ammonium salt even at 0.45 M NaCl, about the lowest salt concentration that can be used without the exchanger coming off. RPC-6, using the same conditions finally adopted for RPC-5, gave essentially the same results as RPC-5.

Resolution in RPC-5 is strongly dependent on temperature but insensitive to flow rate between 0.327 ml/min/cm² (0.1 ml/min) and 3.27 ml/min/cm² (1.0 ml/min). At 35–40° C, two fully separated peaks are obtained for wild-type tyrosyl-tRNA; at 24° C only

partial separation occurs; and at 10° C there is no separation of the two tyrosyl-tRNA peaks. Decreasing the flow rate from 1.0 to 0.1 ml/min does not alter the result. Final conditions adopted are 38° C, a 100-ml linear gradient from 0.55 to 0.60 M NaCl, and a flow rate of 1.0 ml/min.

Cochromatographic experiments with wild-type and *su(s)*² tyrosyl-tRNA indicated the presence of two peaks in the suppressor mutant as well as in wild type. In separate chromatograms the two peaks were separated from wild-type and suppressor tRNA; each peak was rechromatographed and emerged from the column unaltered. These RPC-5 results are consistent with the results of recent experiments with RPC-2 columns, the counterpart of RPC-5 in the chromosorb-supported RPC series: two peaks, in similar proportions as in RPC-5, were obtained for both wild-type and *su(s)*² strains at 30 and 20° C.

Using hydroxylapatite columns, only the first peak is obtained from the *su(s)*² strain in cochromatographic runs where the wild-type strain gives two peaks. This is in contrast to the observation that the same *su(s)*² sample on RPC-5 gives two peaks. On rechromatography the second *su(s)*² peak from RPC-5 cochromatographs with the first wild-type peak on hydroxylapatite.

It was previously reported (2) that the second isoacceptor species was absent in the *su(s)*² strain, and the presence of its precursor was postulated in a model assuming that it chromatographs in the position of the first isoacceptor. Our results suggest that this precursor has been separated on RPC-5 columns from the first isoacceptor. The results from RPC-2 do not agree with those of Twardzik *et al.* and indicate that the procedures used in sample preparation may be critical. We are examining the two tyrosyl-tRNA's obtained from the *su(s)*² mutant by RPC-5 chromatography to determine whether different procedures in tRNA preparation and chromatography affect the amount of the presumed precursor that can be obtained.

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PHENOL OXIDASE ACTIVITY AND THE LOZENGE LOCUS OF *DROSOPHILA MELANOGASTER*

Cynthia K. Warner* and K. B. Jacobson

The phenol oxidase system of *Drosophila melanogaster* contains at least five protein components: A₁, A₂, A₃, P, and S (1, 2). The P component serves to activate the three A components (2), the A₁ component has been reported to possess monophenol oxidase (or tyrosinase) activity, and A₂ and A₃ are reportedly diphenol oxidases (2). Peeples and his co-workers (3-5) have shown a correlation between the amount of phenol oxidase activity and the relative phenotypic severity of various mutant lozenge alleles. These workers report that *lz*^g mutants specifically lack monophenol oxidase activity (the A₁ component), that *lz* mutants possess reduced monophenol and diphenol oxidase activities, and that other mutant alleles of the lozenge locus have generally reduced mono- and diphenol oxidase activities.

A reinvestigation of the preceding observations has failed to confirm them. All three of the A components of the *Drosophila* phenol oxidase system show both monophenol and diphenol oxidase activity. The *lz* mutant has at least as much phenol oxidase activity as wild type and may even have more. The *lz*^g mutant has all three A components, although each component appears to have slightly less activity than its wild-type counterpart. An investigation of a number of different lozenge mutants (including *lz*, *lz*^{v4}, *lz*^s, *lz*^g, *lz*^{rfg}, and *lz*^{BS lz⁴⁶ lz^g) has failed to reveal any correlation between phenol oxidase activity and severity of phenotypic expression. A recently isolated spontaneous mutant, *lz*^{rfg}, appears to lack all phenol oxidase activity. We have no explanation for the disparity of these observations and those reported by Mitchell and by Peeples.}

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AN IMMUNOCHEMICAL APPROACH TO GENETIC MAPPING

M. Cohen, Jr.* and K. B. Jacobson

The technique of annealing radioactive, complementary RNA to its chromosome *in situ* and visualizing the hybridized gene by autoradiography has become a powerful genetic tool. Severe limitations are inherent in this technique, however, because of difficulty in producing RNA that is labeled highly enough and because the specific sequences in the chromosomal DNA are often so small a part of the genome. Thus, successful *in situ* hybridization studies have been performed only on highly redundant gene sequences.

Recognizing these limitations, our approach has been to use a large molecule, a virus, as an amplifying marker by coupling it to the annealed, complementary RNA. Then, by reacting with virus antibody tagged with a fluorescent dye, we hope to visualize the gene for the complementary RNA at the light-microscope level. If successful, this approach could extend the scope of *in situ* hybridization to rarer gene sequences. Since the chromosomal location of *Drosophila melanogaster* 5S RNA has been mapped, this system is being used to develop the approach.

Purified *D. melanogaster* 5S rRNA will be modified by periodate oxidation and coupling with N-ε-2,4-dinitrophenyl-L-lysine. Following annealing of this RNA to *Drosophila* salivary chromosomes on glass slides, rabbit antidinitrophenyl gamma globulin will be applied. The latter should serve as a link between the hybridized, modified RNA and the virus (dinitrophenyl-labeled T4 bacteriophage). Treatment with fluorescein-isothiocyanate-tagged rabbit anti-T4 gamma globulin followed by fluorescent microscopy should reveal the location of the annealed RNA. Most of the reagents have been prepared. The only remaining step prior to testing this approach is modification of the 5S RNA.

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AN ELECTROPHORETIC PROCEDURE FOR SEPARATING MOUSE HEMOGLOBINS AND SERUM ALBUMIN

K. B. Jacobson, Carolyn M. Vaughan, and W. L. Russell

A search for mutant forms of mouse hemoglobin and serum albumin is underway (1) to determine whether intragenic changes as well as deletions can occur as a result of ionizing radiation and (2) to measure the frequency of both kinds of mutation. The present

project is conceived with the expectation that only a few mutations may occur in a large number of offspring of irradiated animals. Therefore, a procedure for performing electrophoresis must be established that requires a minimum of sample handling and a simple electrophoresis procedure.

Evaluation of the electrophoresis procedure consisted of a comparison of polyacrylamide gels and starch gels using lysed samples of whole blood. Both gels resolve the hemoglobin of strains 101 and SEC and of their F₁ hybrid, but the two bands separate farther on starch gel and are easier to score.

Two kinds of power supplies were compared, a conventional transformer with adjustable voltage and the ORTEC apparatus that allows the variation of total power by varying both voltage and pulse rate. The latter gives faster and better separation of the protein bands, apparently due to the pulsing circuit; very good separation of the two hemoglobin bands is achieved in 2.5–3 hr. The former power supply gave from poor to fair resolution in 3.6 hr; shorter times were not available because the gels became too hot at higher voltages.

Chemical treatment of the lysed blood is necessary. Whole blood is diluted 10-fold in water and then the following are added: iodoacetic acid (1.6 mg/ml), potassium ferricyanide (0.25 mg/ml), potassium phosphate (pH 7.0, 0.08 M), and sucrose (250 mg/ml). The final dilution of blood is 1/50 and is critical in that 1/25 or 1/100 dilutions are unsatisfactory.

The starch gel consists of 14% starch and 0.001 M EDTA, 0.025 M boric acid, and 0.045 M Tris. The resultant pH is 8.65. The gel mold is designed so that the gel is in direct contact with the buffer in the electrode vessel, thereby eliminating the severe voltage drop that occurs through the paper or cloth wicks that are usually used. Gels are used within 4 hr of preparation; when gels are stored overnight additional bands of hemoglobin are observed. The separation of hemoglobins and albumin occurs in 2.5 hr at 320 V, 75 mA, and 300 pulses per sec.

The screening of the F₁ offspring of the cross between 101 and SEC mouse strains is underway. The above electrophoresis procedure and a hemoglobin solubility test (by R. A. Popp) is used to test each animal.

HETEROLOGOUS CHARGING OF *NEUROSPORA CRASSA* tRNA^{Phe} BY *E. COLI* VALYL-tRNA SYNTHETASE

J. E. Strickland* and K. B. Jacobson

Previously it was shown that reaction of tRNA^{Val} (*E. coli*) with Syn^{Phe} (*Neurospora crassa*) results in for-

mation of Phe-tRNA^{Val} (1). We have now shown that the reaction of opposite symmetry, tRNA^{Phe} (*N. crassa*) and Syn^{Val} (*E. coli*), results in the formation of Val-tRNA^{Phe}.

The reaction of Syn^{Val} (*E. coli*) with unfractionated tRNA (*N. crassa*) produces little or no Val-tRNA^{Phe}, but the enzyme does react if the tRNA has been fractionated on benzoylated DEAE-cellulose. The presence of an organic solvent (dimethyl sulfoxide) increases the extent of production of Val-tRNA^{Phe} by 5- to 10-fold, whereas homologous charging by either Syn^{Val} (*E. coli*) or Syn^{Phe} (*N. crassa*) is inhibited by dimethyl sulfoxide. In studies on the production of Phe-tRNA^{Val} (*E. coli*) by Syn^{Phe} (*N. crassa*), the reaction is markedly stimulated by this same organic solvent (1).

An aspect of this study is the observation that in unfractionated tRNA there is little or no valylation of tRNA^{Phe} by Syn^{Val} (*E. coli*), whereas tRNA^{Phe} fractionated on benzoylated DEAE-cellulose reacts strongly with the enzyme. Presumably this is because the affinity of Syn^{Val} for other tRNA's of *N. crassa* (tRNA^{Val}) allows them to compete more effectively than tRNA^{Phe} for binding sites on the synthetase. In any case, had we looked only at mixed tRNA we would have concluded that valylation of tRNA^{Phe} does not occur under our conditions.

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EFFECT OF AMINO ACID STRUCTURE ON RATE OF HYDROLYSIS OF AMINOACYL-tRNA

J. E. Strickland* and K. B. Jacobson

The chemical hydrolyses of Val-tRNA^{Val} and of Phe-tRNA^{Phe} occur at quite different rates, the former being much more stable than the latter. The stability of the valyl ester relative to the phenylalanyl ester may be considered to be the result of the electronic configuration as influenced by structure of the amino acid or of the tRNA.

We prepared Val-tRNA^{Val}, Phe-tRNA^{Val}, Phe-tRNA^{Phe}, and Val-tRNA^{Phe} and measured their half-lives at pH 8.5 and 22° C. Their respective half-lives in 0.02 M Tris·HCl are 324, 81, 69, and 318 min. These results indicate that the amino acid structure has the predominant influence on the stability of the ester.

Both NaCl and Mg²⁺ are known to alter the configuration of tRNA, and both have been shown to alter the stability of the esters of amino acids with tRNA^{Val}. In 0.2 M KCl the half-life of Val-tRNA^{Val} decreases from 324 to 183 min and that of Phe-tRNA^{Val} decreases from 81 to 66 min. In 0.01 M Mg(OAc)₂ the half-life of Val-tRNA^{Val} decreases from 324 to 147 min and that of Phe-tRNA^{Val} decreases from 81 to 56 min.

When aminoacyl-tRNA synthetases from heterologous sources are used, Phe-tRNA^{Val} and Val-tRNA^{Phe} can be prepared. Studies on these and on their normal counterparts showed that the amino acid structure determines the stability of the ester bond in aminoacyl-tRNA. Nevertheless, it is likely that the tRNA conformation is also able to influence the stability, since changes in ionic strength and magnesium ion concentration also influence the stability of the ester bond.

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REACTION OF CACODYLIC ACID WITH SULFHYDRYL COMPOUNDS

B. Das Sarma* and K. B. Jacobson

Cacodylic acid is commonly used as a buffer in biochemical research and is also used as a commercial herbicide. The reaction of arsenous compounds with sulfhydryl compounds 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 cacodylate or arsenic acid with 2-mercaptoethanol is an exothermic reaction that generates a product with strong absorption at 230 nm. Between 2 and 3 moles of SH are required for complete reaction of 1 mole of cacodylate. The presence of water causes the rate and extent of reaction to decrease; methanol is a suitable solvent in which to conduct the reaction. Comparison of various arsenic compounds showed that there is no reaction of As(V) acids with 2-mercaptoethanol, whereas As(III) acids do react. Dithiothreitol, a bisulfhydryl compound, reacts with As(III) acids to form a white, insoluble product, whereas 2-mercaptoethanol reacts to form an oil. The product of reaction between dithiothreitol and AsO₂⁻ is the most stable of those studied. Studies on these reactions are continuing.

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ALTERED tRNA IN SUPPRESSOR MUTANTS OF YEAST

Jeremy Bruenn* and K. B. Jacobson

Nonsense mutants in yeast can be suppressed by "supersuppressor" mutants. One class of "super-suppressor" mutants consists of mutants at eight loci that all insert tyrosine at a site in iso-1-cytochrome, whose mRNA codon is known to be UAA (ochre suppressor). Other mutants of this same class insert tyrosine at the UAG codon site (amber suppressor). The tyrosine tRNA's from the amber and ochre suppressors were chromatographed on a reversed-phase column (RPC-5). As reported previously, the amber suppressor contains a new chromatographic peak (tRNA_{II}^{Tyr}) that is different from the Tyr-tRNA of wild-type yeast and consists of 22–26% of the total Tyr-tRNA contained in the amber mutant.

Further characterization of the chromatographic properties of the amber suppressor revealed that the main peak of Tyr-tRNA is depleted by approximately 20%; presumably, this means that the main peak actually consists of several species of tRNA^{Tyr}, one of which is altered in the amber suppressor to give rise to tRNA_{II}^{Tyr}.

To determine whether the new Tyr-tRNA peak is directly correlated with the presence of the amber suppressor mutation, the amber suppressor was crossed genetically with a nonsuppressor strain of yeast. The segregation of the amber suppressor was followed genetically and occurred in a 2:2 ratio in each four-spored ascus. All four spores of an ascus were used to establish cultures of yeast, and the tRNA was extracted, charged with tyrosine, and chromatographed. The two spores that exhibited amber suppression also yielded the new peak of tRNA^{Tyr}, and the two spores that did not contain the amber suppressor allele did not yield the new tRNA peak. Further information was obtained from this cross, since the amber suppressor is dominant. In the diploid, heterozygous for amber suppressor, the amount of tRNA_{II}^{Tyr} is about 13% of the total or approximately half the amount in the haploid organism containing the amber suppressor. Thus it is demonstrated that the locus for amber suppressor mutants of yeast determines the presence of an altered tyrosine tRNA. The translational function of tRNA_{II}^{Tyr} has not been tested.

Tyr-tRNA from an ochre suppressor mutant was also examined chromatographically. The ochre suppressor contained a new tyrosyl-tRNA peak but at a different chromatographic position from that of the amber suppressor peak. Correspondingly, the main peak of

tRNA^{Tyr} was not depleted in the same way as it was in the amber suppressor strains. This is tentative evidence that the ochre suppressor locus, at one of the eight loci for supersuppressors, is also a structural locus for tRNA^{Tyr}.

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ISOLATION AND CHARACTERIZATION OF RIBONUCLEOTIDE REDUCTASE FROM *EUGLENA GRACILIS*

F. D. Hamilton

Studies with a partially purified extract of *Euglena gracilis* have shown that the enzyme responsible for deoxyribonucleotide synthesis in this organism is a cobamide-dependent ribonucleoside triphosphate reductase. This reductase has been purified more than 300-fold from *Euglena* extracts and is free of both phosphatase and kinase activities. The enzyme will reduce all four ribonucleoside triphosphates. With the partially purified enzyme, CTP reduction is maximally stimulated by ATP and to a lesser extent by dATP, dCTP, and dGTP at concentrations of 1 μ M. CTP reduction is inhibited by high concentrations of ATP (0.1 mM) and by 1 μ M dTTP. In addition, dTTP strongly inhibits the reduction of GTP, ATP, and UTP.

The kinetics of ribonucleotide reduction with the enzyme indicates that the reductase of *Euglena* is subjected to allosteric regulation, as are the reductases of *Lactobacillus leichmannii* and *E. coli*.

ASCORBIC ACID SULFATE METABOLISM IN THE BRINE SHRIMP

F. J. Finamore, A. L. Golub, and Rose P. Feldman

The natural occurrence of a sulfated derivative of vitamin C was first demonstrated in this laboratory in acid-soluble extracts of *Artemia salina* embryos. Recently we have undertaken a study of the fate of this compound during development of *Artemia*.

We have observed that dormant embryos have an ascorbic acid sulfate concentration of approximately 2 μ moles per gram of protein and no measurable ascorbic acid. During development the concentration of ascorbic acid sulfate falls sharply at the time the larval shrimp emerge and hatch from their shells, and concurrently the concentration of ascorbic acid increases. Although this temporal relationship suggests conversion of ascorbic acid sulfate to ascorbic acid during emergence, we have found that about three times the quantity of

ascorbic acid appears as can be accounted for by removal of the sulfate moiety from ascorbic acid sulfate. Consequently we attempted to study the origin of ascorbic acid and ascorbic acid sulfate using $^{14}\text{CO}_2$, D-[U- ^{14}C]glucose, D-[U- ^{14}C]glucuronic acid, and [U- ^{14}C]trehalose in sterile incubation with embryos and larvae. To date we have not been able to demonstrate incorporation of ^{14}C into ascorbic acid or ascorbic acid sulfate using any of these precursors. These results indicate that ascorbic acid sulfate and ascorbic acid are not synthesized *de novo* during prelarval and early larval stages of development, and that indeed ascorbic acid sulfate may serve as a source (but perhaps not the only one) of ascorbic acid in the brine shrimp.

We are currently investigating the possibility that ascorbic acid sulfate is converted to another closely related, but distinctly different, compound during emergence of the embryos. We have isolated and purified a compound that exhibits some chemical characteristics similar to ascorbic acid and others that are similar to ascorbic acid sulfate. We are now in the process of identifying this substance and its relationship to the ascorbate derivatives.

ASCORBIC ACID SULFATE. CHEMICAL SYNTHESIS AND METABOLISM

A. D. Bond*

Preliminary studies revealed an important biological function for the ascorbate sulfate synthesized chemically and isolated from brine shrimp cysts. Procedures for its synthesis were modified to provide quantities sufficient for biological studies, and others were developed for synthesis of isotopically labeled compound.

Inconsistencies in physical constants previously reported for this substance were noted. Consequently, very pure samples were prepared, the barium salt was crystallized, and careful analyses were made. An error in extinction coefficients, which affected spectrophotometric determinations of ascorbate sulfate concentrations, was corrected. X-ray analysis of the barium salt revealed the compound to be 2-sulfonato ascorbate, not the 3-isomer as reported by other investigators in the field.

Several procedures were developed for economical synthesis of ascorbate 2-sulfate labeled with ^{35}S or ^{14}C . Quantities of the ascorbate [^{35}S]sulfate were prepared for biological studies in our laboratory, and small samples were provided for testing in other laboratories.

Human skin fibroblasts were cultured by standard procedures and presented with ascorbate sulfate in their growth medium. Cells cultured in the presence of ascorbate sulfate grew at slightly slower rates than controls. Cultures at a rapid rate of growth ($3/4$ saturation density) failed to utilize a significant quantity of ascorbate [^{35}S]sulfate or of [^{35}S]sulfate over a 24-hr period. Cultures maintained for 5 days after confluency with a medium supplemented with 250 $\mu\text{g}/\text{ml}$ of sodium ascorbate, then presented with the isotopes, utilized up to 50% of the ascorbate [^{35}S]sulfate but still very small amounts of [^{35}S]sulfate for a 24-hr period. Under these latter conditions, cultures also converted 1-[^{14}C]ascorbic acid into other metabolic intermediates.

Only very small amounts of [^{35}S] were detected in acid mucopolysaccharides. The majority was found either unchanged or in low-molecular-weight fractions. Small amounts of label were found in ascorbate sulfate isolated from cultures reared on [^{35}S]sulfate. However, much more was found in an unidentified fraction similar to ascorbate sulfate and formed when cells were labeled with [^{35}S]sulfate, ascorbate [^{35}S]sulfate, or 1-[^{14}C]ascorbate. Experiments are presently in progress that will identify this latter species.

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CONTROL OF NUCLEIC ACID BIOSYNTHESIS IN *ARTEMIA SALINA*

E. A. Hiss

The recent work of Brutlag *et al.* (1) and Wickner *et al.* (2), among others, has suggested that the initiation of DNA synthesis is primed by the synthesis of a short chain of RNA. Evidence that this system may be generalized to all procaryotes and phages has been accumulating rapidly. Preliminary results in this laboratory indicate that such a phenomenon may be operative in the brine shrimp, *Artemia salina*, as well. When *Artemia* DNA was preincubated in the presence of *E. coli* RNA polymerase and all the ribonucleoside triphosphates, followed by addition of an *Artemia* extract and deoxynucleoside triphosphates, a low but significant incorporation of [^3H]TTP into an acid-insoluble product was observed. Additional experiments are underway to demonstrate whether or not the observed activity follows the model of procaryotic replication. Previous work in this laboratory suggests that such a system is feasible, since repeated attempts to demonstrate DNA polymerase *in vitro* by other means have been unsuccessful.

In addition we have recently demonstrated RNA polymerase activity in extracts of *Artemia*. This polymerase appears to have multiple forms, similar to the sea urchin system described by Roeder and Rutter (3). Unlike *E. coli* polymerase, the activity of the brine shrimp system is inhibited by various low concentrations of both NaCl and KCl (0.2 M KCl is stimulatory to bacterial polymerase).

If DNA synthesis is demonstrated to be primed by *Artemia* RNA polymerase *in vitro*, then it is conceivable that only one of the forms of RNA polymerase is initiating replication. Further experiments have been designed to test this possibility.

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METABOLISM OF NUCLEIC ACID PRECURSORS IN *ARTEMIA SALINA*

Gary Van Denbos* and F. J. Finamore

Attempts to elucidate the nature of the metabolic pathways involved in nucleic acid biosynthesis during development in brine shrimp indicate the following. (1) P^1, P^4 -diguanosine 5'-tetrphosphate is not a direct precursor of DNA adenine but probably goes through the main nucleotide (nucleoside) pool. (2) The purine and ribose moieties of exogenously supplied purine nucleosides are not incorporated together into their respective nucleotide pools as one might expect; rather, there appears to be an exchange of the purine and ribose moieties (through glycosidic bond cleavage) even at the triphosphate level. (3) None of the purine nucleotide pools appears to be the direct intact (Pur-Rib- α P) precursor of the purine mononucleotides of RNA and DNA.

These observations contradict the traditionally accepted view of purine metabolism. However, they may also reflect a high degree of compartmentalization as well as a reluctance on the part of nauplii to incorporate exogenously supplied compounds into the pathway of nucleotide metabolism.

With this in mind, a procedure has been developed that permits analysis of older brine shrimp (juveniles) that have been depleted of their endogenous food supply and are dependent on exogenous nutrients for sustenance.

Quantitative and qualitative analyses of the incorporation of labeled purine compounds into the acid-soluble pools of juvenile brine shrimp have shown

them to be metabolically similar to nauplii. Of particular note is the observation, made using nauplii, that the purine and ribose moieties of exogenously supplied uniformly labeled [14 C]adenosine remain intact in the formation of AMP (i.e., Pur/Rib = 1.0) but do not do so in the synthesis of ATP, where Pur/Rib = 6. This suggests an atypical mechanism for the synthesis and/or utilization of ATP, involving cleavage of the glycosidic (Pur-Rib) linkage.

It is of interest to follow the metabolism of [$U-^{14}$ C]adenosine into the AMP and dAMP of RNA and DNA as well as the acid-soluble pool. Quantitative and qualitative comparison of the 14 C activity and especially the ratio of purine to ribose in these components may provide valuable insight into the mechanisms of nucleic acid synthesis.

The conversion of uniformly labeled purine nucleosides, as well as deoxynucleosides, into the purine mononucleotides of RNA and DNA is currently under investigation. The extent to which the ratios of purine to ribose in the purine mononucleotides of RNA and DNA reflect those of the acid-soluble nucleotides will provide valuable insight towards understanding the nature, i.e. level of phosphorylation, of the precursors involved in nucleic acid synthesis.

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EFFECT OF SIGMA FACTOR AND DINUCLEOTIDES ON THE TRANSCRIPTION OF PHAGE T4 DNA

D. J. Hoffman and S. K. Niyogi

Preliminary studies with phage T4 DNA have shown the ratio of [$\gamma-^{32}$ P]ATP to [$\gamma-^{32}$ P]GTP incorporation to be dependent on the ratio of sigma factor to core RNA polymerase. Dinucleotides appear to stimulate overall RNA synthesis at low substrate levels in a selective manner that depends on the ratio of sigma to core enzyme. These effects were examined in the absence of KCl, since we found KCl to be inhibitory at reduced substrate levels. With core RNA polymerase alone, GpA and GpG are found to be stimulatory. With the addition of a limited amount of sigma, GpA, GpG, GpU, and UpA are all effective stimulators of overall RNA synthesis. In the presence of increasing amounts of sigma factor or with holoenzyme, the dinucleotides CpA, UpA, ApU, and GpU are most effective. There seems to be a gradual transition away from dinucleotides capable of influencing 5'-GTP initiation to those influencing 5'-ATP initiation in the presence of sigma.

Different combinations of stimulatory dinucleotides have been found to exhibit little additivity in the amount of stimulation, suggesting that the complementary DNA sequences recognized by the holoenzyme are probably in close proximity. [γ - ^{32}P]ATP and [γ - ^{32}P]GTP studies add support to these findings. From these results it is possible to postulate several promoter sequences.

DIFFERENTIAL EFFECTS OF SIGMA FACTOR, IONIC STRENGTH, AND RIBONUCLEOSIDE TRIPHOSPHATE CONCENTRATION ON THE TRANSCRIPTION OF PHAGE T4 DNA

D. J. Hoffman and S. K. Niyogi

The effects of concentration of the sigma subunit of *E. coli* B RNA polymerase on the transcription of phage T4 DNA have been examined under high and low salt and substrate conditions. With ribonucleoside triphosphate (NTP) levels of 30 μM or less, even low concentrations of KCl (less than 0.05 *M*) had an inhibitory effect on overall RNA synthesis. Sigma factor was found to stimulate quite well at low (10 μM) NTP concentrations as well as at moderate ones (250 μM). At normal NTP levels (250 μM) 0.2 *M* KCl had a stimulatory effect of only 50% on core polymerase, a 2-fold effect on holopolymerase, and a 6-fold effect on core polymerase with a restricted amount of sigma present.

Increased ionic strength affected the rate of chain initiation, having a minimal effect on reactions catalyzed by core polymerase, but quite effective when sigma was present. With core polymerase, ATP and GTP initiation occurred equally, but mainly ATP rather than GTP initiation was stimulated by sigma. Increased ionic strength (0.2 *M* KCl) also preferentially stimulated ATP initiation in the presence of sigma.

Substrate concentration studies indicated a greater dependence on ATP than GTP in the presence of sigma. The concentration curves for UTP were similar to ATP under low and high salt conditions with the exception of an initial lag in the case of ATP and rather high K_m 's for ATP.

NATURE OF THE PROMOTER REGIONS IN T4 AND T7 PHAGE DNA'S FOR *E. COLI* RNA POLYMERASE

S. K. Niyogi and Brenda H. Underwood

Complexes of RNA polymerase (in the absence or presence of sigma) and ^{32}P -labeled phage DNA were treated with pancreatic DNase and snake venom phos-

phodiesterase to remove the unbound regions. After phenol extraction the protected regions were purified by dialysis, hydroxylapatite chromatography, and ethanol precipitation. The size of both the "core" enzyme-protected and holoenzyme-protected regions, as determined by end-group analysis, ranged from 20 to 22 residues. From chromatographic analysis and non-complementarity of A/T and G/C ratios, the DNA pieces appear to be single stranded. The promoter regions in the presence of sigma have a higher (A+T)/(G+C) ratio than in the absence of sigma. Furthermore, the promoter regions of T4 DNA do not hybridize to T7 DNA and *vice versa* (although they hybridize to their homologous DNA's), suggesting that RNA polymerase recognizes different regions in these two heterologous phage DNA's.

THE ROLE OF PHAGE-M13-SPECIFIC RNA IN DNA REPLICATION

Sankar Mitra

It was pointed out earlier (1) that rifampicin, a specific inhibitor of RNA synthesis, inhibits replication of M13 phage in certain mutants of *E. coli* HfrC and HfrH, named r41 and H491, respectively. These mutants are moderately resistant to rifampicin. RNA polymerases have been partially purified from these strains and are also found to be more resistant to rifampicin than those of the parent strains. It has been established that at concentrations of rifampicin that hardly affect the growth rates of these bacteria (30 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$ for r41 and H491, respectively) phage yield is drastically reduced in the presence of the drug. The inhibition is more complete when the drug is added at later times after infection. This is interpreted as due to increased permeability of the drug into the cells late after infection. The increased permeability is also shown by a comparison of growth inhibition of uninfected and infected bacteria at different drug concentrations. On the other hand, the phage synthesis is not inhibited in another rifampicin-resistant mutant, P4X6R1, at the drug concentration used above. The RNA polymerase of P4X6R1 is about ten times more resistant to rifampicin than those of r41 and H491.

Rifampicin inhibition of M13 replication is reversible, and the inhibition is not due to prevention of release of phage particles or their maturation. Messenger RNA isolated after pulse-labeling of H491 cultures with [^3H]uridine was isolated from uninfected cells, infected cells, and infected cells pretreated with rifampicin, and the RNA was hybridized with M13 replicative-form (RF) DNA fixed on nitrocellulose

membrane discs. Rifampicin inhibited phage-specific mRNA synthesis by 80%.

M13 DNA, which has a single-stranded (ss) structure, replicates by going through double-stranded RF DNA as the intermediate. RF and ss DNA can be separated by band sedimentation in sucrose after removal of the host DNA by Hirt's procedure with sodium dodecyl sulfate in 1 M NaCl (2). The rates of synthesis of ss and RF DNA's were measured by labeling infected cells with radioactive thymidine. Rifampicin inhibited replication of both ss and RF DNA's, whereas chloramphenicol inhibited replication of ss DNA but not significantly that of RF DNA. On the other hand, chloramphenicol drastically reduces the rate of total DNA synthesis in the infected cells. Rifampicin prevents the initial acceleration of total DNA synthesis immediately after infection, which is presumably due to RF DNA replication (3). Thus, at least in RF DNA replication, it appears that rifampicin acts directly without subsequent protein synthesis. Parental RF DNA synthesis from the infecting ss DNA, which has been shown by Brutlag *et al.* (4) to be dependent on the action of host RNA polymerase is, however, rifampicin-resistant in H491.

These results indicate that (1) M13 DNA controls synthesis of the major amount of its own RNA and (2) this RNA (or RNA's) is directly involved at least in RF DNA replication.

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DNA POLYMERASE IN BACTERIOPHAGE-T5-INFECTED *E. COLI*

R. K. Fujimura and Barbara R. Bussell

Studies of DNA synthesis in bacteria infected with a conditional mutant of bacteriophage T5 that is defective in phage-induced DNA polymerase at high temperatures led us to conclude that there is at least one other DNA polymerase functional in T5-infected cells. An analysis of the DNA polymerases in such a system was carried out, using an *E. coli* polI^- mutant as host.

Isolation of two polymerases. DEAE-chromatography of cell extracts fractionated two different enzyme activities. Enzyme I is active toward DNase-treated DNA and is insensitive to temperature. It is inhibited by high salt concentration. Enzyme II is active toward

denatured DNA and is temperature sensitive. Thus it is the enzyme reported to be the T5-induced polymerase. Its activity is enhanced by high salt.

Enzyme I also induced by phage T5. Phosphocellulose chromatography of enzyme I shows that it elutes much later from the column than *E. coli* polII . It cannot be *E. coli* polIII , since if it were so it should elute earlier. There is a possibility that enzyme I is *E. coli* polII that has been modified by phage infection. The effect of $(\text{NH}_4)_2\text{SO}_4$ on the activity of enzyme I is very similar to its effect on *E. coli* polII . In chloramphenicol-treated infected cells, both enzymes I and II are absent. The elution profile for chloramphenicol-treated infected cells is similar to that of uninfected cells.

Effect of dBrUTP as a substitute for dTTP. *In vivo* studies have shown that the residual DNA synthesis occurring under nonpermissive conditions in cells infected with the conditional mutant of T5 is refractory to dBrUrd, the analogue of dThd. The isolated enzymes I and II both utilize dBrUTP as a substitute for dTTP. Thus the refractiveness does not reside with the polymerase.

Effect of T5 infection on E. coli polII. The quantity of polII isolated from the infected cells is much less than from the noninfected cells. Thus it is either degraded or modified to the T5 enzyme I.

Comparison of polymerase from soluble and insoluble fractions. Membrane-bound polymerases were solubilized by sonication or by Triton-X100 treatment. The enzymes solubilized by such treatments were not different from soluble fractions obtained before such treatments.

INITIATION OF REPLICATION OF BACTERIOPHAGE T5 DNA

R. K. Fujimura

Initiation of synthesis is one of the important steps at which DNA replication is controlled. The early part of DNA replication was studied in a mutant of bacteriophage T5 that induces a temperature-sensitive polymerase that utilizes denatured DNA as its template *in vitro*. The host was *E. coli* polI^- . The important advantage with this system, as observed *in vitro*, is that replication can be stopped or started almost instantly by raising or lowering the temperature.

Cells infected with phage T5 were gently lysed, and the membrane-bound DNA-synthesizing system was characterized with the following results. (1) There are membrane-bound and soluble polymerase activities. The membrane-bound one has activity without addition of exogenous DNA. The DNA formed *in vitro* using the

endogenous DNA-enzyme complex at the membrane is T5 DNA, as shown by DNA-DNA hybridization tests. The amount of polymerase bound to the membrane is about the same whether the infected cells are incubated at the permissive temperature or the nonpermissive temperature. (2) Immediately after adsorption, most of the parental phage DNA is bound to the membrane, and after incubation for 20 min at the nonpermissive temperature most of the parental phage DNA is in a complex with polymerase at the membrane. This DNA-enzyme complex is specifically formed as T5 polymerase is induced. In the presence of chloramphenicol or when T5 DNA is added to a cell lysate of noninfected cells, there is hardly any detectable parental phage DNA bound to the membrane. (3) When the infected cells are incubated at the nonpermissive temperature for 20 min and then pulse-labeled with [³H]thymidine for various lengths of time as the temperature is shifted down to the permissive temperature, the size of the newly replicated DNA increases with the time of the pulse. Even at a short pulse time, the product is not covalently linked to the template. The ratio of pulse-labeled product to template is highest in the membrane fraction.

THYMIDINE METABOLISM IN *ARTEMIA SALINA*

C. G. Mead

A study has been undertaken to test the suggestion of Werner (1) that the 5'-nucleoside triphosphates are not the true precursors for *in vivo* DNA replication. *Artemia salina* was chosen as a test organism because previous evidence indicated that there was little if any repair replication carried out by this organism.

It has been established that thymidine is incorporated into DNA whereas thymine is not. Labeled thymidine mono-, di-, and triphosphates have been demonstrated in the acid-soluble pool after administration of labeled thymidine. There are no other labeled compounds detectable in the triphosphate region of a DEAE column chromatogram. There are, however, additional labeled compounds that appear in the nucleoside region of the chromatogram. Thymidine mono-, di-, and triphosphates are not labeled when the label is introduced as thymine. Thymine probably does enter at least some cells, however, since unidentified labeled compounds are eluted in the nucleoside region of the chromatogram.

The phosphorylation of thymidine *in vivo* is also being investigated. *Artemia salina* DNA can be labeled with ³²P introduced as inorganic phosphate. Thymidine

mono-, di- and triphosphates isolated from the acid-soluble pool, however, are not labeled with ³²P when the organism is grown on inorganic ³²PO₄. Furthermore, the 5'-TMP isolated from ³²P-labeled DNA is labeled with ³²P. The discrepancy in these data is currently being investigated.

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FATE OF HUMAN LYMPHOBLASTOID DNA TAKEN UP BY HUMAN SKIN CELLS IN CELL CULTURE

P. C. Kao, Elliot Volkin, M. Helen Jones,
James D. Regan, and W. H. Lee

The mechanism by which exogenous mammalian DNA is incorporated into human cells *in vitro* is under investigation by several biochemists at the present time. A clear understanding of this mechanism may make gene therapy possible in the future. We used the characteristics of light sensitivity and heavy buoyant density of DNA substituted with bromodeoxyuridine (BrdUrd) to elucidate whether human lymphoblastoid DNA is incorporated into recipient cells in an intact form or in a degraded form.

In light-sensitivity experiments, donor DNA from human lymphoblastoid cells was labeled with [¹⁴C]thymidine (dThd) and incubated with recipient human skin cells in the presence or absence of [³H]BrdUrd. After incubation, the recipient cell DNA was isolated, and the size of labeled DNA was analyzed by alkaline sucrose gradient centrifugation before and after exposure to 313-nm light. Regan *et al.* (1) have shown that BrdUrd-containing DNA is degraded to smaller-molecular-weight polynucleotides upon exposure to light at this wavelength.

In the DNA isolated from the cell culture in the presence of [³H]BrdUrd, cosedimentation of [³H]BrdUrd- and [¹⁴C]dThd-labeled DNA was observed. In addition, an aliquot of this isolated DNA was exposed to light of 313 nm and once again cosedimentation was observed, although in this case the size of the labeled DNA was much smaller. On the other hand, when the DNA was isolated from a culture grown in the absence of [³H]BrdUrd, the size of the labeled DNA was not changed by exposure to 313-nm light. These results suggested that either the donor DNA was degraded to mononucleotides that were reutilized along with light-susceptible [³H]BrdUrd nucleotide precursors, or the donor DNA was degraded to smaller pieces and rejoined, with BrdUrd in the gaps.

In the buoyant-density experiments, two donor DNA's (a heavier [³H]BrdUrd-substituted DNA and a

light [^{14}C]dThd-labeled DNA) were incubated together with the recipient cells. The DNA of the recipient cell was extracted and analyzed by alkaline CsCl equilibrium centrifugation. Both ^{14}C and ^3H sedimented at a position of intermediate density. However, this shift of density did not occur when only the [^3H]BrdUrd-substituted DNA and an excess amount of light-density nucleoside thymidine, instead of [^{14}C]dThd DNA, were incubated with recipient cells.

These results suggest that donor DNA is degraded to somewhat smaller polynucleotide pieces and then re-joined, with the gaps filled with light-density mononucleotides, the amount of which is not enough to shift the heavy, BrdUrd-substituted donor DNA.

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

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

Definitive experiments have been carried out which demonstrate that the donor RNA from blast cells is taken up at some macromolecular level, rather than as exogenous low-molecular-weight breakdown products, by the recipient skin cells. It is extremely difficult, however, in analyzing this RNA in the recipient cell to make the distinction between that which represents fairly intact donor polymer and that which arises by breakdown of the donor RNA and incorporation of the products in *de novo* RNA synthesis in some internal cellular compartment. A solution to this question is important, since the kinds of RNA found during the uptake period appear to be mRNA and/or nuclear-DNA-like RNA.

Various lines of evidence brought to bear on this problem are consistent with a mechanism of uptake that selects only certain species of RNA from the donor pool. However, it is still possible to explain the data by a mechanism involving degradation and resynthesis into skin-cell RNA if it is assumed that these degradation products do not readily mix with the nucleotide pool of the recipient cell. In general, a number of different experimental approaches, mostly involving differential isotopic labeling of lymphoblastoid donor RNA and nucleoside precursors, reveal that the RNA containing the donor RNA label is chemically and physically distinguishable from the RNA formed *de novo* from precursors.

ORGANELLE SYNTHETASES OF *EUGLENA*

W. E. Barnett and D. R. Joseph

Eucaryotic organelles (mitochondria and chloroplasts) are generally considered to have evolved from procaryotic endosymbionts. There is much circumstantial evidence for this position, a major part of which is that organelles contain a unique translational apparatus for the synthesis of proteins. It is clear from previous studies in this lab that both mitochondria and chloroplasts (from *Neurospora* and *Euglena*, respectively) contain aminoacyl-RNA synthetases that are distinct from their cytoplasmic counterparts. We have recently been characterizing the synthetases from mitochondria and chloroplasts of the same organism, *Euglena* B.

All of the evidence to date indicates that the chloroplast and mitochondrial synthetases are identical (although clearly different from the cytoplasmic synthetases). Since it appears unlikely that chloroplasts and mitochondria would have arisen from the same endosymbiont, these observations indicate that the observation *per se* of translational macromolecules unique to organelles does not indicate a procaryotic origin for organelles.

REGULATORY MUTANTS OF *EUGLENA* CONSTITUTIVE FOR CERTAIN SPECIES OF INDUCIBLE tRNA

Diane J. Goins and W. E. Barnett

In wild-type *Euglena* B the chloroplast tRNA's are light-inducible — i.e., they are present only in cells grown in the presence of light. In an attempt to understand the control mechanism(s) involved in the regulation of these tRNA's we have found a mutant ($G_1\text{BU}$) that contains fully derepressed levels of chloroplast isoleucine and methionine tRNA's when grown in the dark. Other chloroplast tRNA's (such as those for phenylalanine and serine) do not appear to be constitutive in $G_1\text{BU}$, indicating that there are groups of tRNA's under independent, coordinate-control genes.

Quantitative studies using partially purified chloroplast and cytoplasmic isoleucyl-tRNA synthetases indicate that $G_1\text{BU}$ is completely derepressed for the isoleucyl-tRNA in the dark. Another mutant has been found that shows partial derepression of these same tRNA's in the dark.

Studies with regulatory mutants such as these should lead to an understanding of how the transcription of chloroplast tRNA's is controlled and, more specifically as a first step, should elucidate the groups of chloroplast tRNA's that are coordinately controlled.

ION-EXCLUSION CHROMATOGRAPHY. A NEW METHOD FOR CHROMATOGRAPHIC SEPARATION

R. P. Singhal and W. E. Cohn

Investigations of the structures of macromolecules, such as nucleic acids, proteins, and carbohydrates, require analytical systems for the identification and assay of the primary building blocks, i.e. the base-containing components, amino acids, and sugars. Among many kinds of chromatography, ion exchange has been a predominant favorite since its introduction in 1949 (1). This arises partly from its ability to be scaled up or down, from tracer levels to manufacturing, and partly from its susceptibility to continuous quantitative monitoring, which eliminates a time-consuming assay of the separated substances.

Although the phenomenon of ion exclusion was observed and used over 20 years ago for the separation of ionized from nonionized substances, the principle seems not to have been exploited for any analytical or chromatographic purposes, nor have separations within the "excluded phase" of ionized substances been achieved, except in a few unrecognized instances.

Ion exclusion is a manifestation of the Donnan effect. We have examined its use in chromatography, taking the common nucleosides as model compounds (2), as influenced by several separation parameters — pH, temperature, ionic strength, organic solvents, and flow rate (3). The results indicate that ionizable substances can be *partially* excluded from the internal liquid of a similarly charged ion exchanger according to their degree of ionization, which in turn can be controlled by the pH and ionic strength of the eluant in a manner similar to ion exchange. Thus the principle can be applied to achieve analytical separation of the (partially) ionized substances while the nonionized substances are simultaneously resolved by partition chromatography.

The results indicate that ion-exclusion chromatography with respect to "base analysis" has practical advantages (speed, dilute volatile eluants, small plate heights, satisfactory resolution) over comparable forms of ion-exchange chromatography (4, 5). The method successfully resolves mixtures of picomole amounts of nucleic acid components at base or nucleoside levels on cation-exchange (e.g. Aminex A-6) and anion-exchange (e.g. Aminex A-25) columns at alkaline and acid pH's, respectively. The substances (both ionized and non-ionized) are easily resolved, even when there are only minor differences in their structure (pK's, isomers, an additional methyl group).

Ion-exclusion chromatography, although explored here with the common nucleosides as model compounds, offers equal promise for the separation of the components of other macromolecules such as proteins, carbohydrates, etc.

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NUCLEIC ACID SEQUENCING

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

We have modified the automated nucleic acid sequenator described in the previous report (1) to improve instrument reliability. The modifications include a change in reactor design so that two previously separate steps can be carried out simultaneously (the elimination step and the enzymatic dephosphorylation). This change permits the simplification of the program from 50 steps to 7 steps. Because of these changes, the stability of the dialysis membrane has become limiting. We have tried a variety of commercially available cellulose-based membranes, but they all decompose under the combined action of periodate and amine. We have fabricated polyacrylamide membranes that can retain RNA at room temperature but not at the higher temperatures required for optimal chemistry. We are examining some commercial polyethylene-backbone polymers as possible separation membranes.

Although the overall path to elimination was known prior to our construction of the sequenator, the specific organic chemistry was not well understood. The previous kinetics and studies of intermediates were inadequate to explain the variety of anomalous observations (2, 3); and adenylic acid, the model substance used by most investigators, is not an accurate model for the reaction with oligonucleotides (4).

The gaps in previous knowledge lay in assuming that a single elimination pathway was operating for all substrates and under all reaction conditions. We have shown that this is not true (4). A thumbnail description of the chemistry includes multiple equilibria in the reaction solution (potentially 12 compounds of significance) between the various aldehydes, hemialdals, carbinolamines, hemialdimines, and their corresponding hemialdiminals and aldimines. With these compounds, three major pathways are available for elimination of

the phosphoric group. They include paths that utilize an aliphatic aldimine or quaternized hemialdiminals. We have designated the third path "abortive elimination," since phosphoric ester cleavage is preceded by an ether cleavage that releases the base-containing fragment. The phosphoric ester cleavage is considerably slower under these conditions. Support for this abortive elimination comes from our isolation of an intermediate in which the phosphate has migrated from the 5' to the 1' position, as well as from the kinetics of the overall elimination reaction.

Our kinetic studies show that the chemical pathways suggested by others are not quantitatively important (4). The best analytical approach still seems to be further oxidation of the elimination product(s) with excess periodate to yield the unsubstituted base. This is rapid and quantitative, and the resulting products are readily analyzable in subnanomole quantities by the standard procedures we have developed.

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CHEMICAL PROBE OF tRNA STRUCTURE AND FUNCTION

R. P. Singhal

Previous modification studies (1, 2) indicate that the reaction of tRNA with bisulfite (HSO_3^-), converting cytidine residues to uridines, depends strongly on time, pH, and temperature. Modification of glutamate tRNA (*E. coli*) with HSO_3^- for 24 hr (where the reaction reaches a plateau at 20° C) results in the conversion of only six reactive cytidines out of the eleven that appear in nonhydrogen-bonded positions in the usual "cloverleaf" structure. The modified tRNA^{Glu} has a relatively unfolded secondary structure (from hyperchromicity and from exclusion volume on gel columns) and shows no glutamate acceptance activity under standard (3) and modified* charging conditions.

In order to localize the cytidines responsible for tRNA synthetase recognition or binding in this tRNA, modification with HSO_3^- was carried out at shorter reaction times. As previously reported (1), the modified cytidines were selectively deaminated at pH 9, the altered tRNA was fragmented by phage T1 ribonuclease, and the modification sites in the molecule were identified. The four cytidines located in the small arm and the pseudouridine loop and at the 3'-terminus

were modified to various degrees in 6- and 12-hr reaction times, whereas the three residues in the anticodon loop were totally converted to uridines in 6 hr. The tRNA that was modified at 6 hr had completely lost its ability to charge glutamic acid, and the first melting temperature was reduced somewhat (6° C). However, a 3-hr modification resulted in 80% conversion of the three reactive cytidines located in the anticodon loop and 40% of that present in the small arm. While this modification showed 82% loss of glutamate-charging ability, no apparent alterations in conformational parameters from those of the unmodified species were noted. The results strongly suggest that the reactive cytidines of the anticodon loop of this molecule are involved in determining the ability of this tRNA to charge amino acids.

Modifications with two other chemicals (cyanogen bromide and *p*-chloromercuribenzoate), which selectively alter the only thionucleoside (5-methylaminomethyl-2-thiouridine) present in this tRNA (in the anticodon), were performed to determine whether the cytidines or a specific form of the anticodon is necessary for recognition by tRNA synthetase. Reaction with cyanogen bromide at pH 8.8 and 20° C for 10 min, followed by an acid (pH 4.7) treatment of the modified tRNA^{Glu}, resulted in selective conversion (75–100%) of the thionucleoside to the corresponding uridine derivative and an 80% loss of the glutamate-charging ability. No other bases were altered. Similarly, *p*-[¹⁴C]chloromercuribenzoate was incorporated (pH 8.0, 45° C, 60 min) to the extent of 54 and 58% of the thionucleoside in two separate experiments; the complex was stable in the presence of Mg^{2+} . No other bases were altered. The modified tRNA showed a loss of about 55% in charging ability.

It thus appears that in this tRNA (1) the three cytidines of the anticodon loop are most easily modified (80% at 20° C in 3 hr) with an equal loss of amino-acid acceptance ability, whereas a total conversion of these three residues into uridines results in a complete loss of charging ability; (2) other "free" cytidines in positions 45 (small loop), 56 (T-ψ-C loop), 75, 74, and 72 (amino-acid stem) are decreasingly reactive in the order given; (3) modification of the thionucleoside located in the "wobble" position of the anticodon also results in equal loss of the glutamate-charging ability. Apparently a chemically unaltered anticodon loop of the molecule is required for aminoacylation to occur.

Five of the 21 nonreactive cytidines (24-hr modification) are in nonhydrogen-bonded sections of the tRNA in the "cloverleaf" arrangement, leading to the

conclusion that these sections are not as exposed as this arrangement might, *a priori*, indicate but are buried in the three-dimensional structure in the modification medium.

*Amino-acid charging assays for the modified tRNA's were also performed with additional ATP, CTP, transferase enzyme, and different concentrations of tRNA synthetase in the reaction mixtures, and also with different reaction intervals ranging from 0.5 to 2.0 hr. However, glutamate acceptance of the modified tRNA's remained identical to the values obtained with the standard assay conditions (3).

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AN IMPROVED PROCEDURE FOR THE SYNTHESIS OF 2'-O-METHYLRIBONUCLEOSIDE 5'-DIPHOSPHATES

B. C. Pal and Diane G. Schmidt

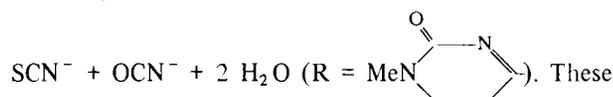
We needed a large amount of 2'-O-methylribonucleoside 5'-diphosphates to make poly(2'-O-methylribonucleic acid), using polynucleotide phosphorylase, for inhibition studies on the reverse transcriptase of Rauscher leukemia virus. 2'-O-Methyladenosine 5'-diphosphate had previously been prepared by Rottman and Johnson (1) with an overall yield of 1.9% by the following route: adenosine \rightarrow 2'-O-methyladenosine (Am) (step 1) \rightarrow AmMP (step 2) \rightarrow AmDP (step 3). We improved the yield of 16.7% by the following modifications. In step 1, the yield of Am was doubled by isolating the compound by chromatography on silicic acid instead of Dowex-1. In step 2, direct phosphorylation of Am with POCl₃ in triethyl phosphate (2) led to AmMP in 77% yield (vs. 21% in this step by following Tener's procedure). It was difficult to remove P_i from AmMP by DEAE-Sephadex chromatography, and its removal is essential before step 3. This was accomplished quantitatively by treating with a slight excess of Ba(OH)₂ in the cold and precipitating excess Ba(OH)₂ by CO₂. Synthesis of 2'-O-methyluridine 5'-diphosphate and 2'-O-methylcytidine 5'-diphosphate by the same procedure is in progress.

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MODIFICATION OF tRNA WITH CYANOGEN BROMIDE

B. C. Pal and Diane G. Schmidt

Cyanogen bromide has been used to modify the thiopyrimidine moieties in tRNA to thiocyanatouracil derivatives, which may possibly be intermediates in further degradation to uracil compounds. We have investigated the mechanism of this reaction, using 1-methyl-4-thiouracil (methyl analog of 4-thiouridine). 1-Methyl-4-thiocyanatouracil (I) was prepared in 70% yield by treating 1-methyl-4-thiouracil with cyanogen bromide in alkali. When the preparation is attempted in alcoholic medium, a considerable amount of bis(1-methyl-4-thiouracil) disulfide is formed, especially when NaOMe is replaced by NEt₃. In agreement with an earlier observation, compound I is degraded slowly but quantitatively to 1-methyluracil and thiocyanic acid in acid. In contrast with earlier findings (1), compound I is converted almost immediately and quantitatively in alkali to 1-methyl-4-thiouracil, 1-methyluracil, thiocyanate, and cyanate in a 1:1:1:1 ratio according to the following scheme: $2 \text{ RSCN} + 4 \text{ OH}^- = \text{RO}^- + \text{RS}^- +$



results indicate that the conversion of 4-thiouridine moieties in tRNA to uridine can be achieved quantitatively by subjecting the cyanogen-bromide-treated tRNA to an acidic pH rather than an alkaline pH, where the desired conversion is only 50%.

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SCISSION OF PURINE AND PYRIMIDINE DISULFIDE BONDS BY CYANIDE

B. C. Pal and Diane G. Schmidt

The discovery of thiouridines in tRNA opened the possibility that the disulfide bond plays a part in the structure of nucleic acids, parallel to its role in protein structure. Although the existence of disulfide bonds in native nucleic acids has yet to be demonstrated, one can be formed by iodine oxidation of tRNA^{Tyr}, which has two adjacent 4-thiouridine moieties (1). The possible importance of such disulfide bonds led us to investigate the cleavage of bis(1-methyl-4-thiouracil) disulfide (methyl analog of the disulfide of 4-thiouridine, a minor base in tRNA) and bis(9-methyl-6-thiopurine) disulfide by sodium cyanide, using spectrophotometry and chromatography. The first step in both the cases is

similar: $RSSR + CN^- \rightarrow RS^- + RSCN$ (R = purine or pyrimidine derivative). In the second step, the thiocyanatopyrimidine is decomposed quantitatively into equimolar amounts of the thio- and oxo derivatives while the thiocyanatopurine is converted into only the thio- derivative. Synthesis and spectral characterization of these two purine and pyrimidine disulfides and their corresponding thiocyanates have been completed.

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MULTIPLEXED ANALYTICAL INSTRUMENT INTERFACE TO PDP-11

Mayo Uziel, S. S. Stevens, and C. O. Kemper

Interfacing equipment has been installed to allow automatic data collection from several analytical instruments by the Division's PDP-11. As installed, the equipment permits either of two data channels (or both) to be connected to any one of four nucleoside analyzers producing alternate readings at two preselected wavelengths and one analyzer producing up to four readings at four preselected wavelengths. These will be used for routine analysis in nucleic acid sequence and modification studies. Data source identification can be manual (by noting multiplex switch settings) or automatic (e.g. by interleaved unique test voltages at the various instruments).

In order to maintain the microvolt sensitivity and noise immunity available at the computer's analog-to-digital converter port, channel multiplexing between instruments is accomplished with a network of self-cleaning, low-noise switches. As a further check on multiplexer reliability, the data analysis programs (now being developed) will contain a baseline measuring routine that can report data behavior typical of a noisy or malfunctioning data channel.

TOXICOLOGY INFORMATION RESPONSE CENTER PROGRAM*

D. G. Doherty, Martha W. Gerrard, Kathy C. Miller, Helen S. Warren, Helga Gerstner, and Florence Holland

The Toxicology Information Response Center at the Biology Division was formed as a branch of the Toxicology Information Program to aid in providing toxicology information services to the general scientific community and the public. The center responds to questions with bibliographies and data tailored to the

subject. It develops comprehensive annotated bibliographies related to timely chemical problems in the area of environmental pollutants, provides state-of-the-art reviews, and is involved in toxicology data extraction and data-base building.

The center's first full year of operation has been one of growth and development. Adequate space has been renovated to accommodate the center's needs, and a staff of five full-time and three half-time employees has been recruited, as well as a Ph.D. toxicologist, who will join the program in August. We have acquired a specialized library of 100 books, 1000 reports and journals, and all the necessary hardware to assess computerized information and reproduce material. Our personnel have been trained in assessing such computerized information files as the *Chemical Abstracts* series, *Toxicon*, and *Medline*. We have interacted in supply and exchange of information with many of the 15 other information centers in Oak Ridge. The requests for information have built up rapidly from an initial 10 per month to the current level of about 60. We have completed 380 in the past year, many of them covering the whole of scientific literature. A computerized system with keyworded indexes has been devised and is in operation to keep track of all requests. The staff has produced annotated bibliographies on pyrethrum, citrus oils, heptachlor, EDTA, toxaphene, and insecticides as enzyme inhibitors, which are in press. A comprehensive bibliography on polychlorobiphenyls (PCB's) and related chlorophenyls has been produced by Griffith E. Quinby,[†] and a review of this subject is in progress. Articles being processed for printing are "Metabolism of Foreign Compounds: An Annotated Bibliography" by Anthony Verbiscar[†] and "Toxicity and Health Threats of Phthalate Esters, Review of the Literature" by John Autian.[†]

Our data-base building and related activities have been carried out both by our group and in collaboration with the Mathematics Division and the Environmental Information Systems Office (EISO) of ORNL. Approximately 2000 bibliographic citations have been entered by EISO into our Toxquest File, as well as the 11,000 citations of the Hayes pesticide file. Some 1000 items of test data from Section II of *Clinical Toxicology of Commercial Products* have been entered, the format design has been completed, and the test file has been completed and checked. Additional data for program design purposes have been collected from *Pesticide Petitions* and *Food Additive Petitions*, and a contract has been negotiated with Hercules Chemical Co. for toxicology information from the published literature in their files.

*Supported by the Toxicology Information Program, National Library of Medicine, National Institutes of Health.

†Consultant.

THE ENVIRONMENTAL MUTAGEN INFORMATION CENTER*

D. G. Doherty, J. S. Wassom, M. M. Nawar, Elizabeth S. Von Halle,† Ida C. Miller,† M. D. Shelby,‡ and Mary Medill-Brown‡

The Environmental Mutagen Information Center (EMIC) is completing its third year of operation at the Biology Division. EMIC was commissioned to collect and organize the literature of chemical mutagenesis and to develop automated programs through which interested individuals would have ready access to information. The center prepares and issues annual indexed literature surveys and special subject bibliographies on compounds of current environmental interest (caffeine, hallucinogenic drugs, nitroso compounds, etc.).

EMIC recently completed a move to new quarters along with the Toxicology Information and Response Center, a move which has proved beneficial to both centers in that we share library resources, equipment, and information.

EMIC's efforts to date have been concentrated primarily on accumulating publications for its data bank. Seven thousand citations have been selected for entry into the computer, the majority of which have been published since 1968. From these 7,000 references, bibliographic data and agent and organism keywords are being recorded and sent to the computer. After this initial processing, the entry is then submitted to either tabular or conventional abstracting. Tabular abstracting is a new approach for use in the management of

mutagenesis data. This makes it possible to segregate essential data under different column headings, thus summarizing the essential information in the citation. These data can be further segregated as to compound and printed in table form. The information in each column of the table can be queried individually or in combination with data under other headings. Items not tabularly abstracted have concise written abstracts prepared for them along with appropriate descriptor terms. Using this tabular abstracting format, EMIC is presently completing a report on the mutagenicity and teratogenicity of food additives in fulfillment of an FDA-funded project.

EMIC not only maintains an active relationship with the Toxicology Information Center but also with 14 other ORNL information centers. Liaison with the environmentally oriented centers is maintained through ORNL's Environmental Information Systems Office (EISO).

EMIC receives many requests for copies of its annual literature surveys and its special subject bibliographies. Even though we presently do not solicit specific questions for information, we do receive them at a rate of two to four per week. These are answered as promptly as time permits. Such services will be made much easier when our computer can be used to satisfy these requests. This capability should be available this year, and active solicitation for requests for information can then be made.

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BIOPHYSICS AND CELL PHYSIOLOGY

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Growth and Regeneration

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Molecular Biology

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Excited-State Biophysics

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J. W. Longworth
M. L. Randolph
S. S. Stevens

Microbial Photosynthesis

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Photosynthesis

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Plant Physiology

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Cellular Radiobiology

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SURVIVAL OF MOUSE EMBRYOS FROZEN TO -196 AND -269°C

D. G. Whittingham,* S. P. Leibo, and Peter Mazur

Attempts prior to 1971 to freeze mammalian embryos were unsuccessful. Then, in 1971, D. G. Whittingham at Cambridge reported the successful freezing of certain stages of early mouse embryos to -80°C for 30 min by suspending them in 7.5% solutions of polyvinylpyrrolidone (PVP) and freezing them at $60^{\circ}/\text{min}$ (1). However, none survived longer than 30 min, and two-cell embryos did not survive at all. We therefore decided to explore the cryobiological factors that might affect the ability of embryos to survive freezing. In these more recent experiments, unfortunately, the embryos were not able to survive freezing in PVP. We turned our attention then to another protective additive, dimethyl sulfoxide (DMSO). Previous studies (2, 3) have shown that cooling rate is an important parameter; and previous thermodynamic and kinetic analysis (4) indicated that objects the size of these embryos ($80\ \mu\text{m}$ diameter) would have to be cooled at $\sim 1^{\circ}/\text{min}$ or less to avoid intracellular freezing, which is nearly always a lethal event. First attempts to freeze eight-cell embryos in 1 M DMSO at rates of 0.4 to $40^{\circ}/\text{min}$ yielded 10% survival or less. Suspicious that deleterious osmotic events might be injuring these large multicellular objects during rapid thawing (used because it has almost always been better than slow thawing), we tried slow thawing at 4 or $20^{\circ}/\text{min}$. This solved the problem. If cooling was between 0.4 and $2^{\circ}/\text{min}$ and warming was 4 or $20^{\circ}/\text{min}$, over 50% of one-, two-, and eight-cell embryos and blastocysts survived freezing to -80 , -196 , and -269°C for up to 8 days. Cooling at $6^{\circ}/\text{min}$ or faster killed all embryos, even if the warming was slow. The above survivals are based on the ability of more than 3000 frozen-thawed embryos to undergo embryological development in culture. In addition, about 1000 frozen-thawed embryos were transferred into foster mothers. About two-thirds of the recipients became pregnant; and in these, 43% of the embryos developed into mature living fetuses or living mice. The reason for terminating some pregnancies was so that we might determine how many embryos had developed into mature fetuses and how many had implanted but not developed further.

One implication of these findings is that the storage of mutant strains of mice not in current use but of

potential interest is now possible. When desirable, the stored embryos could be transferred to foster mothers, and the resulting offspring could be used to reestablish the mutant strain. A second implication is that if these procedures proved applicable to embryos of large domestic animals, they would facilitate world-wide dissemination of stock with an optimal genetic background for a given use or a given geography. Finally, and more generally, the success of cryobiological theory (2) in suggesting the proper approach to the successful freezing of these sensitive embryos increases the likelihood that ways can be found to freeze complex mammalian systems that are of direct medical interest.

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PERMEABILITY OF THE BOVINE RED CELL TO GLYCEROL IN HYPEROSMOTIC SOLUTIONS AT VARIOUS TEMPERATURES

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

A central tenet in cryobiology is that low-molecular-weight protective solutes such as glycerol must permeate cells in high concentration in order to protect them from freezing injury. To test this supposition, it is necessary to estimate the amount of solute that has permeated a cell prior to freezing. The amount in bovine red cells was estimated from the flux equation

$$ds/dt = P\gamma A[(\text{activity external solute}) - (\text{activity internal solute})].$$

Solving the equation required estimates of $P\gamma$, the permeability constant for the solute. Estimates for glycerol in bovine red cells were made in two ways: (1) by measuring the time to 50% hemolysis of red cells suspended in isosmotic or hyperosmotic (1–3 M) solutions of glycerol that were hypotonic with respect to NaCl and (2) by measuring the time required for red cells in hyperosmotic solutions of glycerol in isotonic saline-buffer to become susceptible to osmotic shock upon 10-fold dilution with isotonic saline-buffer. The measurements were made at 0, 10, 15, and 20°C . The values by the second technique ranged from 2.3×10^{-6} cm/min to 2.7×10^{-6} cm/min at 20°C , depending on the concentration of glycerol. The values by the first technique were 0–30% lower. Both techniques yielded

about the same activation energy for permeation between 0 and 20° C, 21 kcal/mole. This is equivalent to a halving of the permeation rate for every 5° drop in temperature.

Expressing the flux equation in the formulation of irreversible thermodynamics changed the value of P by less than 10%, probably because σ , the reflection coefficient, is 0.95 at 25° C. Expressing the driving force as the difference in molality or osmolality of glycerol rather than as the difference in activity, however, had somewhat greater effects on the numerical values of P but had no effect on the activation energy.

It is concluded that estimates of P based on differences in activities and on the osmotic shock technique are the least subject to error. The use of the usual irreversible thermodynamic equations to express the flux may be a misleading refinement, in that the assumptions underlying them become questionable for concentrations of glycerol as high as 1, 2, or 3 M .

KINETICS OF PERMEATION OF DIMETHYL SULFOXIDE INTO BOVINE RED CELLS IN HYPEROSMOTIC SOLUTIONS

R. H. Miller and Peter Mazur

The two protective additives most commonly used to protect cells are glycerol and dimethyl sulfoxide (DMSO). As reported last year and in the preceding report, glycerol does not have to permeate to protect bovine red cells from freezing injury. We would like to know whether this is also true for DMSO. The permeability of the bovine red cell to DMSO was estimated by measuring the time required for the cells to hemolyze when placed in hyperosmotic solutions of DMSO that were hypotonic with respect to NaCl. The cells were found to be about 150 times more permeable to DMSO than to glycerol. For example, it takes 1760 min for cells in 1 M glycerol at 0° C to undergo 50% hemolysis, whereas it takes only 12 min for cells in DMSO. However, the temperature coefficient for the permeation of the two solutes is similar. In both cases, the rate doubles with a 5° rise in temperature, which is equivalent to an activation energy of about 20 kcal/mole. Unfortunately, the permeation of DMSO is so rapid even at 0° C that it may not be possible with our present techniques to determine the relation between the amount of DMSO in the cell and the degree of protection from freezing injury.

TIME-DEPENDENT SENSITIVITY OF BOVINE ERYTHROCYTES TO CHANGES IN GLYCEROL CONCENTRATION AT SUBZERO TEMPERATURES. SIMULATION OF FREEZING DAMAGE

S. P. Leibo and Peter Mazur

We have shown previously (1) that exposing bovine erythrocytes to glycerol at 20° C for 1 and 30 min yielded similar survivals after freezing to -196° C at 50°/min. But exposing the cells to glycerol at 20° C for intermediate times prior to freezing produced dramatic but transient decreases in survival. This decrease is concentration-dependent, the time at which it occurs increasing with increasing glycerol concentration. Seeking an explanation of this transient increased sensitivity to freezing, we believe that we have uncovered a phenomenon that may play a major role in freezing damage of erythrocytes.

Armed with a detailed mathematical and experimental analysis of the rate of glycerol permeation in the bovine erythrocyte (2), we are able to describe the state of the cell with respect to intracellular glycerol concentration and cell water volume as a function of time in glycerol at 20° C. Based on this information, we have sought to test the applicability of the following argument to freezing damage in the bovine erythrocyte. (1) When a solution freezes, the solute concentration increases as water is removed in the form of ice. (2) When a cell is frozen in such a solution, it will be exposed to increased solute concentration, requiring the loss of cell water in response to the osmotic pressure gradient. The response must occur at *subzero temperatures*. The reverse, of course, will occur during thawing.

The test was to ask the following question: Can bovine erythrocytes suspended in concentrated glycerol solutions at 20° C withstand a second hyperosmotic exposure at subzero temperatures? Experimentally, the test consisted of suspending washed erythrocytes in 1 and 2 M glycerol at 20° C for various periods of time. The suspensions were supercooled at subzero temperatures, e.g. -5° C, mixed with prechilled 6 M glycerol in isotonic saline, held briefly, and warmed to 20° C, and the amount of hemolysis was then measured. Conceptually, this treatment may be considered a simulation of the events during freezing. The events during thawing were simulated by first performing the above treatment and then diluting the suspensions, still at subzero temperatures, with prechilled glycerol solutions at the same concentrations that were present initially.

Controls consisted of suspensions exposed to glycerol concentration changes with no temperature change, i.e. at 20° C, and suspensions exposed to temperature changes, i.e. 20 to -5° C, with no concentration changes.

Briefly, we have found that the response of bovine erythrocytes suspended in concentrated glycerol solutions and then exposed to changes in glycerol concentration at subzero temperatures, in the absence of any freezing, parallels rather accurately the response of these cells when frozen to -196° C and then thawed. One interpretation of these results is that the erythrocyte membrane undergoes a transition below 0° C, rendering the cell incapable of tolerating volume changes required by large changes in the osmotic pressure of the suspending solution.

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VISUALIZATION OF FREEZING DAMAGE IN FREEZE-CLEAVED YEAST AND HAMSTER TISSUE-CULTURE CELLS

Harvey Bank* and Peter Mazur

Freeze-cleaving can be used as a direct probe to examine ultrastructural alterations due to freezing. We examined the thesis that at least two factors, which are oppositely dependent upon cooling velocity, determine the survival of cells subjected to freezing. According to this hypothesis cells cooled at rates exceeding a critical velocity are killed by freezing internally; but cells cooled at rates less than this critical velocity do not form intracellular ice and are killed by prolonged exposure to high concentrations of solute. To test this hypothesis, we froze the yeast *Saccharomyces cerevisiae* at five rates ranging from 0.5 to 75,000°/min. The frozen samples were then cleaved and replicated simultaneously in order to minimize artifacts due to handling. We found that the ultrastructure of rapidly cooled cells indicated the presence of intracellular ice, whereas that of slowly cooled cells showed marked dehydration. In order to confirm the presence or absence of intracellular ice, we froze cells at 1 or 1500°/min, rewarmed them for 5 min to -20, -30, or -40° C, and then rapidly re-cooled them to -196° C. If intracellular ice is present, this warming should cause small ice crystals to coalesce into larger crystals by the process of migratory recrystallization. Cells rapidly cooled to -196° C and rewarmed to -20° C showed large, intracellular, angular, crystalline-like bodies; cells rewarmed to -30° C showed few structural changes; and

cells rewarmed to -40° C were similar in morphology to those not rewarmed. Slowly cooled cells, on the other hand, showed no evidence of intracellular ice either before or after warming. These results support the view that intracellular ice is present in rapidly cooled cells and is absent in slowly cooled cells. And they support the view that recrystallization of intracellular ice is responsible for the high sensitivity of rapidly cooled yeast to slow warming.

A similar investigation was also carried out on Chinese hamster tissue-culture cells. Previous studies (1) have shown that when glycerol is present as the protective additive, the survival of frozen-thawed Chinese hamster tissue-culture cells increases as the cooling velocity is increased from 1 to 200°/min. However, the rapidly cooled cells are much more sensitive to warming rate than are the slowly cooled cells. It has been hypothesized (1) that cooling rates which produce high sensitivity to warming rate also produce intracellular ice. We are using the freeze-cleaving technique to test this hypothesis. Hamster cells suspended in 1.25 M glycerol were cooled to -196° C at 50, 200, 2000, and 75,000°/min and then cleaved and replicated. Preliminary results confirm our hypothesis. Cells cooled at 5°/min show no evidence of intracellular ice, whereas cells cooled at 200°/min or higher clearly do.

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FREEZING INJURY IN CHINESE HAMSTER TISSUE-CULTURE CELLS

Peter Mazur and S. P. Leibo

According to one of the popular theories of freezing injury (1, 2), damage to slowly frozen cells should be proportional to the concentration of electrolytes produced during freezing. The theory argues that substances like glycerol and dimethyl sulfoxide (DMSO) protect to the extent that they colligatively reduce electrolyte concentration. That is to say, according to theory, plots of cell survival vs. the concentration of electrolyte produced during freezing should follow a single curve regardless of the concentration of protective additive present. From published phase diagrams of glycerol-NaCl-H₂O systems and DMSO-NaCl-H₂O systems, it is possible to determine the concentration of NaCl in a solution as a function of temperature. This could then be compared to our existing data on the survival of cells in glycerol and

DMSO as a function of temperature, permitting us to construct desired plots of survival vs. electrolyte concentration. The results of such plots are in clear disagreement with the predictions of the theory. The survivals do not fall on a single curve. The one weak point in this conclusion is that the survival experiments were performed with cells in Hanks' balanced salt solution (which contains ions in addition to NaCl), whereas the published phase data are for NaCl alone.

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STUDIES ON ISOLATED EUKARYOTIC NUCLEI

D. E. Olins, Ada L. Olins, and Everline B. Wright

Studies have been directed at the influence of solvent parameters on the various levels of organization of nucleohistone in eukaryotic nuclei. Isolated rat liver and chicken erythrocyte nuclei undergo swelling and a decondensation of chromatin when divalent cations are washed out and monovalent cations are present at low concentrations (e.g., $K^+ \leq 0.02 M$). By employing low-angle X-ray diffraction, and electron microscopy on fixed and sectioned nuclei, we have obtained evidence for the model of chromatin condensation and decondensation shown in Table I. Further, we have shown that the dispersed₁ state exhibits circular dichroic spectra very similar to isolated chromatin, whereas condensed nuclei reveal spectra with apparent nuclear shadowing effects.

We are presently examining the reactions of various bifunctional reactive chemicals with histones and non-histones as a function of solvent composition (and, hence, nuclear morphology). We have already obtained evidence that glutaraldehyde (an aliphatic dialdehyde) reacts most rapidly with the lysine-rich histones, probably leading to polymer networks of cross-linked lysine-rich histones. Swelling and decondensation of nuclei leads to a marked reduction in glutaraldehyde-

fixed products, possibly reflecting the loss of histone-histone contracts broken by the separation of nucleohistone fibers at low ionic strengths.

CELL DIVISION IN RADIATION-INDUCED BACTERIAL FILAMENTS

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

One approach to the regulation of bacterial cell division is to seek naturally occurring factors that can initiate division in cells blocked in some particular step of the division process. We have applied this approach to *E. coli* 1899NM. After irradiation, cells of this strain continue to grow and synthesize macromolecular constituents but are blocked in the initiation of cross-septum formation. Irradiated cells grow into very long multinucleoid filaments (50–100 cell equivalents) but do not divide or form macrocolonies. These filaments can, however, be induced to divide by the addition of an extract from normal cells. This provides the basis for a convenient assay for division, since filaments do not form macrocolonies unless stimulated to divide (1). We have shown previously that the active material is heat labile and associated with a large particulate fraction (2). Attempts to characterize the material further by isolation have been unsuccessful; attempts to obtain soluble, active preparations result in loss of biological activity. We have now established that the activity is associated with a membrane fraction and that extracts prepared from radiation- or drug-induced filaments are inactive.

Figure 1 shows that factors that stimulate cell division in radiation-induced filaments cosediment in a sucrose gradient with succinic dehydrogenase, an enzyme localized in the inner or cytoplasmic membrane. If the material in the membrane fraction is a normal physiological regulator of cell division, the failure of the filaments to divide could be attributed either to a deficiency of the regulator or to an altered membrane

TABLE I. Model for chromatin decondensation

	Chromatin states		
	Condensed	Dispersed ₁	Dispersed ₂
	no M ²⁺ ————→ low M ⁺	no M ²⁺ ————→ very low M ⁺	
Superhelix of nucleohistones (i.e. 110-, 55-, and 38-Å reflections)	present	present	absent
Nucleohistone fiber diameter (Å)	~150	~150	100

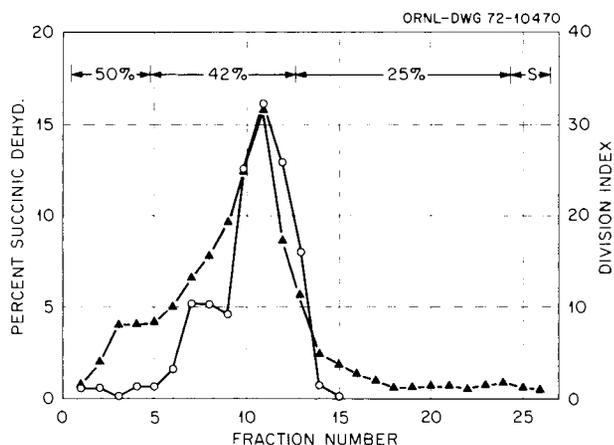


Fig. 1. Succinic dehydrogenase activity (▲---▲) and a division-stimulating activity (○---○) after fractionation of extract on a sucrose gradient. Extract (3 ml) was layered over a 25%-42%-50% step gradient and centrifuged for 8 hr at 25,000 rpm in a Spinco SW-27 rotor. Division index is the ratio of macrocolonies formed by irradiated cells in the presence of extract to number of colonies formed without added extract and is a measure of cell-division stimulation in filaments.

that requires a higher concentration of the hypothetical regulator to "trigger" cross-wall formation. We have therefore tested extracts of radiation-induced and drug-induced (penicillin and phenylethyl alcohol) filaments for division-promoting activity. Such extracts are at least 10 times less active, on a dry-weight basis, than similar extracts of normal cells. Cells of a temperature-sensitive mutant that filaments when grown at elevated temperatures yielded active extracts under permissive conditions and inactive extracts when grown at the nonpermissive temperature. It appears, therefore, that the membrane fraction of filaments is deficient in the stimulating factor.

Several observations suggest that the active material itself is a diffusible substance and not a membrane fragment. Whole cells seeded in agar can act as a source of the factor. When irradiated test cells are overlaid in agar on solidified agar containing extract, recovery can be observed several millimeters into the upper layer. Active low-molecular-weight material cannot be recovered from incubated extracts, however. The material may be relatively unstable when released from the membrane, and this would account for inactivation of the factor when the membrane is solubilized by detergents.

We have evidence for a membrane-associated factor capable of inducing cell division in irradiated filaments. Since the factor is lacking in extracts of filaments, we propose that the failure of the filament to divide and

form macrocolonies is the result of the failure of the cell to reach a threshold concentration of the factor.

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REGULATION OF CELL DIVISION IN BLUE-GREEN BACTERIA

L. O. Ingram and W. D. Fisher

Two classes of cell-division mutants are readily recovered from the blue-green bacterium *Agmenellum quadruplicatum* following mutagenesis. One class (serpentine mutants) consists of cells that propagate as multinucleoid filaments that appear to be defective in the initiation of septum formation. The second class (septate mutants) is defective in the very terminal stages of cell division; cross-septa form but the daughter cells do not separate immediately, and long chains of cells result.

A comparison of the cell wall of normal and mutant cells: Cell-wall defects have been suggested as one possible defect in filamentous cell-division mutants (1). We have therefore analyzed abundance, composition, and cross-linking of peptidoglycan from serpentine and septate mutants and normal cells. The peptidoglycan isolated from all three organisms contained the same components (alanine, diaminopimelic acid, glutamic acid, muramic acid, and glucosamine) in similar proportions. Chromatographic analyses of lysozyme digests of the peptidoglycan from the three types of cells suggested the presence of a highly cross-linked peptidoglycan compound of C-4 units. The only differences detected among the three types of cells were small differences in the relative abundance of peptidoglycan. However, these small differences can be attributed at least in part to differences in overall morphology. Thus no gross alteration in the peptidoglycan of the mutants was revealed by these studies.

Evidence for a positive regulating factor in cell division: The presence of a putative positive effector of cell division, a cellular product capable of initiating cell division in filamentous mutants, was suggested by analysis of the growth pattern of one of the mutant strains (SN12). In dilute suspensions of this organism, cell division lags behind mass increase, and the cells form filaments. Subsequently, these filaments divide into cells of unit cell lengths as the cell density in the culture increases. Cultures of SN12 can be propagated as filaments by frequent addition of fresh medium, or

they can be cultured as cells of unit length in high-density suspensions. Conditions that would be expected to favor the localized accumulation of metabolic products, e.g. inadequate stirring, also antagonize filament formation. We therefore attempted to recover from spent medium material(s) capable of inducing cell division in SN12 filaments.

Spent medium from the SN12 mutant (or the parent organism) was dried by flash evaporation and extracted with 80% ethanol. The ethanol extract was then evaporated to dryness and resuspended in water. Addition of small amounts of this material to dilute suspensions of the SN12 mutant substantially reduced the mean filament length throughout the growth cycle. Comparable extracts of fresh autoclaved medium were inactive. For further characterization of the factor from spent medium, a different mutant (SN29) was used as an assay system. The organism is a nonlethal, temperature-conditional, cell-division mutant. Over the temperature range 35–44° C, the mean filament length is determined by the growth temperature. The assay is considerably simplified, since filament length can be easily controlled by the growth temperature.

The degree of stimulation of the cell is proportional to the amount of extract added over a narrow concentration range. At higher concentrations, filaments divided into cells of approximately the dimension of parental cells within about one generation time (4 hr). Lower concentrations also produced a burst of cell division and a decrease in mean filament length. This resulted in populations of filaments with a shorter mean length, intermediate between the parent cell and the untreated filaments. The extract inhibits growth only at high concentrations, and further purification reduces the growth-inhibitory effects. The active material is dialyzable and heat stable, and the activity can be extracted with relatively polar solvents. Gel filtration gives a molecular weight in the region of 1100. The activity is not removed by activated charcoal or cation-exchange resins but is tightly bound to anion-exchange resins.

As a working hypothesis, we propose that SN12 and SN29 are impaired in the production and/or accumulation of some regulating factor required for the initiation of cell-wall and cell-membrane invagination. Positive regulation at this level does not preclude additional positive or negative regulators of cell division acting at various other points in the cell cycle. The accumulation of the regulatory factor in spent medium suggests that, rather than converting the effector to an inactive form, the cells release it into the surrounding medium.

The regulation of cell-wall and membrane invagination by an effector recovered from the medium suggests a possible mechanism for the regulation of this one step in the cell division process. The initiation of cell-wall invagination requires a critical threshold concentration of the effector that produces a localized membrane alteration (increase in permeability) at any one potential division site and a loss of the effector from the site into the medium. Assuming that filaments have multiple potential divisions and that the effector is lost as a result of a localized permeability change at the one site, such a model is consistent with several properties of populations of filaments. (1) Populations have different mean filament lengths. This could result from differences in the rate of accumulation of the factor. (2) Cell divisions are not localized at one region but occur at various points along the filament, resulting in a wide range of cell sizes in the population. (3) The addition of suboptimal concentrations of the putative effector results in a new and shorter mean filament length.

Stimulation of cell division by membrane-active agents: We have proposed (see above) that the initiation of cell-wall and cell-membrane invagination is controlled by a diffusible substance acting on the cell membrane. Therefore, we investigated the effects of agents reported to decrease or increase the stability of cellular membranes on cell division in multinucleoid filamentous mutants.

Filamentous mutants can be stimulated to divide into unit-cell equivalents by the addition of nontoxic concentrations of agents that decrease the stability of cellular membranes (i.e. dimethyl sulfoxide, short-chain alcohols, sodium oleate, and lysolecithin). Long-chain alcohols, which have a stabilizing effect on cellular membrane, enhance filamentation and antagonize the stimulation of cell division by membrane labilizers such as ethanol and lysolecithin. These results are further indirect evidence for the involvement of the physical state of the membrane in the regulation of cell-wall and cell-membrane invagination. We have suggested previously that cell division is initiated by a positive effector released into the medium after the initiation of the division process. In our model, the membrane-labilizing agents could act to lower the threshold for the positive effector, and membrane stabilizers would elevate the threshold. Alternatively, membrane-labilizing agents could be functionally similar to the positive effector and could initiate the division event directly.

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SELECTIVE INHIBITION BY 2-DEOXYADENOSINE OF DNA SYNTHESIS IN A BLUE-GREEN BACTERIUM

L. O. Ingram and W. D. Fisher

Selective inhibitors have proved useful tools for studying DNA synthesis in bacterial organisms such as *E. coli* (1). However, blue-green bacteria are insensitive to high concentrations of several commonly employed inhibitors (1), and no selective inhibitor of DNA synthesis has been reported for any blue-green bacterium. The study of DNA synthesis in these organisms is further hampered by their failure to incorporate efficiently exogenously supplied nucleic acid precursors (2). In this report we describe the selective inhibition of DNA synthesis in *Agmenellum quadruplicatum* BG1 by deoxyadenosine, an established inhibitor in other systems (3). We also show that at lower concentrations tritiated deoxyadenosine can be used to label the organism's DNA.

Addition of deoxyadenosine at a concentration of 10 $\mu\text{g/ml}$ inhibits DNA synthesis in *A. quadruplicatum*. Based on colorimetric assays, DNA synthesis proceeds at the same rate as in untreated cells for 2 hr and then ceases. The accumulation of RNA is unaffected, and the overall growth rate is only slightly depressed. Concentrations above 10 $\mu\text{g/ml}$ of exogenously added deoxyadenosine are increasingly growth inhibitory and stimulate DNA degradation. There is a concomitant rapid decrease in viability.

At low concentrations (2–4 $\mu\text{g/ml}$), labeled deoxyadenosine is incorporated into TCA-insoluble material. From 60 to 75% of the label can be rendered acid-soluble by deoxyribonuclease.

Our results suggest that deoxyadenosine may be a useful and reasonably specific inhibitor of DNA synthesis in blue-green bacteria.

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DIVERSITY OF CRUSTACEAN SATELLITE DNA'S

Dorothy M. Skinner and Wanda G. Beattie

In the DNA's of 10 crustacean species examined, there are nonmitochondrial satellites that constitute from as little as 1% to as much as 20% of the total. The satellites are of diverse composition (1). (A+T)-rich satellites are found in the DNA's of a freshwater crayfish, *Procambarus* (a); five marine crabs, *Cancer borealis* (b), *C. pagurus* (c), *Libinia* (d), *Callinectes* (e),

and *Maia* (f); and two land crabs, *Gecarcinus* (g) and *Cardisoma* (h). Five of these (b, c, d, g, and h) consist of more than 90% alternating adenylate and thymidylate residues and are extracted by phenol (2). From melting properties, we calculate that the three others (a, e, and f) contain 20–25% G+C residues. These three satellites are insoluble in phenol, and two of them (a and f) have biphasic thermal dissociations. They also show interstrand bias in base composition and separate into two distinct bands in alkaline CsCl; these reassociate rapidly to their original densities on neutralization and heating to 60° C, indicating that they contain highly repetitive sequences.

(G+C)-rich satellites make up 3, 1, and 10%, respectively, of the DNA of two crabs, *Gecarcinus* (g) and *Pagurus* (i), and the lobster, *Homarus* (j). Two of these (from i and j) separate into four and three components in alkali. The sharp monophasic bands observed in neutral CsCl thus have two components. This is confirmed by the biphasic nature of their thermal dissociations. The lower T_m of the (G+C)-rich satellite of *Pagurus* is identical to that of a synthetic polymer of alternating G and T residues paired with A and C residues (74° C in 0.15 M sodium chloride–0.015 M sodium citrate) (3). By centrifugation through alkaline CsCl preparative gradients we have purified all four of the single strands of the *Pagurus* satellite. The most dense of the four has the same densities in neutral ($\rho = 1.771 \text{ g/cm}^3$) and alkaline ($\rho = 1.828 \text{ g/cm}^3$) CsCl as does the synthetic polymer of alternating G and T. The least dense of the four appears to be the A,C complement to the G,T strand, since its density in alkaline CsCl is the same as in neutral CsCl ($\rho = 1.726 \text{ g/cm}^3$). Reassociation studies on the purified components show that the least and most dense are complementary, as are the two components of middle densities. To our knowledge this is, with one exception, the first naturally occurring DNA in which the individual strands consist almost entirely of only two bases. The sole exception, of course, is the "crab poly[d(A-T)]." Studies are in progress to determine the base composition of these and other satellites by direct chemical analysis and by nearest-neighbor analysis.

We wish to emphasize the widely divergent properties of the several (A+T)-rich and (G+C)-rich satellite DNA's isolated from the 10 crustaceans we have examined. By hybridization experiments we have shown that many of the sequences (3–10%) of the (G+C)-rich satellite of *Gecarcinus* are homologous to cryptic sequences in the DNA's of five species of crabs and the lobster (4). It is apparent that DNA satellites are nearly ubiquitous among the crustacea, but every species has an individual

pattern of satellite DNA components. Main-band DNA's of all crustacea examined appear to be virtually identical to each other.

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CRITICAL PERIOD FOR REGENERATION IN PREMOLT CRUSTACEA

Dorothy M. Skinner and Wanda G. Beattie

Our studies on the initiation of precocious molts (1) in several species of crustaceans (2) by the loss (autotomy) of a critical number of limbs have been extended. Using this method to trigger molts, we are able to maintain a constant population of premolt animals throughout the year. Animals stimulated to undergo precocious molts by removal of their eyestalks (containing the neurosecretory cells, which produce and store the molt-inhibitory hormone) have high mortality; in three species of animals with which we and others have worked, no specimens survive both the premolt period and ecdysis. In contrast, with our new method, the survival rate is as good as that for normal premolt animals. In addition to four species of marine crabs (2) and the land crab *Gecarcinus lateralis* (1), the land crab *Cardisoma guanhumi* also responds to the treatment.

We have caused partially regenerated limbs to autotomize at different times during D_0 , the stage in the premolt period when most, if not all, regeneration occurs. We find that animals which lose regenerates before a certain critical time in the premolt period re-regenerate some or all of the autotomized appendages before proceeding to the subsequent stage in the premolt period. This results in a significant increase in the duration of the premolt period. Conversely, animals at a later stage in D_0 molt within 2–25 days after the removal of regenerates but show no signs of re-regeneration.

Our observations indicate that even if a single "on" switch is thrown to initiate the premolt period (loss of molt-inhibitory hormone by eyestalk removal, autotomy of a critical number of limbs, etc.), the ordered series of events leading to normal ecdysis do not subsequently proceed on an invariant time schedule. The whole premolt process appears to be self-monitored in such a way that subsequent events cannot proceed

until early events reach a critical stage of completion, so the *duration* of the critical period can be greatly extended. In fact, we think that animals that do not re-regenerate are already in stage D_1 (signaled by the separation of the epidermis from the old exoskeleton) when their regenerating limb buds are autotomized. Clearly, there is no turning back. The interacting controls are such that the animals do not initiate the re-regeneration process.

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IN VITRO STUDIES ON THE FORMATION OF CRUSTACEAN EXOSKELETON

Virginia S. Mayo and Dorothy M. Skinner

The crustacean exoskeleton is an acellular, four-layered, protein-chitin complex that is synthesized and secreted by the epidermis at a particular stage of the animal's life cycle, the premolt period. In preparation for ecdysis (when the animal escapes from the old exoskeleton), during the premolt period the epidermis separates from the old exoskeleton and secretes new layers of exoskeleton beneath the old. The old exoskeleton is largely (more than 75%) resorbed by the time it is discarded at ecdysis. We are studying the formation of the exoskeleton at various stages of the premolt cycle by examining the newly synthesized proteins of the epidermis of the land crab *Gecarcinus lateralis*.

Epidermal tissue can be maintained *in vitro* in Eagle's minimal essential medium, with the salt concentrations adjusted to those characteristic of *Gecarcinus* hemolymph and with 10% fetal calf serum added. The newly synthesized proteins can be labeled for 20 hr with radioactive amino acids and identified on SDS-polyacrylamide gels. During the premolt stages when exoskeleton is being actively synthesized (stage D_1) and secreted (stage D_2), protein synthesis is greatly increased and discrete bands of radioactive peptides can be identified on the gels. These proteins are not synthesized in the epidermis at other stages of the molt cycle, when exoskeleton is not being formed, suggesting that they are either exoskeletal proteins or function in the formation of the new exoskeleton. Our initial experiments indicate that, with one conspicuous exception, most of the newly synthesized proteins are of low molecular weight.

Autoradiographs of integument tissue (epidermis plus connective tissue) from various stages labeled with [^3H]leucine for 20 hr *in vitro* show that during active

exoskeleton formation the radioactive amino acids are specifically incorporated into proteins of epidermal cells, and there is concentration of label at the innermost area of the new exoskeleton. This also suggests that the radioactive proteins specific for premolt stages that have been identified on polyacrylamide gels may, at least in part, be exoskeletal proteins.

Long-term culture of integumentary tissue plus exoskeleton from postmolt animals indicates that additional exoskeletal layers are laid down *in vitro*. In these experiments, 20% crab serum from postmolt animals is added to the cultures, and after 10 days *in vitro* the tissue has secreted new layers of endocuticle at 30–100% of the rate of synthesis *in vivo*. Now that these culture conditions are established for *in vitro* synthesis of exoskeleton, studies are in progress to maintain premolt epidermal tissue *in vitro* and to determine the effect of ecdysterone and various inhibitors of macromolecular synthesis on cellular differentiation of epidermis and exoskeleton formation.

RESPIRATORY CONTROL IN ULTRAVIOLET-IRRADIATED *E. COLI* LOSS OF PYRIDINE NUCLEOTIDES TO THE SUSPENDING MEDIUM

P. A. Swenson and R. L. Schenley

We previously demonstrated that when *E. coli* B/r is grown in a minimal medium with glycerol as a carbon source and irradiated with 520 ergs/mm² at 254 nm, respiration ceases about 60 min after irradiation (1). The observation that transcription and translation of the irradiated genome are required for the cessation of respiration led us to the general hypothesis that UV interferes with a normal metabolic control system. By inactivating a repressor or its gene, UV permits the excessive synthesis of protein(s) involved in the control of respiration. In unirradiated cells the hypothetical protein would normally be present only in small amounts.

The cessation of respiration is accompanied, and perhaps caused, by the disappearance of pyridine nucleotides from irradiated cells (2). We have used a niacin-requiring mutant of *E. coli* B/r to label with ¹⁴C the pyridine nucleotides of these cells and to trace their fate after irradiation. The pyridine nucleotides go into the suspending medium of the irradiated cells and retain full biological activity. The loss of pyridine nucleotides from irradiated cells seems to be selective and not due to an indiscriminate loss of small molecules from

cellular pools. Some amino acids are lost from cells after irradiation and some are retained.

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DEATH THROUGH RESPIRATORY FAILURE OF A FRACTION OF ULTRAVIOLET-IRRADIATED *E. COLI* CELLS

P. A. Swenson and R. L. Schenley

When *E. coli* B/r cells are grown on a glycerol-containing medium and irradiated with UV (520 ergs/mm²) to 0.5% survival, they respire for about an hour, and then respiration ceases for several hours. The survivors begin to divide about 120 min after UV, as indicated by an exponential increase in viability, i.e. ability to form colonies. Incubation of the irradiated cells with 5-fluorouracil (FU) (0.5 µg/ml) causes respiration to continue, and there occurs within an hour of irradiation a large (60-fold) increase in viability (1).

We tested the effect of caffeine (1.0 mg/ml), an inhibitor of DNA repair, on the viability time-course curves of irradiated cells during incubation in the absence and presence of FU. In the absence of FU, caffeine completely inhibits the viability increase, which we attribute to cell division. In the presence of FU and caffeine, an increase in viability (20-fold) still takes place, which indicates that the increase does not involve completion of repair of DNA while the cells are in liquid medium. Our interpretation is that a large number of irradiated cells do not carry out complete repair of their DNA because of their inability to respire.

When respiration is favored by FU, the potential for repair and division appears; however, division must wait until repair is complete. A delay in repair of the DNA of FU-treated cells is indicated by a plateau after the initial rise in the viability time-course curve. A second rise then takes place, which is interpreted as cell division because it is prevented by caffeine. An important point is that caffeine, which slows down the repair of DNA, is present only in the liquid medium. Therefore those cells, with and without FU, that have the potential to complete repair and to divide will do so when removed from the caffeine and put on plates.

We conclude that one cause of death to *E. coli* B/r cells by UV irradiation is respiratory failure, and that repair capabilities of this strain are much greater than the usual survival curves indicate.

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THERMAL REACTIVATION OF ULTRAVIOLET-IRRADIATED *E. COLI* CELLS BY MAINTENANCE OF RESPIRATION

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

We have found that respiration does not cease in *E. coli* cells grown on minimal medium containing glycerol when they are irradiated with UV (520 ergs/mm²) and incubated at 42° C rather than 37° C. We suggest that the thermal treatment inactivates the respiratory-control protein that we postulate to be synthesized in excess by irradiated cells. As in the case of maintenance of respiration with 5-fluorouracil treatment (1), pyridine nucleotide levels remain high in irradiated cells given thermal treatment.

The respiratory responses of strains B/r and B of *E. coli* are identical, but the kinetics of viability for irradiated cells incubated at 42° C are quite different. Strain B/r, which showed a 30-fold increase in viability during the first 2 hr after irradiation, had a viability response similar to that shown by both strains when respiration was maintained by 5-fluorouracil treatment; i.e., the initial increase was followed by a long plateau and then by a second increase, which we interpret as cell division. The initial increase, which we term thermal reactivation, is not prevented by caffeine. The reactivated cells would otherwise die of respiratory failure. As with 5-fluorouracil treatment, thermal treatment maintains respiration and ensures that the repair capabilities of these cells are realized (2). The long plateau, which follows the initial increase in viability, was eliminated by shifting the irradiated cell cultures from 42° C to 37° C at 60 min after UV. These results indicate that, in addition to promoting respiration, thermal (42° C) treatment inhibits division of irradiated B/r cells.

Strain B showed a smaller initial increase in viability than did B/r, and this increase was followed by a decrease before cell division. With a combination of thermal and 5-fluorouracil treatment, the irradiated strain B cultures responded as they did with 5-fluorouracil treatment alone. The loss in viability in thermally treated strain B cultures was prevented by pantoyl lactone, a stimulator of cell division in radiation-induced filaments in this strain. This fact indicates that loss of viability in strain B results from inhibition of steps leading to cell division.

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IN VITRO LABELING OF DNA FROM SUCROSE GRADIENTS

R. J. Wilkins

The total amount of cellular DNA that can be sedimented through a 5-ml alkaline sucrose gradient is restricted to about 200 ng, so each fraction collected from the gradient contains only a few nanograms of DNA. The task of measuring this amount of DNA is very formidable unless the cells have been grown in the presence of radioactive precursors of DNA. This difficulty has largely limited the use of alkaline sucrose sedimentation to the study of DNA damage produced in cells grown in culture.

Several methods of detecting nanogram quantities of DNA dissolved in an alkaline solution of sucrose and salt are being investigated. The most promising method is the chemical iodination (1) of DNA with ¹²⁵I. Although iodine is bound to a large fraction of the cytosine residues in DNA, nonspecific binding of iodine to protein and other impurities results in a very high background noise, which can only be reduced by extensive washing or filtration. The method has been successfully employed to label DNA from *E. coli* after lysis and sedimentation through alkaline sucrose. The sedimentation profile is essentially the same as that obtained with *E. coli* that have been prelabeled with [³H] thymidine. Current work is aimed at concentrating the DNA before iodination so that the background noise can be reduced and the method can be made more accurate and practical.

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REPAIR OF DNA IN THE ABSENCE OF NORMAL DNA REPLICATION

J. E. Donnellan, R. S. Stafford, and R. B. Setlow

Repair of radiation damage has been observed in most living systems, ranging from parasitic bacteriophage to mammalian cells *in vivo*. A common indication of repair has been the study of replication of regions of old strands of DNA, usually by observing the incorporation of BrdUrd into light DNA as opposed to incorporation into newly formed heavy DNA. Recently a sensitive method for observing this incorporation has been perfected by Regan *et al.* (1). This technique relies on the lability of strands of DNA containing BrdUrd to 313-nm light. Thus newly formed strands of DNA containing much BrUra are obliterated by irradiation at this wavelength, whereas old repair-replicated strands are shortened and old unrepaired strands are not altered. Most studies of repair replication have been

performed on systems fully capable of normal DNA replication. *Bacillus subtilis* W168 vegetative cells exhibit normal repair of pyrimidine dimers. Spores of this organism, on the other hand, have an entirely different photochemistry and repair mechanism. During development of bacterial spores into vegetative cells the photochemistry changes to that of cells before normal DNA replication has begun. We have used the more sensitive BrUra technique to investigate the repair replication of DNA during the period when DNA synthesis does not occur.

Spores of *B. subtilis* labeled in their nucleic acids were allowed to develop for 90 min, and appropriate samples were irradiated with 100 ergs/mm² of 254-nm light. Samples of the germinated spores were allowed to grow either in BrdUrd or in thymidine for an additional 60 min, during which time protein and RNA would be synthesized but no DNA. Finally samples were exposed to various doses of 313-nm light, and the DNA was analyzed on alkaline sucrose gradients. The samples exposed to 254-nm light showed a size range dependent on dose, but the unirradiated control was unaffected, indicating that repair replication occurred in the irradiated cells in the absence of normal DNA replication.

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CHEMICAL MEASUREMENT OF DARK REPAIR IN DNA FROM ULTRAVIOLET-IRRADIATED CELLS BY ITS COMPETITIVE INHIBITION OF HIGH-INTENSITY FLASH PHOTOREACTIVATION

W. R. Proctor* and J. S. Cook

We have adapted a chemical assay for photo-reactivating enzyme so that, by a competitive method, we can measure quantitatively the dark repair of pyrimidine dimers in DNA from unlabeled cells. The substrate is [³H] thymine-labeled *E. coli* DNA treated with acetophenone and light. More than 30% of the label in this DNA is in thymine-thymine dimers, and nearly none is in other photoproducts. The standard photoreactivating enzyme is a preparation from yeast. The enzyme binds to the dimers in the dark, and such bound dimers may be monomerized by a single flash of 1 msec duration from a xenon photographic flash lamp. Dimers in unlabeled UV-irradiated DNA, either double- or single-stranded, added to the reaction mixture provide competitive binding sites and thereby reduce the dimer-to-monomer conversion in the labeled DNA. For quantitative measurements the assay requires less than 0.01 μg of labeled DNA and less than 1 μg of

competing DNA irradiated with doses commonly used in biological experiments. We have used the technique to measure the repair of dimers in *Micrococcus luteus* (*lysodeikticus*), which for technical reasons cannot be measured directly. After a UV dose of 1000 ergs/mm² at 254 nm, *M. luteus* was incubated in the dark in a complete medium at 34° C. At intervals, samples were removed and the endogenous repair system was heat-inactivated at 70° C for 60 min. DNA was released by lysozyme treatment and assayed as described. Under these conditions, the half-time for *in vivo* repair was found to be 7 min, and no detectable dimers remained in either double- or single-stranded segments after 40 min.

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POSTREPLICATION REPAIR OF DNA IN ULTRAVIOLET-IRRADIATED *E. COLI*

R. D. Ley* and R. B. Setlow

In UV-irradiated bacteria, DNA synthesized before the completion of excision repair is in small pieces (1). These pieces correspond to the distance between pyrimidine dimers and seem to be separated by gaps that subsequently are filled in. There is some evidence to support the notion that gaps in daughter strands of DNA synthesized on UV-irradiated DNA templates are filled in (postreplication repair) with parental DNA by a recombinational mechanism (2). Our preliminary experiments with selective photolysis of BrUra-substituted DNA indicate that, in addition to any recombinational event that may occur, a large amount of *de novo* synthesis also is involved in the gap-filling process. UV-irradiated (60 erg/mm² at 254 nm) *E. coli* B(3) thy⁻ cells were pulse-labeled with [³H] thymidine, incubated with nonradioactive BrdUrd, and subsequently exposed to 313-nm radiation. The low-molecular-weight DNA (~15 × 10⁶ as determined in alkaline sucrose gradients) synthesized during the pulse was converted to a higher molecular weight (~40 × 10⁶) during the postpulse incubation in BrdUrd. However, upon exposure to 313-nm radiation, the repaired DNA was converted back to a low molecular weight. Presumably, during postreplication repair BrdUrd is incorporated into the gaps between adjacent, low-molecular-weight pieces of the daughter strands, and exposure to 313-nm radiation selectively photolyzes these BrUra-containing regions. Based on the measured sensitivity of BrUra-substituted DNA to 313-nm radiation, we estimate that the repair of gaps in daughter

strands involves the resynthesis of a region $\sim 10^4$ nucleotides in length.

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RAPID REPAIR OF LESIONS INDUCED BY 313-NM LIGHT IN BROMOURACIL-SUBSTITUTED DNA OF *E. COLI*

R. D. Ley* and R. B. Setlow

Ultraviolet irradiation of BrUra-substituted DNA *in vitro* or *in vivo* results in the formation of large numbers of single-strand breaks, as revealed by sedimentation in alkali. The majority of the breaks are alkali-labile bonds (1). UV irradiation makes relatively few breaks in unsubstituted DNA. At 313 nm the ratio of the sensitivities of substituted to unsubstituted DNA is at a maximum.

Various strains of *E. coli* K12 were labeled with [^3H]BrdUrd and exposed to 313-nm radiation. They were either lysed immediately on alkaline sucrose gradients or incubated 10 min and then lysed. The number-average molecular weights, and hence the numbers of breaks per 10^8 daltons, were computed from the gradient profiles. (Unirradiated cells showed ~ 1 break per 10^8 daltons.) The results are shown in Table I. Postirradiation incubation of the irradiated cells causes the rapid disappearance (90% in 10 min) of the breaks. This rapid "repair" also occurs in two radiation-sensitive mutants (*uvrA* and *polA*) of *E. coli*, but not if the cells are lysed prior to postirradiation incubation. The nature of the repair system involved

with the lesions in BrUra-substituted DNA is not known.

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ACTION OF AN ULTRAVIOLET-SPECIFIC ENDONUCLEASE ON IRRADIATED BROMOURACIL-CONTAINING DNA

W. L. Carrier and R. B. Setlow

An endonuclease partially purified from *Micrococcus luteus* attacks native DNA containing UV-induced cyclobutane pyrimidine dimers and makes approximately one single-strand break per dimer. We have observed not only that UV irradiation results in the production of chain breaks (alkali-labile bonds) in BrUra-substituted DNA but also that the DNA is attacked by the UV endonuclease. However, cyclobutane pyrimidine dimers involving BrUra residues have not been isolated from irradiated DNA, and the nature of the endonuclease-susceptible photoproduct is not known. In typical experiments we used a hybrid *E. coli* DNA containing fully substituted [^3H]BrUra in one strand and [^{14}C]Thy in the other. The DNA was irradiated with 1.2×10^5 ergs/mm 2 at 313 nm (a wavelength that makes few dimers in unsubstituted DNA), treated with the endonuclease, and analyzed for chain breaks by centrifugation in alkali. The irradiation made 4.5 breaks per 10^6 daltons, and endonuclease treatment resulted in the appearance of three additional breaks in the BrUra strand and no breaks in the Thy strand. On the other hand, irradiation of the DNA at 280 nm (a wavelength that makes both dimers and BrUra products) followed by endonuclease treatment results in breaks in both strands. The action of the

TABLE I.

Strain	Number of breaks per 10^8 daltons		Reduction (%)
	After exposure to 10^4 erg/mm 2	After 10 min postirradiation incubation	
<i>uvr</i> $^+$ (AB2497)	18	3	86
<i>uvr</i> $^+$ (AB2497) Lysate*	18	17	6
<i>uvrA-6</i> (AB2500)	22	2	91
<i>polA1</i> (P3478)	12	1.5	88

*Cells were irradiated at 313 nm and lysed prior to incubation for 10 min at 37 $^\circ$ C.

endonuclease on both strands was inhibited in DNA that contained pyrimidine dimers. The presence of cysteamine during irradiation inhibits drastically the direct production of breaks in BrUra DNA by UV but enhances the number of endonuclease-derived ones, so that the sum of the two is constant. Thus we infer that although -SH compounds may react with uracyl radicals in irradiated BrUra DNA and so prevent the formation of products that lead to chain breaks, such compounds result in the formation of base sequences susceptible to UV endonuclease.

FREQUENCY OF γ -RAY-INDUCED SINGLE-STRAND BREAKS AND ALKALINE-LABILE BONDS IN λdv DNA OF *E. COLI* MINICELLS IRRADIATED UNDER AEROBIC AND ANOXIC CONDITIONS

M. C. Paterson*

Chromosomeless minicells are formed by misplaced cell fissions near the polar extremities in a mutant strain of *E. coli* K12. λdv DNA can be introduced into minicells by segregation from a λdv -containing derivative of the original mutant, and these DNA molecules can be isolated from minicells as covalently closed circles (CCC) of molecular weight 10^7 . Consequently, analysis of alkaline and neutral sedimentation patterns permits an accurate determination of the effect of pH on the numbers of single-strand breaks induced in DNA of minicells by ^{60}Co γ -irradiation. That is, it is possible to measure the fraction of γ -ray-induced strand breaks that require an alkaline environment for expression. The kinetics for the appearance of single-strand breaks produced in λdv DNA of minicells by γ -rays is first order, as indicated by an exponential decline in the fraction of DNA as CCC as a function of dose. When γ -rays are delivered to an air-equilibrated (i.e. aerobic) minicell suspension, the efficiency of strand breakage is 29 eV per break when assayed on alkaline gradients and 36 eV per break when assayed on neutral gradients. Irradiation of nitrogen-equilibrated (i.e. anoxic) minicell preparations yields 99 eV per break at alkaline pH and 152 eV per break at neutral pH. Thus, although the total number of single-strand breaks measured in alkali is enhanced 3- to 4-fold when minicells are irradiated under aerobic conditions, the fraction of these scissions requiring an alkaline environment for expression is almost twice as great in minicell preparations irradiated in the absence ($\sim 35\%$) as in the presence ($\sim 21\%$) of oxygen.

Similar sedimentation studies on purified superhelical DNA preisolated from γ -ray-damaged minicells confirm the existence of radiation-induced alkali-susceptible regions in λdv DNA. In addition, the data suggest that physicochemical events occurring during DNA isolation convert bonds weakened but not broken by irradiation into (1) directly induced strand interruptions assayable at neutral pH and (2) alkali-sensitive lesions.

Alkali-labile bonds in plasmid DNA of λdv^+ minicells irradiated under anoxic and aerobic conditions do not disappear during incubation and, therefore, are presumably unsuitable substrates for DNA repair enzymes operative in minicells.

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MAGNITUDE AND REPAIR OF γ -RAY-INDUCED BREAKS IN THE DNA OF HUMAN CELLS

R. B. Setlow and James D. Regan

The numbers of breaks in human DNA irradiated *in vivo* can be measured from the sedimentation patterns of irradiated, radioactively labeled DNA in alkali. Such breaks are normally repaired rapidly. We have observed that quinacrine (10^{-4} M) prevents repair of strand breaks in human fibroblasts, and that when the quinacrine is removed from the growth medium, repair takes place quickly. Quinacrine not only inhibits repair but also acts as a sensitizer, approximately doubling the numbers of breaks per rad. The energy absorbed by cells irradiated in medium containing 10% calf serum is 20 eV per break, whereas in 10% serum- 10^{-4} M quinacrine it is 10 eV per break.

The numbers of bases inserted per chain break can be estimated by the BrUra-photolysis technique (1, 2). For γ -irradiated DNA approximately one BrUra is incorporated per initial chain break. If the breaks are kept open by quinacrine and then permitted to rejoin by removal of the drug, there is still only about one BrUra per break. Thus, surprisingly, during the inhibition of strand-break repair by quinacrine there is no enlargement, by exonuclease action, of the gap representing the strand break.

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THERMAL ENHANCEMENT OF DAMAGE TO DNA IN MAMMALIAN CELLS*

B. V. Bronk,[†] R. J. Wilkins, and James D. Regan

Elevation of temperature above 37° C during and after treatment of Chinese hamster and human cells with methylmethane sulfonate (MMS) has been found greatly to enhance damage to cellular DNA. This is indicated by reduction in strand length of the DNA, as measured by sedimentation in alkaline sucrose gradients. The enhancement of damage increases slowly with increase in incubation temperature from 37° C to 42° C and very rapidly thereafter. Inactivation of an essential step in the repair system is implicated by experiments indicating that effective repair of DNA is reduced or halted above the critical temperature. The sudden onset of the effect at about 42° C suggests thermal inactivation of an essential repair enzyme. Studies are being pursued to determine whether thermal enhancement of DNA damage can be observed with other methods of treatment, such as irradiation of the cells or suicide by incorporation of radioactive isotopes into the DNA.

*Part of this research is being done in collaboration with E. Ben-Hur of Brookhaven National Laboratory.

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AN ENDONUCLEOLYTIC ACTIVITY FROM *MICROCOCCUS LUTEUS* THAT ACTS ON γ -RAY-INDUCED DAMAGE IN PLASMID DNA OF *E. COLI* MINICELLS

M. C. Paterson* and R. B. Setlow

The initial step in excision repair, a multienzymatic error-correcting mechanism known for its versatility, is a single-strand incision near the lesion. This step is mediated by an endonuclease (frequently referred to as UV-specific endonuclease) that presumably recognizes a change in the secondary structure of the sugar-phosphate backbone or a chemical modification in one of the heterocyclic bases.

Several studies have indicated that extracts of *M. luteus* contain an endonuclease exhibiting the properties expected of the incision enzyme *in vivo* (1, 2). We have found that the same extract also acts on superhelical, covalently closed circular (CCC) λ dv DNA isolated from γ -irradiated minicells of *E. coli*. The introduction of nicks into isolated CCC DNA by an endonuclease in the extract results in relaxed circles. The two circular DNA species are easily distinguishable by their sedimentation properties in alkaline sucrose.

The frequency with which the endonuclease-susceptible lesions are produced in superhelical DNA is only marginally enhanced when ⁶⁰Co γ -rays are administered to an aerobic (1.1 lesions per 10⁶ daltons per krad) rather than an anoxic minicell suspension (1.0 lesions per 10⁶ daltons per krad). The ratio of endonuclease-sensitive defects to single-strand scissions induced by γ -irradiation in air is approximately 1:3. The nuclease-sensitive lesions disappear from γ -irradiated minicells during postirradiation incubation, presumably as a consequence of excision repair. Since the addition of UV-irradiated calf-thymus DNA depresses the ability of the *M. luteus* extract to attack not only UV-damaged *E. coli* DNA (a known substrate for the UV-specific endonuclease) but also γ -ray-injured λ dv DNA, we conclude that physicochemical alterations induced by both types of radiation are recognized by the UV-specific endonuclease or a similar endonuclease.

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THE ACTION OF AN ENDONUCLEASE FROM *MICROCOCCUS LUTEUS* ON DNA IRRADIATED *IN VITRO* WITH γ -RAYS

R. B. Setlow and W. L. Carrier

UV damage to DNA is repaired by an excision mechanism in which the first step is an endonuclease attack near a photoproduct (pyrimidine dimer) on the polynucleotide. An enzyme from *Micrococcus luteus* (UV endonuclease) that is a repair endonuclease has been purified and characterized (1). Heretofore, similar enzymic activities against γ -irradiated DNA have not been reported, although there are indirect biological and biochemical data indicating that an excision system may act on base damages in DNA affected by ionizing radiation (2). We describe an endonuclease activity toward DNA irradiated *in vitro*.

E. coli DNA labeled with [³H]- or [¹⁴C]dThd was sedimented in alkaline sucrose gradients, and from the distribution of radioactivity in the gradient the number-average molecular weight (M_n) was determined. The number of single-strand breaks per dalton is given by $1/M_n$. The DNA, irradiated anoxically or in air, and unirradiated controls were treated with a partially purified extract from *M. luteus*, and the numbers of breaks per dalton introduced by the extract were determined as a function of dose. For DNA irradiated

in air there is about one endonuclease-sensitive site per chain break introduced by γ -rays, and for anoxic irradiation there are about two endonuclease sensitivities per γ -ray break. A large excess of UV-irradiated DNA inhibits the endonuclease activity toward γ -irradiated DNA. These data indicate not only that base damage to DNA exposed to ionizing radiation is subject to excision repair (and by inference under genetic control), but that base damage is greater with anoxic radiation, and that the endonuclease described can be used as a sensitive test for the quantitation of base damage and its repair.

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SEPARATION OF UV ENDONUCLEASE FROM γ -ENDONUCLEASE

W. L. Carrier and R. B. Setlow

An extract from *M. luteus* contains endonuclease activity toward UV-irradiated (pyrimidine dimers) and γ -irradiated DNA (base damage?). We have separated the UV-specific endonuclease from the γ -specific endonuclease by chromatography on DEAE-cellulose. This procedure separates the two endonucleases, which elute at 0.01 and 0.3 M salt, respectively. The UV endonuclease is free of the γ -endonuclease, but the fraction containing γ -endonuclease contains some activity toward UV-irradiated DNA. We are continuing the study of the two activities on irradiated DNA.

PROTEIN CHEMISTRY (INTRODUCTION)

F. C. Hartman

The major interest in the protein chemistry laboratory is the elucidation of the structure of enzymic active sites by chemical modification. The most widely applicable method of selectively modifying active-site residues is affinity labeling, in which a protein reagent is designed to resemble the substrate and thus have an affinity for the substrate-binding site. In addition to their value in the characterization of active sites, highly selective enzyme reagents are potentially useful as chemotherapeutic agents, biodegradable insecticides and herbicides, birth control agents, and markers for X-ray crystallographic analyses of proteins. Recent problems under investigation are outlined in the reports that follow.

I. CHARACTERIZATION OF THE ACTIVE SITE OF YEAST TRIOSE PHOSPHATE ISOMERASE

I. Lucille Norton and F. C. Hartman

Catalytic functionality of a glutamyl γ -carboxylate in rabbit-muscle triose phosphate isomerase has been implicated on the basis of its selective esterification by the substrate analog chloroacetol phosphate, with concomitant inactivation (1). As a further test of the functional significance of the active-site glutamyl residue, we have characterized the site of yeast triose phosphate isomerase that is modified by chloroacetol phosphate.

The reaction of chloroacetol phosphate with yeast triose phosphate isomerase is quite analogous to the corresponding reaction with isomerase from rabbit muscle: (1) With high molar ratios of reagent to enzyme, loss of activity is pseudo-first-order. (2) Competitive inhibitors protect against inactivation. (3) One mole of reagent per mole of catalytic subunit is covalently incorporated. (4) Autoradiograms of peptide maps confirm a high degree of selectivity in the modification. (5) The stability (labile toward base, acid, and hydroxylamine) of the reagent-protein bond is that of an ester. (6) The reagent can be used to demonstrate the presence of two active sites per molecule of enzyme.

By successive ion-exchange chromatography and gel filtration, the peptide containing the reagent moiety has been isolated from a peptic digest of chloroacetol phosphate-inactivated yeast triose phosphate isomerase. The sequence of this peptide — Ala-Tyr-Glu-Pro-Val-Trp — is identical to that of the active-site peptide isolated from the rabbit muscle enzyme. Sequence homologies between the segments containing the active-site glutamyl residue in these two diverse species, rabbit and yeast, provide further evidence that the carboxyl group is functional in catalysis.

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II. IDENTIFICATION OF THE SULFHYDRYL GROUP OF RABBIT-MUSCLE ALDOLASE ALKYLATED BY CHLOROACETOL PHOSPHATE

D. W. Salter* and F. C. Hartman

Previous work from this laboratory showed that chloroacetol phosphate, a reactive analog of dihydroxyacetone phosphate, competitively inhibits aldolase at pH 7.0 and irreversibly inactivates the enzyme at pH

10.0 *via* alkylation of one SH group per catalytic subunit (1). To determine whether this SH group is one of the same groups shown by previous chemical studies to be necessary for maximal aldolase activity, we have isolated the peptide containing the acetol phosphate moiety. The inactivated aldolase was treated with [^3H]NaBH $_4$, thereby reducing the carbonyl of the incorporated reagent and introducing a stable radioactive marker; the enzyme was then digested with trypsin. Isolation of the S-alkylated peptide was achieved by successive chromatography of the tryptic digest on Dowex-50 and DEAE-cellulose. Its amino acid composition is identical to that of the N11 cysteinyl peptide described by Sajgó (2): Lys $_1$, Ser $_1$, Asn $_1$, Gln $_1$, Gly $_1$, Ala $_3$, Cys $_1$, Leu $_2$. The cysteinyl residue of this peptide corresponds to the residue in aldolase that is protected by substrate from a variety of modifications (3-5). Thus, our results with a reactive substrate analog provide additional evidence for the presence of a SH group in the active-site region of rabbit-muscle aldolase.

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X-RAY CRYSTALLOGRAPHIC STUDIES OF YEAST TRIOSE PHOSPHATE ISOMERASE. EVIDENCE OF MOLECULAR SYMMETRY

S. W. Hawkinson,* C. H. Wei, F. C. Hartman, I. Lucille Norton, and J. R. Einstein

Crystallographic structure studies of triose phosphate isomerases (TPI's) from several vertebrate species have been in progress elsewhere for several years. Yeast TPI has about the same molecular weight ($\sim 56,000$) and subunit weight ($\sim 28,000$) as rabbit muscle TPI but is quite dissimilar in amino acid composition and specific activity, so comparative structure studies are of interest. TPI from bakers' yeast has been crystallized here in two forms. The first is orthorhombic with unit-cell dimensions $a = 161$, $b = 62$, $c = 47$ Å and four molecules in a unit-cell volume of $474,000$ Å 3 . The space group has been shown to be $P2_12_12_1$ on the basis of the systematic absences $h00$ for h odd, $0k0$ for k odd, and

$00l$ for l odd. The asymmetric unit is one molecule. A 15° precession photograph of the $h0l$ zone, with reflections out to 3-Å spacings, is shown in Fig. 1.

We have recently found a second, entirely different, rhombohedral crystal form of the same enzyme, having chunky, diamond-shaped crystals in contrast to the thin, elongated prisms of the orthorhombic form. From precession photographs we have obtained the following crystal data. Referring the crystal to hexagonal axes, $a = 136$ and $c = 75$ Å, the unit-cell volume $V = 1,211,000$ Å 3 , and there are nine molecules in the unit cell. The systematic absences $-h + k + l \neq 3n$ for all data uniquely indicate the space group to be $R32$, with 18 asymmetric units per unit cell. Thus the asymmetric unit is one molecular subunit, not an entire molecule as for the orthorhombic form, and the two subunits of a molecule are related by a crystallographic twofold rotation axis of symmetry. It may therefore be concluded that the two molecular subunits are nearly identical, if not completely so. (One can not be certain that the subunits are exactly identical, since diffraction data depend only on the ordered portions of the crystal structure, so that differences between subunits in regions that are disordered or subject to very large thermal motion would be "invisible.")

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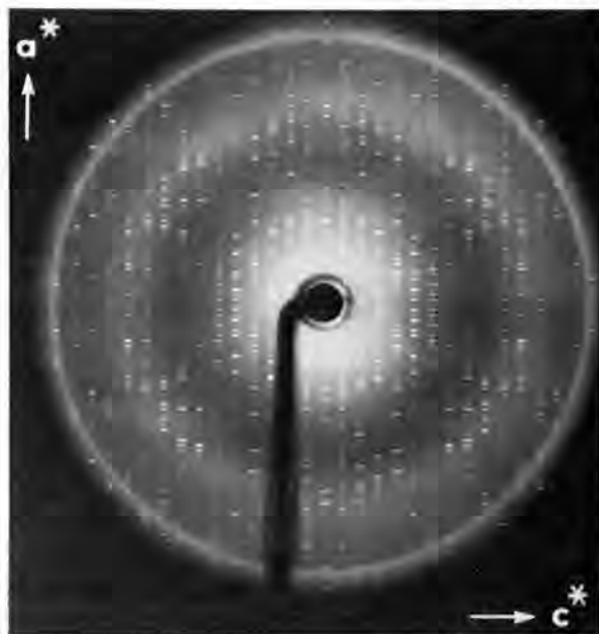


Fig. 1. A 15° precession photograph of the $h0l$ zone of an orthorhombic crystal of yeast triose phosphate isomerase.

Referring the new crystal form to rhombohedral axes, we obtain the primitive (smallest) unit cell with dimensions $a = 83 \text{ \AA}$, and $\alpha = 111^\circ$, a volume of $404,000 \text{ \AA}^3$, and containing three molecules. Thus, the longest true period (83 \AA) is about half the longest period (161 \AA) for the orthorhombic form. Given, in addition, that in the rhombohedral form the structure to be determined is only half a molecule, and that the rhombohedral crystals are comparatively chunky, it is evident that this form is much superior to the orthorhombic form for structure studies. We are currently investigating the crystallization conditions for the rhombohedral form.

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CRYSTALLOGRAPHIC STUDIES OF THE VARIANT HALF OF A κ BENCE-JONES PROTEIN

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

Elucidation of the three-dimensional structures at high resolution of immunoglobulins may depend in part on results obtained from structure studies of immunoglobulin fragments, since crystals of entire immunoglobulin molecules have proved so far to be highly sensitive to radiation (1). We are studying crystals of the variant half (V_L) of a κ Bence-Jones protein (LEN), kindly supplied by Dr. A. Solomon of the University of Tennessee Memorial Research Center, who obtained the material by proteolytic cleavage of the Bence-Jones protein from a single patient with multiple myeloma. We have previously described a monoclinic crystal form with space group P2, unit-cell dimensions $a = 66$, $b = 38$, $c = 44 \text{ \AA}$, $\beta = 90^\circ$, a unit-cell volume of $108,000 \text{ \AA}^3$, and having two molecules (mol. wt., $\sim 11,500$) per asymmetric unit. A second type of crystals obtained from Dr. Solomon were very large ($> 1 \text{ mm}$) but gave diffraction photographs that, previously, could not be indexed. We have now succeeded in doing so with an Elliott rotating-anode microfocussing generator, a precession camera with a 75-mm crystal-to-film distance, and thin crystals, so as to resolve extremely (and unexpectedly) close reflections in the diffraction pattern. The crystals are trigonal, with unit-cell dimensions $a = 43$, $c = 283 \text{ \AA}$, and a unit-cell volume of $457,000 \text{ \AA}^3$. The systematic absences for $000l$ of $l \neq 3n$ and the symmetry of the diffraction data indicate the space group to be $P3_1 12$, with six asymmetric units per unit cell. Given the assumption that these two crystal forms of the same protein have roughly equal values for

the ratio of crystal volume to protein weight, there are ~ 17 molecules per unit cell in the trigonal crystals. The space-group symmetry indicates the number to be $6n$, where n is an integer. We have therefore concluded that there are 18 molecules per unit cell, or three per asymmetric unit. The assumption of either two or four molecules per asymmetric unit would imply values for the above ratio at the extreme limits of the range observed for crystals of globular proteins, and would therefore be highly unlikely.

Since the trigonal crystals have an extremely large cell dimension, and since the structure to be determined is three molecules rather than two as in the monoclinic crystals, this crystal form is the less suitable for structure work. We are working on the crystallization of the monoclinic form and will initiate a search for heavy-atom derivatives.

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ISOLATION, PURIFICATION, AND CRYSTALLIZATION OF TWO PHYTOTOXIC PROTEINS, RICIN AND ABRIN

C. H. Wei

The highly toxic proteins ricin and abrin, isolated from *Ricinus communis* and *Abrus precatorius*, respectively, have been reported to exhibit a strong inhibitory effect on protein biosynthesis in Ehrlich ascites tumors of mice (1, 2). When properly applied, these antitumor substances might be effective in the modern chemotherapy of certain human cancers.

1. *Ricin*: Ricin has been purified and crystallized previously (3, 4) as tiny needles unsuitable for X-ray work. As a result of intensive efforts, we now have succeeded in obtaining crystals large enough (some with a length of approximately 1.5 mm) for X-ray investigations. A 5% acetic acid extract of the defatted meal of *Ricinus communis* was first partly purified by chromatography on column of DEAE-Sephadex A-50 with 0.005 M sodium acetate buffer (pH 5.8) as eluant. It was purified further by CM-cellulose chromatography with a gradient elution of the material using 0.01–0.2 M phosphate buffer (pH 6.5). The purified ricin, contained in the most toxic peak, was dialyzed against 0.005 M phosphate buffer (pH 6.7) containing 10^{-6} M CuSO_4 . After several recrystallizations, plate-like (monoclinic parallelepiped) crystals usually appeared

inside the dialysis bag within a week. The yield was 0.14% of the defatted beans. Homogeneity of the ricin crystals obtained was indicated both by ultracentrifuge sedimentation patterns (obtained by A. P. Pfuderer, Biochemistry Section) and by the results of disc gel electrophoresis. The sedimentation constant, S_{20} , of the protein was measured to be 4.6 svedberg.

X-ray precession photographs, obtained with an Elliott rotating-anode generator and a copper target, have indicated that the diffraction pattern extends beyond 3-Å spacings at room temperature. The crystals are monoclinic with cell parameters $a = 177.3$, $b = 57.3$, $c = 92.2$ Å, and $\beta = 105.67^\circ$. The volume of the unit cell is 9.02×10^5 Å³. Systematic absences indicate that a probable space group is C2. A search for isomorphous crystalline heavy-atom derivatives is in progress. Further investigations of the effect of this protein on normal and tumor cells are in progress in the laboratory of W. K. Yang of the Carcinogenesis Section.

2. *Abrin*: Although abrin has been reported to crystallize as fine rod-shaped crystals (5), these are not suitable for X-ray study. In our experiments, *Abrus precatorius* was homogenized in 5% acetic acid followed by step-wise ammonium sulfate fractionation. Purification of abrin by chromatography on various media including DEAE-Sephadex A-50, Sephadex G-100, and CM-cellulose with various elutants has yielded products of high toxicity but not yet an electrophoretically homogeneous protein. Rod-shaped crystals up to 0.2 mm in length were obtained, however, from the eluate of a DEAE-Sephadex A-50 column. We hope to continue the search for better purification methods and larger crystals.

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AUTOMATED MICRODENSITOMETRY OF X-RAY DIFFRACTION PHOTOGRAPHS

J. R. Einstein and B. W. McClelland

The intensities of X-ray reflections in diffraction experiments are measured one at a time with standard diffractometers, but many intensities can be recorded simultaneously on X-ray film. The development of computer-automated scanning microdensitometers allows rapid and accurate measurement of the inten-

sities of the reflections on film. An Optronics scanning microdensitometer has been interfaced to the Biology Division's PDP-11 computer, with electronic circuits designed by E. K. Cottongim of the Computer Applications Engineering Department, Plant Engineering Division, UCNC. Optical densities of up to 20 million points per film can be measured at rates up to 9000/sec, stored temporarily on disk, and retrieved for processing. X-ray reflections can thereby be measured at rates up to 20/sec. Computer programs have been written to allow flexible control of the data collection by the user. Part of these programs constitute a "handler," which is being integrated into the time-sharing monitor of the computer, so that the scanning microdensitometer can be used simultaneously with all other functions of the computer. The actual user programs are being written in BASIC language to allow the data collection to be controlled conversationally from a computer terminal.

CRYSTAL AND MOLECULAR STRUCTURE OF BARIUM 2-O-SULFONATO-L-ASCORBATE

B. W. McClelland and J. R. Einstein

The location of the sulfate group in the molecule of "ascorbic acid sulfate," a compound of widespread biological distribution, has been the subject of some controversy. A. D. Bond of the Biochemistry Section has provided several kinds of chemical evidence strongly indicating that the sulfate group is in the 2 position, not the 3 position as had previously been generally accepted. X-ray crystallographic studies of the barium salt of a synthetic material, kindly provided by A. D. Bond and F. J. Finamore and shown to be identical to ascorbic acid sulfate from animal sources, now demonstrate conclusively that the sulfate group is indeed in the 2 position.

Barium 2-O-sulfonato-L-ascorbate dihydrate crystallizes from water or water-methanol solution in space group P1 with $a = 5.201(1)$, $b = 6.951(1)$, $c = 8.732(1)$ Å, $\alpha = 99.54(1)$, $\beta = 93.29(2)$, and $\gamma = 109.12(1)^\circ$. With $Z = 1$, $d(\text{calc}) = 2.43$, while $d(\text{meas}) = 2.44(3)$ g/cm³. An Oak Ridge Computer-Controlled Diffractometer was used to measure 1423 independent data out to a minimum spacing of 0.76 Å. All nonhydrogen atoms were located from a three-dimensional Patterson. The structure, subsequently including all hydrogen atoms, was refined using full-matrix least-squares procedures to a final discrepancy index (R) of 0.008. Standard errors on the lengths of bonds between nonhydrogen atoms are in the range 0.002–0.004 Å. An ORTEP drawing of the molecular structure is shown in Fig. 1.

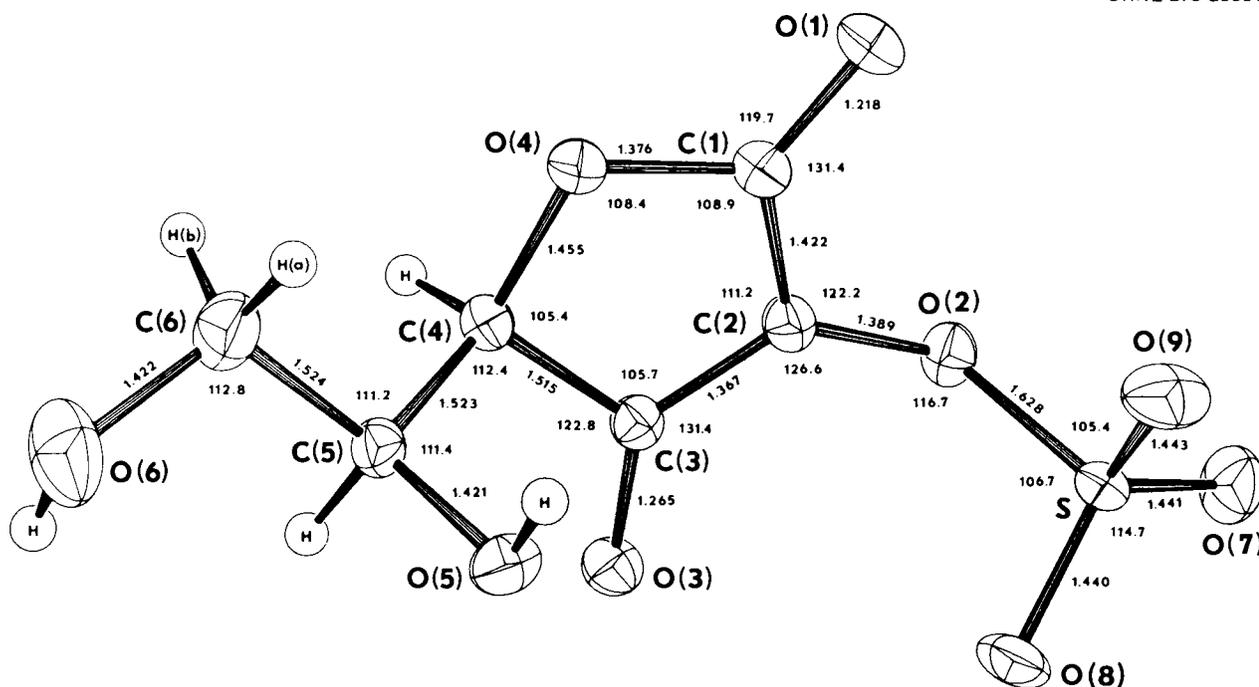


Fig. 1. Molecular structure of the 2-O-sulfonato-L-ascorbate anion, with interatomic distances in Å and bond angles in degrees. The nonhydrogen atoms are represented by 50% probability ellipsoids of thermal displacement.

The values of the structural parameters are generally in good agreement with those expected from previous structural studies on related compounds. It is of interest that the length of the S-O sulfate ester bond found in this study, 1.628(2) Å, is significantly greater than the largest, 1.611(3) Å, reported for any other sulfate ester. The molecular packing is complex, with all six hydrogens bonded to oxygen involved in hydrogen bonding, and all but two oxygens coordinated to the barium ion. An unusual feature of the packing, apparently not heretofore observed, is the presence of a hydrogen bond between two oxygens in the same barium coordination polyhedron.

SEDIMENTATION OF DNA IN SUCROSE GRADIENTS

M. L. Randolph

The need to validate assumptions behind simple calculations of molecular-weight averages derived from sedimentation of DNA in sucrose gradients increases as the use of this technique increases and as interpretations become more quantitative.

The basic differential equation for idealized sedimentation is

$$dr/dt = M \omega^2 r (1 - V\rho) / f\nu, \quad (1)$$

where r is the radius; t is the duration of sedimentation; M , V and f are the mass, partial specific volume, and frictional constant for the sedimenting molecules; ω is the angular velocity; and ρ and ν are the density and viscosity of the solute. Conventionally, one treats M , ω , V , ρ , f , and ν all as constants and hence obtains an exponential dependence of r on t . In sucrose gradients, however, ρ and ν , by experimental design, depend appreciably on r . Empirical approximations to these dependencies at 20° C for 5–20% sucrose solutions have been inserted into equation (1). For convenience, we define X as (distance sedimented)/(tube length). For various values of particle density ($1/V$) from 1.5 to 1.75, the distance sedimented depends linearly on $\omega^2 t$ to within 0.2% for Beckman rotors SW-39, SW-50.1, and SW-56, when $0.1 \leq X \leq 0.9$. For sedimentation of a given molecular species with constant $\omega^2 t$, M , V , and f to $X \cong 0.5$ in these rotors, the variation in X is less than 2% regardless of rotor for any particle density from 1.5 to 1.75. Thus when these assumptions hold, for these rotors and a given molecular species, a linear relation exists between distance sedimented and $\omega^2 t$; and if results are calculated in terms of X , these rotors can be used almost interchangeably. Since molecular weights are usually estimated from the relation $M \sim S^a$, where a is typically taken as 2.6, the errors in molecular-weight estimates may be three times as great as those cited here.

COMPARISON OF X-RAY EFFECTS IN LOG-PHASE AND STATIONARY-PHASE *HAEMOPHILUS INFLUENZAE*

M. L. Randolph and Jane K. Setlow

Responses of *Haemophilus influenzae* cells to ionizing radiation (X-rays) received at different phases of cell growth have been compared in several ways. Reduction of colony formation and frequency of single-strand breaks in the DNA are about equal under the two conditions, but cellular degradation of DNA after irradiation is more marked for stationary-phase cells. Furthermore, the molecular-weight distribution of unirradiated single-stranded DNA from stationary cells appears more nearly monodisperse than does that from log-phase cells. These results suggest that multiple replicating forks are present in log-phase cells but absent, or less frequent, in stationary-phase cells. The degradation results, however, are also consistent with an enzymatic deficiency in the stationary-phase cells.

PHYSICAL STUDIES ON THE INTERACTIONS OF HEAVY METAL IONS WITH NUCLEIC ACIDS

R. O. Rahn, David Spears,* T. A. Carlson,†
and S. Lindenbaum‡

Photoelectron spectroscopic studies of a variety of metal ions, such as Hg^{2+} , Ag^+ , Cu^{2+} , and Pt^{2+} , that bind to the bases in DNA is currently underway. These studies are designed to provide information about the nature of the bond between the metal ions and the bases. Information regarding the site of the binding and the involvement of sigma electrons in the bond can be obtained. To date, studies with Hg^{2+} have been disappointing because of the small shift in the energy levels of Hg^{2+} upon binding to DNA. The reason for this small shift is that Hg^{2+} binds by displacing protons, and H^+ and Hg^{2+} have similar electronegativities. The lack of significant difference in electronegativity is not favorable for obtaining large shifts in the photoelectron spectrum of the bound metal ion. We hope that other metal ions will give more favorable results.

A program to determine thermodynamic data for complexes of heavy metal ions with DNA has been initiated. We have obtained some preliminary values for the heats of reaction of Ag^+ and Hg^{2+} binding to DNA. For Ag^+ , $\Delta H = 5030$ cal/mole; for Hg^{2+} , $\Delta H = 6890$ cal/mole. We are carrying out equilibrium dialysis measurements to obtain binding constants that, to-

gether with the heats of reaction, will allow us to obtain values for the free energy.

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FLUORESCENCE ASSOCIATED WITH THE BINDING OF QUINACRINE TO DNA AND POLYTENE CHROMOSOMES

R. O. Rahn and Mary A. Handel*

Recently it has been suggested that the bright fluorescent bands that occur upon staining chromosomes with quinacrine are due to the association of quinacrine with the (A-T)-rich DNA in these bands. This suggestion was based on the observation that poly[d(A-T)] enhances quinacrine fluorescence, whereas poly(G,C) quenches it. We have worked out the details for analyzing the influence of small amounts of DNA on the fluorescence of quinacrine. Microgram quantities of satellite DNA were isolated from *Rhynchosciara*. Hybridization experiments have shown that this DNA, which is nearly exclusively poly[d(A-T)], is associated with the region of the chromosome that stains brightly. Preliminary experiments have not been successful in establishing that the satellite DNA has any capacity for enhancing the quinacrine fluorescence. Larger quantities of the satellite have been prepared in order to determine more definitely its ability to enhance fluorescence.

Since Hg^{2+} and Ag^+ can bind to the DNA in a chromosome, it was of interest to see whether these ions could influence the fluorescence of quinacrine-stained chromosomes. These ions are very good at quenching fluorescence by means of the heavy-atom effect. Chromosomes that were first treated with Ag^+ and then stained showed rapid bleaching of the fluorescence upon exposure to the excitation light. High concentrations of Hg^{2+} quenched the fluorescence completely. Treatment of stained chromosomes with either Ag^+ or Hg^{2+} led to an accentuation of the fluorescence banding and may prove to be a method for improving cytological analysis of chromosomes.

HEAT-INDUCED CHAIN BREAKAGE IN DNA COMPLEXED WITH Hg^{2+}

R. J. Brake* and R. O. Rahn

Interstrand crosslinks are formed when Hg^{2+} binds to DNA. These crosslinks prevent strand separation at

elevated temperatures, so DNA complexed with Hg^{2+} can be kept at 100°C for 15 min without appreciable disruption of the double-strand helix. However, large decreases in the viscosity occur following heating, and it was of interest to correlate these viscosity changes with molecular-weight changes, using radioactively labeled *E. coli* DNA. Viscosity changes and chain breaks (both single- and double-strand) were measured in DNA after heating in the presence of Hg^{2+} for various times at different temperatures. The correlation between the lowering of the viscosity and the decrease in molecular weight due to double-strand breaks was excellent. Furthermore, the increase in the number of double-strand breaks as a function of the time of heating followed a sigmoid curve, suggesting that the double-strand breaks originated from individual single-strand breaks. Therefore, we rule out the possibility that shearing is responsible for the viscosity decrease.

Studies were carried out with purine-labeled DNA to see whether extensive depurination occurred during heating in the presence of Hg^{2+} . No evidence was obtained that purines were lost or that any chromophore damage had occurred, as determined spectrophotometrically. Since amounts of Hg^{2+} beyond that necessary to saturate all the DNA binding sites were shown to have a large influence on the thermal degradation of DNA, we conclude that unbound Hg^{2+} can facilitate hydrolytic attack of the sugar-phosphate backbone. Further studies of concentration dependence are planned to determine the extent to which bound and unbound Hg^{2+} influence breaking. The results of these studies may be applicable to the specific degradation of certain portions of a DNA molecule.

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TRIPLET PHOTOSENSITIZATION OF DNA. DEPENDENCE ON OXYGEN AND HEAVY METAL IONS

R. O. Rahn and L. C. Landry

Triplet sensitization is a useful means of obtaining DNA that contains thymine dimers and has had widespread application recently in photobiological studies. We have been concerned with the production of single-strand breaks during sensitization. The existence of these breaks has only recently been reported, and it was of interest to determine the dependence on factors such as the presence of oxygen and the nature of the sensitizer. We measured thymine dimers and chain breaks in DNA subjected to 313-nm radiation in the presence of three different triplet sensitizers. The

TABLE I. Triplet sensitization with and without oxygen

Sensitizer	Relative no. of dimers		No. of dimers/break	
	+O ₂	-O ₂	+O ₂	-O ₂
Acetophenone	1	3	55	245
Acetone	1	7	16	54
Benzophenone	0.2	1.2	3	8

relative number of dimers and dimers per break for sensitization with and without oxygen present is given in Table I. Oxygen quenches the triplet of the sensitizer and reduces both the dimer yield and the number of chain breaks. However, as indicated in Table I, the number of dimers per chain break is about 3- to 4-fold lower in the presence of oxygen. These results support the hypothesis that chain breaks are not formed from the triplet state of the excited base but proceed by a way of a free-radical attack on the backbone. As with ionizing radiation, oxygen favors breakage following this attack. Presumably, oxygen participates by way of a peroxide intermediate. In order to learn more about the mechanism involved, we have initiated studies using H_2O_2 as a source of hydroxyl radicals capable of making chain breaks in DNA. With acetophenone as the sensitizer and without oxygen present, we observed that Ag^+ increased dimerization 4-fold and that Hg^{2+} quenched dimerization 5-fold. Furthermore, chain breaks were virtually eliminated with Ag^+ present but were greatly enhanced by Hg^{2+} . The effects of these two metal ions were maximal when all the binding sites on the DNA were saturated. We conclude that in order to make the maximum number of thymine dimers but keep chain breaks to a minimum, photosensitization is best done with acetophenone as the sensitizer, in the presence of Ag^+ , and in the absence of oxygen.

BINDING OF Ag^+ TO DNA. PHOTOCHEMICAL AND LUMINESCENCE STUDIES

R. O. Rahn and L. C. Landry

Ag^+ binds to the bases in DNA and enhances the phosphorescence intensity and the yield of thymine dimers 20- to 30-fold. Hence, Ag^+ is a valuable tool for studying the interaction of UV light with DNA. We have been concerned with interpreting these effects in terms of where the Ag^+ binds to the DNA and what the influence is of Ag^+ binding on the individual bases themselves. Studies on homopolynucleotides show that all of the bases contained in DNA have their phosphorescence enhanced by Ag^+ . In addition, their

fluorescence is simultaneously quenched. This is explained in terms of a heavy-atom effect. With poly(dT), the enhancement of phosphorescence parallels the enhancement of thymine dimerization. From this we conclude that dimers are being formed from the triplet state in poly(dT):Ag.

In DNA, the enhancement of dimerization and phosphorescence also paralleled each other, and we conclude that the enhancement of the thymine triplet state is preferentially favored by the binding of Ag^+ to DNA. Other workers have shown that Ag^+ binds to DNA in two successive stages or complexes. The first type of complex involves G-C residues, and the second type involves A-T residues. We observe enhancement of thymine dimerization even during the first stage of binding, when supposedly G-C residues are being complexed. To verify that Ag^+ binds preferentially to the G-C residues under the conditions of our experiment, we carried out a competitive experiment using unlabeled polynucleotides with various ratios of A-T to G-C to compete for Ag^+ bound to DNA. The loss of Ag^+ from the labeled DNA was monitored by measuring the reduction in the rate of thymine dimerization. We observed that binding was 50 times greater to G-C than to A-T residues. Hence, the enhancement of the thymine triplet state during Type I complexing means either energy transfer occurs from G-C to T or else the Ag^+ is bound in such a way that there is contact between Ag^+ and T.

The enhancement of the thymine triplet state is about 2-fold greater during Type II than during Type I complexing, as indicated by the sharp increase in both phosphorescence intensity and yield of thymine dimers as Type II complexing begins. With triplet sensitization, no such difference in the rate of dimerization occurs going from Type I to Type II complexing. Presumably, during the lifetime of the triplet state, Ag^+ migration occurs and there is no distinction between Type I and Type II binding. This result reflects the sensitivity of photochemistry as a probe for the binding of metal ions.

ANALYTICAL USE OF Ag^+ -ENHANCED DNA PHOSPHORESCENCE

R. O. Rahn

The development of a technique for spectroscopic detection of nanogram quantities of DNA would allow rapid determination of the distribution of the DNA in a sucrose gradient. We have attempted to use the strong phosphorescence from DNA complexed with Ag^+ as a means of detecting low levels of DNA. Working with

DNA in frozen water solutions, we can easily detect 100-ng amounts of DNA. The limiting factor in being able to detect 10-fold lower amounts is background contribution from the water and from the sample tubes used. By using a higher-grade quartz tube and triply distilling the water, we hope to reduce background level to the point where we can detect 10 ng of DNA.

PHOSPHORESCENCE SPECTRA OF TYROCIDIN B AND C

Carl Beyer* and J. W. Longworth

Phosphorescence spectra of tyrocidin B and C are distinctively composed of two overlapped tryptophan spectra with different Stokes' shifts and decay times. One component has its first fine-structure band at 408 nm, the second at 417 nm. The proportions of these two situations depend on the particular solvent used — ethanol, trifluoroethanol, glycerol, glycerol:water, etc. We know from the separate fluorescence anisotropy study of tyrocidin C that there is complete transfer between the two tryptophan residues; and like the tyrocidin B, tyrocidin C shows simply two overlapped spectra. We suggest that the different spectra are associated with distinct conformations and that solvents alter the amount of a given conformation.

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PHOSPHORESCENCE OF LACTALBUMIN-LYSOZYME FAMILY

J. W. Longworth

Bovine α -lactalbumin, hen's egg-white lysozyme, and human leukaemic lysozyme constitute a family of proteins that possess very similar molecular structures, though their primary compositions are significantly different. Hen lysozyme is peculiar in its phosphorescence decay, in that the decay can be decomposed into two contributions. Five-sixths (there are six tryptophanyl residues in the enzyme) of the phosphorescence intensity decay with a lifetime of 1.15 sec, the remainder at 3.95 sec. The cow protein α -lactalbumin has a phosphorescence decay lifetime of 4.90 sec, whereas human lysozyme has a single lifetime, which is 1.35 sec. Since the primary sequences of these three proteins are available, a simple question to ask would be what tryptophan residues are in common between hen and human lysozyme that are absent in cow lactalbumin. There are vicinal sequences of two aromatic residues in both hen and human lysozyme, but

there are no such sequences in lactalbumin. There are four tryptophans in lactalbumin, five in human, and six in hen lysozymes. When complete molecular structures are available for these three enzymes, perhaps the cause of this exceptional influence on the phosphorescence decay behavior will be patent.

FLUORESCENCE ANISOTROPY DEPOLARIZATION AND PROTEIN ROTATORY RELAXATION

J. W. Longworth

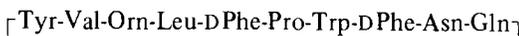
The Perrin relationship $A_0 = A(1 + \tau_{fl}/\tau_c)$ defines a rotatory correlation time (τ_c) that relates to molecular hydrodynamical properties under special circumstances. Debye, following Einstein's analysis of Brownian motion, demonstrated $\tau_c = \eta V/RT$, where V was the hydrodynamic molar volume. I have utilized the intrinsic tryptophanyl fluorescence of proteins as my orientational probe, which meets the special restrictions when there are several emissive tryptophan residues; one may question the validity of this approach when there are only a few emissive chromophores with highly defined orientation in the rotational ellipsoid axis system. A_0 is measured by making T/η approach zero with the simple procedure of working in viscous solvents (glycerol:water) and at low temperatures (220° K). A was measured for several proteins in water solutions at room temperature. Independently, τ_{fl} was determined from single-photoelectron delayed coincidences. The molar volume (V) can be estimated using literature values with the relation $V = M\bar{v}(f/f_0)^3$. M is taken from the amino acid sequence analyses, \bar{v} is available either experimentally or theoretically from composition, and f/f_0 is known from either hydrodynamic or molecular crystal structure. Thus it is possible to calculate the depolarization ratio A/A_0 . In the examples investigated, the measured depolarization is greater than the calculated values. A depolarization is also found in glycerol:water solutions at 296° K, and equally well a significant increase in depolarization ratio occurs in aqueous solution at 273° K. My conclusion is that there are in addition more rapid correlation relaxation means available to the chromophores – that is, these side chains possess motional freedom.

ENERGY TRANSFER IN TYROCIDIN B AND C

Carl Beyer* and J. W. Longworth

Tyrocidin B and C are cyclical decapeptides that are known to possess a β -turn conformation with internal

hydrogen bonds. The sequences are



and



for B and C, respectively. A tyrosine fluorescence from tyrocidin B can be detected readily in trifluoroethanol or water–acetic acid solvents, but there is a negligible contribution in ethanol or glycerol. Fluorescence anisotropy measurements in glycerol at 220° K disclose a tyrosine-to-tryptophan transfer in B and a tryptophan-to-tryptophan transfer in C. Wavelength dependence of the fluorescence yield of tyrocidin B was determined in water–acetic acid and found to be consistent with little tyrosine transfer, but no dependence was found in ethanol, suggesting complete transfer of electronic energy. Inspection of space-filling models of tyrocidin B set in the decapeptide β -turn conformation suggests a tyrosine-to-tryptophan distance commensurate with the critical distance calculated from Förster's theory of electronic energy transfer. For a highly efficient transfer, it is apparent that the orientational factor must approach 4. Equally well, for negligible transfer to occur the orientational factor must approach zero. Solvent-dependent conformational properties are well established for tyrocidin structures by both circular dichroism and nuclear magnetic resonance of related molecules. Therefore we propose that only slight modification in conformation will occur, leading to the appropriate orientation factors – features readily observed in a molecular model.

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PROTEIN FLUORESCENCE LIFETIMES

J. W. Longworth

I have estimated the fluorescence lifetimes of several proteins dissolved in water upon excitation at 280 nm from a single-photoelectron delayed coincidence. The fluorescence quantum yield for excitation at 280 nm is available from the literature, and I have corrected the measured values for the screening fraction of tyrosyl absorption, taking into account the amount of energy transferred from tyrosyl to tryptophanyl residues. I found that there is a constant ratio of τ/Φ with a value of 17.5 nsec. This number is referred to as the natural lifetime and constitutes the absorption strength of the fluorescent electronic transition. A comparable τ/Φ plot

can be obtained with indole derivatives, taking literature values for both yield and fluorescence lifetime, and this too exhibits a constant ratio, where the natural lifetime lies between 13 and 15 nsec. The observation of a common transition strength, though the proteins chosen exhibit significantly different Stokes' shifts, has now to be reconciled with our understanding of the cause of the Stokes' shift in proteins. Clearly our model must possess a common fluorescent transition, so it favors a generalized solvation model, where different Stokes' shifts reflect simply different solvation correlation times *vis-à-vis* a fluorescence lifetime. (Relationships of the form $\bar{\nu} = \bar{\nu}_\infty + (\bar{\nu}_0 - \bar{\nu}_\infty)$, $\tau_c/\tau_c + \tau_{fl}$ prevail.)

ENERGY TRANSFERS WITHIN PROTEINS

J. W. Longworth and C. A. Ghiron

Transfers of electronic energy can occur within a protein between the tyrosine (Tyr) and tryptophan (Trp) residues. Transfers can be demonstrated by showing that Tyr absorption sensitizes Trp fluorescence; the phenomenon is detected by determining the relative quantum yield of Trp fluorescence at several exciting wavelengths. A quantitative interpretation of the yield's wavelength variation relies on the absorption properties of individual monomers. We find for proteins with a large disulfide content that it is essential also to include the absorption of the disulfide to account adequately for the observed dependency of the yield on the wavelength. With dipeptides and simple oligopeptides it is feasible to show Tyr-Trp and Phe-Tyr transfers by these procedures, but with proteins the method is restricted to Tyr-Trp (with rare exception).

Intertyrosine and intertryptophan transfers can also take place within proteins, but of course they are not disclosed by the above procedure. Rather, fluorescence anisotropy measurements are performed. Since in general no two residues within a protein possess identical coordinates but have different orientations, a transfer between two residues causes a depolarization that is detected by comparison with monomer behavior. The ratios of the fluorescence anisotropy of RNase A, insulin, and bovine pancreatic trypsin inhibitor to NAcTyrNH₂ differ greatly from unity, showing that there is intertyrosine transfer in the proteins. The ratio is constant for all excitation wavelengths between 270 and 200 nm. Though the fluorescence anisotropy spectrum for Trp possesses significant fine structure, undergoing wide variation in values between 270 and 300 nm, an essentially constant anisotropy ratio is found between trypsin and NAcTrpNH₂; inter-

tryptophan transfers occur within this protein. For subtilisin BPN' the ratio alters with wavelength, approaching the monomer anisotropy at 310 nm. The wavelength variation for the anisotropy ratio indicates that Tyr-Trp transfers cause depolarization where Tyr absorbs. Fluorescence anisotropy measurements disclose transfers between like residues and among the different aromatic residues of a protein.

TRANSFERS OF SINGLET ENERGY WITHIN TRYPSIN

C. A. Ghiron and J. W. Longworth

Transfers of singlet energy within trypsin were investigated by measuring the fluorescence absorption anisotropy of its tryptophanyl residues. A ratio of the anisotropy of trypsin to that for N-acetyl-L-tryptophanamide was determined between 310 and 250 nm. The ratio had an average value of 0.7 in the region 305–295 nm, whether the trypsin anisotropy was measured at 228 or 298° K, although trypsin dissolved in 5 M guanidine-HCl at 228° K showed no fluorescence depolarization (i.e., the anisotropy ratio was ~1). Thus, there is an extensive conformation-dependent Trp-Trp energy transfer in trypsin. The ratio of anisotropies of trypsin at 304 and 270 nm was used to estimate Tyr-Trp energy transfer. Ratios of 2.0 and 1.9 were obtained at 298° K for the native and guanidinium-unfolded enzyme, respectively. The comparable value for N-acetyl-L-tryptophanamide was 1.8. This indicates little Tyr-Trp transfer. As confirmation, we find that there is no difference in the excitation spectra of native and unfolded trypsin at 298° K. The fluorescence was entirely from tryptophan, while the wavelength dependencies of the quantum yield were the same. When experiments were performed at 228° K, the 304- to 270-nm anisotropy ratios were 2.7 for native and 2.0 for unfolded trypsin. This indicates a conformation-dependent exaltation of the Tyr-Trp transfer efficiency. We conclude that Tyr-Trp singlet energy transfer in trypsin occurs with appreciable efficiency only at low temperatures.

ELECTRONIC ENERGY TRANSFERS IN YEAST TRIOSE PHOSPHATE ISOMERASE

J. W. Longworth and F. C. Hartman

The triose phosphate isomerase of yeast is a dimeric enzyme with a composition molar mass of 56.9 kg/mole. It consists of closely related subunits with approximately 266 amino acids (1). An active-site peptide has been identified with a sequence

ValAlaTyrGlu*ProValTrp (2), which is identical with a sequence from the active site of the rabbit isomerase (3). A selective esterification of a single carboxyl of Glu* is caused by 3-chloroacetyl-1-phosphate; the modification inactivates the enzyme. Fluorescence anisotropy spectra have been determined in 70% glycerol at 296 and 220° K and in water at 296° K. Identical spectra were obtained in the two solvents at 296° K. Modification of the enzyme by the affinity label quenched the tryptophanyl fluorescence but did not affect either the Stokes' shift or lifetime of the fluorescence. The anisotropy ratio of the protein to N-acetyl-L-tryptophanamide in glycerol at 220° K was constant for all excitation wavelengths between 250 and 300 nm. The depolarization ratio for the native enzyme was 0.63 ± 0.08 , and 0.82 ± 0.05 for the inactivated enzyme. The depolarization is attributed to intertryptophan electronic energy transfers between the three tryptophanyl residues of each subunit. The lesser depolarization ratio observed from the inactivated enzyme is fully consistent with the quenching of the Trp residue in the active-site peptide. Cooling the glycerol solutions to 220° K altered significantly the anisotropy spectra. No significant depolarization is found between 295 and 310 nm, suggesting there is now little intertryptophan transfer at this temperature. Below 295 nm significant depolarization is found; a depolarization ratio for excitation at 270 nm vs. 305 nm for the native enzyme is 0.58, for the inactivated enzyme it is 0.70, and for both the active and inactivated enzyme in 5 M guanidinium hydrochloride it is 0.80. The additional depolarization observed at 270 nm is attributed to energy transfer from tyrosine to tryptophan; this occurs at 220° K but not at 296° K. Comparable temperature effects have been observed in several proteins, although there are other proteins for which no temperature effects are detectable. A wide variety of possibilities for transfer with or without temperature dependence prevail, depending on the specific protein investigated.

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SUBTILISIN FLUORESCENCE. AN EXCEPTION TO A RULE

J. W. Longworth

Subtilisin Carlsberg, the serine protease of *Bacillus subtilis* has one Trp and 13 Tyr residues. The majority

fluorescence is from tyrosine, with negligible tryptophan contribution. Although phosphorescence has a significant tryptophan emission component characterized by fine-structure peaks and a decay constant, fluorescence absorption anisotropy spectra determined at 313 nm and >385 nm are characteristic of tyrosine and tryptophan, respectively. Both spectra are depolarized compared to monomer spectra, suggesting Tyr-Tyr and Tyr-Trp transfers. The related protease of *B. amyloliquefaciens* subtilisin BPN' has three Trp and 10 Tyr residues. The luminescence is predominantly from Trp, with a small Tyr component. The fluorescence absorption anisotropy spectrum shows typical tryptophan fine structure but is depolarized where tyrosine absorbs, indicative of Tyr-Trp transfers.

TYROSINE FLUORESCENCE SENSITIZATION BY PHENYLALANINE IN DIPEPTIDES

C. A. Ghiron and J. W. Longworth

Teale (1) found that the excitation spectra of insulin and RNase showed in the 260-nm region a contribution of phenylalanine excitation to the tyrosine fluorescence of these proteins. However, Cowgill (2) found that the dipeptides Tyr·Phe and Tyr·Gly have identical excitation spectra for tyrosine fluorescence (i.e., light energy absorbed by the phenylalanine was not transferred to the neighboring tyrosine residue). We have reinvestigated this transfer of electronic energy by two measuring methods: a wavelength dependency of the quantum yield of the acceptor and a depolarization in the fluorescence absorption anisotropy. We find an appreciable difference between the excitation spectra of L Phe·L Tyr and NAcL TyrNH₂ at room temperature. The fluorescence observed was entirely from tyrosine, yet the wavelength dependence of the quantum yield was the same for both compounds. An anisotropy depolarization of the tyrosine fluorescence of NAcL Phe·L TyrNH₂ as compared with NAcL TyrNH₂ occurred at wavelengths shorter than 270 nm, where phenylalanine absorbs. We conclude that phenylalanine-to-tyrosine energy transfer occurs in dipeptides.

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PHENYLALANINE-TO-TYROSINE ENERGY TRANSFER IN Ile⁵-ANGIOTENSIN II

C. A. Ghiron and J. W. Longworth

Smeby *et al.* (1) proposed that the tyrosine (Tyr) and phenylalanine (Phe) residues, necessary for pressor

activity of angiotensin II, are contiguous in the three-dimensional structure of this octapeptide. Their proposal implied that there would be efficient transfer of energy from Phe to Tyr; as a consequence, this process should be demonstrable by fluorescence measurements. Phe absorption contributes to Tyr fluorescence of Ile⁵-angiotensin II (between 250 and 270 nm). There was a depolarization of the Tyr fluorescence anisotropy of angiotensin compared to NaCl TyrNH₂ at <270 nm. Hence, light energy absorbed by Phe was transferred to Tyr prior to emission from Tyr. Exciting angiotensin with 280-nm light caused fluorescence to come entirely from Tyr, but on excitation with 252-nm light there was fluorescence from Phe in addition. Therefore, the energy transfer process is not completely efficient. We estimate that the transfer is <50% efficient. From Förster's theory we calculate the distance between Phe and Tyr in angiotensin to be >1.0 nm. This is to be compared with 1.3 nm, the value estimated for the end-to-end distance of a tetrapeptide with a random conformation (derived by Flory), and 0.75 nm, the distance if the octapeptide were an α -helix.

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ARE THE LIFETIMES OF PROTEIN EXCITED STATES EXPONENTIAL?

J. W. Longworth and S. S. Stevens

Molecular structures of several proteins have shown us that individual tryptophanyl residues are "in" or "out" of the structure. Significant alteration of the excited-state environment would be expected to produce a multiplicity of excited-state properties. No clear indication is found in the bandwidths of emission spectra. We have recently measured phosphorescence decays from several proteins over a wide intensity range (10^{-3} to 10^{-4} of the initial intensity). Even though the proteins are known to possess their tryptophanyl residues in more than one environment, the decay is fitted well with a single exponential. Two proteins — hen lysozyme and avidin — have been found that do not exhibit this behavior, which lends credence to our methods.

Fluorescence decay measurements do not necessarily follow the above behavior. More than a single exponential is found, and a simple fitting with two exponential values is commonly observed; but examples have been measured in which this is not the case and a single lifetime suffices. Values for the short lifetime are 1–6 nsec, and for the long lifetime they are 8–17 nsec.

The long-lived component can make up as much as 25% of the intensity but typically amounts to 5% or less. The origin of the long tryptophanyl component is not yet established. Its lifetime and fraction of the intensity depend on conformation, solvent, and temperature. In our preliminary studies we have chosen to use only the lifetime value for the initial intensity decay to 0.1.

The answer to the question posed: Triplet states, they are; the singlet state, they can be but probably are not.

DELAYED-COINCIDENCE TIMING SPECTROSCOPY. A TALE OF A TAIL

S. S. Stevens and J. W. Longworth

A crucial element in application of delayed-coincidence methods to measurement of fluorescence lifetimes is the flash lamp, which is used to excite the fluorescent solutions. Two troublesome features of nanosecond arc-discharge flash lamps have attracted our attention. First, lamps optimized for brief output tend to render themselves inoperable after a few million flashes. Following a suggestion in laser engineering literature, we have with some difficulty fabricated arc electrodes of zirconium metal. This material's unusually high resistance to sputtering gives us satisfactory lamp output for at least 10^9 discharges, an impressive operational life. (Since we conscientiously turn off our lamp after we use it, 10^9 discharges represent only about 1 second of electric power use by the arc.) At one discharge per second, our lamp should last a century or more.

Our discharge is in hydrogen or air, and in either gas, in addition to the prominent hump from the photo-multiplier late pulse, a pronounced tail from a long-lived emission interferes with data analysis. The tail, which amounts to 3% of the intensity and decays with an 11-nsec lifetime, has resisted all efforts (clean lamp fabrication, exhaustive degassing, use of high-purity filler gas, etc.) to remove it. As our data analysis progresses from straight edge to deconvolution, the late pulse hump and the tail will become more troublesome. Eventually, means must be found to clean the data numerically if they cannot be improved physically.

DATA-ACQUISITION FACILITY

S. S. Stevens, C. O. Kemper, and M. L. Randolph

Installation of the common equipment of an automated data-acquisition system for the Biology Division is essentially complete. The hardware, based on a DEC PDP-11, includes magnetic disk and magnetic tape bulk storage, a 300-character-per-second paper tape reader, two

user terminals (with provision for expansion to 15 as needed) and provision for 32 analog data-input channels and 16 digital data-input channels. The system will operate with an extended BASIC time-sharing monitor, to allow an experimenter immediate access to his data while other experiments are in progress.

Seven devices (amino acid analyzers, scanning microdensitometer, etc.) are already connected to the processor, and engineering to interface several other devices is in progress. As of July 1, about 25 persons had system passwords and were using the facility for data acquisition or processing.

AUTOMATED AMINO ACID ANALYSIS

F. C. Hartman, S. S. Stevens, and C. O. Kemper

A Beckman 120C Amino Acid Analyzer has been connected to the Division's PDP-11 time-shared data-acquisition computer via two channels of the computer's analog-to-digital converter port. The interface permits the computer to store photometric data from the analyzer with <0.1% error, at a program-selectable data rate during the course of an amino acid analysis.

The data are stored in disk files by the computer during a run and processed automatically when the analyzer run is terminated. The processing program first searches the data for baseline sections, to which a regression line is fit. Then a floating average is used to search the data for absorbance peaks; for each peak, the elution time (referred to the start of the run) and the area of the peak are computed. Linear time shift and regression algorithms are used to match the data peaks to a stored collection of standard peaks, and a table is printed to show identity and elution time for each peak, with the ratios of sample peak area to standard peak area (i.e. concentration of each component in the sample being analyzed).

The program includes options for data and peak inspection, permanent data storage on magnetic tape, and standard peak calibration. Total data analysis takes about 10 minutes, perhaps 10 times faster than hand reduction of the data.

EXCITED-STATE ENERGY TRANSFER. GENERAL THEORY AND MODEL SYSTEMS

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

We are interested in the behavior of mobile excited states, or excitons, in photosynthetic systems and in

biopolymers. Last year's report (*1*) detailed our extensive work on the theory of incoherent, or hopping, excitons in linear polymers and indicated our interest in the doped polyacenes as model systems for the study of exciton quenching. We have extended our one-dimensional theory to two and three dimensions and have shown how the extended theory can be applied to the doped polyacenes.

We have generalized the concept of an exciton quencher to include the case of partially reversible quenching. We find that whenever there is slow back-transfer from the quencher to the host, the detailed time dependences of the host and of the quencher change considerably from what they are when there is no back-transfer. Under uniform initial excitation, except in one-dimensional cases or with slow back-transfer, the excitation decay is a single exponential to within very small correction terms (of order $c^{2/3}$ in three dimensions, where c is the fractional quencher concentration). This phenomenon, which we call "zero-mode dominance," is as strong for lattices with quenchers on random lattice sites as it is with quenchers that are periodically located. In three dimensions the decay rates for these two situations differ only by the order of $c^{1/2}$, but in two dimensions the rate with random quenchers is 25% less than the rate with periodic quenchers for c between 10^{-4} and 10^{-6} .

We also showed quite generally that a three-dimensional lattice in which the nearest-neighbor energy-transfer rate along one lattice direction — call it βF — is much less than the other two rates — each of which is F — still behaves kinetically as if it were an isotropic three-dimensional lattice, as long as $\beta \gg c$. An analogous conclusion holds for an anisotropic two-dimensional lattice.

Some of our conclusions, particularly those regarding randomly located quenchers and anisotropic energy-transfer rates, are contrary to commonly held notions based on less rigorous arguments. We have thus been able to use our results to rule out a number of proposed hypotheses that attempted to account for the anomalous exciton-quenching kinetics in doped polyacenes (*1*). In addition, we have been able to use our results on back-transfer to put forward a new hypothesis to account for the anomaly, one which confirms our conjecture of last year (*1*).

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FLUORESCENCE DEPOLARIZATION IN CHLOROPHYLL SYSTEMS

R. M. Pearlstein and R. P. Hemenger

The polarization of fluorescence from an array of interacting molecules contains, in principle, at least two kinds of information. These concern the degree of orientational order of the emission dipoles in the array and the strength of the intermolecular coupling. For chlorophyll in a viscous solvent, one usually assumes there is no order, orientational or otherwise, among the emission dipoles; all of the information is then about the energy-transfer rates. For chlorophyll arrays *in vivo* it is our contention that the energy-transfer rate is so high that information regarding the coupling-rate constants is obscured by the contribution from the orientational order of the emission dipoles.

Our results on the depolarization of chlorophyll fluorescence *in vitro* are based on a new approach to the use of Förster theory in this situation. We have derived for the first time an expression for the time-dependent depolarization (or rather a closely related quantity called "fluorescence anisotropy"). The logarithm of this expression is a series in powers of the square root of time for impulsive initial excitation. The series converges rapidly enough to allow straightforward comparison of theory with anticipated experimental results. We have also shown that the time-dependent depolarization, which is a function of both time and chlorophyll concentration, is related by a simple integral transform to the steady-state depolarization, which depends only on concentration. Thus, our theoretical results provide the basis for a potentially powerful method of determining energy-transfer rate constants and of testing the assumption of Förster energy transfer by time-dependent experiments without the use of impractically higher concentrations. Such determinations may be of great importance in photosynthesis, because current dogma in the latter field assumes that the Förster mechanism of energy transfer applies to chlorophyll *in vivo*; this assumption has never been satisfactorily tested even *in vitro*.

We have also examined, theoretically, arrays of dipole moments that should serve as models for chlorophyll *in vivo*. For an array of randomly oriented dipoles with extensive hopping-type excitation transfer, whose mean pairwise rate is much greater than that of fluorescence, we conclude that the steady-state polarization is less than the ratio of the latter rate to the former. For photosynthetic units this ratio is about 10^{-3} , which is an order of magnitude smaller than the smallest

observed polarization. We therefore conclude that all fluorescent chlorophyll *in vivo* possesses some degree of orientational order.

These considerations, and our analysis of the results of several experiments by a number of investigators, lead us to propose a model for the red oscillators (dipoles) of chlorophyll *in vivo*. We suppose that there are two distinct sets of emitters, one in or almost in the lamellar plane, the other tilted out of plane somewhere near a so-called "magic angle." The first set would thus give a highly polarized emission for either random or aligned lamellae, but the second would give high polarization only for aligned lamellae. We tentatively assign the first set to Photosystem I and the second to Photosystem II. We must stress that although this model does not uniquely account for existing data, it is certainly one of the simplest possible of such models.

MANGANESE-CATALYZED OXIDATION OF WATER

N. A. Nugent and W. A. Arnold

Manganese has been shown to be an essential element in the photo-oxidation of water by the chloroplasts of green plants. Experimental evidence indicates a catalytic role for this metal (1). Experiments in this laboratory have concentrated on the chemistry of manganese and the conditions required for the oxidation of water. The catalytic properties of manganese-containing chloroplast membranes have also been studied.

Manganese (IV) oxide has been shown to be an excellent catalyst when coupled to an electron acceptor whose redox potential is greater than 1.0 V. The redox potential for maximal oxygen evolution from water in these systems is 1.2 V. When coupled to chlorophyll, manganese (IV) oxide catalyses a photo-oxidation of water, producing molecular oxygen in the presence of electron acceptors such as benzoquinone ($E_h = 0.25$ V). Oxygen rates of about 5% of the maximum chloroplast rate have been observed.

Experiments are underway in which we are attempting to oxidize water with chloroplast membranes in the dark, using high-potential electron acceptors. These experiments are expected to yield information concerning the electronic state of the manganese catalyst in the chloroplast membrane.

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PHOTOSYNTHESIS

W. A. Arnold and J. R. Azzi

Green plants, while doing photosynthesis, emit two different kinds of light: prompt fluorescence, with a lifetime of something like 0.5 nsec, and delayed light, which lasts up to several hours. Although their emission spectra seem to be the same, both come from excited chlorophyll. The time dependence tells us that the two light emissions are made by quite different mechanisms.

The intensity of the prompt fluorescence is a number of times brighter than the intensity of the delayed light. This ratio, reported by various investigators, varies from 10 to 1 million. Since the intensity of the prompt fluorescence is not the same function of the exciting light intensity as the intensity of the delayed light, we see that the ratio depends on the exciting light. Since the intensity of the delayed light falls with the time in the dark, the ratio also depends on when the delayed light is measured.

Last year we reported (1) that a few hundred volts per centimeter applied across a chloroplast suspension made the delayed light a number of times brighter. An auxiliary experiment has shown that the electric field does not effect the intensity of fluorescence. This new phenomenon gives us a method of measuring delayed light in the presence of fluorescent light and under continuous excitation. Past experiments have shown that both the intensity of the fluorescent light and that of the delayed light are proportional to the exciting light intensity when it is below saturation. We have done such experiments (2).

We find that the prompt fluorescence is something like 140 times the delayed light when measured in this way, and the value remains constant while the exciting light is changed more than 1000 times.

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INTERACTIONS OF 5-FLUORODEOXYURIDINE AND THYMIDINE UNRELATED TO DNA SYNTHESIS

A. H. Haber, O. J. Schwarz,* L. W. Evans,† and L. L. Triplett

5-Fluorodeoxyuridine (FdUrd) has been widely used as an inhibitor of DNA synthesis. When phosphorylated to 5-fluorodeoxyuridine 5'-monophosphate, it inhibits the activity of thymidylate synthetase, which catalyzes the formation of thymidylate from deoxyuridylate.

Most organisms can phosphorylate thymidine to thymidylate but are unable to synthesize thymidine *de novo*. Consequently, depletion of the thymidylate pool is the mechanism by which FdUrd inhibits DNA synthesis. This depletion of the thymidylate pool can be reversed by addition of thymidine. In developmental biology, it is commonly assumed that counteraction by thymidine of FdUrd-induced growth inhibition proves that the growth inhibition resulted entirely from specific inhibition of DNA synthesis. We found, however, that the uptake of [¹⁴C] FdUrd into germinating wheat seedlings is reduced by thymidine. This apparent competition for uptake into the tissue occurs for FdUrd concentrations (10^{-4} M) that produce approximately half the inhibition of DNA synthesis and of growth observed after treatment with concentrations that completely inhibit DNA synthesis. Hence, the apparent alleviation by thymidine of FdUrd-induced growth inhibition may result from reduced uptake of FdUrd into the tissue. The question of whether the growth inhibition itself is caused completely by inhibition of DNA synthesis is still left open.

Very high concentrations of FdUrd can be shown to produce effects on wheat seedling growth and lettuce seed germination that are unrelated to DNA synthesis. Heavily gamma-irradiated wheat and lettuce seeds germinate and grow without DNA synthesis. The inhibition of germination and growth by FdUrd in these systems obviously is unrelated to inhibition of DNA synthesis by FdUrd, since there is no DNA synthesis to be inhibited (1, 2). Although these nonspecific growth effects are found after treatment with FdUrd at concentrations (10^{-2} M) higher than typically used, they are nevertheless pertinent here in one important respect: FdUrd inhibition of growth or germination can be alleviated by thymidine. Since thymidine reverses the nonspecific effects of the unusually high concentrations of FdUrd, it is conceivable that thymidine might in some instances also reverse any possible nonspecific effects from treatment with the lower concentrations of FdUrd typically used to inhibit DNA synthesis.

In conclusion, (1) thymidine interferes with the uptake of FdUrd in systems in which FdUrd reduces DNA synthesis and growth, and (2) thymidine appears to reverse FdUrd-induced growth inhibition in systems in which, irrespective of any of these chemical treatments, there is no DNA synthesis. Consequently, reversal of FdUrd-induced growth inhibition by thymidine is not generally conclusive evidence for (even though it is consistent with) the assumption that the

growth inhibition results entirely from inhibition of DNA synthesis.

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DISTINGUISHING BETWEEN CHEMICAL REGULATION OF SENESCENCE AND CHEMICALLY INDUCED LETHALITY IN LEAF TISSUE

A. H. Haber and L. L. Triplett

A difficulty in using chlorophyll loss to study chemical regulation of leaf senescence is the inability to distinguish between chemical effects on (1) retarding senescence and (2) inducing lethality. From studies of γ -ray-induced lethality, we developed criteria for distinguishing killing from retarded senescence (1). In killed tissue, chlorophyll is maintained at a high level if the tissue is kept in darkness but is rapidly bleached out if the tissue is illuminated. By contrast, when physiological senescence is retarded, as by cytokinins, the chlorophyll loss is retarded in either light or darkness. The effects of light and darkness on killed tissue are opposite from their respective effects on living, slowly senescing tissue (2). Using the apical leaf tissue of wheat, we studied actions of many inhibitors of DNA, RNA, and protein synthesis in light or darkness. Some inhibitors give effects not relevant to senescence, because they kill the tissue. Among these (with lethal concentrations in parentheses) are actinomycin D (100 μ g/ml), cycloheximide (3 μ g/ml), *p*-fluorophenylalanine (300 μ g/ml), 5-fluorouracil (100 μ g/ml), nalidixic acid (1000 μ g/ml), phenethyl alcohol (300 μ g/ml), and puromycin (100 μ g/ml). At concentrations up through 1000 μ g/ml the following were nonlethal and also ineffective in altering the time course of senescence: chloramphenicol, cytosine arabinoside, 5-fluorodeoxyuridine, hydroxyurea, streptomycin, and tetracycline.

The lack of effect of most inhibitors of DNA synthesis is consistent with our finding that in this system there is no DNA synthesis (3). Thus this system, which does not involve ionizing radiation, can be used to test the specificity of chemical inhibitors of DNA synthesis in a manner similar to the irradiated systems we have used previously (4). The lethality resulting from phenethyl alcohol is consistent with the non-specific effects observed after treatment with this

inhibitor of DNA synthesis in our irradiated systems (4). In general, sublethal concentrations of inhibitors of RNA or protein synthesis had little or no effect on the long-term, normal physiological senescence in this nongrowing apical leaf tissue.

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GROWTH AND DEVELOPMENTAL RESPONSES OF WHEAT SEEDLINGS TO MASSIVE BUT NONLETHAL DOSES OF IONIZING RADIATION

D. E. Foard and Rhonda F. Irwin

"Monon" wheat seedlings that receive doses in excess of 5 krad of γ -rays 3 days after sowing grow thereafter to a limited extent; no new organs are initiated, and growth in length ceases within 7–10 days after irradiation. Three-day-old seedlings that receive doses of 0.5–1 krad γ -rays are greatly, but not completely, suppressed in growth in length and in organ initiation. Such irradiation sharply curtails growth in length of all seminal roots and causes a swelling of the root apex, superficially similar to that caused by colchicine treatment. Cells of the root apex proper and of the root cap are often devoid of contents. Within such parent roots, often very near the necrotic tip, arise branch roots from the pericycle, typically the tissue of origin of lateral branch roots. These branch roots have typical anatomy, and although their rate of growth is less than those of unirradiated controls, it is sufficient for functioning of the whole plant. Details of the initiation of leaf primordia and growth of shoot parts of the irradiated seedlings are currently under study.

PROTEIN QUALITY AND EMBRYO SIZE IN WHEAT GRAIN

D. E. Foard and Rhonda F. Irwin

Storage proteins in the embryos of cereal grains are nutritionally far richer than proteins stored in the endosperm. Thus, a potentially important approach to improvement of plant protein quality is to increase the size of the embryo relative to the size of the endosperm. Such an approach could supplement the use of the very few mutants in which the synthesis of nutritionally unsatisfactory proteins is repressed with a

concomitant increased synthesis of nutritionally rich proteins – e.g. the opaque-2 mutation in maize, which produces kernels with decreased prolamin but increased albumins and globulins (*J*). We have recently begun to measure embryo and endosperm size in a wide variety of wheat species and cultivars. Thus far we have data only for embryo size. Of the diploid group investigated, *Triticum boeoticum*, a wild form, has a slightly larger embryo than the cultivated *T. monococcum*. *Triticum aethiopicum*, a cultivated tetraploid species (or variety of *T. turgidum*), has the largest embryos of any species of any level of ploidy so far observed. Some samples of this species regularly have individual embryos with a volume in excess of 8 mm³. The largest embryos observed so far in the allohexaploid bread wheats are in the cultivar Lemhi, in which embryos occur with volumes slightly in excess of 2 mm³ per embryo. We are currently measuring endosperm volumes in order to obtain the ratio between volumes of embryo and endosperm in the grain.

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WOUND REGENERATION, AUXIN, AND PEROXIDASES IN TOBACCO*

G. P. Howland and I. M. Sussex†

The kinetics of wound regeneration and peroxidase induction have been studied in the pith of intact tobacco plants (*Nicotiana tabacum* var. Wisconsin-38). Severing a small portion of the vascular elements leads to the establishment of a wound callus, which later develops into a wound cambium. This is followed by the differentiation of lignified elements, which ultimately form organized vascular strands around the wound through the pith. During the same period, specific peroxidase isozymes (as detected by starch-gel electrophoresis) appear in a narrowly defined region of the pith next to the wound. These isoperoxidases have not been detected previously in the pith, although they have been found in samples of pith tissue cultured *in vitro* on media supplemented with auxin.

The disruption of normal auxin transport has been assayed by monitoring the movement of [¹⁴C] indole acetic acid. Five “non-pith” peroxidases appear both above (4-fold accumulation of auxin) and below the wound (more than 75% reduction in auxin). These latter peroxidases are therefore not regulated in their appearance by the change in the local concentrations of auxins. However, one peroxidase (A_6) does seem to be regulated in its appearance by the local auxin concen-

tration, since it is detected prematurely in the region below the wound site where the auxin concentration is reduced.

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PROTEIN POLYMORPHISM AND GENIC HETEROZYGOSITY IN A POPULATION OF THE PERMANENT TRANSLOCATION HETEROZYGOTE *OENOTHERA BIENNIS**

G. P. Howland and D. A. Levin†

In *Oenothera biennis*, chromosomal translocations (resulting in a ring of 14 chromosomes at diakinesis) and a balanced lethal system have established a state of permanent structural heterozygosity. These factors, combined with self-fertilization, insure that the progeny will be identical to their parent, barring mutation. Thus, this type of organism is uniquely equipped to accumulate and retain heterozygosity. Starch-gel electrophoresis and enzyme histochemistry were employed to examine allelic polymorphism and heterozygosity in a large population of *O. biennis* that occupies an ecologically heterogeneous locality isolated from conspecific populations. Data on 19 separate enzyme loci disclosed a level of polymorphism (26% of the loci) not unlike that observed in several other natural populations. However, due to the extreme uniformity found in this population, all individuals examined were shown to carry these polymorphic alleles in a heterozygous configuration. Since the species is a colonizer, it is likely that the population was founded by a single individual and retained this genotype by virtue of the genetic system. Because of this genetic system, the proportion of heterozygosity per individual (26%) is higher than that reported for any other natural population. Additionally, three individuals (n = 452) were found to carry a rare allele for phosphoglucose isomerase-1 (PGI-1), and three others a variant for PGI-2. These two rare alleles may represent recent mutations that have appeared in this population.

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CYTOKININS IN RELATION TO MITOTIC INHIBITION IN GAMMA-PLANTLETS

W. R. Jordan III and A. H. Haber

In this project we are testing the hypothesis that the absence of cell division in gamma-plantlets is related to the inability to produce endogenous cytokinins in normal amounts. Two complementary approaches are used.

1. *Endogenous cytokinin*: Preliminary to a comparison of the cytokinin content of normal wheat seedlings with that of gamma-plantlet seedlings growing without cell division, a method of measuring and chromatographically characterizing the extracted cytokinins has been partially worked out. Homogenized wheat seedlings are extracted twice with 4 ml/g 95% ethanol at 4° C. It has been found that equilibration of the active material between the homogenate and the liquid phase occurs within 30 min. The extracts are recovered by centrifugation, combined, and evaporated *in vacuo* at 45° C to an aqueous residue, which is then extracted with four half-volumes of ether at pH 2.9. The resulting aqueous phase, freed of ether by boiling briefly, is active in the tobacco callus test; but the responses to various concentrations of this material suggest that it contains interfering substances as well. The pH of this solution is adjusted to 3.3, 15 ml of 10% AgNO₃ per 10 g of seedling fresh weight is added, and the mixture is stored overnight at 4° C. At least part of the active material is then present in the precipitate that forms and can be solubilized by stirring in 10 ml of 0.2 N HCl at 45° C. After the HCl has been removed by adding water and evaporating several times, the residue is active, does not interfere with the assay, and is suitable for paper chromatography.

2. *Effects of exogenous cytokinins on gamma-plantlets*: In efforts to induce cell division in gamma-plantlets, seedlings were grown in axenic culture on nutrient medium containing a range of concentrations of auxins [indoleacetic acid, (2,4-dichlorophenoxy)acetic acid, and *p*-chlorophenoxyacetic acid], kinetin, gibberellic acid, and coconut milk. The epiblast-coleorhiza of wheat seeds enlarged abnormally on media with high auxin concentrations, as reported previously (1), but in irradiated seedlings these enlargements stopped growing within about 2 weeks, suggesting mitotic inhibition, whereas the swellings from unirradiated seedlings continued to proliferate into a large callus. Growth of gamma-plantlets is unaffected by kinetin, which also fails to restore cell division, as shown by cell counts. As in leaf and coleoptile tissue from unirradiated seedlings, kinetin treatment of

gamma-plantlets does appear to counteract the growth inhibition resulting from very high concentrations of auxin. In this instance also, however, kinetin fails to restore cell division in gamma-plantlets.

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MORPHOLOGICAL LOCALIZATION OF DNA SYNTHESIS IN WHEAT LEAF TISSUE

Paula J. Thompson,* A. H. Haber, L. L. Triplett, and T. S. Handwerker†

During growth of the wheat leaf, division of tissue cells is localized at the base. The apical part of the leaf of a week-old seedling undergoes neither cell division nor expansion. In many plant tissues, however, despite the absence of cell division, DNA synthesis can continue. We therefore determined, with respect to the longitudinal axis of the leaf, the morphological regions in which DNA synthesis occurs. Since wheat has enzymes for phosphorylating thymidine to thymidylate (1), and since these enzyme activities persist with aging (2), we used presence or absence of incorporation of radioactive thymidine into DNA as an indication of the presence or absence of DNA synthesis. From week-old wheat seedlings, the first leaf, which is about 14 cm long, was cut into 2-cm sections. Incorporation of radioactive thymidine into DNA occurred only in the most basal section. Similar results were found for dark-grown tissue incubated with the label in darkness. Approximately 1/4 to 1/2 of the total label taken up into the basal tissue was incorporated into DNA during a 4-hr incubation. Using CsCl density-gradient centrifugation of DNA extracted from light-grown and dark-grown unlabeled tissue, we found two peaks, presumably corresponding to nuclear and plastid DNA. The labeled DNA from dark-grown, dark-incubated basal tissue was found to have a sharp peak at a density of 1.700, corresponding to one of the two peaks in our experiments and to the nuclear DNA peak at a density of 1.702 found for wheat by Wells and Ingle (3). By contrast, light-grown apical tissue incubated in the light or dark-grown apical tissue incubated in the dark did not incorporate significant radioactivity into DNA, despite uptake of the labeled thymidine into the tissue to an extent comparable with the uptake into the basal tissue. The absence of thymidine incorporation into apical tissue was not influenced by kinetin concentrations effective in retarding leaf senescence. We conclude that the actions of light and of cytokinin in retarding senescence of this tissue, previously shown to

be independent (4), can each occur in absence of DNA synthesis.

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MECHANISMS OF THYMIDINE PHOSPHORYLATION IN WHEAT

O. J. Schwarz* and A. H. Haber

We have shown that germinating wheat, both gamma-plantlet and unirradiated, develops an enzyme activity in which ATP can serve as a phosphate donor in the production of thymidylate from thymidine (1). This finding, together with other biochemical data, suggested that the active enzyme was thymidine kinase (1). Recently it has been reported that in some systems AMP serves as the phosphate donor in the phosphorylation of thymidine in a reaction catalyzed by a nucleoside phosphotransferase (2). In preparations from both gamma-plantlet and unirradiated control wheat seedlings, AMP is more effective than ATP as a phosphate donor in the conversion of thymidine to thymidylate. The same preparations also transfer phosphate from phenylphosphate to thymidine to form thymidylate, further demonstrating the existence of a phosphotransferase enzyme. By the use of snake venom 5'-nucleotidase, it was shown that for each of the three phosphate donors used (AMP, ATP, phenylphosphate), all the thymidylate formed was in fact 5'-thymidylic acid. In both gamma-plantlets and unirradiated controls, enzyme activity utilizing AMP for thymidine phosphorylation appears between 12 and 24 hr after sowing, whereas the enzyme activity utilizing ATP does not appear until 24–36 hr. We therefore suggest that the nucleoside phosphotransferase pathway is a major mechanism of thymidine phosphorylation in wheat. Since ATP is converted to AMP in the enzyme preparations, it remains for future studies to determine the actual contribution of thymidine kinase in the phosphorylation of thymidine.

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ULTRASTRUCTURAL CHANGES ASSOCIATED WITH AUXIN-INDUCED TUMORS OF THE COLEORHIZA EPIBLAST IN WHEAT

Patricia L. Walne* and A. H. Haber

When wheat is germinated in high concentrations of auxin, the coleorhiza epiblast grows in an excessive and disorganized manner and resembles a callus. This increased growth involves cell division and a greater dry weight. Wheat was germinated in 1 mM indoleacetic acid or water, and the coleorhiza epiblasts were fixed after 1, 2, 3, and 4 days. In contrast to the controls, auxin-treated tissues developed many lipoidal bodies after 1 day; these diminished in number by 2 days. Additionally, the following changes, resulting from auxin treatment, became progressively more evident with time — cytoplasmic vacuolation; appearance of myelin figures, polyribosome configurations, and extensive profiles of rough endoplasmic reticulum; and sloughing of cytoplasmic contents into and the accumulation of electron-dense granules in vacuoles.

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CELLULAR RADIOBIOLOGY GROUP

Alexander Hollaender

International activities: The group conducted an important symposium on "Gene Expression and its Regulation" at La Plata University in Argentina. It was an extremely successful meeting, with about 350 people attending, and the proceedings have already gone to press. During this year the proceedings of two previous symposia have appeared. The proceedings of the symposium on "Fertility of the Sea" held in Sao Paulo, Brazil in 1969 and of the symposium on "Visual Processes in Vertebrates" held in Santiago, Chile in 1970 (the tenth anniversary of this series of symposia) are both important publications in their respective fields.

The symposium planned for 1972 will be held in Cali, Colombia, at the Universidad del Valle on November 27–December 1 and will deal with "Fundamental Approaches to Plant and Animal Improvement." Plans are also well underway for the 1973 symposium in Bahia, Brazil on "Physiologic and Genetic Aspects of Reproduction." Continued support of these symposia at a somewhat reduced rate is assured by the Ford Foundation.

Cooperative activities: The cooperative activities first established with the University of Tennessee in Knoxville through the UT-Oak Ridge Graduate School of

Biomedical Sciences have extended to other areas. The Biology Division, the Oak Ridge Associated Universities, the University of Tennessee in Knoxville, and the UT Memorial Research Center have cooperated in developing a pattern for a Cancer Research Center. A Planning Grant has been obtained from the National Cancer Institute, and the work is now in progress.

We have also been very much involved in plans for an Area Health Education Center, which is urgently

needed. It will possibly be set up at the University of Tennessee Memorial Research Center, with heavy involvement of the Oak Ridge National Laboratory. A grant from the Carnegie Foundation and the Commonwealth Fund has been obtained.

In addition, we have been active in obtaining support for the Black Colleges Program, which is under the direction of Dr. Frank Hamilton and is part of the Graduate School for Biomedical Sciences.

GENETICS AND DEVELOPMENTAL BIOLOGY

W. E. Barnett

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

^aPostdoctoral investigator

^bVisiting investigator from abroad

^cConsultant

^dLeave of absence

^eVisiting investigator

STUDIES ON CHANGES IN CELL CYCLE PARAMETERS AND CELL SIZE IN CHINESE HAMSTER CELL CULTURES WITH DAILY RENEWAL OF MEDIUM

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

Last year we reported results of combined autoradiographic and microphotometric studies on the changes in cell cycle parameters and cell size in cultures of Chinese hamster cells grown from culture initiation to decline without change of medium (1). The same parameters have now been followed in cultures grown with daily renewal of medium. Basically the same general changes in cycle parameters and to some extent cell size were seen, but the changes occurred more slowly than when the medium was not renewed. Specifically, there was a major increase in the fraction of the cell cycle occupied by G₁ even when the cell number was increasing exponentially, and the rate of this change was slowed only somewhat by daily renewal of medium. The major conclusion is that cell cycle parameters undergo continuous change during cell growth. A standard set of parameters can only be given if the cell line, medium, conditions of culture, and age of culture are specified. It is not sufficient to specify broad culture stages such as lag, log, and stationary phase. The data from the various experiments is most easily described in terms of two partially independent cycles, the DNA replication and mitotic cycles, with the rate of growth in cell mass being determined partially independently of either of these cycles.

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PROPERTIES OF SENESCENT BACTERIAL CULTURES

Alice A. Hardigree and H. I. Adler

Our interest in bacterial cell growth and division prompted an investigation of bacteria in the senescent phase of growth. Aerated nutrient-broth cultures of four strains of *E. coli* K-12 have been kept at constant volume and temperature (37° C) for several weeks, with daily determinations of cell viability by plating on nutrient agar and periodic determinations of X-ray and

heat resistance. In all strains studied thus far, cell viability declines rapidly during the first 6 to 10 days (to 5% of the original maximum titer of $\sim 4 \times 10^9$ cells/ml), then much more slowly thereafter (to 0.5% of the original titer after an additional 9 weeks).

The most thorough studies have been done with an Hfr strain of *E. coli*. In this strain the sensitivity of very old cultures to heat (60° C) and X-irradiation (250 kVp) is somewhat greater than that of cells sampled from early stationary phase.

We have attempted to restore viability of cells from old cultures (23–27 days) by making various additions to the culture (e.g., AMP, cyclic AMP, deoxyribose, nutrient broth, phosphate buffer, and water as control). The most effective single chemical used thus far has been AMP. Addition of AMP (0.001 M) brought the titer of a culture from 1.5×10^7 cells/ml to 1×10^9 cells/ml (a 100-fold increase) in 24 hr, after which the titer slowly declined. Titer was increased 30-fold at a concentration of 0.00025 M AMP.

We conclude that cells maintained over long periods of time in nutrient medium at 37° C undergo changes in their response to ionizing radiation and heat, and lose their ability to divide when plated onto nutrient agar. The ability to divide can be restored by treatment with certain chemicals. We will determine if the same agents influence sensitivity to ionizing radiation and heat.

RADIATION RESISTANCE OF MINICELL-PRODUCING *ESCHERICHIA COLI* STRAINS

Alice A. Hardigree, G. G. Khachatourians,*
D. R. Stallions, and H. I. Adler

The original *E. coli* minicell-producing strain, P678-54, was discovered during a search for X-ray-resistant mutants which had retained the same UV resistance as the parent strain. Since that time other minicell-producing strains have been produced by means of conjugation and mutation techniques. We are currently attempting to see if similar radiation responses occur in these strains when compared to their parent strain.

Increased X-ray resistance has now been observed for minicell-producers in two other strains. $\chi 1260$, a temperature-sensitive, minicell-producing strain obtained by conjugational techniques, is more resistant than its parent strain BUG-6; also, a minicell-producing strain of *Salmonella typhimurium* isolated after treatment with a mutagenic agent is more resistant than its parent strain.

We are anxious to determine if the properties of forming minicells and increased X-ray resistance are due to the same gene or genes. To this purpose, we are currently performing conjugation experiments in order to determine the precise map location of the gene(s) controlling these two properties.

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CHROMOSOME TRANSFER UNDER ANAEROBIC CONDITIONS IN *ESCHERICHIA COLI*

D. R. Stallions and Roy Curtiss III*

Conjugal transfer of both chromosomal and extra-chromosomal genetic material has been shown to occur in the mammalian intestinal tract (1, 2). The frequency of this transfer approaches the frequency obtained in *in vitro* matings (1, 2). Data indicating that conjugal genetic transfer did not occur at significant levels in *Escherichia coli* in an anaerobic environment has been reported (3). These results create an enigma since the mammalian intestinal tract is largely anaerobic (4). Because of the medical importance of the transfer of resistance transfer factors and the paradox concerning the gaseous environmental requirement for bacterial conjugation, we reinvestigated the effect of anaerobiosis on bacterial conjugation.

We conducted bacterial matings in environments of atmospheric air or nitrogen. We utilized a minimal medium used in the earlier experiments (3) and a complex medium in the matings. We have found that the anaerobic restriction of recombinant formation (3) applies only to anaerobic matings conducted in a synthetic mating medium. Anaerobic conditions have no effect upon the recombinant levels in matings carried out in a rich medium. We have shown that the anaerobic restriction of bacterial chromosome transfer (3) is limited to a condition not normally found in nature. The conjugal transfer of genetic information between bacteria can occur at significant levels in an anaerobic environment provided that the mating medium is nutritionally rich. The anaerobic condition of the intestinal tract is not a limiting factor in *in vivo* bacterial conjugation as the intestinal contents provide a nutritionally suitable medium (4).

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CONJUGAL REPLICATION OF DNA

R. G. Fenwick, Jr.*

During the past year I have been continuing a study of conjugal DNA replication, or that DNA synthesis which occurs during conjugation by *Escherichia coli*. To do this, cells carrying the R factor R64-11 and which are thermosensitive for vegetative DNA synthesis were used as donors and minicells were used as recipients. When such matings are conducted at 42° C there is a stimulation of DNA synthesis as measured by incorporation of radioactive thymidine and the DNA being synthesized is that of the R factor which is being transferred. Nalidixic acid, which inhibits vegetative DNA replication and conjugal transfer, inhibits this conjugal DNA synthesis and, in addition, physically damages the R-factor molecules as measured by the loss of covalently closed, circular R-factor DNA. The addition of the drug also causes the R factors which normally are cytoplasmic to become membrane bound. The mating system has also been used to study the requirement for RNA and protein synthesis during conjugation. I found that the donor cells are capable of transferring each R factor once when protein synthesis is inhibited by chloramphenicol. This is true even when protein synthesis is inhibited prior to initiation of mating, indicating that donor cells have all proteins required for one round of conjugal transfer before they are exposed to recipient cells or minicells. Protein synthesis is required, however, to transfer a second copy of a particular plasmid. On the other hand, conjugal transfer of the R factor is completely inhibited when RNA synthesis is inhibited by rifampin before the mating is initiated. Rifampin, like chloramphenicol, also inhibits multiple rounds of transfer when it is added after the start of a mating. These results demonstrate that initiation of conjugal replication, like vegetative replication, requires synthesis of RNA which is not translated into a protein. The nature of this RNA is under investigation.

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FATE OF CONJUGALLY TRANSFERRED DNA IN *ESCHERICHIA COLI* MINICELLS

G. G. Khachatourians,* R. J. Sheehy,[†] and Roy Curtiss III[‡]

DNA-deficient minicells produced by an *E. coli* F⁻ strain can act as recipients for conjugally transferred

DNA. Forty to 50% of the single-stranded [³H]dThd-labeled Hfr DNA transferred to minicells is degraded at 37° C in 3 hours into (1) cold trichloroacetic-acid-soluble material and (2) low-molecular-weight material as judged by sedimentation through alkaline sucrose gradients. These findings indicate the presence of both exo- and endonucleolytic activities. Physiological conditions of the minicell-producing cultures and their growth stages influence degradation of single-stranded DNA. Exonuclease activity is greater in minicells from stationary phase cultures. Both nuclease activities are temperature dependent. 2,4-Dinitrophenol inhibits both activities while divalent cations, especially Ca²⁺, stimulate them. Degradation of single-stranded DNA could be minimized when incubation is carried out in buffers containing 0.5 M NaCl or lacking divalent cations. Exonucleolytic degradation products found in the acid-soluble fraction have been identified as thymine by chromatographic analysis.

We have also examined minicells from *recA*⁻, *recB*⁻, *endI*⁻, *dnaA*⁻(TS), and *dnaB*⁻(TS) mutants for their ability to degrade conjugally transferred DNA. Only *recB*⁻ minicells were found to have reduced DNA degradation activity.

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MOLECULAR STUDIES ON ENTRY EXCLUSION IN *ESCHERICHIA COLI* MINICELLS

R. J. Sheehy,* Clifton Orr,† and Roy Curtiss III‡

Minicells produced by abnormal cell division in a strain of *Escherichia coli* K-12 have been employed here to investigate the phenomenon of "entry exclusion." When purified minicells from strains containing F' and/or R factors are mated with [³H] dThd-labeled Hfr or R⁺ donors, the recipient minicells can be conveniently separated from normal-sized donors following mating, and the products of conjugation can be analyzed in the absence of donors and of further growth of the recipients. Transmissible plasmids or episomes are transferred less efficiently to purified minicells derived from strains carrying similar or related extrachromosomal elements than to strains without them. Measurements of DNA degradation and determination of weight-average molecular weights of DNA following its transfer indicate that degradation of

transferred DNA, or transfer of smaller pieces of DNA, cannot account for the comparative reduction in transfer to entry-excluding recipients. Therefore, we conclude that entry exclusion operates to prevent the physical entry of DNA into recipients expressing the exclusion phenotype. The R-produced repressor (product of the *drd*⁺ gene), which represses fertility (i.e., ability to act as donor), partially reduces exclusion mediated by R and/or F factors in matings between strains carrying homologous elements.

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EVIDENCE FOR THE PRESENCE OF F AT THE LEAD REGION OF CONJUGALLY TRANSFERRED Hfr DNA

Louis Glatzer* and Roy Curtiss III†

Studies on the DNA transferred to F⁻ minicells by Hfr strains of *Escherichia coli* were initiated to determine whether or not a portion of the F episome is present on the lead region of the donor chromosome. The techniques of DNA duplex-annealing on filter discs and direct electron microscopic observation of DNA heteroduplexes were utilized to answer the question. Single-stranded, lead-region DNA transferred to minicells was extracted, purified, and duplexed with purified F DNA. The results show that up to 50% or more of the recoverable lead-region DNA is homologous to F. Hybridization between [³H] thymidine-labeled lead-region Hfr DNA and F DNA is competitively reduced by the addition of unlabeled F DNA, lead-region Hfr DNA, and Hfr chromosome DNA to the hybridization mixture but not by the addition of F⁻ chromosome DNA. Since the lead-region, single-stranded Hfr DNA has a weight-average molecular weight of about 25 × 10⁶ daltons and F has a single-stranded molecular weight of about 32 × 10⁶ daltons, we infer that about 40% of F is proximally transferred by Hfr donors. In other words, the transfer origin on F is about 40% of the length of F away from the site on F that recombined with the chromosome during integration of F to yield an Hfr donor. This finding is supported by the observation of forked configurations when lead-region and F DNAs are heteroduplexed in solution and subsequently examined with the electron microscope.

Contrary to these positive results, genetic experiments designed to thoroughly test for restoration of fertility of poor donors by integration of the lead region were negative. This indicates that F sequences present at the lead region do not code for genes critical to the transfer properties of the Hfr strain used in this study or are not integrated at measurable frequencies.

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FUNCTIONAL TRANSCRIPTION AND TRANSLATION IN PLASMID-CONTAINING MINICELLS OF *ESCHERICHIA COLI* K-12

Anne C. Frazer* and Roy Curtiss III†

We have been interested in the use of minicells (1) in examining transcription and translation of plasmid genes (2). Such experiments require a knowledge of how RNA polymerase functions in minicells. The most important question to answer is whether minicells indeed synthesize functional protein and then to evaluate the regulation of such synthesis. For this study we used minicells containing the complex *Col-trp* plasmid.

Plasmid-containing minicells were assayed for derepression of anthranilate synthase, the first enzyme encoded by the plasmid-born tryptophan operon. Both the rate of change in anthranilate synthase levels and the rate of total *de novo* protein synthesis were determined for derepressed (no Trp) and repressed (50 $\mu\text{g/ml}$ Trp) purified minicell suspensions. Anthranilate synthase levels rose 1.5- to 3-fold in suspensions derepressed for 30 min, while no change occurred in repressed suspensions. The kinetics of derepression were biphasic showing a rapid increase in enzyme levels during the first 10–15 min followed by a more gradual increase. Under the conditions employed, the initial rate of derepressed anthranilate synthase synthesis was $2.1 \pm 0.5 \times 10^{-6}$ μmoles of anthranilate produced per min per mg protein per ml minicell suspension per min derepression. Using the data of Morse and Yanofsky (3) we can estimate that this corresponds to 3 to 4 molecules of enzyme synthesized per minicell. These results demonstrate that plasmid-containing minicells synthesize functional protein and that this synthesis is subject to repression and derepression similar to that observed in cells.

In addition, Barbara A. Hamkalo, in a collaborative study, has successfully visualized by electron micros-

copy (4) long polysomes in the material extruded from osmotically shocked minicells. This is further evidence that transcription and translation in *Col-trp*-containing minicells is similar to that in cells.

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BROMODEOXYURIDINE RESISTANCE AND ULTRAVIOLET SENSITIVITY IN CHINESE HAMSTER CELL LINES

Diana B. Smith

The BrdUrd resistance of some Chinese hamster cell lines grown in culture was found to be correlated with increased UV sensitivity. BrdUrd has an effect on normal somatic cells of suppressing differentiation and transcription, but it was incorporated into the DNA of some immediate BrdUrd-resistant and UV-sensitive cells. An explanation for this is that a repair process determines differentiation in normal cells as well as repairing UV damage. It is assumed that the UV repair process is excision repair, which is detectable but difficult to measure in rodent cells, and not recombination repair. Based on Werner's theory (1) that the replicase and polymerase have different precursors, normal DNA synthesis is conceived of as involving both precursors in specific types of incorporation. Thymidine enters the two precursor pools, TMP-X and TTP. The repair enzymes are capable of switching precursors at recognition sites for initiation of transcription, thereby increasing the rate of transcription and causing a stable inherited differentiation. In somatic cells in culture, the insertion of the incorrect precursor (TTP \rightleftharpoons TMP-X) at these sites may give rise to epigenetic traits which behave as revertible "mutants." BrdUrd resistance is due to the cells' relative inability to incorporate BrdUrd into these specific sites, and may sometimes itself be an epigenetic trait, as any interference with thymidine metabolism (and BrdUrd is an analogue of thymidine) may predispose a cell to acquisition of epigenetic traits.

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THE INDUCTION OF TRANSLOCATIONS IN MOUSE SPERMATOGONIA BY X-RAYS

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

Following irradiation of the testis, even at the lowest doses used here, the majority of spermatogonia are killed, and the stem cells, which are comparatively more radioresistant, remain to repopulate the testis. Symmetrical translocations induced in spermatogonial stem cells can be observed in spermatocytes following this repopulation of the testis.

Using information obtained in preliminary experiments, we sampled animals at a time when it was known that the first wave of cells reached spermatocyte metaphase I. The interval between irradiation and sampling is dose-dependent, so time of sampling varied from about 5 weeks after 50 r to about 12 weeks following 600 r.

In order to reduce possible error due to clones of aberrant cells being scored, only 50 cells were scored from each testis. Symmetrical translocations were scored as multivalent configurations at spermatocyte metaphase I, and a total of 600 or 800 cells were analyzed at each dose point. Partial-body X-ray doses of 50, 100, 200, 300, 400, 500, 600, or 1200 r were given at a dose rate of 100 r/min.

The dose-response curve for symmetrical translocations, over the dose range of 0 to 400 r, is nonlinear, and gives a best fit to the equation $Y = a + bD + cD^2$, with a sizeable two-track component (Fig. 1). At doses above 400 r the curve shows some saturation, and subsequently a decrease in yield, so that at 1200 r the yield is the same as that at 300 r.

These data differ from those published elsewhere (1, 2), which showed linear dose-response curves. It is known that there is a variation of translocation yield with time after irradiation, and if the timing of this variation is dose-dependent, as might be expected from data for other systems, then using a single sampling time, or a very narrow range of sampling times, would result in a distorted dose-response curve being obtained. However, we feel we have circumvented this problem in our experiments by always sampling when the first wave of cells reached spermatocyte metaphase.

A quadratic dose-response curve is expected when it is remembered that a dose-rate effect and a decrease in yield for split doses have been observed for reciprocal translocations induced in mouse spermatogonia (3, 4).

Using these and other data obtained in this laboratory, we can conclude that (1) the shape of the dose-response curve for asymmetrical translocations induced in leukocytes of the mouse is the same as that

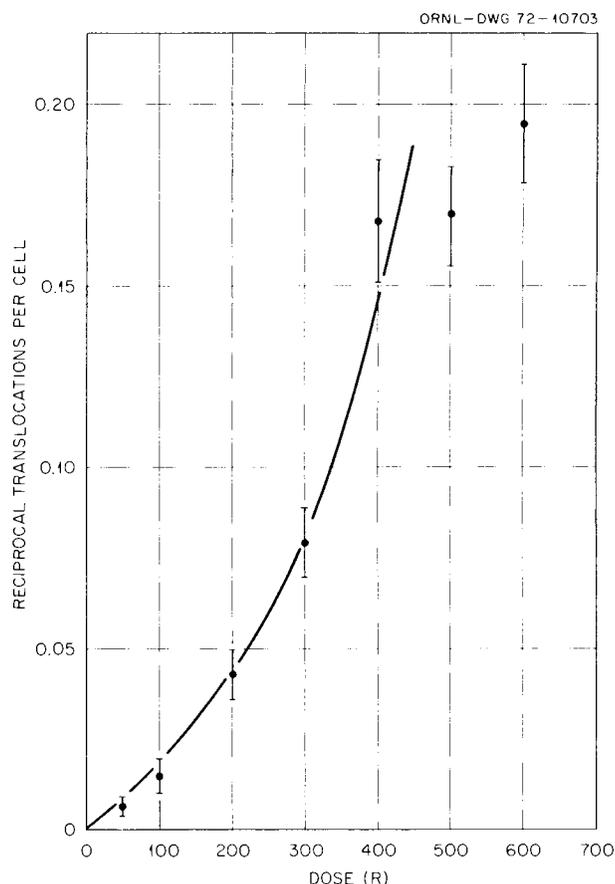


Fig. 1. Dose response curve for reciprocal translocations observed at spermatocyte metaphase I.

The curve was fitted over the range 0 to 400 r, as it saturates at doses above 400 r.

for asymmetrical translocations induced in spermatogonia and observed in spermatocytes; (2) a strong selective pressure operates during testicular repopulation, as the yield of translocations observed in spermatocytes is only about 30% of that assumed to have been induced in spermatogonia; and (3) estimates of the X-ray-induced translocation frequency in human spermatocytes can be made.

In conjunction with these experiments, we are studying the proportion of translocations in the mouse which are transmitted to F_1 progeny. With these data we are hopeful of being able to estimate the genetic hazard of radiation to man.

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CHROMATID ABERRATIONS INDUCED BY HYCANTHONE IN HUMAN LEUKOCYTES*

R. J. Preston and F. G. Pearson

Hycanthone is a drug which has been used fairly widely for the treatment of schistosomiasis. As this drug has been reported to be mutagenic in bacteria and in mammalian cells in culture, this experiment was designed to determine whether or not it induced chromosome aberrations in human leukocytes.

Cells were treated with hycanthone methanesulfonate (0.4 or $0.8 \times 10^{-4} M$) in the dark for 4 hr at 18, 12, or 6 hr prior to fixation, so that cells in the G_2 and S phases would be treated. All cultures were fixed 52 hr after initiation. Tritiated thymidine ($0.05 \mu\text{Ci/ml}$ of 1.0 Ci/mmole) was present during the period of hycanthone treatment so that, following autoradiography, it could be determined which cells were in the S phase at this time. Control cultures, to which only tritiated-thymidine had been added, were run in parallel with the hycanthone series so that corrections could be made for those aberrations induced by the tritiated thymidine.

The results are shown in Table I. In order to determine the number of aberrations induced by hycanthone alone, the yield obtained in the relevant control group must be subtracted from the hycanthone group. It can be seen that hycanthone induces all types of chromatid aberrations in both labeled and unlabeled cells, i.e. in S and G_2 cells. However, it does not appear to be effective at all treatment times. There appears to be no difference in aberration yields between the hycanthone and control groups when the treatment was given 12 hr before fixation. There is, however, a

significant difference between control and hycanthone treatments, particularly at the higher hycanthone concentrations, when the drug was added at 6 or at 18 hr before fixation.

Although the results are somewhat difficult to interpret, it seems reasonable to conclude that hycanthone induces chromatid aberrations in both S and G_2 cells at the concentrations used. However, it should also be added that these concentrations are at the very least 10–15 times as high as those used for clinical treatment of schistosomiasis. It is unlikely that at clinical doses hycanthone would induce an aberration yield significantly different from the "spontaneous" yield.

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GENETIC HAZARDS OF IONIZING RADIATIONS: CYTOGENETIC EXTRAPOLATIONS TO MAN BY INTERSPECIFIC COMPARISONS

J. G. Brewen, R. J. Preston, and D. G. Gosslee*

A practical end point of measuring mutation and translocation production by ionizing radiation in the mouse is the extrapolation of the data to estimate the genetic hazard to man from ionizing radiation. The assumption has often been made that the mouse can serve as a model for predicting effects in man, since both organisms are mammals. It has become apparent, however, that different mammals have different quantitative responses to ionizing radiation and consequently it appears that direct comparisons between mouse and man may not be justified.

TABLE I. Yields of chromatid aberrations following hycanthone treatment

Time of hycanthone treatment prior to fixation (hr)	Labeled cells (%)	No. of cells	Hycanthone concentration ($\times 10^{-4} M$)	Chromatid plus isochromatid deletions		Interchanges	
				Labeled	Unlabeled	Labeled	Unlabeled
2–6	49	125	0	9	0	0	0
	58	150	0.4	6	8	1	2
	56	150	0.8	15	43	22	49
8–12	99	150	0	29	0	0	0
	99	150	0.4	31	0	1	0
	94	150	0.8	29	0	0	0
14–18	99	150	0	40	0	5	0
	100	150	0.4	69	0	6	0
	100	150	0.8	101	0	12	0

Earlier studies on the production of chromosome aberrations in the peripheral leukocytes of various species show that the quantitative response is quite heterogeneous among the various species. Based on our earlier results (1) that show no significant difference in the quantitative response of somatic and germinal cells, it would appear that the heritable damage produced in germ cells is also variable among these species.

The present report presents data that show that the quantitative differences in chromosome aberration production among six species is not entirely random, and also that these data can be used to make extrapolative predictions on the genetic effects of ionizing radiation in man.

Peripheral leukocytes from six mammals were irradiated with various doses of 250 kv X-rays, cultured by standard procedures, and analyzed for chromosome aberrations. The species, X-ray doses, and corresponding yields of chromosome aberrations are summarized in Tables I and II. Regression analysis of the

aberration yields showed that both dicentric and deletions increase with dose according to the model $Y = bD + cD^2$ for all species except *Wallabia bicolor*. In this latter instance the best model representation is $Y = cD^2$. The "best fit" models for dicentrics are plotted in Fig. 1. Further analysis showed that when a term $(N - 1)$ for chromosome arm number was included in the model [$Y = (N_i - 1)(b_i D + c_i D^2)$] the great majority of the interspecific variations could be accounted for. Consequently, the yields of dicentrics were plotted against chromosome arm number at each of four doses (Fig. 2). From these fits it appears that there is a linear relationship between the probability of forming a dicentric and the number of chromosome arms available to participate in an aberration. Thus man, with an arm number of 81, is twice as sensitive to the production of dicentrics as the mouse, with an arm number of 40.

Since dicentrics are one of two possible interchange events that are thought to occur at equal frequencies, they can be used as a direct measure of the rate of

TABLE I. Yields of dicentric chromosomes per 100 cells (\pm S.E.) at each dose employed in the different species studied*

Dose (r)	<i>Wallabia bicolor</i>	<i>Cricetulus griseus</i>	<i>Mus musculus</i>	<i>Sus scrofa</i>	<i>Homo sapiens</i>	<i>Saguinus fuscicollis</i>
0	0	0	0.3 \pm 0.3	0	0	0
50	0.3 \pm 0.3	1.6 \pm 0.6	2.3 \pm 0.9	1.3 \pm 0.7	4.3 \pm 0.8	6.5 \pm 1.8
100	1.0 \pm 0.6	6.0 \pm 1.2	6.7 \pm 1.5	10.0 \pm 1.8	13.2 \pm 1.5	12.7 \pm 2.0
150	5.4 \pm 0.9			19.7 \pm 2.6	20.8 \pm 1.9	34.3 \pm 3.4
200	9.5 \pm 1.5	12.6 \pm 1.6	14.3 \pm 2.2	27.0 \pm 3.0	33.7 \pm 2.4	50.3 \pm 4.1
300	23.0 \pm 2.8	30.3 \pm 2.8	32.3 \pm 3.2	61.0 \pm 4.5	67.5 \pm 3.4	80.0 \pm 5.2
400	51.3 \pm 4.1	51.4 \pm 3.8	65.0 \pm 8.0	92.7 \pm 5.6	120.5 \pm 4.5	

*A minimum of 300 cells were analyzed at each experimental point except the 400 r dose in the case of *M. musculus*, where 100 cells were analyzed.

TABLE II. Yields of deletions per 100 cells (\pm S.E.) at each dose employed in the different species studied

Dose (r)	<i>Wallabia bicolor</i>	<i>Cricetulus griseus</i>	<i>Mus musculus</i>	<i>Sus scrofa</i>	<i>Homo sapiens</i>	<i>Saguinus fuscicollis</i>
0	2.6 \pm 0.6	0.5 \pm 0.4	0	0	0.3 \pm 0.2	0
50	5.3 \pm 1.1	2.4 \pm 0.7	2.0 \pm 0.8	4.7 \pm 1.2	4.2 \pm 0.8	5.0 \pm 1.6
100	6.7 \pm 1.5	8.3 \pm 1.4	3.0 \pm 1.0	7.7 \pm 1.6	6.0 \pm 1.0	11.0 \pm 1.9
150	15.1 \pm 1.5			22.0 \pm 2.7	8.8 \pm 1.2	24.3 \pm 2.8
200	23.8 \pm 2.4	15.8 \pm 1.8	16.7 \pm 2.4	32.3 \pm 3.3	15.8 \pm 1.6	32.0 \pm 3.3
300	50.0 \pm 4.1	38.8 \pm 3.1	29.3 \pm 3.1	69.3 \pm 4.8	33.8 \pm 2.4	53.7 \pm 4.2
400	114.7 \pm 6.2	61.4 \pm 4.2	64.0 \pm 8.0	105.0 \pm 5.9	61.8 \pm 3.2	

formation of the other interchange event. This other interchange is commonly called a reciprocal translocation and is, unlike a dicentric, transmissible from one generation to the next. Hence, based on the dicentric data, we conclude that man is twice as sensitive as the mouse to the production of heritable translocations.

The deletion data, however, show that mouse and man are equally sensitive. Since a significant portion of the so-called "specific-locus" mutations in the mouse

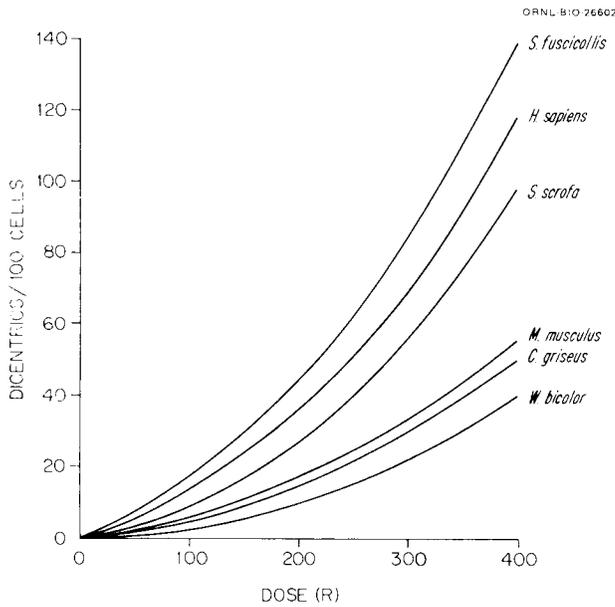


Fig. 1. Best-estimate regression curves for dicentric production in six mammalian species.

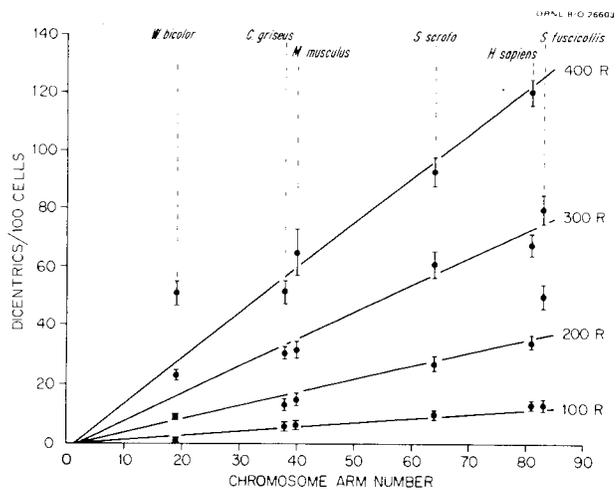


Fig. 2. Best-estimate regression curves for dicentric yield vs. chromosome arm number in six mammalian species.

Observed yields are plotted with standard errors at each of the four doses considered.

are associated with minute deletions, it appears that man and mouse are equally sensitive to the production of this class of mutation by X-rays.

Concurrent with the studies on chromosome aberration production in peripheral leukocytes, studies are being conducted on the production of reciprocal translocations in spermatogonia stem cells. A complete dose-response curve has been done for the mouse (2) and preliminary data are available on the Chinese hamster. Figure 3 shows a log-vs.-log plot of aberration yield against dose for dicentrics in man, mouse, and Chinese hamster; and reciprocal translocations in mouse and Chinese hamster up to a dose of 400 r.

Two features of these data deserve discussion. First, the yields of reciprocal translocations in the mouse and Chinese hamster are equal, as are the yields of dicentrics

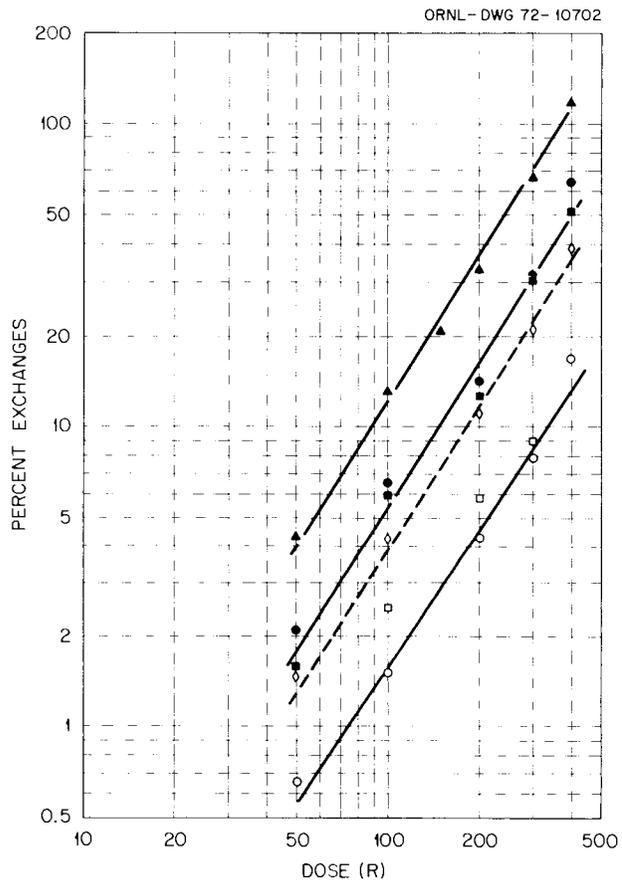


Fig. 3. Dose response curves of interchange aberrations vs. dose in the peripheral leukocytes and primary spermatocytes of the species studied.

Dicentrics in human peripheral leukocytes (▲); dicentrics in Chinese hamster (■) and mouse (●) peripheral leukocytes; reciprocal translocations in Chinese hamster (□) and mouse (○) primary spermatocytes; and predicted reciprocal translocation in human primary spermatocytes (◇).

in the two species. Thus, based on the yield of dicentrics and the chromosome arm number, a prediction from mouse to hamster is borne out to be accurate. The same extrapolative prediction concerning reciprocal translocations in man might be made with a certain degree of confidence. This is done in Fig. 3.

Second, the yields of reciprocal translocations produced in spermatogonia stem cells and recovered in primary spermatocytes are only 30% of the yield of dicentrics seen in peripheral leukocytes. This discrepancy is seen in both the mouse and Chinese hamster. Since both exchange classes are expected to be produced in equal frequencies in both tissues, it appears that there is a strong selection operating against germ cells carrying a translocation. This possibility is currently being investigated. If it is shown to be true, the only apparent obstacle to making a direct comparison from mouse to man is the possibility that the selection process may be different in the two species.

*Mathematics Division, ORNL.

1. J. G. Brewen and R. J. Preston, *Biol. Div. Ann. Progr. Rept.*, June 30, 1971, ORNL-4740, p. 60.
2. R. J. Preston, J. G. Brewen, and W. M. Generoso, this report, p. 69.

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

Diana B. Smith

Cells resistant to BrdUrd, an analog of thymidine, were found to be UV-sensitive and are reported

elsewhere. In order to see if exogenous thymidine had an analogous effect on survival and on mutation to 8-azaguanine resistance, experiments involving post-treatment with or without thymidine were conducted. The results of these experiments using a fibroblast wild-type cell line (V79-4) show that thymidine at 10 $\mu\text{g}/\text{ml}$ slightly decreased survival and enhanced mutation frequencies (Table I). Whole fetal calf serum contains thymidine as well as substantial amounts of purines and other molecules. The use of dialyzed serum has a noticeable effect on survival and mutation.

The experimental procedure was to inoculate 100 or 10^5 cells in 100 mm plates containing 10 ml medium. After 2 hr to allow attachment, the plates were rinsed with saline and then UV irradiated in 1 ml saline with two 15-watt germicidal lamps at a dose rate of 10 ergs/sec as determined with a Jagger meter. The saline was replaced with medium, plus or minus thymidine. Forty-two hours later 10 $\mu\text{g}/\text{ml}$ 8-azaguanine was added to the plates containing 10^5 cells. The plates were scored for number of surviving clones 10–14 days later.

The amount of thymidine used was not sufficient to cause a significant difference in plating efficiency or clone size in control plates. The effect of larger, but sublethal, doses of thymidine has not been determined for survival and mutation induction, but may give quite different effects at slightly inhibitory, but nonlethal, doses.

The results indicate that factors affecting thymidine metabolism are important in mammalian cell survival and mutation recovery after UV irradiation. Genetic or epigenetic traits affecting thymidine utilization should

TABLE I. The effect of exogenous thymidine (10 $\mu\text{g}/\text{ml}$) on survival and mutation frequencies after UV irradiation

Medium	UV dose (ergs/mm ²)	dThd	Survival fraction	Mutants	Survivors	Mutants/10 ⁵ Survivors
RPMI1640 +	0	–	100.00	7	453,000	1.55
5% whole fetal calf serum	100	–	44.20	52	968,600	5.37
	0	+	100.00	13	535,850	2.43
	100	+	41.46	113	907,700	12.45
RPMI1640 +	0	–	100.0	5	442,260	1.13
5% whole fetal calf serum	100	–	35.50	23	505,760	4.55
	0	+	100.00	26	457,000	5.69
	100	+	34.40	73	471,600	15.48
RPMI1640 +	0	–	100.00	2	910,000	.22
5% dialyzed fetal calf serum	50	–	81.54	5	742,000	.69
	100	–	30.32	4	276,000	1.45
	0	+	100.00	10	1,032,000	.97
	50	+	72.29	12	746,000	1.61
	100	+	18.60	16	192,000	8.33

also show a differential survival to UV — the direction depending on the site of the defect.

TWO FORMS OF REPAIR IN HUMAN DNA

James D. Regan and R. B. Setlow

Using the 5-bromodeoxyuridine photolysis method (1, 2), we have examined repair in human cell DNA after damage by ultraviolet (UV) radiation and after damage by ionizing radiation. The repair processes generated by these two agents differ markedly in our assay. UV radiation appears to induce a lesion, namely a pyrimidine dimer, which results in a topological perturbation of the normal DNA configuration. This perturbation is presumably acted upon by the UV-endonuclease which causes a single strand break in the DNA. Following this break there is extensive excision of bases resulting in a gap amounting to ~100 nucleotides. The gap is filled, presumably by DNA polymerase, and then closed by action of a ligase. UV repair may extend over 20 hr after the initial radiation damage.

DNA strand breaks are induced, directly or indirectly, by ionizing radiation. There is no extensive excision of bases. Sixty minutes after irradiation the DNA has returned to control molecular weight values.

Thus significant differences exist in the DNA repair sequence induced, depending on the agent which causes

the initial damage and perhaps the topological configuration of the lesion. These two repair schemes are diagrammatically represented in Fig. 1.

1. J. D. Regan, R. B. Setlow, and R. D. Ley, *Proc. Nat. Acad. Sci. U.S.A.* **68**: 708–712 (1971).
2. J. D. Regan, R. B. Setlow, M. M. Kaback, R. R. Howell, Edmund Klein, and Gordon Burgess, *Science* **174**: 147–150 (1971).

DNA CHAIN ELONGATION AND JOINING AFTER ULTRAVIOLET IRRADIATION IN NORMAL HUMAN AND XERODERMA PIGMENTOSUM CELLS

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

DNA synthesized by UV-irradiated mammalian cells is initially in smaller pieces than that synthesized by unirradiated cells. The shorter segments elongate and join during incubation after irradiation. This process is inhibited by 10^{-3} M hydroxyurea. To determine if the mechanism of chain elongation and joining involves *de novo* DNA synthesis, the cells were incubated in bromodeoxyuridine after UV irradiation, photolysed by 313 nm radiation, and the DNA sedimented in alkaline sucrose. If *de novo* DNA synthesis is involved, the bromodeoxyuridine will be incorporated into the gaps between the small pieces during chain elongation and joining, and the gaps would be reopened by photolysis to yield the short chains present immediately after UV irradiation. Our results for normal human fibroblasts agree with this expectation. This repair process was also examined in cells from three patients with xeroderma pigmentosum — a disease characterized by extreme sensitivity to sunlight. One patient had uncomplicated xeroderma pigmentosum; the second had de Sanctis-Cacchione syndrome; and the third was one having normal unscheduled DNA synthesis but xeroderma pigmentosum pathology. All three types of cells were able to elongate and join the discontinuous DNA strands synthesized after UV irradiation by a process involving *de novo* DNA synthesis.

DNA REPLICATION IN HUMAN CELLS TREATED WITH METHYL METHANESULPHONATE

Steven N. Buhl and James D. Regan

Human cells treated with methyl methanesulphonate (MMS) have reduced rates of DNA synthesis and synthesize DNA in smaller segments than untreated cells. Normal human and xeroderma pigmentosum cells

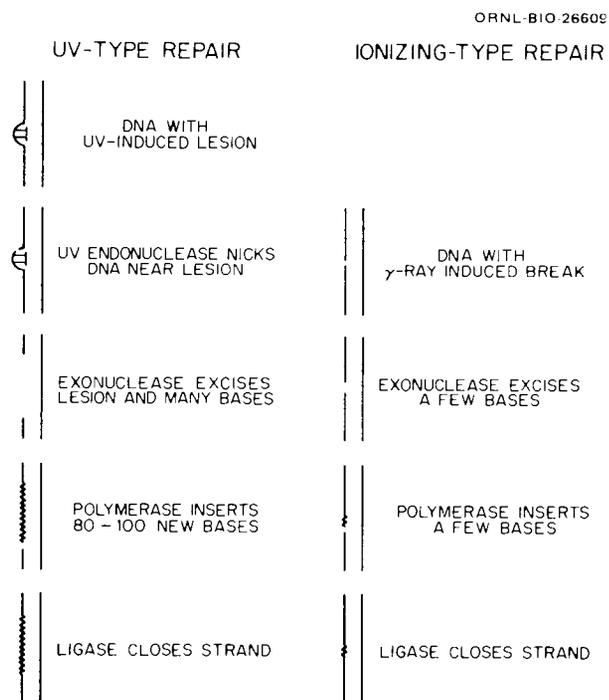


Fig. 1. Schematic diagrams that illustrate two types of repair in human cells.

were incubated in [^{14}C]thymidine for 24 hr, 10^{-4} M MMS for 1 hr, and pulse labeled for 30 min with [^3H]thymidine at various times following the treatment before lysis and sedimentation in 5–20% alkaline sucrose gradients. The $^3\text{H}/^{14}\text{C}$ ratio, which measures relative amounts of DNA synthesis, decreased with increasing time after treatment with MMS. The units of DNA synthesized within the first 2 hr of the treatment were of equal size and of intermediate molecular weight in the treated and untreated cells, whereas 2–4 hr after treatment the unit of DNA synthesized by treated cells was much smaller than the unit synthesized by untreated cells. However, the pulse label 24 hr after treatment with MMS was again incorporated into DNA units of intermediate molecular weight in both treated and untreated cells. Incubation of pulse labeled cells in nonradioactive medium for 24 hr before sedimentation allowed for elongation and joining of these intermediate and short segments into high molecular weight DNA in treated and untreated cells.

BIOCHEMICAL ANALYSIS OF HUMAN GENETIC DEFECTS

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

The main research effort in the initial phase of this study — a search for undescribed inborn errors of metabolism in man — consists of two general areas: (1) the screening and chromatographic analyses of selected high genetic risk patients, and (2) the isolation and identification of metabolites presumably involved in genetic lesions. The principal experimental material is serum and urine of mentally retarded children.

At this point, over 200 samples have been channeled through the entire screening process. This consists of preliminary screening using various colorimetric tests to exclude previously described genetic disorders, cation exchange chromatography of serum and urine for ninhydrin-positive substances, and anion exchange chromatography for UV-absorbing compounds.

Out of these samples, a number of suspect cases have been detected and are being investigated in depth. The secondary step involves identification of unknown accumulants by further ion exchange chromatography, UV spectroscopy, gas chromatography, and mass spectroscopy. Much of the preliminary work was carried out with the cooperation of the Body Fluids Analysis Program at ORNL. At this point, however, we have been able to set up a comparable gas chromatographic facility and preparative chromatographic system within

the Biology Division. In addition, the screening capability will be increased by the completion of a second high-resolution UV analytical system.

A partial summary of the results to date includes a sample showing a massive urinary accumulation of a metabolite tentatively identified as a quinoline derivative; another, apparently deficient or extremely low in uric acid; and, in addition, a large number of samples showing various accumulates as yet unidentified. A number of samples from patients with known disease states or under known drug therapy have been useful; for example, the chromatographic position and characteristics of urinary L-DOPA derivatives have been defined by use of a sample from an individual using this medication.

The initial results show the feasibility of the UV system as a screening tool for abnormal urinary compounds. Subsequent activities will attempt to extend these observations to the metabolic basis for the presumptive genetic lesion.

REPAIR OF RADIATION-INDUCED DAMAGE TO THE DNA OF *NEUROSPORA CRASSA*

T. E. Worthy* and J. L. Epler

A method has been described for labeling a specific pyrimidine in the DNA of *Neurospora crassa* (1). In cells grown in the presence of [$5\text{-}^3\text{H}$]uridine, more than 97% of the radioactivity associated with the DNA had been incorporated into cytosine. The specific activity of the labeled DNA was 3×10^3 cpm per μg . The DNA was isolated by elution from hydroxyapatite columns with phosphate buffer (0.40 M, pH 6.8).

This procedure was used to demonstrate and characterize the excision repair system in *N. crassa*. Ultraviolet-induced pyrimidine dimers are removed with approximately first-order kinetics. The repair system, based on behavior in the presence of known inhibitors of repair, resembles that found in mammalian systems.

The labeling procedure was utilized to study the repair of single-strand breaks in the DNA induced by gamma irradiation. Repair appears to be dependent on incubation in growth medium; incubation in buffer resulted in little or no repair. The kinetics of repair of single-strand breaks is first-order; greater than 90% of the breaks were rejoined in the first 5–10 min of incubation. The efficiency of strand breakage in air is approximately the same as reported for both bacteria and mammalian cells.

The seven available radiation-sensitive mutants of *N. crassa* have been examined for photoreactivation exci-

sion repair and strand-rejoining repair. From this screening, UVS-6 was selected and the influence of this gene on the frequency of nonreciprocal recombination determined.

*Predoctoral investigator, Institute of Radiation Biology, University of Tennessee and Graduate Laboratory Participant under appointment from ORAU.

1. T. E. Worthy and J. L. Epler, *J. Bacteriol.* **110**: 1010–1016 (1972).

INDUCTION OF AUXOTROPHIC MUTATIONS BY TREATMENT OF CHINESE HAMSTER CELLS WITH 5-BROMODEOXYURIDINE AND BLACK LIGHT*

E. H. Y. Chu,[†] N. C. Sun,[†] and C. C. Chang[†]

Treatment of an aneuploid Chinese hamster cell line (V79) in tissue culture with 5-bromodeoxyuridine and black light, with or without prior exposure of cells to another mutagen, led to the isolation of auxotrophic mutants, each exhibiting a specific nutritional requirement for glycine, uridine, purine, or a combination of glycine, hypoxanthine, and thymidine. Sixty-one mutants that could not utilize exogenous galactose were also isolated. Various lines of evidence indicate that the combined treatment of BrdUrd and visible light, which was originally thought of primarily as a selective procedure, is an effective method for inducing mutations in mammalian cells.

*This research was jointly sponsored by the National Cancer Institute and the U.S. Atomic Energy Commission.

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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 thermotolerant cells were treated with ethyl methanesulfonate, plated at 37° 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 which 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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AMINO ACID SEQUENCE OF THE BETA CHAIN OF SINGLE HEMOGLOBIN FROM C57BL, SWR, AND NB MICE

R. A. Popp and E. G. Bailiff

C57BL is a common strain of laboratory mice and is often used as a reference for comparison in genetic studies. C57BL hemoglobin is electrophoretically homogeneous in starch gels in contrast to the diffuse hemoglobin pattern obtained for many other strains of laboratory mice. The sequence of amino acids in the beta chain of C57BL hemoglobin is given in Table I. Comparative studies were done for the majority of residues in the beta chains of the single hemoglobins from SWR and NB mice. They were indistinguishable except for an inversion of two amino acids at residues $\beta 72, 73$. Whereas the C57BL beta chain has a serinyl-aspartyl sequence, both SWR and NB beta chains have the aspartylserinyl sequence. A minimum of four base-pair substitutions are required to change the code words for the reverse sequences obtained; i.e., AG_C^U GA_C^U to GA_C^U AG_C^U . Although the beta chains of the single hemoglobins from these three strains of mice each appear to be homogeneous, the Hbb^{sC57BL} gene is clearly an allele of the Hbb^{sSWR} or NB gene.

TABLE I. Amino acid sequence of the beta chain of hemoglobin from C57BL mice

					5					10					15
1	Val	His	Leu	Thr	Asp	Ala	Glu	Lys	Ala	Ala	Val	Ser	Gly	Leu	Trp
16	Gly	Lys	Val	Asn	Ala	Asp	Glu	Val	Gly	Gly	Glu	Ala	Leu	Gly	Arg
31	Leu	Leu	Val	Val	Tyr	Pro	Trp	Thr	Gln	Arg	Tyr	Phe	Asp	Ser	Phe
46	Gly	Asp	Leu	Ser	Ser	Ala	Ser	Ala	Ile	Met	Gly	Asn	Ala	Lys	Val
61	Lys	Ala	His	Gly	Lys	Lys	Val	Ile	Thr	Ala	Phe	Ser	Asp	Gly	Leu
76	Asn	His	Leu	Asp	Asn	Leu	Lys	Gly	Thr	Phe	Ala	Ser	Leu	Ser	Glu
91	Leu	His	Cys	Asp	Lys	Leu	His	Val	Asp	Pro	Glu	Asn	Phe	Arg	Leu
106	Leu	Gly	Asn	Met	Ile	Val	Ile	Val	Leu	Gly	His	His	Leu	Gly	Lys
121	Asp	Phe	Thr	Pro	Ala	Ala	Gln	Ala	Ala	Phe	Gln	Lys	Val	Val	Ala
136	Gly	Val	Ala	Ala	Ala	Leu	Ala	His	Lys	Tyr	His	*			

AMINO ACID SEQUENCES OF THE MAJOR AND MINOR BETA CHAINS OF DIFFUSE HEMOGLOBINS FROM BALB/c MICE

R. A. Popp and E. G. Bailiff

The ratio of major to minor forms of hemoglobins in strain BALB/c mice is approximately 85/15. Last year we reported (1) four amino acid substitutions within the first 60 residues of the major and minor beta-chain polypeptides. Structural analyses of the remainder of the two beta-chain polypeptides reveal two additional amino acid differences. Table I summarizes the amino acid substitutions found among the mouse-hemoglobin beta chains analyzed to date. The hemoglobin band that migrates more rapidly on starch gels than the major component arises as a consequence of the formation of intermolecular disulfide bonds at the cysteinyl residues at position β 13. The hemoglobin band that migrates more slowly on starch gels than the major hemoglobin contains the minor beta-chain polypeptides, and the major hemoglobin contains the major beta-chain polypeptides. The basis for the different electrophoretic mobilities of the major and minor components of diffuse mouse hemoglobin is that at pH 8.6 the lysinyl residue at β 76 of the minor beta-chain polypeptide is protonated, whereas that histidinyl residue in the major beta-chain polypeptide is not protonated.

1. R. A. Popp and E. G. Bailiff, *Biol. Div. Ann. Progr. Rept.*, June 30, 1971, ORNL-4740, p. 64.

ERROR FREQUENCY FOR HEMOGLOBIN SYNTHESIS IN AGED MICE

G. P. Hirsch and R. A. Popp

A simple procedure has been developed for the isolation of highly purified hemoglobin and the deter-

TABLE I. Positions and kinds of amino acid replacements found in the β -chains of mouse hemoglobins*

Residue Number	β -Chain Polypeptides			
	C57BL	SWR	BALB/c major	BALB/c minor
9	Ala	Ala	Ala	Ser
13	Gly	Gly	Cys	Cys
16	Gly	Gly	Gly	Ala
20	Ala	Ala	Ser	Pro
58	Ala	Ala	Ala	Pro
72	Ser	Asp	Asp	Glu
73	Asp	Ser	Ser	Ser
76	Asn	Asn	His	Lys
77	His	His	Asn	Asn
139	Ala	Ala	Thr	Thr

*For remainder of sequences see Table I, preceding abstract.

mination of the error frequency of protein synthesis *in vivo*. The method utilizes the unique responses of oxyhemoglobin to treatment with potassium ferricyanide and of methemoglobin to potassium cyanide. Since few proteins are affected by these reagents, hemoglobin of different forms can be recovered in good yield from separate regions of the same chromatographic fractionation free from contaminants. The error frequency values are measured for the incorporation of tritiated isoleucine into positions 33 to 64 of the alpha chain by sequencing the alpha-chain fragment containing residues 33 to 141 which contains no "coded" isoleucine. Error frequencies are about 0.5% for phenylhydrazine-induced hemoglobin synthesis.

In addition to error-frequency determinations, the procedure allows estimation of the production of base-substitution somatic mutations. Incorporation of isoleucine into "non-coded" positions can occur in every cell and position by "errors" of the protein

synthetic machinery, but in addition some substitutions can take place by mutation such that individual cells would contain hemoglobin with one-fourth substituted molecules. By comparing the substitution frequencies of "error only" positions with those that contain both sources of substitution after mutation-inducing treatment, mutagenesis can be estimated. The method developed is currently being used to test the error theory of aging and to assay for the accumulation of somatic mutations in adult and old mouse reticuloendothelial stem cells.

RED CELL LIFE-SPAN IN C57BL MICE OF DIFFERENT AGES

Mary W. Francis, R. A. Popp, and L. H. Smith

The hematocrit values and erythrocyte numbers decline with age in strain C57BL mice. This decrease may be due to a shortened circulating life or to a reduced rate of hematopoiesis in aging mice. We wished to determine whether the life-span of erythrocytes produced by aged mice was altered or if the microenvironment of the aged mouse had any adverse effect on circulating erythrocytes produced by younger mice.

⁵¹C-labeled erythrocytes from young (10-week-old) and adult (17-month-old) donors were infused into recipients 3, 7, and 21 months of age. The life-spans of the erythrocytes from both ages of donors were similar in recipients of all three age groups. The $t_{0.5}$ was approximately 18 days and the $t_{0.05}$ was approximately 50 days for each combination studied. Thus, the decreased hematocrit values in aged mice possibly result from decreased hematopoiesis, but it does not seem to be caused by a reduced life-span of erythrocytes produced and circulating in aged mice.

IN VIVO LYMPHOCYTE TRANSFORMATION INDUCED BY H-2D and H-2K ANTIGENS

Diana M. Popp

In vivo lymphocyte transformation was used to permit histological evaluation of lymphocyte response to antigens of the D or K end of the H-2 locus. Congenic lines B10(2R), B10(5R), and B10.A and inbred line B10 were used. Lethally irradiated mice received lymph node lymphocytes differing from the recipients by the gene product of the D or K end only or the D+K end. Tissues removed at daily intervals were used for histologic evaluation and enumeration of large pyroninophilic cells (LPC's) and mitotic figures. For K end and D+K end differences, the appearance of LPC

and mitotic figures in splenic follicles was immediate, peaking by days 3 and 4; the K end only response was $\frac{1}{2}$ to $\frac{1}{3}$ that of the D+K combination. Swelling of the follicles occurs early, LPC's appear in the red pulp on day 3 and follicular disorganization appears by day 4. On day 6, sparsely populated follicles contain pockets of untransformed cells in the periphery. For D end only, the appearance of LPC's and mitotic figures was delayed, peaking at day 5. Splenic follicles retain normal size until day 5 when disintegration of normal architecture occurs. On day 6 untransformed cells are found in the thymic dependent regions. The data show that antigens determined by the extremities of the H-2 locus induce different immune responses. The time and site of transformation suggest a correlation with T and B cell activation.

THE EFFECT OF RADIATION-SENSITIVE MUTATIONS ON THE FREQUENCY OF ULTRAVIOLET-INDUCED MUTATION AT THE *ad-3A* AND *ad-3B* LOCI

F. J. de Serres

The effect of UV was studied on *uvs-2*, *uvs-3*, *uvs-4*, *uvs-5*, *uvs-6*, and *upr-1* in comparison with wild-type strains of *Neurospora crassa*. These experiments showed that *uvs-2* and *upr-1* are more sensitive to mutation-induction than wild-type; whereas *uvs-3*, *uvs-4*, and *uvs-5* are more resistant. In fact, with *uvs-3* it was not possible to demonstrate a significant increase over the spontaneous frequency with any exposure level tested. In all of these *uvs* strains the mutation-induction curves saturated at lower forward-mutation frequencies than in the wild-type strain. Experiments with *uvs-6* showed that it responds exactly the same as the wild-type strain with regard to mutation-induction after UV. Samples of *ad-3* mutants induced in each *uvs* strain have been saved for genetic analysis.

THE EFFECT OF RADIATION-SENSITIVE MUTATIONS ON THE FREQUENCY OF GAMMA RAY-INDUCED MUTATION AT THE *ad-3A* AND *ad-3B* LOCI

M. E. Schüpbach and F. J. de Serres

The effect of sparsely ionizing radiation on mutation-induction in *uvs* strains of *Neurospora crassa* was studied with *uvs-2*, *uvs-3*, *uvs-4*, *uvs-5*, *uvs-6*, and *upr-1* in comparison with wild-type strains. These experiments showed that *uvs-2* and *upr-1* are also more sensitive to mutation-induction than wild-type after

gamma irradiation. Both *uvs-4* and *uvs-5* are more resistant than wild-type, but the difference is not as marked as in the experiments with UV. The experiments with *uvs-6*, however, show that it is much more sensitive than *uvs-2* or *upr-1*. *Uvs-3* appears to show the same sensitivity as wild-type to mutation-induction after gamma irradiation. Samples of *ad-3* mutants induced in each *uvs* strain have been saved for genetic analysis.

THE EFFECT OF DIFFERENT WAVELENGTHS OF ULTRAVIOLET ON MUTATION-INDUCTION IN THE *ad-3* REGION OF A TWO-COMPONENT HETEROKARYON OF *NEUROSPORA CRASSA*

F. J. de Serres and R. B. Setlow

Previous experiments with UV (2537 Å) on a two-component heterokaryon of *Neurospora crassa* have shown that both point mutations and chromosome deletions in the *ad-3* region increase as the square of the UV exposure, and are found in a ratio of about 19:1. After photoreactivation with visible light this ratio changes to about 34:1, showing that point mutations and chromosome deletions result from qualitatively different lesions after UV. Whether these result from qualitatively different lesions to DNA or to other structural components of the chromosome is not known. To investigate this problem further we have irradiated this heterokaryon with four different wavelengths of UV to determine whether different wavelengths produce different spectra of *ad-3* mutations. Irradiation with 2600 Å (DNA absorption peak) was compared with 2800 Å (protein absorption peak) and with irradiations at 2650 Å and 3130 Å to provide additional points of comparison. *Ad-3* mutants were obtained with exposures to the first three wavelengths but not the last. Samples of *ad-3* mutants were reserved for genetic analysis to determine primarily whether the ratio of point mutations to deletions has been altered, but also whether any other differences will be found between the three groups of mutants.

THE EFFECT OF LOW DOSE RATES AND LOW DOSES ON THE SPECTRUM OF X-RAY-INDUCED MUTATIONS IN THE *ad-3* REGION

F. J. de Serres

In previous experiments we compared the effect of X-irradiation at 1000 r/min and 10 r/min at doses of 5,

10, 20, and 40 kr. These experiments showed that there was no effect of dose rate on the induction of point mutations (1-hit events), but that a 100-fold reduction in dose rate resulted in a 10-fold decrease in the frequency of multilocus deletions (2-hit events). At the 10 r/min dose rate, the slope of 2.0 per multilocus deletions obtained at high dose changes to a slope of about 1.0 at low doses. Additional exposures at 1000 r/min and 10 r/min have been performed to investigate these dose-effect curves between 0.1 and 2.5 kr. These additional data should make possible a more realistic comparison of the *Neurospora* and mouse specific-locus data at high and low dose rates. This experiment should make it possible to determine whether a significant fraction of the specific-locus mutants at low doses result from multilocus deletion and whether these mutations show 1-hit kinetics at low doses. Samples of *ad-3* mutants from each dose have been reserved for genetic analysis by means of heterokaryon tests to determine genotype and allelic complementation, and dikaryon and trikaryon tests to distinguish point mutations from multilocus deletions.

DEVELOPMENT OF TEST SYSTEMS BASED UPON DRUG RESISTANCE IN *NEUROSPORA*

G. R. Hoffmann

In screening for mutagenic activity of environmental compounds, it is important to have forward mutation screening systems capable of detecting a variety of genetic alterations. Such a test system, based upon drug-resistance in *Neurospora crassa*, has recently been developed.

Mutants resistant to 8-azaguanine and 5-fluorodeoxyuridine were induced in component-I of heterokaryon-12 (the standard heterokaryon of the *ad-3* test-system). All the mutants tested were found to be recessive when incorporated into heterokaryons.

Conducting mutation experiments in heterokaryons which are heterokaryotic for a resistance marker should allow detection of both point-mutations and deletions for a specific locus.

While selection for forward mutations to 5-fluorodeoxyuridine resistance can be done clearly and simply in homokaryotic strains, background growth in the presence of the inhibitor has prevented effective selection in heterokaryons. Selection for 8-azaguanine-resistance, however, can be carried out in either homokaryons or heterokaryons under carefully defined selection conditions. Preliminary experiments with ethyl methane-sulfonate indicate a considerably higher mutation fre-

quency in the 8-azaguanine system than in the *ad-3* system.

In addition to the development of a new system for the detection of forward mutations, growth experiments and linkage group determinations are being carried out with the new drug-resistant mutants.

DEVELOPMENT OF ASSAY SYSTEMS FOR DETECTION OF SOMATIC MUTATIONS IN VIVO IN MAMMALS

H. V. Malling

Chromosome aberrations in somatic cells are known to increase with aging of mammals and to be induced by treatment with chemical mutagens or ionizing radiation. It is unlikely that point mutations should not be induced simultaneously. However, no screening system has yet been developed to detect point mutations *in vivo* in mammals although several investigators are working on this problem. Attempts have been made to develop a testing system based on cytochemical stainings of dehydrogenases with tetrasolium salt and use of aberrant substrates or coenzymes. If such systems could be developed they would be extremely important in understanding of the role of somatic mutations in aging and cancer, in evaluation of genetic damage after accidental radiation exposure, and in monitoring of the human population.

TESTING FOR THE MUTAGENICITY OF IRRADIATED PAPAYAS FED TO RATS IN A HOST-MEDIATED ASSAY WITH *SALMONELLA TYPHIMURIUM* AS THE INDICATOR ORGANISM

H. V. Malling

Treatment of food with ionizing radiation can both decrease the economical losses associated with either storage or transportation and can also lower the probability of spreading insect pests from one geographical area to another. Most foods are complex mixtures of substances that might undergo chemical changes during irradiation, leading to formation of toxic compounds, some of which might be mutagens or potential mutagens. Thus the large-scale use of irradiation to process food requires adequate scientific data to permit evaluation of its safety for the consumer. A mammal can either detoxify a given compound or it can form mutagenically active metabolites from nonmutagenic compounds. By using the host-mediated assay in which an indicator organism is injected into the peritoneal cavity of rodents, the indicator organism is

exposed not only to the original compound but also to its metabolites. When the indicator organism is recovered and checked for the presence of mutations, it is possible to determine whether any mutagenic compounds or metabolites were present during incubation of the indicator in the host animal.

The histidine-requiring mutant (G-46) of *Salmonella typhimurium* was used as an indicator organism, and the mutants were scored as reverse mutations to histidine-independence. Rats were fed irradiated or nonirradiated papayas for a period of 8–12 weeks prior to injection of the bacteria. Dimethylnitrosamine (DMN) was used as a positive control because this compound had earlier been shown to induce reversions in strain G-46 in the host-mediated assay, but not *in vitro* under nonhydroxylating conditions. The present experiments showed no increase in the frequency of histidine revertants of *S. typhimurium* incubated in the peritoneal cavity of rats being fed diets of irradiated papayas over nonirradiated controls. The number of revertants per 10⁸ survivors in the positive DMN control was 39 and 30 times greater than the number of reversions in the normal-food control.

MUTAGENICITY OF HYCANTHONE

Tong-man Ong and F. J. de Serres

Hycanthone, a chemical compound useful in the treatment of schistosomiasis, is attracting wide concern because of its potential carcinogenic and mutagenic hazard to treated individuals in the human population. The mutagenicity of this compound was tested in the *ad-3* system of *Neurospora crassa*. The results show that hycanthone is mutagenic. The mutation frequency is increased about 20-fold over the spontaneous mutation frequency when the conidia are treated with 0.3 mM of hycanthone.

SUPPRESSORS OF A MINUTE IN *DROSOPHILA MELANOGASTER*

Carroll Nix

Suppressors of *M(2)173* were produced in *Drosophila melanogaster* by irradiation, with the idea that most would be tandem duplications and that some would be large enough to include region 56E-F of the salivary map. Since 56E-F is presumed to be the locus for the 5S RNA genes, these large duplications were of interest for dosage studies using DNA-RNA hybridization.

Out of six suppressors recovered, only two proved to be large tandem duplications; the other four presumptive duplications were not cytologically discernible. In

TABLE I. Crossover frequency*

	$nw^D Su$	$Su Pu^2$	$Pu^2 Pin^{Yt}$	$Su Pin^{Yt}$	$nw^D \cdot Pin^{Yt}$	N
Su^1	3.21	0.19	10.21	10.40	13.61	529
Su^3	8.60	3.39	14.71	18.33	26.70	442
Su^5	9.05	1.90	17.14	19.05	28.10	420
Su^6	9.15	0.304	13.41	13.72	22.87	328

*Genetic position according to D. L. Lindsley and E. H. Grell, *Genetic Variations of Drosophila melanogaster*, Carnegie Inst. Wash. Publ. No. 627, 1968. nw^D , 2-83.0; $M(2)I73$, 2-92.3; Pu^2 , 2-97.0; Pin^{Yt} , 2-107.3.

order to locate these suppressors, a crossover test was used. Females of genotype bw Suppressor/ $nw^D Pu^2 Pin^{Yt}$ were crossed to $M(2)I73/SM5$ males. Only $Su/M(2)I73$ progeny were scored. Although the numbers are small, it can be seen from Table I that all four suppressors mapped very close to $M(2)I73$. Except in the case of Su^1 there was little or no effect on crossing over. In the case of Su^1 there was approximately a 50% reduction; however, it remains to be seen what is causing the reduced crossing over.

In addition to mapping the $M(2)I73$ suppressors, other experiments were done in order to localize $M(2)I73$ on the salivary map. By using various Y -autosome translocations with different autosomal break points (I), it was possible to make a deletion map of the salivary region 56D-57B.

It was shown that $M(2)I73$ is located in the region defined by the breakpoints 56F-57A. It is also of interest that deficiencies for the region 56E-56F and 56D-56F do not show a Minute phenotype. Thus hypoploidy for the presumptive 5S RNA genes does not give a dominant phenotype.

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GENETICS OF AN ENZYME WITHOUT NATURAL VARIANTS IN *DROSOPHILA MELANOGASTER*

E. H. Grell

Before this work, the development of a genetics of an enzyme in *Drosophila* was limited to enzymes in which inherited variants could be found. In surveys of genetic variability, it has been found that about 50% of the enzymes are variable as judged by gel electrophoresis. That leaves 50% with which there is no easy starting point for a genetic study. The most direct method of obtaining a genetic variant is to induce one with a mutagen. If the enzyme can be detected on gel

electrophoresis, then a mutant enzyme with altered migration on the gel can be selected. This is straightforward theoretically, but in practical terms it requires marking and following all three major chromosomes. Any partial location would be helpful. There is often an electrophoretic difference between enzymes from *D. melanogaster* and *D. simulans*. They are closely related species, but the F_1 hybrids are sterile. A genetic locus can be assigned to a chromosome arm on the basis of data obtained from X-ray-induced partial hybrids. The genetic locus of one of the isozymes of glutamic-oxaloacetic transaminase (GOT) was assigned to the left arm of the second chromosome on the basis of the partial hybrids. This location was used in mutant searches by treating attached-2L flies with ethyl methanesulfonate. F_1 males with the treated 2L were selected and mated to raise an F_2 . One F_2 offspring from each F_1 male was selected and electrophoresed on acrylamide gel to look for mutant enzyme.

From a total of 320 flies examined by electrophoresis, one culture with a GOT mutation was recovered. The mutant GOT migrates more rapidly to the anode than does the standard *D. melanogaster* GOT. Not all flies with the marked chromosome in that culture carried the mutant, so the father was mosaic for mutant and nonmutant germ cells. The genetic locus of the mutation is near the tip of the left arm of the second chromosome, with a map position of 2.5. The salivary gland chromosome location was determined from testing flies with fragments of 2L added to the normal chromosome complement. Fragments without the gene for the enzyme were judged to be broken distal to the gene, and those with the gene were judged to be broken proximal to the locus. The gene appears to be in the last band to section 22A or the first two bands of region 22B.

Mutants may be induced in any enzyme that can be detected on gel electrophoresis. Some procedures are more successful than others. In the search for a GOT mutant, F_2 flies were examined; in a previous search for

alcohol dehydrogenase (ADH) mutants, F_1 flies were examined. The search for a GOT mutant yielded one in 320 cultures; the search for the ADH mutant yielded one in 5800 cultures. Several of the F_1 flies from the ADH experiment obviously carried mutant tissue, but the mutation did not extend into the germ cells and so the offspring did not have it. Testing of F_2 flies provides for resolution of mosaicism and nearly all that are detected can be recovered. The use of the attached chromosomes has the advantage that two genes are treated in each sperm, and each fly tested represents two treated genes rather than only one. The testing of 320 flies represented the testing of 640 genes. The tests are laborious so that the factor of 2 is a significant magnification of effort. Mutants of this GOT enzyme with a greater variety of effects may now be sought. Stocks have been constructed to detect mutations which inactivate the enzyme as well as those that alter its electrophoretic migration.

THE SUPPRESSOR OF FORKED LOCUS OF *DROSOPHILA MELANOGASTER*

E. H. Grell

In previous work, the suppressor of forked locus was known from one mutant allele, and was recognized by its effect on forked mutants, restoring them to a nearly normal phenotype. It also has an enhancing effect on the phenotype of apricot (w^a), lightening the eye color of apricot to white. It was known to have one other property: when suppressor of forked, $su(f)$, is heterozygous with a deficiency of the locus, the flies have short bristles similar to the phenotype of the bobbed mutant. In order to study this locus a series of its mutations was induced with ethyl methanesulfonate (EMS).

A total of eight $su(f)$ mutations were induced. They may be divided into three categories: (1) recessive lethals, (2) short bristled, and (3) normal bristled. The three recessive lethals are suppressors of forked when they are heterozygous with a viable allele of $su(f)$. Their presence in the homozygous or hemizygous state prevents the survival of the fly into the adult stage. The three recessive lethals are not identical and have various amounts of wild-type activity for the apricot enhancer. Even the recessive lethal with the least amount of normal activity of the apricot enhancer is not equivalent to a deficiency. The two small-bristled mutants are more vigorous than bobbed mutants, neither being strong enhancers of apricot. The heterozygotes of the small-bristled mutants with a deficiency of either of the two more extreme recessive lethals are also lethal. The

normal-bristled suppressors are variable in their suppression of forked. Some homozygotes suppress only about half of the forked bristles on a fly, while others suppress most forked bristles.

The $su(f)$ locus has two properties that were previously unknown: (1) it can mutate to small-bristled mutants and to recessive lethals, and (2) its function is necessary for the viability of the fly. The strong apricot enhancement of the original $su(f)$ is unusual in that none of the present series is a more than moderately effective enhancer. In the present series the suppression of forked was the object of selection, and to obtain enhancers of apricot one would have to select for such enhancers.

The genetic location of $su(f)$ is adjacent to the bobbed locus, the locus of the 18 and 28S RNA subunits of ribosomes. Unlike bobbed, it is an ordinary sex-linked gene in that it is not present on the Y chromosome as is bobbed. Unlike bobbed, the $su(f)$ locus shows strong dosage compensation between male and female. A male with one dose of $su(f)$ appears to have normal phenotype, but a female with only one dose of $su(f)$ has small bristles. Similarly, the new EMS-induced small-bristled recessives are viable as males but lethal when heterozygous with a deficiency in females.

MEIOTIC EXCHANGE WITHOUT THE SYNAPTONEMAL COMPLEX

Rhoda F. Grell, H. L. Bank,* and George Gassner III†

The synaptonemal complex is a tripartite structure associated with chromatin material. It is normally restricted to meocytes and has been functionally interpreted by many investigators as playing a role in meiotic exchange. The evidence for this assumption is circumstantial, resting primarily upon the presence of the complex in primary meocytes whenever genetic or cytological evidence indicates that exchange is occurring. In the higher Diptera, meiotic crossing over has been eliminated in the males of virtually all species with the exception of *Drosophila ananassae* where in some strains exchange has been restored. An electron microscope examination of the testes of *D. ananassae* males was undertaken to determine if the complex is present. In conjunction with the cytological investigation, concurrent genetic studies measured the frequency of crossing over in such males.

The frequency of crossing over between the markers pe and stw on chromosome 3 was $10.1 \pm 0.7\%$ in the male and $37.0 \pm 1.3\%$ in the female, in good agreement with published reports of 13.1% and 38.0%, respec-

tively (*I*). The electron microscope studies were carried out independently in two laboratories. In the first study (H.L.B.), ten thin serial sections ($\sim 500\text{\AA}$), preceded and followed by $1\text{-}\mu$ sections of the gonadal tissue, were cut. Identification of both thick sections as primary spermatocyte by light microscopy was followed by careful examination of the thin sections for the presence of the complex. Among a total of 272 cells from third instar larvae and 225 cells from late pupae, no structure identifiable as a synaptonemal complex was found. In the second study (G.G.), phase-contrast light microscopy was used to locate primary spermatocytes in 4- to $5\text{-}\mu$ sections, following which electron micrographs were made of three to five adjacent $600\text{--}700\text{\AA}$ sections. A total of 168 micrographs of 53 cells were recorded. In no case were synaptonemal complexes found. In both studies, the complex was readily located in the female gonad.

The demonstration of high levels of crossing over in the absence of the complex implies that it is not an inseparable feature of meiotic exchange. In light of this new evidence, a reevaluation of the functional significance of the complex would seem to be in order.

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HEAT-INDUCED RECOMBINATION IN THE PROXIMAL REGION OF CHROMOSOME 3

Rhoda F. Grell

The heat-sensitive period for enhancement of crossing over in the *X* and second chromosomes and for its induction in chromosome 4 coincides with the time of DNA replication in the oocyte (*I*, *2*). Further, the intensity of the heat response resembles the "interchromosomal effect" in that the greatest increases occur in distal and proximal segments. The proximal region of chromosome 3 has now been examined by exposing groups of developing females, approximately marked to measure exchange (*st in ri p^D/Sb*) to a $35 \pm 0.5^\circ\text{C}$ heat treatment for 12 hr, beginning at 114 hr after egg laying and at 6 hr intervals thereafter up to 168 hr.

As in the *X* and 2, the maximal response observed in chromosome 3 occurs in the region spanning the centromere between *ri* and *p^D* where crossing over is increased from the control level of 1.3 ± 0.24 to 19.3 ± 1.29 and 19.6 ± 0.97 with treatments initiated at 138 and 144 hr, respectively. The magnitude of this

response greatly surpasses that found elsewhere in the genome. It exceeds that elicited by the interchromosomal effect, which also induces its greatest response between *ri* and *p^D*.

The heat-sensitive period for the proximal region of chromosome 3 (treatments at 120–156 hr) coincides well with the known time of DNA synthesis in the oocyte (132–156 hr). A comparison of the responses of the centromeric regions of *X*, 2, and 3 discloses that they are temporally distinct, with the *X* peaking at treatment at 132 hr, chromosome 3 at 138 and 144 hr, and chromosome 2 at 156 hr. The degree of response varies so that for *X*, 2, and 3 the increases are 2-, 3.5-, and 15-fold, respectively.

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INDUCTION OF CROSSING OVER IN CHROMOSOME 4

Rhoda F. Grell

Normally, the fourth chromosome does not undergo exchange in the diploid female of *Drosophila*; in triploid females it does so with a low frequency. The ability of elevated temperature to enhance crossing over elsewhere in the genome suggested the possibility that heat treatment might be an effective method for inducing recombination in the 4. Utilizing the "pupal system" (*I*), temperatures 7° or 10°C above control levels were applied to developing females beginning at 96 hr after egg laying and continuing at ~ 6 hr initiation intervals until 168 hr. Treatment was given for 12 or 24 hr. The females carried either a single mutant marker in each 4 or two markers in one 4 and none in the other. The markers chosen, *ci^D* and *spa^{P01}*, were located at the proximal and distal ends of the 4, respectively, so as to provide a maximal intervening length for recognition of exchange.

Crossover progeny were recovered for parental markers in both the *cis* and *trans* configuration. Reciprocal products of exchange were obtained in \sim equal frequencies. The effective period for exchange induction was localized to treatments beginning from 132 to 150 hr after egg laying. It was therefore coextensive with the known period of DNA replication in the oocyte (132–156 hr) and coincident to a large degree with the sensitive period for enhancement of exchange (126–162 hr). During the effective period crossing over averaged 0.2–0.3%.

Of considerable interest was the recovery of anomalous products presumably associated with crossing over

since their high frequency virtually excluded a mutational origin. The new phenotypes included an alteration from spa^{P01} to the less extreme spa allele in $1/4$ of the male crossovers and induction of sterility in $1/3$ of the male crossovers. The alterations are provisionally interpreted as products of unequal crossing over between contiguous duplications and/or asymmetrical exchange between noncontiguous duplications.

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ANALYSIS OF CATALYTIC FACILITATION BY MULTIENZYME COMPLEXES: ANALOG COMPUTER SIMULATION

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

A multienzyme system may be defined as a group of two or more metabolically consecutive enzymes. A number of catalytically important functions have been suggested for observed aggregated multienzyme systems, or multienzyme complexes (1,2). In particular, we have considered the reduction of steady-state transition time (1) as a potential advantage of the aggregated state. With regard to metabolic flux, the capability of undergoing rapid, efficient transitions between states may be as significant in terms of regulation as the maintenance of the steady state *per se*. Substantiation requires comparison of *in vitro* transition-time data for existing multienzyme complexes with that for the corresponding unaggregated systems. When the latter do not exist, a suitable analytical representation for the hypothetical unaggregated system must be furnished. Consequently, we have developed an artificial simulative model which we anticipate will function in sufficient approximation when applied to the study of certain dynamic properties of multienzyme systems.

As a first approximation one may obtain transition-time data for a given (unaggregated) multienzyme system via application of Michaelis-Menten kinetics. However, beyond the case of simple two-enzyme systems the set of differential equations becomes increasingly less tractable. Consequently, we have developed a modular analog computer system for simulating the kinetic function of multienzyme systems. The basic module employs Michaelis-Menten kinetics and is readily adaptable to a number of regulatory conditions, e.g., reversibility and feedback inhibition. The computer model has thus far been extended to systems of up to five enzymes, and a wide range for substrate concentrations and rate constants (e.g., K_m , V_{max}) has been ensured. Kinetic data are easily obtainable for transitions between steady states, as well as equilibrium states. Transition-time data obtained from the com-

puter are in good agreement with theoretical calculations for simple two-enzyme systems.

As a prelude to and justification for subsequent studies with more complex systems, we are currently applying the computer model to an existing two-enzyme system. The two-step conversion of *N*-(5'-phosphoribosyl) anthranilate to indole-3-glycerol phosphate in the tryptophan biosynthetic pathway is catalyzed by two separate enzymes in *Saccharomyces cerevisiae*. Thus, upon obtainment of the respective enzymic rate constants for this system, the theoretical predictions of the computer model may be compared directly with actual *in vitro* data for the representative unaggregated enzyme system. Satisfactory correlation will then warrant the application of the model to transition-time analysis for the same two-step reaction system in *Neurospora crassa*, wherein the two reactions are catalyzed by the anthranilate synthetase complex.

A more complicated system for potential application of the computer model is afforded by the *arom* complex of *N. crassa* (3). This complex catalyzes five sequential reactions. Figures 1 and 2 display a five-enzyme system roughly representative of the *arom* complex sequence. The plots were obtained for a steady-state transition initiated at time zero. In Fig. 1, all K_m 's and all V_{max} 's are, for simplicity, set equal; in Fig. 2, K_{-3} is then increased 100-fold. Of particular importance are the lags in accumulation of interme-

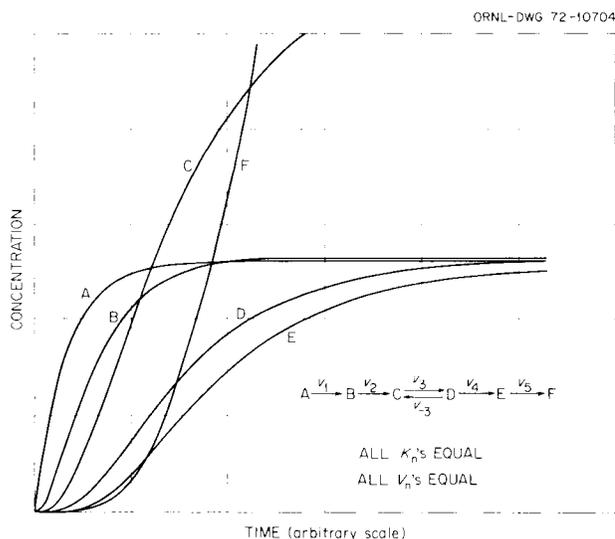


Fig. 1. Analog computer simulation of a five-step monolinear multienzyme system with one reversible reaction (monomolecular reactions).

Steady-state transition. All K_m 's equal, all V_{max} 's equal. (K_n denotes K_m for n th reaction, V_n the V_{max} for n th reaction, and v_n the velocity of the n th reaction.)

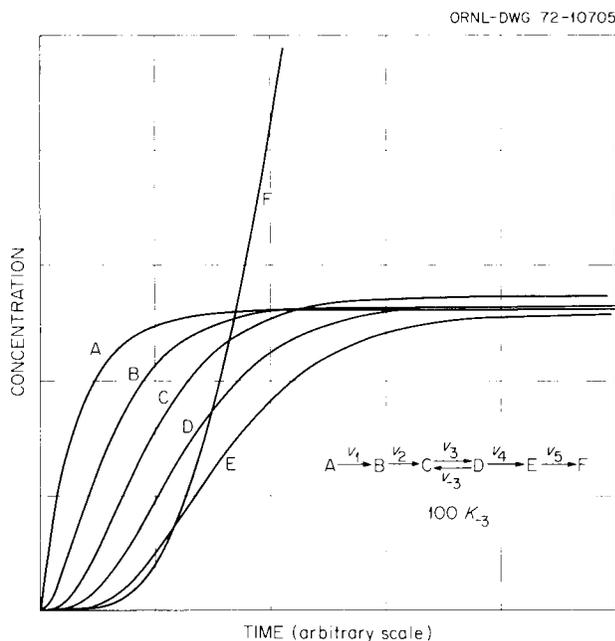


Fig. 2. Same as Fig. 1, except K_{-3} increased 100-fold.

diates and final product. Significant reduction of these lag times (i.e., reduction of transition time) is a unique possibility as a result of catalytic facilitation by multienzyme complexes.

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THE *arom* COMPLEX OF *NEUROSPORA CRASSA*: PRELIMINARY KINETIC STUDIES

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

The *arom* multienzyme complex of *N. crassa* and other organisms catalyzes a sequence of five reactions giving rise to such important ultimate end products as the aromatic amino acids and nicotinamide adenine dinucleotide. Specifically, the complex converts 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) to 3-enolpyruvylshikimate 5-phosphate (EPSP), which is the immediate precursor of the important branch-

point compound, chorismate. We have initiated kinetic studies aimed at elucidating the nature of possible catalytic facilitation by this complex.

We have developed essentially two types of assay for the overall activity of the *arom* complex. The first is a *dissected* assay wherein DAHP is converted to EPSP, the reaction stopped at desired time points, and the accumulated EPSP is converted enzymatically to anthranilate; the anthranilate is then determined spectrofluorometrically. The second type is a *continuous* assay wherein the conversion of DAHP to anthranilate is followed continuously; this is expedited by the incorporation of an excess of EPSP→anthranilate coupling enzymes into the total reaction mixture.

Previous studies (1) indicated the possibility of catalytic facilitation by the *arom* complex. By use of a continuous assay, it was observed that the overall rate was tenfold greater when conducted with initial substrate, as opposed to the rate conducted with an intermediate-substrate, shikimate. Also, substrate-competition experiments indicated preferential catalysis with initial substrate. Results were mostly consistent with compartmentalization effects.

Our present studies are of a preliminary nature. Results thus far with the overall assay indicate linearity for initial rates of EPSP synthesis with time. Furthermore, the rates are linear essentially from time zero, apparently precluding any significant lag in accumulation of final product. This latter result might be interpretable as a manifestation of catalytic facilitation, possibly due to activation of the complex by the initial substrate and/or compartmentalization of intermediates.

Development of assays and kinetic studies have employed partially purified *arom* complex from a chorismate synthetaseless mutant. We are currently extending our research to wild type, with which most of our subsequent studies will be conducted. Radioisotope trapping techniques afford the possibility of examining the compartmentalization of intermediates. Also, upon obtainment of V_{max} and K_m for the individual enzymic activities of the complex, a steady-state transition-time analysis (2) is feasible. An analog computer model (2) might simulate the corresponding five-enzyme unaggregated system.

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1. F. H. Gaertner, M. C. Ericson, and J. A. DeMoss, *J. Biol. Chem.* **245**: 595 (1970).
2. G. R. Welch and F. H. Gaertner, this report, previous abstract.

CHORISMATE SYNTHASE. MULTIPLE FORMS OF THE ENZYME IN *NEUROSPORA CRASSA*

K. W. Cole and F. H. Gaertner

As reported previously (1), chorismate synthase in *N. crassa* was found to exist in two molecular forms. Since this enzyme catalyzes the main branch-point reaction in the biosynthesis of the aromatic amino acids, it was conceivable that two forms of the enzyme were involved in some specific structural and/or regulatory relationship with the phenylalanine-tyrosine and tryptophan branches of the aromatic system. To test this possibility we separated the two enzymic forms by DEAE-cellulose chromatography and rechromatographed aliquots of each on separate scaled-down versions of the original column. In addition, sucrose gradient analyses were performed on each, as well as kinetic analyses of the enzyme reaction.

Prior to rechromatography on DEAE-cellulose, the first enzyme had a sedimentation coefficient of up to 10S. After rechromatography, the sedimentation coefficient of the first enzyme decreased to 8S or the value obtained for the second enzyme. Moreover, the position occupied by the first enzyme in the DEAE-cellulose chromatogram shifted to that occupied by the second enzyme. Kinetically, the two enzymic forms were indistinguishable. Both required TPNH as a cofactor, both were activated by the substrate 3-enolpyruvylshikimate 5-phosphate and by diaphorase, and both were unaffected by the end products tyrosine, phenylalanine, and tryptophan. Finally, a mutation affecting chorismate synthase was shown to completely eliminate both activities which indicated they share common polypeptide chains. Although we have not ruled out the possibility that the two forms of chorismate synthase accommodate the aromatic branch point in some way, our data do not support this conclusion. Rather, they indicate that some of the enzyme undergoes a structural alteration during the course of purification, and that enzyme which elutes first from DEAE-cellulose is structurally a larger, more unstable version of the second enzyme.

1. F. H. Gaertner and K. W. Cole, *Biol. Div. Ann. Progr. Rept.* June 30, 1971, ORNL-4740, p. 70.

STUDIES OF TRYPTOPHAN PYRROLASE IN *NEUROSPORA CRASSA*

D. A. Casciano and F. H. Gaertner

In *N. crassa*, tryptophan is degraded to metabolites which are either excreted or utilized in the biosynthesis

of nicotinyl coenzymes. Although some information is available concerning most of the enzymes involved in the biosynthesis of nicotinamide adenine dinucleotide from tryptophan, significantly no direct information is available concerning tryptophan pyrrolase, the first enzyme in the pathway. This lack of knowledge has been due primarily to the inability to assay tryptophan pyrrolase in an *in vitro* system. In the present report we describe an assay which detects tryptophan pyrrolase in osmotic lysates of a wall-less variant of *N. crassa* and some observations we have made concerning this enzyme. The analysis is based on the quantitative conversion of the product of tryptophan pyrrolase, formylkynurenine, to the ethyl-acetate extracted fluorescent product, anthranilate, by means of two readily isolated coupling-enzymes from *N. crassa*, kynurenine formamidase and an inducible kynureninase (1). Anthranilate was identified by spectral and chromatographic methods. Our results indicate that (1) tryptophan pyrrolase is a constitutive enzyme activated by its substrate, (2) heme and reducing agents (compounds required to detect rat-liver tryptophan pyrrolase) are not required for maximal activity, (3) *N*-acetyl tryptophan cannot substitute for tryptophan in activation of the enzyme, and (4) the enzyme appears to be unaffected by distal end products. However, under certain conditions as yet undefined, NADPH has been shown to inhibit activity up to 90%.

In addition, we have succeeded in detecting tryptophan pyrrolase activity in wild-type *N. crassa* by producing protoplasts utilizing snail-gut enzyme. This technique is now being used to characterize several tryptophan-niacin auxotrophs.

1. F. H. Gaertner, K. W. Cole, and G. R. Welch, *J. Bacteriol.* 108: 902 (1971).

COMPARISON OF THE ENZYMES CATALYZING TRYPTOPHAN TO NICOTINAMIDE ADENINE DINUCLEOTIDE IN *NEUROSPORA CRASSA* AND MAMMALIAN LIVER

D. A. Casciano, C. McDermott,* and F. H. Gaertner

The aromatic amino acid tryptophan is converted to the essential coenzyme nicotinamide adenine dinucleotide (NAD) by way of a series of nine biosynthetic reactions. This same pathway of reactions appears to exist in *N. crassa* as exists in most mammals, including the mouse. To obtain a better understanding of the nature of these metabolic sequences in the life process, we are investigating, in a comparative manner, the regulation and cellular organization of the enzymes which catalyze the metabolism of tryptophan.

We have begun an investigation to characterize the appearance of three enzymes (tryptophan pyrrolase, kynurenine formamidase, and kynureninase) of the tryptophan-to-NAD pathway, with mouse liver as a model system. At birth these enzymes are at a low level but increase rapidly in specific activity around five days after birth. Nine days after birth, they are present at adult levels. We are attempting to determine whether the developmental emergence of the enzymes is coordinately regulated, and whether these enzymes may be prematurely evoked by their substrates and/or hormone(s).

In addition, preliminary experiments characterizing kynureninase suggest that the mammalian system has a single enzyme which, from excretion studies and kinetic analyses, is functionally more correctly termed a hydroxykynureninase. The enzyme in *N. crassa* exists in two forms (1), one of which is inducible and has been characterized as a degradative enzyme which functionally may be correctly termed a kynureninase, the other of which is constitutive and has properties comparable to the mammalian biosynthetic hydroxykynureninase.

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1. F. H. Gaertner, K. W. Cole and G. R. Welch, *J. Bacteriol.* **108**: 902, (1971).

THE BIOGENESIS OF MELANOSOMES IN *XENOPUS LAEVIS* OOCYTES

James N. Dumont

The appearance of pigment in developing amphibian oocytes is one of the most striking events of oogenesis. Cytological studies aimed at examining the biogenesis of these pigment-containing organelles or melanosomes have been conducted. Although visible pigmentation does not appear until Stage III (0.45–0.60 mm diameter) of oocyte development, the genesis of melanosomes begins much earlier when the oocytes are in Stages I and II (0.20–0.45 mm). The initial phases of melanosome biogenesis are marked by localized accumulations in the peripheral cytoplasm of smooth membranous elements which are interpreted as cisternae of smooth endoplasmic reticulum. As oocyte growth proceeds, the tips of many of these cisternal elements enlarge and come to contain a small core of electron-dense material. These structures are termed premelanosomes. Initially this material of the premelanosomes is homogeneous, but later it condenses into dense 200 Å particles which are at first randomly

arranged, but eventually become evenly spaced and aligned into rows. These events occur prior to the deposition of melanin. During Stage III of oocyte development, melanin synthesis and deposition in the premelanosomes begin. During this time the dense particles become arranged into a hexagonal pattern and form the framework for melanin deposition. Cytochemical tests for the localization of tyrosinase (i.e., the DOPA reaction), an enzyme essential for melanin synthesis, reveal that the Golgi complexes, which are negative prior to this stage, now exhibit a highly positive reaction. Furthermore, the Golgi complexes, which in early stages lie deep within the cytoplasm, now move into close proximity to the developing melanosomes. DOPA-reaction product is also localized within membrane-bounded cisternae near the zones of premelanosomes. It is difficult to determine if the premelanosomes contain reaction product since they also contain electron-dense melanin. This has been resolved by cell fractionation and biochemical techniques and is reported elsewhere (1). It seems clear, therefore, that the framework components of the melanosome are initially synthesized in elements of smooth endoplasmic reticulum and that tyrosinase is provided by the Golgi complexes to the premelanosomes.

1. J. J. Eppig and J. N. Dumont, this report, p. 88.

CYTOCHEMICAL LOCALIZATION OF CYTOCHROME OXIDASE IN *XENOPUS LAEVIS* OOCYTES

T. G. Wilson* and James N. Dumont

Cytochrome oxidase, a component of the mitochondrial electron transport chain which transfers electrons from cytochrome c to molecular oxygen, has been cytochemically localized in the mitochondria of *Xenopus laevis* oocytes using the method of Seligman *et al.* (1). This method uses 3,3'-diamino-benzidine (DAB) as the localizing agent. Either fresh or formaldehyde-fixed oocytes were incubated in a buffered solution of DAB for 1 to 4 hr, fixed in glutaraldehyde and postfixed in osmium tetroxide, and prepared for observation in the electron microscope.

Reaction product was observed in the mitochondria of both fresh and fixed oocytes. Little reaction product was observed in oocytes incubated 1 or 2 hr, but heavy deposits of reaction product occurred with longer incubation periods. Reaction product was found in the intracrystal spaces of the mitochondria and on the outer membrane, although deposition was not continuous

across the outer membrane. Mitochondria devoid of reaction product were frequently found in close proximity to mitochondria containing reaction product. The presence of these nonreacting mitochondria, although appearing normal morphologically, suggests that a percentage of mitochondria in an oocyte may be functionally inactive. No reaction product was observed when KCN, an inhibitor of cytochrome oxidase, was added to the incubation mixture.

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1. A. M. Seligman, M. J. Karnovsky, H. L. Wasserkrug, and J. S. Hanker, *J. Cell Biol.* 38: 1, (1968).

TYROSINASE ACTIVITY IN ISOLATED OOCYTE PREMELANOSOMES

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

Tyrosinase activity and melanin pigment synthesis can be stimulated in *Xenopus* oocytes which are in specific stages of their development by injecting the mature female with gonadotrophin. The tyrosinase activity has been localized cytochemically in oocytes by the DOPA-reaction in the distal cisterna of the Golgi complex and in an anastomosing network of smooth-surfaced tubules associated with the Golgi complex. However, it was difficult to ascertain by cytochemical means whether the premelanosomes of *Xenopus* oocytes contain tyrosinase activity since it is very difficult to see the difference between DOPA-reaction product and naturally deposited melanin. Therefore, a method was developed for the isolation and purification of oocyte premelanosomes, and tyrosinase activity was measured in them. This method employs a combination of differential and density gradient centrifugation in sucrose. Table I shows the tyrosinase activity in melanin granules isolated from oocytes in various stages of development.

The data indicate that premelanosomes isolated from Stage III and IV oocytes contain significant tyrosinase activity. This activity appears to decrease in the later stages of melanization.

Further experiments have indicated that the tyrosinase can be released from the premelanosomes by treatment with 1% Triton X-100. Centrifugation after detergent treatment pellets the premelanosomes, but leaves about 85% of the enzyme activity in the supernatant. The specific activity of the enzyme in the supernatant is very high (3,482,000 cpm/mg protein). Thus this procedure will be a very useful first step in the tyrosinase purification necessary for future experimentation.

TABLE I. Tyrosinase activity in isolated melanin granules

Oocyte stages	cpm per 50 μ l prep.	cpm per μ g protein	cpm per μ g melanin
III & early IV	2750	1275	29.1
Mid-late IV	784	3636	4.6
V	47	217	.7

THE *IN VITRO* MAINTENANCE OF *XENOPUS* OOCYTES IN VARIOUS STAGES OF DEVELOPMENT AFTER OVARIAN DISSOCIATION WITH COLLAGENASE

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

In order to study the progress of various cytological and biochemical events occurring during the growth and differentiation of oocytes, it became necessary to develop a system wherein large numbers of oocytes in all stages of development could be maintained *in vitro* for at least 3 days. We have found that ovaries can be dissociated by incubating small pieces in 0.2% collagenase in solution OR-2 (developed by R. A. Wallace). Oocytes of all stages are thus liberated from ovarian tissue except for the adherence of a few follicle cells. The oocytes may then be grouped according to stage and placed in solution OR-2 containing 1–5% dialyzed *Xenopus* serum. Oocytes thus maintained at 20° C remain in good morphological condition for up to 5–7 days. We have also found that, although the free amino acid pools of such oocytes are depleted by about 60% after only 1 day maintenance in OR-2, protein synthesis remains constant after 3 days in the solution. The use of collagenase to dissociate the ovary has some adverse effects on the uptake of yolk proteins *in vitro*. However, the incorporation of amino acids and uridine, the ability of progesterone to bring about germinal vesicle breakdown, the synthesis of proteins, and the ultrastructure of the oocytes are not affected by collagenase.

CELL CULTURE STUDIES OF FOLLICLE CELLS OF *XENOPUS LAEVIS*

Marsha Segal and James N. Dumont

Oocytes of *Xenopus laevis* are surrounded by a layer of follicle cells, the precise function of which is unclear. However, in an attempt to elucidate the function of these cells, a procedure for obtaining a pure cell culture has been devised. Late Stage IV and Stage V oocytes are dissected from the ovary into solution OR-2. The

oocytes are placed in a solution of trypsin-EDTA at 30° C for 10 min. They are then transferred to a culture dish containing a tissue culture medium consisting of 70% L-15 and 10% fetal calf serum. The follicle cells detach from the oocytes and attach to the culture dish in 24–48 hr. The oocytes are then removed and fresh media is added. A culture obtained in this manner has been maintained *in vitro* for about 2 weeks. The cells can also be grown in the tops of collagen-coated BEEM capsules and fixed and sectioned for electron microscopy.

The ultrastructure of these cells at various times after primary culture is currently being studied. Phase contrast microscopy reveals that follicle cells from frogs that have received an injection of HCG contain refractile granules, while those from unstimulated frogs (i.e., no injection of HCG) do not contain such granules. Cytochemical techniques are being used to study the nature of these granules, but it is believed that they contain steroids.

AMINO ACID POOLS IN DEVELOPING OOCYTES OF *XENOPUS LAEVIS*

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

Many of the metabolic processes of developing amphibian oocytes are aimed at supplying the components necessary to ensure the successful development of progeny. We have examined the accumulation of free amino acids in the developing oocytes of *Xenopus laevis*. Knowledge of these amino acid pools is important for several reasons. First, it aids in the understanding of nitrogen metabolism, particularly protein synthesis, in the oocyte. Second, it is important pragmatically for use in experiments involving labeled amino acids where pool size must be considered. Third, it provides further information on the accumulation of metabolic material for possible use during oocyte development and subsequent embryogenesis.

The sizes of amino acid pools in developing oocytes of various stages have been determined for normal and for human chorionic gonadotrophin (HCG)-stimulated *Xenopus laevis*. The total amino acid pool increases 20- to 30-fold in oocytes in Stages II through V. This increase is commensurate with the increase in size of the oocyte. In addition, the pool is greater (5% in Stage II and 29–38% in Stages III through V) in oocytes from the HCG-stimulated frogs. Glutamic acid accounts for approximately 35–45% of the total amino acid pool in all stages of oocytes. In none of the stages, however, are there detectable amounts of either proline or cysteine.

THE ROLE OF NEURAL RETINA IN TRANSFORMATION OF IRIS INTO LENS

Tuneo Yamada

In our efforts to establish an *in vitro* system of Wolffian lens regeneration, we succeeded in obtaining lens development accompanied by gamma crystallin synthesis from the normal newt iris cultured in contact with the retinal complex of frog larvae (1). No lens is formed by the normal newt iris cultured alone under the same condition. Since then attempts have been made to simplify the system.

The culture medium used in the original experiments was 72% Medium 199 and 8% fetal calf serum. It has been found possible to obtain well-differentiated lens from the newt dorsal iris combined with frog larval retinal complex in 80% Defined Medium A₂ + APG (Holems) without the addition of serum. Thus macromolecular components are not required in the medium for obtaining the tissue transformed *in vitro*.

The retinal complex used in the original experiments was composed of neural retina as the main component and pigment retina, sclera, and choroid as the protective layers. The neural retina can be surgically separated from other layers and cultured. However, without protective layers the neural retina tends to show degenerative changes during the long culture required for tissue transformation. If the normal newt dorsal iris is cultured in contact with the separated neural retina, well-differentiated lens can be formed from the former, in spite of the degenerative condition of neural retina. The experiments show that the neural retina is able to support lens formation in conformity with the classical hypothesis.

The iris is composed of the epithelium of pigmented cells, and iris stroma. It is possible to separate the epithelium from the stroma surgically. When a piece of the dorsal epithelium is placed on the retinal complex of frog larvae and cultured, the former produced well-differentiated lens. This shows that cells forming the new lens come from the iris epithelium in agreement with our earlier results, and that either the iris stroma has no role in lens formation by dorsal iris epithelium or its role can be substituted by factors available in the retinal complex. The weight of other evidence is in favor of the second alternative.

1. T. Yamada and D. S. McDevitt. *Abstr. of Papers, 11th Ann. Meet., Amer. Soc. Cell Biol.*, 1971, p. 334.

INFLUENCE OF THE PITUITARY ON THYMIDINE LABELING OF NEWT DORSAL IRIS EPITHELIUM

T. G. Connelly and Tuneo Yamada

We have confirmed earlier reports that hypophysectomy retards the progress of Wolffian lens regeneration. Our results show that hypophysectomy 3 days prior to lentiectomy significantly retards, by 2 Sato stages, the progress of lens regeneration at 21 days. A 10-day period of hypophysectomy beginning 5 or 10 days after lens removal has no effect on regeneration. Growth of the regenerate, as indicated by lens diameter, is not affected by hypophysectomy. In animals hypophysectomized 3 days prior to lens removal, thymidine labeling of iris epithelial nuclei in both dorsal and ventral irises at 5 days after lentiectomy is significantly lower (5- to 6-fold) than in controls. At 7 days the difference is only 1.5- to 1.7-fold and is no longer statistically significant (Table I). A similar trend was noticed in an *in vitro* system when irises were cultured with or without a pituitary. Thus, the percentage of labeled nuclei in irises cultured with pituitary for 5 days was slightly higher than that in irises cultured without

pituitary for the same length of time. After 7 days *in vitro* the total amount of labeling drops in both types of culture and the values lie closer together (Table I).

These data suggest that when lens regeneration *in vivo* is initiated in the absence of the pituitary, the thymidine-labeling index of iris epithelial nuclei may be reduced due to (1) a decrease in the number of cells reentering the cell cycle, (2) delay in reentry of iris epithelial cells into the cell cycle, and (3) lengthening of the cell cycle time. These effects could result in a general delay of regenerative progress without a retardation in regenerate growth. Although the trend in labeling indices in irises cultured with or without pituitary is similar to that seen in hypophysectomized or sham-operated animals *in vivo*, the reduction in the quantitative difference may indicate that explanation itself is sufficient stimulus to raise thymidine incorporation to normal levels regardless of the presence of the pituitary. This aspect of the involvement of the pituitary in thymidine incorporation by iris epithelial nuclei is currently under investigation.

SERUM GLUCOSE LEVELS OF NORMAL, SHAM-OPERATED, AND HYPOPHYSECTOMIZED NEWTS

T. G. Connelly and Aida Goldstein

Hypophysectomy of adult newts prior to lens removal is known (1) to retard the progress of regeneration over a 21-day period as compared to sham-operated animals, and (2) to alter the thymidine-labeling index of iris epithelial nuclei 5 and 7 days after lentiectomy *in vivo* and *in vitro*. Since hypophysectomized (hypoxed) animals do not normally eat well, both these and the sham-operated control animals were starved for the duration of the experiments. Overton (1) reported that starvation reduces the mitotic index in urodele larvae. Therefore, the possibility existed that some of the effects observed were due to an indirect effect of hypophysectomy on metabolism leading to an enhanced degree of "starvation," rather than to a direct effect on the regenerating cells. As a first approach to this problem, we investigated the changes in serum glucose levels in hypoxed and sham-operated newts after varying periods of starvation.

The results show that starvation for 10 or 15 days decreases serum glucose levels in normal, sham-operated, and hypoxed animals to a similar extent (30%), suggesting that (at least up to 15 days starvation) hypophysectomy has no effect on the blood glucose levels (Table I). During this time, the blood protein

TABLE I. Thymidine labeling of iris epithelial nuclei in the presence or absence of the pituitary

Experimental conditions	Mean percentage of labeled nuclei \pm S.E.	
	Dorsal	Ventral
<i>In vivo</i> (5 days)		
Sham	9.08 \pm 1.57 (4242)*	6.99 \pm 1.22 (4129)
Experimental	1.56 \pm 0.54 (4408)	1.64 \pm 0.66 (4308)
<i>In vivo</i> (7 days)		
Sham	23.73 \pm 4.41 (3583)	18.78 \pm 4.05 (3760)
Experimental	14.64 \pm 3.45 (3816)	11.88 \pm 3.16 (3688)
<i>In vitro</i> (5 days)		
With pituitary	43.68 \pm 5.78 (5488)	
Without pituitary	31.47 \pm 4.28 (5455)	
<i>In vitro</i> (7 days)		
With pituitary	28.31 \pm 2.84 (6709)	
Without pituitary	32.03 \pm 4.38 (6912)	

*Figures in parentheses = total nuclear sections counted.

TABLE I. Effect of hypophysectomy on blood glucose levels

	mg/100 ml glucose	μ mole glucose/mg protein
Fed animals		
Normal	50.0	0.0722
10-Day starved		
Normal	35.6 \pm 4.6	0.0451
Sham	34.9 \pm 2.8	0.0503
Hypoxed	35.6 \pm 4.1	0.0541
15-Day starved		
Normal	30.8 \pm 4.9	0.0333
Sham	27.8 \pm 2.6	0.0503
Hypoxed	23.8 \pm 0.9	0.0503

level remains essentially constant. Further studies on glucose levels are being carried out on newts subjected to longer periods of starvation as well as to various hormone treatments.

1. J. Overton, *J. Exp. Zool.* 115: 521–560 (1950).

TISSUE INTERACTIONS AND LENS REGENERATIVE ACTIVITY OF NEWT IRIS *IN VITRO*

T. G. Connelly and Tuneo Yamada

Recent experiments have extended our earlier investigation of the lens regenerating ability of normal newt iris influenced by other tissues *in vitro* (1). Our results (Table I) indicate that the ability to support lens

regeneration *in vitro* is shared to a certain extent by a variety of tissues. The most effective ones tested are newt or frog pituitary and frog tadpole retina (2, 3). Frog kidney, frog adrenal plus kidney, and newt spinal ganglion all appear to have similar abilities to enhance depigmentation and lens fiber formation, but are less effective than pituitary tissue. Combination of irido-corneal complexes with newt kidney or muscle results in an increase in the number of cases exhibiting depigmentation, but does not greatly enhance the formation of lens fibers when compared to irido-corneal complexes cultured alone. When whole rings of iris trimmed of ciliary epithelium are cultured alone the incidence of depigmentation decreases markedly. In the presence of pituitary, removal of both ciliary epithelium and stroma from the iris epithelium does not interfere with its ability to produce a lens *in vitro*. This result confirms the classical notion that iris epithelial cells, but not other cell types, are transformed into lens cells in Wolffian lens regeneration. Finally, separation of the iris and the pituitary by insertion of a nucleopore filter reduces the frequency of lens fiber formation but does not affect depigmentation of the iris in such cultures.

These data indicate that the factor(s) responsible for supporting lens regeneration *in vitro* is either not unique to neural retina or can be substituted by some other factors from other tissues. They also suggest that the presence of the ciliary epithelium and cornea in

TABLE I. Response of newt iris to various frog and newt tissues *in vitro* after 20–21 days of culture

Tissue added	No depigmentation (Stages 1–2)	Depigmentation only (Stages 3–5)	Lens formation (Stages 6–10)
Frog pituitary*	0	2/20	18/20
Newt kidney*	6/16	10/16	0/16
Newt muscle*	18/47	27/47	2/47
Frog kidney*	0/16	12/16	4/16
Frog adrenal plus kidney*	2/20	13/20	5/20
None*	46/69	22/69	1/69
Dorsal iris epithelium (stroma and ciliaries removed) plus frog pituitary	1/6	0	5/6
Iris rings without ciliaries or cornea			
Plus newt pituitary	4/20	2/20	14/20
Cultured alone	19/20	0	1/20

*Irido-corneal complexes

irises cultured without other tissues may enhance the ability of iris epithelial cells to undergo depigmentation. Experiments investigating the apparent differences between the regeneration-enhancing activity of frog and newt kidney, and the possible influence of retinal tissue regenerated from ciliary epithelium in irido-corneal complex cultures on depigmentation in irises cultured alone, are presently in progress.

1. T. G. Connelly and J. R. Ortiz, *Biol. Div. Ann. Progr. Rept.*, June 30, 1971, ORNL-4740, p. 72.
2. T. Yamada, *Biol. Div. Ann. Progr. Rept.*, June 30, 1971, ORNL-4740, p. 71-72.
3. T. Yamada and D. S. McDevitt, *Abstr. of Papers, 11th Ann. Meet., Amer. Soc. Cell. Biol.*, 1971, p. 334.

DEDIFFERENTIATION OF IRIS EPITHELIAL CELLS *IN VITRO*

J. R. Ortiz* and Tuneo Yamada

Morphological aspects of dedifferentiation of iris epithelial cells (EC's), which occurs before those cells become transformed into lens cells in Wolffian lens regeneration, were studied with the electron microscope *in vivo* (1). According to those results, the normally smooth cell surface is activated after lentectomy to develop extensive projections. The slender tips of those projections containing melanosomes are removed from the cells and taken up by migrating macrophages. Repetition of such removal leads to complete loss of the melanosome population which characterizes the differentiated state of EC's. To obtain information on the control mechanism of such a dynamic process occurring in individual cells, it is essential to establish a method of observation of individual cells engaged in the process. Efforts along this line are reported here.

The dorsal iris of the newt is composed of the iris epithelium and iris stroma. When explants from the dorsal iris of the adult newt *Notophthalmus viridescens* are placed on a cover slip in the culture medium, stroma cells first migrate out of the explant. By 4 days of culture, the EC's which are heavily pigmented emerge from the explants. Behavior of individual EC's in the mixed cell population was followed by time-lapse cinematography, as well as by light microscopy of living and fixed material. The data indicate that EC's, which are spherical when spread on the glass surface, send out projections (while its surface is in constant movement). The change in the cell shape is accompanied by irregular locomotion. EC's often come in contact with stroma cells, which are of a fibroblast-type shape, and are also very active in movement. During culture, EC's discharge fragments of the cytoplasm containing melanosomes. Such free fragments remain in active locomotion and

exhibit shape changes for a certain period of time. After repeated shedding of cytoplasmic material, the level of pigmentation of EC's becomes reduced during the course of 5-day culture. When the culture is extended for 10 days a gradual decrease in activity of EC's occurs.

When the dorsal irises are placed in culture, 80% of them release free EC's. However, if the stroma is removed from the dorsal iris, either mechanically or after a 5-min treatment with 0.2% collagenase in isotonic saline with phosphate buffer, and the epithelium alone is cultured, 21% of the explants release a limited number of EC's. Thus, the presence of stroma in the explant seems to enhance emergence of EC's in culture. Furthermore, EC's in the absence of stroma cells did not show active locomotion, formation of projections, or their shedding. EC's can be obtained by dissociating the iris epithelium with 1% trypsin in Ca- and Mg-free saline with phosphate buffer, filtering through Swinnex filter, and treating with trypsin inhibitor. The population of EC's thus obtained again fails to show active locomotion, formation of projections, or their shedding.

These data as a whole seem to suggest the possibility that cell surface changes of EC's, which start soon after lentectomy and lead to dedifferentiation of the cells, are dependent on stroma cells. The hypothesis will be further tested and if confirmed, the mechanism of the cell interaction will be studied.

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1. J. N. Dumont and T. Yamada, *Develop. Biol.* (in press).

CHANGES IN MEMBRANE SIALIC ACID CONTENT OF IRIS EPITHELIAL CELLS DURING WOLFFIAN LENS REGENERATION IN THE ADULT NEWT

Aida Goldstein and Tuneo Yamada

During the early or dedifferentiation stage of Wolffian lens regeneration in adult newts, dorsal iris epithelial cells show an ordered process of events leading to depigmentation. These include enhanced RNA and DNA synthesis, invasion of macrophage, mitosis, and cytoplasmic "shedding," whereby melanin is lost from the cells destined to become the new lens fiber cells. Zalik and Scott (1) reported that iris epithelial cells placed in an electric field show a drop in electronegativity around 8-12 days after lentectomy, just at the time the cells are undergoing mitosis and cytoplasmic "shedding." One possible source of membrane negative

charge is the carboxyl group of sialic acid, which normally occurs as the terminal sugar of mucoproteins and lipids. Since it is conceivable that membrane changes play an important role during the dedifferentiation process, studies were initiated to determine whether there is a change in membrane sialic acid content of iris epithelial cells, or whether the total amount of sialic acid remains the same and the loss in electronegativity is due to masking of the negative charge. The problem was approached in two ways: one by autoradiography of cells labeled *in vitro* with [^3H]glucosamine and the other by measuring the sialic acid content chemically.

For autoradiography, whole iridio-corneal complexes from both eyes of three to five newts were incubated in 72% L-15 culture medium containing 8% fetal calf serum and 5 $\mu\text{c}/\text{ml}$ [^3H]glucosamine for 5 hr at 21°. The iridio-corneal complexes were then rinsed in 80% PBS, fixed, embedded, and sectioned at 5 μ . Sections were rinsed 5 min each in cold 10% TCA and 5% TCA to remove soluble label, and in some cases the sections were bleached in 10% H_2O_2 for 18–20 hr at room temperature. These were then dipped in 0.2% PPO, coated with emulsion, and exposed for 7–10 days. After development and staining, the sections were examined for the amount of grains in the membrane under dark-field illumination. Preliminary results indicate that 8- to 9-day regenerates show enhanced incorporation of label into the membrane as compared to unoperated (control) animals. Results for 2- to 10-day regenerates are presently under analysis to determine at what day the enhanced incorporation first appears. Cytochalasin B, reported to prevent mucoprotein synthesis and inhibit microtubule formation, was added to some irises during incubation with [^3H]glucosamine to see if it prevents incorporation of label into the membranes. These results will shortly be analyzed.

To determine chemically the sialic acid content of iris epithelial cells, 90 dorsal irises were removed and placed directly into 80% PBS at room temperature. The epithelial cells were removed from the underlying stroma by letting the tissue sit in 80% PBS for ~5 hr at room temperature, which loosens the cells from the stroma, and then by a combination of passing the tissue rapidly in and out of a small-bore capillary tube and rapid mixing on a vortex mixer. The stroma settles out and the epithelial cells are pipetted off. Both are collected by centrifugation at 3000 rpm for 10 min at 3° C. Although the iris epithelial cells are free from stroma, the stroma always contains a varying amount of epithelial cells. Neuraminidase-sensitive sialic acid was obtained by incubating epithelial cells and stroma for 2

hr at 37° C in 0.2 ml neuraminidase solution containing 0.03 units neuraminidase in 0.1 *N* acetate buffer, pH 5.2, and 2 mM CaCl_2 . At the end of the incubation time, cells were removed by centrifugation at 7000 rpm for 10 min and the supernatant was frozen until assayed. To obtain sialic acid that is not neuraminidase-sensitive, the cells were then incubated in 0.2 ml of 0.1 *N* sulfuric acid for 1 hr at 80° C. Nonsoluble material was centrifuged down and the supernatant stored frozen until assayed. Sialic acid was determined by the method of Warren (2).

These experiments are presently in progress, with only a small amount of data available. Early results showed that incubation of cells for 2 hr at 37° C without enzyme, but with the acetate buffer, does not release any sialic acid, confirming that the release of sialic acid is due to the action of the enzyme. Control as well as 19- and 21-day regenerates appear to have ~44% of the sialic acid that is sensitive to neuraminidase, but the total amount of sialic acid is ~20% higher in the control. Besides determining the sialic acid content of 3-, 7-, 10-, and 15-day regenerates, experiments will also be carried out to determine whether trypsin and collagenase release sialic acid-containing peptides. This is important since one or both of these enzymes are commonly employed to disrupt tissues for cell cultures.

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ROLE OF *N*-ACETYLGLUCOSAMINIDASE IN DEDIFFERENTIATION OF IRIS CELLS IN WOLFFIAN LENS REGENERATION

Victor Idoyaga-Vargas and Tuneo Yamada

The cells of the dorsal iris epithelium of the adult newt regenerate the lens after lensectomy. During this tissue transformation the iris epithelial cells undergo a series of changes detected at the cellular and molecular levels, culminating in the production of lens-specific proteins. Conspicuous among these changes are alterations in the properties of the cell membrane that result in the partial elimination of cytoplasm and complete loss of pigment granules. The elimination of overt differentiation is accompanied by the ability to differentiate in a new direction. The reversion of iris cells to this less committed status is termed dedifferentiation (1). Our present purpose is to study the possible role of *N*-acetylglucosaminidase, a lysosomal enzyme, during dedifferentiation.

Alteration of enzyme activity during regeneration in dorsal and ventral irises: On DNA basis, the activity of

glucosaminidase in the dorsal iris increases progressively from days 4 to 12, remains at about the same levels up to day 19, and then declines to its initial values. During the period of highest activity, the amount of micro-moles of *p*-nitrophenol liberated per milligram of DNA per hr is about fivefold higher than control (unoperated animals). This period coincides with the time in which cytoplasm loss and depigmentation are occurring. Also, in the ventral iris the activity increases, but to a lesser extent.

The localization of enzyme activity: A method was worked out for a separation of a fraction enriched in iris epithelium from that enriched in iris stroma using collagenase. When irises of unoperated animals (controls) were used to obtain the stroma- and epithelium-enriched fractions, the activity was twofold higher in the former on a protein basis. The 8-, 12-, and 17-day series yielded comparable results. Further work on DNA basis is progressing. Preliminary experiments to determine the intracellular localization of the enzyme were carried out with the stroma- and epithelium-enriched fractions. Both fractions were homogenized and spun down for 10 min at 3000 rpm. The sediment contained nuclei (as checked by phase microscopy) and the supernatant was devoid of them. They were therefore termed the "nuclear" and "cytoplasmic" fractions. The fractionation was done separately for the dorsal and ventral irises. Controls and 4-, 8-, and 12-day regenerates were analyzed. The results revealed a consistently higher activity (on a protein basis) of the cytoplasmic fractions.

In further experiments, whole irises from control animals were spun down at 114,000 *g* for 60 min. The sediment and the supernatant were then analyzed for enzyme activity. It was found that 90% of the activity was present in the high-speed sediment. When 4- and 12-day regenerates are submitted to high-speed centrifugation as before, there is a dramatic increase of activity in the supernatant. The increase amounted to up to 69% in the 12-day dorsal iris. These results suggest that a solubilization of the enzyme occurs during dedifferentiation.

Preliminary experiments currently in progress indicate that there is an enzyme release into the medium during regeneration. Values up to 25% of the total activity were found in the low-speed supernatant of dorsal irises from lentectomized animals, spun down without homogenization. Half the amount of these values was obtained with irises from control animals. The variation of enzyme activity during regeneration indicates that there is a close association between *N*-acetylglucosaminidase activities and the process of dedifferentiation of

iris cells. The pattern of alteration in the activity of the enzyme parallels the pattern of invasion by macrophages in the iris (2). Further, the regenerated or normal lenses which are free of macrophages show very low values of enzyme activity. The histochemical detection of the enzyme will tell whether or not macrophages are the main source of the enzyme activity.

The higher activity in the "cytoplasmic" rather than in the "nuclear" fractions of the normal iris, combined with a virtual absence of enzyme activity in the high-speed supernatant of the same tissue, suggests that glucosaminidase is bound to a particulate fraction in the normal condition. Further purification procedures will ascertain the possible localization of the enzyme in the lysosomes.

As indicated by recent work, cell membrane alterations may play a key role in the dedifferentiation process (Dumont and Yamada, in press; Zalik and Scott, in press). Since glucosaminidase releases *N*-acetylglucosamine from mucopolysaccharides (3), it may be actively involved in altering the mucopolysaccharides of the cell membrane during dedifferentiation.

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STUDIES OF CELL AFFINITY IN THE WOLFFIAN LENS REGENERATING SYSTEM

H. A. Grunz and Tuneo Yamada

Ultrastructural and cell-electrophoretic studies have indicated that during transformation of newt iris epithelial cells into lens cells after lentectomy, progressive changes occur at the cell periphery (1, 2). It is proposed to test possible changes in cell affinity during the transformation by confronting the cells engaged in transformation either with normal iris epithelial cells or normal lens epithelial cells, and by characterizing the pattern of cell aggregation obtained. The following tissues were employed: (1) the depigmented part of the newt dorsal iris, 13 and 15 days after lentectomy; (2) dorsal iris of unoperated newt; (3) dorsal iris of unoperated frog; (4) lens epithelium of unoperated newt; and (5) lens epithelium of unoperated frog. Tissues were disaggregated in 1% trypsin in Ca- and Mg-free saline with phosphate buffer. Disaggregation was completed in L-15 culture medium with the aid of specially constructed pipettes. Filtration through a

Swinnex filter yielded mostly single cells devoid of cell clusters. Aggregation of different cell types was conducted by introducing the following combination of dissociated cell populations into an Erhlemeyer flask and placing it on a gyratory shaker moving at a speed of 50 rpm: (1) + (2), (1) + (3), (1) + (5), (2) + (3), and (4) + (5). The reason that frog cells as well as newt cells were used is that frog cells can be easily distinguished from newt cells by their nuclei, and help identify the regenerate cells (1) in cell aggregates, while tissue-specific affinity is expected to be common to both species.

As soon as 10 min after the start of shaking, aggregates of 10–20 cells were observed. It is known that such primary aggregation is not cell-type specific (3). So the suspension was kept shaking for 24 hr, when cell-type specific aggregation is expected to be completed. Aggregates are then collected, fixed, and embedded in EPON. Examination of aggregate is in progress.

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FEULGEN-DNA MICROSPECTROPHOTOMETRY OF WOLFFIAN LENS REGENERATION

Stella W. Perdue and H. A. Grunz

Lentectomy of the adult newt eye induces DNA synthesis in the dorsal iris epithelium, followed by transformation into a new lens. Relative amounts of nuclear DNA during Wolffian lens regeneration will lead to further elucidation of the system involved. Cell squashes were prepared from testes in spermatogenesis, normal lens epithelium, normal lens fibers, regenerating lens epithelium, and regenerating lens fibers and then were stained by the Feulgen procedure for measurement on the Zeiss UMSP-1. Very early, uncondensed sperm were measured to establish the 1C amount of Feulgen-DNA. Preliminary results indicate that normal lens epithelium nuclei are of the 4C amount of Feulgen-DNA and that the nuclei of the normal and regenerating lens fibers degenerate from the 4C amount of Feulgen-DNA. Twenty- and 32-day lens epithelium regenerates have nuclear, Feulgen-DNA values mainly of the 4C amount with a few of the 8C amount, and intermediate values between 4C and 8C. Polyploidy or polyteny has not yet been established.

UMSP measurement of nuclei from the iris epithelium is impossible with the heavily pigmented melanosomes present. A bleaching procedure compatible with the Feulgen procedure for DNA had to be found. A

modification of the potassium permanganate-oxalic acid bleaching procedure will satisfy this requirement. Cell squashes of 6-, 7-, 13-, and 15-day regenerating iris epithelium; of 13- and 15-day regenerating lens epithelium; and of normal iris epithelium and normal lens epithelium have been prepared and will be compared.

ELECTRON MICROSCOPY OF BACTERIAL AND BACTERIOPHAGE GENETIC ACTIVITY

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

The structural aspects of ribosomal RNA transcription in *Escherichia coli* and *Salmonella typhimurium* were found to be quite similar. In both cases each rRNA region is composed of two contiguous short to long ribonucleoprotein fibril gradients, corresponding to the 16S and 23S ribosomal RNA genes. There are about six of these doublet regions per genome and they appear to be randomly scattered along the *E. coli* and *S. typhimurium* chromosomes. In contrast to these microorganisms, the rRNA regions of *Bacillus subtilis* are not all randomly arranged along the chromosome. The contents of rapidly-growing cultures that are osmotically shocked and prepared for electron microscopy as previously described (1) exhibit a closely clustered arrangement of two or three rRNA regions, while the remaining rRNA genes are scattered. This clustering agrees with transformation and DNA-RNA hybridization data.

After infection of an appropriate bacterial host by bacteriophage T7, transcription and translation of phage genes can be studied. Shortly after infection, host transcription (and thus translation), is inhibited by a phage-induced function. Therefore, it is possible to look selectively at phage genetic activity as well as phage maturation. Figure 1 shows a typical preparation 8 min after T7 infection under conditions where cells lyse at about 12 min. T7 polyribosomes appear in bunches, in association with membrane fragments (arrow). The membrane-associated translation complexes seen in T7-infected cells might at least partially explain the exceedingly long half-lives of phage-induced mRNA's (20 min vs. 3 min). The pale phage heads in Fig. 1 are lying in pools of DNA and are probably being filled with phage genomes; the dense particles are mature T7 phage. A study of cells infected with several T7 mutants is in progress to selectively visualize different aspects of the infection cycle.

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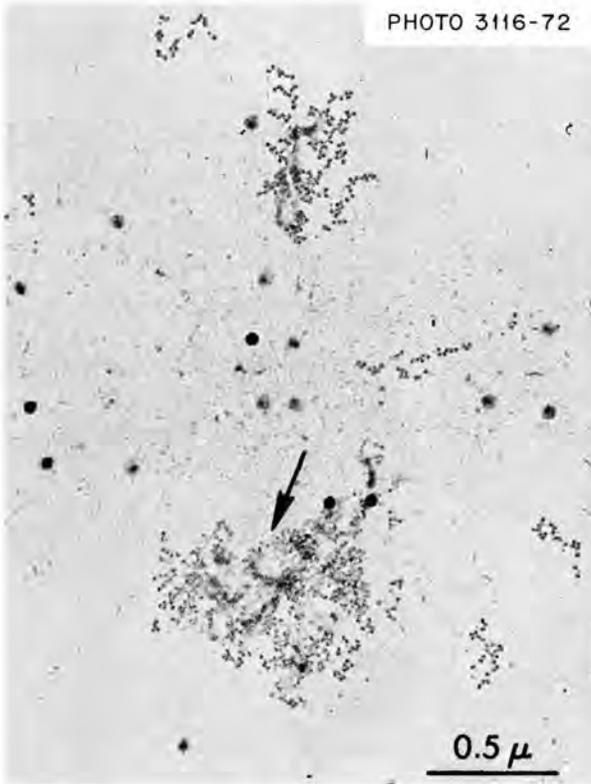


Fig. 1. Extruded contents from an *E. coli* cell infected with bacteriophage T7.

Preparation was made 8 min postinfection. Arrow indicates membrane-associated polyribosomes.

STRUCTURAL ASPECTS OF YEAST GENETIC ACTIVITY

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

We are studying the structural aspects of nuclear and mitochondrial genetic activity in *Saccharomyces cerevisiae*. This system, although a lower eukaryote, may be an ideal model for electron microscopy of eukaryotic transcription and translation because of the small amount of DNA/nucleus (3–5 *E. coli* equivalents), the large number of well-characterized mutants, and the ease and relative rapidity of cell growth. In addition, the large mitochondrial DNA molecule, as compared to higher eukaryotes (20 μ vs. 5 μ), affords a greater probability of revealing at least portions of mitochondrial DNA upon osmotic shock of the organelle.

Using wild-type yeast (from which cell walls are removed by glucalase treatment), temperature-sensitive osmotic mutants (obtained from Dr. L. Hartwell), and sorbitol-dependent mutants (obtained from Dr. D.

Schlessinger), we have made preliminary observations on yeast nuclear genetic activity. Figure 1 illustrates a partially spread region of nuclear contents. The nuclear pores are quite evident and serve as landmarks. The spread nuclear material exists as a deoxyribonucleoprotein axis with a somewhat beaded appearance. At higher magnification, the axis appears to have unevenly spaced short fibrils attached, presumed to be sites of RNA synthesis. The tightly wound nuclear material with densely stained knobs at the ends of attached fibrils (arrow) may be active yeast ribosomal RNA precursor genes, by analogy with the structure of these genes in amphibians and mammals. When these regions are somewhat stretched, it is possible to see that, unlike the higher eukaryotes, untranscribed spacer DNA between adjacent matrix units is not present.

Cytoplasmic polyribosomes are easily visualized, and are typically less than 20 ribosomes in length, arguing against the presence of polycistronic mRNA translation in these cells.

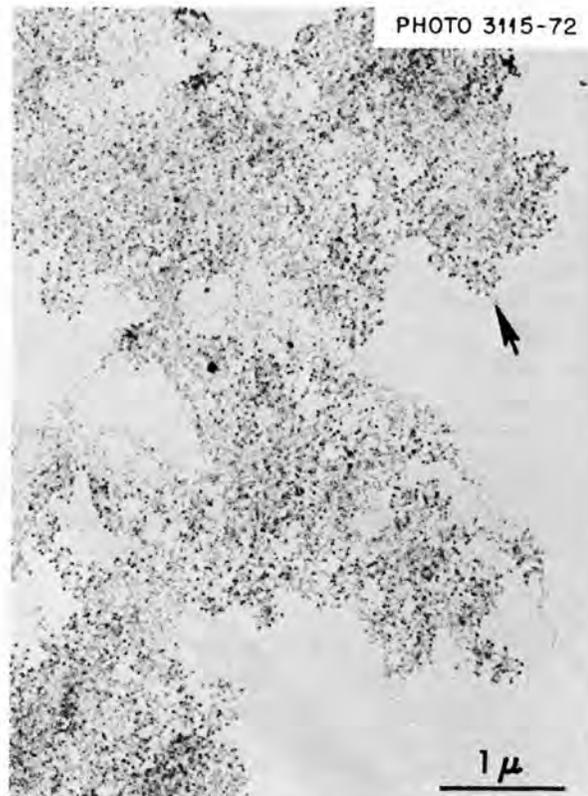


Fig. 1. Portion of spread nuclear contents from *Saccharomyces cerevisiae* cell.

Region suggestive of active ribosomal RNA precursor genes is shown (arrow).

ELECTRON MICROSCOPIC STUDIES OF TRANSCRIPTION IN MAMMALIAN CELLS

Aimée H. Bakken and O. L. Miller, Jr.

This project involves attempts to isolate and disperse nuclear contents of various mammalian cell types for ultrastructural studies of active genetic loci. Preparative procedures involve testing of various combinations of detergents and high pH treatments for solubilizing plasma membranes and nuclear envelopes without degradation of nuclear contents, followed by dispersal of chromatin in distilled water (adjusted to pH 9) before fixation and centrifugation onto a carbon support film for electron microscopy. Best results have been obtained thus far with a commercial dishware detergent, JOY (Proctor and Gamble).

Preliminary studies have utilized two cell types: HeLa cell cultures, because extensive biochemical information on RNA metabolism is available for this cell; and mouse oocytes, where successful mapping of genetic activity might conceivably provide a tool for studying some types of early embryonic defects.

We believe we now have the first pictures of transcription in a human cell line. Both chromosomal RNA and active rRNA genes of HeLa cells have been visualized.



Fig. 1. Dispersed HeLa cell chromatin with attached ribonucleoprotein fibrils.

Chromosomal RNA molecules are widely spaced on the genome (Fig. 1) and no gradients of ribonucleoprotein fibrils have been observed. Ribosomal RNA genes are about 3.6μ long (Fig. 2), somewhat shorter than the 4.5μ predicted by the molecular weight of the rRNA precursor. This foreshortening probably occurs due to localized denaturation of the DNA duplex at the transcription sites of the 130 or so RNA polymerases



Fig. 2. HeLa ribosomal RNA genes showing gradients of nascent ribonucleoprotein fibrils containing RNA molecules in successive stages of maturation.

reading each gene. Similar to rRNA genes of amphibians, HeLa rRNA genes have intergene spacers. Chromosomal RNA synthesis during mouse oogenesis has been visualized, but no rRNA genes have been detected so far.

Further technical refinements plus the use of cells with biochemically defined genetic systems should open the way for identification of specific active genes, in addition to rRNA genes, and eventually allow ultrastructural mapping of chromosomes.

A SURVEY FOR INHIBITORS OF PROTEIN INCORPORATION BY ISOLATED AMPHIBIAN OOCYTES

R. A. Wallace and Ti Ho

A variety of both some of the more commonly used metabolic inhibitors and several substances previously

found to influence protein incorporation by other types of cells were tested for their effects on protein incorporation by *Xenopus* oocytes *in vitro* (Fig. 1). With the exception of the uncouplers and the sulfhydryl reagent iodoacetate, the metabolic inhibitors at concentrations of 10^{-3} M or less had little influence on sequestering activity. Protein incorporation was severely depressed without apparent cytotoxic manifestations by the presence of vinblastine (which disrupts microtubular structures), by a variety of sulfhydryl agents (particularly the arsenicals) and, at high concentrations, by negatively charged macromolecules. Cycloheximide, at concentrations which essentially abolished all protein synthesis, reduced protein incorporation by only 40–50%. Since protein uptake is mediated by micropinocytosis at the cell surface (1), the cycloheximide data implies the existence of a considerable membrane precursor "pool."

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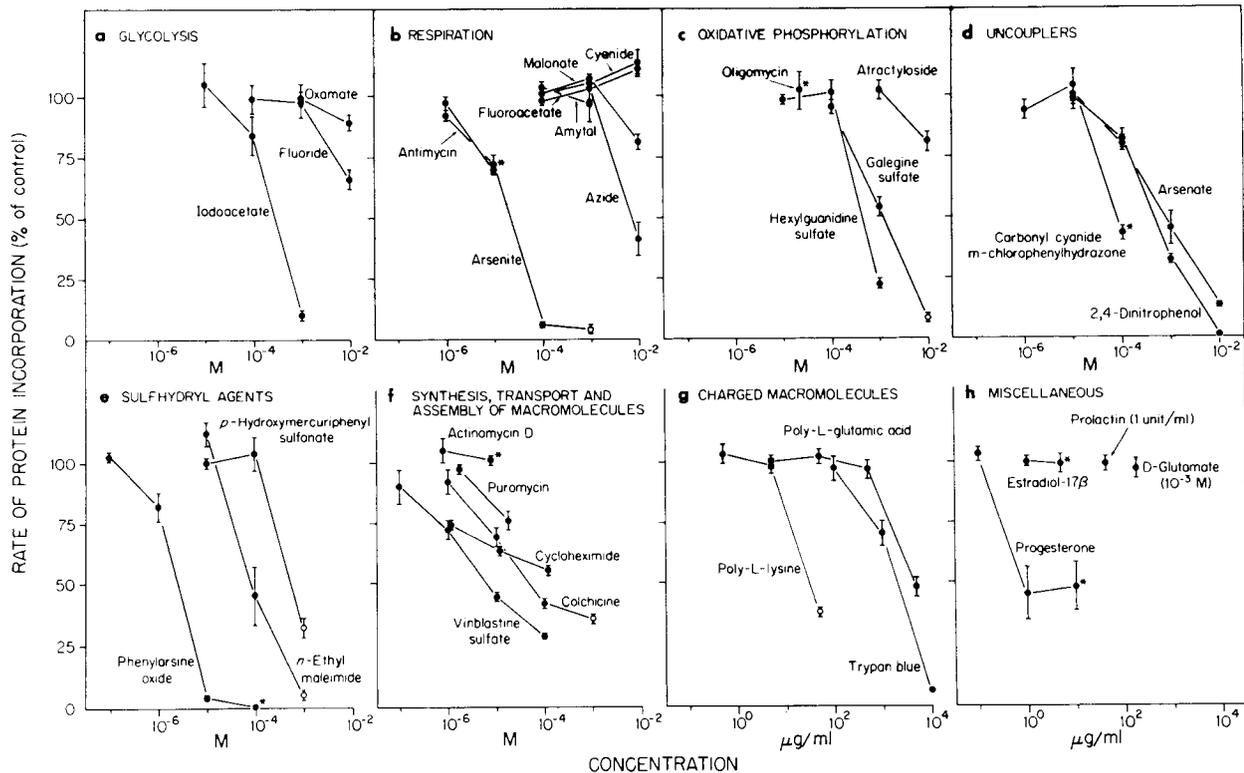


Fig. 1. Rate of protein incorporation by oocytes in the presence of different amounts of various inhibitors.

The inhibitors are classified according to their metabolic effects on other systems (a-d, f), chemical reactivity (e), and macromolecular change (g), or as miscellaneous (h). The rates indicated are relative to those observed in the absence of any inhibitor and each point represents 19-36 oocytes derived from at least two different females. Asterisk indicates the presence of 0.5% ethanol as a solvent for the additive. Oocytes generally appeared normal after incubation, ●; oocytes generally appeared abnormal (discoloration, blistered surface, etc.), ○.

Taken together, the evidence supports a preliminary model whereby protein-binding sites reside within positively charged areas of the surface membrane. An essential component of these sites would be reduced vicinal sulfhydryl groups that react with external protein by thio-disulfide interchange. Subsequent internalization and transport of the bound protein would require both the insertion of new or recycled membrane protein into the oolemma and the integrity of a microtubular system.

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OPTIMUM INCUBATION CONDITIONS FOR ISOLATED AMPHIBIAN OOCYTES

R. A. Wallace, D. W. Jared, J. N. Dumont, and Marsha Sega

Oogenesis involves a number of distinctive processes, among which is an inordinate cellular growth. The major part of this growth is generally limited to a relatively well-defined period and is characterized by the deposition of a large amount of proteinaceous material (1). During this period, a yolk protein precursor (vitellogenin) is synthesized external to the ovary and is subsequently taken up into growing oocytes by micropinocytosis (2). In order to study in detail the processes involving the oocyte, we developed a method for individually incubating isolated, growing oocytes from *Xenopus laevis* (3). Over the past several years, we have refined our incubation procedure by assessing the effects on protein incorporation of each individual ion in the incubation medium and of various additives. Protein uptake was found to be markedly sensitive to temperature and the ionic composition of the medium, with calcium being a particularly important component. Magnesium, phosphate, and additional energy sources were of only minor significance. Based on these observations, we have reformulated several slightly improved versions of the standard incubation medium.

A stereological analysis of micropinocytotic pits and vesicles, by isolated oocytes incubated for up to 24 hr, was also performed and indicated that the number of micropinocytotic pits and vesicles, if anything, was greater than that found *in vivo*. This important observation implies that pronounced changes in the rate of protein uptake which we have observed in the presence of inhibitors or altered incubation conditions represent perturbations of the normal sequestering mechanism rather than some other minor or less significant process.

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A CYCLIC AMP-STIMULATED PROTEIN KINASE FROM AMPHIBIAN OVARY AND OOCYTES

R. A. Wallace and Andrea Tenner*

Previous work (1) has documented that extracts and purified preparations derived from amphibian ovary and eggs promote the transfer of phosphate from the terminal position of ATP to certain proteins. Casein, lipovitellin, and particularly phosvitin served as the most effective substrates tested in the presence of the ovarian protein kinase. The suggestion was made at the time, therefore, that the phosphorylation of yolk proteins may be an important mechanism in the formation of yolk inclusions within the oocyte. A more recent biochemical study (2) on isolated oocytes undergoing yolk formation would seem to preclude this possibility, however, since the yolk proteins (lipovitellin and phosvitin) did not appear to be labeled up to 48 hr after $^{32}\text{P}_i$ was supplied to the oocytes. Other unidentified proteins did become labeled, however, providing evidence for endogenous protein phosphorylation. Because of these findings we have initiated a new study to explore the significance of protein kinase in the amphibian oocyte.

Protein kinase activity was found in extracts and purified preparations (ammonium sulfate fractionation, DEAE-cellulose chromatography) of ovary and oocytes of *Xenopus laevis*. Chromatographically purified preparations promoted a phosphorylation of proteins at a linear rate for at least 30 min regardless of substrate. At low concentrations of substrate (0.2 mg/1 ml), histone was more readily phosphorylated than either casein or phosvitin. Furthermore, cyclic AMP promoted a 3- to 10-fold increase in the rate of histone phosphorylation, whereas the phosphorylation of casein and phosvitin remained unaffected. Cyclic AMP-stimulated histone kinase activity was promoted by Mg^{2+} and Co^{2+} and was inhibited by Ca^{2+} , Cu^{2+} , and *p*-hydroxymercuriphenyl sulfonate. At high concentrations (3.0 mg/ml), phosvitin served as a good substrate for a cyclic AMP-independent phosphorylation catalyzed by the same purified preparations that displayed cyclic AMP-stimulated histone kinase activity. The relationships between the two catalytic activities remains to be determined.

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MOLECULAR BASIS FOR THE TRANSFORMATION DEFECTS IN MUTANTS OF *HAEMOPHILUS INFLUENZAE*

N. K. Notani,* Jane K. Setlow, Vijaya R. Joshi,† and D. P. Allison

To determine the molecular basis of transformation defects in *H. influenzae*, the fate of genetically marked, ^{32}P -labeled, heavy DNA was examined in three mutant strains (rec_1^- , rec_2^- and KB6) and in wild type having ^3H -labeled DNA and a second genetic marker. Transforming cells upon lysis with digitonin followed by low-speed centrifugation are separable into the supernatant fraction, containing mainly the unintegrated donor DNA, and the pellet, containing most of the resident DNA along with integrated donor DNA. Electron micrographs of digitonin-treated cells also indicate that the resident DNA is trapped inside a cellular structure, but that cytoplasmic elements such as ribosomes are extensively released. DNA synthesis in digitonin-treated cells is immediately blocked, as is any further integration of donor DNA into the resident genome. Isopycnic and sedimentation analysis of supernatant fluids and pellets revealed that in strains rec_2^- and KB6 there is little or no association between donor and resident DNA, and thus there is negligible transfer of donor DNA genetic information. In these strains, the donor DNA is not broken into pieces of lower molecular weight as it is in strain rec_1^- and in the wild type, both of which show association between donor and recipient DNA. In strain rec_1^- , although some donor DNA atoms become covalently linked to resident DNA, the incorporated material does not have the donor-DNA transforming activity.

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NEW TRANSFORMATION DEFECTIVE MUTANTS OF *HAEMOPHILUS INFLUENZAE*

K. L. Beattie

A previously described (1) method for enriching *Haemophilus influenzae* cultures for transformation-deficient mutants was used to isolate sixteen mutants which take up DNA normally but have transformation

frequencies ranging from 2×10^{-1} to 4×10^{-3} that of wild type. Unlike in the rec_1 and rec_2 mutants, which were previously isolated in this laboratory, recombination of temperature-sensitive HP1c1 phages can occur in all of the new mutants. Therefore these mutants must be blocked in a step (or steps) in transformation which is not required for phage recombination. Alternatively, in phage recombination this step could be controlled by phage genes. Like the rec_2 mutant, but unlike the rec_1 mutant, all of the new mutants have wild-type sensitivity to UV irradiation, and lysogenic cultures of these mutants can be induced by mitomycin C to produce phage. The spontaneous rate of phage induction in some of the new mutants appears to be three to five times higher than in wild-type strain Rd and the rec_1 and rec_2 strains. Like the rec_2 mutant, some of the new mutants appear to be slightly sensitive to the alkylating agent methylmethanesulfonate (MMS), but not nearly as sensitive as the rec_1 mutant. As in the rec_1 and rec_2 mutants, transfection is depressed in all of the new mutants.

To study the molecular basis of the transformation defects in the new mutants, a gentle lysis method was used which, following exposure of competent cells to ^{32}P - and heavy isotope-labeled transforming DNA, allows separation of the cellular material into a pellet fraction containing nearly all of the resident DNA and integrated transforming DNA, and a supernatant fraction containing the unintegrated DNA (2).

In all of the mutants there is some initial association of donor radioactivity and biological activity with the pellet fraction. Only two of the new mutants show an increase in donor radioactivity in the pellet fraction as a function of time of incubation in the nongrowth competence medium. Unlike wild type, none of the new mutants show an increase in donor DNA biological activity in the pellet fraction with time. The nature of the pellet-associated donor DNA is being analyzed by velocity and equilibrium centrifugation techniques.

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EVIDENCE FOR THE INVOLVEMENT OF A SPECIFIC UV ENDONUCLEASE AND A RECOMBINATION GENE IN DNA MAINTENANCE BY *HAEMOPHILUS INFLUENZAE*

J. E. LeClerc* and Jane K. Setlow

The following mutants have been used for an investigation of the effect of combinations of repair defects

on UV sensitivity: (1) UV1, about 20 times more UV-sensitive than wild type, defective in excision of UV-induced pyrimidine dimers because of lack of the specific UV endonuclease; (2) UV2, with the same UV sensitivity as UV1, excision-defective because of a block in excision following the action of the UV endonuclease; and (3) *rec*₁⁻, recombination-defective and about four times more UV-sensitive than wild type, in spite of normal excision, because of inability to fill gaps in DNA synthesized after UV irradiation. Wild-type and UV2 cells transformed to streptomycin resistance following exposure to *rec*₁⁻ DNA carrying a streptomycin-resistance marker are cotransformed to *rec*₁⁻ at a frequency of 4%. The *rec*₁⁻ transformants of wild type have the same UV sensitivity as the original *rec*₁⁻ mutant. The UV2 *rec*₁⁻ double mutants are extremely UV-sensitive, about 80 times more sensitive than wild type, and four times more than the UV2 parent, indicating that the effect on UV sensitivity of the UV2 and *rec*₁⁻ mutations together is additive. Among 360 UV1 cells transformed to streptomycin resistance with *rec*₁⁻ DNA, only one was *rec*₁⁻. However, this transformant has the same response to UV-induced killing as the *rec*₁⁻ cells, and thus contains a functional UV endonuclease. The fact that no UV1 *rec*₁⁻ double mutants were found indicates that this combination is inviable. This conclusion is strengthened by the inability to detect, out of 112 single colony isolates tested, a cell more UV-sensitive than *rec*₁⁻, when *rec*₁⁻ cells were transformed with UV1 DNA and selected for antibiotic markers linked to the UV1 gene. It is concluded that either the UV endonuclease or the *rec*₁⁺ gene product must be present for DNA maintenance.

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A DOUBLE *REC*⁻ MUTANT OF *HAEMOPHILUS INFLUENZAE*

Joanne M. Nickol* and Jane K. Setlow

Two different *Rec*⁻ mutants isolated in this laboratory, *rec*₁⁻ and *rec*₂⁻, have been crossed by transformation to produce a *rec*₁⁻*rec*₂⁻ strain, for the purpose of gaining some understanding of the interactions between the two *Rec* genes. The double mutant was selected by making use of the fact that the *rec*₁⁻ mutation causes UV sensitivity as well as a recombination defect, whereas the *rec*₂⁻ strain has wild-type UV sensitivity. Investigation has been made of the radiobiological and transformation properties of the double mutant. The phenotypic effect of the *rec*₁⁻ mutation, which normally causes sensitivity to X-rays and the alkylating

agent methyl methanesulfonate (MMS), as well as to UV radiation, is partially suppressed by the presence of the *rec*₂⁻ mutation, in that the sensitivity is either intermediate between the two strains (following UV irradiation), or as low as that of the *rec*₂⁻ strain (following treatment with X-rays or MMS). Although the *rec*₁⁻ mutation alone causes a deficiency in filling single-strand gaps in DNA synthesized after UV, the double mutant is able to carry out this process, but less efficiently than the *rec*₂⁻ strain. Similarly, the double mutant is inducible following lysogenization with the phage HP1c1, although the *rec*₁⁻ mutation alone makes the cell noninducible.

It has been shown that *rec*₂⁻, but not *rec*₁⁻, contains a block to DNA-DNA association that must precede recombination, whereas *rec*₁⁻ is apparently deficient in converting associated DNA molecules to completed recombinants (*I*). If the residual transformation in *rec*₂⁻ cells is the result of a very small fraction of cells that can permit normal DNA-DNA association, the transformation frequency of the double mutant would be expected to be the product of the frequencies of the single mutants. However, the transformation frequency of the double mutant is like that of the *rec*₂⁻ strain, about 10⁻⁷ that of wild-type cells, whereas the *rec*₁⁻ strain transforms about ten times more efficiently than *rec*₂⁻. Thus it appears probable that the residual transformation in *rec*₂⁻ takes place by an entirely different mechanism from normal recombination.

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I. N. K. Notani, Jane K. Setlow, Vijaya R. Joshi, and D. P. Allison, this report, p. 100.

A MUTANT STRAIN OF *HAEMOPHILUS INFLUENZAE* WITH LOW TRANSFORMATION FOR A SINGLE MARKER

N. K. Notani*

H. influenzae strain N19, originally isolated as a UV-sensitive mutant, was previously shown to be transformed to novobiocin resistance (*nov*) with an efficiency about two orders of magnitude lower than that of another, closely linked marker, streptomycin resistance (*str*) (*I*). In order to determine whether or not this phenomenon was the result of decreased ability of N19 cells to integrate the *nov* marker, DNA containing the *nov* and *str* markers, labeled with ³²P and heavy isotopes, was introduced into ³H-labeled N19 cells and at intervals the DNA was extracted, subjected to isopycnic analysis and fractions assayed for

transforming ability on wild-type cells and on N19. The results indicated that the *nov* marker was incorporated into the N19 genome with normal efficiency, as judged by the ability of hybrid DNA molecules to transform wild-type cells, but not N19 cells, to novobiocin resistance. Even when the N19 cells were grown for 5 hr following transformation, the incorporated *nov* marker was present and able to transform wild-type cells, although only a very small fraction of the N19 cells become novobiocin resistant. These results suggest that N19 contains a mutation which usually prevents the expression of the incorporated *nov* marker.

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A GENETIC ANOMALY IN *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow and N. K. Notani*

Construction of genetic maps of bacterial genomes by transformation depends on measurements of transformation frequencies of single and multiple genetic markers. Two markers are considered to be linked when the frequency of transformation of two markers together is considerably higher than the product of the frequencies of transformation of the two markers measured separately. We have investigated a case in which there are inconsistencies in linkage measurements between three drug markers which confer resistance to streptomycin (*str*), kanamycin (*kan*), and novobiocin (*nov*) in *H. influenzae*, and which have been previously shown to be in that order on the chromosome. The method we have used was to transform one or two of these markers into a cell containing the two other markers, or a single one of the three. The transformed cell was isolated as a single colony, grown up and lysed, and single and multiple transformation frequencies from the DNA of the lysate were measured on an unmarked recipient cell. About 150 different lysates were assayed in this way to determine linkage between the three markers. When the *kan* locus in the first transformation was nonhomologous (with kanamycin-resistant recipient cells and DNA from kanamycin-sensitive cells, or *vice versa*), the transformant obtained showed the usual linkage between the outside markers, *str* and *nov*, but in some cases the *kan* marker failed to show linkage with the *str* and *nov* markers, as judged from the single and double transformations obtained

with the DNA of the original transformant. In some transformants the *kan* marker appeared linked to one of the outside markers, but not the other.

One obvious interpretation of these results is that the *kan* marker can be located either between *str* and *nov*, or in some other position on the chromosome. However, the possibility that when *kan* appears unlinked this marker is in an aberrant position on the chromosome has been eliminated by the discovery that the same lysate which shows no linkage between *kan* and *nov* when assayed on wild-type *H. influenzae* (strain Rd) shows good linkage between these markers when assayed on the UV-sensitive strain, N19. Thus it appears that the *kan* marker is indeed in the expected site, close to *nov*, but fails to cotransform with the *nov* or *str* marker in wild-type cells, possibly because of small regions of inhomology between the donor DNA and wild-type recipient cells, on one or both sides of the *kan* marker, which results in a high probability of crossover at these sites. This hypothesis would explain why there can be linkage between the outside markers, *str* and *nov*, but not between one or both of these markers and the inside *kan* marker.

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THE HOMOLOGY BETWEEN THE DNA'S OF *HAEMOPHILUS INFLUENZAE* AND *H. PARAINFLUENZAE*

M. E. Boling

The filter-disc method for hybridization was used to measure the degree of homology between the DNA's of *H. influenzae* and *H. parainfluenzae*. An equation was derived, describing the relation of the homology between the DNA's and the amount of hybridization observed. Where H is the portion of each genome from either strain which is homologous, d is the amount of DNA on the disc, s is the amount of radioactively labeled DNA to which the discs are exposed, and K is the constant representing the ratio of the efficiencies of hybridization between homologous DNA's and heterologous DNA's, the equation is $H = \sqrt{Kd_o s_o / d_x s_x}$. The subscripts o and x denote homologous and heterologous exposures, respectively, which result in the same amount of annealing. Assuming that K = 1, the homology between the two strains was found to be 0.44. It was also demonstrated that those sequences which the strains have in common represent the same fraction of their genomes, suggesting that the two genomes are the same size. This was found by approximating the homology separately for each strain, by

exposing one, radioactively labeled, DNA to the other DNA which was in excess on a disc. Under these conditions almost all the labeled DNA which can find a homologous sequence will anneal. Approximately half the DNA annealed in each of the reciprocal exposures.

MOLECULAR EVENTS ACCOMPANYING THE FIXATION OF GENETIC INFORMATION IN *HAEMOPHILUS* HETEROSPECIFIC TRANSFORMATION

N. K. Notani* and Jane K. Setlow

Heterospecific transformation between *Haemophilus influenzae* and *H. parainfluenzae* was investigated by isopycnic analysis of DNA extracts of ³H-labeled transforming cells which had been exposed to ³²P-labeled, heavy transforming DNA. The density distribution of genetic markers from the resident DNA and from the donor DNA was determined by transformation assay of fractions from CsCl gradients, both species being used as recipients. About 50% of the ³²P atoms in *H. parainfluenzae* donor DNA taken up by *H. influenzae* cells are transferred to resident DNA, and only a small amount of the label is lost under conditions of little cell growth. There is less transfer in the reciprocal cross, and almost half the donor label is lost. In both crosses the transferred donor material transforms for the donor marker considerably more efficiently when assayed on the donor species than on the recipient species, indicating that at least some of the associated ³²P atoms are contained in relatively long stretches of donor DNA. When the transformed cultures are incubated under growth conditions, the donor marker associated with recipient DNA transforms the donor species with progressively decreasing efficiency. The data indicate that the low heterospecific transformation between *H. influenzae* and *H. parainfluenzae* may be due partly to events occurring before association of donor and resident DNA, but results mostly from events which occur after the association of the two DNA's.

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THE DEFECTIVE PROPHAGE IN *HAEMOPHILUS INFLUENZAE*

M. E. Boling, D. P. Allison, and Jane K. Setlow

It has been previously postulated that wild-type *H. influenzae* contains a defective prophage. We have succeeded in purifying the phage following induction of

wild-type cells with mitomycin C. Electron microscopic examination has shown that the phage is morphologically similar to the *H. influenzae* phages HP1c1 and S2, with a tail and tail fibers, except that it appears to have a defective tail plate. We have failed to lysogenize a strain of *H. influenzae* which does not carry the defective prophage with this phage, using the inducibility of the cells as a criterion for the presence of prophage. We subsequently found by electron microscopy that the phage is unable to attach to cells, presumably because of the tail plate defect.

The molecular weight of the DNA of the defective phage is approximately the same as those of the DNA's of phages HP1c1 and S2, as measured by sedimentation velocity. However, the defective phage, unlike that of HP1c1, apparently does not contain sticky ends, as judged by the lack of multiple forms of the DNA which are readily found in preparations of HP1c1 DNA. Some of the defective phages contain bacterial DNA, since we have found that it is possible to transform competent cells with DNA extracted from defective phage which was purified following induction of genetically marked cells. Transduction is not possible with these phages because of the attachment defect.

The homology between the three phage DNA's has been measured by the disc method. Whereas more than 90% of the HP1c1 and S2 genomes are homologous, the defective phage shows little homology to either of the others.

PHAGE DNA REPLICATION FOLLOWING INFECTION OF *HAEMOPHILUS INFLUENZAE* BY WHOLE PHAGE HP1c1

N. K. Notani,* Jane K. Setlow, and D. P. Allison

The *H. influenzae* phage system is particularly suitable for a study of phage DNA replication, because we have found that infected cells treated with digitonin release most of the phage DNA, but only a small fraction of cellular DNA. Thus the intracellular phage DNA may be readily separated from most of the bacterial DNA.

It was previously observed that phage HP1c1 contains DNA which is capable of circularization (1). We have found three forms of intracellular phage DNA following infection with ³²P-labeled phage: linear molecules, open circles, and twisted circles, as judged by alkaline and neutral velocity sedimentation, density sedimentation with ethidium bromide, and electron microscopy. Pulse label during intracellular phage development preferentially enters the twisted circles, and then may be chased into slower-sedimenting form. There may be

a fourth form of intracellular phage DNA which is present in small amounts, since we have observed that the peak of biologically active transfecting DNA is always between the twisted and circular forms on velocity gradients. This may represent linear dimer molecules, which were previously shown to be very efficient for transfection (1). Although it is known that bacterial DNA markedly depresses plaque formation by phage DNA infection (2), the amount of bacterial DNA on the gradients, as measured by transformation by a genetic marker in the original host cell, is too small to have an appreciable effect on the transfection from gradient fractions.

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THE MECHANISM OF TRANSFECTION OF *HAEMOPHILUS INFLUENZAE* BY PURIFIED PHAGE DNA

N. K. Notani* and Jane K. Setlow

The efficiency of transfection in *H. influenzae* under the best of conditions is around 10^{-2} per input phage DNA molecule, and has been shown to be considerably lower in *rec*₁⁻ cells with a defective recombination mechanism (1). It was previously postulated that recombination of phage DNA was required for most successful transfection in wild-type cells. To investigate this possibility further, we have infected wild-type and *rec*₁⁻ cells with ³²P-labeled phage DNA. At intervals the infected cells were treated with digitonin to release the intracellular phage DNA, which was then subjected to sedimentation velocity measurements and also used for transfection to measure its biological activity. After uptake of phage DNA into both strains there is extensive degradation, as evidenced by the appearance of slower sedimenting material and some loss of acid-insoluble counts, as well as decrease in transfecting ability of the DNA. Some of the phage DNA in wild-type cells about halfway through the latent period appears in rapidly sedimenting forms, with a wide distribution of molecular weights. This material is not present in infected *rec*₁⁻ cells, and thus may represent some type of recombinant phage DNA which is the precursor to phage production in wild-type cells. The structure of these molecules is currently under investigation.

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BREAKAGE OF PARENTAL DNA STRANDS IN *HAEMOPHILUS INFLUENZAE* BY 313-nm RADIATION AFTER REPLICATION IN THE PRESENCE OF 5-BROMODEOXYURIDINE

K. L. Beattie

The preferential breakage of BrUra-containing DNA compared to unsubstituted DNA by 313-nm radiation has been utilized to determine the extent of DNA repair after UV irradiation of mammalian cells (1) and the length of repaired regions in the DNA of UV-irradiated bacteria (2), and has also been used to study postreplication repair of DNA in UV-irradiated mouse cells (3). In an attempt to use 313-nm-induced strand breakage to detect repair replication in a thymine-requiring mutant of *Haemophilus influenzae*, it was found that 313-nm irradiation of untreated cells grown in the presence of BrdUrd after labeling with [³H]dThd resulted in a significant amount of breakage of the ³H-labeled DNA strands. Breakage of the ³H-labeled strands was about 0.6% as efficient as that of fully BrUra-substituted DNA, and occurred at a much higher frequency than in DNA of cells not exposed to BrdUrd. During growth in the presence of BrdUrd, susceptibility to 313-nm-induced breakage of the ³H-labeled DNA strands increased, reaching a maximum in about one generation, and it decreased to zero during subsequent growth for one generation in medium containing dThd instead of BrdUrd. Heat denaturation of DNA extracted from [³H]dThd-labeled cells grown in the presence of BrdUrd eliminated 313-nm-induced breakage of the ³H-labeled strands.

It is concluded that breakage of the ³H-labeled DNA strands resulted from reaction with photoproducts in the base-paired, BrUra-containing strands, rather than from photolysis of BrdUrd incorporated into parental strands. The lack of significant 313-nm-induced breakage of [³H]dThd-labeled DNA in *E. coli* after growth in the presence of BrdUrd (2) may reflect differences in the conformation of the DNA in *H. influenzae* and *E. coli*.

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MUTATION FIXATION AND DNA SYNTHESIS IN *HAEMOPHILUS INFLUENZAE*

R. F. Kimball and Jane K. Setlow

The use of DNA from *Haemophilus influenzae* lysed at various times after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) has allowed us to follow the apparent fixation of the induced mutations. The DNA from cells lysed immediately after treatment with the mutagen produces very few drug-resistant mutants when used in a transformation assay; DNA from cells lysed 30 min or longer after treatment produces nearly the same number of mutations by transformation as would have been found in the treated bacteria themselves (1). Most if not all DNA synthesis is delayed much more than 30 min in the treated bacteria, suggesting that a complete round of DNA synthesis was not required. An attempt was made in a ^{32}P "suicide" experiment to see if a smaller amount of DNA synthesis was required for the fixation. The MNNG-treated bacteria were incubated with ^{32}P during the fixation period, and the frozen lysates were held until nearly complete decay of the isotope had occurred. The expectation was that mutant regions of DNA would be preferentially destroyed by ^{32}P decay if a small amount of DNA synthesis was indeed a prerequisite for mutation fixation. No loss of mutagenic action could be demonstrated in the lysates. Unfortunately, the test is not sensitive for very short segments of newly synthesized DNA since tests with normal replication showed that, under our conditions, only about one in a thousand of the phosphorus atoms in newly synthesized DNA was the radioactive isotope. Thus all we can say is that any new DNA synthesis involved in mutation fixation must have involved regions appreciably less than 1000 nucleotides. A definitive test will require methods that allow the detection of much shorter segments of newly synthesized DNA.

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THE LACK OF MUTAGENICITY OF HYCANTHONE IN *HABROBRACON SERINOPAE* FEMALES

Roger H. Smith

Hycanthone, an antischistosomiasis agent, has been shown to be mutagenic in some systems (1, 2, 3) but not in others (4). *Habrobracon serinopae* females were fed hycanthone mixed in sucrose to determine whether or not dominant or recessive lethal mutations were

induced in oocytes in meiosis I and mitotically active oogonia. Virgin females produce haploid progeny parthenogenetically, therefore the entire genome can be screened for mutations in the following generation.

Virgin females of the *p* (plum eye color) stock were starved at 28° C for approximately 3 days, or until their abdomens showed dorsal-ventral flattening. These females were divided into five groups of 24 females each: (1) fed 40% sucrose (control); (2) fed 40% sucrose and X-irradiated, 3 kr (501 r/min, 250 kVp at 30 ma with 3mm Al); (3) 0.1 *M* hycanthone; (4) 0.05 *M* hycanthone; (5) 0.01 *M* hycanthone (all in 40% sucrose). The females were observed while they fed to verify that each female did indeed eat the mixture. The crop expands as the wasp feeds. All groups of females, with the exception of (3), fed to full capacity, and they rubbed their mouth parts with their front tibiae, indicating an aversion to the mixture.

All the females were set individually with host larvae, *Anagasta kuhniella*, for oviposition immediately after being fed. Oviposition was allowed continuously for 10 days, with each female being transferred each day at approximately 9 A.M. and 3 P.M. to a new stender dish containing a fresh host. Egg counts were made after each transfer, and hatchability was scored 36–48 hr later. Adult survival was recorded 8–10 days after oviposition. The entire experiment was carried out at 28° C \pm 1/2° C and 50–60% relative humidity.

A total of 28,319 eggs were oviposited by the females in this experiment. The pattern of egg production showed no significant difference between females fed sucrose and those fed sucrose mixed with the three concentrations of hycanthone (Fig. 1). The pattern produced by females irradiated with 3kr is typical for cells going through oogenesis (5). The depression in egg production from day 3 to day 7 indicates the cells that were transitional oogonia at the time of exposure to X-rays. The recovery after day 7 indicates the cells that were the more resistant oogonia at the time of irradiation. Only the eggs from the irradiated group show a significant reduction in hatchability (Table I). There are no obvious differences between the control group and the three groups of females fed hycanthone.

The pattern of egg production by females fed the hycanthone indicates that there was no detrimental effect on the ability of these females to produce eggs. This lack of reduction in the average number of eggs per female is a good indicator that there was no change in general physiology (6). The absence of the typical depression like that seen for the irradiated females and the high hatchability of haploid eggs from females fed hycanthone show that no dominant or recessive lethal mutations were induced.

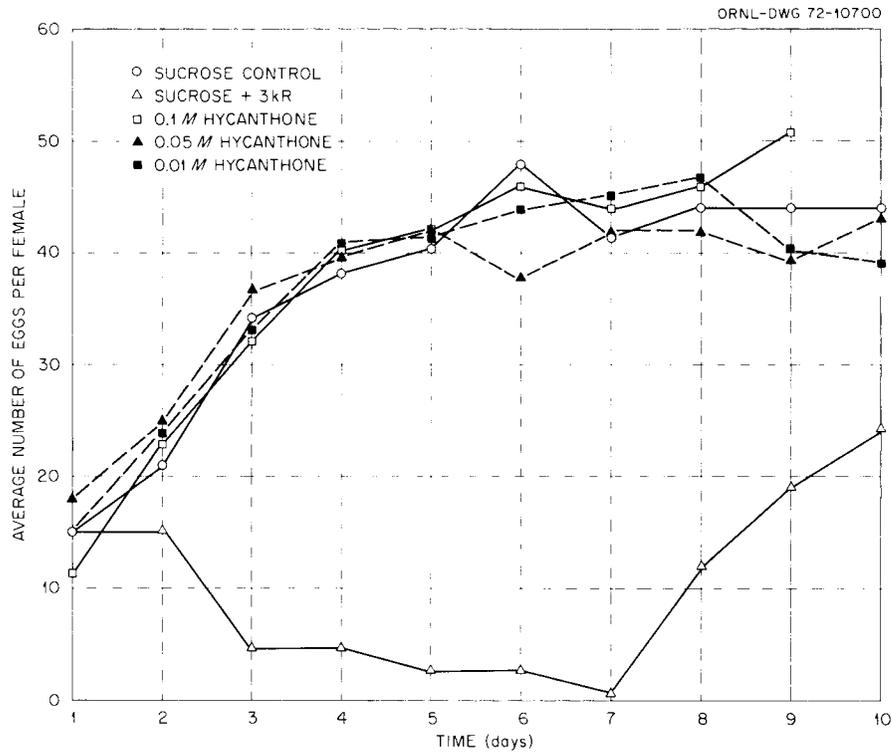


Fig. 1. The pattern of egg production for virgin females after a single feeding of 40% sucrose (○); sucrose + 3kr (△); and 0.1 M hycanthone (□), 0.5 M hycanthone (▲), and 0.01 M hycanthone (■) in 40% sucrose.

TABLE I. The number of eggs and the frequency of hatchability of eggs from virgin female *Habrobracon* fed hycanthone or X-irradiated with 3kr

Day	Control		3kr		0.1 M		0.05 M		0.01 M	
	No. of eggs	Hatch frequency								
1	350	0.860	331	0.508	201	0.920	390	0.936	291	0.955
2	484	0.888	324	0.731	377	0.939	535	0.907	436	0.890
3	719	0.940	105	0.533	542	0.935	782	0.931	635	0.934
4	764	0.955	86	0.651	608	0.956	837	0.949	743	0.954
5	819	0.956	43	0.721	631	0.946	885	0.936	748	0.957
6	724	0.898	55	0.412	646	0.954	823	0.930	788	0.957
7	772	0.784	25	0.320	616	0.896	818	0.906	765	0.817
8	792	0.872	207	0.517	648	0.914	782	0.897	792	0.824
9	712	0.896	362	0.688	644	0.910	755	0.864	642	0.924
10	752	0.896	558	0.762	504	0.915	830	0.877	640	0.881
Total eggs	6880		2096		5426		7437		6480	

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GENE DOSAGE IN *HABROBRACON*

Roger H. Smith and Janet J. Eide*

Gene dosage can be studied very easily in *Habrobracon serinopae* because sex is determined by ploidy and by a single locus. Females are diploid, and haploid males are produced parthenogenetically by virgin females. By the appropriate crosses, diploid males can be produced that are genetically distinguishable from the haploid males. Therefore it is possible to determine whether or not a diploid individual makes more gene product than a haploid individual or whether or not dosage compensation occurs. In this study electrophoretic and microdensitometric measurements were made of the esterase system to compare enzyme activity between the three classes of individuals.

Crosses were made between strains with similar sex alleles (x^a , x^b) in order to obtain all three classes of individuals. Standard wild-type ($H\ddot{s}$) males were crossed to females homozygous for plum eye color (p). The progeny from this cross were wild-type females ($+/p$, x^a/x^b), wild-type males ($+/p$, x^a/x^a , or x^b/x^b), and plum eye males (p , x^a , or x^b). In addition, female and haploid male progeny from $H\ddot{s}^+$ and p females were used for comparison. After being allowed to feed on *Anagasta kuhniella* larvae, the females in each group were removed from the hosts so they would store eggs. Each group was then set for oviposition for 3 hr. To obtain an adequate number of offspring for each end point, each group was set for several oviposition periods. The progeny were allowed to develop at 28°C. At 5, 6, 7, 8, 10, and 15 days after oviposition, collections of 10 animals were made. Each group of ten animals was weighed and homogenized in 1 ml of 50% sucrose and 0.075 M tris-sulfate pH 9.0 homogenizing solution. In some cases 10 animals were not obtained and the amount was adjusted accordingly. The samples were then centrifuged at 33,000 rpm for 24 min and the supernatant was frozen at -80°C.

The ORTEC 4200 and 4100 electrophoresis systems were used to obtain zymograms of the *Habrobracon*

esterases. The procedure of Smith and Reindollar (1) was followed for electrophoretic runs and the reaction mixtures to stain the gels. The ORTEC 4300 Scanning Microdensitometer[†] was employed to obtain automatic densitometric visual and digital readings of all esterase peaks. A comparison was made between identical samples to check for experimental errors. Differences ranged from 1 to 7%; therefore we had confidence in making comparisons between the different classes of animals. Protein determinations were made on each sample to obtain a value for relative enzyme activity per microgram of protein.

Figure 1 is a plot of total esterase activity per μg of protein over the five developmental periods. For all three classes of individuals enzyme activity decreases from the early pupal period up to adulthood and then increases in the adult stage, especially in females. The pattern is similar for the two major esterase bands, EST A and EST B. All esterase bands are present in diploid females, diploid males, and haploid males, and only in

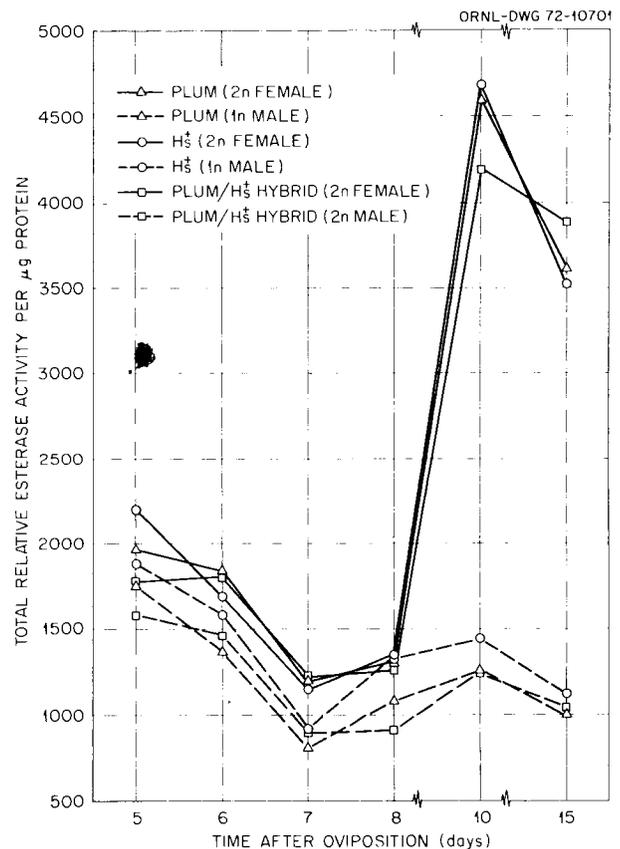


Fig. 1. Total relative activity of all esterases per μg protein in different developmental stages of *Habrobracon*.

the adults is there a significant quantitative change in enzyme activity.

These results indicate that the esterase activity is the same in haploid males and diploid males from day 5 of development to day 15, 7 days after adulthood. Therefore dosage compensation occurs for these gene loci. Also, the pattern and quantity of esterase activity is the same for the female until adulthood, but then increases threefold, showing a difference in sex and not between ploidy.

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GENETIC METHODS FOR INSECT CONTROL

Roger H. Smith and R. C. von Borstel*

Smith (1) proposed that conditional lethal mutations induced by mutagens would be useful in the control of insect pests. The special characteristics of mutations that would be useful for population regulation were described in detail. Most of these were based on the results of gene action studies performed on temperature-sensitive lethal mutations in *Habrobracon* and results reported for *Drosophila* (2). With the theoretical basis of population control by conditional lethal mutations established, we collected the methods that could be used to place the mutations into a pest population.

Dominant conditional lethals can be used in the same way as the sterile-male method. The pest population is overflowed with pests carrying the lethal mutations which result in the death of all F₁ progeny. This method may be most useful in species that cannot tolerate the high dosages of radiation or chemosterilants needed to induce 100% dominant lethal mutations in sperm. Other benefits which might be attained in different species of insects are the release of both males and females, selection of dominant conditional lethals that have little or no effect on mating ability, and the use of complementing double heterozygotes instead of a single homozygote which might be weak and unable to compete with wild individuals.

Recessive conditionally lethal mutations of course could not be used by overflowing. These mutations would have to replace the wild-type alleles in the population to be effective. Therefore the lethal allele would have to be given a selective advantage over the natural allele. This could be done by linking the conditional lethal to a complex translocation so that as

the translocated-type chromosomes replace the natural chromosomes (3) the conditional mutation thereby replaces the natural alleles. The same could be accomplished by linking the lethal mutation to a meiotic drive chromosome or to a gene that conveys a selective advantage to its possessor, such as "supermaleness," reduced development time, higher egg production, and so on. A necessary requirement for this general method is that the replacement of the natural alleles occurs before the environmental conditions change from permissive to restrictive.

These ideas, both original and published, have been presented in a review manuscript which has been accepted for publication (4).

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ADMINISTERING MUTAGENS TO *HABROBRACON* BY THE VACUUM INJECTION TECHNIQUE

Roger H. Smith

Sega and Lee (1) developed a method of reduced air pressure to administer ethyl methanesulfonate (EMS) to *Drosophila*. This technique is now employed to deliver EMS to *Habrobracon*. It has an advantage over feeding a chemical mutagen in a honey or sucrose mixture because *Habrobracon* females normally have to be starved before they eat and then they resorb their most mutagen-sensitive oocytes (Metaphase I). Also, of course, this new method is much faster than individual injections by a syringe and needle.

We are using the vacuum system described by Sega and Cumming (2). Presently a range of reduced air pressures and EMS concentrations are being tried to establish a dose-response curve based on EMS concentration, time under vacuum, and degree of reduced air pressure. We have established that 5 λ to 0.5 λ EMS, a 4-hr recovery period from the time of initial vacuum to the time of oviposition, and an air pressure of 9.5 pounds of mercury per square inch are about the best parameters to obtain dose-response data for survival and mutation in Metaphase I oocytes.

This procedure should allow us (1) to do a comparative study between EMS and X-radiation for the

induction of mutations at specific loci and temperature-sensitive mutations in the entire genome for different stages of the cell cycle in oogenesis, (2) to make more accurate estimates of chemical dosages delivered to the insects (2), and (3) hopefully to reduce the variability of the delivered dosage between animals and between experiments.

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CHARACTERIZATION OF TWO MUTATOR STRAINS IN YEAST

Dorma J. C. Gottlieb*

A method has been developed in this laboratory for determining spontaneous reversion rates in yeast with a reproducibility of better than 30% from experiment to experiment (1). In order to gain a better understanding of the spontaneous mutation processes in yeast, we have induced and selected "mutator" genes. A preliminary report on the procedure and results of such a search has been published (2).

The purpose of this study was to characterize two of these mutator strains and try to determine their mode of action. These two strains, provisionally designated VA-3 and VA-105, were chosen because they exhibit the highest spontaneous reversion rates (measuring reversion from lysine-dependence to independence) obtained thus far.

Both strains have been crossed to a "nonmutator" strain and the spontaneous mutation rates of the resulting diploids were determined. Based on the results of these experiments, it was concluded that in both cases the mutator character is recessive.

Data on the segregation of the mutator characteristic in heterozygous diploids indicates that with VA-3 a single gene is responsible for the increased mutation rate. In VA-105, it appears that there are two separate mutator genes, one of which is allelic to VA-3.

The spontaneous mutation rates were determined by measuring the reversion rates of the ochre-suppressible allele, *lys1-1*. All reversions were mutants of the super-suppressor type, which is not true of the parental strain. As a further test, the reversion rates for a second ochre-suppressible allele, *arg4-17*, were determined with the two mutator strains as well as with the parental. The results obtained were essentially the same as with *lys1-1*. In the mutator strains, the reversions were all Class I Set 1 super-suppressors, which have been shown to involve the genes coding for tyrosine tRNA's.

It is unknown whether the mutator activity of these genes affects only super-suppressors or is a more generalized effect. Preliminary results with VA-105 show an effect on the reversion rate of a nonsense mutant, *his1-7*. Experiments are in progress to measure more accurately the effect of VA-105 on reversion rate of *his1-7*, and to determine if these mutators have an effect on the reversion of *thr3-10* (suggested to be a frameshift mutation).

The effect of the mutators on forward mutations is also being determined by measuring the rates of induction of canavanine-resistant mutants. By looking at the effect of the mutators on several different types of mutations, it may be possible to determine the type of lesions that is responsible for the increased spontaneous reversion rates observed in these strains.

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MAMMALIAN GENETICS

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
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Effects of Radiation on Mammalian Gametogenesis

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Mammalian Comparative Mutagenesis

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EFFECT OF THE INTERVAL BETWEEN IRRADIATION AND CONCEPTION ON X-CHROMOSOME LOSS IN FEMALE MICE

W. L. Russell, Patricia R. Hunsicker,
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and Georgia M. Guinn

The interval between irradiation and conception has a marked effect on the frequency of specific-locus mutations induced in female mice. In offspring conceived at intervals longer than 7 weeks after irradiation, no significant increase over control mutation rate was seen (1). The purpose of the present experiment is to test whether the interval between irradiation and conception has a similar effect on an entirely different type of genetic damage, namely, X-chromosome loss.

Mature F₁ hybrid female mice from a cross of 101 and C3H strains were exposed to 400 r of gamma radiation, at approximately 0.006 r/min, from a ¹³⁷Cs source. After the completion of exposure, the females were mated to males carrying the sex-linked gene *Greasy* (*Gs*). The offspring were scored for presumed *Gs*/O females which were checked by breeding tests and chromosome counts. Chromosome counts of the mothers of these females were also made, to exclude any cases where the parent was already X/O.

Since the number of offspring from matings occurring in the early weeks after irradiation is not yet large, this report is restricted to results from offspring conceived more than 7 weeks after irradiation. In a total of 6152 female offspring in this group, there were five cases of X-chromosome loss (0.081%).

The observed frequency is not statistically significantly different from the three X-chromosome losses in 7065 females (0.042%) scored in the controls in this experiment, nor from the 13 X-chromosome losses in 20,823 females (0.062%) in the combined controls from this and other experiments.

Even if the slight, but nonsignificant, increase over the control frequency should later turn out to be real, it is clearly still going to be very much less than the frequency of from 85 to 102 cases of X-chromosome loss observed in 13,359 females (from 0.64 to 0.76%) born from early matings of mothers that had been exposed to 400 r of 80 r/min irradiation (2).

It appears that the effect of the interval between irradiation and conception on X-chromosome loss in female mice is similar to that observed for specific-locus

mutations. If the results can be applied to the human, they indicate that the hazard from this type of genetic damage, like that from the entirely different type of damage represented by specific-locus mutations, may be very small, or even nonexistent, for all offspring conceived later than a certain interval after irradiation.

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TRITIUM-INDUCED MUTATIONS IN THE MOUSE

R. B. Cumming, W. L. Russell,
Elizabeth M. Kelly, and Marva F. Walton

Having developed a workable system for using the specific-locus method to detect mutations induced by tritiated water in mice (1), we have expanded the work in this program. To date, 14 groups of 18 male mice each have passed through the specially designed isolators following injection with tritiated water. The first two groups received 0.75 mCi, and the remaining 12 groups 0.50 mCi, per gram of body weight. The males were mated to T-stock females, and their offspring were scored for mutations at seven specific loci.

A total of five specific-locus mutations have been observed in 3842 offspring from germ cells that were in postspematogonial stages throughout their exposure to radiation from the tritium. A computation of the mean radiation dose per germ cell indicates that the induced mutation rate in these postspematogonial stages is close to that expected from an equal dose of externally applied radiation.

In 8487 offspring from germ cells that received almost all of their radiation exposure in spermatogonial stages, eight specific-locus mutations have been scored. On the basis of our present estimate of the mean dose, this mutation rate is more than twice as high as would have been expected from external gamma radiation delivered at a comparable dose rate. With the small sample of mutations, and some uncertainties about dose estimation over the long periods of exposure involved, it is questionable whether the difference is statistically significant. However, a departure from expectation in the direction which raises the possibility that tritium may turn out to be more of a hazard than anticipated certainly warrants the collection of additional data.

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X-RAY-INDUCED SPECIFIC-LOCUS MUTATION RATES IN NEWBORN AND YOUNG MALE MICE

P. B. Selby*

The specific-locus mutation frequency resulting from 300 r of X-irradiation delivered at approximately 80 r/min has been determined for the reproductive cells present in male mice 0, 2, 4, 6, 8, 10, 14, 21, 28, and 35 days of age. All irradiated mice were F₁ hybrids from a cross of strains 101 and C3Hf. Each mouse was irradiated at one of the above ages, and then, after growing up, was paired with a female of the T stock, which is homozygous for seven recessive marker genes. The offspring produced by these irradiated males throughout their lifetimes were observed for mutations at the seven genetically marked loci.

Two different approaches were taken according to the goals desired and the sample sizes planned to achieve those goals. The experiment on day-0 males was designed so that a large enough sample of offspring would be observed for specific-locus mutations to provide a reliable estimate of the actual mutation frequency, providing, of course, that the mutation rate was not so low that few mutations would be obtained.

The objective of the remaining nine experiments, on males ranging from 2 to 35 days of age, was to test whether or not there might be a much higher mutation rate during some particular stages of development. While a slightly increased mutation frequency in some transitional stage of development might have little effect on an estimate of genetic hazard to man, a greatly increased mutation rate in some stage would warrant concern. In order to explore this possibility, smaller samples of offspring from each of the nine age groups were observed for mutations. Although these samples were smaller, they were, nevertheless, large enough to reveal, through calculation of the upper 95% confidence limit of the mutation rate at each stage, whether or not any of the stages tested had a much higher specific-locus mutation rate. Large enough samples were collected so that there was a good chance that the upper 95% confidence limit of the mutation rate at each age would be less than four times the point estimate of the adult spermatogonial mutation rate if the mutation frequency were not, in fact, higher than that of the adult.

Sixteen specific-locus mutations were found in 55,456 offspring sired by males irradiated on day 0.

The point estimate of this mutation frequency is 13.7×10^{-8} mutations/locus per r. In comparison, the point estimate of the spermatogonial mutation frequency for similarly irradiated adults is 29.1×10^{-8} mutations/locus per r (1). The mutation frequency of the newborn male is significantly lower than that of the adult, $P = 0.011$ (for a one-tailed test).

As was mentioned, the nine groups from day 2 to day 35 were only tested for the presence of a much higher mutation rate. The closer intervals at the earlier ages were chosen because of the more rapid changes in the immature testis during that time (2). Although the testis appears mature histologically at 35 days of age (2), it was not known whether it would have the same mutational response as the older males used in earlier specific-locus experiments.

Of the nine age groups from day 2 to day 35, the point estimates of only two were higher than the adult, the highest being 1.8 times the adult. However, neither of these two higher point estimates was statistically significantly higher than the adult rate. Only one of the nine groups had an upper 95% confidence limit higher than three times the point estimate of the spermatogonial mutation frequency in the adult. Even this group's upper 99% confidence limit was less than four times the point estimate of the adult. It therefore appears highly unlikely that the mouse testis passes through any stages of development between birth and adulthood during which the germ cells in it are very much more responsive to the induction of specific-locus mutations than those of the adult. If the data from all nine ages are combined, there are 43 specific-locus mutations in 77,895 offspring. This is a mutation frequency of 26.3×10^{-8} mutations/locus per r, which is very similar to the point estimate of the spermatogonial specific-locus mutation frequency, 29.1×10^{-8} mutations/locus per r (1), found for the similarly irradiated adult. This does not, however, exclude the possibility that a few of the ages tested may have a somewhat different mutation rate.

It is not known exactly when the transition in mutation frequency, from lower in the newborn testis to higher in the adult, occurs. However, the data suggest that the change has occurred by day 8 (3).

Six of the 16 mutations found on day 0 occurred as three clusters of two mutations each. This frequency of clusters, 3 in 13 independent occurrences of mutations, is significantly higher than the frequency of clusters, 0 in 40 independent occurrences, that Russell (4) found in the similarly irradiated adult, $P = 0.012$ (Fisher's exact test). Clusters are thought to be indicative of relatively decreased survival of germ cells following

irradiation. In view of this, it is interesting that there was also a statistically significant increase in permanent sterility among the males irradiated with 300 r on day 0 compared to that in adults irradiated with as much as 1000 r. Whereas 4.6% of the males irradiated with 300 r on day 0 were permanently sterilized, only 0.7% of adult males exposed to 1000 r were permanently sterilized, the latter incidence not being statistically significantly different from controls (5).

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A SEARCH FOR RADIATION-INDUCED MUTATIONS AT THE HEMOGLOBIN LOCUS IN THE MOUSE

W. L. Russell, Carolyn Vaughan,
and K. B. Jacobson

An electrophoretic procedure for separating mouse hemoglobins has been worked out that will facilitate the scoring of blood samples from a large number of animals (1). This is now being used, along with subsequent examination by R. A. Popp of the same blood samples by a solubility technique, to detect mutations at the hemoglobin locus in the mouse. The two main purposes of this project are: (1) to determine the radiation-induced mutation rate for a clearly defined biochemical variant, and (2) hopefully to obtain enough mutations to characterize the nature of radiation-induced variants, specifically the relative frequency of intragenic changes and deletions. The hemoglobin locus is ideally suited for this second purpose.

The 101 and SEC mouse strains differ at the hemoglobin locus, and their electrophoretic patterns are easily distinguishable from each other and from their F₁ hybrid. Offspring from a cross of these strains, following irradiation of one parent, are now being screened. The total number examined so far is small, since the parental strains are still being expanded. No mutations have been observed.

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ABSENCE OF MUTAGENIC EFFECT OF HYCANTHONE IN MICE*

W. L. Russell and Elizabeth M. Kelly

Hartman *et al.* (1) have reported that hycanthone, an antischistosomal drug, is mutagenic in *Salmonella*. On the basis of this, they expressed alarm at the widespread clinical testing of the compound in humans. We have conducted a specific-locus mutation test of hycanthone in mice. Adult wild-type males were injected intraperitoneally with 150 mg/kg of the compound (hycanthone methanesulfonate) and mated to our T stock which is homozygous for seven recessive marker genes. The offspring were scored for mutations at the seven loci. No mutations have been observed in 16,196 offspring (11,660 from cells treated in spermatogonial stages and 4536 from treated post-spermatogonial stages).

The mutagenic ineffectiveness of the compound cannot be attributed to any failure to reach the testis. Using labeled compound, Cumming (2) has detected a high proportion of the injected dose in the testis. Furthermore, Oakberg (3) has shown that when hycanthone is injected into mice at doses ranging from 24 to 150 mg/kg enough of the compound reaches the testis to be effective in killing some of the spermatogonia.

An exact comparison of fate and metabolism of hycanthone in mouse and man has not been reported. However, the preliminary data obtained by Cumming give no indication that the mouse can eliminate the compound faster than man can. It is, therefore, provisionally assumed that 3 mg/kg, which is the typical clinical dose in man, would give comparable exposure of the testis to the drug in mouse and man. It is further assumed that, if hycanthone were mutagenic, the mutation rate would probably drop faster than linearly with injected dose. Thus, dividing the mutation rate at 150 mg/kg by 50 would probably overestimate, and almost certainly not underestimate, the mutation rate at 3 mg/kg.

Taking the upper 95% confidence limit of the zero mutation rate observed with 150 mg/kg, subtracting the control rate (obtained from other experiments), and dividing by 50, gives an estimated upper limit for the induced rate of only 6% of the mouse spontaneous mutation frequency. The same calculation with the upper 99% and 99.9% confidence limits of the observed zero frequency gives estimated upper limits for the induced rates that are 9% and 15%, respectively, of the spontaneous rate.

Thus, if hycanthonone is capable of inducing any gene mutations at all in mouse germ cells, the frequency from a dose of 3 mg/kg is unlikely to be more than a small fraction of the spontaneous rate.

*Research jointly sponsored by the Food and Drug Administration and the U.S. Atomic Energy Commission.

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FURTHER GENETIC ANALYSIS OF MUTATIONS OBTAINED IN SPECIFIC-LOCUS EXPERIMENTS

Liane B. Russell

We have earlier (1, 2) reported on certain aspects of the detailed analysis of mutations obtained during specific-locus mutation-rate experiments. This work employed primarily methods of complementation analysis in the *d-se* region of linkage-group II (chromosome 9). By utilizing over 100 independent mutation detected by their phenotypic effects in combination with the test-stock markers *d* and *se*, we were able to reveal the existence of 16 complementation groups spanning 8–9 functional units. The work in the *d-se* region has this year continued along a variety of avenues and an additional complementation program has been instituted for the *c-locus* region of linkage-group I (chromosome 7) (3).

Continuing analysis of the *d-se* region is yielding basic information that has practical applications along a number of lines. (1) By providing data concerning the relation of the nature of the genetic changes to a number of biological and physical variables in the mutagenic treatment, it aids in the interpretation of experiments in mutagenesis from the point of view of human risks. (2) It has provided data on the number of functional units per crossover unit (namely, about 20) and hence on the genome size. This is being used by the U.N. Committee on the Effects of Atomic Radiation for calculations of genetic risks in humans. (3) It is providing material for estimating heterozygous effects of various types of mutations (4). (4) It has provided genetic material for other studies in mutagenesis, namely the screening of a chromosomal region for mutations induced within it (5).

Close to 300 additional combinations of mutants from newly available stocks were tested this year, and these tests have revealed at least two additional functional units and two additional complementation

groups. A third "skipping" type of mutation is indicated by the new data, this one involving *d*. The two previously found "skipping" mutations that involve *se*, as well as other *se*-lethal mutants are being analyzed for their effect on recombination of the outside markers *d* and *tk* (normal recombination ~6%). One of the "skipping" mutants has been found to inhibit recombination almost totally and may be an inversion spanning 2–6 map units.

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HETEROZYGOUS EFFECTS ON VIABILITY OF *d* OR *se* MUTATIONS THAT INVOLVE A KNOWN MINIMUM NUMBER OF LETHAL FUNCTIONAL UNITS

Liane B. Russell

Since mutations could exert most of their effect on populations while in a heterozygous state, it has always been of interest from the point of view of genetic risk in humans to determine the heterozygous viability effects of induced genetic alterations. Along these general lines, studies have been carried out (1) in which the number, location, or nature of induced mutations were not known, and which may therefore be considered empirical in nature. By contrast, only limited reports are available on the heterozygous effects of mutations detected by the specific-locus method. Thus, it has been noted that most *s-locus* lethals (2) and the majority of *Df(d se)* mutations (3) yield heterozygotes that are of obviously reduced stature; that small *s-lethal* heterozygotes may be less viable than normal (2); and that certain *Df(d se)*'s may affect heterozygous viability as well as reducing transmission (4).

The complementation analysis of the *d-se* region (5) has now provided us with more accurate information concerning the genetic nature of various *d* and *se*-lethals, and data have therefore been compiled that shed light on their heterozygous viability effects. Each mutation has been outcrossed for many generations to nonirradiated stocks; therefore there is a relatively high probability that any other induced mutations that were present elsewhere in the genome (except closely linked to the specific-locus mutation being studied) have been lost.

Data are here summarized on ratios at weaning age in matings segregating for lethals involving the dilute (*d*) or short-ear (*se*) loci. It should be pointed out that any heterozygous viability effects that might occur later in life would not be detected by this measure, nor would other types of fitness effects, such as reproductive capacity. Moreover, in the absence of contemporary control matings utilizing nonlethal alleles at the same loci, the data can hope to detect only effects of large magnitude.

Segregation in three different kinds of crosses was recorded at weaning age (25–32 days) (6). In the case of *se*-lethals (*se^l*), the cross tabulated in Table I is

+ *se^l/d* + × + *se^l/d* + and the lethal-bearing heterozygotes are therefore wild-type in phenotype, while the nonlethal segregants are dilute. The expected proportion of lethal-bearing heterozygotes, if these are fully viable, is 66.7%, assuming dilution has no adverse effect on survival to weaning, and slightly higher than 66.7% if dilution does have a slightly adverse effect.

In the case of *d*-prenatal lethals (*d^{pl}*), one rather limited set of results (Experiment 1 in Table II) comes from crosses of *d^{pl}+/d se* × *d^{pl}+/d se* or *d^{pl}+/+ se* × *d^{pl}+/+ se*. In each case, the lethal-bearing heterozygotes, in contrast to the remaining segregants, are not short-eared, which could possibly bestow on

TABLE I. Viability of *se*-lethal heterozygotes*

Mutant symbol	Minimum number of lethal "functional units" involved	Offspring classified (No.)	Proportion of <i>se^l</i> -heterozygotes in progeny at weaning age (%)
32DTD	1 (32DTD is a "skipping" mutation)	1127	66.4
52CoS	1	1471	66.8
5RD300H	1	654	65.3
207K	2	3852	59.2
70FBF _o	2+ (70FBF _o is a deficiency of >2 cM)	197	39.6

*Determined from percentage of wild-type among total viable progeny from cross of + *se^l/d* + × + *se^l/d* +.

TABLE II. Viability of *d*-prenatal lethal heterozygotes

Mutant	Minimum number of lethal "functional units" involved	Experiment 1*		Experiment 2 [†]	
		Offspring classified (No.)	Proportion of <i>d^{pl}</i> -heterozygotes at weaning age (%)	Offspring classified (No.)	Proportion of <i>d^{pl}</i> -heterozygotes at weaning age (%)
1FDF _o Hr _e	1	245	63.7	1003	48.0
7G	1	365	72.9	1741	52.2
19DThW _b	1	164	67.7	1082	51.7
3FAF _y _c	2	175	60.0	2071	51.8
3FR60H _c	2	155	63.2	1513	54.2
19R145H	2	108	74.1	1832	50.9
21R75M	2	141	64.5	1226	49.9
9R250M	2	170	73.5	2322	49.6
3FrS	2	205	68.3	2216	48.4
19Z _b	2	1377	66.5	1656	47.9
8DVT	2	638	68.8	2051	47.5
3FDF _o Hr _e	2	41	48.8	731	36.8

*Crosses of *d^{pl}+/d se* × *d^{pl}+/d se* and *d^{pl}+/+ se* × *d^{pl}+/+ se*. Proportion of *d^{pl}* heterozygotes determined from percentage of non-short-eared animals among total viable progeny.

[†]Alternating crosses of *d^{pl}+/d se* × *d^{op}+/+ se* and *d^{pl}+/+ se* × *d^{op}+/d se*: the + *se/d se* and *d^{pl}+/d^{op}*+ segregants are omitted when calculating proportion of *d^{pl}*-heterozygotes among remainder of the progeny. Results from the two alternating crosses are averaged.

them a slight advantage. The expected proportion of d^{pl} heterozygotes, if these are fully viable, is thus at least 66.7%. The other, more extensive, set of d^{pl} results (Experiment 2 in Table II) comes from alternate crosses of $d^{pl}/d\ se \times d^{op}/+\ se$ and $d^{pl}/+\ se \times d^{op}/d\ se$, with calculations based on only the d^{pl} - or d^{op} -bearing progeny (i.e., omitting $d^{pl}/d^{op}+$, which dies before, or at, weaning, and omitting $+ se/d\ se$). The crosses therefore alternate a situation in which the lethal-bearing heterozygotes are dilute and the others are wild-type with the opposite situation — thus canceling out any slight viability effect of dilute that might exist. The expected proportion of d^{pl} -heterozygotes is 50%. While the genetic background in none of the crosses is uniform, any modifiers probably distribute themselves randomly among the segregants.

The data for *se*-lethals (Table I) show a variety of results. No clear-cut heterozygous effect on viability by weaning age is demonstrated in the three mutations that involve a minimum of one lethal unit. On the other hand, there is apparently more than 27% loss of *se^l*-heterozygotes prior to weaning in the case of 207K which involves two or more lethal units; and about 67% loss of heterozygotes in the case of 70FBFo which is a deficiency of over 2 centiMorgans in length. In the *d*-lethal data (Table II), major heterozygous viability effects are apparent in the case of one of 12 mutations studied, namely 3FDFoHr_e, where 42% of the heterozygotes appear to be lost. Whether this mutant is a deficiency of intermediate size, such as 70FBFo (see above), will be difficult to determine, since, in the testing of d^{pl} -lethals, the closest marker available to the "left" of *d* (namely, *sg*) is 4.5 centiMorgans distant. No clear-cut heterozygous viability effects (in excess of 10% loss) could be demonstrated for the other 11 mutations studied. However, as has been pointed out, the method is suitable only for detecting effects of large magnitude. The results indicate that such major heterozygous effects can be lacking in the case of small deficiencies, including those that have a minimum of two lethal units in addition to the marker cistron. Whether or not more subtle effects, or effects on later viability or on productivity, are present would have to be determined in additional experiments.

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INDUCTION OF MUTATIONS IN A GIVEN CHROMOSOMAL SEGMENT

Liane B. Russell and Clyde S. Montgomery

Methods that have been proposed to detect mutations in certain chromosome segments include the scanning of short distances from markers by a backcross or intercross scheme (1) or the use of inversions (2). Both require three successive crosses, with tedious mating and bookkeeping procedures. We have this year developed a more practical method that utilizes as tools some of the presumed small deficiencies characterized by the complementation analysis of the *d-se* region (3).

Mice homozygous for the standard markers *d* and *se* are irradiated (so far, we have used 600 r to spermatogonia) and mated to wild type. The F₁ progeny are crossed to a test stock carrying, in repulsion, two adjacent deficiencies, one including *d* and the other *se*, which together span a region of 2–10 map units in linkage group II. (The maximum length is derived from the finding that the outside markers *sg* and *tk* are not involved in the deficiencies.) If a lethal has been induced in one of the marked segments, either *d* or *se* progeny will be lacking, except for rare crossovers. Similarly, if a grossly visible mutation has been induced, it will show up in the *d* or *se* progeny. As soon as an F₁ has produced at least three each of normal *d* and *se* progeny, it is discarded as nonmutant; but if fewer than this number have been found in 25 offspring, the appropriate segregants are saved for further testing. (Note: A detectable lethal cannot be farther from a marker than 6 centiMorgans — the *se-tk* distance — and a lethal thus situated would permit only 2% of the progeny to exhibit the marker phenotype.) Each tested F₁ represents one irradiated chromosome.

During this year, stocks for the experiment were produced, the irradiations were carried out, and a large number of F₁ were collected in both irradiated and control groups. To date, over 1000 of these F₁ have been set up in the test cross and the testing has actually been completed on a small batch of these. We estimate

that about 7000 F₁ in each group will need to be tested to give meaningful results. It is hoped that, in addition to yielding certain mutation-rate information, we shall detect new functional units in the *dse* region and recover further useful genetic material.

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ANALYSIS OF FUNCTIONAL UNITS FOR LETHALITY AND GLUCOSE-6-PHOSPHATASE ACTIVITY IN THE *c*-LOCUS REGION OF THE MOUSE

Liane B. Russell, D. L. DeHamer,*
Clyde S. Montgomery, and K. Bruce Jacobson

Complementation testing of *c*-locus mutants (linkage group I, chromosome 7) that have arisen in W. L. Russell's specific-locus mutation-rate experiments was begun this year, using 32 independent mutations that were originally detected by their *c^{ch}/c*-like phenotype in combination with the test-stock marker *c^{ch}*, and were in subsequent testing found to be lethals when homozygous. Crosses for more than half of the 528 possible combinations of these mutants have been made. In addition, the mutants are being tested with the nearby markers *tp* and *sh-1*.

Among the 32 mutants used are three that were earlier sent to Gluecksohn-Waelsch and homozygotes for which have been found, by her and by Cori's group, to be deficient for glucose-6-phosphatase (*I*). Consequently, offspring from those of our crosses that yield albino are being tested for G6Pase activity, using Cori's method.

Even though this experiment is still in relatively early stages it is already clear that we are finding at least three situations, both with respect to lethality and G6Pase-activity: no complementation, complete complementation, and partial complementation (the last may be of several degrees). On a simple, formal hypothesis, we can thus postulate functional lethal units on *both* sides of *c*, and functional units that control G6Pase-activity on both sides of *c*. It is still too early to determine the relation between G6Pase and lethal units.

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A SECOND X-AUTOSOME TRANSLOCATION INVOLVING LINKAGE GROUP VIII IN THE MOUSE

Liane B. Russell and Clyde S. Montgomery

X-autosome translocations [T(X;A)'s] in the mouse have been rare and valuable tools in providing evidence concerning activity of the mammalian X chromosome. Where there are several independent T(X;A)'s involving the same autosome, the value of the material becomes enhanced by the opportunity for comparative work, e.g., studies of the same loci in relation to different rearrangement points. This has been done to advantage with five T(X;1)'s (1, 2). The discovery, and exploration of basic properties, of a second T(X;8) will now allow a similar type of work to be performed with another set of chromosomes.

A translocation between the X chromosome and LG VIII (chromosome 4), named R7, was found following neutron irradiation to spermatozoa. Breakpoint location in LG VIII has been determined to be centromere-*wi-b-m*-R7. Recombination between *b* and R7 is ~20%. In 3-point crosses, *wi-b* recombination was ~6%, and *b-m* recombination ~11%, indicating no crossover inhibition in these regions. Breakpoint location in the X has not yet been firmly established; *Ta*-R7 recombination is between 3 and 20%. Experiments with other X markers are in progress.

X-inactivation affects the autosomal *b* and *m* loci, so that R7 heterozygous females with appropriate combinations of markers are recognizable by a variegated phenotype. Since *b* appears to be an amorph, *b* R7/*b* + females are nonvariegated brown. + R7/*wi* + females do not show any signs of the *wi* phenotype; this neurological mutant may not be suitable for grossly revealing mosaicism.

As was the case with the T(X;1)'s studied (1), viability and weight are reduced in heterozygous R7 females; survival to weaning is depressed by ~8%, and weaning weight by at least 12%. Unbalanced segregants from R7 crosses die during embryonic life, and R7 females are thus "partially sterile." Heterozygous males are completely sterile due to cessation of spermatogenesis in pachytene. In other respects, males are normal and are nonvariegated.

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DIVISION OF SPERMATOGONIAL STEM CELLS IN THE MOUSE

E. F. Oakberg and Patricia D. Tyrrell

Identification of those type A spermatogonia which occur as single, isolated cells with oval, dark-staining, uniformly granular nuclei as the stem cells (A_s spermatogonia) has made study of their cycle properties feasible. Previous experiments had indicated that duration of their cell cycle was variable, and that it could be as long as 207 hr.

In the present experiments, (101 × C3H) F_1 hybrid mice were given either a single injection of 10 μ Ci or 10 daily injections of 10 μ Ci (total of 100 μ Ci/mouse) of [3 H] thymidine. Animals were killed at intervals from 1 to 414 hr after injection, and autoradiographs of 4- μ tissue sections were prepared.

At one hour, the percentage of labeled cells was 46% for single, and 89% for multiple, injections; at 414 hr, these values were 2.4 and 24%, respectively. Several deductions may be made from these observations. Labeling after the single injection was higher than previously observed for all type A cells in controls, but comparable to that for cells surviving X-radiation (1), suggesting a long S period for A_s spermatogonia. Repeated injections failed to label all A_s cells, but long-term labeling suggested that each injection labeled a proportionate number of long-cycling cells, since the frequency at 414 hr after 10 injections was one order of magnitude higher than observed for a single injection. An average of the last eight time intervals gave 30.8% labeling for multiple and 3.48% for the single injection, confirming the 414-hr value. Thus kinetics of stem cells does not appear to be affected by multiple injections; only more cells are labeled. Labeling, in reference to stage of the cycle of the seminiferous epithelium at 414 hr (two cycles of the seminiferous epithelium) after the single injection, indicates that stem cells arise primarily from divisions at stages I, II, and IX of the cycle, with a smaller contribution at stages X-XII. Labeling at stages III-VIII initially was low, and did not persist for long

time intervals. Divisions of labeled A_s spermatogonia were observed at all time intervals, and gave rise to both single and paired labeled cells, confirming our previous observations on the process of stem cell renewal and differentiation.

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CYTOTOXIC EFFECTS OF HYCANTHONE ON MOUSE SPERMATOGONIA*

E. F. Oakberg and Patricia D. Tyrrell

Interest in the possible mutagenicity of hycanthone, an antischistosomal agent, stimulated an experiment on possible effects of this chemical on spermatogonial survival. Spermatogonial killing would demonstrate effective levels of the drug and/or toxic metabolites in the cells of primary interest in mutation induction in mammals.

A dose-response curve, utilizing both intraperitoneal and intramuscular injections of (101 × C3H) F_1 hybrids at doses of 23.6, 37.5, 75, 118, and 150 mg/kg of hycanthone base was run. A second group of SB hybrid mice was injected with 150 mg/kg. Animals were killed 72 hr and 5 days after injection, the testes were fixed in Zenker-formol, and 5- μ sections were stained in PAS-hematoxylin. Surviving cells were enumerated in 99 tubule cross-sections distributed according to the frequency distribution of tubule stages in control mice.

Only half the controls and the 150 and 37.5 mg/kg treatments have been scored. There was no significant difference between intraperitoneal and intramuscular injection at these doses, so the data of Table I were combined to give more precise estimates of survival.

Killing of spermatogonia was demonstrated, with increasing sensitivity paralleling spermatogonial differentiation (Table I). Thus the stem cells, A_s spermatogonia, are the most resistant cell type just as in the case of radiation. Even at the 150 mg/kg dose, 82% of A_s (stem) spermatogonia survived in the 101 × C3H,

TABLE I. Survival of spermatogonia of the mouse 72 hr after injection of hycanthone, expressed as percentage of control

Mouse strain	Dose (mg/kg)	Cell type treated					
		A_s	Aa1-A ₁	A ₁ -A ₂	A ₃	A ₃ -A ₄	A ₄ -In
101 × C3H	37.5	94.21	105.30	89.23	87.11	85.69	87.67
101 × C3H	150	81.57	62.42	29.63	9.06	6.35	1.21
SB	150	87.62	81.69	59.15	40.82	27.49	12.42

and 88% in the SB strain, whereas survival of A_4 -In cells was 1.21 and 12.42%, respectively.

At 37.5 mg/kg, stem cells also were more resistant, but response of differentiating cell types was uniform. At present, there is no explanation of the 105% observed for Aal- A_1 spermatogonia, since this still may be a sampling effect that will disappear when the remaining controls are scored. Preparation and injection of chemical in the two experiments were identical, and the most likely difference between SB and 101 X C3H mice is one of genetic constitution.

It is of interest to compare these results with the X-ray doses required to give comparable effects in the 101 X C3H hybrids. 150 mg/kg is equivalent to about 100 r, and 37.5 mg/kg to ~8 r of X-rays. Thus a fourfold change in dose of hycanthone has resulted in about a 12-fold difference in X-ray dose required to give a comparable effect. This suggests a much steeper dose-response curve for the chemical.

*Research jointly sponsored by the Food and Drug Administration and the U.S. Atomic Energy Commission.

CYTOLOGICAL CAUSES OF F_1 MALE STERILITY AFTER MUTAGENIC TREATMENT

N. L. A. Cacheiro and Margaret S. Swartout

A cytological study was made of the testes of sterile F_1 males found after mutagenic treatments in order to determine if there was any correlation between the various types of spermatogenic block and chromosomal

abnormalities, or if other genetic or physiological mechanisms might play a major role in F_1 sterility.

The males used in the present study were F_1 sons from three experimental series. One was an X-ray series (of L. B. Russell) in which the sterile sons came from matings in the second week after 200 r. Two series (of R. B. Cumming) involved ethyl methanesulfonate (EMS) treatment of 250 mg/kg given to males; in one of these series this was the only treatment, while in the other the animals were prefed with butylated hydroxytoluene (BHT). In the two EMS series, as in the X-ray series, the sterile sons came from presterile-period matings.

Altogether 42 sterile sons were studied from the three series: 13, 17, and 12 respectively. Sterility of these F_1 males had been established as follows. If, after 4 weeks of mating to a single female, no pregnancy ensued, the male was mated to three new females for 8 weeks. After that, if no young were born, the F_1 male was considered sterile.

One of the testes of each sterile male was processed using a squash technique and aceto-orcein staining; the other was fixed in Bouin's, sectioned, and stained with hematoxylin and eosin. The sections were used to evaluate the stages of spermatogenesis present and to determine where the blockage occurred. The squashes were used for mitotic and meiotic analyses.

The stages at which spermatogenesis is blocked, and their relation to cytological findings and type of mutagenic treatment, are shown in Table I. Altogether,

TABLE I. Relation of stage of spermatogenic block and cytological findings in sterile sons derived after various mutagenic treatments

Chromosome sizes (mitosis)	Latest spermatogenic present	Chromosome behavior (diakinesis)	Treatment			Total
			X-rays	EMS	EMS + BHT	
None visibly abnormal	Pachytene	?	4 [†]	1 [†]		5
	Diakinesis	T*	1	1		2
	Spermatozoa	T No X-Y pairing	3 1 [†]		2	7
Some visibly abnormal	Pachytene	T	1	8	1	10
	Diakinesis	T	1	1	5	7
	Spermatozoa	T T(Y-A)	2	4 1	3	10
?	Sertoli	?			1 [†]	1
Total F_1			13	17	12	42

*T = Translocation.

[†]Diagnosis of translocation could not be made.

17 males had some spermatozoa, 9 did not go past diakinesis, 15 were blocked in pachytene, and 1 had Sertoli cells only.

Presence of a translocation was deduced as follows: In cases where diakinesis was found, the presence of quadrivalents, or trivalents and univalents, or more complex figures was considered diagnostic. Of the 26 males where spermatogenesis proceeded to or beyond diakinesis, 24 had such diagnostic figures, one being a Y-autosome translocation. In cases of pachytene blockage, translocations were assumed to be present if the mitotic complement included an abnormally long and/or an abnormally short chromosome. This was the case in 10 of the 15 pachytene-blocked males. This leaves only eight males (footnoted in Table I) in the total of 42 sterile ones in which diagnosis of translocation cannot positively be made – partly due to the nature of the material – but in which translocations could presumably be present. In two of these eight, failure of X-Y pairing was observed, possibly indicating a Y- or X-deficiency.

In a comparison of the mutagenic treatments, it appears that EMS may produce a higher percentage of sterile sons with unequal translocations than does irradiation. Thus, in the two EMS series, 23 of 28 (82%) had visibly abnormal chromosomes in mitosis, while this proportion was 4 of 13 (31%) for X-rays. The sample sizes would, however, have to be increased to confirm this indication.

It is clear from these results, and from the earlier ones of Lyon and Meredith (1), that autosomal translocations, and not only X-autosomal translocations (2), can induce complete male sterility. In an attempt to determine whether a cytological difference could be detected between these translocations and those causing male semisterility, we analyzed the semisterile males from the same two EMS series (Table II). It is apparent that unequal translocations occur with considerably higher frequency in sterile than in semisterile sons.

TABLE II. Relation of sterility or semisterility to mitotic abnormalities in sons of EMS-treated males

	Mitotic complement	
	Visibly abnormal	Not visibly abnormal
Sterile males	23	5
Semisterile males	4	24

Summary.

1. Male sterility in F_1 progeny of males treated with mutagens is, in a very high percentage of cases, due to

autosomal translocations. It had earlier been found that reciprocal X-autosome translocations apparently always cause male sterility. In addition to the autosomal-translocation cases, we also found a Y-autosome translocation and two possible cases of Y- or X-deficiencies among sterile F_1 's.

2. A comparison between translocations causing male sterility and those causing male semisterility indicates that a much greater proportion of the former have breaks near the proximal or distal ends of chromosomes.

3. The stage in spermatogenesis at which blockage occurs may be at any time between pachytene and sperm formation, with no clear correlation with type of aberration present. No detailed cytological analysis can be made in the case of pachytene blockage.

4. It may be speculated that breaks near the ends of chromosomes, possibly near the centromere, may involve heterochromatin, and that the sterility may in some way be related to this.

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CYTOLOGICAL STUDIES OF MOUSE X-AUTOSOME TRANSLOCATIONS USING QUINACRINE-MUSTARD FLUORESCENCE AND THE GIEMSA TECHNIQUE FOR REVEALING CENTROMERIC HETEROCHROMATIN

N. L. A. Cacheiro, Margaret S. Swartout, and Liane B. Russell

Seven of the eight known reciprocal X-autosome translocations in the mouse occurred at this laboratory (1), and six of these have been available for varied cytological studies. In past work, we have analyzed mitotic (2) and meiotic (3) karyotypes by conventional staining techniques. The recent development of cytological methods using quinacrine-mustard (QM) fluorescence and the special Giemsa technique for the characterization of centromeric heterochromatin has provided superior new tools for additional cytological study and for its correlation with genetic findings. These cytological tools are now being used in studies of four X-autosome translocations involving linkage group I (= chromosome 7) and two involving linkage group VIII (= chromosome 4). All of these T(X;A)'s have earlier been shown (2) to have one excessively long chromosome and one very small one, which in some cases is the smallest member of the complement.

QM fluorescence in mitosis: The binding pattern of QM allows identification of the 19 autosomal pairs and the sex chromosomes of the mouse according to a recently derived standard karyotype (4). Since translocations do not alter the pattern, but merely rearrange it (5), the technique can be used to identify what chromosomes are involved in the translocation, to locate the breakpoints at least roughly within these chromosomes, and to match chromosomes to linkage groups. Our work with six T(X;A)'s is aiming toward these objectives.

Mitoses were obtained from kidney tissue cultures of mature heterozygous T(X;A)'s and stained with QM. Karyotypes were prepared from at least two males and two females from each of the translocation stocks, using the new chromosome classification (4). Determination of breakpoint location is facilitated when one break occurs in a bright area of one chromosome and the other in a dull area of the other, producing a banding contrast after rejoining. Where this tool is not available, determinations are less accurate, and estimates of relative locations are influenced by other

available facts, such as relative sizes of translocation products and configurations at diakinesis (3).

By matching the excessively long and very short chromosomes to nonpaired chromosomes in the complement, it was easily verified that the chromosomes involved were indeed those that would have been predicted on the basis of linkage information, namely #7 and X for the T(X;1)'s, and #4 and X for the T(X;8)'s. The short translocation product was in all cases found to be distinguishable from the Y or the smallest two autosomal pairs (chromosomes #18 and #19).

In R2 and R3, the extralong chromosome consists of the centromere and major portion of chromosome 7, plus 80–90% of the distal portion of the X. Conversely, the small translocation product has an X centromere and short proximal X portion, plus a short distal portion of chromosome 7. In R3, the small translocation product is shorter than pair #19; but in R2 it is intermediate between pairs #18 and #19 in length. The approximate break locations of R2 and R3 are shown in Fig. 1, with those in the X being considerably less

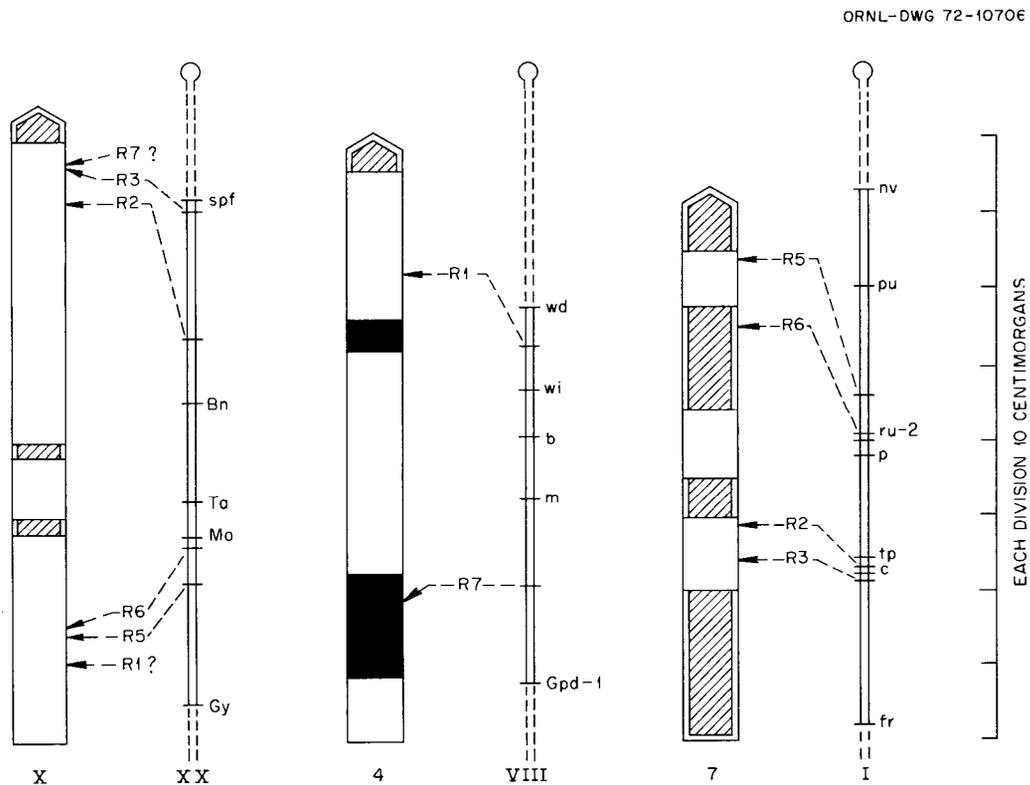


Fig. 1. Cytological fluorescence banding patterns and partial genetic maps of chromosomes involved in various X-autosome translocations.

certain than those in #7 (since there is little, if any, banding pattern in the proximal part of the X).

In R5 and R6 we find the converse situation from R2 and R3 in that the extralong chromosome consists of the X centromere and a major proximal portion of the X plus about 80–90% of the distal portion of #7. Approximate break locations are shown in Fig. 1.

The two T(X;8)'s represent two reciprocal situations. In R7 (as in R2 and R3) the long chromosome has an autosomal centromere and proximal portion, while in R1 (as in R5 and R6) the long chromosome has an X centromere and proximal portion. In R1 as well as R7, both of the translocation products can be easily identified by their banding patterns.

Fig. 1 also shows partial genetic maps of the linkage groups involved, including the genetic location of breakpoints where this has been determined (6). The comparison of cytologically and genetically located breakpoints serves to place a number of loci within certain morphological sections of chromosomes.

Use of the Giemsa technique for locating centromeric heterochromatin in diakinesis: In earlier work (3), we determined the proportion of rings, Type-A and Type-B chains, univalent-plus-trivalents, and bivalent-plus-bivalents found in the different T(X;A) stocks. Complete interpretation of the data was hampered by the inability to identify the centromeres in these different configurations. The use of new Giemsa-staining techniques has made possible a renewed attack on the problem.

The occurrence of at least one crossover in one or more of the arms of the pachytene cross will result in different and identifiable configurations at diakinesis or metaphase-1. This material can therefore be used to derive a crossover frequency at the cytological level, and thus an estimate of segment lengths involved in the translocations.

The method is also being applied to autosomal translocations.

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FERTILITY OF EMS-INDUCED TRANSLOCATIONS IN MALE MICE*

W. M. Generoso and Sandra W. Huff

The efficiency of low doses of chemicals, relative to high doses, in inducing genetic damage to mice and the shape of the dose-effect curve may vary markedly from one chemical to another. In males, the most efficient way of studying the effectiveness of low doses of chemicals to induce chromosomal breakage is by screening male progeny of treated males for translocation heterozygosity. This is accomplished by testing them for sterility or semisterility. Semisterility is almost always associated with induced reciprocal translocations, and there are strong evidences that sterility in F₁ males from EMS-treated male parents has the same basis (1, 2).

In the course of our study on dose effects of ethyl methanesulfonate (EMS) we accumulated information on the fertility of male translocation heterozygotes. A total of 149 EMS-induced presumed translocations were recovered. Of these, 51 were completely sterile and 98 were semisterile. None of the sterile ones were cytogenetically analyzed because, in all cases, the testes were markedly smaller than normal and, for most males, spermatogenesis did not progress to diakinesis. All of the semisterile translocations were verified cytogenetically and all were transmissible to the next generation.

Two sets of fertility data (Tables I and II) are available for the semisterile F₁ progeny. The first set was obtained by caging each one with a young female (about 12 weeks old) of the (SEC × C57BL)F₁ strain for the production of at least four litters. The second set was obtained by mating each one to six virgin (C3H × C57BL)F₁ females which were killed during pregnancy for uterine analysis.

TABLE I. Average fertility of semisterile males based on four litters

Litter no.	Number of semisterile males	Litter size (avg.)	Percentage of normal males*
1	98	4.2	46.3
2	98	4.9	43.6
3	98	4.7	40.9
4	96	4.8	42.0
			43.2

*970 normal males.

TABLE II. Average fertility of semisterile males based on six pregnant females killed

Class	Number of males	Implants (avg.)	Living embryos (avg.)	Dead implants (%)
Semisterile	98	9.3	4.3	54.2
Normal	39	10.7	10.2	4.8

On the basis of live births the average fertility of semisterile translocation heterozygotes was 43.2% that of normal males. This is comparable to the result obtained on killed females. The average number of living embryos is 42.2% that of normal controls. It should be mentioned that there are translocation lines whose fertility is markedly higher than 50%. Data on sacrificed females shows that fertilization involving sperm carrying unbalanced chromosome constitution leads to death of embryos, in the main either shortly before or after implantation. This is indicated by the fact that reduction in the number of living embryos is principally attributable to an accompanying increase in dead implantation. However, a small proportion of embryonic loss may occur in early cleavage stages, as indicated by a slightly lower number of implantations in the semisterile group.

Data on the fertility of translocation heterozygotes from the triethylenemelamine dose-response and X-ray experiments will become available in the near future. This information will be correlated with the cytogenetic analysis. A good understanding of the nature of translocations induced by a wide variety of agents will be very helpful in the development of a procedure for routine screening of chemicals.

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EFFECT OF DOSE IN THE INDUCTION BY TRIETHYLENEMELAMINE OF CHROMOSOME ABERRATIONS IN THE MOUSE*

W. M. Generoso, W. L. Russell, Sandra W. Huff, and Katherine T. Cain

Experiments on dose effects of ethyl methane-sulfonate (EMS) and triethylenemelamine (TEM) in the induction of chromosomal aberrations have so far revealed two important points (1, 2). First, the shape of

the dose-effect curves in the induction of dominant-lethal mutations and reciprocal translocations suggests that the effectiveness of EMS is proportionately much lower at low doses than at high doses. The EMS dominant-lethal dose-response curve is clearly not linear — it is markedly concave upward. The EMS translocation dose-response curve also showed that there is a more rapid increase in the number of translocations with dose than would be expected on the basis of dose-square kinetics. The TEM dose-effect curve for dominant-lethal mutations, on the other hand, approaches linearity and showed that this compound is proportionately more effective at low doses than EMS. Secondly, the comparative EMS study between dominant lethals and translocations showed that translocations are a much more reliable and sensitive end point than dominant-lethal mutations for measuring induced chromosome breakage. The subject of this report involves the progress of the TEM translocation dose-effect study.

Twelve-week-old (101 × C3H)F₁ male mice were injected intraperitoneally with various concentrations of TEM and mated with normal females during days 1½ to 15½ after injection. This period corresponds to the mid-spermatid stage which was found to be most sensitive for dominant-lethal induction with TEM. F₁ male offspring were fertility tested for translocation heterozygosity (3).

TABLE I. TEM dose-effect in the induction of translocations in male mice

Treatment	Dose	Number of F ₁ males tested	Number of presumed translocations
TEM	.0125	181	1 (.55%)
	.025	126	4 (3.17%)
	.05	217	10 (4.61%)
	.1	189	32 (16.93%)
	.2	43	12 (27.91%)
Control		2601	3 (.0012%)

Results to date are given in Table I. The amount of data is not yet sufficient for an accurate determination of the dose-effect curve but it already seems clear that, as for EMS, translocations are a more sensitive and accurate indicator of chromosome breakage. In the dominant-lethal dose-response study, clear induction was detected only beginning at the dose of .05 mg/kg. The translocation data, on the other hand, indicate that the dose of .025 mg/kg already induces a detectable

increase in chromosome breakage. Testing of F₁ males for translocation heterozygosity is continuing and, upon completion, we will be able to establish the dose-effect curve as well as find out whether the lowest dose of .0125 mg/kg induces a detectable increase in translocations.

*Research jointly sponsored by the Food and Drug Administration and the U.S. Atomic Energy Commission.

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INDUCTION OF CHROMOSOMAL ABERRATIONS IN MOUSE SPERMATOGONIA*

W. M. Generoso, R. J. Preston, and Katherine T. Cain

It has been generally known that certain alkylating chemicals are very effective in the induction of chromosomal breakage in the late spermatogenic stages of the mouse. Nevertheless, the ability of these chemicals to induce chromosomal breakage in spermatogonia has not been well studied; mostly cytogenetic and dominant-lethal studies on chemical effects in spermatogonia are available and their results have been conflicting. The type of data which has been lacking is on heritable chromosomal aberrations. Obviously, the most important genetic effects in terms of hazards are the transmissible ones. Thus it is essential to find out what is the actual incidence of specific chromosomal aberrations transmitted to the first generation progeny. To this end we are studying three compounds that are known to induce chromosomal breakage in postspermatogonial stages. We are testing their ability to induce heritable translocations, and correlating this result with that obtained from testicular cytogenetic analysis. Results on spermatogonia to date include (1) effects on cell killing and repopulation — this has been accomplished indirectly by observing the induction of sterility and the length of time required for the recovery of fertility — and (2) frequency of induced translocations as observed in the primary spermatocytes stage.

Twelve-week-old (101 × C3H)F₁ male mice were injected intraperitoneally with various concentrations of triethylenemelamine (TEM), tris(1-aziridinyl)phosphine oxide (TEPA), or cyclophosphamide. Each male was caged with a female of the (SEC × C57BL)F₁ or (C3H × C57BL)F₁ strains 42 days after treatment. For TEPA and TEM, four randomly selected males per dose

TABLE I. Fertility effects of chemicals and X-rays after treatment of spermatogonia*

Treatment	Dose	Number of males	Number of permanently sterile males	Appearance of first litter (avg. in days)
Cyclophosphamide	350	36	0	27.7
	400	22	0	28.9
TEM	3.0	32	0	41.2
	4.0	47	0	43.1
TEPA	20	23	0	47.8
	25	32	0	55.6
	30	14	1	78.2
X-ray	150	54	0	24.4
	300	47	0	28.6
	600	42	0	60.9
	1200	48	1	122.4
HBSS [†] (control)		29	0	24.6

*All males were mated 42 days after treatment.

[†]Hanks' balanced salt solution.

were killed for cytogenetic analysis soon after the first litter was born. In the case of cyclophosphamide, four males were sacrificed 7 months after pairing. Spermatocytes were prepared using the air-drying method of Evans *et al.* (1). Approximately 1000 male progeny per chemical were weaned and are presently being fertility-tested for sterility or semisterility.

The spermatogonial cell-killing effect of the three compounds, as measured by induced sterility, is shown in Table I. For comparison, X-ray data from our spermatogonial translocation experiment, which is in progress, are also included. On the basis of the highest doses, cyclophosphamide has the shortest sterile period, with TEM and TEPA about the same. One interesting comparison between X-rays and the chemicals is that the magnitude of spermatogonial cell-killing effects of the three chemicals, relative to the dominant-lethal effects in postspermatogonial stages, is in marked contrast to that found with X-rays. The dose of 600 r of X-rays induces about 50% dominant lethals in the most sensitive postspermatogonial stages and a sterile period of 36.3 days. In the case of TEM, only 0.2 mg/kg of TEM is required to induce 50% dominant lethals in the postspermatogonial stages. It should be noted that a TEM dose of 4.0 mg/kg induced a shorter sterile period than 600 r of X-rays. A similar situation holds true for TEPA and cyclophosphamide.

Another interesting comparison between X-rays and chemicals is shown by the cytogenetic data. Although these results are still preliminary, it is clear that all three chemicals are ineffective compared with X-rays (2) in the induction of translocations in mouse spermatogonia

TABLE II. Induction of translocations in mouse spermatogonia, scored in spermatocytes, following treatment with various chemicals

Chemical	Dose (mg/kg)	Number of cells scored	Translocations	
			Number	Percent
TEM	0.05	80	0	0.0
	1.6	100	2	2.0
	2.0	400	9	2.25
	4.0	350	4	1.14
TEPA	20	100	1	1.0
Cyclophosphamide	350	200	8*	4.0

*This is a maximal number because there were two instances of the same translocation occurring in successive cells. Only chain configurations were observed, which might indicate a bias in scoring, or might be a correct observation.

that are scorable in spermatocytes (Table II). These results are in general agreement with those of Leonard *et al.* (3), Gilliavod and Leonard (4), and Cattnach and Williams (5). The obvious question is: what does the cytogenetic frequency mean in terms of the frequency of translocations among the F₁ progeny? Very little is known about this relationship from studies with X-rays and none at all is known from those with chemicals. Thus, obtaining the frequencies of heritable translocations in both X-ray and chemical experiments will help in the understanding of this relationship.

*Research jointly sponsored by the Food and Drug Administration and the U.S. Atomic Energy Commission.

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STUDIES ON THE INDUCTION OF CHROMOSOMAL ABERRATIONS IN MICE BY HYCANTHONE*

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Hycanthone methanesulfonate (Etrenol, Winthrop) has been shown to be effective for the treatment of schistosomiasis in both animals and man. The apparent efficacy of this drug, its ease of administration, and its low toxic side effects have generated widespread enthusiasm for its use in field trials on human patients.

Hycanthone has been a subject of considerable controversy because of several reports that this drug is an effective mutagen in a number of test systems, but ineffective in others, and may be a genetic hazard to the human population. In the mouse, Russell *et al.* (1) found that hycanthone is ineffective in inducing specific-locus mutations in males. The present report provides additional information on the possible mutagenicity of hycanthone in mice. The effectiveness of this drug to cause chromosomal damage, measured in terms of dominant-lethal effects, in male and female germ cells was investigated.

In males, the dominant-lethal effect of hycanthone was studied for all stages in spermatogenesis. Two treatment protocols were used. In the first type of treatment, 12-week-old males from two different hybrid strains, (101 × C3H)F₁ and (SEC × C57BL)F₁, were given a single injection of 150 mg/kg of hycanthone. For the two strains, this dose caused one death out of 38 treated and four deaths out of 37 treated, respectively. In the second type of treatment, only (101 × C3H)F₁ males were used. They were injected with 125 mg/kg of hycanthone daily for 5 days, the total dose being 625 mg/kg. With this treatment, one death was observed out of 12 mice. Each male was caged with two virgin females immediately after receiving either the single 150 mg/kg dose or the last 125 mg/kg dose. Females were checked for presence of vaginal plug every morning and each mated female was replaced by a fresh one. Mated females were killed for uterine analysis 12–15 days after the plug.

Effects of hycanthone in females were studied by first subjecting 12-week-old (SEC × C57BL) mice treated with a dose of 175 mg/kg to the total reproductive capacity test. Fertility effects observed in the first litter were then analyzed by conducting a dominant-lethal test on matings that occurred 1/2 to 4 1/2 days after treatment. This test was done on another strain of females, (C3H × C57BL)F₁, and at three different doses of the drug – 175, 150, or 100 mg/kg.

In both the male and female experiments, the drug was dissolved in cold (about 5° C) Hanks' balanced salt solution (HBSS) and administered intraperitoneally. The injectable solution was maintained cold by keeping it in an ice water bath. Control mice were given a comparable volume of cold HBSS.

Results in males show no significant effect of hycanthone in inducing chromosomal aberrations throughout the entire spermatogenic cycle. This conclusion was based upon large numbers of females mated during 50 days after treatment of males. Six groups of experimental and control males were involved in the study, and an average of 260 females were mated per group.

Analysis was done by pooling data into consecutive two-day periods (average of 11 mated females per period). The single dose of 150 mg/kg did not induce any detectable increase in dominant-lethal mutations nor any other fertility effects in the two strains of males. Similarly, with the repeated treatment, no detectable increase in dominant-lethal mutations was observed throughout spermatogenesis, but complete sterility of treated males was observed on matings that occurred 37½ to 41½ days after treatment. Such infertility is attributable to the cell-killing action of hycanthone on differentiating spermatogonia (2).

Results on two strains of female mice show that hycanthone has a marked effect on fertility. The dose of 175 mg/kg induced a marked reduction in the size of the first litter, but not the subsequent litters, of (SEC × C57BL)F₁ females in the total reproductive capacity experiment. The dominant-lethal analysis on (C3H × C57BL)F₁ females revealed that hycanthone's fertility effect is attributable to an increase in the frequency of dead implantations. Doses of 100, 150, and 175 mg/kg induced 15.0, 27.7, and 30.0% dead implantations, respectively, as compared to two control frequencies of 3.7 and 5.9%.

Obviously, the question that needs to be asked, but which is very difficult to answer at the present time, is: what causes the increase in dead implantations and in turn lowers the size of the first litters? One possible explanation is that the increase in dead implantations may be due to the early toxic effects of the drug, since at two higher doses some animals died within 2 hr after treatment. But even if no deaths occurred, as with the 100 mg/kg dose, this explanation might hold true. The other possibility is that the increase in dead implantations was due to induced dominant-lethal mutations in oocytes in large follicles, since chemicals known to be mutagenic produce a similar response. It must be emphasized that, in experiments on females, the distinction between genetic and toxic effects as the cause of the increase in dead implantations is very difficult to make, since the females themselves receive the treatment.

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CHEMICAL INDUCTION OF SEX-CHROMOSOME LOSS IN FEMALE MICE*

W. M. Generoso, Katherine T. Cain,
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Our inability to determine whether the fertility effect of hycanthone in female mice (see above report) is due

to induced genetic damage clearly illustrates the necessity for further studies of the basic mechanisms involved in the chemical induction of chromosomal aberrations in female mice, as well as the need for a more reliable genetic assay system for evaluating mutagenic hazards of chemicals in mouse oocytes. Although certain chemicals are very effective in bringing about pre- and postimplantation death following prefertilization treatment of females (dominant-lethal-type response), only indirect evidences are presently available for a genetic basis of damage to oocytes. Thus it would certainly be highly desirable to determine the relationship of the presumed dominant-lethal response to heritable chromosome damage. For these reasons, we are studying the ability of certain chemicals to induce sex-chromosome loss and reciprocal translocations in the mouse oocytes.

X+/X+ females of the (SEC × C57BL)F₁ strain were injected intraperitoneally with either 75 or 50 mg/kg of isopropyl methanesulfonate (IMS) dissolved in cold Hanks' balanced salt solution (HBSS). Control mice were given a similar volume of HBSS. The general procedure for testing for X-chromosome loss follows that outlined by Russell *et al.* (1). Immediately after injection, females were caged individually with *Greasy* males (X_{gs}/Y). Female progeny from this mating were scored for *Greasy* phenotype (presumed X_{gs}/0), while male progeny were fertility-tested for translocation heterozygosity. Presumed X_{gs}/0 females were tested genetically by mating them to *sparse fur* males (X_{spf}/Y). The appearance of *sparse fur* female progeny (X_{spf}/0) is taken as the confirmation. IMS was initially used in this study for two reasons. First, this compound is very effective in inducing dominant-lethal-type response in the (SEC × C57BL)F₁ females and, second, even at high doses, treated females can produce several litters (2, 3).

Only sex-chromosome-loss data are presently available, and data for the two doses were pooled together because of low numbers of progeny scored so far. Already, however, there is clear indication that IMS induces sex-chromosome loss on oocytes in the advanced stages of follicular development. Among the first litters of treated females, born within 30 days after treatment, four XO females (tested) were recovered out of 675 female progeny scored. Among progeny scored in the second and subsequent litters, on the other hand, there appears to be no appreciable increase in sex-chromosome loss. Only one presumed XO (still untested) was found out of 4365 female progeny tested. In the controls, no XO individual was found out of 1777 female progeny tested so far.

Obviously, more data are needed to have a better estimate of the rates of sex-chromosome-loss induction both in the first and in later litters. However, the results so far obtained already provide the first direct clear-cut evidence for chemically induced chromosomal aberrations in the mouse oocytes and warrant a more extensive study on the nature of induced sex-chromosome loss — that is, whether it is due to chromosomal breakage or to chromosomal nondisjunction. This is a very important question from a practical standpoint, as sex-chromosome aneuploidy constitutes a good proportion of human chromosomal abnormalities.

*Research jointly sponsored by the Food and Drug Administration and the U.S. Atomic Energy Commission.

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FURTHER REFINEMENTS IN THE MOLECULAR DOSIMETRY OF EMS-ETHYLATION OF MOUSE SPERM DNA*

R. B. Cumming and Marva F. Walton

One serious problem in comparing the mutagenic responses of different genetic test systems to a particular agent has been the lack of a common system of measuring the dose. The measurement of the number of ethylations per cell produced in mature sperm by intraperitoneal injection of ethyl methanesulfonate (EMS) at doses which produce genetic damage has been reported (1). Refinements in techniques have been developed which allow more precise measurement of ethylation by EMS of the DNA of mouse sperm. The procedure involves using [¹⁴C] thymidine to label the sperm DNA and treating the mice with ethyl-2-[³H]methanesulfonate, which has a specific activity 50 times greater than that used in a previous experiment (1).

Sperm-cell suspensions are disrupted in a medium containing 2-mercaptoethanol and urea which releases part of the DNA into solution. The DNA is purified by centrifugation in a CsCl gradient, and samples are collected on filter paper discs, which are oxidized by combustion to allow the accurate assay of the radioactivity from each of the two isotopes. We now estimate about 5×10^5 ethylations per sperm at a dose of 200 mg/kg. Twenty percent of these ethylations have

been found to occur in the DNA, giving 10^5 ethylations per mouse sperm genome at this administered dose.

*Research jointly sponsored by the Food and Drug Administration and the U.S. Atomic Energy Commission.

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POSSIBLE NONRANDOM ATTACK OF MOUSE SPERM DNA BY ETHYL METHANESULFONATE*

G. A. Sega, R. B. Cumming, and Marva Walton

The total ethylations per mouse sperm cell following injection with isotopically labeled ethyl methanesulfonate (EMS) at various doses has been determined (1). We have now established procedures for releasing DNA from the refractory sperm heads of the mouse. Using these procedures and the double labeling technique of Sega and Lee (2), in which the sperm DNA is labeled with [¹⁴C] thymidine followed by *in vivo* treatment with [³H]EMS, ³H/¹⁴C ratios have now been determined for whole sperm cells and for sperm-cell DNA in the mouse.

Samples are oxidized by combustion and ³H and ¹⁴C activities are quantitatively separated from the resulting combustion products and assayed separately. The results so far appear to indicate nonrandom ethylation of the sperm genome. This effect seems to be dose-dependent, with higher EMS doses giving a larger shift in the ³H/¹⁴C ratio for sperm DNA as compared to whole sperm. The ³H/¹⁴C ratio is between 2 and 12 times higher for the DNA than it is for the whole sperm. This indicates that the fragments of DNA recovered are richer in [³H]ethyl groups than the genome as a whole.

We are now involved in additional experiments to double check on this apparent nonrandom ethylation of sperm DNA. Mouse sperm is doubly labeled with [³H] deoxycytidine and [¹⁴C] thymidine, or any other appropriate pair of labeled deoxynucleosides. When the label is incorporated into mature sperm, part of the mice are held as controls and part are injected with various doses of unlabeled EMS. A change in the ³H/¹⁴C ratio between the whole sperm and the sperm DNA not found in the controls would indicate non-random breakage of DNA by EMS and can also be used to determine what DNA bases are preferentially ethylated.

*Research jointly sponsored by the Food and Drug Administration and the U.S. Atomic Energy Commission.

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DOSE AND METABOLIC FATE OF HYCANTHONE IN MICE*

R. B. Cumming

Hycanthone, the hydroxymethyl metabolite of lucanthone, is a drug which appears to be highly effective in combating the seriously debilitating parasitic disease, schistosomiasis, without producing significant levels of obvious toxic side effects. In widespread clinical trials in Latin America and Africa the drug has been administered, usually as the methanesulfonate at 3 mg of base per kg of body weight in a single intramuscular injection, to several hundreds of thousands of individuals. From a clinical point of view this agent is seen as a particularly potent weapon against one of the world's most important public health problems. However, questions have been raised about potential side effects which are more subtle but no less critical than immediately observable toxicity. These include mutagenicity, teratogenicity, and carcinogenicity. Hycanthone has been reported to be mutagenic in bacteria by Hartman *et al.* (1) and in other test systems, but years of experience in radiation mutagenesis and in studies with model chemical mutagens have shown that tests in

mammalian germ cells are required to provide any reasonable basis for estimating human hazards. Moreover, it is important with chemical mutagens to compare the metabolic processing of a test chemical in the genetic test organism with that in man to help in the difficult problem of such interspecies comparisons.

Preliminary results are available from a study on the metabolic fate of hycanthone administered to mice as the methanesulfonate by intraperitoneal injection. The experimental conditions were identical to those used in a genetic test reported by Russell and Kelly (2) and in a spermatogonial survival test by Oakberg and Tyrrell (3). A variety of tissues are being examined in this study, but data from only the blood and testis are reported at this time. The animals were injected with generally labeled tritiated hycanthone kindly supplied by Sterling Winthrop Research Institute, and tissues or tissue components were assayed for radioactivity at various times after injection. Four dose levels were used, ranging from 150 mg/kg ($\mu\text{g/g}$) to 6.23 mg/kg of hycanthone base. The highest dose is that at which our genetic studies were performed (see Russell and Kelly, 2). Some of the data are presented in Table I. Included also, for comparative purposes only, are some data on

TABLE I. Tissue concentration at various times after injection

Organism	Dose ($\mu\text{g/g}$)	Tissue	15 min		1 hr		2 hr		4 hr		24 hr	
			$\mu\text{g/g}$	%*	$\mu\text{g/g}$	%*	$\mu\text{g/g}$	%*	$\mu\text{g/g}$	%*	$\mu\text{g/g}$	%*
Mouse	150 i.p.	Blood	111.75	74.5	56.25	37.5			19.65	19.65	16.15	10.77
		Testis	119.85	79.9	100.05	66.7			27.45	27.45		
	75 i.p.	Blood	42.22	56.3	16.27	21.7			9.75	9.75	7.58	10.11
		Testis	65.70	87.6	42.37	56.5			17.92	17.92		
	37.5 i.p.	Blood	17.96	47.9	8.14	21.7			5.47	5.47	3.54	9.45
		Testis	29.62	79.0	17.10	45.6			8.47	8.47		
	6.23 i.p.	Blood	2.38	38.2	1.74	28.0			0.75	0.75	1.10	17.64
		Testis	2.78	44.6	3.28	52.7			0.35	0.35		
Rat [†]	3 i.m.	Blood	0.22 [‡]	7.33 [‡]	0.13	4.33	0.07	2.33	0.03	0.03	<0.01	<0.33
		Plasma	0.20 [‡]	6.66 [‡]	0.13	4.33	0.06	2.00	0.02	0.02	<0.01	<0.33
Monkey [†]	3 i.m.	Blood	0.64	21.33	0.41	13.66			0.07	0.07	<0.01	<0.33
		Plasma	0.65	21.66	0.43	14.33			0.08	0.08	<0.01	<0.33
Human [†]	3 i.m.	Plasma					0.36	12.0	0.26	0.26	0.0	0.0
					0.18	6.0	0.36	12.0	0.23	0.23		

* $\frac{\mu\text{g per g of tissue}}{\mu\text{g injected per g of body weight}} \times 100.$

[†]Data from studies performed by the Sterling Winthrop Research Institute.

[‡]30 min.

other mammalian species — data supplied by the Sterling Winthrop Research Institute from their own studies on the metabolism of this drug.

The data show that an appreciable proportion of the injected whole-body dose level of the drug can be detected in the testis shortly after injection, and that blood levels are somewhat lower than testis levels for a wide range of doses and times. The drug is slowly cleared and is greatly reduced in the tissues after 4 hr.

All the other species about which we have some information, including humans, do not reach as high blood levels as do mice, and the drug is cleared more rapidly and completely than in mice. Blood and plasma levels do not differ significantly, so the human plasma levels may be compared with the mouse blood levels. Dose probably does not make a great difference in the proportion retained because, at the 24-hr period, similar percentages are retained in the blood of the mouse

regardless of dose, and this is much higher than for any other species. The difference in injection route is probably not important because Oakberg (3) has found no significant differences in biological effect between i.p. and i.m. injection.

Based on these preliminary data, it appears that for a given administered dose the mouse receives much higher tissue levels of the drug for longer periods of time than do other species for which there are data.

We thank the Sterling Winthrop Research Institute for their cooperation in this study.

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PATHOLOGY AND IMMUNOLOGY

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Mammalian Radiation Recovery

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Radiation Recovery of Hemopoietic Cells

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Experimental Hematology

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Pathological Effects of Neutrons, High-Energy Radiation, and Chemicals

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IMMUNE STATUS AT 12 MONTHS IN THYMECTOMIZED RADIATION CHIMERAS

C. C. Congdon, Helen Payne,
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The immune status of BC3F₁ mice at 12 months after thymectomy, irradiation, and syngeneic bone marrow transplantation was measured using anatomic, humoral, and cellular measures of immunity. Body weight at the 12-month interval averaged 19 g compared to 39 g in normal control mice. An extreme wasting syndrome in certain mice contributed to low average weight in the experimental group. Spleen and peripheral lymph node weights were likewise lower in the experimental mice compared with control animals. Autopsy and cellular pathology study failed to explain the extreme wasting in certain experimental mice. Thymus-dependent areas of lymphatic tissues were depleted of lymphocytes in the experimental group, but the number of germinal centers was equal to control values. Tail skin allografts at the 12-month interval showed an average mean rejection time of 20 days in thymectomized chimeras. Control mice rejected at 10 days. Both primary and secondary humoral antibody production were deficient in the experimental group for rat red blood cell antigens. In control mice the mean primary and secondary log₂ titers were 8 and 14, compared to 1 and 1 at the third weekly bleeding after antigen in the thymectomy group. One other piece of data about the thymectomized radiation chimera is the primary *in vitro* response of dispersed spleen cells to sheep red blood cell antigens at 2–3 months after thymectomy. Hemolytic plaque formation was about one-half control values in the short-term culture situation.

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MODIFICATION OF GRAFT-VERSUS-HOST DISEASE

R. E. Toya

Graft-versus-host disease is the major obstacle to the clinical use of bone marrow transplantation as a protective measure following lethal radiation. If the immunopoietic competency of donor and recipient animals could be altered, so that the immune system would be depressed and unable to respond to foreign antigens, this obstacle could be eliminated. Previous

studies indicated that the erythropoietic stimulating compound, phenylhydrazine, induced the expected hemopoiesis but also appeared to be altering the immunocompetent cells of the donor animals. This "*in vivo* cell separation" allows hemopoietic cells to predominate over immunocompetent cells, thus reducing the graft-versus-host disease. The approach is to study the acute graft-versus-host disease observed in lethally irradiated F₁ hybrid mice after the injection of parent spleen cells and also mixtures of parent bone marrow and lymph node cells. Phenylhydrazine treatment to the parental donor allows the use of the spleen as a hemopoietic repopulating agent, and the acute graft-versus-host disease is largely eliminated.

As another means of reducing mortality in irradiated mice treated with foreign hemopoietic tissue, which contains sufficient immunocompetent cells to induce graft-versus-host disease, we have looked at the use of blocking antibody, which is present in sera obtained from long-term rat-mouse radiation chimeras. Results indicate that blocking antibody aids survival of these chimeras, perhaps by interfering with cells involved in the initiation of graft-versus-host disease.

THE DISTRIBUTION OF TRANSPLANTABLE HEMOPOIETIC COLONY-FORMING CELLS IN THE SPLEEN*

K. L. Mossman[†] and A. L. Kretchmar[‡]

The statistical distribution of colonies in the spleen colony assay for hemopoietic stem cells does not follow the Poisson series, as originally assumed (1). Extensive work in our laboratory, using C3H mice, indicates that the variance of the distribution obtained is smaller than expected by the Poisson distribution. A more satisfactory fit of the data is obtained using the binomial distribution. Data examined from other laboratories were also compatible with the binomial model in at least two other strains of mice. An interpretation consistent with our present findings is that, while hemopoietic stem cells are necessary in initiating colony growth, a more important factor is the number of niches in the spleen capable of supporting the development of a gross nodule.

In the course of our investigation, two exceptions have been noted in which the distribution of colonies is neither Poisson nor binomial. Both C57BL/6 mice and 3-day preirradiated C3H mice exhibit a distribution in which the variance is larger than the mean. These findings are particularly interesting because (1) the distribution of colonies among spleens in C3H mice given radiation and bone marrow on the same day is

best fit by the binomial model, (2) the 3-day preirradiated C3H mice yield a lower value for the assay, and (3) the C57BL/6 strain has been shown to exhibit poor growth of colonies. An explanation for the larger variance, consistent with our model, is that a less than full complement of niches competent to support the growth of a gross nodule is present in the spleen. This factor could increase the variance of the distribution by introducing an additional random event, *viz.* "competent niche."

*Work performed in collaboration with the Mammalian Recovery Group.

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RADIOPROTECTION BY PHENYLHYDRAZINE

L. H. Smith and T. W. McKinley, Jr.

We have continued to study the biostimulant radioprotector phenylhydrazine (PhNHNH₂), which when injected 7, 8, or 9 days before exposure to X-rays increases the LD_{50/30} of mice about 20% (1). Although results of our studies suggest that protection is associated with marked alterations of hemopoietic tissue resulting from the action of PhNHNH₂ on erythrocytes, there are indications that other factors may be involved. Possibilities include (1) increase in radiosensitivity of hemopoietic stem cells and (2) stimulation of the reticuloendothelial (RE) system.

The first possibility seemed untenable because we found that the *in vitro* radiosensitivity of bone marrow and spleen cell colony-forming units (CFU) of PhNHNH₂-treated mice was normal. However, since *in vitro* conditions may be inadequate for rapid repair of radiation injury, we determined the *in vivo* radiation sensitivity of hemopoietic stem cells from PhNHNH₂-treated mice by using the endogenous spleen CFU technique. Mice were given either saline or 3 mg of PhNHNH₂·HCl. Seven days later they were exposed to 450–1100 r, and 8 days thereafter the mice were killed and the endogenous spleen colonies were counted. The D₃₇'s for saline- and PhNHNH₂-treated animals were 96 ± 8 and 110 ± 11 r, respectively. Thus, the radioresistance of drug-treated mice was increased by a factor of 1.15. However, the statistical significance of the difference is questionable (P > 0.3). Notably, the radiation inactivation curve for drug-treated mice was shifted to the right about 180 r, suggesting a larger

number of CFU at risk at the time of radiation. This value is higher than the increase of 130 r in the LD_{50/30} for PhNHNH₂-treated mice, which suggests that the endogenous spleen-colony method somewhat overestimates the protective effect of PhNHNH₂ in terms of animal survival.

The second possibility (stimulation of the RE system) is not amenable to direct experimental attack. However, indirect evidence was obtained which suggests that the RE system plays little, if any, role in radioprotection by PhNHNH₂. Mice were given the drug followed by transfusions of different numbers of erythrocytes on days 1 and 2 after drug injection. Seven days after drug injection, the mice were exposed to 850 r, and 30-day survivors were scored. As the number of erythrocytes injected was increased, 30-day survival was progressively decreased and was almost zero after transfusion of large numbers (2.8 × 10⁹) of erythrocytes. Thus, the more the erythrostimulating effect of PhNHNH₂ was countered by erythrocyte transfusion, the smaller was the radioprotection; and we conclude that radioprotection is a direct function of the degree of erythropoietic stimulation. Since there is no reason to believe that transfused erythrocytes would alter stimulation of the RE system by PhNHNH₂-damaged erythrocytes, we also conclude that stimulation of the RE system is not involved in radioprotection by this drug.

RE stimulation by PhNHNH₂ results directly from drug-damaged erythrocytes. We attempted to mimic this stimulation by injecting PhNHNH₂-damaged erythrocytes into mice before irradiation. Radioprotection was not observed in X-irradiated mice given damaged erythrocytes 7 days before exposure, an observation which supports the contention that the radioprotective effect of PhNHNH₂ is not the result of RE system stimulation. However, significant protection occurred when drug-damaged erythrocytes were given 24 hr before irradiation, an observation which requires further investigation.

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ATTEMPTED IMPLANTATION OF A FUNCTIONING PANCREATIC ISLET GRAFT INTO MICE WITH EXPERIMENTALLY INDUCED DIABETES

R. C. Kramp* and C. C. Congdon

Although the major interest of the Mammalian Recovery Group is transplantation of foreign hemopoietic tissue, homotransplantation of other organ systems is frequently considered promising, because in

an irradiated animal bearing a foreign marrow graft the immune system is depressed. Studies were initiated to see whether the endocrine portion of the pancreas could be grafted into mice made diabetic by injection of either alloxan or streptozotocin (specific β -cell cytotoxic agents). The first phase of the study involved the isogenic implantation of islets into alloxan diabetic mice.

Alloxan studies: It has been reported that the diabetic state produced in mice with alloxan may or may not be permanent. Spontaneous recovery from diabetes occurs in individual animals, although the event may occur after several weeks. To ensure that recovery from alloxan diabetes following implantation of the pancreas was due to a successful take of a functioning graft and not due to spontaneous recovery, a study was performed in which the diabetic state in mice receiving various doses of alloxan was followed for a period of 5 months. The degree of diabetes was measured in terms of blood and urine glucose and of body weight changes. It was found that a dose of 80 mg alloxan per kg body weight administered to nonstarved mice produced a relatively healthy animal in which hyperglycemia (>200 mg glucose/100 ml blood) was maintained for a period of over 10 weeks in all animals. It was concluded that mice given 80 mg/kg could be used in our implantation studies with certain reservations. First, a very large control group of sham-operated animals must be included in the experimental study with the implanted group. Secondly, a return to normoglycemia (≤ 150 mg/100 ml) following the hyperglycemic state produced by alloxan should be demonstrated within 4–6 weeks in order to rule out spontaneous recovery. Individual blood glucose levels in normal mice ranged between 94 and 150 mg/100 ml.

Implantation study: The technique for implanting islet tissue was basically as follows: Ligation of the pancreatic ducts of the donor was performed 3 months prior to removal and implantation into subcutaneous sites of recipient mice. The histological appearance of the ligated pancreas at this time showed obliteration and fatty tissue replacement of the exocrine compartment, while the islets remained intact and stained positively with aldehyde fuchsin. Each recipient received ligated pancreatic segments from four donor animals to insure that a sufficient amount of islet tissue was implanted. Alloxan was administered to recipient animals 3 days prior to implantation, and the course of diabetes in the implanted and sham-implanted controls was followed.

The results, showing blood glucose levels immediately before and 35 days after implantation, are given in Fig.

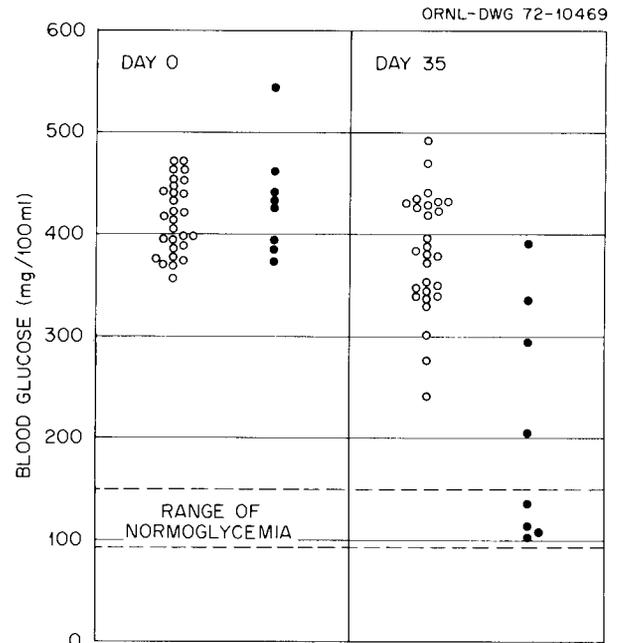


Fig. 1. Blood glucose levels in implanted (●) and sham-implanted (○) groups immediately before and 35 days after implantation of pancreatic islet tissue.

1 for both implants (eight animals) and sham-implanted controls (28 animals). Four out of eight implanted animals showed complete recovery from alloxan diabetes 35 days after implantation, manifested by absence of glucose in the urine, increase in body weight over controls, and a return to normoglycemia (103–135 mg/100 ml). The blood glucose levels of 28 sham-implanted controls were all above 240 mg/100 ml at 35 days.

Results of this study suggest that isografts of pancreatic tissue containing functional islets can ameliorate alloxan-induced diabetes, providing the exocrine compartment of the pancreas is atrophied by ligation of pancreatic ducts in the donor prior to implantation.

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ON THE FATE OF STEM CELLS IN TRANSPLANTED MARROW THAT FAIL TO FORM COLONIES

Joan Wright Goodman and Sarah G. Shinpock

When bone marrow is transplanted into an irradiated mouse, a certain number of the stem cells present in the inoculum give rise to colonies in the host's spleen (1). When the transplantation is isogenic and the cell dose is

small, the number of colonies that can be counted is a measure of the absolute number of stem cells – or more accurately, colony-forming units (CFU) – present in the inoculum. This measure can be used as a basis for evaluating the performance of the same kind of marrow transplanted to genetically different recipients. In many donor-host combinations a standard marrow dose produces comparable numbers of spleen colonies in isogenic and nonisogenic recipients. There are other combinations, however, in which relatively few colonies are expressed. Because the recipients in such combinations have been exposed to a lethal dose of irradiation and because no histologic evidence can be found that host-derived immunologic reactions are occurring (2), the basis for poor growth does not appear to be immune rejection of a portion of the transplanted CFU.

One (or more) of the following possibilities could account for a deficient expression of CFU in poor-growth combinations. (1) Stem cells fail to localize to the usual extent in the recipient's spleen. (2) They localize normally, but for lack of an essential nutrient or lock-and-key fit, they do not initiate division. (3) They localize in abnormal splenic sites where the microenvironment is not favorable for hemopoietic differentiation. We reasoned that if either the second or third statement were true and that under those circumstances the stem cells remained viable, we should be able to rescue them after a period of time and allow them to express themselves by putting them back into an isogenic environment.

Two experiments were designed to test the possibility of rescuing B6 stem cells unexpressed in B6D2F₁ recipients by retransplantation after 4 hr into irradiated B6 mice. In neither experiment was "full" (assessed in terms of B6 → B6 spleen retransplantation into B6) rescue achieved. One-half to three-fourths of the CFU either had not localized in the spleen or had become trapped in such a way that they were damaged, killed, or irreversibly turned off as far as further proliferation was concerned. When B6 thymocytes were transplanted with B6 marrow to F₁ recipients, the number of CFU rescued after 24 hr was equivalent to the number found in B6 → B6 chimeras. Further studies are being conducted to find out whether the initial localization of CFU is faulty (the first possibility above) in B6 → F₁ transplantation, and if so, how the presence of thymocytes alters the pattern.

HEMOPOIETIC DIFFERENTIATION PATTERNS IN CHIMERIC SPLEENS. EFFECT OF LYMPH NODE LYMPHOCYTES

Nancy L. Basford and Joan Wright Goodman

Because the thymus contains immunocompetent cells, the question of the relationship of graft-versus-host (GVH) reactivity to augmentation of hemopoiesis arose. One approach to studying this question was to inject lymph node lymphocytes (LNC) instead of thymocytes into irradiated recipients. With ⁵⁹Fe uptake as an endpoint, eight experiments showed that LNC produced no augmentation (1). However, when marrow-plus-LNC chimeric spleens were examined histologically, unusually extensive granulopoiesis was found. Experiments were undertaken to evaluate the differentiation patterns of hemopoietic colonies in spleens of B6D2F₁ mice treated with various combinations of marrow, LNC, and thymocytes from B6 mice. Results obtained showed the following. (1) Radiation controls showed no colonies, and both red and white pulp areas were empty. Plasma cells were present in large numbers, apparently "uncovered" by the irradiation. (2) Marrow when given alone produced mostly small, erythropoietic colonies. The white pulp was quite atrophic, and red pulp was relatively empty. (3) Lymphocytes when given alone produced a few small colonies that were all granulopoietic. In these spleens, the white pulp was full and contained many dividing cells. (4) Lymphocytes given with marrow produced a significantly greater number of colonies than marrow alone, and the differentiation pattern was shifted dramatically toward the granulopoietic line compared with the predominant erythropoiesis produced by marrow alone. There was diffuse infiltration of the red pulp by lymphocytes, and there was evidence of GVH, necrosis, epithelioid cell proliferation, and full white pulp containing transforming cells. (5) Thymocytes given with marrow produced the greatest number of colonies and the largest colony size. The pattern of differentiation was shifted somewhat toward the granulopoietic line, but erythropoiesis was still predominant. The red pulp in these spleens was full compared to that in mice treated with lymphocytes and marrow. White pulp appeared to be regenerating.

Further studies are in progress to document the apparent ability of LNC to alter the normal differentiation pattern of transplanted marrow.

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PLATEAU IN RESPONSE CURVE FOR THYMOCYTE-INDUCED AUGMENTATION OF MARROW GROWTH

Linda L. Pritchard*

Parental bone marrow cells do not always grow well when transplanted into lethally irradiated F₁ hybrid recipients. In mouse strain combinations exhibiting this poor growth phenomenon, marrow growth can be augmented by transplanting parental thymocytes within a few days of grafting the marrow (1). Donor marrow, not thymus, provides the hemopoietic precursor cells.

In these experiments, lethally irradiated (C57BL/6 × DBA/2)F₁ (B6D2F₁) males, 12–16 weeks old, were given intravenous injections of C57BL/6 (B6) marrow cells and thymocytes suspended in phosphate-buffered saline. Eight days later, recipient spleens were removed and fixed in Tellyesniczky's solution; macroscopic nodules (2) were counted under a dissecting microscope.

The degree of growth augmentation observed depends on the number of thymocytes injected. For bone marrow doses ranging from 3×10^4 to 3×10^5 live nucleated cells, the number of nodules increases linearly with increasing thymocyte dose up to a thymus:marrow ratio on the order of 1000. At higher thymocyte doses, a plateau is reached where further increasing the thymocyte dose does not result in an increase in the number of spleen nodules. Thus there exist a limited number of thymocyte-sensitive hemopoietic precursor cells in the marrow. Furthermore, for a given marrow dose, the plateau in response occurs at just that number of nodules produced by the same marrow dose when transplanted into isologous (B6) recipients. In other words, it is possible to abolish poor growth of B6 marrow in B6D2F₁ recipients by adding a sufficient number of B6 thymocytes.

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RESPIRATORY MYCOPLASMA INFECTION IN THE MOUSE

C. B. Richter, J. A. Franklin,
Carole S. King, and F. S. Shults

Although experience with chronic respiratory infections in the rat is usually extrapolated directly to the

mouse, there appears to be little basis for this. Studies in our laboratory have shown several important differences between the disease processes and clinical manifestations in the two species. (1) Spontaneous disease beginning in early life and progressing in severity, as seen in the rat, does not occur in the mouse. (2) Increasing mortality with age from natural infection does not occur in the mouse. (3) Colony enzootics so commonly seen in rat colonies do not occur in mouse colonies, even when they are housed in the same environment with infected rats. (4) Although infection in both species results in primarily a suppurative process, cavitating abscesses, as seen in the rat, do not occur in the mouse. (5) Mycoplasma lung infections in the mouse appear to be closely related to other stress-inducing insults such as virus infections, experimentation, etc. (6) The adult mouse is capable of completely resolving experimental infection; however, it is not known whether or not the rat can do this.

Respiratory mycoplasma infections in the mouse are important and do produce chronic infections of long duration. Experimental infection causes deciliation of bronchial epithelium as early as 48 hr and subsequent cytological exfoliation. During acute stages of infection, bronchial epithelial labeling indices, as measured autoradiographically after [³H] thymidine injection, may reach 35 times control levels and persist as high as 10 times for at least 120 days. However, pathology studies have shown that practically 100% of the survivors have completely resolved the infection by 1 year, if not the residual morphologic changes such as bronchiectasis, atelectasis, epithelial metaplasias, and lobar atrophy. Electron microscopy and fluorescent antibody studies have clearly demonstrated that the mycoplasmas are extracellular parasites on the ciliated epithelium of the bronchi and trachea. They are strongly cell associated, and at least one means of replication *in vivo* may be a budding process at the organism-cytoplasm interface, resulting in a minimum reproductive unit of about 100–140 nm in diameter.

Experience has shown that mouse respiratory mycoplasmas are much more fastidious and difficult to isolate than those of the rat. Currently four serological methods of identification are being studied to determine interrelationships between isolates from the two species: fluorescent antibody, growth inhibition, complement fixation, and agar-gel diffusion. Work in this area has just begun, but early results show some differences in growth-inhibiting response and distinct differences in precipitating antibody. On the other hand, complement-fixation antigens seem to be commonly shared.

KINETICS OF PARAINFLUENZA VIRUS INFECTION IN THE LUNG

C. B. Richter, Carole S. King,
and J. A. Franklin

Sendai virus is a paramyxovirus related to Newcastle Disease, mumps, human parainfluenza, Simian virus 5, and others. Like the other members of the group, Sendai is an RNA virus capable of causing high morbidity and potentially high mortality in the host species. This is particularly true among colonized mice, although serotitres against this virus can be demonstrated in other species, such as rats and hamsters. The objectives of this study are to establish which cell populations of the lungs are infected and to determine the time sequences of viral replication, the sequence of host cell damage and repair, and the acute and residual pathology of the infection. Serologic studies have shown that significant hemagglutinin inhibition titres are present within 10 days (1:80) after experimental infection, with peaks seen at 20 days (1:320) and persisting at high levels for at least 120 days. This indicates that immunity conferred by this infection is very durable. Latency experiments have shown that serologically recognizable antigen persists as long as 60 days after experimental infection when serial blind passages of lungs from recovered animals are tested; thus, recovered animals may remain effective carriers for some extended periods.

Electron microscopy has shown virus budding from infected cells as early as 3 days following experimental infection. Virions can be seen replicating in the nucleus in a small percentage of cells, but the cytoplasm is the primary site for replication. Time sequences of viral replication in specific cell populations in infected mice will be studied by fluorescent antibody techniques and thin frozen sections. Autoradiographic studies on infected lungs have shown 100-fold increases above control for crude labeling indices of bronchial epithelium by the tenth day of infection when [³H] thymidine is injected. This time period is right after the period of maximum cell lysis and represents maximum cell regeneration. Cell regeneration continues at levels above normal for several weeks after infection, although acute-phase pathology is usually completely resolved by the 20th day. Residual scarring and metaplasias of epithelium may persist for several months afterwards. Bronchial epithelial squamous metaplasia is an outstanding resolution-phase lesion and appears to be a result of excessive repopulation following the lytic phase. These metaplastic foci are frequently confused for tumors or carcinoma *in situ*.

LIFE-SPAN PATHOLOGY STUDIES ON PATHOGEN-FREE RATS

C. B. Richter, F. S. Shults, and R. L. Hendren

Two strains of pathogen-free rats are being used in this study of normal survival times and baseline pathology: F344/Bd[SPF] inbred and SD:Bd[SPF] outbred. These animals are placed into the pathology program as randomly selected retired breeder pairs from the production barrier. They are left pair-mated for life and are observed daily for clinical signs of illness. Those which are terminally ill are killed and subjected to thorough autopsy covering all body systems. Animals that die unnoticed are excluded from the pathology study; however, all demographic information is recorded. It is anticipated that a minimum of 200 autopsies from each strain will be required to establish significant trends in more common disease processes and exclude other diseases, notably tumors, as normal processes which might interfere with interpretation of cancer induction studies. In addition, the study should show specific diseases that may become useful cancer experiment models.

To date, only limited information is available because few animals have reached full life expectancy, particularly in the SD:Bd strain. However, several trends are apparent in the F344/Bd strain already: (1) low incidence of mammary tumors among multiparous females; (2) high incidence of pituitary tumors; (3) high incidence of testicular tumors, primarily interstitial cell type; and (4) high incidence of myelomonocytic leukemias in old animals. Current experiments are in progress to study the transplantability of these tumors to young and adult rats on the same strain. These tumors may be useful models for the study of immunotherapy of leukemia.

No statement is possible yet on life-span because of the limited data available. However, range of age at death has been 24–42 months. The animals remain remarkably free of infectious disease at death.

DIFFERENTIAL AGING IN THE CAPACITY OF SPLEEN AND BONE MARROW CELLS TO INDUCE GRAFT-VERSUS-HOST REACTIONS

W. J. Peterson, T. Makinodan, G. B. Price,*
and M. G. Chen†

Studies on the lymphohematopoietic system have shown that the capacity to make humoral antibody decays with age. In contrast, measurements of age-related changes in the capacity to initiate a cell-mediated immune response have been variable (1). For

example, in certain mouse strains which develop autoimmune disease, the retention of foreign skin grafts has been correlated with a decay in cell-mediated immune responsiveness, whereas in nonautoimmune strains the ability to reject skin homografts may or may not change with age (2). Another age-related change is seen in the number of stem cells in the lymphohematopoietic tissues. The number of hematopoietic colony-forming units in the spleen of old mice is considerably less than in young mice. On the other hand, the number of colony-forming units in the bone marrow does not change with age. Hence, when two tissues perform a similar function, an age-related change may be seen in one and not in the other. Therefore, studies have been undertaken to compare spleen and bone marrow cells in their capacity to initiate cell-mediated immune responses as a function of age, using the graft-versus-host (GVH) reaction assay. The first report showed that the capacity of bone marrow cells to initiate the GVH reaction does not change with age (3). The purpose of the present study was to assess the capacity of spleen cells to initiate the GVH reaction.

Accordingly, spleen cells from BC3F₁ mice whose histocompatibility specificity is H-2^{bk} were transfused into sublethally X-irradiated (450 r) BALB/c mice whose histocompatibility specificity is H-2^d. A daily check for dead recipient mice was performed, and the percent mortality was calculated. The results show a correlation between percent mortality and spleen cell number. Thus, 50×10^6 , 25×10^6 , 12.5×10^6 , and 6.25×10^6 spleen cells from young BC3F₁ donors killed 100, 90, 61, and 11% of the recipient mice, respectively. Further studies showed an age-related decay in the capacity of spleen cells to induce the GVH reaction. Thus, 50×10^6 cells from old donors killed 64% of the recipients, whereas the same cell dose from young donors consistently killed 100% of the recipients. This age-related change is amplified at smaller spleen cell doses. Thus, 12.5×10^6 cells from old donors killed 8.3% of the recipients whereas this number of cells from young donors killed 61% of the recipients. The death rate for recipients treated with spleen cells from young donors is considerably greater than that of recipients treated with spleen cells from old donors. The underlying cause for the differential aging of spleen and bone marrow cells to induce the GVH reactions is unknown, but a change in the respective microenvironments is likely to be a contributing factor. Continuing studies using the parental strains have been initiated in an attempt to better understand the nature of the immunologic decay.

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ABSENCE OF AGE-RELATED IMPAIRMENT OF IMMUNE RESPONSE IN ADHERENT CELLS FROM SPLEENS OF OLD MICE

Margaret L. Heidrick* and T. Makinodan

Spleen cells from aged mice when cultured *in vitro* with sheep red blood cells (SRBC) generate fewer antibody-forming cells than cells from young mice. To further define the nature of the decrease in response with age, spleen cell suspensions from young and old mice were separated into two populations on the basis of their ability to adhere to plastic petri dishes, and their activity was assessed in various combinations with SRBC. Old adherent cells cultured with young nonadherent cells responded fully to the antigen, whereas old nonadherent cells combined with young adherent cells gave a response comparable to unseparated old spleen cells. Similar results were obtained when adherent cells were exposed to SRBC, washed free of the antigen, then recombined with the nonadherent cells. The results demonstrate that the adherent cells of old spleens are indistinguishable from those of young spleens in their ability to initiate antibody response, but the activity of nonadherent cells of old spleens is impaired. Hence it is concluded that the impairment of the humoral immune response observed in old mice reflects a decrease in the activity of the nonadherent cell population.

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DIFFERENCE IN THE MAGNITUDE OF AGE-RELATED CHANGES IN CELL-MEDIATED IMMUNE ACTIVITIES *IN VIVO* AND *IN VITRO*

Sarah A. Goodman*

It has been postulated that one of the factors which may contribute to senescence is a decline in cellular

and/or humoral immune competency. It is well established that the humoral immune response in mice decreases sharply during senescence. The data concerning cell-mediated immune responsiveness, however, are not as clear-cut and in some cases are conflicting, even though this system is of particular interest because of its postulated role in immune surveillance.

Discrepancies in the data on the cell-mediated immune response may arise from several sources, including different methods of assay and the use of different species and mouse strains with different life-spans. Furthermore, laboratory studies of the effects of senescence on cell-mediated immune responses have classically been performed *in vivo* with systems which measure homograft rejection and transplantation disease, both of which are difficult to quantitate; therefore, the specific and direct influence of one cell type on another is impossible to measure in these systems.

For these reasons age-related changes in cell-mediated immune responsiveness of a long-lived hybrid mouse (BC3F₁, H-2^{bk}) were studied both *in vitro* and *in vivo*. The former assay measured the ability of sensitized spleen cells to kill allogeneic target cells (Mastocytoma P815 grown in CD2F₁, H-2^{dd}) *in vitro* and the latter, resistance to death caused by proliferation of either allogeneic or syngeneic tumor cells in unsensitized mice. When irradiated tumor cells were used as immunogens, the *in vitro* response of old cells was reduced to only 60% that of the sensitized young cells. However, mortality due to proliferation of the same dose of unirradiated allogeneic tumor cells was severely increased with age. More than 98% of the old mice (28–32 months) died within 20 days, in contrast to no deaths in 3-month-old mice. Subsequent quantitative studies relating mortality to the number of tumor cells injected revealed that old mice are 1000 times less resistant than young mice. This is in sharp contrast to the difference in relative *in vitro* activity, which was at best only 2-fold. These data indicate that although the immune surveillance mechanism, as measured by the ability of sensitized spleen cells to kill allogeneic target tumor cells *in vitro*, declines with advancing age, the decline in this capacity is not sufficient to account for the decreased resistance to the growth of allogeneic tumor cells *in vivo*. Therefore, the observed increase in neoplastic conditions as a function of age must involve other factors than the ability to kill target cells *in vitro*. These factors, which have yet to be determined, may be humoral or environmental.

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CHANGE IN PROPORTION OF T AND B LYMPHOCYTES IN AGING MICE AND ITS SIGNIFICANCE TO HUMORAL IMMUNE ACTIVITY

Mary E. Halsall and T. Makinodan

Aging mice can exhibit a 20-fold decrease in humoral immune activity; 10% of the decrease is due to the humoral environment and 90% to the cells of the immune system. The latter is due primarily to decrease in the proliferative capacity of thymus-derived (T) and bone-marrow-derived (B) cells. The proportions of T and B cells in the spleen may shift with age, but the significance of this shift has not been established. Currently, ficoll gradients are being used to separate young and old spleen cell suspensions into T- and B-enriched populations, and the number as well as the anti-SRBC activity of enriched T and B cells in various combinations and proportions are being assessed *in vitro*. The results reveal that (1) the percentage of T-enriched cells increases with age; (2) not only is there an optimal ratio of T and B cells to generate a maximum response for both the young and the old, but the optimal ratio is about the same for both, and (3) old T and B cells perform better in combination with young B and T cells, respectively. These findings show that there is a third factor that influences the activity of the immune system and changes with age – a regulatory factor that controls the relative levels of T and B cells.

AGE-RELATED CHANGES IN HYDROLASE ACTIVITY OF PERITONEAL MACROPHAGES

Margaret L. Heidrick*

Macrophages are of prime importance for protection against invading organisms and possibly for initiating an immune response. The lysosomal enzyme activity of these cells is related to their ability to destroy invading organisms. To assess changes in the functional capacity of macrophages with age, the activity of three hydrolytic enzymes (cathepsin D, beta-glucuronidase, and acid phosphatase) was measured in peritoneal macrophages from mice of various ages. Assays were done on the cells from the peritoneal cavity that adhered to plastic petri dishes after 24 hr culture *in vitro*. This cell population was macrophage-like in morphology, and 95–99% of the cells phagocytized carbon particles. The total activity of the three enzymes increased slightly with advancing age. The ratio of membrane-bound to free enzyme changed more dramatically, with macrophages from old animals having a proportionately higher level of free enzyme. The results

suggest a change with age in the enzyme metabolism rate and/or an alteration in the lysosomal membrane structure.

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A MODEL APPROACH TO IMMUNOLOGIC REJUVENATION OF THE AGED

E. H. Perkins and T. Makinodan

The marked increase in the incidence of cancer and autoimmune diseases and the dramatic decrease in humoral and, to some extent, cell-mediated immune capacity in aged mice are convincingly established. According to the immunologic theory of aging, mature adults remain healthy and are free of self-destructive autoimmune diseases because, presumably, immunological surveillance by a normally functioning, highly efficient immune system continually protects against infectious agents and aberrant (cancer) cells. Reconstitution of a damaged or exhausted immune system by injection of genetically compatible immunocompetent cells (immunologic rejuvenation) is a promising approach for restoration of immune activity and should enhance general health and vigor by increasing resistance to infections and to growth of malignant or benign cancer cells, thus markedly improving the quality of the final quarter of life.

Using this model, spleen cells from young adult mice previously immunized with *Salmonella typhimurium* were transferred to either young adult or old syngeneic recipients before or after storage at -196° C. This was thought to be an appropriate model test system, because mortality due to infection generally increases with advancing age and benign infections are often difficult to control in the aged. The susceptibility of recipient mice was then determined by challenging them at increasing time intervals after reconstitution with lethal doses of the virulent organisms. The findings demonstrate the following. (1) Immunologic rejuvenation of both young and old mice is possible with immunocompetent cells from specifically immunized donors. Young mice were not susceptible to a 100-fold greater challenge dose of organisms, and the marked increase in susceptibility of aged BC3F₁ mice to *S. typhimurium* infection was completely eliminated by adoptive transfer of "immunized" spleen cells. (2) Long-term survival (>45 weeks) and protective capacity of these cells against *S. typhimurium* infection can occur even in nonirradiated recipient mice. (3) Storage at -196° C does not impair their protective capacity. This demonstrates the feasibility of establishing reserve

banks of frozen, viable immunohematopoietic cells to meet clinical disease crises in later life or to be used to reconstitute these systems following destruction by accidental exposure to radiation, toxic chemicals, or deleterious environmental mutagens. In view of the complexity of human histoincompatibility reactions, banking a reserve of an individual's own optimally functioning immunohematopoietic cells or tissue for later immunological rejuvenation hopefully offers a rational and realistic approach to this complex and unresolved problem.

POSSIBLE IMMUNE ORIGIN OF AGE-RELATED PATHOLOGICAL CHANGES IN A LONG-LIVING STRAIN OF MICE

C. P. Peter

Many of the diseases in the aged have been shown to have an association with various immune deficiency states. It has been suggested that as a result of the decline of humoral and cell-mediated immune potential with age aberrant clones of lymphoid cells appear, which in turn react with the host's own tissues, resulting in various autoimmune diseases. Furthermore, many viruses produce life-long infections, which are transmitted regularly from mother to offspring. These infected offspring develop various pathological processes that are thought to be immunologic in nature. The present study was undertaken to examine age-related diseases of possible immunologic origin in long-living hybrid mice (BC3F₁). The types of pathological processes of possible immunologic origin detected in BC3F₁ mice are (1) glomerulosclerosis, (2) amyloidosis, (3) polyarteritis and arterial hyalinization, and (4) perivascular lymphoid and plasma cell infiltration of various tissues.

Mild to moderate glomerulosclerosis was present in about 50% of the animals examined that were over 18-20 months of age. The severity of the lesion appeared to increase with age. Immunofluorescent studies have demonstrated the deposition of mouse gamma globulins in the glomeruli of 38 of 44 mice over 20 months of age. Ten of 18 mice tested also showed deposition of murine leukemia viral antigen. Therefore, it appears that the glomerular lesion is primarily due to deposition of antibody gamma globulin or an antigen-antibody complex. Electron microscopy revealed changes similar to those induced by immune complexes. Amyloidosis and arterial lesions were very rare in this hybrid. Widespread lymphoid and plasma cell infiltration in the kidney, liver, lung, and pancreas were found in approximately half of the mice examined. In

about 10–15% of the mice the infiltration of the pancreas was so marked as to cause an atrophy of the acinar cells. These changes are similar to those which have been described in immunologic diseases of various other species.

These observations suggest that at least certain of the age-related diseases in this strain are immunologic in nature. In the case of glomerulosclerosis the evidence is direct. It is very possible that age-related diseases of immunologic nature occur more frequently in long-living strains of mice than in short-living ones.

DELAYED EFFECTS OF VARIOUS IMMUNOSUPPRESSIVE TREATMENTS ON THE LIFE-SPAN AND PATHOLOGY OF LONG-LIVING MICE

C. P. Peter, T. Makinodan, and W. J. Peterson

Short-term effects of various immunosuppressive treatments have been studied very extensively. However, very little is known about the long-term effects that such insults may have on life-span and pathology. Therefore, a comprehensive series of studies was initiated to evaluate the effects of splenectomy, thymectomy, X-irradiation, and cortisone acetate and cytoxan treatments on life expectancy, pathology, and immune competence. This report concerns only the effects on life expectancy and pathology. BC3F₁ mice with long life-span (mean, 31 months; maximum, 45 months) were treated at ages ranging from 2 to 24 months.

In general, the life expectancy was not altered by these treatments, except in the case of X-irradiation and in the case of thymectomy before 3 months of age. In the former case there was a marked reduction in the life-span. However, in the case of thymectomy the only alteration observed was a higher rate of mortality beginning about 32 months of age. Spontaneous death in approximately 80% of the mice of this strain is the result of various neoplastic diseases (reticulum cell sarcoma, hepatoma, subcutaneous sarcoma, and pulmonary adenoma and adenocarcinoma). Thymectomy, especially before 3 months of age, and cortisone acetate and cytoxan treatments resulted in a significant increase in death due to nonneoplastic diseases. These were primarily in the form of bronchopneumonia. There was also a slight but significant increase in the incidence of reticulum cell sarcoma in thymectomized animals. X-irradiation caused a significant increase in the incidence of endocrine tumors, lymphoid tumors, and nonneoplastic processes. This observation concurs with previous observations in other strains of mice.

These results suggest that these immunosuppressive treatments have a significant long-lasting effect on the pattern of pathology and possibly on life-span in this long-living strain of mice. Many of these effects can be explained on the basis of decreased immune capacity (humoral and cellular). This immune-deficient state in turn resulted in a higher incidence of both neoplastic and nonneoplastic diseases.

EFFECTS OF SENESCENCE ON THE KINETICS OF ERYTHROID PROGENITOR CELLS

M. G. Chen* and Lynda F. May

We have recently reported age-related changes in the concentrations, but not total numbers, of two types of hematopoietic stem cells — *in vivo* spleen colony-forming units (CFU) and *in vitro* agar colony-forming units — in the femoral bone marrow of a long-lived hybrid mouse. In addition, a marked decrease in the clonability of CFU from young (3 months) or old (27 months) bone marrow was found in the spleens of old, lethally irradiated syngeneic recipients as compared to young recipients. This was partly attributable to a decrease in the “f” factor or plating efficiency in the old recipients (1).

Price and Makinodan (2) have reported that the old thymus-derived and old bone marrow-derived cells involved in the primary immune response to sheep erythrocyte antigen are less capable of growth and proliferation than the respective young populations. Because of the similarities in the proliferative events involved in the immune response and in hematopoiesis, we investigated possible effects of senescence on erythropoiesis.

Erythrocyte progenitors were measured in intact young or old hypertransfused animals after stimulation with erythropoietin (3) and by heterochronic transplantation of young or old donor bone marrow into young or old lethally-irradiated, syngeneic recipient mice (4). No significant differences in the 72-hr ⁵⁹Fe uptakes were seen between intact young and old mice given the same amounts of erythropoietin over a range of doses. Similarly, closely equivalent frequencies of erythroid progenitors in young and old marrow were determined by limiting-dilution studies in which small numbers of bone marrow cells were transplanted into lethally irradiated young recipients, which were then assayed for erythrocyte ⁵⁹Fe uptake 7 days later. However, transplantation of larger numbers of cells produced significantly greater isotope uptakes from equivalent quantities of young as compared to old donor cells. Cell transfer into old recipients resulted in

markedly lower ^{59}Fe uptakes, regardless of the age of the donor material. These findings are compatible with a defect in the proliferative capacity of old progenitor cells and a deficiency in the environment of the old animal for erythroid maturation and proliferation.

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CULTURE OF HEMATOPOIETIC STEM CELL POPULATIONS IN MILLIPORE DIFFUSION CHAMBERS

M. G. Chen* and Lynda F. May

A basic tenant of any theory concerning the regulation of hematopoiesis is that humoral factors operate at the level of the early progenitor cells. However, direct evidence concerning the nature of these "ecological factors" is lacking because of the anatomical and physiological complexities involved in *in vivo* studies. Alternatively, *in vitro* culture of hematopoietic tissue to this end has not been notably successful because of stem cell death early in the period of culture and failure of self-renewal among surviving stem cells. Therefore, we have been engaged in developing a controlled, accessible, closed system for culture of hematopoietic stem cells and their progeny *in vivo* that would allow study of some of these regulating factors.

The kinetics of two hematopoietic stem cell populations (spleen and agar colony-forming-units) and their progeny have been studied by an *in vivo*, yet closed, Millipore diffusion chamber technique. Chambers containing bone marrow of syngeneic (mouse) or xenogeneic (human) origin were implanted into the peritoneal cavity of X-irradiated (600 r) recipient mice or untreated controls and assayed for numbers of stem cells and progeny at various times after implantation. Stem cell proliferation similar to that seen after irradiation was observed, and this was enhanced by soluble factors from the irradiated recipient mice. The rate of cellular proliferation was inversely proportional to the number of cells cultured, and extent of proliferation was related to the size of the chamber. Significant erythropoiesis did not occur. Preliminary studies on human bone marrow cells in chambers cultured in mice demonstrated the feasibility and applicability of this technique to clinical investigations.

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DEFECTIVE IMMUNOPOIESIS IN AKR MICE. GENETIC CONTROL OF THE IMMUNE RESPONSE AT THE LEVEL OF PROLIFERATION

C. F. Gottlieb, E. H. Perkins, and T. Makinodan

We have shown that immunohematopoiesis is defective in the AKR mouse (1). When identical aliquots of spleen or bone marrow cells from either AKR or C3H donor mice were injected into lethally irradiated AKR and C3H recipient mice, the response in terms of plaque-forming cells (PFC) or colony-forming units (CFU) was always lower in the AKR recipient. This noncellular defect appears unrelated to donor-host incompatibility, AKR thymus (or thymic factors), and leukemia virus.

Continued investigation revealed two significant observations. Limiting-dilution analysis of AKR spleen cells cultured with sheep erythrocytes in cell-impermeable diffusion chambers has shown the frequency of antigen-sensitive units to be about 0.5×10^{-6} , with no difference between the AKR and C3H recipients. Therefore, the difference in peak direct PFC response observed when identical aliquots of cells from a mixture of AKR spleen cells and antigen were cultured in AKR and C3H recipients can be attributed to a difference in the burst size (number of PFC generated per antigen-sensitive unit) in the two recipients. The calculated burst sizes in the AKR and C3H recipients are 500 and 2150 PFC per unit, respectively, in the chamber system and 310 and 2250 in the spleen-cell transfer system.

Experiments designed to explore the genetics of this proliferative defect have used F_1 and F_2 progeny of the AKR and C3H parental lines, as well as the two F_1 backcrosses (2). In these experiments, mice from six groups (two parental, F_1 , F_2 , and two F_1 backcrosses) were used as recipients of identical aliquots of a mixture of AKR spleen cells and antigen. The response of the AKR spleen cells was then measured in the six environments. The results of these experiments suggest that a single autosomal gene locus is responsible for the deficient immunopoiesis in the AKR recipient. Although other stem cells were not assayed in the genetic study, the pattern exhibited by the spleen weights of the recipients was identical to the PFC response, suggesting that the same locus also influences the response of other stem cells.

These results now establish a third level of genetic regulation of the humoral immune response — at the level of proliferation of stimulated cells, distinct from the structural gene control of the variable region of the antibody molecule and from other genetic loci controlling the antibody response. In the latter case, the low response has been attributed either to defective recognition of antigen or to differences in the frequency of immunocompetent precursors for the antigen tested.

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LATE SOMATIC EFFECTS OF LOW DOSES OF GAMMA RADIATION

L. J. Serrano, G. E. Cosgrove, Lou Satterfield, W. C. Klima, H. C. Swann, J. M. Yuhas, E. B. Darden, Jr., M. C. Jernigan, and J. B. Storer

The purpose of this experiment is to determine the shape of the dose-response curve for late radiation effects such as carcinogenesis, leukemogenesis, and life-shortening in mice. Since low radiation doses have been employed, it has been necessary to use large sample sizes. In the first phase of the experiment, 15,000 female mice and 2,000 male mice of the RFM strain were used. These animals were raised and maintained under "barrier" conditions. Gamma rays were delivered from a ^{137}Cs source at a dose rate of 41 r/min. Doses used were 0 (controls), 10, 25, 50, 100, 150, and 300 r. This phase of the experiment is nearly complete. In July 1972, fewer than 20 mice remained alive and fewer than 100 autopsy cases remained to be evaluated histologically. The data for the male mice are complete and preliminary analyses have been done. The pathology data for the females will be encoded on an on-line computer for analysis. These analyses should be completed by the end of 1972.

The purpose of the second phase of this experiment is to determine how much less effective gamma rays are when the dosage is delivered at a low rate. A total of 7000 female RFM and 7000 BALB/c mice have been set up. Most of the mice were exposed to gamma radiation at a rate of 10 r/day. Dosages employed were 0 (controls), 50, 100, 200, and 400 r. Smaller groups were exposed at 40 r/min to doses of 50 and 200 r to determine whether the response of the animals has changed between phase I and phase II of the experiment (the two phases could not be run concurrently). Setouts are essentially complete, but it will

be another 3 years before the population dies out and an analysis can be made.

THE EFFECT OF PREVIOUS PARITY AND IRRADIATION ON THE LIFE-SPAN AND DISEASE INCIDENCE IN THE RF FEMALE MOUSE

G. E. Cosgrove and L. C. Satterfield

In previous radiation survival experiments using the RF mouse, it was noted that exposure to sublethal whole-body radiation results in a marked increase in the incidence of ovarian tumors. In those experiments only virgin females of different ages were used. In the experiment reported here, the effect of previous parity is being studied. The endpoints used are life-span and the incidence of various diseases as seen at necropsy at the time of natural death. In addition, the findings might be of interest because multiparous female mice have been repeatedly exposed to fetal antigen, and this might alter their susceptibility to subsequent development of neoplasms.

The RF female mice were obtained from a barrier-maintained pathogen-free colony at about 6 months of age and transferred to a conventional colony. Half the mice were virgin females and the rest had borne two or more litters. Those selected for irradiation were exposed to 150 r of whole-body X-rays at a dose rate of approximately 85 r/min, while the remainder were maintained as unirradiated controls. Thus, there were four experimental subgroups (Table I) with about 125 mice per group. At the time of natural death necropsies were performed and findings recorded. Computer storage and retrieval methods were used for analysis of the information.

Some of the major findings are indicated in Table I. The life-shortening effect of 150 r was mild in both

TABLE I. Survival time and disease incidence in RF mice

	Nulliparous		Multiparous	
	0 r	150 r	0 r	150 r
Mean survival time (<i>days</i>)	627	555	607	585
Total neoplasms (% <i>incidence</i>)	85	84	67	78
Total lymphoma/leukemia	70	70	59	60
Thymic lymphoma	13	25	7	16
Myeloid leukemia	0	4	0	3
Reticulum cell sarcoma	57	41	52	41
Tumor, lung	12	13	14	13
Tumor, ovary	10	32	3	30
Glomerulosclerosis	80	67	75	68

nulliparous and multiparous mouse groups, and previous parity itself did not significantly affect survival. Neoplasms of all types were more frequent in nulliparous mice, but radiation did not greatly alter the incidence. The higher incidence of thymic lymphoma in nulliparous mice – irradiated and control, and the higher incidence of ovarian tumors in nulliparous controls accounted for most of this difference. Radiation increased the incidence of thymic lymphoma, myeloid leukemia, and especially ovarian tumor. However, the incidence of ovarian tumors in multiparous irradiated mice was not different from that in irradiated nulliparous mice.

It seems, then, that previous parity does not affect the subsequent incidence of postirradiation ovarian tumors but is accompanied by some reduction in thymic lymphomas.

RELATION OF DOSE TO RELATIVE BIOLOGICAL EFFECT OF FAST NEUTRONS FOR THE INDUCTION OF LENS OPACITIES

E. B. Darden, Jr., K. W. Christenberry, J. M. Yuhas, and J. J. Beauchamp*

Due to problems of sample size, if for no other reason, detection of statistically significant effects of ionizing radiation below doses of a few rads is usually not feasible in experimental animals. Important decisions about the effects of low doses in man have been made on the basis of extrapolations from observations at much higher doses, without a really adequate theoretical basis for doing so. By analyzing the response in an intact mammalian system, the mouse lens, in which dose effects can be assayed at doses 10-fold or more below conventional experimental levels, we hope to gain more insight into the mechanism of response in more radioresistant mammalian systems, in order to put extrapolation on a sounder basis.

A brief summary of preliminary results was given previously (1). Lens opacities in mice exposed to fission neutrons or X-rays were scored on the basis of the percentage of the lens affected (2). For a given dose level and type of radiation the mean percent opacity, $Y(t)$, observed at time t after treatment can be closely approximated by the reciprocal relation $Y(t) = a + bt^{-1}$. The parameters a and b are estimated from the observed values of $Y(t)$ and t . The latent period t_1 , before the appearance of opacification, is then $-b/a$. Total response is defined as the area under the $Y(t)$ curve – $R = \int_{t_1}^{t_{\max}} (a + bt^{-1}) dt$ – between t_1 and a given time t_{\max} , say 400 days, for each treatment group and for the unirradiated control group. We

represent the radiation component of the area (Δ) by $R - R_0$, since the area for each irradiated group will necessarily include a component of senile opacification approximated by R_0 , the area under the control curve. Over the dose range studied, the relation between Δ and the corresponding dose D for either neutrons or X-rays can be described by a quadratic expression in log dose – $\Delta = \alpha_0 + \alpha_1 \log D + \alpha_2 (\log D)^2$ – where the coefficients α_0 , α_1 , and α_2 are estimated from the values of Δ and D for each treatment group. We can then determine RBE by either graphical or analytical means as a function of dose.

The results show that the log RBE of neutrons plotted against the log dose of either neutrons or X-rays is almost a linear function over the experimental range tested. The slopes are ≤ -0.05 and ≤ -1 for neutrons and X-rays, respectively. Plotted against neutron dose, for example, the RBE varies from about 6 to 55 over an 800-fold range in dose (0.2–160 rads). Our results appear to be in harmony with recently proposed theoretical considerations (3) related to the expression of the fundamental injury events in subcellular sites produced by recoil protons and electrons, but further analysis is necessary to confirm this.

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LATE SOMATIC EFFECTS OF ACUTE FRACTIONATED AND PROTRACTED EXPOSURE TO FISSION NEUTRONS AND GAMMA RAYS

E. B. Darden, Jr., L. J. Serrano, J. B. Storer, and M. C. Jernigan

Effect of mode of delivery upon biological response is generally greater with low-LET radiation (X- and γ -rays) than with predominantly high-LET radiation such as fast neutrons. RBE is consequently higher for lower dose rate and is often further increased at low doses because of differences in the dose-response curves for high- and low-LET radiations. Generally speaking, concern about the possibilities of increased malignancy and other delayed effects resulting from exposure of the general public to low levels of ionizing radiation still applies primarily to low-LET radiation. With the increasing use of small transuranium sources in medicine and industry, particularly ^{252}Cf as a substitute for radium in radiotherapy, a more urgent need exists now

to improve our understanding of the delayed hazards associated with exposure to densely ionizing radiation. Definitive information is scanty, even in laboratory animals.

The experiment in progress was set up to evaluate the relative influences of acute, fractionated, and protracted exposure to fast neutrons and γ -rays on late radiation effects in mice, principally changes in longevity, induction of neoplasia, and lens opacification. A brief description of the study was given earlier (1), but important modifications have been made since then. To extend the spectrum of diseases observed, we are including both the RFM and the BALB/c strains. About 9000 mice, divided equally between strains, are being committed to the neutron study, the irradiation phase of which is about half completed. For RBE determinations, the neutron-irradiated groups will be compared with mice receiving acute and protracted ^{137}Cs γ -ray exposure in the Low (Medium) Level Experiment rather than with mice from Dr. Yuhas' project as originally planned. This change enables all the mice in the RBE study, particularly those in the protracted exposure groups, to be maintained under similar background conditions.

For the acute and fractionated exposures, mice receive up to 196 rads of fission neutrons at a dose rate of 24.5 rad/min either at one time or in fractions given approximately biweekly in the ORNL Health Physics Research Reactor. For the protracted irradiations, mice beginning at 10 weeks of age receive corresponding neutron doses in our ^{252}Cf exposure facility at a dose rate of 1 rad/day. As a control for changes in radiosensitivity with age, half of the acutely exposed replicates are irradiated at 10 weeks of age and the remainder at a later age, to correspond to the age of the mice at the end of protracted exposure.

Neutron irradiations were begun in the fall of 1971. The original ^{252}Cf source was slightly under strength, leading eventually to a reduction in the planned irradiation output. In the spring of 1972, we were able to exchange this source for a stronger (1100 μg) source permitting a return to our original schedule.

The limited observations to date, based largely on the induction of thymic lymphoma, do not reveal any pronounced difference in effectiveness between acute and protracted neutron exposures. The results are thus consistent with findings of others — that in most systems studied, the effects of high-LET radiation are relatively independent of dose rate.

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EFFECT OF FAST NEUTRONS AND X-RAYS ON THE MOUSE EMBRYO IN THE EARLY PRENATAL PERIOD

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We previously reported (1) initial results of survival studies in mice prenatally irradiated with X-rays or fission neutrons (ORNL Health Physics Research Reactor). Replicates were irradiated within the first day of conception, and the uterine contents were examined just prior to term for live and dead embryos. Dose-survival curves for single exposures approximated simple exponentials, with $D_0 \sim 16$ rads and ~ 76 rads for neutrons and X-rays, respectively, giving an RBE of nearly 5. Preliminary neutron studies using acute and protracted exposures did not show any effect of dose rate on survival. The observations thus suggested that embryo death following neutron irradiation was probably the result of a single irreparable injury event. Some mice were allowed to come to term, and the offspring are being observed for delayed effects.

We are continuing to investigate radiation parameters likely to affect survival. In further studies with neutrons, survival was not affected by whether radiation occurred in the morning or afternoon nor by splitting the dose between these times. With X-rays, survival was slightly favored by irradiation in the morning as compared to the afternoon. With either type of radiation, increased survival was observed after treatment in the early evening as compared to the afternoon but was not observed if the dose was split between afternoon and evening exposures.

The results provide further evidence that a single injury event with little or no repair is responsible for embryo death when irradiation occurs during the preimplantation stage. Efforts are now being made to correlate the results with morphologically observed changes in the zygote and early embryo.

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EFFECTS OF ACUTE THROMBOCYTOPENIA ON THE DNA CONTENT AND MORPHOLOGIC DISTRIBUTION OF MEGAKARYOCYTES

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Megakaryocyte changes have been studied in rats made thrombocytopenic by a single intravenous in-

jection of 0.2 ml of antiplatelet serum (APS). The DNA content per megakaryocyte was measured on Feulgen-stained squash preparations of bone marrow using the two-wavelength method. The average amount of DNA per megakaryocyte was increased at 18 hr but not at 12 hr. This initial ploidy shift was paralleled by an increase in the percentage of immature megakaryocytes, first discernible at 18 hr. The DNA content per megakaryocyte and the morphologic distributions remained altered through 96 hr but were normal 120 hr after APS. Furthermore, a greater average number of megakaryocytes per high-power field (18–60 hr after APS) in marrow sections and an augmented mitotic index (18–36 hr after injection) in the same preparations indicated that an influx of precursor cells into the immature megakaryocyte compartment accompanied the ploidy shift. Other morphologic evidence as well as cholinesterase data described in the previous annual report suggest that this influx is the result of a pulse stimulus rather than a gradual or a continual one. Acute thrombocytopenia, therefore, appears to cause both an influx of precursor cells into the immature megakaryocyte compartment and an increase in the DNA content per cell.

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PLATELET PRODUCTION AFTER ACUTE THROMBOCYTOPENIA IN RATS

T. T. Odell, Jr., C. W. Jackson,* and J. R. Murphy

Platelet production was studied in rats made thrombocytopenic by intravenous or intraperitoneal injection of antiplatelet antiserum (APS). Various amounts of APS were injected in different groups to vary the degree and duration of thrombocytopenia. The route of injection did not appear to influence the response materially. In agreement with earlier reports, the rate of platelet production was increased after induction of thrombocytopenia. Moreover, our results indicate that the time between injection of APS and increased production and the changes in rate of production differ with degree and duration of thrombocytopenia. After a single moderate dose of APS sufficient to reduce the platelet count to about 20–40% of the count before treatment, the peripheral platelet count began to rise within about 24 hr, and the rate of increase (about 260,000/mm³/day) was only a little greater than the normal production rate in control rats (about 230,000/mm³/day). When the platelet count was depressed to 3% or less by APS, platelet production began

to increase sometime between 24 and 48 hr, and the rate was approximately double that of controls. After more protracted thrombocytopenia (3–6 days) brought about either by a single large dose or by repeated injections of APS, the rate of platelet production was as great as 3–5 times that of controls. Thus the rate of platelet production after induction of thrombocytopenia by APS was proportional to the degree and duration of thrombocytopenia.

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AUTORADIOGRAPHIC STUDIES OF LABELING OF MEGAKARYOCYTES WITH ³⁵SO₄

T. T. Odell, Jr. and D. K. Beeman

Rats were injected intravenously with 1 μCi of Na₂³⁵SO₄ per gram of body weight. Some rats were given a cold sulfate chase 30 min after injection of the hot sulfate. Rats were killed and bone marrow samples were taken at intervals during the 4 days after injection. Autoradiograms of marrow smears were made, and the labeling index of immature and mature megakaryocytes was scored. The labeling index of megakaryocytes in rats that received the cold sulfate chase was somewhat lower than in rats without the chase, but the pattern was similar in the two sets of data. Eight hours after injection of ³⁵SO₄, about 75% of the mature megakaryocytes in the chased rats were labeled, and the labeling index declined to a very low level by 72 hr. The labeling index of the immature megakaryocytes was about 45% at 8 hr and then declined essentially to zero by 48 hr. This disappearance of labeled cells is consistent with other data that indicated a maturation time for megakaryocytes of between 48 and 72 hr. The results also suggest that more mature megakaryocytes are more active in sulfate uptake than are young ones. In addition, the results are useful in understanding more fully experiments in which ³⁵SO₄ is used for studying the production rate of blood platelets and for assaying thrombopoietic agents. They indicate, for example, that sulfate-labeled platelets are released to the circulation for about 48 hr after a single injection of Na₂³⁵SO₄.

THE MECHANISM OF RADIATION-INDUCED LIFE SHORTENING AND ITS RELATION TO ACCELERATED AGING

H. E. Walburg, Jr.

The repeated observation that many diseases of old age develop prematurely in irradiated animals led to the

simplified hypothesis that radiation causes life shortening by accelerating the aging process. Although such phenomena as hair depigmentation, lens cataracts, and female infertility occur strikingly earlier in irradiated populations, they appear to develop by entirely different mechanisms, suggesting a lack of homogeneity between radiation-induced life shortening and the aging process. The principal support for the hypothesis of accelerated aging, however, derives from analysis of survival experiments, where all causes of death appear to be advanced in time by radiation. If the force of mortality rises more rapidly and brings forward the age of onset of all diseases normally occurring in the population without altering the incidence of such diseases, it can be judged to cause accelerated aging (1). Since in early experiments all causes of death appeared to be advanced in time by radiation, the necessary criteria seemed fulfilled. Unfortunately, the experimental conclusions were based on an analysis of observed mean ages at death for animals dying with specific diseases. It has recently been demonstrated that if the true incidence of one early-occurring disease increases, then the observed mean ages at death of all later-occurring diseases are reduced (2). Since the principal radiation-induced disease is thymic lymphoma (an early-occurring lethal disease), it is not clear whether the decreases in mean age of death of the later-occurring diseases are true radiation acceleration of these diseases or a statistical artifact as noted above. Since the final incidences of late-occurring diseases and their mean ages of death are seriously affected by mortality rates preceding the onset of such diseases, a meaningful analysis requires an estimation of the net probability of death due to a specific disease, i.e. the probability of death if the specific disease is the only risk acting on the population (3). Demonstration of an accelerated aging effect of radiation requires that the net probability of death and its associated mean age at death be altered for all diseases occurring in a population at risk and that the time of occurrence be uniformly advanced for all such diseases. When the mean age of death for data adjusted properly for competing risks is examined, it can be seen that some diseases are accelerated by radiation and some are not. It is clear from at least two experiments, one a reanalysis of data from a large experiment conducted in the 1950's which has been cited in support of the accelerated aging hypothesis and the other a more recent small experiment, that only neoplastic diseases are affected by irradiation at moderate to low doses, i.e. 300 rad and below. Figure 1 shows the net probability of dying from nonneoplastic diseases for LAF₁ mice

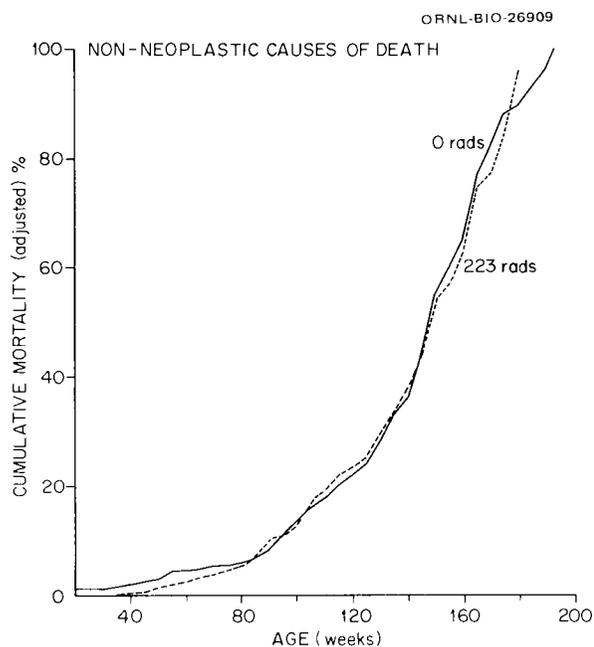


Fig. 1. Cumulative mortality for LAF₁ male mice dying from nonneoplastic diseases. ---, 180-270 r X-radiation; —, no radiation.

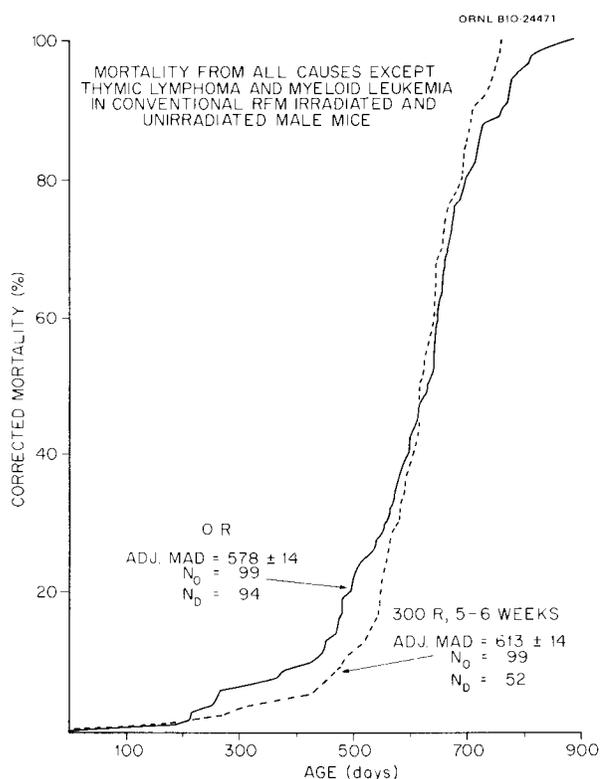


Fig. 2. Cumulative mortality for RfM male mice dying from diseases other than thymic lymphoma and myelogenous leukemia (deaths prior to 60 days of age not included). ---, 300 r X-radiation, 5-6 weeks; —, no radiation.

either unirradiated or exposed to 180–270 rads gamma rays during Operation Greenhouse, a field test of the effectiveness of atomic bomb radiations for induction of delayed somatic effects (4). It is clearly seen that radiation at this dose level does not alter the net probability of death due to nonneoplastic diseases. Figure 2 shows similar data for a small experiment with RFM mice that died due to causes other than thymic lymphoma and myelogenous leukemia, the principal causes of death in irradiated populations of this strain. It is clear that at an exposure of 300 r other causes of death are uninfluenced by the radiation exposure. These data suggest that (1) radiation-induced life shortening is due to the induction of some but not all diseases, thus nullifying the accelerated aging hypothesis, and (2) only neoplastic diseases appear to be induced or accelerated by radiation. While these conclusions require considerable substantiation, they form the basis of a new hypothesis for explanation of the mechanism of radiation-induced life shortening.

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INFLUENCE OF MICROBIAL FLORA ON DEVELOPMENT OF SPONTANEOUS NEOPLASMS IN MICE

H. E. Walburg, Jr.

It is clear from early studies that tumors can be induced in gnotobiotic animals by chemical carcinogens and oncogenic viruses. Casual observation of germfree mice, however, suggested that these animals were less susceptible to the development of spontaneous neoplasms than their conventional counterparts. Nonetheless, spontaneous tumors were repeatedly reported in germfree mice, and the number and diversity of tumors increased as more animals were examined at advanced ages. Unfortunately, these observations were qualitative in nature; quantitative assessment of differences between germfree and conventional mice was not possible. Recently, attempts have been made to quantitate differences between the development of spontaneous neoplasms in gnotobiotic and in conventional animals in this laboratory. Data from these studies indicate that the development of some neoplasms is influenced by the microbial environment, while that of others is not. Conclusions from the pilot studies with ICR mice have already been reported (1),

and preliminary data from experiments with RFM mice have also been reported (2). This report consists of a final analysis of the data from the RFM experiments and preliminary data from the experiments with C3H mice. The principal spontaneous neoplasm in RFM mice is reticulum cell sarcoma, and the net probability of dying from reticulum cell sarcoma is significantly higher in conventional than in germfree mice ($P < 0.001$). Thymic lymphosarcoma, a less commonly occurring spontaneous hemopoietic neoplasm in RFM mice, occurs in germfree mice at a higher incidence and earlier than in their conventional counterparts, but the differences are not significant, presumably due to the small number of cases observed. In female RFM mice the development of ovarian tumors and of all endocrine tumors when the various types (e.g. breast, ovarian, adrenal, thyroid) are pooled does not appear to be affected by the microbial environment. In RFM mice the development of lung tumors, which are generally small papillary adenomas only rarely contributing to the death of the animal, does not appear to be significantly different between germfree RFM mice and their conventional counterparts. Likewise, for RFM mice the development of all tumors other than endocrine and lung tumors is not significantly different, either for those tumors causing death or for those occurring as incidental findings at death. Further, the incidence of malignant tumors in RFM mice is not significantly affected by microbial environment. Thus, it appears in untreated RFM mice that while lymphomas are affected by the microbial environment, nonhemopoietic neoplasms are not.

Preliminary results from germfree and conventional C3H mice suggest a similar lack of effect among nonhemopoietic tumors. Endocrine tumors of female C3H mice show no effect of the microbial environment for either mammary or ovarian tumors. The development of liver tumors appears to have inconsistent patterns in our experiments. There is no significant difference for the development of liver tumors between germfree and conventional C3H male mice. Although the cumulative mortality adjusted for competing risks is not different to 900 days of age, after this time germfree mice develop considerably fewer tumors than their conventional counterparts. Germfree C3H females develop liver tumors at a significantly earlier age ($P < 0.05$) than their conventional counterparts. Roe and Grant (3) have reported that germfree mice develop fewer hepatomas than their conventional counterparts. Therefore, it appears that the development of hepatomas in germfree and conventional C3H mice is variable, and further studies will be required.

It has been clearly shown that there are no differences in susceptibility to oncogenic virus between gnotobiotic and conventional mice, and all germfree mouse strains show evidence of oncogenic virus in their tissues. Thus it does not appear that the differences in incidence of hemopoietic neoplasms in germfree and conventional animals can be explained by a difference in the presence of or susceptibility to oncogenic virus. The immune competence, both humoral and cellular, of gnotobiotic animals has been extensively studied. These studies demonstrate that germfree animals have a slightly but consistently greater immune competence than their conventional counterparts. Such an increase in immune competence might explain a general reduction in the total numbers of neoplasms in germfree animals but not the selective pattern which is seen. Further, it has been demonstrated that nutritional deficiencies can result in a reduction in the incidence of neoplasms. The effect of nutritional deficiencies does not appear to be selective, and thus it cannot explain the reduced incidence in some but not other tumors. The lack of a consistent response in the development of spontaneous hepatomas in C3H mice may, however, be related to nutrition, since these tumors are particularly sensitive to dietary alteration. The microbial environment influences the amount of DNA synthesis in some cellular compartments, principally those producing granulocytes, lymphocytes, and intestinal epithelial cells, but not in all. Thus the effect of the microbial environment on the incidence of reticulum cell sarcoma in RFM mice may be related to antigenic stimulation of the reticulum cells in lymphatic tissue. While the relative importance of various host factors in influencing the ultimate expression of neoplasia is not clear, studies on the role of the microbial environment in the pathogenesis of spontaneous neoplasms may help to clarify the mechanisms of carcinogenesis.

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2. H. E. Walburg, Jr. and G. E. Cosgrove, *Biol. Div. Ann. Progr. Rept. Dec. 31, 1967*, ORNL-4240, p. 217.
3. F. J. C. Roe and G. A. Grant, *Int. J. Cancer* **6**, 133 (1970).

IMMUNOLOGICAL STUDIES OF SARCOMAS ORIGINATING FROM REPEATED ANTIGENIC STIMULATION

R. L. Tyndall, J. A. Otten, and L. H. Jordan

A previous report described the origin of transplantable reticulum cell sarcomas (RCS) resulting in-

directly from repeated antigenic stimulation. The sarcomas arose in mice inoculated as newborns with normal spleen cells from adult mice that had been inoculated twice weekly with sheep erythrocytes (1). Because of the increased incidence of neoplasia in human transplant recipients subjected to constant antigenic insult and the relative paucity of experimental information concerning neoplasms associated with such insult, we undertook an immunological characterization of one of these sarcomas (L1).

Immunization of adult BALB/c mice with X-irradiated L1 sarcoma cells was sufficient to protect mice against subsequent challenge with viable L1 cells. Immunization with another transplantable RCS (L5) did not protect mice against challenge with L1 cells (Table I). When sera (0.1 ml) or spleen and lymph node cells ($\sim 1 \times 10^6$) from L1 immune mice were incubated *in vitro* (37° C, 60 min) with 1×10^4 viable L1 cells and subsequently inoculated into normal recipients, the immune sera, but not the cells, was capable of neutralizing the L1 sarcoma cells. These results indicated that the immune protection capable of destroying the L1 sarcoma, unlike most tumors, is primarily associated with the humoral rather than the cellular compartment.

Because human leukemic tissue extracts have been shown to enhance synergistically the effects of selected stocks of both Friend and Rauscher leukemia viruses, we tested the ability of several samples of human leukemic tissues to immunize mice against challenge with the L1 sarcoma. Immunization with each of three such samples markedly protected mice against subsequent challenge with L1 cells (Table I). In addition, sera from mice immunized with leukemic tissue from human donors has been effective in neutralizing L1 cells when mixed *in vitro* prior to challenge of normal mice. Two samples of blood cells from normal human donors were ineffective in immunizing mice against L1 challenge (Table I).

Whether the protection afforded mice immunized with human leukemic tissues and subsequently challenged with L1 sarcoma cells is specific for human neoplastic tissues and whether perchance it indicates common antigens between the L1 sarcoma and human leukemic cells is the current concern of this study.

1. R. L. Tyndall, P. C. Estes, and J. A. Otten, *Transplantation* (in press).

TABLE I. Inhibition of transplantable reticulum cell sarcoma (L1) by prior immunization with X-irradiated tumor cells*

X-irradiated cells used for immunization	No. tumors/no. mice inoc.	Tumor incidence (%)
L1	0/9	0
L5	9/9	100
Human leukemic cells		
Sample 1	0/9	0
Sample 2	3/12	25
Sample 3	0/12	0
Normal human blood cells		
Sample 1	4/4	100
Sample 2	5/5	100
None	24/24	100

*Adult mice were immunized with two inoculations of X-irradiated tumor cells. Mice were challenged 8–12 days after the second immunization with viable tumor cells. Experiments were terminated 25 days after tumor challenge.

RAMIFICATIONS OF THE PSEUDOPREGNANT RESPONSES OF TUMOR-BEARING ANIMALS

R. L. Tyndall and J. A. Otten

The isoenzymatic similarities between fetal and leukemic murine tissues were described previously (1). Likewise, the preferential localization of ^{67}Ga in both fetal and leukemic mouse tissues was noted (2). In view of such similarities, we also showed that identical alterations in serum protein profiles were consistently observed in pregnant, leukemic, and scalped mice (3). Moreover, as also recently noted, a common antibody has been detected in sera from pregnant, scalped, and tumor-bearing rats (3). Thus not only are there similarities between fetal and neoplastic tissues *per se*, but some common host responses have been observed in sera of animals bearing such tissues. While these observations thus far are of both theoretical and possibly diagnostic value, a more important question is whether the pseudopregnant responses of animals bearing malignant tissues affect in any way the outcome of the neoplasia. To this end we are currently studying, in cooperation with Drs. Clapp, Serrano, and Nettesheim, the effects of parity and/or immunization with fetal tissues on a variety of spontaneous, viral, and chemically induced tumors.

Thus far neither multiparous mice nor mice immunized with fetal tissues have shown any significant alteration in response to chemically induced pulmonary adenomas or to leukemias induced by Rauscher leukemia virus. The immunosuppressive effects of such agents, however, may be prejudicial to any repressive

effect of parity on immunization. Conversely, in studies of spontaneous thymic lymphoma in RFM mice, where no immunosuppressive effect would be indicated, interim data indicate a significant inhibition of thymoma in multiparous compared to comparably aged virgin mice (4). Consequently, studies are now being directed to investigating the effect of parity or scalping on spontaneous tumor incidences and transplantable tumors in which immunosuppressive side effects do not grossly complicate the experimental results.

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4. L. J. Serrano, this report, p. 150.

REGULATION OF LEUKEMIA VIRUS REPLICATION BY FUSION WITH NONPERMISSIVE CELLS*

R. W. Tennant, Lorraine M. McGrath,
R. E. Hand, Jr., and F. E. Myer

Some strains of mouse sarcoma viruses are able to transform human cells, but most strains of mouse leukemia viruses do not replicate in cultured human cells. Three principal causes for the nonpermissiveness of human cells have been tested. First, the cells may lack specific virus receptors; second, the cells could be deficient for functions required for virus replication; or

third, the human cells could exert a positive control over some viral functions. In order to test these possibilities, the fate of Moloney virus was followed in heterokaryons of nonpermissive WI-38 and permissive 3T3 cells by a technique of simultaneous autoradiography and fluorescent-antibody assay. Virus synthesis could not be detected in fused synkaryons of WI-38 cells nor in heterokaryons of WI-38 and 3T3 but did occur in 3T3 synkaryons. These results suggest that nonpermissiveness of the human cells for Moloney leukemia virus is dominant. Further, five cloned cell lines of human-mouse hybrids obtained from Dr. Howard Green were tested for permissiveness for Moloney leukemia virus. The cells contained less than the full complement of human chromosomes and were all permissive for virus synthesis. The results tentatively suggest that some human gene functions can inhibit or restrict Moloney leukemia virus replication.

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DISEASE PATTERN RELATED TO PARITY OF RETIRED BREEDERS

L. J. Serrano

Pregnancy is a significant challenge to most of the organ systems of a female, and it may influence a female's response to degenerative diseases of aging. To examine this possibility, female breeders of strains RFM and BALB/c mice having given birth to 0-6 litters were grouped by parity at time of retirement and maintained until death; they were then necropsied. So far, less than half have died.

Tyndall's findings (1) that serum protein profiles in both pregnant and leukemic mice are identical suggested that pregnancy may have a significant influence on the time of occurrence and the incidence of spontaneous leukemia.

A preliminary analysis of the available data shows that after age 300 days, the incidence of thymic lymphoma is higher in mated but nulliparous females than in females that have produced litters.

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ORDER OF BIRTH AS AN EXTRANEIOUS FACTOR IN EXPERIMENTS

L. J. Serrano

The mother's age or parity at the time of an animal's birth may influence its adaptability to environmental or

experimental stresses. Strong (1) compared mice born in the first to twelfth litters and found that litter order was related to survival time and to latent period for induction of tumors. Incidence of spontaneous tumors and leukemias, immune response, longevity, and anomalies have been related to maternal influences also. When a breeding colony is established to produce animals for an experiment, the first mice into the experiment will be from early litters of young mothers, and the last mice in will be from late litters of older mothers; or smaller experiments may get mice predominantly from one litter order and thus have a nonrepresentative sample. We need to answer the question, "Are responses to the stress of irradiation also modified by order of birth?"

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. Most of the mice in the higher dose group have died, but other results are not available yet.

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OVARIECTOMY AND LIFE-SPAN IN IRRADIATED FEMALES

L. J. Serrano and J. B. Storer

Differences between males and females in life shortening after irradiation have been demonstrated by others. The same difference was seen during analysis of data from the Low (Medium) Level Experiment. In males, the life shortening could be accounted for by diseases such as leukemia, but in the females there was a residual nonspecific shortening. To elucidate the role of ovarian hormones on this effect, ovariectomized females of the RFM and BALB/c strains will be irradiated with a ¹³⁷Cs source at 50, 100, and 200 rad and maintained until death. Input of mice is expected to be complete during the year.

CARCINOGENIC EFFECTIVENESS OF GAMMA RAYS AS A FUNCTION OF DOSE RATES BETWEEN 1 r/DAY AND 1 r/SECOND

John M. Yuhas and Anita E. Walker

It is well established for most radiobiological endpoints that the effectiveness of the exposure is determined, in part, by the rate at which it is administered. For most of these endpoints, effectiveness declines as

the dose rate is lowered, with a maximum effectiveness being reached at dose rates of 1 r/sec and above. The dose rate dependence of effectiveness is not universal, however, since in certain instances lower dose rates can prove more effective than higher ones, or a minimum effectiveness can be observed that is characteristic of all dose rates in a given range. The question being posed by these studies is how does the carcinogenic effectiveness of gamma rays change over the above-mentioned range, and secondly, why do these changes occur.

BALB/c mice are being exposed to graded doses of gamma rays (0 to 392 r) at rates ranging from 1 r/day to 1 r/sec. This provides the basic information on effectiveness. In order to determine what role age-dependent changes in resistance and remaining life expectancy play, we are also exposing mice to single acute doses at ages of up to 300 days. Further, fractionated exposures throughout a period equal to the longest exposure time are being given in order to determine what role dose size plays in the overall effect. In combination, these data will allow us to describe the dose-rate-dependent alterations in terms of those which are due to alterations in the effectiveness of the exposure and in terms of those which are due to alterations in the animal itself.

This later point, alterations in the animal, is being investigated in terms of age-dependent changes in immunologic reactivity and similar alterations in hormonal status. The effects of radiation given under the aforementioned conditions on each of these should provide the opportunity of associating the empirically observed alterations in effectiveness with specific physiologic systems involved in the development of certain neoplasms.

More than 75% of the mice required for these experiments have now been introduced. Through the use of a simplified data-retrieval system, we are able to obtain estimates of effectiveness throughout the entire experiment almost immediately. It is only 11 months since the first animals were introduced into the experiment, and it is already clear that dose rate has a profound effect, and further that the magnitude of the effect depends on the size of the dose. The tumors and other diseases being observed are quite diverse in the BALB/c mouse, and preliminary estimates indicate that we will be able to construct dose-response curves for solid tumors in addition to the more commonly observed radiation-induced leukemias.

CARCINOGENIC EFFECTIVENESS OF CHRONIC GAMMA RAYS DELIVERED DURING THE PRECONCEPTION AND FETAL PERIODS

J. M. Yuhas and Judith O. Proctor

A controversy presently exists in human epidemiology as to whether irradiation received during the fetal period, and in some cases the preconception period, is highly carcinogenic. Retrospective analyses of children exposed as a by-product of their mothers' radiologic examination have indicated a 40% increase in the cancer risk per rad, while similar analyses conducted in samples of children who were exposed as fetuses at the time of Hiroshima and Nagasaki have failed to demonstrate an elevation in the leukemia risk.

It would appear, therefore, that resolution of these inconsistencies might come from experimental animal data, which would either confirm or refute the very high sensitivity of the fetus to the carcinogenic effects of radiation. In order to reduce the selective forces that are brought into play when animals are exposed on a specific day during gestation, we have exposed mating pairs of BALB/c mice to 8 r/day until the litter is born. Each litter receives the same exposure during fetal life and also a variable amount of preconception exposure. Both irradiated and unirradiated males and females are being followed throughout life for cancer and leukemia incidence. Periodic testing for immunologic competence and other functional characteristics is also being conducted.

To date the mortality in the groups that received radiation during the fetal period is actually less than that of the controls. No specific causes of death can be attributed to those that are dying, and presumably the deaths are associated with infectious processes that select out the weaker members of the population. Since radiation during the fetal period has exerted its selective force prior to this time, it might be expected that the general fitness of the irradiated populations would be better. Immunologic competence, as estimated by response to sheep red blood cells, is slightly greater in the irradiated populations.

THE ROLE OF IMMUNOSUPPRESSION IN RADIATION LEUKEMOGENESIS: PREDICTION OF A NONLINEAR DOSE-RESPONSE CURVE*

J. M. Yuhas, R. W. Tennant,
M. G. Hanna, Jr., and N. K. Clapp

We reported previously (1) that RF mice which died with glomerulosclerosis demonstrated only one-half the

leukemia risk expected of them, and further that the presence of this kidney lesion had no apparent effects on the risk of nonlymphatic tumors. We have now shown that glomerulosclerosis is the by-product of a humoral immune response directed against the antigens induced on the cell surface by murine leukemia virus. In brief, animals that can mount this immune response suffer a reduced leukemia risk but do manifest the kidney lesion.

This observation offers the possibility of testing the role of immunosuppression in radiation leukemogenesis in the intact mouse. We have investigated the pathology records of more than 1500 RF mice exposed to graded doses of 60-MeV protons or 300-kVp X-rays. Essentially three questions were posed: (1) Is the efficiency of this immune response, as reflected by the development of glomerulosclerosis, independent of radiation dose? (2) Does the response affect radiation-induced leukemias as well as those which occur spontaneously? (3) Is glomerulosclerosis development inhibited by radiation? Both of the first two questions gave positive answers; i.e., the efficiency of the response is independent of radiation dose, and the radiation-induced leukemias are susceptible to the effects of the response. Figure 1 is a plot of the expected vs. observed glomerulosclerosis patterns in RF mice as a function of

radiation dose. The expected values decrease with increasing radiation dose, since the heavily irradiated animals do not live as long, although the expected and observed values coincide through doses as high as 100 rads. The development of glomerulosclerosis is inhibited at all higher doses.

This indicates that at doses above 100 rads the development of a systemic response that serves to reduce the leukemia risk is inhibited. Therefore, radiation leukemogenesis in the low dose range (through 100 rads) results largely from the induction of malignant transformations, while in the higher dose range not only this factor but a reduction in the ability to react to these transformations contributes to the increased leukemia risk. Therefore, the dose-response curve for radiation induction of leukemia cannot be linear.

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

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AGE-DEPENDENT CHANGES IN IMMUNOLOGIC REACTIVITY AND THEIR ROLE IN DETERMINING DISEASE ONSET AND LONGEVITY

J. M. Yuhas, Judith O. Proctor, and N. H. Pazmino*

It has been proposed that the immunologic system is involved causally in the process of aging in two ways: (1) The accumulation of errors in the ability of the system to recognize "self" and "nonself" leads to autoimmune diseases. (2) The overall degeneration of the system fails to recognize the antigenically foreign nature of infectious agents and tumors, and these then lead to the death of the host. Prior reports from this laboratory (1) have demonstrated that immunologic reactivity does decay with age in the BC3F₁ mouse; further, the presence of certain diseases is associated with an even more profound reduction. It is difficult, however, to conclude from these data that the reduction in immunologic reactivity allowed the pathological conditions to develop, since it is equally possible that the appearance of the pathological condition compromised the ability of the immune system to respond.

In order to resolve this difficulty, we are assaying the ability of mice of four genotypes to respond to a standard test antigen as a function of age, and comparing the age-dependent changes in reactivity to the histopathology of the test animals. These four genotypes (RFM, BALB/c, BC3F₁, and CD2F₁) differ in

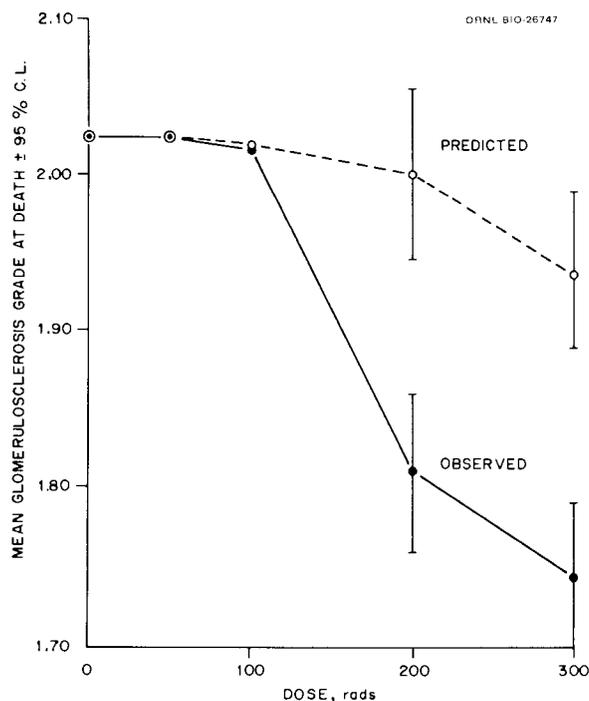


Fig. 1. Severity of glomerulosclerosis at death as a function of radiation dose.

average life-span, tumor incidence, and the ages at which certain diseases appear. If, for example, tumors of the pituitary appear in one genotype only after the immunologic reactivity has declined, it might be proposed that immunologic deficits were responsible for this tumor. Demonstration of the appearance of similar tumors in another genotype at an age characterized by adequate immunologic reactivity would make this interpretation unlikely. Negative evidence, i.e. appearance of a tumor in many genotypes only after immunologic reactivity declines, would not establish the causal relation but would provide a broader base of support for the proposal. In order to take into account the possibility that negative evidence will be observed, we are also testing age-dependent reductions in the ability of these mice to lose actively acquired tumor and viral immunity.

As might be expected from the diversity of responses obtained from different genotypes in other test systems, no single pattern can be used to describe the age-dependent changes in immunologic reactivity for all of the genotypes studied. Further, the rate at which the reactivity declines is not predictive of the average life-span of the animals. This is undoubtedly related to the fact that the causes of death in mice vary strongly as a function of genotype. While the data on the diseases of these mice are still being accumulated, it is clear that in at least one case the disease precedes the decline in immune competence. During the early stages of reticulum cell sarcoma in the RFM mouse, the immunologic reactivity, as assayed by response to sheep red blood cell antigens, is actually elevated relative to that in mice of similar age without this tumor. This does not disprove the possibility of causal relationships but indicates that the problem is much more complex than originally proposed.

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PHARMACOLOGIC EFFECTS OF PHOSPHOROTHIOATE RADIOPROTECTIVE DRUGS: THEIR IMPORTANCE IN TOXICITY AND RADIOPROTECTION

J. M. Yuhas, Judith O. Proctor, and L. H. Smith

Within the most efficient class of radioprotective drugs presently available, the phosphorothioates, there

are wide variations in protective effectiveness that are difficult to relate to the primary structures of the drugs. Similarly, analyses of the distribution and chemical forms of the drugs have failed to account for this variation. Table I gives data for a series of drugs which present this problem. The prototype drug for this series is mercaptoethylamine (MEA); phosphorylation of the sulfhydryl yields WR-638, and all of the others are aminoalkyl derivatives (N-substitutions). Within the aminoalkylaminoethyl series at least, toxicity increases and reaches a maximum when the alkyl group contains at least four carbons. No such relation for protection is obvious, however, since all of the phosphorothioates increase the resistance of the mouse to radiation by a factor of 2, with the exception of WR-2721, which increases resistance by a factor of 2.7. Protection expressed in these terms does not take into account the variations in the amount of sulfur injected in each test dose, so a more meaningful estimate of the protection can be obtained by comparing the observed protection to the amount of sulfur contained in each test dose. Table II shows that increase in resistance per unit of sulfur injected is far superior for the aminoalkylaminoethylphosphorothioates that contain at least three carbons in their alkyl chain. Similarly, these same agents can induce a profound vasodilatation, as estimated by the amount of blood that pools in the spleen by 1 hr after injection. In order to determine whether this vasodilatation accounted for the superior protection these drugs offer, we selected the most efficient one, WR-2721, and tested its radioprotective abilities in splenectomized and/or hypoxic mice. The rationale for these studies was that if pooling of blood in the spleen was inducing peripheral hypoxia, then mice without a spleen would not be protected as efficiently as normal ones, and in the intact animal hypoxia and WR-2721 would show less additivity of protection than in splenectomized animals.

Table III shows the results of these studies. Splenectomy itself had little effect on the radiation resistance of the BC3F₁ mouse, but normal mice are much more responsive to the protective influences of the drug. Both types of mice respond similarly to hypoxia. If the difference between the normal and splenectomized mice in terms of their ability to be protected by WR-2721 was that the latter did not show as great a peripheral hypoxia component following WR-2721 injection, then addition of hypoxia to drug-treated splenectomized mice should generate a larger increase in resistance than would similar treatment of normal drug-treated mice. This has in fact been observed (Table III) and normal and splenectomized mice

TABLE I. Toxicity, radioprotective effectiveness, and pharmacologic effects of selected phosphorothioates

Drug	Formula	Toxic LD ₅₀ (mg/kg)	Test dose (mg/kg)	LD _{50/30} (r ± S.E.)		DRF*	Spleen weight† (mg)	Change in spleen weight‡ (mg)
				Treated	Untreated			
WR-638	NH ₂ (CH ₂) ₂ SH ₂ PO ₃	777 (700–864)§	500	1520 ± 16	734 ± 8	2.07	101 ± 2.8	+4.3
WR-2578	NH ₂ (CH ₂) ₂ NH(CH ₂) ₂ SH ₂ PO ₃	1300 (1233–1445)§	900	1401 ± 24	720 ± 8	1.95	103 ± 2.1	+6.5
WR-2721	NH ₂ (CH ₂) ₃ NH(CH ₂) ₂ SH ₂ PO ₃	704 (665–745)§	500	2030 ± 37	747 ± 6	2.72	128 ± 5.7	+31.0
WR-2822	NH ₂ (CH ₂) ₄ NH(CH ₂) ₂ SH ₂ PO ₃	292 (265–321)§	175	1275 ± 32	738 ± 12	1.73	115 ± 2.8	+18.2
WR-2823	NH ₂ (CH ₂) ₅ NH(CH ₂) ₂ SH ₂ PO ₃	345 (313–381)§	200	1473 ± 47	750 ± 12	1.96	127 ± 3.7	+30.2
WR-2824	NH ₂ (CH ₂) ₆ NH(CH ₂) ₂ SH ₂ PO ₃	343 (304–386)§	200	1550 ± 27	759 ± 18	2.04	117 ± 1.4	+20.2

*Dose reduction factor or ratio of treated and untreated LD_{50/30} values.

†Spleen weight of BALB/c mice 1 hr after injection of the test dose.

‡Difference between spleen weights of control (96.8 ± 2.3) and drug-treated mice.

§95% confidence limits.

TABLE II. Role of the sulfur contained in the test dose of selected phosphorothioates in determining radioprotection

Drug	Test dose (mg)	Percent sulfur	Sulfur per mg	Resistance increase (%)	Increase per mg sulfur
WR-638	12.5	17.8	2.225	107	48
WR-2578	22.5	14.1	3.175	95	29.9
WR-2721	12.5	13.3	1.675	172	103
WR-2822	4.375	12.5	.550	73	133
WR-2823	5	11.9	.60	96	160
WR-2824	5	11.3	.575	104	181

TABLE III. LD_{50/30} values for BC3F₁ mice that were splenectomized, given 5% oxygen during exposure, and/or 500 mg/kg of WR-2721 1 hr prior to exposure*

Experiment	SP-X†	5% O ₂	WR-2721	LD _{50/30} ± S.E. (r)
A	–	–	–	784 ± 13
	+	–	–	778 ± 14
B	–	–	+	2002 ± 50
	+	–	+	1638 ± 41
C	–	+	–	1579 ± 31
	+	+	–	1567 ± 47
D	–	+	+	2208 ± 42
	+	+	+	2119 ± 42

*+, treatment given; –, treatment not given.

†SP-X, splenectomy 4 weeks before irradiation.

given both treatments show almost identical resistances, indicating that hypoxia has replaced that component of the mechanism of action of WR-2721 which was removed by splenectomy.

We conclude, therefore, that the variation in protective efficiency among the phosphorothioates results from two factors: the amount of sulfur that can be safely injected into the animal in the respective chemical forms, and the presence or absence of a pharmacological reaction in injected animals that serves to decrease peripheral oxygen tensions.

RADIOTHERAPY OF EXPERIMENTAL LUNG TUMORS IN THE PRESENCE AND ABSENCE OF A RADIOPROTECTIVE DRUG, S-2-[3-AMINOPROPYLAMINO]ETHYL-PHOSPHOROTHIOIC ACID (WR-2721)

John M. Yuhas, Anita E. Walker,
S. F. Echols,* and Mildred G. Hayes

Our studies on the applicability of WR-2721 to the radiotherapy of solid tumors has continued. In the urethane-induced lung adenoma system the following points have been established. (1) Injection of WR-2721 prior to localized lung exposures increases the resistance of the mice to skin injury and lethality by a factor of 2, whether the exposures are given singly or as a series of weekly fractions. (2) In the low therapeutic dose range (up to 3000 r), WR-2721 offers no protection to the lung tumors themselves. (3) Injection of the drug prior to each of a series of weekly exposures does not protect the tumor. (4) The drug is able to protect the lung tumors if the time between injection and the completion of exposure is grossly prolonged (1–2 hr).

This last point, that given sufficient time the drug could protect the tumor, indicates that the tumor in question does not differ qualitatively from the normal tissues but simply requires longer to respond to the drug. In order to establish that the protection observed was indeed the result of a time dependency, we compared the protection the drug offered these lung tumors when exposure was started 15 or 90 min after injection. As expected, mice that received the drug 90 min before exposure showed not only more but larger tumors than the mice which received no drug or drug treatment 15 min before exposure.

While this time factor would not affect the applicability of WR-2721 to the clinical radiotherapy of solid tumors, we investigated the basis of this time dependency in order to gain better understanding of the basis of the differential protection that this drug offers to normal and tumor tissues. In collaboration with Drs. G. Kollman and B. Sharpiron of the Albert Einstein Medical Center, we are investigating both the amount

and the chemical form of the drug found in normal tissues and in the lung tumors as a function of time after injection. The data obtained to date indicate that the tumors absorb the drug very slowly relative to a series of normal tissues, that the drug is maintained within the tumor in a nonprotective form (either the parent compound or an oxidized derivative), and that the compound is not metabolized by the tumor as rapidly as it is in the normal tissues. A second point is being investigated as a possible explanation of the time dependency for the tumor response to chemoprotection. WR-2721 is able to induce peripheral vasodilatation (1), which reaches its maximum development 1 hr after drug injection. Figure 1 describes the increase in spleen weight due to the accumulation of blood as a function of time following WR-2721 injection. Since the lung adenomas are very small and presumably well oxygenated, it is entirely possible that the induction of systemic hypoxia could protect them at these prolonged times.

In order to stimulate as closely as possible the clinical situation, we are currently investigating the relative protection that WR-2721 offers lung tumors and normal tissues when the exposures are given daily for 10 days.

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1. J. M. Yuhas, J. O. Proctor, and L. H. Smith, this report, p. 153.

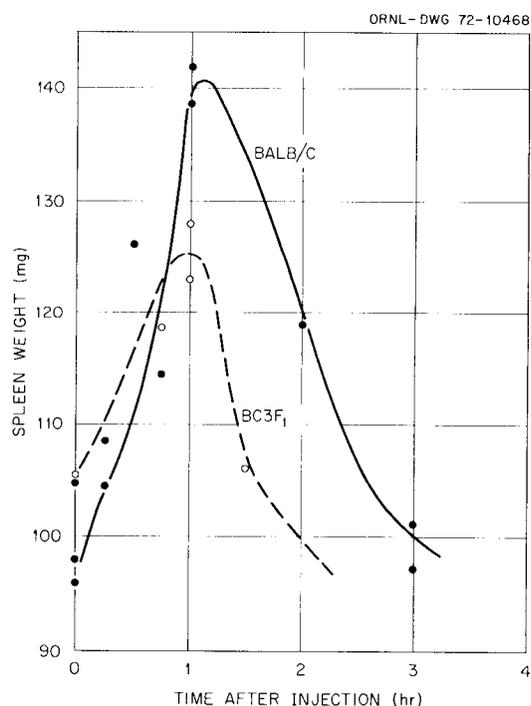


Fig. 1. Change in spleen weight with time after injection of WR-2721.

CARCINOGENESIS PROGRAM*

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Respiratory Carcinogenesis

Paul Nettesheim
T. H. Corbett
Hans Schreiber^b

Mechanisms of Chemical Carcinogenesis

William Lijinsky

Mutagenicity of Carcinogens

F. J. de Serres
E. H. Y. Chu
H. V. Malling
Tong-man Ong^a

Somatic Cell Genetics

E. H. Y. Chu

Repair Mechanisms in Carcinogenesis

R. B. Setlow
J. S. Cook
James 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

F. T. Kenney
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Biochemistry of Viral Carcinogenesis

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RNA Tumor Virus-Developmental Processes

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INHALATION COCARCINOGENESIS STUDIES WITH SYNTHETIC SMOG AND FERRIC OXIDE

Paul Nettesheim and D. A. Creasia

The primary purpose of this study is to determine whether chronic inhalation exposure to Fe_2O_3 and/or synthetic smog increases the respiratory tract tumor response of hamsters to low doses of diethylnitrosamine (DEN) given subcutaneously. The study involved a total of 1348 hamsters; details of the experimental design were reported previously. The experiment has been terminated and approximately 60% of all animals involved in the study have been evaluated histopathologically to date. Some of the preliminary findings are summarized in Table I. The data suggest an enhancement of the DEN-induced tumor response in the distal segment of the bronchial tree and the lung parenchyma, caused by Fe_2O_3 inhalation. There was no effect on the laryngeal and tracheal tumor response. Tumors in the nasal cavity appear to be less frequent in the Fe_2O_3 -exposed hamsters than in the controls exposed to filtered air. Exposure to synthetic smog did not affect the respiratory tract tumor response to DEN. These preliminary findings appear to support the results of other investigators who found that the DEN-induced lung tumor response is enhanced by massive intratracheal injections of Fe_2O_3 .

TABLE I. DEN-induced tumors in hamsters exposed to synthetic smog and Fe_2O_3 particles*

	Nasal cavity	Larynx + Trachea	Bronchus + Lung
Air	13	65 (116)	13 (14)
Smog	11	65 (118)	11 (11)
Fe_2O_3	5	62 (91)	22 (34)

*120 animals have been evaluated histologically in each group. Numbers indicate number of tumor-bearing animals, numbers in parentheses indicate total number of tumors found.

INHALATION COCARCINOGENESIS STUDIES WITH NITROGEN DIOXIDE, PARAFORMALDEHYDE, AND ACROLEIN

Paul Nettesheim, D. A. Creasia, and J. C. Kim

A new series of chronic inhalation studies are being initiated with reactive gases such as nitrogen dioxide,

paraformaldehyde, and acrolein, which are common air pollutants and also gas phase components of tobacco smoke. The major objectives of these experiments are (1) to establish an experimental model which can be used to test different air contaminants for cocarcinogenic activity in the respiratory tract, (2) to determine whether certain classical gaseous respiratory tract irritants exhibit cocarcinogenic activity, and (3) to determine whether multiple events can be identified in the development of respiratory tract neoplasia, similar to multiple stage carcinogenesis of the skin.

Three different carcinogen-animal systems are being used in these studies; carcinogens are applied by intratracheal injection: (1) Benzpyrene (BaP) in gelatin is given to hamsters in 15 large, weekly doses (5 mg each). This is known to produce no, or only very few, respiratory tract tumors. (2) BaP plus Fe_2O_3 is given to hamsters in 15 small, weekly doses (1 mg each). This combination has been shown to produce a definite but small tumor response in 10–20% of the animals. (3) 3-Methylcholanthrene is given to rats in two doses on subsequent days (5 mg each). This has been shown to result in formation of "benign" squamous cell tumors within 6–8 weeks which progress to true neoplasia in 6–8 months.

The following groups, with 100 hamsters each, have been started in recent weeks: (1) 15 × 5 mg BaP plus intermittent NO_2 for life; (2) 15 × 5 mg BaP plus intermittent NO_2 for 15 weeks; (3) 15 × 5 mg BaP, with NO_2 exposure started 4 weeks after last carcinogen injection; and (4) NO_2 exposure only. The entire experimental series will be initiated by spring of 1973.

NITROGEN DIOXIDE AND PARAFORMALDEHYDE-INDUCED CELL PROLIFERATION IN THE RESPIRATORY TRACT OF RATS AND HAMSTERS

D. A. Creasia, J. C. Kim, Elizabeth B. Edwards, and Paul Nettesheim

In our new inhalation cocarcinogenesis study, NO_2 and paraformaldehyde are being tested for their possible cocarcinogenic activity in lung tumor induction with benzpyrene. It was therefore important to determine what kind of histopathological and regenerative changes would occur in various parts of the respiratory tract following exposure to these respiratory irritants. The experiments revealed marked species differences in response to respiratory irritants; e.g., 5 hr exposure to 10 ppm NO_2 causes a marked hyperplasia in the

respiratory bronchiole of hamsters, while in rats 5 hr exposure to 25 ppm NO₂ causes only a very mild hyperplastic response. Electron microscopic studies show that this lesion is largely due to an increase of type II alveolar cells in the alveoli of the respiratory bronchiole. We also found that NO₂ (10 ppm, 5 hr) induces a marked increase in cell proliferation in all segments of the respiratory tract beginning 24–48 hr after exposure, while inhalation of paraformaldehyde (50 ppm, 5 hr) increases cell proliferation only in the upper respiratory tract.

Further studies are under way to investigate the proliferative response of respiratory tract epithelium during multiple inhalation exposures.

RAPID DEVELOPMENT OF BRONCHIOALVEOLAR SQUAMOUS CELL TUMORS IN RATS AFTER INTRATRACHEAL INJECTION OF 3-METHYLCHOLANTHRENE

Hans Schreiber, D. H. Martin,
and Paul Nettesheim

In most available lung cancer models, multiple intratracheal injections of high doses of carcinogens are required for tumor induction, and tumors develop after a long latent period. In this study with specific-pathogen-free F-344 rats, lung tumors were induced within weeks by either single or multiple injections of relatively low carcinogen doses.

Squamous cell tumors of the lung appeared as early as 5 weeks after a single intratracheal injection of 10 mg 3-methylcholanthrene, or 2 weeks after 5 weekly injections of 5 mg of the carcinogen. The bronchioalveolar origin of these tumors was determined on early stages of tumor development. At first these tumors had a regular, well-differentiated histological appearance. Later a loss of differentiation and marked cellular atypia was noted, and finally metastases occurred. Early, well-differentiated tumors, when transplanted, did not grow immediately after transplantation; at 5–8 months, however, rapidly growing squamous cell carcinomas developed at the site of transplantation.

The site of origin and the histogenesis of these squamous cell carcinomas are certainly different from those of bronchogenic carcinoma in man. Nevertheless the early development of macroscopic precursors in this tumor system offers an interesting model for therapeutic trials, and for studying the transition from "benign" prestates to malignancy.

EXFOLIATIVE CYTOLOGY DURING DEVELOPMENT OF BRONCHOGENIC CARCINOMA IN HAMSTERS

Hans Schreiber, G. Saccomanno,* D. H. Martin,
and Paul Nettesheim

The difficulty in diagnosing human lung cancer in its preinvasive stage is regarded as the main reason for the very high mortality from this disease. Exfoliative pulmonary cytology is to date the most promising of all diagnostic procedures. However, the reliability of this technique is still hampered by difficulties in separating premalignant and malignant changes from changes caused by concurrent respiratory infections or by simultaneous exposure to toxic inhalants, e.g. cigarette smoke. An experimental animal system permits one to study cytological and histopathological responses to a single factor without interference from others. The following experiment was designed to study the sequence of cytological events during the development of experimental bronchogenic carcinoma.

Hamsters received repeated intratracheal instillation of 3,4-benzpyrene and Fe₂O₃ at cumulative doses of 10, 25, and 45 mg of carcinogen, providing a pool for serial killing and life-span studies. Cytological specimens were obtained by a recently described method (*1*) at 3- to 4-week intervals. Severe cytological response to the carcinogen was found during and immediately after the intratracheal instillation. This early toxic reaction regressed within a month, and metaplastic cells with gradually increasing signs of atypia appeared. Three degrees of atypia (mild, moderate, and marked) were distinguished. Exfoliated cells conclusive for malignancy were found in all animals 1 month or longer before death. The cytological findings usually correlated well with the specific tumor types that developed in an animal as determined at autopsy.

The histological and cytological material of the hamsters was compared to histopathological specimens and sputum cytology smears obtained from heavy smokers and uranium miners. Striking similarities in the sequential morphological changes leading to bronchogenic carcinoma were found. The response of hamsters to infectious and various toxic agents is now being explored.

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Í. H. Schreiber and P. Nettesheim, *Cancer Res.* 32: 737–745 (1972).

COLONY FORMING ASSAY WITH A SQUAMOUS CELL CARCINOMA DERIVED FROM MOUSE LUNG

Mary L. Williams and Paul Nettesheim

To evaluate the success of therapy for lung cancer and to determine the effect of host factors on lung cancer growth, a tumor transplant system which has the following characteristics is needed: (1) the transplantable tumor should be derived from a respiratory tract cancer and should maintain its state of differentiation on transplantation, (2) it should be possible to transplant the tumor to the lung, and (3) the relative size of the tumor stem cell pool should be measurable.

We recently described the induction of squamous cell carcinomas in the respiratory tract of mice. From one

such carcinoma a large tumor cell pool was established and stored in liquid nitrogen. It was found that on intravenous injection of these tumor cells into isogenic hosts, squamous cell tumors develop within 2–3 weeks; these tumors are found only in the lungs of the recipients. Figure 1 shows the incidence of lung tumor nodules as a function of time and injected cell dose. The tumor system appears to fulfill the criteria described above.

Studies are under way to determine the “plating efficiency” changes in cell proliferation and tumor stem cell pool as a function of tumor growth.

SEPARATION OF MULTIPLE FORMS OF PROTEIN-BOUND METABOLITES OF CARCINOGENIC HYDROCARBONS FROM SEVERAL RODENT SPECIES

T. H. Corbett and Paul Nettesheim

Our findings demonstrate that metabolites of the carcinogenic hydrocarbons 9,10-dimethyl-1,2-benzanthracene (DMBA) and 3-methyl cholanthrene (3-MCA) bind to skin and lung tissue proteins in multiple forms. These metabolites are freed from the proteins by treatment with Raney nickel. Since Raney nickel selectively cleaves carbon-sulfur bonds, the metabolites are most likely bound through cysteine or homocysteine in tissue proteins. The numbers and the relative quantities of the bound metabolites vary greatly between the Fisher rat, the Syrian golden hamster, and three mouse strains. It is possible that the metabolites (used as an indication of a particular pathway of metabolic activation) correlate with species susceptibility to hydrocarbon carcinogenesis for the tissues studied. We found that 3-MCA is eliminated more rapidly from BALB/c mouse lung tissue than from the skin tissue, and that lung tissue enzymes activate a smaller percentage of the hydrocarbon to tissue-bound forms than the skin tissue enzymes. These two factors could account for most of the differences in tumor susceptibility to tumor induction between these tissues. Both lung and skin tissues of the BALB/c mouse metabolized the hydrocarbon DMBA to the same protein-bound metabolites. There were no significant differences in the elimination and gross tissue binding of 3-MCA in the lung tissues of the rat, hamster, and three mouse strains, although large differences exist in their relative lung tumor susceptibilities.

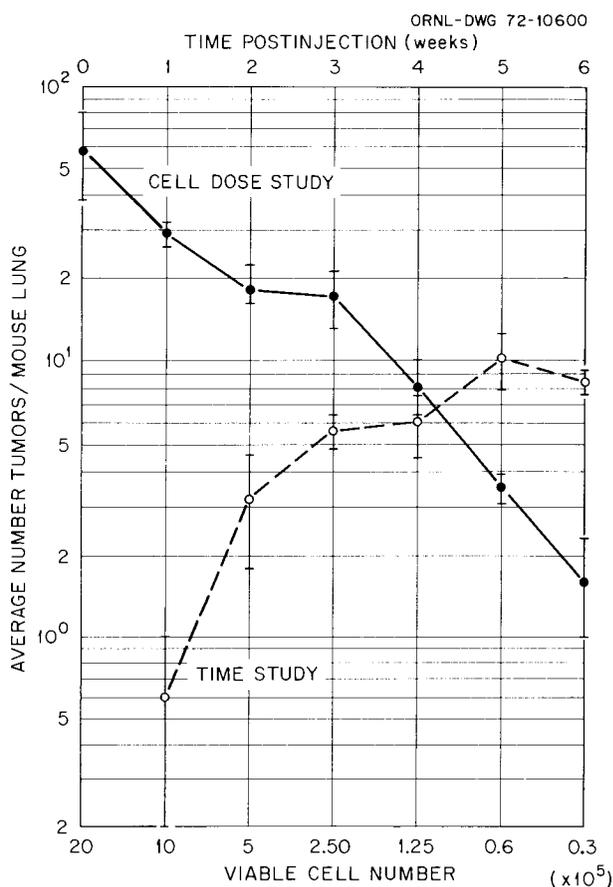


Fig. 1. Tumor incidence as a function of time and number of transplanted tumor cells.

For the time study, 10^5 viable tumor cells were injected. In the cell dose study, recipients were killed 3 weeks after transplantation (5–10 animals per point).

THE EFFECT OF PR8 VIRUS RESPIRATORY INFECTION ON THE BENZPYRENE HYDROXYLASE ACTIVITY IN BALB/c MICE

T. H. Corbett and Paul Nettesheim

The possible role of respiratory infections of viral or bacterial etiology in the pathogenesis of lung cancer has been a subject of debate for many years. In experimental lung tumor models, respiratory infections have been shown to either increase or decrease the tumor response, depending on the type of infectious agent and the type of carcinogen.

Since many chemical carcinogens, in order to react with tissue constituents and manifest their carcinogenic potential, must be enzymatically activated (converted to an electrophile), a change in the activation (or degradation) of the chemical carcinogen might at least in part explain the alteration of the tumor response. This work concerns efforts to detect virally induced changes in the metabolism of a carcinogenic hydrocarbon, benzpyrene (BaP), by measuring the formation of the metabolite, 3-hydroxy benzo(a)pyrene.

The experiments showed that PR8 virus infection in the respiratory tract of BALB/c mice decreases the benzpyrene hydroxylase activity in the lung tissue to 10% of control levels. This suppression was not due to an inhibitor substance in the infected tissue, since mixes of various quantities of the infected and uninfected homogenates were simply additive. The fact that the BaP hydroxylase activity in the liver was not affected by the respiratory disease indicates that the reduction of the enzyme activity is not due to the general debilitation of the animals' health during the acute stage of pneumonia. Neither is the loss of activity likely

TABLE I. Distribution of radioactivity in nose breathing and cannulated hamsters exposed to labeled cigarette smoke

Experiment number	Percent of total radioactivity detected*		
	Head	Trachea and lung	Intestinal tract
<i>Non-cannulated</i>			
23	51.2	39.6	9.2
24A	39.3	53.1	7.6
24B	28.1	56.4	15.5
<i>Cannulated</i>			
23	0.0	95.1	4.9
24A	5.9	89.1	5.0
24B	0.0	93.1	6.9

*Numbers are the means from 5 animals, exposed to ¹⁴C-dotriconcane for 30 sec.

to be caused simply by tissue destruction, since enzyme induction studies showed no differences between infected and uninfected animals after high doses of inducer (2000 µg 3-MCA); the virus infection inhibits induction of the enzyme only with low doses (20 µg 3-MCA/animal).

The marked suppression of benzpyrene hydroxylase activity in lungs of mice suffering from influenza virus pneumonia suggests that respiratory tract infections, such as the one examined, may profoundly affect the metabolism of chemical carcinogens.

MAXIMIZATION OF SMOKE PARTICLE DEPOSITION BY USE OF A NOSE BYPASS DEVICE

J. Kendrick, D. A. Creasia, Anna S. Hammons,
W. L. Maddox,* M. R. Guerin,* and Paul Nettesheim

A number of investigators have postulated that one of the major reasons for the failure to induce lung cancer in laboratory rodents by tobacco smoke is the filtration in the nasal cavities of these obligatory nose breathers. This explanation has been recently contested by experiments suggesting that only a minor fraction of smoke particles is trapped in the nasal passages of hamsters and similar laboratory species.

Experiments were carried out to determine (1) the relative amount of smoke particles trapped in the noses of hamsters exposed to radioactive smoke, and (2) the effect of an intratracheal cannula on smoke particle deposition.

The most recent results from these studies are summarized in Table I. The data indicate that a considerable amount (30–40%) of smoke particles is deposited in the nasal passages and that bypassing the nose with a cannula inserted through the larynx into the trachea considerably increases particle deposition in the lower respiratory tract.

Studies are presently under way to determine the effect of nose filtration on various gas phase components, and to test various nose bypass devices for chronic smoke inhalation studies.

*Analytical Chemistry Division.

STUDIES ON THE REDUCTION OF CIGARETTE SMOKE TOXICITY IN HAMSTERS

D. A. Creasia, Anna S. Hammons, W. L. Maddox,*
M. R. Guerin,* and Paul Nettesheim

One of the major complications in tobacco smoke carcinogenesis studies is the high susceptibility of most

laboratory animals to the acute toxic effects of tobacco smoke. One of the major toxic compounds is carbon monoxide (CO-Hb formation). Experiments were carried out in an attempt to reduce carbon monoxide poisoning. Hamsters were exposed for various lengths of time to either a 0.1% CO-atmosphere (mainstream smoke contains approximately 0.1% CO) or to tobacco smoke. The buildup of CO-Hb in the blood during exposure and the clearance of CO from the blood stream after cessation of exposure were determined by standard techniques. An exposure of 50 min to 0.1% CO in air resulted in a CO-Hb level of 40%; a 50-min exposure to the smoke of 10 cigarettes using the ORNL smoking apparatus resulted in 47% CO-Hb levels.

When hamsters with this CO-Hb concentration were allowed to breathe clean air, the CO-Hb was found to drop to 30% in 30 min and to 5% in 150 min. If the animals breathed 100% O₂ instead, the CO-Hb dropped to 10% in 30 min and to 5% within 50 min.

After completion of these studies, subchronic smoke-inhalation experiments were initiated with and without O₂ therapy between every third cigarette. Hamsters are presently being exposed to 8 (no O₂) or 10 (with O₂) cigarettes per day.

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IN VITRO TESTS OF THE MUTAGENICITY OF PRECARCINOGENS, PROXIMATE AND ULTIMATE CARCINOGENS

Tong-man Ong*

In addition to the mutagenicity tests of the chemical carcinogens reported in our progress report of last year (1), the mutagenicity of 1-naphthylamine (1-AN), 2-naphthylamine (2-AN), 1-naphthylhydroxylamine (*N*-hydroxy-1-AN), 2-naphthylhydroxylamine (*N*-hydroxy-2-AN), 1,2,3,4-diepoxy-cyclohexane (1,2,3,4-DECH), aflatoxin B₂, aflatoxin G₂, hycanthone acetylaminofluorene (AAF₁) and *N*-ethyl-nitrosourea were tested in the *ad-3* system of *Neurospora crassa*.

1. *Naphthylamines*: 1- and 2-AN were not mutagenic in *N. crassa* when conidia were treated (2). The mutagenicity of these two compounds was further tested in the vegetative cultures of *N. crassa*. The results show that 2-AN is mutagenic. There is about a 70-fold increase in mutation frequency over the spontaneous mutation frequency after the vegetative cultures of *N. crassa* were treated with 0.4 mM of 2-AN. The mutation

frequency, however, was only slightly increased after the vegetative cultures were treated with similar concentrations of 1-AN. The mutagenicity of the hydroxylated compounds, *N*-hydroxy-1-AN and *N*-hydroxy-2-AN (presumptive active metabolites of 1- and 2-AN) was also tested. The results indicate that both compounds are mutagenic in *N. crassa* when conidia were treated, *N*-hydroxy-1-AN being more mutagenic than *N*-hydroxy-2-AN.

2. *1,2,3,4-diepoxy-cyclohexane*: The mutagenicity of 1,2,3,4-DECH (a noncarcinogenic compound which is related to the carcinogenic compounds 1,2,4,5-diepoxy-pentane and 1,2,7,8-diepoxyoctane) was also tested. The results show that 1,2,3,4-DECH is not mutagenic under the same experimental conditions in which both carcinogenic compounds were found to be mutagenic.

3. *Aflatoxins*: Aflatoxin B₂ probably is a very weak carcinogen. Aflatoxin G₂ is not carcinogenic. The mutagenicity of both compounds was tested in vegetative cultures of *N. crassa*. The results show that neither compound gave a mutation frequency significantly different from control experiments.

4. *The acetylaminofluorene (AAF) compounds*: Mutagenicity tests were made with the *ad-3* test system in *Neurospora* on the glucuronide of *N*-hydroxy-AAF, but even after extensive tests this compound was found to be nonmutagenic. Samples of the *ad-3* mutants which were obtained previously, after treatment with acetyl-*N*-hydroxy-AAF, have been characterized with respect to their genotype and complementation pattern. *N*-acetoxy-AAF is the only compound known to alkylate the 8-carbon position of guanine in DNA. It will therefore be extremely exciting to characterize these mutants at the molecular level. Preliminary analysis of the acetyl-*N*-hydroxy-AAF-induced *ad-3* mutations has shown that most mutants resulted from point mutations and that extremely few chromosome breaks were induced. The genetic characterization of the *N*-hydroxy-AAF- and acetyl-*N*-hydroxy-AAF-induced *ad-3* mutants has been completed.

5. *Mutagenicity of ultimate carcinogens or strong carcinogens which do not need activation*: Mutagenicity tests were made with the *ad-3* test system in *Neurospora* on *N*-ethyl-nitrosourea. This compound was highly mutagenic. Samples of mutants have been isolated. The genetic characterization of these mutants has been completed. This compound is one in a series of nitrosamides and nitrosamines which are in the process of being tested for their mutagenic specificity to investigate if certain general characteristics can be found for these compounds, which might lead to understanding of their high carcinogenicity.

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1. *Biol. Div. Ann. Progr. Rept. June 30, 1971*, ORNL 4740, pp. 133-134.
2. *Ibid.*, p. 133.

HOST-MEDIATED ASSAY FOR MUTAGENICITY OF CARCINOGENS

Tong-man Ong* and H. V. Malling*

The idea behind the host-mediated system is to combine the ease by which mutations can be measured in microorganisms with the influence of metabolism in higher organisms. In most host-mediated assays the indicator organism is injected into the peritoneal cavity of the host organism. Indicator organisms in the peritoneal cavity of an animal have a limited access to short-lived mutagenic metabolites in the blood stream of the animal. Injection of the indicator organism into the peritoneal cavity may therefore give false negative results. Exploratory experiments are now in progress to overcome this difficulty by injecting the indicator organism directly into the blood stream of the animal. Preliminary experiments with DMN and DEN have shown that, after incubation of *Neurospora* conidia in the peritoneal cavity or in the liver, mutants were obtained only in the conidia samples obtained from the liver. Samples of such *ad-3* mutants have been isolated and the genetic characterization of these mutants is in progress.

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MUTAGENICITY OF LABILE ULTIMATE CARCINOGENS

Tong-man Ong* and H. V. Malling*

Metabolism of carcinogens by the microsomal hydroxylating system yields highly reactive compounds which often have very short half-lives. Microsomes supplemented with NADPH and other requirements were incubated *in vitro* with carcinogens and indicator cells, and the effect of reactive metabolites on mutagenesis was assayed. A histidine-requiring mutant of *Salmonella typhimurium* was used as indicator organism. The mutants were detected as revertants of the histidine-requirement to wild type. The results showed that DMN was highly mutagenic under the experimental conditions and that DEN was a weaker mutagen than DMN. Microsome preparations from rat liver are considerably less active than similar preparations from

mouse liver in converting DMN and DEN to mutagenic metabolites. Microsome preparations of livers from mice kept on a BHT-containing diet gave approximately twice as many revertants with DMN or DEN as the liver preparations from mice on normal food. Improvements in the experimental conditions have increased the yield of revertants induced by DMN to approximately seven times above previous levels.

It has been suggested that metabolic conversion might be necessary for the expression of the carcinogenicity of aflatoxins. These compounds are not mutagenic in resting conidia, but they are mutagenic in the vegetative cultures of *N. crassa*. Experiments using the microsomal test system, with *S. typhimurium* as testing organism, are in progress to determine whether metabolic conversion of aflatoxins to mutagenic metabolites occurs under these conditions.

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CHARACTERIZATION OF *ad-3* MUTATIONS INDUCED BY CHEMICAL CARCINOGENS

F. J. de Serres*

A major effort will go into the genetic characterization of *ad-3* mutants of *Neurospora* induced in experiments with chemical carcinogens. This characterization will not only make it possible to determine whether carcinogens produce similar spectra of genetic alterations, but will also make it possible to identify the genetic alteration produced by these chemicals which is perhaps responsible for their carcinogenic activity. Such genetic characterization, by a series of genetic tests of the *ad-3* mutants of *N. crassa* induced by ethylenimine, 2-AN, *N*-hydroxy-1-AN, 1-phenyl-3,3-dimethyltriazene, 1-phenyl-3-monomethyltriazene, 4-nitroquinoline 1-oxide, 4-hydroxy aminoquinoline 1-oxide, 1,2,4,5-diepoxyoctane and 1,2,7,8-diepoxyoctane is still in progress. The genetic characterization of triethylene-melamine-, *N*-OH-AAF-, acetyl-*N*-hydroxy-AAF-, and *N*-ethyl-nitrosourea-induced *ad-3* mutants has been completed.

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MECHANISMS OF NITROSAMINE CARCINOGENESIS

W. Lijinsky

The program has consisted of continuing studies of the possible relation of nitrosamine formation from

amines and nitrite to human cancer. Particular attention has been given to the reaction of tertiary amines with nitrite in mildly acid conditions (simulating those in the stomach).

Some model *N*-alkyl derivatives of cyclic amines — pyrrolidine, piperidine, morpholine, and piperazine — were shown to form the *N*-nitroso derivatives of the cyclic amines in appreciable yield, with little or no ring opening, except with *N*-methylpyrrolidine. These findings were of value in predicting the formation of nitrosamines from the many drugs that are *N*-alkyl derivatives of cyclic amines.

Most attention has been given to those tertiary amines and related compounds which might be ingested by man, either intentionally or incidentally. A lengthy study of the formation of dimethylnitrosamine (DMN) from trimethylamine and trimethylamine-*N*-oxide (both of which occur in fish) has shown that the molar ratio of nitrite to amine is much more important in determining the yield of nitrosamine than is the absolute concentration of either amine or nitrite. The yield of DMN in 4 hr at 90° C from 0.5 *M* trimethylamine oxide was 40% of theoretical with 2.0 *M* sodium nitrite, 20% with 1.0 *M* nitrite and 2% with 0.5 *M* nitrite. With 0.05 *M* trimethylamine oxide and 0.2 *M* nitrite the yield of DMN was 22% of the theoretical. The yield of DMN from trimethylamine under similar conditions was consistently about half that from trimethylamine oxide. The yields of DMN were lower at 37° than at 90° C, but at concentrations of 0.05 *M* or 0.01 *M* trimethylamine oxide gave approximately 1% of DMN, while trimethylamine gave much smaller yields. Based on these findings, a feeding study of trimethylamine oxide and sodium nitrite in rats has been started.

The drugs that have been studied include aminopyrine, oxytetracycline, disulfiram, tolazamide, chlorpromazine, chlorpheniramine, dexpropoxyphene, lucanthone, quinacrine, and cyclizine. All have been shown to form the predicted nitrosamine in substantial yield at concentrations of 20 mg/ml in the presence of excess nitrite. The yield of nitrosamine at lower concentrations varied considerably from one compound to another. Aminopyrine was the most reactive compound, and gave 30% yields of DMN at concentrations of drug and nitrite of 20 to 50 parts per million. A study of aminopyrine and nitrite fed to rats at several concentrations is in progress. We hope to demonstrate that the results of purely chemical studies on formation of nitrosamines by reaction of tertiary amines with nitrite can be extrapolated to conditions *in vivo*.

Most recently we have studied the reaction of a variety of dialkylcarbamates and tri- and tetra-alkyl-

ureas with nitrite in mildly acid conditions. Many such compounds are herbicides and pesticides, and could be incidental food contaminants. In every case the formation of dialkylnitrosamine was demonstrated, even when the amino compound was only slightly soluble in water. In some instances the reaction of such a compound could be compared with that of its sulfur containing analog. The effect of the sulfur atom was to increase the formation of nitrosamine by one or two orders of magnitude.

Long-term feeding studies of a number of secondary and tertiary amines of environmental interest, together with nitrite, are in progress.

DEFECTIVE REPAIR OF *N*-ACETOXY-2-ACETYLAMINOFLUORENE-INDUCED LESIONS IN THE DNA OF XERODERMA PIGMENTOSUM CELLS

R. B. Setlow and James D. Regan

Human cells are capable of removing a large fraction of UV-induced lesions in their DNA; more than 50% of the cyclobutane pyrimidine dimers are excised in the first 12 to 24 hr after irradiation. But cells from individuals with xeroderma pigmentosum (XP) are incapable of excising dimers, presumably because they lack a functional UV endonuclease, the enzyme that initiates the repair process by nicking the DNA next to a dimer. In view of the reactivity of *N*-acetoxy-AAF with DNA, and since treatment with this carcinogen stimulates unscheduled DNA synthesis in human lymphocytes, we examined repair events in the DNA of human cells after treatment with this agent. As an assay we employed a technique that utilizes the photolysis of BrdUrd incorporated during repair to yield an estimate of the number of repaired regions in the cellular DNA and to indicate the average size of the repaired regions (1). On exposure to 313 nm, the BrUra-containing repaired regions are broken and analysis of the DNA by alkaline sedimentation gives the numbers of breaks. The observed relation between 313 nm fluence and breaks gives an estimate of the numbers and sizes of the repaired regions. Our results indicate that extensive repair occurs in the DNA of normal human cells exposed to *N*-acetoxy-AAF, whereas there is a much lower level of repair in XP cells.

Figure 1 summarizes data from a number of experiments on normal and XP cells treated with *N*-acetoxy-AAF. The data are expressed as differences between the reciprocals of the weight-average molecular weights ($1/M_w$) of cells incubated in BrdUrd and cells incubated

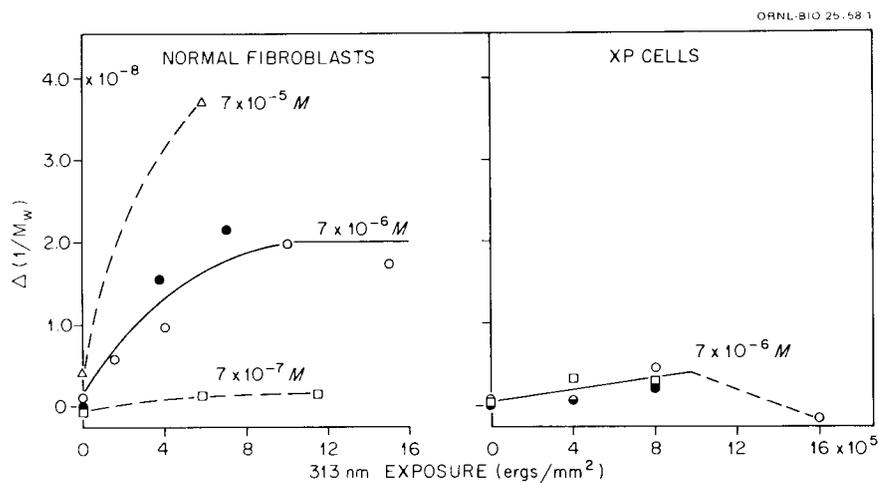


Fig. 1. The effect of 313-nm exposure on cells treated with the indicated concentrations of N-acetoxy-AAF and permitted to repair for 20 hr.

The difference $[(1/M_w)_{\text{BrdUrd}} - (1/M_w)_{\text{dThd}} \equiv \Delta(1/M_w)]$ is a measure of the number of breaks in repaired regions resulting from 313-nm exposure. The different symbols for normal cells represent separate experiments, those for XP cells represent different cell lines.

in dThd (controls). In normal cells, 313-nm irradiation results in large changes in $1/M_w$. In XP cells, however, the changes are minimal.

The curve for normal fibroblasts exposed to 7×10^{-6} M N-acetoxy-AAF is similar to that after exposure to 50 ergs/mm² of 254 nm and indicates that the average repaired region contains ~20–30 BrUra residues and that the regions are spaced $\sim 8 \times 10^6$ daltons apart. These data indicate that fibroblasts and tumor cells from individuals with XP are defective not only in the repair of UV damage to DNA but in the repair of some carcinogen-induced base damage as well. One would expect such cells to be very sensitive to a wide class of chemical carcinogens. The data reinforce the notion that there is a close — perhaps causal — connection among carcinogens, damage to DNA, and the repair of damaged DNA.

The following report summarizes our findings for a number of carcinogens and mutagens.

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CLASSIFICATION OF CHEMICAL CARCINOGENS AND MUTAGENS BASED ON THE FORM OF DNA REPAIR THEY INDUCE

James D. Regan and R. B. Setlow

Using the 5-bromodeoxyuridine photolysis method we have examined a number of chemical agents, including carcinogens and mutagens, with regard to the

type of repair sequence they generate. We find that, among the chemicals thus far investigated, two forms of repair events seem to occur. Some agents induce an ionizing radiation-type repair sequence (short-lived strand breaks with no extensive excision) and some induce a UV-type repair sequence (extensive excision and replacement of bases over a protracted time period). These results are presented in summary form in Table 1.

Of particular interest is the finding that agents which induce the UV type of repair in *normal* human cells cause lesions which go essentially unrepaired in cells from the UV-sensitive disease, xeroderma pigmentosum.

ABSOLUTE ULTRAVIOLET PHOTSENSITIVITIES OF DNA-CONTAINING MAMMALIAN VIRUSES WITH SPECIAL REFERENCE TO KILHAM RAT VIRUS

J. S. Cook, R. W. Tennant, and W. R. Proctor*

Kilham rat virus (KRV), which contains single-stranded DNA of molecular weight 1.7×10^6 , is exponentially inactivated by 254-nm radiation. The mean lethal dose of 120 ergs/mm² produces 0.34 thymine-containing dimers per virion; other photo-products must therefore be responsible for at least two-thirds of the lethality. Since the DNA is single-stranded, i.e., since there are no complementary bases opposite those altered by the radiation, and since the inactivation curve has no shoulder, we conclude that KRV is not subject to host cell repair (hcr).

TABLE I. The characteristics of repair of DNA in human cells treated with carcinogens and mutagens

Agent	Dose Concentration	Length of treatment	Breaks per 10^8 daltons after 10^6 ergs/mm ² of 313 nm	BrdUrd's inserted per lesion	Type of repair
254 nm UV	200 ergs/mm ²		10	25	UV (normal and XP differ ~ 10-fold)
Co ⁶⁰ γ -rays	10 Kr		0.6	1	Ionizing
<i>N</i> -acetoxy AAF	7×10^{-6} M	60 min	4	40	UV (normal and XP differ ~ 10-fold)
4NQO	5×15^{-7} M	90 min	~2		UV and Ionizing?
EMS	10^{-2} M	120 min	~1.0		Ionizing
MMS	5×10^{-5} M	5 min	~0.4		Ionizing?
Propane Sultone	2×10^{-4} M	2 hr	~0.4		Ionizing?
ENU	10^{-4} M	4 hr		crosslinking	Essentially no repair
Mitomycin C	0.5 μ g/ml	7 hr	~0.4		Ionizing?
				crosslinking	
ICR-170	10^{-6} M	1 hr	~1	10	UV-like (normal and XP differ ~ 10-fold)
Urethane	10^{-4} M	1 hr			None – and no detectable damage

Abbreviations: *N*-acetoxy AAF – *N*-acetoxy acetylaminoflourene; 4NQO – 4 nitroquinoline oxide; EMS – ethyl methane-sulfonate; MMS – methyl methanesulfonate; ENU – ethyl nitrosourca.

In an attempt to derive an absolute standard for the presence or absence of host cell repair of mammalian viruses, we have compared our data with other published data for DNA viruses inactivated by radiation at 254–260 nm. We have included a few relevant determinations on *E. coli* and coliphages, and used only data for which doses are given in absolute units. We neglected differences in base composition and assumed that there is no significant redundancy in the viral genomes. Following target theory, we calculate a weighted sensitivity as the inactivation cross section of the virus divided by the molecular weight of the viral DNA; the results are expressed in units of 10^{-24} cm²/photon. We find that all the results from many laboratories can be classified into three nonoverlapping groups: I – most sensitive, includes eight determinations on viruses with single-stranded DNA (2 coliphages and KRV) assayed in either *hcr*⁺ or *hcr*⁻ hosts; weighted sensitivities, 350–740 units; II – intermediate sensitivity, includes six determinations of viruses and cells with double-stranded DNA (3 coliphage and *E. coli* AB 2480) assayed in cells with the minimum (zero?) capacity for repair; weighted sensitivities, 70–160 units; III – least sensitive, includes 16 determinations of mammalian viruses with double-stranded DNAs (5 viruses, 16 host cells) assayed in a variety of rodent and

primate cells; weighted sensitivities, 2–24. Coliphages assayed in *hcr*⁺ cells also fall in the last group.

We conclude (1) that groups I and II exhibit no host cell repair, and that the differences between them reflect an approximately fourfold difference in the intrinsic UV photosensitivity of single-stranded vs. double-stranded intraviral DNAs, and (2) that all of the mammalian viruses containing double-stranded DNA are subject to some degree of host cell repair in the systems thus far reported. This latter includes the measurements of Aaronson and Lytle (1) who compared the survival of SV40 when assayed in normal human cells and in two repair-deficient cell lines from patients with xeroderma pigmentosum. From their data, we calculate the weighted sensitivities of SV40 in normal cells to be 2 and in xeroderma cells to be 9 and 14, respectively. These results suggest, in the light of the classification given above, that although host cell reactivation is reduced (and the virus therefore more sensitive) in xeroderma cells, repair in these cells is not altogether absent.

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ULTRAVIOLET IRRADIATION AND TUMOR INDUCTION IN ANIMALS

R. W. Hart* and R. B. Setlow

Exposure to UV radiation leads to skin tumors in man. The detailed mechanisms involved in the process are not known, but the fact that there is a very high skin cancer incidence at an early age in individuals with xeroderma pigmentosum indicates that the initiating UV damage may be to DNA, because in such individuals there usually is a defect in the enzyme mechanisms that repair DNA damage in the dark. We are attempting to determine whether specific photochemical products — cyclobutane pyrimidine dimers — result in tumor induction by using the knowledge that the reversal of UV damage by photoreactivating treatment is the result of the monomerization of dimers. Fish contain photoreactivating enzyme, and in the hybrid fish *Poecilia formosa* — a gynogenetic species — there are no immunological barriers to transplanting cells from one member of a clone to another. Nutritional, environmental, and breeding requirements were determined for this species in order to obtain mass cultures. The homogeneity within three clones was determined by tissue transplant procedures. The degree of sexual reproduction is very small and is easily detectable since its occurrence is linked to a color marker in the male. Time to tissue rejection was used to determine relationships between the clones in culture and the hybrids.

Striated muscle, liver, cardiac muscle, and intestinal tissue were homogenized to clumps of 3–8 cells/clump. The clumps in suspension were irradiated with various doses of 254 nm and injected into fish from the same clone as the tissue of origin. The degree of tumor induction at a given UV fluence is now being determined. In our initial experiment, a fluence of 700 ergs/mm² resulted, within eight weeks, in the induction of tumors in 20% of the animals kept at 31° C. Tumor induction is now being measured for UV-irradiated cells that have been photoreactivated.

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INDUCTION OF CHROMOSOMAL ABERRATIONS IN HUMAN CELLS BY ULTRAVIOLET RADIATION AND BY THE ULTRAVIOLET-MIMETIC CARCINOGEN *N*-ACETOXY-ACETYLAMINOFLUORENE (*N*-ACETOXY-AAF)

Traute M. Schroeder* and James D. Regan

UV radiation and *N*-acetoxy-AAF induced similar repair sequences in normal human cells and induced

lesions in repair-deficient xeroderma pigmentosum (XP) cells which are essentially unrepaired (1). It is important to relate molecular events in DNA during repair with morphological events such as chromosomal aberrations which may occur in normal and repair-deficient human cells after DNA damage. Attempts to show the effects of UV radiation on normal and XP cells have been made by Parrington *et al.* (2) but the results are not informative as to the extent, the quality, and the sites of chromosome breakage. Preparations of normal human chromosomes after 100–150 ergs/mm² of 254 nm were obtained for breakage analysis. XP cells, however, exhibited no mitotic activity after similar doses. Similarly, good preparations of normal human chromosomes could be obtained after treatment with 10⁻⁶ M *N*-acetoxy-AAF; XP cells exhibited no mitotic activity after treatment with a similar dose of this carcinogen. Comparative analyses of UV- and *N*-acetoxy-AAF-induced chromosome aberrations in normal human cells are being completed.

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CHEMICALLY-INDUCED NEOPLASTIC TRANSFORMATION IN HUMAN CELLS

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Normal skin fibroblasts and cells from a person with xeroderma pigmentosum (XP) were treated for 2 hr with varying concentrations (7×10^{-6} to 7×10^{-8} M) of the carcinogen, acetoxyacetylaminofluorene (acetoxy-AAF). In two experiments colonies of XP cells were detected, among cells treated with 7×10^{-7} M acetoxy-AAF, which showed an unusually high growth rate and an apparent lack of contact inhibition. Also, individual cells of these colonies appeared more stellate in their morphology than the nontreated cells. These observations suggest that these colonies are a result of acetoxy-AAF-induced neoplastic transformation. The normal cells, or XP cells not treated with acetoxy-AAF, did not show any evidence of transformation. The potentially transformed XP cells are currently being tested to determine if they conform to other criteria of transformation. Tests for malignancy are planned, using the immunologically deficient “naked” strain of mice.

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REPLACEMENT OF OUABAIN-BINDING SITES AND RECOVERY OF ELECTROLYTE TRANSPORT IN OUABAIN-TREATED MAMMALIAN CELLS

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Tritiated ouabain binds to HeLa S3 cells and inhibits electrolyte (^{86}Rb) transport by 50% in 30 min at a concentration of $3 \times 10^{-7} M$. Following saturation of all ouabain-binding sites (0.75×10^6 sites/cell after 90 min), cells incubated in ouabain-free medium lose radioactivity with an apparent half-time of 8–12 hr. After subtotal ouabain blockade (50–80% maximum binding and transport inhibition), cells were returned to ouabain-free growth medium for 5–6 hr or about $1/5$ of a generation time. Although less than $1/4$ of the ouabain initially bound was released from these cells, a second titration with [^3H]ouabain revealed a number of new ouabain-binding sites, equal to the number initially present, so that the total was nearly twice the number of sites on control cells held in ouabain throughout. The cation transport rate returned to normal in the same 5- to 6-hr period, an effect which was blocked by cycloheximide. These results appear to be due to the synthesis and insertion of new transport enzymes into the cells' surface in response to altered cation transport during ouabain blockade. Similarly, an enhanced V_{max} for transport, as assayed in a standard medium, can be achieved by growing cells for one generation in a low- K^+ medium, which indicates that our results are due to specific induction of surface enzyme rather than to membrane turnover. The mouse lymphoma cell L5178Y responds in a similar fashion.

Under complete ouabain blockade, or incubation in K^+ -free medium, the cells remain viable by dye-exclusion criteria but show a marked inhibition of protein synthesis. Under these drastic conditions the cells fail to respond, i.e., they synthesize no new transport sites.

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SYNTHESIS OF SPECIFIC MEMBRANE ENZYMES IN HeLa CELLS DURING THE CELL CYCLE

W. R. Proctor* and J. S. Cook

We have initiated studies on the synthesis of components of cell membranes during the cell cycle, with emphasis on those surface enzymes whose activities are

important to intermitotic growth of the cells. Alkali cation transport is mediated by such an enzyme (Na-K-Mg-ATPase), for which the relevant characteristics are: (1) the enzyme is bound to the cell surface; (2) the enzyme regulates the content of intracellular salts which, as the quantitatively most abundant of the osmotically active solutes, are the principal determinants of cell size and change in cell size during growth; and (3) in HeLa cells, the enzyme specifically and tightly binds the cardiac glycoside ouabain, so that with the use of [^3H]ouabain we may count the number of transport sites on the surface of each cell. Our preliminary results show that, following synchronization of HeLa S3 cells by the double-thymidine-block technique, the number of ouabain-binding sites per cell increases sharply at the end of S or beginning of G_2 period. This apparent burst of synthesis of a specific membrane component correlates approximately in time with the synthesis of surface sialic acid (G_2 , ref. 1) in human lymphoid cells and with the peak incorporation of [^3H]fucose into HeLa cell plasma membranes (2).

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A SERINE ANALOG WHICH INHIBITS DNA SYNTHESIS BUT NOT PROTEIN SYNTHESIS IN SERINE-REQUIRING HUMAN LEUKEMIA CELLS*

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Human leukemia cells require serine (1). The effect of two putative serine analogs on DNA and protein synthesis was investigated in human chronic granulocytic leukemia (CGL) cells. The first of these compounds, 1-ethyl, 2-hydroxy cyclohexane carboxylic acid, produced about 30% inhibition of DNA synthesis when employed at a final concentration of $10^{-3} M$ in the presence of 10^{-5} or $10^{-4} M$ serine.

The second of these compounds, α -hydroxy amino cyclohexane carboxylic acid, inhibits DNA synthesis in CGL cells by 90% in 48 hr at $10^{-3} M$ at physiological concentrations of serine. Protein synthesis is inhibited only about 10% by this analog. Thus the analog may be inhibiting one-carbon metabolism (and thereby DNA synthesis) while failing to inhibit protein synthesis by virtue of lack of affinity for aminoacyl tRNA synthetases. Investigation of this possibility is in progress. The

possible therapeutic advantages offered by an analog with this specificity is obvious.

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AUTOGENOUS IMMUNITY TO ENDOGENOUS RNA TUMOR VIRUS ANTIGENS IN MICE WITH A LOW NATURAL INCIDENCE OF LYMPHOMA

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Serological and ultrastructural studies have demonstrated antigenic expression of C-type RNA tumor viruses in normal tissues of both fetal and adult mice of strains with a low as well as high natural incidence of lymphoma. It has been generally assumed that with vertically transmitted tumor viruses, an initial period of tolerance exists to virion- and virus-induced cell-surface antigen. An alternative to this assumption is that some immunity develops to endogenous C-type virus, as demonstrated during the "pre-leukemic" period in AKR mice as well as in NZB mice. Unfortunately, it is difficult to evaluate the functional significance of the innate immunity to MuLV in AKR mice, since this is a strain with a high incidence of lymphoma. The important question is whether the balance of this host factor regulates or is directly associated with RNA tumor virus-induced pathogenesis. This question can best be evaluated by studies in mice with a low incidence of lymphoma.

In our study we attempted to correlate detectable MuLV antigen in the thymus and spleen with the development of immune competence and glomerulosclerosis, and with the incidence of lymphoid neoplasia. We also determined the specificity of kidney-bound antibody by elution and indirect immunofluorescence with cultured AKR cells, spontaneously producing Gross virus. RF mice were selected for these studies because several of their characteristics led us to suspect the presence of some resistance to, or host regulation of, C-type virus expression in this strain.

In the present study, MuLV antigen could be detected by CF tests in approximately 20% of the thymuses tested from 7-day-old RF mice. We assume that the broadly reactive antiserum, as used in the complement fixation test, has a certain degree of resolution with a relatively high quantity of leukemia-virus antigens. Thus the increase in the percentage of thymuses positive for

MuLV antigens can be reasonably interpreted as an increase in the quantity of antigen with age. No detectable viral antigen was measured in the spleen until 30 days of age, and the peak antigen concentration was reached at 50 days. The decrease in antigen in both thymus and spleen definitely correlates with the development of immunologic competence in the spleen, as assayed by *de novo* germinal center formation, antigen localization of germinal centers, and (more specifically) immune elimination of antigens from the serum. Our interpretation was that the developed immunologic competence could also be functional for MuLV. This suggested, further, that immune elimination correlates well with the development of histologically detectable glomerulosclerosis.

Complement fixation tests of the kidney homogenates revealed that 100% of the kidneys from 1- and 1.5-year-old mice were positive for leukemia virus antigens. We then tested the specificity of the kidney-bound IgG for RNA tumor virus antigen. The kidney-bound IgG was positive, as measured by indirect immunofluorescent reaction with AKR spontaneous Gross-virus-replicating cells and AKR thymoma. We conclude from these data that a contributory factor in the glomerulosclerosis which develops in RF mice is a fixation of antigen-antibody complexes resulting from a chronic humoral immune response to endogenous C-type RNA tumor virus.

The role of this innate immunity appears to be beneficial in the RF mouse, as judged from data correlating spontaneous lymphoid neoplasia with severity of glomerulosclerosis. An inverse relationship is established in aged animals. An evaluation was made on the survival data from a control group of 311 RF mice. Animals that died were autopsied and evaluated for incidence and degree of glomerulosclerosis, and for concomitant incidence of lymphoid neoplasia, such as thymic lymphoma and a mixed type B reticulum cell sarcoma. The results indicate an inverse relation between the severity of glomerulosclerosis and the incidence of all lymphoid neoplasia. In fact, animals diagnosed as having severe glomerulosclerosis, compared with those having less severe cases, developed 26% of the expected thymic lymphomas; 61% of expected reticulum cell sarcoma was also obtained in this group.

The question of whether autogenous immunity can regulate pathogenesis in a positive or negative manner may be a function of the quality or quantity of the humoral response. Only in one specific incidence has it been shown that the immune specificity is directed toward Gross virion antigens and there are few data demonstrating development of a virus-neutralizing anti-

body during the course of the response to endogenous (wild-type RNA tumor) viruses. However, the possibility that this autogenous immune response to endogenous RNA tumor virus may be one component of host functions which control or regulate pathogenesis serves as one possible explanation of the diverse lymphoma patterns in various mouse strains in the presence of apparently similar viral burdens.

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IMMUNOLOGIC CROSS-REACTIVITY OF ANTIGENS COMMON TO TUMOR AND FETAL CELLS

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The results of our previous studies have suggested that both a humoral factor and cytotoxic lymphocytes, generated as a result of immunization with isologous fetal tissue but not as a result of neonatal or normal tissue immunization, depressed the growth of Rauscher leukemia virus (RLV)-infected spleen cells and a plasma cell tumor in BALB/c mice. These data strongly suggest that BALB/c fetuses, at approximately $\frac{3}{4}$ of their gestation process, possess antigen(s) common to RLV-infected spleen cells and a plasma cell ascites tumor. Equally important is the fact that fetal specific antigens can immunize adult animals, such that the functional immunity is capable of suppressing the RNA virus-induced tumor growth and pathogenesis, in agreement with recent findings on DNA virus-induced tumors. We have also demonstrated that adoptive transfer of fetal antigen primed cells, as well as postpartum spleen cells, can suppress RLV-induced splenomegaly. Our results do not rule out the possibility that the immunity elicited by embryonal antigen is against a virus-induced transplantation antigen. This is unlikely, however, as the RLV-mediated antigens are not cross-reactive with the endogenous Gross virus that would be present in the fetuses used for immunization.

A major problem in the fetal antigen studies to date is the fact that no true immunologic cross-reactivity has been demonstrated. That is, while it has been demonstrated that immunization with fetal cells is able to suppress the growth of tumors to a limited degree in a variety of strains of animals, no one has demonstrated that immunization with tumor can suppress the effect of embryonal growth. This demonstration is imperative if immunologic cross-reactivity of antigens on tumor and fetal cells is to be established.

Preliminary attempts to alter the growth of subcutaneous embryomas using whole fetal homogenates in

intact hamsters immunized with SV-40 tumors have been unsuccessful in spite of the fact that in this system fetal cell immunization has a suppressive influence on tumor growth. Also, no adequate quantitative *in vivo* assay has been developed which would clearly demonstrate immunologic cross-reactivity between antigens on fetal and tumor cells. This assay will be particularly important at the present time, since it has been recently demonstrated that the common antigen of fetal and tumor cells is distinct from the tumor-specific antigen, at least in chemically induced tumors.

This year we have demonstrated immunologic cross-reactivity between plasma cell tumor and fetal cells of BALB/c mice, and we have developed an *in vivo* assay for demonstrating immunologic cross-reactivity in a variety of tumors and fetal cells. To do this we capitalized on the colony-forming potential, in lethally irradiated adult recipients, of the predominant hemopoietic cells of embryonic liver. We used a modified colony-forming unit (CFU) technique developed years ago for the quantitative evaluation of stem cells in adult bone marrow. This technique involves the adoptive transfer into lethally irradiated recipients of stem cells which seed to the spleen and develop clones which are expressed as gross colonies, countable on the spleen surface. The number of colonies is a linear function of cell dose, both for adult bone marrow cells and hematopoietic cells of embryonic liver. The advantage we have in this system is that the recipients, prior to irradiation and adoptive transfer, can be immunized with either fetal cells or tumor cells, and after irradiation the fetal colonies derived from hematopoietic stem cells of embryonic liver grow in an environment in which humoral antibody produced against the immunization regimen can influence their growth.

Using this technique we first demonstrated the effect of fetal cell immunization on fetal liver colony-forming units (FCFU). It was clear that FCFU are markedly suppressed in recipients immunized with fetal liver cells, less suppressed by recipients immunized with whole fetal homogenates, and not affected in recipients immunized with fetal cell homogenates minus liver cells. Recipients immunized five times with fetal liver cells demonstrated 40% of the expected colonies. These results were significant at $P \leq 0.01$. We then were able to demonstrate that in animals immunized with plasma cell tumor, a 70% suppression of FCFU was obtained compared with unimmunized controls. Also, the degree of suppression decreased as a function of time for the last immunization. These results suggest that the levels or efficiency of the humoral factor diminish as a function of time after immunization. Thus there exists

a common antigen of plasmacytoma and fetal cells, one capable of eliciting a response that is cytostatic and/or cytotoxic to the growth of fetal liver cells in immunized irradiated recipients. No such suppressive effect of plasma cell tumor immunization could be demonstrated against colonies developed from adult bone marrow, nor did nonspecific immunization with sheep red blood cells or homologous red blood cells influence the number of FCFU.

Critical tests of cross-reactivity involved absorption studies. Groups of animals were immunized three times with plasma cell tumors and, simultaneously with the last immunization, at least two groups were injected subcutaneously with either viable or irradiated fetal liver cells; other groups were not subcutaneously injected. One week after the last immunization and the subcutaneous injection of viable or irradiated fetal liver cells, mice were lethally irradiated and 24 hr later were injected for FCFU formation. The results of this study were as follows: (1) A significant difference in FCFU was achieved in animals immunized with plasma cell tumor compared to nonimmunized controls. (2) A similarly significant suppression of FCFU was achieved in plasma cell tumor-immunized animals receiving a subcutaneous booster of irradiated fetal liver cells. (3) Little or no suppression, however, was noted when FCFU was counted in plasma cell tumor-immunized animals receiving a subcutaneous booster of viable fetal liver cells. We tentatively suggested that the embryoma growing subcutaneously, as a result of injection of live fetal liver cells in adult animals immunized previously with plasma cell tumor, is capable of absorbing antibody and does this during the weekly interval between the last immunization and adoptive transfer. Such absorption of antibody does not occur when nonviable irradiated fetal cells are subcutaneously injected; thus, antibody is free to exert its suppressive effect on FCFU.

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CHANGES INDUCED BY LACTIC DEHYDROGENASE VIRUS IN THYMUS AND THYMUS-DEPENDENT AREAS OF LYMPHATIC TISSUE

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To examine the effects of lactic dehydrogenase virus (LDV) on stromal and parenchymal cells of the thymus and thymus-dependent regions of lymphatic tissue, virus was injected either i.p. or intrathymically into male BALB/c mice that had been either thymectomized

and irradiated, adrenalectomized, or not treated. Serum lactate dehydrogenase activity was determined, and the spleen, mesenteric lymph node, and thymus were weighed and prepared for histologic and ultrastructural examination.

The principal events of the infection process took place during the first 48 hr after infection and were: (1) virus particles, seen in ultrastructure, consistently associated with the plasma membrane of phagocytic cells of the reticulum, with some virus-membrane configurations suggesting replication by budding from these cells; (2) eventual degeneration of these phagocytic reticular cells; and (3) cytotoxic degeneration of thymus-derived lymphocytes and their subsequent phagocytosis by macrophages. The cortex of the thymus was markedly depleted of lymphocytes by 4 days after intrathymic virus injection, as opposed to minimal depletion after i.p. injection. Virus particles were directly associated with the stromal cells but not with the thymus-derived lymphocytes, indicating that the latter cytopathic effect was mediated by some intermediate soluble factor. This response also occurred in adrenalectomized animals that were inoculated with LDV, indicating that the intermediate factor is not an adrenal cortical steroid.

Serum lactate dehydrogenase activity increased sharply after i.p. or intrathymic injection of the virus into otherwise untreated animals; it then declined slightly and remained constant throughout the remainder of the experiment. Initially the spleen weights increased, whereas thymus weights decreased, although in thymectomized-irradiated animals the increase in spleen weight was 60% greater, and the serum lactate dehydrogenase activity increased to a peak in half of the time of the controls.

*Participant in the Great Lakes Colleges Association Program.

HISTOPROLIFERATIVE EFFECT OF RAUSCHER LEUKEMIA VIRUS ON LYMPHATIC TISSUE. LACTIC DEHYDROGENASE VIRUS POTENTIATION OF THE ERYTHROID RESPONSE

M. J. Snodgrass, J. M. Yuhas, and M. G. Hanna, Jr.

Lactic dehydrogenase virus (LDV) and erythropoietin potentiate Rauscher leukemia virus-stimulated erythroid hyperplasia in the red pulp of the spleens of BALB/c mice. Studies of the incorporation of ^{59}Fe into the peripheral blood erythrocytes indicated that this stimulation was probably chronic with lactic dehydrogenase virus and an early acute response with

erythropoietin. Both stimulants in combination with Rauscher leukemia virus (RLV) induced about a 60% greater splenomegaly at 25 days than RLV alone when they were injected as little as 1 hr before the leukemia virus. Histologically, the splenomegaly resulted from an enhancement of the erythroid hyperplasia associated with the Rauscher infection. After infection with Rauscher virus, C-type virus particles were found, ultrastructurally, budding from erythroblasts at various stages of development in the splenic red pulp and from reticulocytes in the peripheral blood. Erythroblasts were infected by the leukemia virus at both early and late stages of development, but continued to differentiate at least to the enucleated stage. Thus, the stimulation of erythropoiesis in the splenic red pulp by LDV provides another basis for the synergistic interaction of LDV and RLV, and continued differentiation of the RNA tumor-virus-infected cells of the erythrocytic series raises the question of whether these cells undergo a true malignant transformation during the RLV-induced splenomegaly.

HISTOPATHOLOGY OF *MYCOBACTERIUM BOVIS* (BCG)-MEDIATED TUMOR REGRESSION

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B. Zbar,* and H. J. Rapp*

A comparative histopathological study was performed on inbred guinea pigs at the site of a transplanted syngeneic hepatocarcinoma and in the draining lymph nodes, in the presence and absence of *Mycobacterium bovis* strain BCG. BCG was injected into the growing intradermal tumor 7 days after transplantation, at which time the tumor had metastasized to the first regional lymph node. The histopathology was compared with that of saline-inoculated tumors and with that of animals in which surgical excision of tumors had been performed 7 days after transplantation. In this system, guinea pigs die 60–90 days after intradermal injection of 10^6 hepatocarcinoma cells in the absence of BCG treatment. The results demonstrate that intradermal tumors completely regress after treatment with BCG and that regional lymph node metastases are eliminated. The mechanism is a BCG-mediated granulomatous reaction at both the tumor site and the regional lymph node. As detected both histologically and ultrastructurally, histiocytes appear to be the major effector cells in this reaction. In this syngeneic tumor system it is clear that a conventional lymphoproliferative response of the regional node, in the absence of histiocytosis, is insufficient to inhibit tumor growth. Additionally,

treatment of these transplanted syngeneic tumors in guinea pigs with a single sensitization by vaccinia virus, oxazolone, or turpentine was compared with BCG therapy. Cellular reactions in the regional lymph nodes, which were characteristic of the development of delayed-type hypersensitivity, had no detrimental effects on the tumor and did not alter metastatic growth. The turpentine-induced inflammatory reaction at the tumor site was also ineffective in suppressing tumor growth. Both turpentine and oxazolone treatments, however, enhanced tumor growth in the skin.

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IMMUNE SUPPRESSION AND CARCINOGENESIS IN HAMSTERS DURING TOPICAL APPLICATION OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE (DMBA)

A. K. Szakal and M. G. Hanna, Jr.

Regional and systemic immune responses were correlated with tumor development in Syrian hamsters treated with 7,12-dimethylbenz[*a*]anthracene (DMBA), a potent carcinogen. The results indicate a significant transient depression of regional and systemic humoral immunity, followed by a more lasting suppression of cell-mediated immunity. Papilloma development was associated with maximal suppression of humoral immunity, whereas malignant transformation began at the transitional period between recovery of humoral immunity and depression of cell-mediated immunity. The functional role of depressed humoral and cell-mediated immune responses in tumorigenesis is supported by results of studies on DMBA-induced *in situ* tumor development in ATS-treated, splenectomized, and/or lymphadenectomized animals. Although initial papillomagenesis was primarily contingent on the early depression of humoral immunity, survival of papillomas in the last phases of their development appeared to be regulated through cell-mediated immune reactivity elicited directly in proportion to immunogenicity. It was concluded from these results that a specific chronological relationship between the transient depression of regional and systemic humoral immunity and the more lasting depression of cell-mediated immunity is necessary for successful tumorigenesis. The present results support the contention that a *modulation* of normal humoral (regional and systemic) and cell-mediated immunity is induced by DMBA, and is functional in tumorigenesis.

COMPARATIVE BIOCHEMICAL PROPERTIES OF RNA-DIRECTED DNA POLYMERASES FROM RAUSCHER MURINE LEUKEMIA VIRUS AND AVIAN MYELOBLASTOSIS VIRUS

L. C. Waters, Wen-Kuang Yang, L. G. Hardin, and Chong-kun Koh

Since oncogenic RNA viruses have been isolated from a wide spectrum of animal hosts, ranging from viper to primate and possibly man, a comparison of the properties of the RNA-directed DNA polymerases from these viruses should help to elucidate their phylogenetic evolution. In addition, a highly sensitive enzyme would make it possible to detect and differentiate such viruses. For this purpose, we selected two oncogenic RNA viruses (Rauscher *murine* leukemia virus and *avian* myeloblastosis virus) and employed various parameters to determine whether the polymerases of these two viruses can be distinguished by their biochemical properties. The results demonstrate that the biochemical properties of the two polymerases are so different that a definite distinction can be made.

(1) In glycerol gradient centrifugation, the avian virus polymerase sediments at $\sim 6S$, the murine virus polymerase at $\sim 4S$.

(2) The two polymerases show different chromatographic behavior on hydroxylapatite and phosphocellulose. Phosphocellulose chromatography of the avian virus polymerase yielded two peaks, presumably representing two forms of the same enzyme.

(3) Effects of divalent cations, pH, and temperature on the ability of the two polymerases to utilize various template-primers are significantly different. The template-primers tested include DNase-activated calf thymus DNA, $d(A-T)_n$, $(rA)_n \cdot (rU)_n$, $(rA)_n \cdot (dT)_n$, $(rA)_n \cdot (dT)_6$, $(dA)_n \cdot (dT)_6$, $(rC)_n \cdot (dG)_6$, and $(rI)_n \cdot (dC)_6$; all showed individual characteristic requirements of divalent cations, pH, and temperature for optimum activity with the two polymerases. A minor change in the optimal conditions usually resulted in a marked decrease in the detected polymerase activity.

(4) In kinetic experiments, chain initiation appeared to be the rate-limiting step in the $(rA)_n \cdot (rU)_n$ -dependent dTMP-incorporating activity of the *murine* virus polymerase, whereas this was less apparent with the *avian* virus polymerase.

(5) The $d(A-T)_n$ -dependent activity of the *murine* virus polymerase is markedly inhibited by $(rU)_n$ or $(rG)_n$ in a straightforward manner; however, such inhibition is less marked and shows an apparent biphasic kinetic curve with two K_i 's in the case of the *avian* virus polymerase.

PREPARATION OF RNA-DIRECTED DNA POLYMERASES FROM MAMMALIAN TISSUES INFECTED WITH ONCOGENIC RNA VIRUSES

Wen-Kuang Yang, Chong-kun Koh, and L. C. Waters

It has been well established that RNA-directed DNA polymerase is present within the virion of all oncogenic RNA viruses. There is indirect evidence indicating that this enzyme is essential for viral replication to occur and also for host cell transformation to develop. Purification of the enzyme has been reported from at least three groups of investigators who used isolated virus particle preparations of avian source. In mammalian systems, where the amount of available viruses is limited, no purification of the enzyme has been reported. We have used mammalian tissues infected with oncogenic RNA viruses as sources for the purification of RNA-directed DNA polymerases. There are three purposes for the study. First, we need large quantities of purified polymerase for biochemical characterization as well as for immunological studies of host cell-virus interaction. Second, since low recovery of enzyme after various purification procedures has been noted by many workers, we believe that development of a satisfactory method will be important. Third, to obtain purified viral RNA-directed DNA polymerase from mammalian tissues, it is necessary to differentiate this polymerase from cellular DNA polymerases, especially those shown to have the capacity to utilize RNA templates for DNA synthesis.

Spleens of BALB/c mice infected with Rauscher leukemia virus were fractionated by homogenization and differential centrifugation into subcellular fractions. RNA-directed DNA polymerase activity was detected mainly in the particulate fractions other than nuclear. When compared with virus isolated from plasma, particulate fractions of the infected spleens contained at least 200 times more enzyme activity. This indicated that spleens of the infected mice can be used for the purpose of enzyme purification. Extraction with solutions containing 0.25 M KCl removes cellular DNA polymerases, but not viral polymerase, from the particulate fractions. To keep the RNA-directed DNA polymerase of Rauscher leukemia virus in a solubilized form, both high salt (0.5 M KCl) and detergent (0.5% Nonidet P 40) are required. Successful purification has been achieved by performing Sephadex G100 gel filtration and hydroxylapatite column chromatography for the initial separations of the enzyme in solutions and gradients containing 0.5 M KCl and 0.2% Nonidet P40. Subsequent chromatography on phosphocellulose (0.1–0.5 M KCl gradient and 0.1% Nonidet P40)

resulted in RNA-directed DNA polymerase preparations purified from 200- to 500-fold over the lysate of isolated virus preparations. High yields of the enzyme have been obtained and we think that this is because of the use of high salt and detergent solutions in early stages of purification.

The purified polymerase preparations are similar to the polymerase of the plasma Rauscher leukemia virus in biochemical properties such as molecular weight, optimal conditions for using synthetic template-primers, sensitivity of its DNA-dependent activity to inhibitory single-stranded polyribonucleotides, and enzyme kinetics. Conditions for stabilizing the purified enzyme have been determined. Both chromatographic behavior in various column systems and enzymological properties of the purified enzyme are distinct from those of the two cellular DNA polymerases.

The preparation scheme used for the RNA-directed DNA polymerase from Rauscher virus-infected spleens has been applied in two other tissues, mouse plasma cell tumors infected with unknown "C" type particles and tissues from rats carrying stem cell leukemia. In all cases, the method has given satisfactory purification and yield of polymerases, which are membrane-bound in each tissue.

CELL CULTURES FOR ENZYMOLOGICAL STUDIES OF VIRAL CARCINOGENESIS

Ti Ho, Wen-Kuang Yang, L. C. Waters,
and G. David Novelli

The study of oncogenic RNA virus-host cell interaction requires tissue and cell culture systems. The tissue culture facility of this group was initiated in November 1971 for the purpose of molecular and biochemical investigations. The research activities, many of them at the beginning stage of development, are summarized as follows:

1. *Collection and maintenance of established cell lines*: Various established cell lines have been obtained and adapted to grow in an enriched medium, which contains nonessential amino acids and 10% fetal calf serum in addition to Dulbecco's modified Eagle's medium. All of them have been characterized by karyotype analysis and recloned, when shown to have marked karyotypic heterogeneity. By tritiated thymidine incubation and subsequent sucrose gradient analysis, most cell lines were determined to be free of pleuropneumonia-like organism (PPLO) contamination and stored as stocks in liquid nitrogen. These lines include BALB 3T3, NIH Swiss 3T3, SV-40-transformed BALB 3T3, murine-sarcoma-virus- (MSV) trans-

formed golden hamster HT-1, and four Chinese hamster embryo lines (courtesy of Dr. Yerganin, Children's Cancer Research Foundation, Boston).

2. *Establishment of new cell lines from tumor tissues*: Certain experimental tumors, both transplantable and naturally occurring, have been cultured with good growth, using our standard culture medium. Once established and at the 2-3 passage stage, the cells are stored as stocks in liquid nitrogen. The tumors include two AKR mouse thymomas, a BALB/c mouse reticulum cell sarcoma, MOPC 31C plasmacytoma, and a fibrosarcoma from an adult rat which was inoculated with Rauscher leukemia virus as newborn.

3. *Examination of DNA polymerases in cultured cells*: We are comparing the DNA polymerase profiles in the "normal" and transformed cells from tissue cultures. For this study, the cells have been grown in gram quantity in roller tubes and polymerase patterns analyzed by sucrose gradient centrifugation of the cell extracts. Efforts have also been made to search for RNA-directed DNA polymerase in the MSV-transformed HT-1 cells. Preliminary results, based on our experience with the Rauscher leukemia virus polymerase, indicate that there may not be such polymerase activity in the HT-1 cells.

CHANGES IN DNA POLYMERASE PATTERNS OF EHRlich ASCITES TUMOR CELLS UNDER DIFFERENT GROWTH CONDITIONS

J. E. Strickland,* R. W. Barton, and Wen-Kuang Yang

Transplantable Ehrlich ascites tumor shows a characteristic pattern of DNA synthesis and cell proliferation during passage in mouse hosts. When 5×10^6 cells are inoculated into the animal intraperitoneally, there is a rapid increase in the number of cells engaging in DNA synthesis, reaching 60-80% as determined by autoradiographic analysis. This high percentage persists until 7-8 days after transplantation, when a large number of tumor cells (about 1×10^9) accumulate in the peritoneal cavity of the animal and the percentage of the DNA-synthesizing cells begins to decrease. At 14 days, only 10-30% of the cells incorporate tritiated thymidine. We consider that this system may be used for studying the biochemical mechanisms involved in DNA replication of the mammalian cell.

Extraction of the tumor cells by a solution containing 0.5 M KCl showed two peaks of DNA polymerase activity on glycerol gradient centrifugation. In the extracts from 2-, 3-, 5-, and 8-day tumor cells, the major peak was of larger molecular weight. This peak remained nearly constant until between 8 and 14 days

after transplantation when it decreased to 30–50% of the original activity. The decrease in the DNA synthesis activity of the cells appeared to precede the decrease in the activity of the large molecular weight polymerase, thus suggesting that a cause-effect relationship of the two phenomena may be unlikely. No definite evidence was found for the presence of an inhibitor, which acts on the large molecular weight polymerase, in the tumor cells at 14 days.

Both large and small molecular weight polymerases were isolated from 3- and 14-day tumor cells for comparative studies. There was no difference in template specificity between enzymes from tumor cells at these two different stages of DNA synthesis. The major peak responded well to activated calf thymus DNA and d(A-T)_n, while the minor peak preferred (rA)_n·(dT)_n but also used d(A-T)_n and the activated DNA. Purification of the two polymerases is in progress for further enzymological comparison and for testing possible factors affecting their functions.

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GROWTH OF ASCITES AND SUBCUTANEOUS SOLID FORMS OF MOPC 31C MOUSE PLASMACYTOMA *IN VITRO*

R. J. Brake,* Ti Ho, and Wen-Kuang Yang

Mouse plasmacytoma cells (MOPC 31C) can be grown as an ascitic or subcutaneous tumor in the BALB/c mouse, depending on the route of injection. Several different plasmacytoma cell lines have been established in culture, with the frequent observation that an early pattern of differentiation must be completed before a stable species of tumor cells can be isolated. Classification as plasmacytoma is based on plasma cell morphology and capacity for production of homogeneous immunoglobulin (myeloma protein).

We have successfully cultured MOPC 31C cells from both ascitic and subcutaneous explants, and observed similar morphological instability *in vitro*. Primary ascites cultures indicated that only a small fraction of *in vivo*-grown cells could express proliferative capacity *in vitro*. This subpopulation has given rise to a fast-growing lymphoid cell line, which grows in suspension and is highly tumorigenic upon reinoculation into the mouse. Subcutaneous tumors, on the other hand, gave rise to a variety of cell types when single cell suspensions were prepared from tumor tissue, with or without the use of trypsin. One week after primary culturing fibroblastic and epithelioid cells formed a mixed monolayer, while lymphoid cell clumps were both in suspen-

sion and loosely associated with the attached cells. The spherical lymphoid cells may be removed and cultured as an apparently pure strain, but efforts to isolate the attached cell types have failed. Comparisons of lymphoid cells from the two types of tumor indicate similarities of karyotype and tumorigenicity, although slight differences in clumping tendencies and size distribution are apparent in culture. Comparison to the slower growing attached species has not been achieved.

It is not clear whether these cell types represent distinct populations *in vivo*, or the products of different phenotypic expression of the same cell *in vitro*. Experiments are in progress to determine whether the fibroblastic and epithelioid cells are, in fact, tumor cells or normal, host-derived contaminants. We intend to examine these partially separated cultures for the presence of marker chromosomes and myeloma protein production by immunochemical methods to answer this question.

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SEPARATION OF POLY(A)-CONTAINING RNA OF POLYRIBOSOMES BY AFFINITY THERMOCHROMATOGRAPHY

Kai-Lin Lee, D. R. Joseph,* and F. T. Kenney

The available evidence indicates that glucocorticoid-type hormones induce tyrosine transaminase and few other enzymes by promoting the synthesis of their specific messenger RNAs. However, the evidence is indirect and far from complete. Isolation of the specific mRNAs and assay of these entities will provide direct evidence of the mechanisms involved in regulation of enzyme synthesis by steroid hormones.

Recently we have shown that the hydrocortisone-mediated induction of tyrosine transaminase is highly sensitive to cordycepin, which suggests that mRNA coding for the enzyme contains poly(A) segments. Other investigators have shown that much of the active mRNA of mammalian cells contains such segments. The presence of poly(A) in mRNA should provide a chemical basis for the fractionation of such RNAs. The fractionation has been readily achieved by affinity thermochromatography on poly(U)-Sepharose columns.

Total poly(A)-containing RNA from polysomes of rat liver or cultured H-35 cells was absorbed on poly(U)-Sepharose columns and eluted into several fractions by stepwise decrease in salt concentration (0.5 M to 0.01 M) and increase in temperature (22° C to 50° C). Neither ribosomal RNA nor transfer RNA will absorb

on the poly(U)-Sepharose column at 22° C and 0.5 NaCl. By comparing the elution pattern of standard oligo(A)s with that of poly(A)-RNA of polysomes it was found that the separation of poly(A)-RNA by affinity thermochromatography is dependent on the size of poly(A) segments in the RNA. When adenosine-labeled polysomal RNA was fractionated by this technique, fractions were obtained with varying specific radioactivity, presumably due to differences in the size of poly(A) segments and the rate of synthesis of each RNA fraction.

When partially purified rabbit hemoglobin mRNA was applied to a poly(U)-Sepharose column, a 200-fold further purification was obtained.

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PRESERVATION OF TYROSINE TRANSAMINASE MESSENGER RNA BY CYCLOHEXIMIDE

Kai-Lin Lee, F. T. Kenney, and J. C. Laney

We have previously shown that functional transaminase mRNA can be preserved by cycloheximide in hydrocortisone-treated H-35 cells. The preserved mRNA is rapidly translated after removal of the inhibitor. Since the mechanism by which cycloheximide inhibits protein synthesis is different from that of puromycin or NaF, we carried out similar experiments in which cycloheximide was replaced by puromycin or NaF. Neither puromycin nor NaF preserved tyrosine transaminase-specific mRNA. These results are in accord with the mechanism of action of these inhibitors, and suggest further that essentially all the mRNA preserved by cycloheximide is associated with polysomes. The rapid cessation of the induced synthesis of transaminase 1 hr after cycloheximide removal indicates that mRNA accumulated during the treatment with cycloheximide is degraded after it has been translated.

When cells were preinduced with insulin, then treated with cycloheximide, enzyme levels continued to decrease following removal of the inhibitor. This result indicates that insulin does not increase the mRNA content of the cells, and provides further support for our earlier conclusion that the peptide hormone is a translation inducer.

EFFECT OF CORDYCEPIN ON THE INDUCTION OF TYROSINE TRANSAMINASE IN CULTURED HEPATOMA CELLS

Kai-Lin Lee and F. T. Kenney

Reports from several laboratories have demonstrated the presence of large poly(A) segments in polysomal

RNA from mammalian cells. Two identified messenger RNAs, hemoglobin and ovalbumin mRNAs, have been shown to contain poly(A) (1), but no poly(A) sequence can be detected in histone messenger RNA (2). It is not known whether the mRNA coding for tyrosine transaminase contains poly(A) sequences; such information should facilitate the identification and isolation of this mRNA. To assess this possibility, we tested the effect of cordycepin (3'-deoxyadenosine) on the steroid-mediated transaminase induction. This adenosine analog has been shown to reduce the entry of nuclear mRNA into cytoplasmic polysomes, presumably by interfering with the addition of poly(A) to the mRNA precursors in the nucleus.

The induction of tyrosine transaminase by hydrocortisone in cultured H-35 cells can be completely abolished by cordycepin. The synthesis of poly(A)-containing RNAs of polysomes is also greatly reduced by this drug. However, the synthesis of total cellular RNA, ribosomal RNA, or protein is only slightly affected by this analog. Based on these results, we conclude that the mRNA coding for tyrosine transaminase contains poly(A) segments. This should facilitate its identification and purification.

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GLUCOCORTICOID HORMONE-RECEPTOR INTERACTION

S. E. Lane* and F. T. Kenney

Previous studies have implicated the importance of intracellular receptors in the induction of steroid-mediated enzyme synthesis. The initial events appear to be the formation of the specific steroid-protein complex in the cytoplasm, followed by nuclear uptake and interaction of the steroid with the chromatin. In order to analyze further the role of the receptor protein, we first attempted to obtain additional physicochemical information on the interaction of the cytoplasmic steroid-receptor from cultured H-35 cells.

Employing a charcoal assay to eliminate possible interference from low-affinity binding, a saturation of the binding occurred at approximately 2 to 4 × 10⁻⁷ M hydrocortisone, which is identical to the optimal steroid concentration for tyrosine aminotransferase induction. An affinity constant of ~1 × 10⁻⁶ M⁻¹ was also obtained from these data. Treatment with pronase or high temperature resulted in the release of [³H]-hydrocortisone from the complex, whereas RNase and DNase showed no effect.

Glycerol density gradient centrifugation and acrylamide gel disc electrophoresis revealed that the receptor protein has a sedimentation coefficient of 4S and an electrophoretic mobility similar to a prealbumin. Further studies using the isoelectric focusing-acrylamide gel technique suggests an acidic isoelectric point of the complex, below pH 4. Similar results were obtained with rat liver cytosol.

To correlate the relative effectiveness of steroids other than hydrocortisone in inducing tyrosine aminotransferase with their binding to the cytoplasmic receptor protein, competitive experiments were initiated. The results demonstrated the stereo-specificity of the 11-OH group, since 11 β -OH progesterone was an excellent competitor of hydrocortisone for the receptor, while 11 α -OH progesterone competed poorly. These results can be correlated with induction experiments in which only the 11 β -OH progesterone is active. Other steroids, such as dexamethasone (a potent synthetic glucocorticoid), showed a high affinity for the receptor. These results suggest that effective steroid-protein binding is an essential requirement for enzyme induction.

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MESSENGER RNA TURNOVER IN MAMMALIAN CELLS

C. D. Stiles,* Kai-Lin Lee, and F. T. Kenney

The turnover of mRNA specific for tyrosine aminotransferase (TAT) has been shown to be contingent upon movement of ribosomes along the message during translation in cultured hepatoma cells (1). The rate of synthesis of TAT and alanine aminotransferase (AAT) is inversely proportional to the half-life of the mRNAs coding for these two enzymes. Ribosomal velocity along TAT and AAT mRNA is identical, despite the fact that AAT mRNA has a half-life 15 times greater than that of TAT message. Insulin, which induces *de novo* synthesis of TAT via a translational mechanism, does not alter the rate of ribosome movement along the message and does not alter messenger half-life. On the basis of these observations, a model has been formulated which satisfactorily accounts for the extreme heterogeneity in the observed turnover rates of individual mRNAs in mammalian cells and also sheds light on the mechanism whereby insulin induces *de novo* synthesis of specific hepatic proteins.

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REVERSE TRANSCRIPTASES IN NORMAL AND MALIGNANT CELLS

D. R. Joseph* and F. T. Kenney

It is now well documented that oncogenic RNA viruses contain RNA-dependent DNA polymerases (reverse transcriptases). However, a controversy exists over the presence of reverse transcriptase (RT) in normal and malignant cells. There is presently no satisfactory procedure to determine if these enzymes are present in cells and tissues; assays of crude extracts give ambiguous results. We initiated a study to develop a simple procedure to look for RT in normal, malignant, and virus-infected cells.

DNA-cellulose and polynucleotide-Sepharose were prepared for affinity chromatography by UV activation and cyanogen bromide activation, respectively. Reverse transcriptases from mouse and chicken oncogenic RNA viruses were found to bind to both types of affinity columns, requiring high salt for elution. A 50-fold purification of the Rauscher leukemia virus (RLV) enzyme was achieved on a poly(G)-Sepharose column in one step. The effect of salt, buffers, detergent, Mg²⁺, EDTA, bovine serum albumin (BSA), and dithiothreitol (DTT) on the stability of the RLV enzyme was determined. NP 40, BSA, and DTT stabilize the enzyme, but EDTA inactivates it.

The behavior of normal cellular DNA polymerases on affinity columns was investigated. DNA polymerases from normal mouse liver and spleen were purified by affinity chromatography on DNA-cellulose columns. The high-molecular-weight DNA polymerase (cytoplasmic) does not bind to DNA-cellulose. This enzyme has no RNA-dependent activity. However the small-molecular-weight DNA polymerase, which has RNA-dependent activity, binds to and can be eluted from DNA-cellulose. This enzyme from DNA-cellulose also binds tightly to poly(G)-Sepharose, as do the viral enzymes. *E. coli* DNA polymerase (which also can use RNA as a template) also binds to poly(G)-Sepharose. The high-molecular-weight, DNA-dependent DNA polymerase activity from mouse spleen, isolated by gel filtration, is completely lost on poly(G) columns.

A simple procedure was developed to assay for RT activity in cells. Because mammalian (1) and avian RT (2) are inhibited by poly(G) and have a high affinity for it, poly(G)-Sepharose columns can be used to remove

RNA-dependent DNA polymerases from cellular extracts. We chose virus-containing spleens from RLV-infected mice as a model system. Poly(G)-Sepharose is resistant to nucleases in spleen extracts under the conditions of column chromatography. The cells or tissues to be examined are homogenized in detergent and low salt in the presence of DNase I and pancreatic RNase. After incubation the extract is brought to 0.55 M KCl and centrifuged. The viscous supernatant is diluted to 0.30 M KCl, mixed thoroughly, and the precipitate removed by centrifugation. After a twofold dilution the extract is applied to a poly(G)-Sepharose column and the DNA polymerases are eluted with NaCl. Viral RT can be distinguished from cellular enzymes by their template specificities. Viral enzymes prefer poly(rA·rU) and poly[rA·(dT)₁₀], whereas cellular enzymes prefer DNA and poly(rA·dT). Also, viral RT is inhibited by polynucleotides (1, 2) and cellular enzymes are not.

*NIH Postdoctoral Fellow.

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REGULATION OF VIRUS EXPRESSION

J. N. Ihle,* J. G. Farrelly, R. W. Tennant,
and F. T. Kenney

We have developed a number of techniques and experimental systems in an attempt to determine the biochemical parameters governing expression of the oncogenic RNA viruses. Our first interest was in finding a method to analyze quantitatively the various aspects of virus expression. To this end, we purified the GS-1 antigen from Rauscher leukemia virus to near homogeneity, using a combination of Sephadex chromatography and ethanol precipitation of protein obtained from deoxycholate-disrupted virus. This new technique allows large-scale purifications with relatively good yields as compared to those obtained from previously published procedures. We are now preparing antibodies to these preparations for use in radioimmune assays. These techniques should allow us to measure the initiation of virus expression (as measured by synthesis of the GS-1 antigen as described below) with improved resolution.

Next, we worked out optimal reaction conditions for the protein kinase associated with Rauscher leukemia virus, and plan to study the role of this enzyme in regulation of virus expression.

Finally, we have begun experiments to define conditions that will allow us to detect viral DNA synthesis *in vivo*, following infection. To date we have developed techniques (1) for obtaining large quantities of radioactively labeled viral 70S RNA to use as a probe for viral DNA, and (2) for partial resolution of cytoplasmic and nuclear DNA fractions via sucrose gradient. When completed, these techniques should provide information basic to understanding the mechanism of viral DNA synthesis *in vivo*, and they will also provide a necessary "marker" event during viral infection.

These methods, used with those developed in this laboratory for analysis of the viral reverse transcriptase, will provide sensitive assays for specific virus functions. The assays will be correlated with physiological and genetic data to be obtained as described below.

We have established several experimental systems to determine the general biochemical levels at which regulation of viral expression can occur, and to develop further operational distinctions between the various aspects of viral expression. In particular, in a 3T3 cell system using Moloney leukemia virus, we have confirmed observations by others with Rous sarcoma virus that, when serum-starved cells are infected with virus, a stable "provirus" is formed. The subsequent expression of the provirus is dependent upon addition of serum, followed by cell division. This observation allows one to distinguish early events (provirus formation) from late events (virus expression), and thus study the biochemical requirements of each independently of the other.

We have also established a culture line of AKR cells which, upon treatment with iododeoxyuridine (IdUrd) are "activated" to express endogenous virus (as described by others). This system will allow us to study regulatory mechanisms in virus expression presumably divorced from such events as viral DNA integration and the presence of regulatory proteins in the virion introduced during infection or synthesized from viral RNA immediately after infection. Experimental data, so far, indicates that cell division is required for activation of endogenous viral expression. Using inhibitors of DNA synthesis, we have demonstrated that this requirement is complex. Initially, DNA synthesis is required for activation, suggesting that IdUrd must be incorporated to effect activation. However a second period of DNA synthesis is subsequently required to allow virus expression once activation has occurred. These data provide a correlation between exogenous and endogenous virus expression and suggest that the provirus state and the activated state may be identical. More specifically, they suggest that the requirement for a cycle of cell division in exogenous viral infection is

probably not related to viral DNA integration but is specifically required for subsequent expression of the viral genome.

Lastly, to obtain experimental systems with more predictive value in determining the specific biochemical parameters governing virus expression, we have initiated the production and isolation of temperature-sensitive mutants of Moloney leukemia virus.

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INHIBITION OF MURINE LEUKEMIA VIRUS DNA POLYMERASE BY SINGLE-STRANDED POLYRIBONUCLEOTIDES

J. G. Farrelly, L. E. Roberson, J. N. Ihle,*
R. W. Tennant, and F. T. Kenney

We are continuing our study of polynucleotides as inhibitors of the RNA-directed DNA polymerase of Rauscher leukemia virus. Previously it was determined that single-stranded polyribonucleotides severely inhibited the viral polymerase whether endogenous RNA, DNA, or synthetic polymers such as the alternating copolymer poly[d(A-T)] are used as templates (1). These homopolymers did not inhibit DNA polymerase purified from mouse embryos or from *E. coli*. By further study of inhibition of poly(A) we determined the chain length necessary for the inhibition of the viral polymerase. Poly(A) was subjected to controlled base hydrolysis, and oligonucleotides were produced which subsequently were separated on DEAE-Sephadex. Oligonucleotides up to 12 nucleotides in length were tested as inhibitors of the viral enzyme. It was found that tetramers were ineffective while octomers and larger oligomers were as effective inhibitors as is poly(A).

We have shown that poly(A) inhibits the replication of Moloney leukemia virus (MLV) in mouse embryo cells while it has no effect on cellular DNA synthesis or growth (2). To protect the polynucleotide against the action of cellular nucleases we have prepared 2'-O-methyl poly(A) [poly(Am)] and have tested it as an inhibitor of viral polymerase and of viral replication. Poly(Am) was prepared from 2'-O-methyl ADP by the action of polynucleotide phosphorylase and was separated from unreacted 2'-O-methyl ADP by Sephadex G-150 chromatography. Using poly[d(AT)] as a template, the K_i for poly(Am) as an inhibitor of RLV polymerase (30 μM) was very similar to that for poly(A) - 20 μM (2). Poly(Am) was, however, found to be a more efficient inhibitor of viral replication than

poly(A). At 2000 $\mu g/ml$, poly(A) inhibited replication of MLV in mouse embryo cells to $\sim 90\%$. At 16 $\mu g/ml$ of poly(Am), the extent of inhibition was about 90% while at 48 $\mu g/ml$ inhibition of viral replication approached 100%. We are now preparing large quantities of poly(Am) and other methylated polynucleotides to use in testing whether the inhibitor has an effect on viral functions *in vivo*. We are also conducting tests to determine whether poly(A) or poly(Am) has any effect on the iododeoxyuridine activation of AKR cells and the role of reverse transcriptase, if any, in this process.

*American Cancer Society Postdoctoral Fellow.

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SUPPRESSION OF MOLONEY LEUKEMIA VIRUS SYNTHESIS BY POLYADENYLIC ACID

R. W. Tennant, F. T. Kenney, and F. W. Tuominen*

A previous report from this laboratory (1) demonstrated that the DNA polymerase of Rauscher leukemia virus is strongly inhibited *in vitro* by unprimed, single-stranded polyribonucleotides as a result of competition between the polymers and the active template for the same enzyme-binding site. Thus, we attempted to determine whether these compounds would have any effect on the replication of murine leukemia virus in cell cultures. The results showed that polyadenylic acid reproducibly inhibited Moloney leukemia virus and that the inhibition was not due to any observable effect on the growth and division of the host cells. Treatment with polyuridylic or polycytidylic acid resulted in less inhibition. Furthermore, polyadenylate was effective only when it was added before or during the early stages of infection. The dose-response data did not reveal a direct relationship to the concentration of polyadenylate but rather suggested a complex interaction between cellular uptake of the compound and inhibition of virus replication. The observations support the interpretation that the effect of polyadenylate on leukemia virus replication is due to specific inhibition of viral DNA synthesis.

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SYNERGISTIC EFFECT OF HUMAN TISSUES ON RAUSCHER LEUKEMIA VIRUS FREE OF LACTIC DEHYDROGENASE VIRUS

R. L. Tyndall, J. A. Otten, and R. W. Tennant

The erythroid hyperplasia, immunosuppression, and leukemic syndrome that follow infection with Rauscher leukemia virus (RLV) have been shown to be dependent on the contaminating lactate dehydrogenase virus (LDV) in stocks of RLV (1). Using specially developed stocks of RLV free of LDV, studies were undertaken to test the capacity of human leukemic tissues or tissue extracts to interact synergistically with RLV.

All of three samples of human leukemic tissue preparations synergistically interacted with LDV-free RLV on inoculation of adult BALB/c mice. Neither RLV alone nor human leukemic cells alone produced any disease or viremia, but the combination produced a marked, typical Rauscher syndrome 30 days after inoculation (Table I). In collaborative studies with Dr. R. A. Steeves, of Roswell Park Memorial Institute, cell-free ultracentrifugates of three additional human leukemic tissues were tested for their synergistic interaction with RLV. These samples had been previously tested by Dr. Steeves for their ability to "rescue" the

focus-forming leukemia virus associated with the Friend leukemia virus complex. In combination with our RLV, the one of three samples that was negative in Dr. Steeves' tests was positive. However, the other two samples, positive in Dr. Steeves' tests, were poorly reactive with our LDV-free RLV. Whether such complementary patterns will be maintained in further collaborative experiments is of interest. Studies with blood cells from normal human donors are also under way. These results indicate that some, but not all, such samples also reacted synergistically with LDV-free RLV although the reaction was less pronounced than that with leukemic human cells (Table I).

The degree of the synergistic reaction of normal vs. leukemic human tissues with LDV-free RLV is under study. Whether the basis of the synergism is one of activation of target cells for RLV replication, activation of endogenous LDV, or the unlikely possibility of infectious agents in the human tissues is also being investigated.

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TABLE I. Synergistic effect of various inocula on LDV-free RLV*

Inocula	No. of mice	Time between inoculation and termination of experiments (days)	Avg. spleen wt (mg)	Sera titer of RLV [†] at termination of experiments
RLV	7	25	96	<10
Human leukemic tissue				
Sample 1	8	25	119	<10
1 + RLV	7	25	1323	15 × 10 ⁴
Sample 2	8	27	102	NT [‡]
2 + RLV	4	27	1024	NT
Sample 3	4	25	108	NT
3 + RLV	4	25	1913	NT
Normal human blood cells				
Sample 1	4	27	105	<100
1 + RLV	8	27	367	<100
Sample 2	4	27	108	<100
2 + RLV	8	27	878	11 × 10 ⁵

*RLV and test inocula injected concomitantly in adult BALB/c mice.

[†]As determined by XC test (No. of foci/0.1 ml)

[‡]NT = not tested

A COMMON ANTIBODY IN THE SERA OF PREGNANT AND TUMOR-BEARING RATS

R. L. Tyndall and J. A. Otten

The isoenzymatic and physiologic similarities of fetal and leukemic murine tissues *per se* (1, 2) and the similarities in the electrophoretic profiles of serum protein from mice bearing such tissues (3) prompted a search for antibody(ies) common to sera of pregnant and tumor-bearing animals. If found, it would indicate that tumor-bearing animals might respond immunologically to their tumors as pregnant animals do to fetal tissues, and would provide a model system for studying the ramifications of such a response.

After a thorough screening of a variety of viruses and fetal preparations, a crude antigen, associated with semipurified pellets of RLV from transformed JLS V5 cells, was shown to precipitate a common antibody from sera of pregnant and tumor-bearing rats. The precipitin tests in micro-Ouchterlony plates showed lines of identity when sera from pregnant rats and from rats bearing transplantable sarcomas or leukemia were reacted with the RLV-associated antigen (Fig. 1). Sera

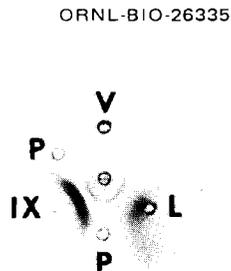


Fig. 1. Precipitin line common to sera from leukemic (L) and pregnant (P) rats but absent in sera from virgin (V) rats.

from virgin rats did not react. These precipitin reactions extend, to a serologic parameter, the previously described similarities in electrophoretic profiles of sera from animals responding to pregnancy and neoplasia. We are currently studying the effects of immunization with the RLV-associated antigen in regard to the level of antibody response obtainable and, most importantly, the effect of such antibody on a variety of spontaneous, viral, and chemically-induced neoplasia.

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CYTOTOXIC FACTORS IN HUMAN SERA ABSORBABLE BY AND DISRUPTIVE TO MURINE LEUKEMIA VIRUSES

R. L. Tyndall, J. A. Otten, and N. D. Bowles

In the course of characterizing sera from normal, pregnant, and leukemic human donors we observed that most human sera were cytotoxic for a variety of cultured mouse cells. Sera at dilutions of $\frac{1}{4}$ or $\frac{1}{8}$ were generally toxic for the murine leukemia virus (MuLV)-infected JLS V6 and JLS V5 cells, and for uninfected BALB/c 3T3 cells and 3T3 cells transformed by the Kirsten sarcoma virus. In view of the prevalent expression of leukemia virus information and/or complete virus in many cultured mouse cells, attempts were made to absorb the cytotoxic factors with MuLV. Both semipurified preparations of Rauscher leukemia virus (RLV) and purified preparations of Gross leukemia virus were capable of absorbing the cytotoxic factors from human sera.

Further studies of the absorbed sera showed them to be precipitable by rat anti-MuLV antisera or monkey anti-RLV antisera but not by normal rat or monkey sera. That the precipitin reaction, in micro-Ouchterlony plates, between MuLV-absorbed sera and anti-MuLV antisera was due to soluble MuLV antigens in the absorbed sera was indicated by a line of identity between absorbed sera and deoxycholate-disrupted MuLV. Heating the human sera for 30 min at 56° C prior to MuLV adsorption abolished the precipitin reaction with MuLV antisera.

These results indicate that normal human sera contains factors disruptive to MuLV. It is of obvious importance to determine if these factor(s) are also disruptive for "C" type viruses of human origin and to determine if their presence or absence is related to human disease. In this regard it is interesting to note that preliminary analysis of sera from humans with leukemia or lymphosarcomas showed diminished reactivity with anti-MuLV antisera following adsorption with MuLV. Whether such differences between sera from normal and diseased human subjects persist in more extensive analysis will be the focus of future studies.

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