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BIOLOGY DIVISION ANNUAL PROGRESS REPORT

Period Ending June 30, 1973

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ANNUAL PROGRESS REPORT
For Period Ending June 30, 1973

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

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OCTOBER 1973

OAK RIDGE NATIONAL LABORATORY
Oak Ridge, Tennessee 37830
operated by
UNION CARBIDE CORPORATION
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U.S. ATOMIC ENERGY COMMISSION

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INTRODUCTION

This volume is devoted primarily to a collection of short summaries of the many research projects in the Division. We hope that it is useful to those who want an overview of our activities. It is necessary, of course, to contact the investigators or their pertinent publications for more complete information.

In addition to the research activities of the Division, it should be noted that many supporting programs are developing rapidly. In spite of uncertainties about the future funding of pre- and postdoctoral training, the University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences continues to flourish and to attract outstanding students who are a credit to the University and to the research laboratories in which they work.

During the course of the year, the Division has been privileged to hear many excellent seminars from visiting investigators as well as its own staff. The Division sponsored a well-received symposium on chromosome structure, and publication of the volume resulting from this meeting is expected shortly. Plans are currently well under way for the next symposium in this series. The topic is "Genetic mechanisms of recombination."

The Division continues to follow closely developments in its sponsoring agencies. Perhaps the most currently significant trend is the movement of the Atomic Energy Commission towards a more generalized energy research and development agency. The Biology Division is well equipped to fulfill its role in this expanded program. Initial activities in this direction are reflected in some of the reports contained in this volume. In the future we expect to become even more heavily involved in biological studies related to the generation and conservation of energy produced by nuclear and nonnuclear means. We expect to continue to have our research cover the entire range of activities from the most basic studies to those that help in the decision making and standard setting required of our supporting agencies.

Howard I. Adler
Director, Biology Division

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KINETICS OF ALBUMIN SYNTHESIS BY CULTURED MOUSE HEPATOMA CELLS

B. E. Ledford and John Papaconstantinou

The differentiation of eucaryotic cells into specific tissues is usually accompanied by the acquisition of morphological and biochemical specificity. In most cases these highly differentiated cells can synthesize large quantities of tissue-specific structural proteins. This is clearly seen in the synthesis of crystallins in the lens,[1] globin in the reticulocyte,[2] albumin in the liver,[3] and other highly differentiated tissues.

The initiation of synthesis of tissue-specific proteins is the basic regulatory event underlying the ability of eucaryotic cells to express specific genes during cellular differentiation. Regulation of protein synthesis encompasses both transcriptional and translational control, and both of these events are important in the initial regulation of differentiation as well as in the regulation of the level of gene expression as is commonly seen in the highly differentiated cell under various conditions of growth and nutrition. We are interested in studying the mechanisms controlling gene expression in eucaryotic cells during differentiation. Cultured cells that retain some of the genotypic properties of the tissue of origin by synthesizing tissue-specific proteins are of particular value in studying the role of factors such as metabolites, hormones, vitamins, or growth factors involved in the regulation of protein synthesis.

In recent years a number of tumor-derived cell lines have been established that synthesize tissue-specific proteins indefinitely. For example, melanomas will synthesize large amounts of pigment,[4] hepatomas synthesize and secrete serum proteins,[5,6] and the induction of tissue-specific enzyme and protein synthesis is observed in hepatomas,[7] neuroblastoma,[8] and leukemia cells.[9] One of these cell lines was established from the mouse hepatoma BW7756 and has been shown to maintain a high level of albumin synthesis in long-term culture.[10] We have used these cultures to study the regulation of tissue-specific protein synthesis. In our studies we have shown that albumin and α-fetoprotein are synthesized and secreted into the culture medium; albumin synthesis occurs at *in vivo* levels and is regulated by the rate of cellular replication.[11]

Identification of albumin as a component of the proteins secreted by Hepa. One of the characteristics of this cell line is that it secretes large amounts of protein into a serum-free medium. To identify and characterize these proteins, a polyacrylamide gel electrophoretic analysis was done. The complex protein solution was resolved into three distinct major bands (I, II, and III) and several minor bands. A qualitatively similar radioactive profile is obtained from the secretion of cells incubated in the presence of [^{3}H]leucine, showing that the proteins are actively synthesized by the Hepa cells.

Identification of the albumin fraction was achieved by the use of specific precipitation with mouse albumin antiserum. Treatment of the radioactive sample with this antiserum specifically eliminates the protein in band I. In addition, this protein has a molecular weight of 67,000, undergoes coelectrophoresis with mouse albumin, and has the solubility properties of albumin.

Rate of intracellular albumin synthesis and secretion. The rate of total protein synthesis, albumin synthesis, and albumin secretion was determined. Incorporation of [^{3}H]leucine into total protein rapidly becomes linear (3-min lag period) and remains linear throughout the labeling period (160 min); [^{3}H]leucine was also incorporated linearly into intracellular albumin for a period of 60 min. After 60 min the labeling of intracellular albumin levels off, indicating that the small intracellular albumin pool has been completely labeled. With respect to secretion, no [^{3}H]albumin appeared in the medium until 45 min after the addition of [^{3}H]leucine. This lag period represents the time required for synthesis and transport through the secretory apparatus of the cell. The slope of the rate of secretion is approximately 10% of that of the total incorporation, which is comparable to the ratio of albumin to total protein synthesis observed in intact rats by Peters and Peters.[3] In addition, the slope of the secretion is approximately equal to the slope of intracellular incorporation, indicating that the rate of secretion and synthesis is comparable.

The effect of cellular growth rate on albumin synthesis. Our preliminary studies have indicated that the rate of albumin synthesis is linked to the rate of cellular growth.[11] Similar observations have been reported by Bernhard et al.[10] Our studies have shown that during rapid growth (logarithmic growth) the cellular rate of albumin synthesis is approximately 20,000 molecules/cell/min. When the cells approach stationary phase (growth rate slows down), there is an abrupt increase in the cellular rate to 60,000 molecules/cell/min, and in stationary phase a further increase to 100,000 molecules/cell/min is reached.

On the basis of these observations, the generation time of Hepa cells in log phase was increased by the

addition of dibutyryl cAMP to the medium, and the rate of albumin synthesis was determined. This treatment increased the generation time twofold and stimulated the cellular rate of albumin synthesis to levels observed in stationary cells.

From these studies we conclude the following: (1) albumin mRNA synthesis is discontinuous; it occurs for a specific length of time during a specific phase of the cell cycle; (2) increasing the generation time increases the duration of the period of mRNA synthesis, thereby increasing the rate of albumin synthesis. We are presently attempting to synchronize the Hepa cells to determine whether our model is correct.

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EXPRESSION OF FETAL GENES BY MOUSE HEPATOMA CELLS IN CULTURE: THE SYNTHESIS OF α-FETOPROTEIN

B. E. Ledford and John Papaconstantinou

Fetal development in a broad spectrum of animal species including man and rodents is accompanied by the synthesis of a fetal-specific α-globulin, α-fetoprotein (αFP).[1-4] This α-globulin is a major component of fetal serum, and the yolk sac cells and fetal liver parenchymal cells are the site of synthesis of αFP.[4,5] The concentration of αFP peaks during embryonic development and drops rapidly in the early postnatal period; αFP is virtually absent in the serum of adult animals.

The synthesis of αFP is reinitiated in adult animals during liver regeneration, in animals afflicted with a primary hepatocellular carcinoma, in embryonic teratocarcinoma, and in viral hepatitis.[5] It is of interest to us that the level of αFP can range from a low level of 0.5% of the fetal level in regenerating liver to 100% of the fetal level in hepatocellular carcinoma.[5] We believe that this range of variation of αFP levels requires transcriptional and/or translational controls, and we propose to use the synthesis of αFP as a model for our studies on the control of tissue-specific protein synthesis.

The mouse hepatoma cell line, Hepa, secretes a variety of serum proteins.[6,7] Of these, albumin has been identified as a major component of the secreted proteins. In this phase of our research program, we have established that a second major component of the secreted proteins is αFP.

Identification of α-fetoprotein as a component of the proteins secreted by Hepa. When Hepa-secreted proteins are treated with antiserum to adult mouse serum, one major component of the mixture is not precipitated. The electrophoretic mobility of this protein places it just behind the albumin fraction, that is, in the position characteristic of αFP.

In order to identify this protein, a monospecific antiserum to it was prepared. This antiserum was used in an immunoelectrophoretic analysis of adult mouse serum, fetal serum, and Hepa secretion. The antiserum reacted only with protein from fetal mouse serum and from Hepa secretion. None of the protein was detectable in adult mouse serum.

An estimation of the molecular weight of this fetal-specific protein was obtained from SDS–acrylamide gel electrophoresis. A molecular weight of 70,000 was obtained. This is in excellent agreement with the value obtained for human αFP.[8] From these studies, we conclude that in addition to albumin one of the major components of the Hepa secretory proteins is αFP.

To date, our studies with the mouse hepatoma cell line, Hepa, have indicated that these cells have the ability to express both adult-specific (albumin synthesis) and fetal-specific (αFP synthesis) characteristics. We propose to study the mechanism of control of synthesis of these proteins in replicating and stationary cells and in synchronized cells.

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HEMOGLOBIN SYNTHESIS IN A MURINE LEUKEMIA CELL LINE

P. R. McClintock,* John Papaconstantinou, and R. A. Popp

We are in the process of studying the *in vitro* differentiation of a clone of murine leukemic cells. This clone (clone 745), first described by Friend et al.,[1,2] acquires some erythroid characteristics when cultured in the presence of low concentrations of dimethyl sulfoxide (DMSO). After 48 hr of exposure to 1–2% DMSO, the cells begin to synthesize hemoglobin and continue to do so for at least 72 hr longer.

The induction of hemoglobin synthesis requires at least one round of mitosis.[3,4] Hemoglobin synthesis does not begin if the DMSO is added to cultures that are in stationary phase, and it can be delayed by postponing the first two cell divisions with 1 m*M* $N^6,O^{2'}$-dibutyryl adenosine 3′:5′-cyclic monophosphate. No induction is seen when the cells are treated with erythropoietin (0.2 unit/ml) or etiocholanolone (10^{-7} *M*), indicating that these cells are derived either from cells beyond the proerythroblast stage or from nonerythroid cells.

At present we are examining the hemoglobins produced by these cells to determine which of the globin chains are being synthesized.[5] This is being done by isotopic labeling and partial sequence analysis. Results obtained so far indicate that there is no detectable synthesis of embryonic or fetal globins.

In the future we plan to study the mechanism of induction by DMSO and the time of synthesis of hemoglobin, globin mRNA, and the structural genes for globins with regard to phases of the cell cycle. We will also examine events taking place at the nuclear level, especially with regard to alterations in RNA patterns and chromosomal protein patterns.

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SYNTHESIS OF MITOCHONDRIAL DNA IN SPERMATOCYTES OF *RHYNCHOSCIARA HOLLAENDERI*

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

The morphological changes seen in the mitochondria of insect spermatocytes are highly specific for these cells and occur at specific stages during spermatogenesis. In a variety of insects, these changes are first evident when the mitochondria appear as aggregates of small granular particles located in specific regions of the cell. As development of the spermatocyte proceeds, mitochondria acquire different shapes such as spheres, rods, or threads and are scattered throughout the cytoplasm.[1] Such specific mitochondrial differentiation is observed in both *Rhynchosciara hollaenderi*[2] and the closely related dipteran, *Sciara coprophila.*[3,4] In the initial stages of spermatogenesis, the mitochondria appear as discrete granules closely associated with the nucleus; they enlarge, occupying practically the entire cytoplasmic area, and then are arranged along the spermatid flagellum, where they ultimately fuse to form a single mitochondrial derivative extending from the nucleus to the tip of the tail of the mature spermatid.

In a previous publication,[5] we presented evidence for a specific pattern of DNA synthesis concurrent with the morphological changes in the spermatocyte mitochondria. The synthesis of a circular DNA (ρ = 1.681 g/cm^3) is first detectable when the mitochondria become visible as granules aggregated around the nuclear envelope. As the mitochondria increase in size, the synthesis of this DNA becomes a major part of the total DNA synthesized, and when meiosis is initiated, 80% of the total DNA synthesized is this circular DNA. Since mitochondrial DNA is usually extracted as circular molecules and since the circular DNA we have described is preferentially synthesized during mitochondrial differentiation, studies were undertaken to determine whether this DNA is associated with the mitochondria and to characterize the physicochemical properties of this circular DNA.

An attempt was made to make a pure preparation of spermatocyte mitochondria and to characterize the DNA from this fraction. Because of their unique morphology, we were unable to purify these mitochondria by any of the standard procedures. Cells were homogenized and an 800-g pellet was prepared. Light and electron microscopy indicated that the pellet consists of mitochondria and membrane fragments. A DNA preparation of this pellet indicated that main band DNA (chromatin) is also present in the pellet. To

determine if the circular DNA was localized in the mitochondria, the 800-g pellet was treated with DNase to remove DNA associated with the membrane fragments or adhering to the outside of the mitochondria. After this treatment, only circular DNA and a linear satellite (nicked circular DNA) were resolved from the DNA extracted. We conclude from these studies that the mitochondria are the source of the circular DNA extracted from *Rhynchosciara* spermatocytes.

One of the more interesting observations we have made in these studies is that the circular DNA synthesized in *Rhynchosciara* spermatocytes has a contour length of 9 μm, which is equivalent to a molecular weight of 18×10^6 daltons. To date, mitochondrial DNA of all metazoan species examined is in the form of circular molecules with a contour length of approximately 5 μm. This is also true with respect to insects, where mitochondrial DNA isolated from *Musca* has a length of 4.6 μm to 5.6 μm[6] and from thoracic muscles of *Schistocerca gregaria* has a contour length of 4.74 μm.[7] In *Rhynchosciara* spermatocytes, however, the only circular DNA we detect is 9 μm in contour length. The possibilities exist therefore that (1) all mitochondrial DNA of *Rhynchosciara* has a contour length of 9 μm, (2) only the mitochondria of the spermatocytes have DNA of 9 μm contour length, or (3) the mitochondrial DNA of the spermatocytes represents circular dimers of a 4.5-μm form. Such circular dimers occur in a low frequency in some normal tissues.[8] Nass[9] has shown that 8% of the mitochondrial DNA in L-cells harvested in logarithmic growth are circular dimers and that interlocked or catenated forms are more commonly observed. In stationary L-cells the circular dimer frequency increases to 82%. Since the *Rhynchosciara* spermatocytes are also in a prolonged stationary phase, it is possible that this status favors dimer formation. We are presently engaged in extracting mitochondrial DNA from dividing spermatocytes, somatic tissues, and oocytes to answer the possibilities posed above.

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

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THE EFFECT OF pH ON THE BUOYANT DENSITY OF RABBIT DNA IN Ag^+-Cs_2SO_4 GRADIENTS

E. F. DuBrul and John Papaconstantinou

The theoretical basis for the specific binding of Ag^+ to DNA was developed by Jensen and Davidson[1] in their studies on the binding of this metal ion by various DNA's over a pH range from 5.6 to 8.0. These authors showed that the binding of Ag^+ to DNA is pH dependent and that GC-rich DNA's bind more Ag^+ than do AT-rich DNA's. In addition it was shown that as more Ag^+ is added to the DNA its buoyant density increases, thus making possible good separation between GC-rich and AT-rich DNA's in Cs_2SO_4 equilibrium density gradient sedimentation. On the basis of this work, Ag^+-Cs_2SO_4 isopycnic density gradients have been used effectively for the isolation of specific sequences in eucaryotic DNA.[2-4] When this Ag^+-Cs_2SO_4 technique was applied to the fractionation of eucaryotic DNA at pH's greater than 8.0, the results indicated that Ag^+ binds preferentially to AT-rich segments of the genome, whereas at pH's lower than 8.0, Ag^+ appears to bind preferentially to GC-rich segments. For example, Corneo et al.[2,3] have fractionated mammalian DNA into main band and satellites at pH 9.2 and found that some satellites that appear to be AT-rich in Ag^+-Cs_2SO_4 are in fact GC-rich on the basis of T_m and buoyant density in CsCl. In addition,

Suzuki et al.[4] have localized the DNA sequences complementary to silk fibroin mRNA in DNA fractionated by Ag^+-Cs_2SO_4 equilibrium density gradient sedimentation at pH 9.2. The fibroin mRNA is GC-rich, yet it hybridizes to DNA in the less dense region of these Cs_2SO_4 gradients; the fibroin genes are GC-rich on the basis of CsCl analysis. Suzuki et al.[4] suggest that this GC-rich fibroin DNA exhibits unusual behavior in Ag^+-Cs_2SO_4. We have also found that the DNA complementary to putative globin mRNA (52.7% GC) is localized in the less dense region of a Ag^+-Cs_2SO_4 gradient at pH 9.2.[5] However, when the entire gradient was analyzed, it become apparent that the positions of AT-rich and GC-rich DNA's were reversed in the Cs_2SO_4 gradient. Furthermore, we have been able to demonstrate that the positions of AT-rich and GC-rich DNA in a Ag^+-Cs_2SO_4 density gradient are pH dependent and that the positions can be reversed by changing the pH. Our observations explain the reversed position of high GC containing silk fibroin genes[4] and rabbit globin genes[5] localized in Ag^+-Cs_2SO_4 gradients at pH 9.2.

It is not clear what controls the amount of silver bound per unit of DNA, but one is led to think that the arrangement of bases in the DNA and the tertiary structure of the DNA are equally as important as the overall base composition. This is strongly suggested by the results of Corneo et al.[2,3] in their studies of guinea pig satellite DNA in which they found that satellites with the same GC content behave differently in Ag^+-Cs_2SO_4. Thus, fractionation of a genome in Ag^+-Cs_2SO_4 at different pH's may prove useful in separating DNA molecules that have similar GC content but in which the bases are differently arranged.

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ASCORBIC SULFATE METABOLISM AND ITS RELATION TO VITAMIN C

F. J. Finamore, A. D. Bond, and Rose P. Feldman

The natural occurrence of a sulfated derivative of vitamin C was first demonstrated by this laboratory in extracts of *Artemia salina* embryos; recently it has been identified as a metabolite in rat, guinea pigs, fish, rabbits, and man. Our laboratory has succeeded in chemically synthesizing a pure product that is identical to the naturally occurring compound, and, in collaboration with J. R. Einstein and B. R. McClelland of this Division, the crystalline compound was subjected to x-ray analysis. The results obtained from x-ray crystallography indicate both the synthetic and natural products to be ascorbic-2-sulfate and not the 3-isomer as most investigators supposed.

Our particular approach to the function of this sulfated derivative of vitamin C has taken several directions. First, we have found that ascorbic sulfate cannot substitute for ascorbic acid in preventing scurvy in guinea pigs. These results suggest the animal apparently does not contain an effective sulfatase for the conversion of ascorbic sulfate to ascorbic acid. Secondly, during development of *Artemia salina,* we observed that the concentration of ascorbic sulfate falls sharply at the time of emergence and hatching concomitant with an increase in the concentration of ascorbic acid. However, the quantity of ascorbic acid that appears is twice what can be accounted for by the removal of the sulfate moiety from ascorbic sulfate. To date we have not been able to demonstrate, either *in vivo* or *in vitro*, the existence of the sulfatase that is necessary to convert the sulfated derivative to free ascorbic acid. This latter observation prompted us to consider another possibility, namely, that ascorbic sulfate may not be a *direct* precursor of ascorbic acid in brine shrimp and that its disappearance is due to the fact that it is converted to another but distinctly different compound. Our search for such a modified derivative led us to the isolation and purification of a compound that exhibits many chemical characteristics that are identical to ascorbic acid itself. Data from mass spectroscopy and elemental analysis indicate the compound has a molecular weight of 204 and an empirical formula of $C_8H_{12}O_6$. The similarities between this compound and ascorbic acid can be summarized as follows: both reduce Ag; they have the same p*K* (4.2) and therefore behave similarly during anion exchange column chromatography; their Rf's are identical in four different solvent systems in thin layer chromatography; they both are destroyed by charcoal treatment; they are enolic in nature; each contains four OH groups. As far as differences are concerned, these may be listed as follows: both ultraviolet and infrared spectra are different; the unknown compound is stable to alkali whereas ascorbic acid is not.

We are currently in the process of making a structural assignment to this new and potentially interesting relative of ascorbic acid.

THE USE OF DINUCLEOSIDE MONOPHOSPHATES IN THE ELUCIDATION OF RNA INITIATION (DNA PROMOTER?) SEQUENCES DURING T4 DNA TRANSCRIPTION

D. J. Hoffman and S. K. Niyogi

The effects of dinucleoside monophosphates on the transcription of phage T4 DNA by *E. coli* RNA polymerase have been examined at various concentrations of the sigma subunit and extremely low ribonucleoside triphosphate concentration. (1) The results show that labeled specific dinucleoside monophosphates are incorporated as chain initiators. (2) When the ratio of sigma factor to core enzyme is small, there is a general stimulation by most 5′-guanosyl dinucleoside monophosphates. (3) When the ratio is increased or holoenzyme is present, ApU, CpA, UpA, and GpU are the most effective stimulators. (4) At high concentrations of sigma, only certain adenosine-containing dinucleoside monophosphates (ApU, CpA, UpA, and ApA) stimulate the reaction. (5) Competition hybridization studies indicate that the dinucleoside monophosphate (ApU, CpA, UpA, and GpU)-stimulated RNA's are of the T4 "early" type, suggesting that these compounds act at T4 "early" RNA initiation (DNA promoter?) regions. (6) Studies involving both combinations of stimulatory dinucleoside monophosphates and competitive effects of these compounds on chain initiation by ATP and GTP suggest that the stimulatory dinucleoside monophosphates act as chain initiators and may recognize part of a continuous sequence in a promoter region. (7) Studies involving the incorporation of ^{3}H-labeled stimulatory dinucleoside monophosphates and certain selected trinucleoside disphosphates support the above conclusions. A possible, although speculative, T4 RNA initiation (DNA promoter?) sequence that is compatible with our results is given below:

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

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

DIFFERENTIAL EFFECTS OF σ FACTOR, IONIC STRENGTH, AND RIBONUCLEOSIDE TRIPHOSPHATE CONCENTRATION ON THE TRANSCRIPTION OF PHAGE T4 DNA WITH THE RIBONUCLEIC ACID POLYMERASE OF *ESCHERICHIA COLI*

D. J. Hoffman and S. K. Niyogi

The effects of concentration of the σ subunit of *Escherichia coli* B RNA polymerase on the transcription of phage T4 DNA have been examined under high and low salt and substrate concentrations. The following conclusions were reached:

1. At normal substrate level, KCl stimulates the polymerase reaction with T4 DNA template largely by promoting σ activity, even when the quantity of σ factor present is very small. (In the case of calf thymus DNA template, where σ had little stimulatory effect, KCl had no enhancement effect.)
2. σ factor stimulates the initiation of chains beginning with ATP rather than with GTP. This is evident both from substrate-dependence studies and incorporation studies using [γ-^{32}P] ATP and [γ-^{32}P] GTP.
3. The σ-dependent ATP initiations were further promoted by KCl.
4. At extremely low substrate levels, an inhibitory effect of KCl is predominant although σ remains stimulatory. This inhibition occurs at the level of chain initiation.

PROPERTIES OF A NEW RIBONUCLEASE FROM *E. COLI*

S. K. Niyogi and Brenda H. Underwood

Several years ago a new ribonuclease activity was isolated from *E. coli.*[1] We have now purified this enzyme further and shown that it is distinct from RNase I, RNase II, RNase III, and polynucleotide phosphorylase. This enzyme has a marked specificity for oligoribonucleotides as substrates, the activity being inversely proportional to the chain length of the oligoribonucleotide. The enzyme activity is inhibited by secondary structure; oligoribonucleotides combined with complementary polynucleotides are poorly attacked below the T_m of the complex and efficiently above the T_m. The products are 5′-nucleotides liberated from the 3′-end of the substrate.

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1. A. L. Stevens and S. K. Niyogi, *Biochem. Biophys. Res. Commun.* 29, 550 (1967).

ROLE OF *E. COLI* dna GENES IN THE REPLICATION OF M13 PHAGE AND ITS DNA

Priyatosh Mukherjee, D. R. Stallions, and Sankar Mitra

Temperature-sensitive mutants of *E. coli* in dna genes A, B, C(D), E, and G have been tested for their ability to support M13 phage DNA synthesis at the restrictive temperature. M13 phage replication required dna A and B gene functions only early after infection, gene A product being necessary later than the gene B product.

The products of genes C(D), E, and G are continuously required for phage replication.[1] The gene A product is required for M13 progeny replicative form (RF) DNA synthesis. The progeny single-strand (SS) DNA synthesis is more completely inhibited in dna C(D) mutants at the restrictive temperature than the RF. The synthesis of both SS and RF is depressed in the dna E mutant at the restrictive temperature. In dna G mutant, at the restrictive temperature, much less label was incorporated in the M13 RFII peak than in M13 RFI and SS, as compared with that in the control at the permissive temperature. Thus all the five known dna gene functions necessary for host DNA replication are also required for M13 DNA replication, although for the parental M13 SS DNA to RF replication only the dna E product is necessary.[2]

1. S. Mitra and D. R. Stallions, *Virology* **52**, 417 (1973).
2. R. Schekman et al., *Proc. Nat. Acad. Sci. U.S.* **69**, 2691 (1972).

TRANSCRIPTIONAL CONTROL OF M13 DNA SYNTHESIS

Sankar Mitra

Further studies of the role of M13-specific messenger RNA in the early replication of M13 replicative form (RF) have been carried out.[1,2] The inhibition of progeny M13 phage RF and single-stranded (SS) DNA synthesis by rifampicin in the rifampicin-resistant host *E. coli* H491 is reversible. Both chloramphenicol (100 μg/ml) and rifampicin (200 μg/ml) inhibited M13 SS and RF synthesis, but the kinetics of inhibition of M13 RF synthesis showed a lag for chloramphenicol and none for rifampicin. On the other hand, there was a slight lag in the total acid-insoluble incorporation of [^{3}H] amino acids in the presence of rifampicin, unlike in the presence of chloramphenicol. These data indicate that rifampicin inhibits M13 DNA synthesis directly over and above through inhibition of synthesis of M13-coded proteins necessary for DNA replication.

Finally, M13 RF was found to contain alkali-labile nucleotides after pulse-labeling with [^{32}P] PO_4 after removal of nascent mRNA chain by heat denaturation and equilibrium ultracentrifugation in Cs_2SO_4. The alkali-labile nucleotides were absent in RF isolated from rifampicin-treated cells.

1. S. Mitra, *Biol. Div. Annu. Progr. Rep. June 30, 1972*, ORNL-4817, p. 20.
2. S. Mitra, *Virology* **50**, 422 (1972).

ELECTRON MICROSCOPIC OBSERVATION OF M13 DNA REPLICATION INTERMEDIATES

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

The precise mechanism of replication of M13 RF and synthesis of SS from RF is not completely understood. It has been suggested that both SS and RF replicate by a rolling-circle mechanism in φX174, whose DNA is very similar in structure to that of M13. A simple direct method to identify the intermediates would be by electron microscopy. Preliminary results from fractionated M13 DNA pulse labeled with [^{3}H] thymidine, and after removal of host DNA, led to the following tentative conclusions: (1) M13 RF has a supercoiled ring structure which invariably has a denatured region (bubble) when supercoiling was removed in the presence of formamide. The linear molecules, which were obtained by cutting the rings at one specific point with the restriction enzyme of *Hemophilus influenzae* Rd, have no such bubble. (2) There is no evidence so far of replicating molecules with D-loops as obtained by Cairns in the case of circular *E. coli* chromosome or by Tomizawa and by Inman in the case of λ phage DNA. (3) Some RF molecules had one tail, and a very few molecules had two tails, but the tails were invariably single-stranded. The tails were absent in M13 mutant of gene 5, which is essential for SS DNA synthesis. (4) Concatenate oligomers of M13 RF were very commonly seen early after infection. (5) A denaturation map of M13 RF molecule corresponding to AT-rich clusters along the DNA has been established.

It is not clear how M13 RF replicates, but it is very likely that SS DNA is synthesized by the rolling-circle mechanism.

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BACTERIOPHAGE T5-INDUCED DNA POLYMERASE INVOLVED IN DNA DUPLICATION AND REPAIR

R. K. Fujimura and Barbara R. Bussell

DNA polymerases were extracted from *E. coli* mutants pol A^- and pol A^-B^- infected with phage $T5_{ts53}$. The infected cells do not replicate DNA at 43° because the phage-induced DNA polymerase is temperature sensitive. The analysis *in vivo* indicated that repair synthesis still occurs at 43°, which depends on phage-induced protein. DEAE-cellulose chromatography separated *E. coli* DNA polymerases from T5-induced DNA

polymerase. Further purification of the fraction with *E. coli* enzymes with phosphocellulose chromatography resolved it into *E. coli* DNA polymerase II and III and two other minor peaks. All the *E. coli* enzymes present in noninfected bacteria were present in an infected mutant of *E. coli.* These *E. coli* enzymes are apparently nonfunctional after the phage infection because there was no detectable DNA synthesis (duplication or repair) *in vivo* when chloramphenicol was added soon after the infection. The peak from DEAE-cellulose with T5-induced DNA polymerase activity was purified further and shown to consist of two activities, the temperature-sensitive activity with denatured DNA and the temperature-stable activity with activated DNA. It is proposed that this polymerase activity detectable with activated DNA at 43° is responsible for repair synthesis observed *in vivo* at the high temperature.

T5-induced DNA polymerase from wild-type phage-infected bacteria showed activity to two types of template. However, preliminary analysis of DNA polymerases extracted from *E. coli* su$^-$ infected with T5 containing an amber mutation in the structural gene for T5 polymerase showed no T5-induced polymerase activities with either template. *In vivo* analysis of such a system showed no residual synthesis. These data support the data with the temperature-sensitive mutant, indicating that T5-induced DNA polymerase is involved in both DNA duplication and repair.

PROPERTIES OF BACTERIOPHAGE T5-INDUCED TEMPERATURE-SENSITIVE DNA POLYMERASE

R. K. Fujimura and Barbara R. Bussell

Temperature-sensitive DNA polymerase induced by bacteriophage T5 has been purified so that only a single band is visible in SDS–acrylamide gel electrophoresis. This DNA polymerase is temperature sensitive when assayed with denatured DNA as template but temperature stable when assayed with nicked DNA as template. The temperature-sensitive DNA polymerase activity with denatured DNA and the temperature-stable activity with activated DNA are both irreversibly inactivated at the same rate by heating at 50°. Thus the gel electrophoresis data and the heat inactivation data indicate that the polymerase activities to two kinds of template reside in one protein.

In cruder preparations, the temperature-stable DNA polymerase activity to nicked DNA is more difficult to demonstrate because of the presence of nuclease(s) that rapidly degrade the nicked DNA. At 25° there is synthesis with a slightly detectable amount of the nuclease activity, but at 43° both template and product are degraded rather rapidly. Thus the results give only an apparent temperature sensitivity. With denatured DNA, the inhibition of polymerization at 43° occurs without significant degradation of the template.

DNA SYNTHESIS IN *ARTEMIA SALINA*

C. G. Mead

Attempts to demonstrate *in vitro* DNA synthesis in *Artemia salina* have all been unsuccessful. Analysis of the DNA precursor pool of *Artemia* at the time of DNA synthesis indicates that the deoxyribonucleoside triphosphates may not be the immediate precursors of DNA. The data indicating this to be the case come from experiments showing that the thymidine portion of thymidine triphosphate is incorporated into DNA whereas the alpha-phosphate is not. Examination of the acid-soluble pool after labeling with [^{3}H] thymidine and inorganic $^{32}PO_4$ indicates that the thymidine mono-, di-, and triphosphates are labeled with [^{3}H] thymidine but are not labeled with ^{32}P in the alpha-phosphate position. The 5′TMP isolated from the DNA is labeled with [^{3}H] thymidine and ^{32}P. These data suggest a phosphate exchange with some intermediate between the deoxynucleoside triphosphate and the DNA product. The relationship between the time after onset of DNA synthesis and this discrepancy is being investigated.

Preliminary evidence has been obtained for the existence of at least two new compounds that have the properties of poly-phosphorylated deoxynucleoside derivatives. Neither of these compounds can be labeled with [^{3}H] thymidine, and both are labeled with ^{32}P from inorganic $^{32}PO_4$. Both compounds are from DEAE cellulose in the region of the deoxynucleoside triphosphates and have the properties of charcoal absorbability, periodate resistance, and phosphate acid-lability, and at least one of these compounds is attacked by snake venom diesterase. The identity of these compounds is as yet unknown.

BIOCHEMICAL BASIS FOR THE LETHAL EFFECT OF 6-MERCAPTOPURINE AND AZATHIOPRINE (IMURAN) ON HUMAN CHRONIC LYMPHOCYTIC LEUKEMIA CELLS *IN VITRO*

C. G. Mead, F. S. Goswitz,* and James D. Regan

Goswitz[1] has shown that human chronic lymphocytic leukemia (CLL) cells are extraordinarily sensitive to 6-mercaptopurine and azathioprine when maintained *in vitro.* Interestingly, these agents have not been effective

against CLL cells *in vivo*. We have attempted to define the biochemical basis for the lethal effect of these purine analogs on CLL cells *in vitro* by a series of experiments involving incubation of human CLL cells with labeled purine precursors and preformed purines. Cells grown in medium containing labeled adenosine for 24 hr incorporate the label into the purine ribonucleotides of the acid-soluble pool and into the purine ribonucleotides of the RNA. Labeled glycine is not incorporated into either the acid-soluble purine nucleotides or RNA. In summary, these experiments have shown no abnormalities or defects in purine synthesis in human CLL cells cultivated *in vitro*. Furthermore, experiments by Goswitz indicated that the addition of adenosine or adenine to the culture medium does not spare the effect of 6-mercaptopurine or azathioprine on these cells.

Experiments with isotopically labeled 6-mercaptopurine and azathioprine are presently in progress in an attempt to determine the fate of these compounds in human CLL cells.

*Medical Division, Oak Ridge Associated Universities.

1. F. S. Goswitz, 1973, unpublished experiments.

FATE OF HOMOLOGOUS AND HETEROLOGOUS DNA'S AFTER INCORPORATION INTO HUMAN SKIN FIBROBLASTS

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

Increasing research is being carried out on the uptake of selected DNA molecules by cells in culture as an initial step in potential gene therapy. Thus it is hoped that, by insertion into recipient cells of either naked DNA or DNA in viral particles, the cells will acquire functional genes for a lacking enzyme. Some few reports have claimed a high degree of success in this regard, wherein the ultimate gene product of the foreign DNA was produced. These await confirmation from other laboratories. Many others have tried, by use of isotopically labeled DNA, to demonstrate some integration of exogenous DNA into the DNA of the recipient cell. Interpretation of these latter experiments is extremely difficult because of the possible analytically inaccessible biochemical pathways the foreign DNA may undergo after uptake. One of the more obvious deleterious pathways involves the degradation of DNA inside the recipient cell to precursors that are readily used for cellular *de novo* DNA synthesis. In the present report we attempt to evaluate whether homologous DNA and heterologous DNA remain reasonably intact in the recipient cell or whether they are largely degraded in the cell to precursors reutilized for *de novo* DNA synthesis.

Both homologous DNA from a human lymphoblastoid cell line and heterologous DNA from bacteriophage T7 are taken up in macromolecular form by human skin fibroblasts in culture. Experiments utilizing the heavy buoyant density and ultraviolet-light sensitivity of DNA containing 5-bromouracil reveal that the homologous DNA is retained in the recipient cells in relatively undegraded form for over 20 hr. Similar experiments with heterologous DNA from bacteriophage T7 show that this DNA undergoes extensive degradation after entry into the fibroblasts and that the products of degradation are readily incorporated into DNA synthesized *de novo*.

THE INTEGRITY OF HUMAN LYMPHOBLASTOID RNA AFTER INCORPORATION BY HUMAN SKIN FIBROBLASTS

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

We have found that homologous human DNA from a lymphoblastoid cell line, unlike heterologous phage T7 DNA, is retained for many hours in polymeric form by human skin-cell fibroblasts. This report addresses the question of the stability of RNA from the same homologous source in the skin fibroblast cells.

RNA from a human lymphoblastoid cell line is taken up at the macromolecular level by human skin fibroblasts in culture. Most of the donor RNA is found in the nuclei of recipient cells, apparently associated there with deoxynucleoprotein. Two techniques were designed to determine whether the RNA undergoes gross degradation intracellularly, with resynthesis of the degradation products into newly synthesized fibroblast RNA. The first method involves a comparison of the chromatographic profiles from methylated albumin kieselguhr columns of ^{3}H-labeled donor RNA with ^{14}C-labeled RNA formed *de novo* from the simultaneous incorporation of nucleosides. The other method utilizes the ability of 5-fluorouridine (FUrd) to substitute for uridine in RNA. A nearest-neighbor analysis was made of the RNA in the recipient cells after the uptake of [^{3}H]FUrd together with donor ^{32}P-labeled RNA or of $^{32}PO_4$ together with donor [^{3}H]FUrd-labeled RNA. The results of these experiments indicate that the RNA taken up retains its polymeric form for some hours and is not present in significant amounts as a result of breakdown-resynthesis inside the cell.

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ON THE NUCLEAR AND CYTOPLASMIC ORIGIN OF ORGANELLE AMINOACYL-RNA SYNTHETASES

L. I. Hecker, R. J. Reynolds,* Arlee P. Teasley, J. A. Schiff,† and W. E. Barnett

The chloroplasts of *Euglena* contain a unique set of aminoacyl-RNA synthetases. These synthetases have been shown to be light inducible; that is, exposure of dark-grown cells to light results in a rapid increase in the level of the chloroplast synthetases. We have also found that there are low levels of chloroplastic synthetases in the aplastidic mutant W_3BUL that contain no chloroplast DNA. Thus it appears that the chloroplast synthetases are coded by nuclear genes.

These observations afford an opportunity to ask a basic question about organelle compartmentalization: Is the nuclear coded mRNA transported into the chloroplast for translation, or is the nuclear mRNA translated in the cytoplasm and the finished protein (synthetase) compartmentalized?

Using two antibiotics, streptomycin and cycloheximide, which specifically inhibit translation on 70s and 80s ribosomes, respectively, we have followed the kinetics of induction of the chloroplast phe-tRNA synthetase and have shown that it is translated on cytoplasmic ribosomes (80s) and apparently immediately transported into the organelle.

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†Department of Biology, Brandeis University, Waltham, Massachusetts.

ORGANELLE SYNTHETASES OF *EUGLENA*

L. I. Hecker, C. E. Nix, and W. E. Barnett

We have continued our efforts to identify and characterize the chloroplastic and mitochondrial aminoacyl-RNA synthetases of *Euglena*. Efforts to distinguish chromatographically (or by acylation specificity) between the mitochondrial and chloroplastic ile-, val-, and ser-tRNA synthetases have all failed. These results indicate that the two organelle enzymes for each of the above amino acids are quite similar. We have, however, succeeded in separating the two organelle phe-tRNA synthetases by the use of extremely shallow gradients and hydroxyapatite chromatography. Thus the latter observation demonstrates that in at least one case the chloroplastic and mitochondrial synthetases are distinguishable and suggests that separation of the two enzymes for ile, val, and ser may be possible with higher resolution chromatography and/or electrophoresis.

AUTOMATED SEQUENTIAL DEGRADATION OF RIBONUCLEIC ACIDS

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

A. The instrument described earlier[1] is capable of controlling the wet chemistry and enzyme digestion to greater than 99% yield. The mechanical components on the other hand have less reliability and limit the usefulness of this particular machine. Further engineering input should solve the "reliability" problem.

B. The chemistry of the elimination process is first order under a limited set of conditions (pH, structure of the amine, and temperature). This permits prediction of yields as a function of time and temperature.

The stringent control of the conditions for elimination arises from the complexity of the reaction. We have established that three different pathways, including two different mechanisms, contribute to the elimination process (Fig. 1). Optimal conditions are obtained between pH 5.5 and 6.5, using ornithine as a catalyst.

C. We have prepared an immobilized alkaline phosphatase that is superior to our earlier preparations and has useful properties for incorporation into an automated enzyme digestion apparatus. The enzyme is attached to a modified agarose by a "diazo" linkage. Depending upon the coupling conditions, up to 2 mg of enzyme have been attached per milliter of agarose beads. The material has a pH optimum near pH 8 and is inhibited by high concentrations of ornithine. The enzyme activity is maintained at least two weeks at 37°.

D. We have used a combination of dialysis and ultrafiltration to purify the RNA for each stage of the cycle and to recover the released base for analysis. Since we remove the excess periodate before this step, we can use commercially available cellulose membranes. The limiting factor in the use of channel-type dialyzers (ultrafilters) is the washout volume. This latter volume (the amount of wash water needed to obtain better than 99% yield) is dependent upon the flow pattern from the enzyme vessel through the ultrafilter. We have determined that ideal "plug flow" is dependent to a large extent on the geometry of the enzyme vessel preceding the ultrafiltration step.

1. M. Uziel, A. Jeannine Bandy, and A. J. Weinberger, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, p. 24.

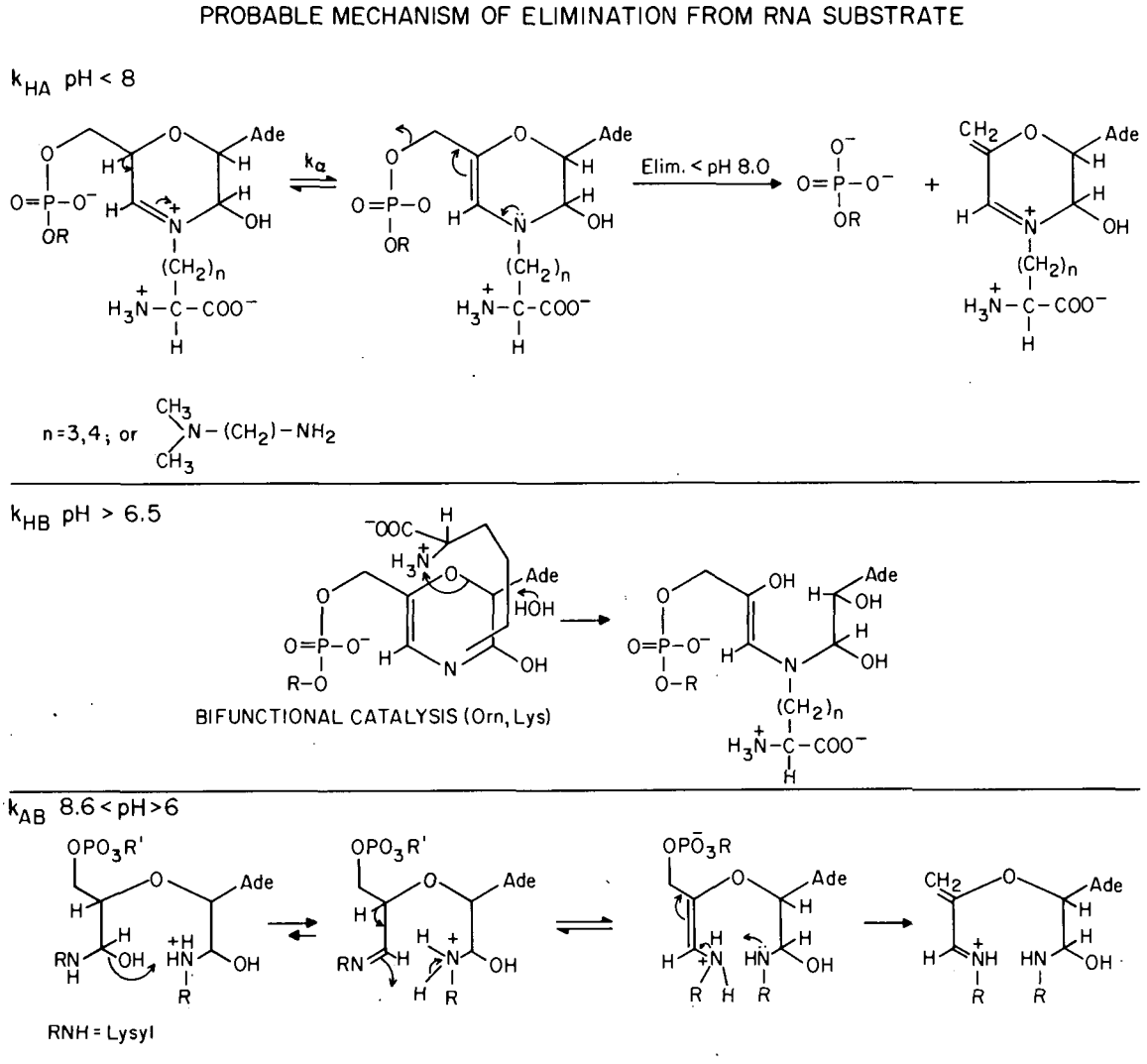

Fig. 1. **Top panel** illustrates the mechanism common to all the primary amine-catalyzed reactions: general acid catalyzed rate determining step governed by a prior equilibrium formation of the conjugate acid form of the carbinolamine. **Middle panel** presents the probable mechanism of ether cleavage catalyzed by an intramolecular process using lysine or ornithine. **Bottom panel** illustrates our interpretation of the bell-shaped component of the pH optimum using lysine as the catalyst. The rate-determining step(s) uses both the conjugate acid and conjugate base forms of the (2')carbinolamine.

MULTIPLEXED ANALYTICAL INSTRUMENT INTERFACE TO PDP-11

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

The routine quantitative analysis of chromatographic data from instruments that monitor at one, two, or four wavelengths can be performed with a combination of seven programs that initiate and collect the data, separate the data according to wavelength, and then calculate the areas in absorbance units. The programs can process linear absorbance data as well as percent transmission data. In addition, the program is adjustable for the voltage output of the instrument to be monitored.

The system is sensitive to absorbance values as small as 0.001 absorbance unit and up to ca. 3 absorbance units. The accuracy is superior to hand calculations using the approximations to Gaussian distribution.

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2′-O-METHYLURIDINE 5′-DIPHOSPHATE AND 2′-O-METHYLCYTIDINE 5′-DIPHOSPHATE

B. C. Pal, Diane G. Schmidt, and K. L. Beattie*

We needed a large amount of 2′-O-methyluridine 5′-diphosphate to make poly(2′-O-methyluridylic acid), using polynucleotide phosphorylase, for inhibition studies on the reverse transcriptase of Rauscher leukemia virus. 2′-O-Methyluridine 5′-diphosphate had previously been prepared by Dunlap et al.[1] by deamination of 2′-O-methylcytidine 5′-diphosphate. We have prepared this compound in an overall yield of 18% by the following route:

2′-O-methyluridine (Um) → UmMP (step 1) →

UmDP (step 2).

In step 1, direct phosphorylation of Um with $POCl_3$ in triethyl phosphate[2] led to UmMP in 73% yield. Some workers[3] have found difficulty in adopting this procedure for the preparation of the mononucleotide. When the reaction mixture is neutralized by strong alkali, cyclic phosphates are formed. This complication can be avoided by treating the reaction mixture with triethylammonium bicarbonate instead. In step 2, UmMP was converted into UmDP following the morpholidate procedure of Khorana.[4] The UmDP was found to be very unstable even when kept in the freezer. The CmDP was obtained in an overall yield of 29% in a similar manner. Both UmDP and CmDP prepared in this way were found to be suitable substrates for polynucleotide phosphorylase.

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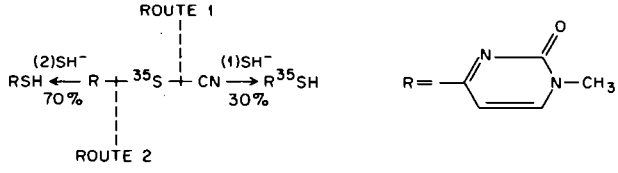
*Student at the UT–Oak Ridge Graduate School of Biomedical Sciences.

1. B. E. Dunlap, K. H. Fridrici, and F. Rottman, *Biochemistry* **10**, 2581 (1971).
2. M. Yoshikawa, T. Kato, and T. Takenishi, *Tetrahedron Lett.* **50**, 5065 (1967).
3. I. Tazawa, S. Tazawa, J. L. Alderfer, and P. O. P. Ts'o, *Biochemistry* **11**, 4931 (1972).
4. J. G. Moffatt and H. G. Khorana, *J. Amer. Chem. Soc.* **83**, 649 (1961).

MECHANISM OF REDUCTION OF 1-METHYL-4-THIOCYANATOURACIL BY BISULFIDE: A ROUTE TO *IN VITRO* LABELING OF tRNA'S WITH ^{35}S

B. C. Pal and Diane G. Schmidt

4-Thiouridine in tRNA's is converted into 4-thiocyanatouridine by CNBr, and we have shown that 4-thiocyanatouridine is reduced back to 4-thiouridine by SH^-. If the last reaction takes place by the scission of the pyrimidine ring–S bond, then it will be possible to incorporate the ^{35}S label in tRNA for biological studies by using $^{35}SH^-$. The mechanism of this reaction has been investigated by using ^{35}S-labeled 1-methyl-4-thiocyanatouracil.



It was found that 70% of the reaction proceeds by ring-S fission. $R^{35}SCN$ was prepared by the following route:

$$RSH \xrightarrow{CNBr} RSCN \xrightarrow{^{35}SH} R^{35}SH \xrightarrow{CNBr} R^{35}SCN.$$

We have successfully prepared ^{35}S-labeled *E. coli* $tRNA^{fMet}$ and have demonstrated that the label is exclusively in the 4-thiouridine moiety of the tRNA.

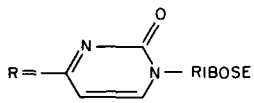
SYNTHESIS AND CHARACTERIZATION OF 4-THIOCYANATOURIDINE

B. C. Pal and Diane G. Schmidt

4-Thiocyanatouridine has been proposed as the intermediate formed in the CNBr-initiated modification of *E. coli* transfer RNA's containing 4-thiouridine. This rather unstable intermediate has not been satisfactorily characterized so far. We have been able to prepare this compound in quantitative yield by the following scheme:

$$RSSR + CN^- \text{ (buffered at pH 7)} \xrightarrow{\text{Step 1}} RSCN + RS^-$$

$$\uparrow \text{CNBr} \quad \text{Step 2}$$



It was found essential to carry out the reaction with CN^- at pH 7; otherwise the 4-thiocyanatouridine formed is immediately decomposed under alkaline conditions of the unbuffered cyanide solution. The 4-thiocyanatouridine prepared in this way was desalted by running it through a column of G-10. In acid, it is completely converted into uridine, whereas in alkali it is converted into uridine and 4-thiouridine in 7:2 ratio. It is reduced completely to 4-thiouridine by HS^-. Thus one should be able to modify the 4-thiouridine moiety in tRNA to 4-thiocyanatouridine and regenerate the original tRNA using HS^-.

Table 1. Modification of Glu tRNA

$^2S \rightarrow NC^2S\text{-}\langle\rangle\rightarrow O=\langle$

Modification		Loss of ^{2}Srd	Acceptance	
	Mg^{++}	(mole/tRNA)	pmole/A_{260}	% Loss
None		None	1250	0
CNBr	–	0.80	242	79
CNBr	+	0.78–0.85	249	79

CHEMICAL PROBE OF tRNA STRUCTURE AND FUNCTION

R. P. Singhal

Previous studies[1,2] indicate that the modification of glutamate tRNA (*E. coli*) with bisulfite under carefully controlled reaction conditions, where cytidine residues are transformed into uridines, results in specific modifications of three cytidines located in the anticodon region of the molecule. This modification showed 82% loss of glutamate-charging ability – a loss parallel to the extent of the modification (80%) of the three reactive cytidines. To test the hypothesis that a chemically unaltered anticodon loop of the tRNA is required for aminoacylation to occur, the thiouridine residue located in the "wobble" position was selectively modified.

Results of the modification with two other chemicals, cyanogen bromide and *p*-chloromercuribenzoate, are shown in Tables 1 and 2 respectively.

The modification of the thio residue of $tRNA^{Glu}$ with cyanogen bromide is more complete than with the mercury compound and remains unaffected by the presence of magnesium ions in the environment. The inactivity of the mercury reagent toward the 4-thiouridine residue, located in the dihydrouridine stem of $tRNA^{fMet}$, is perhaps due to an enhanced affinity of 4-thiouridine for the magnesium ion. The results further suggest that the anticodon region of tRNA is exposed, and possibly it is involved (e.g., recognition or binding with tRNA ligase) in the amino-acid charging mech-

Table 2. Modification of tRNA with *p*ClHg [^{14}C] Bz

$Srd \rightarrow HOOC\text{-}\langle\rangle\text{-}Hg\text{-}S\text{-}\langle$

tRNA	Mg^{++}	^{14}C incorporation		Acceptance	
		pmole Hg/A_{260}	%	pmole AA/A_{260}	% Loss
Glu		None		1140	0
	–	620	54	549	52
	+	560	49	684	40
fMet		None		1720	0
	–	517	30	1728	0
	+	92	5	1737	0

anism. The exposed nature of the anticodon and my earlier observation[1] that the cytidine of the pseudouridine loop can react are in confirmation of the three-dimensional structure of $tRNA^{Phe}$, recently analyzed at 4 Å resolution by Kim et al.[3]

1. R. P. Singhal, *J. Biol. Chem.* **246**, 5848 (1971).
2. R. P. Singhal, *Biol. Div. Annu. Progr. Rep. June 30, 1972*, ORNL-4817, p. 26.
3. S. H. Kim, G. J. Quigley, F. L. Suddath, A. McPherson, D. Sneden, J. J. Kim, J. Weinzierl, and A. Rich, *Science* **179**, 285 (1973).

ION-EXCLUSION CHROMATOGRAPHY. CATION AND ANION EXCLUSION ON ANION AND CATION EXCHANGERS

R. P. Singhal and W. E. Cohn

Ion-exclusion chromatography, previously demonstrated for the separation of anionic substances on cation-exchange material,[1] has been investigated for the separation of cationic substances on anion-exchange material and for the resolution of strong anions on cation-exchange column.

A mixture of nucleosides, derived from ribonucleic acid and including cations as well as uncharged species, can be resolved rapidly and efficiently at an acid pH on columns of an anion exchanger if the latter is comprised of small and uniformly sized beads (Aminex A-25, 17-μm diameter). The plate heights calculated from the results ($\cong$0.2 mm) are superior to those seen in ion exchange on the same columns and are comparable to those reported earlier[1] for anion exclusion on cation-exchange columns of similar physical characteristics. The charged (cationic) substances, if not retarded by partition effects, appear within elution volumes less than the liquid content of the column, thus justifying the term "exclusion," and respond to pH changes and to the Donnan effect in a manner predictable from their pK values and the ionic strength of the medium. An uncharged substance (e.g., ^3H_2O), in the absence of partition effects, will appear at one liquid column volume. Partition effects are shown by retardation beyond one liquid column volume and by further retardation with increased ionic strength ("salting out"), and these effects can be manipulated, together with pH and the addition of organic solvents, to maximize resolutions and reduce the time for a given separation.

A mixture of ribonucleotides containing phosphate groups as strong anions can be resolved on cation-exchange material (Aminex A-6) by a combination of anion-exclusion and partition phenomena. In a solution of low ionic strength at acid pH (e.g., 10 mM ammonium formate, pH 3.50), the primary phosphate group (anion) of each nucleotide is completely ionized, and amino groups (cations) of cytidylate and adenylate are variably ionized. Results indicate that these small differences in net anionic changes and differences in the lipophilicity of purine and pyrimidine bases permit both anion-exclusion and partition chromatography. Using this principle, mixtures containing both nucleotides (5′- or 2′- and 3′-phosphates at acid pH) and nucleosides[1] at alkaline pH can be resolved on cation-exchange columns. This provides an attractive method for end-group and base-composition analyses, both at the same time.

A direct comparison between ion-exclusion and ion-exchange separations (for example, see ref. 2) on identical columns confirms the superiority of the former in several practical aspects (e.g., smaller plate heights leading to better resolution, shorter analysis times, and the use of dilute, volatile eluants).

1. R. P. Singhal, *Arch. Biochem. Biophys.* **152**, 800 (1972).
2. R. P. Singhal and W. E. Cohn, *Biochem.* **12**, 1532 (1973).

ANION-EXCHANGE CHROMATOGRAPHY ON REVERSED-PHASE COLUMNS: ISOLATION AND ASSAY OF NUCLEOSIDES, NUCLEOTIDES, AND OLIGONUCLEOTIDES FROM NUCLEIC ACIDS AND FROM CYTOPLASM

R. P. Singhal

Reversed-phase ion-exchange chromatography, developed specifically for the separation of transfer and other species of ribonucleic acids, can be utilized[1] in the same manner as the more conventional polystyrene- or cellulose-based ion-exchange and ion-exclusion chromatographic procedures for the resolution of smaller entities, such as nucleosides, nucleotides, and oligonucleotides.

The separations in this reversed-phase anion-exchange chromatography are achieved by (1) nonionic interactions (partition, hydrophobic interactions, hydrogen bonding, van der Waals forces) between the solutes (e.g., nucleotides, tRNA's) and the immobilized alkyl groups and (2) the anion-exchange properties of the immobilized quaternary ammonium derivatives. The nonionic interactions appear to be somewhat more effective, and the anion-exchange properties less so, in reversed-phase chromatography than in polystyrene exchange chromatography. That ionic forces do play a

significant role in reversed-phase chromatography is evident by the fact that the desorption of nucleotides requires a higher salt concentration at alkaline than at acid pH because of the ionization of the secondary phosphate and hydroxyl groups. Also, most substances on reversed-phase columns appear in the reverse of the order of their net anionic charges. The nonionic forces in this chromatography, as in polystyrene ion exchange to some extent, differentiate between compounds that seem to bear almost identical charges.

The major 5′-nucleotides and several peaks corresponding to the minor components are resolved in a single volatile eluant on a short column in less than 1 hr. The chromatography of an alkali hydrolysate of tRNA's on a larger column indicates that 2′- and 3′-nucleotides can be resolved (except cytidylates) by appropriate adjustments of ionic strength and flow rate. On the other hand, each pair of isomers can be eluted as a single peak by a different adjustment of these two parameters. This provides a tool for the rapid assay of the major and minor nucleotides as well as of the nucleoside residue derived from the 3′-terminus (which appears before cytidylate) and the nucleotide of the 5′-terminus of RNA's (pGp from tRNA's and pppGp from the precursor RNA's, both appearing after guanylate).

The chromatography of a pancreatic ribonuclease digest of tRNA at alkaline pH provides homogeneous peaks of most compounds, from the pyrimidine mononucleotides to the long-chain oligonucleotides that are difficult to resolve on DEAE-cellulose columns. The higher net anionic charge at alkaline pH provides greater anion-exchange properties, and the pH of 9.8 permits a separation of uridine isomers.[2] Since the peak widths are narrow (small plate heights), analytical sensitivity is enhanced. This is apparent from the separation of less than 1 mg (14 A_{260nm} units) of a ribonuclease digest of glutamate tRNA.

Figure 2 indicates chromatography of the acid-soluble components on the reversed-phase column with a linear gradient of ammonium acetate at pH 4.4. Twelve out of a total of 32 detectable peaks have been identified by their absorbance ratios at 260 and 280 nm, their net anionic charges at pH 4.4, their bases (purine, pyrimidine), and their elution positions with respect to reference compounds. Peaks 2, 10, 14, and 18 appear to contain pseudouridine plus thymidine, uridine, inosine, and guanosine phosphates, either alone (peak 2) or as derivatives. A concave gradient elution beginning with low anionic strength (less than 0.15 *M* acetate ion) improved the resolutions of the early-appearing, overly crowded peaks.

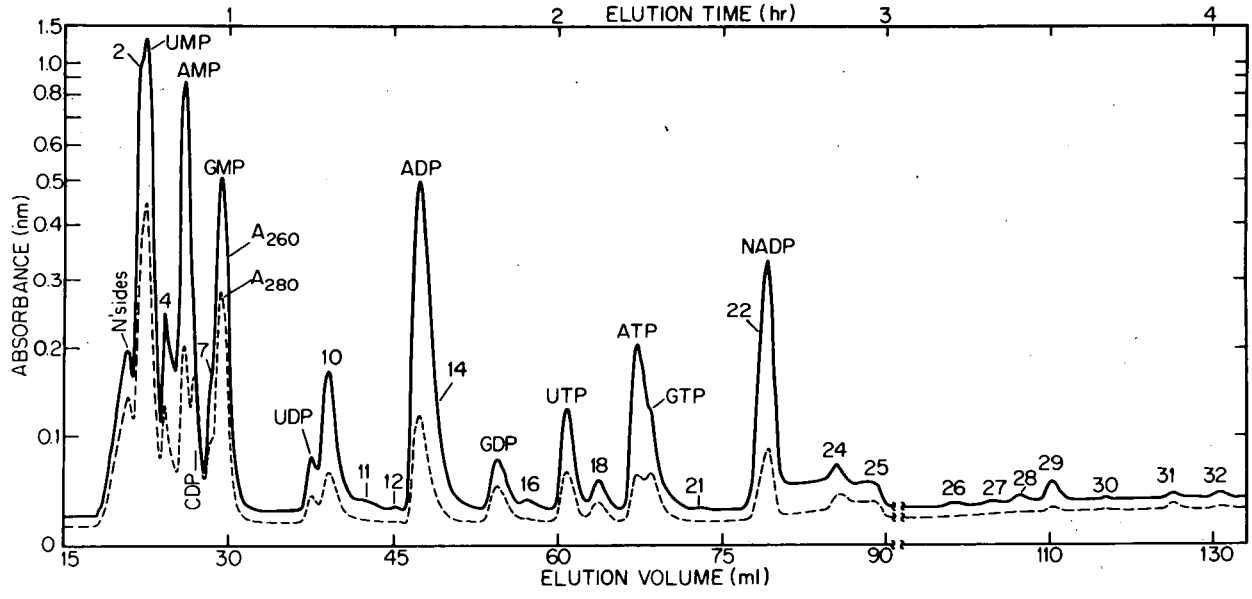


Fig. 2. Mapping of $HClO_4$-soluble ultraviolet-absorbing compounds present in a rat liver homogenate by reversed-phase chromatography. Inert plastic beads (polychlorotrifluoroethylene) coated with a quaternary ammonium derivative [methyl-trialkyl (C_8–C_{10}) ammonium chloride] were packed in a 98-cm by 6.35-mm column and eluted with a linear gradient of 0.15 *M* to 0.93 *M* ammonium acetate (plus acetic acid), pH 4.4 (100 ml of each component) at 50°C with a flow rate of 0.5 ml/min.

In conclusion, this ion-exchange chromatography, which is based upon anion exchange and nonionic interactions between nucleic acid components and a matrix consisting of quaternary ammonium with hydrophobic side chains, yields satisfactory and rapid separations of nucleic acid components and related substances by simple volatile eluants.

1. R. P. Singhal, *Biochim. Biophys. Acta* **319**, 11 (1973).
2. R. P. Singhal and W. E. Cohn, *Anal. Biochem.* **45**, 585 (1972).

PURIFICATION OF A RIBONUCLEOSIDE TRIPHOSPHATE REDUCTASE FROM *EUGLENA GRACILIS*

F. D. Hamilton

A ribonucleoside triphosphate reductase has been purified to homogeneity from *Euglena gracilis.* Purification has been achieved through the use of ammonium sulfate fractionation, pH 5 precipitation, and DEAE-cellulose chromatography. The final step of the purification procedure involves preparative gel electrophoresis in an acrylamide gel system. The purified enzyme is active with either CTP, ATP, UTP, or GTP as a substrate. Deoxyadenosylcobamin and dithiothreitol are obligatory cofactors for the *in vitro* reaction. CTP, ATP, and UTP reduction are stimulated by the addition of the appropriate deoxyribonucleoside triphosphate. Experimental results indicate that the *Euglena* reductase is subject to allosteric regulation by deoxyribonucleotides.

PURIFICATION OF ^{5}U-METHYLTRANSFERASE FROM *ESCHERICHIA COLI*

L. R. Shugart and Barbara H. Chastain

Purification of ^{5}U-methyltransferase by DEAE-cellulose column chromatography results in an enzyme preparation that is extremely labile and contains both contaminating nuclease activity and RNA.[1] This material has proved unsatisfactory for the study of the effect of stepwise methylation on methyl-deficient tRNA molecules.

To date the most promising purification procedure appears to be Sephadex column chromatography. On an analytical scale we have been able to obtain ^{5}U-methyltransferase preparations that are stable for several months at 4°C, with no contaminating RNA and with greatly reduced RNase activity. The enzyme is now being obtained on a preparative scale.

1. L. R. Shugart, J. G. Farrelly, and M. P. Stulberg, *Bacteriol. Proc.*, p. 163 (1971).

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

L. R. Shugart

In *E. coli* with relaxed control over RNA synthesis (rel$^-$) and an auxotrophic requirement for methionine (Met$^-$), the tRNA's synthesized during methionine starvation are deficient in methylated nucleosides. The tRNA's isolated from these starved bacterial cells are, however, a mixture of the newly synthesized molecules and the normal, fully methylated species present prior to such treatment.

A quantitative analysis of the methylated nucleoside content of normal and methyl-deficient tRNA's from an rel$^-$ mutant indicates that the tRNA's synthesized under conditions of methionine starvation are not completely undermethylated.[1,2]

Selective methylation during methionine starvation of this organism could be the result of a loss of certain tRNA methyltransferase activities by the organism and/or a reflection of the affinity of the enzymes for the precursors of methylation.

1. L. Waters, L. R. Shugart, W. K. Yang, and A. Best, *Arch. Biochem. Biophys.* **156**, 780 (1973).
2. K. R. Isham and M. P. Stulberg, "The Content of Modified Bases in Undermethylated Phenylalanine tRNA," this report.

THE CONTENT OF MODIFIED BASES IN UNDERMETHYLATED PHENYLALANINE tRNA

K. R. Isham and M. P. Stulberg

Last year,[1] we reported on initial experiments concerning the content of modified bases in mixed, undermethylated tRNA from *E. coli.* We have now completed an analysis of modified bases from a highly purified and undermethylated $tRNA^{Phe}$ (UU-$tRNA^{Phe}$). The most marked difference between normal and UU-$tRNA^{Phe}$ is present in the modified adenosine adjacent to the 3′ end of the anticodon. Normal $tRNA^{Phe}$ contains N^6-(Δ^2-isopentenyl)-2-methylthioadenosine in this position; however, the UU-$tRNA^{Phe}$ completely lacks the methylthio group in this modified adenosine. As expected, the ribosylthymine is absent in UU-$tRNA^{Phe}$. However, the other methylated base, 7-methyl guanosine, seems to be low and in significant amounts in both normal and undermethylated species. We are now attempting to correlate the undermethylation present in isolated UU-$tRNA^{Phe}$ with *in vitro* methylation by isolated tRNA methyltransferases.[2]

This lack of modification in UU-tRNA^{Phe} can be due to a required sequential modification dependent on prior methylation or the result of methionine starvation on the ability of the cell to replace rapidly turning-over modification enzymes.

1. K. R. Isham, A. N. Best, and M. P. Stulberg, *Biol. Div. Annu. Progr. Rep. June 30, 1972*, ORNL-4817, p. 12.
2. L. Shugart and B. Chastain, "Purification of ^{5}U-Methyltransferase from *Escherichia coli*," this report.

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

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

Last year,[1] we described an *in vitro* protein synthesis system which we were constructing from ascites tumor cells and encephalomyocarditis (EMC) virus, representing the synthesis system and messenger respectively.

We have made cell-free extracts from the ascites cells and isolated RNA from the virus grown on the cells. Incorporation of amino acids into protein (presumably capsid protein) has been accomplished under the direction of EMC RNA and poly U. Presently we are seeking optimal conditions for maximal activity. Linear rates of incorporation have been observed for 150 min with no dimunition of rate indicated for longer time periods.

We plan to isolate protein synthesis components from aging, irradiated, and possibly other altered cells to test their function in the rate, extent, and fidelity of specific protein synthesis in this *in vitro* system. Those mouse tissues that demonstrate age-dependent, reduced levels of tRNA methyltransferases will be the first to be examined for the integrity of their tRNA.

*Supported by a USPHS Postdoctoral Fellowship.

1. M. P. Stulberg and K. R. Isham, *Biol. Div. Annu. Progr. Rep. June 30, 1972*, ORNL-4817, p. 13.

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 of the "Y" base of phenylalanine tRNA. The presence of an intact "Y" base adjacent to the anticodon is apparently necessary to maintain the proper conformation of the anticodon loop of this tRNA.[1] Previous work from this laboratory has shown that the "Y" base is modified in phenylalanine tRNA from senescent wheat leaf tips.[2,3] An attempt was made to purify sufficient altered "Y" base to determine the structure by mass spectrometry; however, the lability of the excised altered "Y" base precluded this. Altered "Y" base in phenylalanine tRNA was also found in wheat leaf tips from wheat that had been grown in the presence of 3-aminotriazole, an agent that prevents chloroplast development.

The emphasis of the work now is to determine at what stage of development phenylalanine tRNA with altered "Y" base appears. Phenylalanine tRNA is being purified from one-day through seven-day wheat leaves. The work has also been extended to tobacco callus tissue in culture where protein synthesis and cell division can be controlled by cytokinin and auxin. The effects of the various growth conditions on "Y" base are being examined in this system.

*Supported under a training grant by NICHD.

1. R. Thiebe and H. G. Zachau, *Eur. J. Biochem.* 5, 546–55 (1968).
2. L. R. Shugart, *Exp. Gerontol.* 7, 251–62 (1972).
3. B. L. Whitfield and L. R. Shugart, *Biol. Div. Annu. Progr. Rep. June 30, 1972*, ORNL-4817, p. 13.

ROLE OF TRANSFER RNA IN REGULATING ENZYME FUNCTION

J. B. Bell and K. Bruce Jacobson

The number of isoacceptor tRNA's is known to vary with developmental stage of eucaryotes, but little is known about the mechanism that regulates the quantity of any isoacceptor tRNA. If a crude preparation of tRNA is made from a log-stage culture of wild-type *Saccharomyces cerevisiae* and aminoacylated with either labeled phenylalanine or tyrosine, only one isoaccepting species of tRNA for each of these two aromatic amino acids is resolved by RPC-5 chromatography. However, in an auxotrophic mutant, XB109, multiple isoacceptors for either phenylalanine or tyrosine can be found. We have shown that growth conditions play a role in the number of various isoaccepting forms of tRNA observed. For example, the patterns of isoacceptors differ in preparations of tRNA made from cultures differing in the extent of aeration.

The first enzyme in the biosynthetic path for phenylalanine and tyrosine in yeast is subject to feedback inhibition by these amino acids. The enzyme is 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP)

synthetase. Meuris[1] has recently reported that $tRNA^{Phe}$ and $tRNA^{Tyr}$, as well as the two free amino acids, inhibit this enzyme. Inhibition can be obtained with uncharged $tRNA^{Phe}$, but $tRNA^{Tyr}$ must be charged to cause the observed inhibition. We are currently studying DAHP synthetase to repeat the observation of Meuris. Subsequently we shall test which, if any, of the multiple isoaccepting forms for $tRNA^{Phe}$ and tyrosyl $tRNA^{Tyr}$ will inhibit DAHP synthetase. Our observation of multiple isoaccepting forms of these tRNA's, coupled with the observation that a tRNA may interact with an eyzyme to inhibit its activity, affords a chance to test for possible functional roles of isoaccepting forms of tRNA in regulating processes other than translation.

Yeast possesses two isofunctional DAHP synthetases, one sensitive to feedback inhibition by phenylalanine and the other to tyrosine. Study of this system is facilitated by the use of two mutants obtained from Meuris. The mutants lack, respectively, one or the other of the two isofunctional DAHP synthetases. So it is possible, for example, to study the effect of $tRNA^{Phe}$ on the activity of the phenylalanine-sensitive form of the enzyme without unacceptable background activity from the other form of the enzyme.

1. P. Meuris, *Mol. Gen. Genet.* **121**, 207–18 (1973).

TYROSYL-tRNA SYNTHETASE IN *DROSOPHILA MELANOGASTER*

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

The mechanism of suppression of the vermilion locus in *Drosophila* apparently depends on the tRNA isoacceptors for tyrosine which are present in flies carrying the *su(s)*[2] mutation. Several lines of evidence indicate that the two isoacceptors present in *su(s)*[2]*v* flies ($tRNA^{Tyr}_{su(s)A}$ and $tRNA^{Tyr}_{su(s)B}$) are not the same as those present in wild-type flies ($tRNA^{Tyr}_{SAM\ I}$ and $tRNA^{Tyr}_{SAM\ II}$). One of the indications that $tRNA^{Tyr}_{su(s)B}$ differs from $tRNA^{Tyr}_{SAM\ II}$ is the existence of a synthetase which can charge the latter but not the former. Chromatography of tyrosyl-tRNA synthetase on DEAE cellulose can give two fractions – one activity which aminoacylates $tRNA^{Tyr}_{SAM\ I}$, $tRNA^{Tyr}_{SAM\ II}$, and $tRNA^{Tyr}_{su(s)A}$ and a second activity which aminoacylates the three preceding tyrosyl tRNA's as well as $tRNA^{Tyr}_{su(s)B}$.

We have attempted to characterize the conditions necessary for the specific aminoacylation of $tRNA^{Tyr}_{su(s)A}$, as opposed to $tRNA^{Tyr}_{su(s)B}$. Nineteen synthetase preparations have been made in this study. All of the enzymes were fractionated on DE-23 columns using NaCl. In three cases the enzymes were eluted from the columns with a linear gradient (0 *M*–0.4 *M* NaCl). This method of elution destroyed the enzyme activity in all three cases. In ten enzyme preparations only one form of enzyme, which eluted from the DE-23 column in 0.1 *M* NaCl, was obtained. In all cases the enzymes so prepared could not distinguish $tRNA^{Tyr}_{su(s)A}$ from $tRNA^{Tyr}_{su(s)B}$ and aminoacylated both isoacceptors. Finally, in six instances, the tyrosyl-tRNA synthetase was obtained in two forms. In all six cases, one form of the enzyme eluted from the DE-23 column with 0.3 *M* NaCl and aminoacylated both $tRNA^{Tyr}_{su(s)A}$ and $tRNA^{Tyr}_{su(s)B}$. Four of the enzyme preparations yielded a second form eluting from the column in 0.1 *M* NaCl, and two of them yielded a second form eluting in 0.2 *M* NaCl. All six of these enzyme forms eluting in the lower salt concentrations possessed specificity of charging in that they could charge $tRNA^{Tyr}_{su(s)A}$ but not $tRNA^{Tyr}_{su(s)B}$. An extensive study of storage temperatures, homogenization methods, and chromatography conditions has failed to indicate the reason (or reasons) for the variability of synthetase activities.

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TRANSFER RNA–GENE MAPPING

Maurice Cohen, Jr.,* and K. Bruce Jacobson

Genetic mapping by hybridizing tritiated RNA to chromosomal DNA *in situ* has been useful in localizing redundant genes; rare sequences cannot be detected in a reasonable time, however, if the RNA is labeled with tritium. Because of this limitation we have approached the mapping of transfer RNA genes in *Drosophila melanogaster* by chemically labeling the purified tRNA with ^{125}I. Since this isotope has a shorter half-life and the beta-particle energy is comparable with that of tritium, chromosomal DNA annealed to iodinated RNA may be visualized by autoradiography.

Ribosomal 5s RNA (*Drosophila melanogaster*) has been iodinated and successfully hybridized to the locus known to contain the DNA complementary to this RNA. Transfer RNA's are presently being purified by reversed-phase chromatography and will be iodinated and annealed to chromosomal DNA in the same fashion.

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PHENOL OXIDASE ACTIVITY IN *DROSOPHILA*

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

In an earlier report on the lozenge locus and phenol oxidase activity in *Drosophila melanogaster,* we were unable to correlate the severity of the lozenge phenotype with the amount of phenol oxidase activity.[1] We did find that a spontaneous lozenge mutant isolated by R. F. Grell, lz^{rfg}, had no phenol oxidase activity at all. We are interested in the biochemical mechanism of suppression in *Drosophila,* and we attempted to make a suppressor of lz^{rfg} by EMS mutagenesis but were unable to do so. Screening of a variety of *Drosophila* stocks resulted in identification of three more mutants which have a phenol oxidase banding pattern on acrylamide gels that can be distinguished from wild type, namely, speck, black cells, and tyrosinase-1.

The *su(s)* mutation not only suppresses the vermilion phenotype in *Drosophila* but enhances the effect of certain lozenge alleles; so we tested the effects of four different *su(s)* alleles [$su(s)^2$, $su(s)^{e1}$, $su(s)^{e6}$, and $su(s)^{x1}$] on both *lz* and lz^g phenol oxidase patterns. The appropriate stocks were prepared and the enzymatic activities assayed, but in no case did we observe a significant alteration in the characteristic enzyme patterns.

We are also trying to locate the structural gene for tyrosinase in *Drosophila simulans.* Hybrid *melanogaster–simulans* flies homozygous for a particular *D. melanogaster* chromosome or chromosome arm have been constructed and the phenol oxidase activity analyzed. Analyses of such hybrids for chromosomes 1, 2L, 2R, 3L, and 4 have failed to locate the structural gene for tyrosinase on any of these chromosomes. Presently, 3R hybrids are in preparation and will be analyzed when they are available.

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1. C. K. Warner and K. B. Jacobson, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, p. 14.

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

Audrey N. Best and G. David Novelli

Previous studies by L. C. Waters have demonstrated that transfer RNA isolated from *E. coli* cells that have been exposed to chloramphenicol (CAP) for several hours yield altered profiles when chromatographed on reversed-phase columns. Analysis of the nucleoside content of tRNA synthesized in the presence of CAP suggests that these tRNA's are immature forms capable of being converted to mature forms on removal of CAP. The major deficiencies in modification occur with 4-thiouridine and dihydrouridine, although several minor methylated nucleoside components decrease in content during CAP treatment.[1]

We are expanding this investigation to isolate and purify some of these altered species. The proposed method of separation of an individual tRNA species is by the formation of an aminoacyl-tRNA-GTP-protein elongation factor Tu complex. The isolated species can be tested for the nature of alteration. If the separation of an individual *E. coli* tRNA species can be effected, we believe this method would be satisfactory for the purification of mammalian tRNA's. We know that elongation factor Tu from *E. coli* can form a complex with GTP and human placental aminoacyl tRNA. Some preliminary studies on the complex formation have been completed. We have isolated and purified the components of the aminoacid incorporation system of *E. coli.* This project is proceeding in cooperation with the Macromolecular Separations Group of the ORNL Chemical Technology Division.

1. L. C. Waters, L. Shugart, W. K. Yang, and A. N. Best, *Arch. Biochem. Biophys.* **156,** 780–93 (1973).

EXAMINATION OF SOME HIGHLY PURIFIED TRANSFER RNA'S FROM *ESCHERICHIA COLI.* DIFFERENCES IN AMOUNT OF MINOR COMPONENTS AND PRESENCE OF A PHOTOPRODUCT IN "NORMAL" tRNA'S

R. P. Singhal and Audrey N. Best

During the last decade the Oak Ridge National Laboratory has supplied samples of several purified and partially purified preparations of *E. coli* tRNA's to numerous laboratories, where these samples have been used in studies ranging from crystallization to the recognition site of amino acid:tRNA ligase. The evaluation of the results of these studies depends largely on how closely the tRNA sample conforms to the published structures. Hence, these tRNA's are appropriate models for investigation of the extent to which their compositions correspond to the base compositions derived from the published sequences of the major component.

During the isolation of tRNA's, a loss of 4-thiouridine due to oxidation, or to a photochemical reaction between 4-thiouridine and cytidine, had been suspected. Waters et al.[1] recently reported that tRNA synthesized in the presence of chloramphenicol lacks a full complement of modified nucleosides. Such reports raise questions as to whether tRNA's prepared from normally grown cultures always contain their full complements of modified nucleosides and whether or not a tRNA sequence intellectually reconstituted from its purified oligonucleotides represents a true picture of the molecules present in the tRNA preparation. To answer these questions, the nucleoside composition of 80 to 100% pure arginine, formylmethionine, glutamate, phenylalanine, and valine tRNA preparations from *E. coli* were assayed by two sensitive analytical methods: ion-exclusion column chromatography of nucleosides[2,3] and thin-layer separation[4] of *in vitro*-labeled nucleoside derivatives.

The results indicate that the incomplete recovery of a given minor component is accompanied by increased amounts of the parent nucleoside, and preparations appear to contain a heterogeneous population of tRNA's where the macromolecules differ in the extent of modification by methylation, isomerization, and thiolation.

In some of these preparations, the low amounts of certain of the rare modified nucleosides indicated that these nucleosides may be mixtures of completely and partially modified (mature and immature) tRNA's, possibly reflecting the times in the growth cycle at which they were harvested and adding incomplete maturation to the known causes of chemical heterogeneity. This observation must be taken into account in interpreting work with these widely used preparations.

A compound that is formed by a photochemical cross-linkage of cytidine and 4-thiouridine residues located in the amino acid and dihydrouridine stems of tRNA, respectively, was present in all five tRNA preparations. Some 35% of the tRNA's containing 4-thiouridine appeared to have been modified by this photochemical reaction during the course of their isolation. The formation of the photoproduct was responsible for equivalent losses in cytidine and thiouridine.

While neither of the two analytical systems employed can alone detect and quantitate every one of the many nucleoside species present in tRNA's, the column procedures[2,3] had the practical advantages of speed, scale, and range of chromatographic conditions (pH, temperature) without loss of versatility.

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2. R. P. Singhal, *Arch. Biochem. Biophys.* **152**, 800 (1972).
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ISOLATION OF BIOLOGICALLY ACTIVE INDIVIDUAL tRNA'S

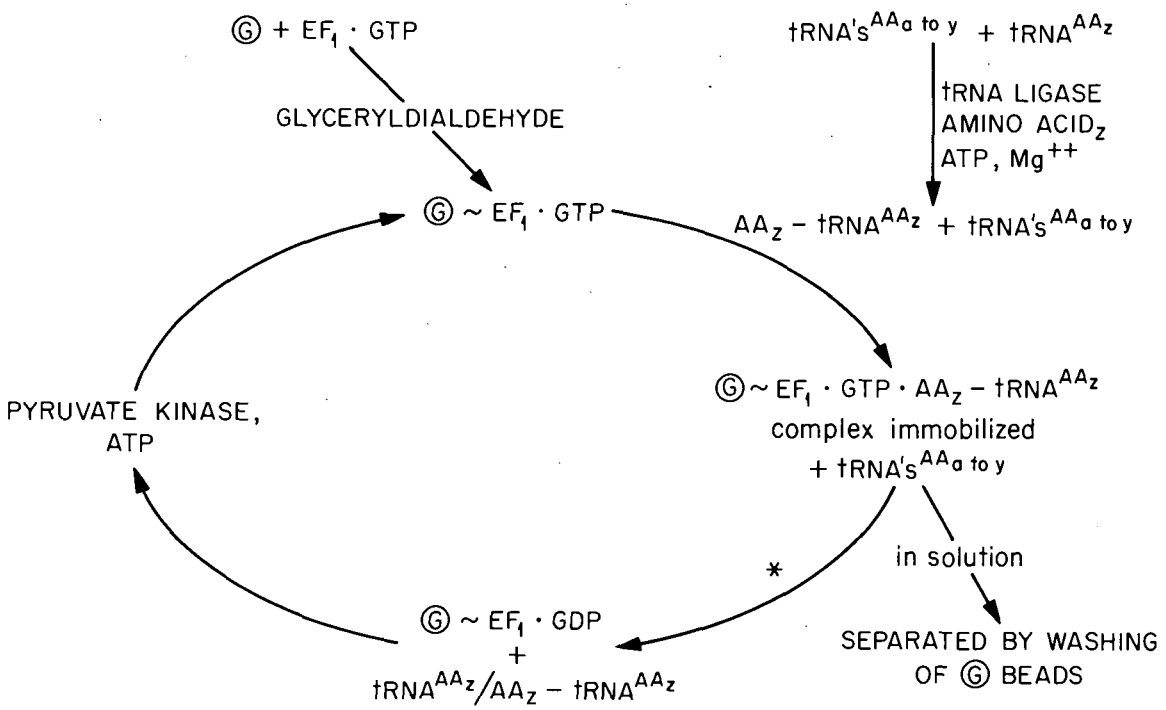
R. P. Singhal and G. David Novelli

The introduction of reversed-phase chromatography provided sensitive separations of large quantities of specific tRNA's. Significant differences in normal and cancer- and drug-induced tRNA's have been noticed. Since the tRNA's of these pathological samples are present in small amounts, differ in minute details, and are not resolved by earlier innovations, other methods for tRNA separation are sought.

Reactions of the scheme which follows essentially indicate a complex formation between a specific protein, called an elongation factor, and any aminoacylated tRNA. By making use of this reaction, we seek to explore isolation of tRNA's present in minute or major amounts in normal or in pathological tissue, such as cancer cells.

Results indicate that the elongation factor-1 of *E. coli* (Tu), and also of mammalian tissue (rat liver, EF_1), can chemically be immobilized onto inert support (such as glass beads) by Schiff-base coupling of free amino (ϵ-amino of lysine or N-terminal) groups of the elongation protein via a dialdehyde with alkyl amines that are bound to the inert support. By using [^{14}C]Glu-$tRNA^{Glu}$ and [^{32}P] mixed-tRNA's, such immobilized elongation proteins from both bacterial and mammalian sources were found active in the complex formation with aminoacylated tRNA's only, as expected, and inactive to uncharged tRNA's.

Despite difficulties in removing unbound tRNA's that have affinity to adsorb to the untreated glass surface contained with the alkylamine glass, preliminary data indicate that the tRNA's specific to a single amino acid can thus be prepared. Currently, investigations are under way for an inert support that could exclude any unspecific binding of tRNA's.



Notes to the scheme: G, alkylamine bound to glass beads; EF_1, elongation factor$_1$ of *E. coli* (Tu) or of mammalian cells (EF_1); and AA, amino acid; a to z, specific amino acids. Asterisk indicates tRNA specific to a single amino acid, bound to the bead, is recovered by breaking the complex. This is achieved by exchanging GTP of the complex with free GDP or by deaminoacylating the tRNA under high salt conditions. The net result in both cases is the recovery of immobilized Tu·GDP or Tu·GTP, which can be used again, and the isolation of a purified $tRNA^{AAz}$.

SUPPRESSOR MUTATIONS AND ALTERATIONS IN THE CHROMATOGRAPHIC PROFILE OF TRANSFER RNA IN *BACILLUS SUBTILIS*

Shigemi I. Simms and G. David Novelli

In a collaborative study with Manley Mandel, we have examined the tRNA profiles of 16 of the 20 aminoacyl tRNA's of certain strains of *B. subtilis* containing certain suppressor mutations. Mandel started with Frank Young's highly transformable strain *B. subtilis* B151. By transformation he moved all the genetic markers necessary to construct strain 129. This is now the wild-type suppressor-negative (Su–) parent. The phenotype of the parent is His^-, Met^-, sporeforming, and unable to support the growth of a defective phage. The addition of suppressor 1 (Su 1^+) to the parent yields strain 135, and the addition of suppressor 3 (Su 3^+) yields strain 173. At all other loci these strains are genotypically identical.

The tRNA from Su 1^+ cells and Su 3^+ cells were cochromatographed in separate experiments against the tRNA from the Su– parent. In each case both tRNA's were aminoacylated with the same amino acid, but carrying either ^3H or ^{14}C as the label. These were cochromatographed over reversed-phase (RPC-5) columns using the appropriate NaCl gradient that would effect the best resolution of isoaccepting species. For many of the amino acids, we found the same number of isoaccepting species of tRNA in both of the suppressor-carrying strains that were in the same chromatographic position as those from the suppressor-negative parent. However, there seemed to be some variation in the quantitative amounts of the individual isoacceptors between the parent and the suppressed strains. The significance of these quantitative differences has not been evaluated at this time.

The most significant difference seen in these experiments was the appearance of qualitatively new peaks of isoacceptor tRNA in the tRNA's from the suppressor-carrying strains that were absent in the parent. Specifically, Su 1^+ cells exhibited a new peak of leucyl-tRNA and a new peak of lysyl-tRNA and of phenylalanyl-tRNA that are absent in the tRNA from the parent. The Su 3^+ cells exhibited only the new peak of lysyl-tRNA.

Further work, which will involve the isolation of the new peaks of tRNA free from all other species and

subsequent nucleotide analysis, will be required before it can be determined whether these new peaks of tRNA represent structurally new species coded for by the suppressor genes or whether they are structurally similar species as in the wild type that have not had all of their bases modified. Correlation of any physiological properties bestowed upon the cells that have these new tRNA's will depend upon studies being conducted in Mandel's laboratory.

MATURATION–MODIFICATION OF PRECURSOR tRNA

Jan Dijk* and R. P. Singhal

Ribonucleic acids play important roles both as short-lived messengers and as stable species like ribosomal or transfer RNA's. The RNA transcribed from genes (DNA segments) are longer than actual sizes of mRNA's or stable RNA's. The function of these "extra" segments in post-transcriptional molecules, such as precursor tRNA's, is unclear. A wide spectrum of precursor tRNA's from *Escherichia coli* were examined for structural differences and for degrees of modification of minor bases at early stages of maturation.

When protein synthesis in *E. coli* (Met^-, RC^{rel}) is inhibited by methionine starvation or chloramphenicol, precursor tRNA accumulates and is pulse-labeled with [6-^{3}H]uridine *in vivo*. Pre-tRNA and 5S RNA are eluted as a single peak between ribosomal and transfer RNA peaks on Sephadex G-100, indicating similar size (about 120 nucleotides). Chase experiments show that the presumed pre-tRNA disappears after 45 min; they indicate that it is converted to tRNA. When the pre-tRNA–5S RNA mixture is incubated with a cell extract of *E. coli* Q-13, about 50% is converted to RNA with a size similar to tRNA. In DNA·RNA hybridization experiments, the mature tRNA replaces the DNA-bound pre-tRNA. These results indicate that the middle peak of the 5S size from the Sephadex column contains precursor tRNA's.

The pre-tRNA is resolved into 3 to 5 distinct peaks after the 5S RNA peak in reversed-phase chromatography. [^{3}H]pre-tRNA and [^{3}H]tRNA, after short labeling (1 to 10 min) and when analyzed at the nucleoside level by anion-exclusion chromatography, both show an increase with time in the extent of uridine modification, such as isomerization, methylation, and thiolation. Moreover, when the extents of uridine modifications in pre-tRNA and tRNA are compared, a nearly constant ratio is observed (1:4 for both ψrd and rThd).

The nucleotide composition of pre-tRNA, resolved in eight individual peaks on a reversed-phase column, indicates a similar composition (adenylate, 19%; cytidylate, 27%; guanylate, 33%; uridylate, 21%) of seven peaks, which differ (adenylate, 21%; cytidylate, 22%; guanylate, 27%; uridylate, 30%) from one peak. Two-dimensional maps of oligonucleotides, obtained by pancreatic ribonuclease digestion of pre-tRNA peaks, show similarity in positions of several small oligonucleotides, but considerably differ in the structure of long-chain tRNA fragments.

The results are summarized: (1) pre-tRNA's are relatively stable molecules; (2) the modification of minor bases occurs even before the removal of the "extra" segment of nucleotides; (3) the base compositions of a wide variety of pre-tRNA's do not appear to differ appreciably; (4) in DNA·RNA hybridization experiments, the mature tRNA can replace the DNA-bound pre-tRNA molecules; (5) complete modification is not a prerequisite for maturation, contrary to the earlier report;[1] and (6) the distinct peaks of pre-tRNA on RPC-5 can be regarded as one or more precursor species with different degrees of modification.

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*Supported in part by NATO grant N 81-37.

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IN VITRO PROTEIN SYNTHESIS

Helen G. Sellin

The *in vitro* protein-synthesizing system from *E. coli*, developed in previous years, was used by D. Kelmers in Chemical Technology Division to monitor T7mRNA purification. While T7mRNA has proven remarkably stable under a variety of conditions, retaining its ability to direct lysozyme synthesis *in vitro*, all attempts to separate it from contaminating rRNA have been uniformly unsuccessful to date.

We have also attempted to increase the efficiency of lysozyme synthesis in the enzyme-ribosome purified system by the addition of extra amounts of some of the various known components. Addition of T7 or *E. coli* RNA polymerase has not improved T7 DNA-directed lysozyme synthesis. Examining T7mRNA-directed lysozyme synthesis, no effect was seen with elongation factors prepared by A. N. Best. Aliquots of the earliest steps during release factor preparations did stimulate, but this is probably due to the presence of ribosomes in these fractions. More purified fractions of release factors had no effect. Initiation factors are required if

salt (NH_4Cl)-washed, but not buffer-washed, ribosomes are used. Ribosomes treated with puromycin to remove tRNA must be salt-washed to remove puromycin. Such puromycin-treated ribosomes are used routinely.

Examining the enzyme preparation, which is a high-speed supernatant passed over DEAE cellulose, Sephadex G-25, and G-100, a high level of amino acid (leucine) synthetase activity is found. The enzyme preparation has an A_{280}/A_{260} = ca. 0.6 to 0.7, but the A_{260} absorbing material extracted with phenol is not chargeable.

LOW MOLECULAR WEIGHT RNA METABOLISM IN PHYTOHEMAGGLUTININ-STIMULATED HUMAN LYMPHOCYTES

G. D. Griffin

Human peripheral blood lymphocytes can be stimulated to transform into blast cells that subsequently divide by addition of the plant mitogen, phytohemagglutinin, to cultures of these cells. The process of transformation is characterized by large increases in protein and RNA synthesis, followed by DNA synthesis which begins about 30 hr after addition of the mitogen to the cultured cells. Extensive gene derepression seems to take place during the transformation process. Although ribosomal RNA synthesis accounts for a large percentage of the total RNA synthesized during transformation, significant quantities of 4S sedimenting RNA are also synthesized throughout the transformation process.[1] Low molecular weight RNA is the predominant species synthesized during the first hours after PHA addition to lymphocyte cultures. Experiments have been undertaken to examine two aspects of the transformation process: (1) the possible changes which may occur in 4S RNA species (particularly isoaccepting tRNA species) during the changeover from the resting cell to the blast form and (2) a possible regulatory or "primer" role of small molecular weight RNA in the induction of DNA synthesis.

Lymphocytes are separated from whole blood of human donors by sedimentation in dextran solutions and passage over a glass bead column, which removes phagocytic cells, leaving a cell population of greater than 90% lymphocytes. Cultures of these cells are incubated with radioactive RNA precursors at various times after stimulation with phytohemagglutinin (PHA), and RNA is extracted and analyzed by reversed-phase chromatography using the RPC-5 system. Chromatographic profiles of RNA from lymphocytes shortly after stimulation with PHA are similar to RNA profiles obtained from resting lymphocytes, although a quantitative shift in total distribution can be run by about 4 hr after PHA addition. By 12 to 18 hr after PHA addition, RNA profiles show considerable quantitative differences from the resting state, and this particular profile pattern persists until at least 60 hr after PHA addition. Nucleotide analysis (kindly provided by Ram Singhal) of radioactive RNA labeled with uridine indicated very similar extents of conversion to cytidine for a given period of labeling, independent of the time after PHA addition when the label was introduced. Polyacrylamide gel electrophoresis of RNA preparations showed that the RNA used for analysis was at least 90% 4S material. Centrifugation of lymphocyte DNA labeled by short pulses of radioactive thymidine in cesium sulfate solutions has been employed to look for an RNA primer covalently attached to newly synthesized DNA. No indication of such a hybrid species has yet been found in the PHA-stimulated lymphocyte.

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1. H. L. Cooper, *J. Biol. Chem.* **243**, 34 (1968).

STUDIES OF ALTERATIONS OF DNA-DEPENDENT RNA POLYMERASE OF *E. COLI* FOLLOWING T4 PHAGE INFECTION

Audrey L. Stevens and Diane D. Crowder

Upon infection of *E. coli* with T4 phage, a new program of transcription takes place. To accommodate the new program, DNA-dependent RNA polymerase of *E. coli* is modified in several ways.[1] We have found that four new small proteins are formed after infection and are bound to the enzyme.[2] Studies with mutants of T4 phage show that two of the new binding proteins may be involved in the formation of "late" T4 mRNA that forms after DNA replication starts in the phage-infected system. Gene 55 and gene 33 mutants of T4 phage failed to make late T4 mRNA, and one of the small proteins (M.W., 22,000) is missing from the polymerase following T4 phage 55^- infection and another (M.W., 12,000) following T4 phage 33^- infection.

RNA polymerase from two T4 phage-infected systems has been studied in detail. One infection system involves a T4 DO amber mutant, and polymerase (Enz^+) containing all four new binding proteins is isolated from it. The other system involves a T4 gene 55 mutant, and polymerase ($Enz\ 55^-$) lacking the 22,000 M.W. binding protein but containing the other three is isolated from it. The content of the new binding proteins and that of the sigma subunit (σ) of the polymerase have been determined at different stages of the purification procedure. Both Enz^+ and $Enz\ 55^-$

contain substantial amounts of σ at early purification stages, but their activity with T4 DNA as a template is low, suggesting that the σ activity is reduced on the enzymes. (σ has been shown by others[1] to highly stimulate polymerase activity with T4 DNA as a template.) Considerable σ is lost during the purification procedure, particularly on density gradient centrifugation. Following phosphocellulose chromatography of Enz^+ and Enz^-, and also polymerase from normal *E. coli*, σ-containing fractions and core enzyme fractions are obtained. Assays of these fractions show that: (1) the σ-containing fraction of Enz^+ is slightly stimulatory to the core enzyme fractions with T4 DNA as a template, but it strongly inhibits the activity of normal σ in stimulating their activity; (2) the normal σ-stimulated activity of Enz^+ and Enz 55^- core enzymes is inhibited at both low and high salt by the σ fraction of Enz^+, while the normal σ-stimulated activity of normal core enzyme is inhibited at high salt. The results show that Enz^+ contains an anti-σ activity which may be a modified σ. Enz^+ contains more of the inhibitory material than Enz 55^-, suggesting a possible correlation of its presence with the gene 55 function. The activities of Enz^+ and Enz 55^- with T4 DNA are very salt-sensitive, in marked contrast to normal enzyme activity.

Using a crude membrane fraction from T4 phage-infected cells, the effect of the enzymes described above on late T4 mRNA synthesis has been studied. The core enzyme fraction of Enz^+ stimulates late mRNA synthesis in the system. Other enzyme fractions are only 10 to 20% as effective under the conditions that have been used.

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THE BIOLOGICAL CONVERSION OF CHOLESTEROL TO ESTRONE*

Jan Dijk and G. David Novelli

Cholesterol dehydrogenase in *Nocardia*. When *Nocardia* is grown on a mineral medium containing glycerol as a carbon source, the only cholesterol-converting activity present is a cholesterol dehydrogenase which converts cholesterol into cholestenone ($\Delta^5 \rightarrow \Delta^4$, 3-OH → 3-O).

Since it had been reported that at cell yields higher than 1 g/liter the specific activity of the enzyme would decrease (*Meth. Enzym.*, Vol. I, p. 678), several modifications of the originally described growth medium were tried.

The enzyme is assayed by incubating *Nocardia* cells with cholesterol and measuring the amount of cholestenone formed. The specific activity is expressed as micromoles of cholestenone formed per hour per milligram of protein (the latter is determined by the Lowry reaction).

It was found that cells could be taken up to 3 g/liter when 0.1% of an acid casein hydrolysate was added to the medium; under these conditions the specific activity was about 1.0 to 1.4 units.

Pilot experiments were carried out for a culture in the 300-liter fermentor. The main problem was the tendency of the cells to concentrate in a floating layer which had to be broken up by suddenly increasing the stirring rate. Under these conditions the cell yield was more than 4 g/liter, while the specific activity was still maintained around 1.3 units.

A large batch of cells was grown in the 300-liter fermentor and shipped to San Diego. The specific activity was approximately 1.1 units.

Conversion of cholesterol into estrone by *Nocardia*. Cells have been grown on mineral medium supplemented with 0.5 g/liter cholesterol and 0.05% casein hydrolysate for more than a week. The insolubility of cholesterol prevents accurate measurements of the cell growth; they seem to grow very slowly.

Supernatants and cells taken at increasing time intervals were extracted with organic solvents. The presence of cholesterol in the cell preparations again presented difficulties. The extracts were analyzed by thin-layer chromatography (TLC). All samples turned out to be negative; only in cells after 46 hr of growth was a spot found which showed all the characteristics of estrone. The presence of large amounts of interfering material in the extracts prevented the application of larger amounts to the TLC. It is estimated that approximately 2% of the cholesterol has been converted into estrone.

In order to identify and quantitate the intermediates involved in the cholesterol-estrone conversion, experiments with ^{14}C- or ^{3}H-labeled cholesterol should be carried out. A major problem in these experiments is the presence of large amounts of insoluble cholesterol, which dilutes the specific activity of the radioactive cholesterol to undesirably low levels.

Systematic experiments are now being performed to find the minimal amount of cholesterol needed for the cells to induce the enzymes for the conversion. Apart from cholesterol as a substrate, cholesterol hydrogen succinate (more soluble than cholesterol) and cholesterol acetate are tried.

In the meantime, a satisfactory TLC system for the separation and identification of intermediates has been tested.

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*Work performed in collaboration with the Chemistry Department, University of California, San Diego, under Interagency Agreement NSFAG442, AEC40-382-72.

ISOLATION OF A HEART-RATE-DEPRESSING PHEROMONE FROM CROWDED GOLDFISH

Peter Pfuderer and A. A. Francis

Several species of fish, when crowded, excrete into their water a substance or substances that can be extracted from the water by organic solvents. These factors have been shown to inhibit the fish's growth and reproduction when in a small body of water. These inhibitors have also been shown to be species specific or family specific.

We have found that a family-specific growth inhibitor could be extracted from the water of crowded goldfish using trichloroethane, chloroform, or carbon tetrachloride. These extracts also contained a family-specific heart-rate-depressant activity which could be identical to or similar to the growth inhibitor. We have set up an assay to measure the heart-rate depression of a goldfish in water and have used this assay to follow the purification of this crowding pheromone in both goldfish and carp.

The heart-rate-depression activity behaves as a diglyceride on column chromatography. This heart-rate pheromone is a low-molecular-weight lipid with a very unique ultraviolet spectrum, having a maximum at 272 nm.

The heart-rate-depressant activity of the pheromone is completely reversed by the addition of low concentrations of atropine to the fish water, implying that the activity is mediated through the nervous system. We can speculate that the growth- and reproduction-inhibiting pheromone or pheromones could also act through the nervous system, perhaps controlling pituitary functions, and that this control mechanism may be found in higher vertebrates also, where growth, reproduction, and heart rate are all known to be inhibited with age.

THE EFFECT OF PHTHALATE ESTERS ON THE HEART RATE OF THE GOLDFISH

Peter Pfuderer and A. A. Francis

Chloroform extracts of tissues of cyprinidont fishes from the TVA lakes were found to contain substances capable of acting as a heart-rate depressant on the goldfish when dissolved in that fish's water. Three of these compounds have been isolated, and two of them have been identified by mass spectroscopy as benzyl butyl phthalate and dioctyl phthalate. These compounds were concentrated primarily in the liver and to a lesser extent in the brain, heart, and kidney tissues as assayed by the change in the goldfish heart rate. The heart-rate-depressing activity of these compounds may be confined to the cyprinidont fishes, since no effect has yet been observed in fish of other species. Low doses of phthalate esters produced corresponding depressions of the heart rate, which were reversible. High doses (0.003%) of these compounds have been observed to lower the heart rate from approximately 120 beats/min to 40–50 beats/min in about 10 min. Atropine (24 μg/ml) reverses this effect, which may indicate the primary effect is on the central nervous system.

BIOPHYSICS AND CELL PHYSIOLOGY

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RESPONSE OF HUMAN RED CELLS TO FREEZING AND THAWING AS A FUNCTION OF THE PERMEATION OF PROTECTIVE SOLUTES

R. H. Miller and Peter Mazur

Previous studies have demonstrated that protective compounds such as glycerol do not have to penetrate bovine red cells in order to confer protection during freezing.[1] Cow red blood cells exposed to glycerol for a few seconds at 0°C and then frozen survived as well as cells held for 30 min at 20°C. Survival was equal despite the fact that intracellular glycerol concentrations differed by a factor of 200 in the two instances.

Current experiments involving human red blood cells have yielded similar results. Permeability coefficients for glycerol were determined by measuring the time to hemolysis in hypotonic media and the time to susceptibility to osmotic shock in isotonic media. The human cell differed from the cow cell by being 45 times more permeable to glycerol at 20°C and 300 times more permeable at 0°C. The increased difference at 0°C reflects the much lower activation energy required for glycerol permeation in the human red blood cell.

Survival (as measured by percentage of unhemolyzed cells) of human red blood cells held in 2 *M* glycerol for 0.2 to 10 min ranged from 70 to 85%. It is suspected that this slow increase in survival with time in glycerol may reflect the upward slope of a transient dip in survival similar to that noted with bovine red blood cells. Reasons for discounting glycerol permeation as causative stem from experiments in which cells were suspended in 1.404 *M* sucrose (isosmotic with 2 *M* glycerol) for periods of 0.5 to 10 min. Even though sucrose is a nonpermeating compound, survivals of 85% were obtained. These results indicate that permeation of the additive is not necessary for protection during freezing and that sucrose protects the cell as well as does glycerol.

The above survival measurements were made on cells still suspended in glycerol or sucrose. More recent experiments have been concerned with returning frozen-thawed cells to an isotonic saline environment prior to making hemolysis measurements. Red blood cells suspended in 2 *M* glycerol for 0.5 to 15 min prior to freezing and returned to isotonic saline for measurement have yielded survivals of 78 to 89%.

1. P. Mazur, S. P. Leibo, and R. H. Miller, *Cryobiology* 8, 383 (1971).

SURVEY OF PRESERVING MAMMALIAN CELLS BY FREEZING

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

The most commonly accepted procedure for preserving the viability of nucleated mammalian cells by freezing is to suspend them in about 10% dimethyl sulfoxide (DMSO) or glycerol, cool the suspensions at about 1°C/min, store them in liquid nitrogen, and warm them at moderate to rapid rates (100° to 500°C/min).

It has been shown that for many cell types these procedures yield sufficient viable cells to initiate a new culture, although the actual percentage of survival is frequently very low. This, in turn, raises the specter of the freezing itself producing a selection for undesired mutants that may be present in the frozen stock. To reduce these problems and to seek procedures permitting the long-term storage of cell stocks by freezing, we have initiated a survey of the freezing sensitivity of a variety of mammalian cell types.

As a result of a detailed analysis of freezing sensitivity of hamster tissue-culture cells, we have shown that the most important variables that determine the ultimate survival of frozen-thawed cells are (1) the cooling rate and (2) the nature and concentration of the protective compound. Therefore, our approach is to suspend the cells to be tested in 1 *M* DMSO, one of the more effective protective compounds known, and then to cool the suspensions at rates from 1.5° to 1000°C/min to −196°C. The frozen suspensions are then warmed rapidly at about 600°C/min, since rapid warming of single-cell suspensions is almost always as good as and usually better than slow warming. The thawed suspensions are then assayed by procedures appropriate for each cell type.

Thus far, we have measured survival after freezing and thawing in 1 *M* DMSO of HeLa cells, assayed by colony-forming ability, and of mouse lymphoma cells by dye-exclusion. We have observed a survival maximum of 65% for HeLa cells frozen at 77°C/min, and a broad survival plateau of about 80% to 90% for mouse lymphoma cells frozen at 2° to 60°C/min. These studies are presently being extended to several other types of cells.

PLASMOLYSIS INJURY AS A FACTOR IN FREEZING INJURY IN PLANT TISSUE-CULTURE CELLS

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

Research on freezing injury in higher plants has been handicapped by the difficulty in interpreting results for

whole plants and whole plant organs. Plant tissue cultures, however, can be used to quantitatively assess the relationship between survival and the various cryobiological parameters. The present study is making use of suspension tissue cultures of *Haplopappus gracilis*. From these cultures one can isolate single cells and small clumps containing one to six cells, and the viability of these cells can then be determined by colony formation on agar.

Haplopappus is extremely sensitive to freezing injury; survival of cells frozen in distilled water dropped from 70% at −2°C to 1.6% at −3°C. Sucrose and DMSO conferred some protection, but not very much. Since slow freezing would dehydrate the cell and produce plasmolysis, it was decided to determine the effects of nonpenetrating plasmolyzing agents on these cells.

The time course of survival at 20°C in either 1 *m* sucrose, 1.08 *m* glycerol, or 1 *m* mannitol showed an initial rapid decrease in survival followed by a more gradual decrease in survival over a 2-hr period. Examination of the kinetics of plasmolysis under a microscope revealed that it began within 30 sec after addition of the plasmolyzing agent and was essentially complete within 90 sec after addition. The decrease in survival in sucrose was not prevented by the slow stepwise addition of sucrose to the final 1 *m* concentration or by the slow stepwise dilution from 1 *m* sucrose.

The concentration of sucrose that produced incipient plasmolysis was between 0.45 and 0.50 *m*, values similar to the threshold values for injury. The time course of survival in 0.25, 0.4, and 0.5 *m* sucrose was essentially the same as that of cells in distilled water. But in 0.6, 0.8, 1.0, and 2.0 *m* sucrose, cell survival drops with time; and in 1.0 and 2.0 *m* sucrose, it exhibits an initial rapid drop.

During freezing, these injurious concentrations would be reached between −2°C and −4°C, temperatures that correspond fairly closely to those that were observed to produce lethal injury during freezing.

EFFECT OF PHYSIOLOGICAL STATE OF *NEUROSPORA CRASSA* IN RESPONSE TO PHYSICAL STRESS

J. L. Leef*

The research for the past year has been concerned with a "physiological characterization" of *Neurospora crassa* protoplasts formed by the action of the digestive juice from the snail *Helix pomatia*. Effects of various concentrations of the digestive juice on physiological functions of the cells were determined, including viability, time to germination or colony formation, respiration, ability to withstand osmotic shock, the degree of viability after such a shock, and changes in ultrastructure. Comparisons have been made between untreated conidia and three types of protoplasts: conidial, hyphal, and a genetic variant of *Neurospora crassa* which largely lacks a cell wall. The results indicate that all of the physiological parameters studied are influenced to variable degrees by both concentration of and length of exposure to the digestive preparation. In addition, tremendous batch variability was observed with the digestive juice. The first batches used had no effect on the viability of *Neurospora crassa* conidia after lengths of incubation up to 24 hr. However, the same concentration of digestive juice from another batch exerted a cytotoxic effect on the conidia after only 5 min of exposure. In the latter case, the conidia were not converted to protoplasts but killed outright by minimal exposure to the digestive juice. The toxicity was not removed following dialysis or partial purification by column chromatography.

I am now studying the effects of various freezing and thawing regimes on the viability of germinating conidia. Mazur has shown that different cell types require different cooling and warming velocities to achieve optimum viability following freezing and thawing. It is expected that these optimum values will change for the conidial population as it passes from the dry state to hydrated and finally to germinated. In addition, the effect of various low temperature preservatives will be examined with these three basic cell populations. Low temperature preservatives are broadly classified as either intra- or extracellular agents. It seems probable that each basic cell population will have different requirements for additives. The type and concentration of preservative that is most effective on hydrated conidia will not necessarily be the same for germinated conidia.

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

Peter Mazur and S. P. Leibo

Increasing evidence indicates that formation and recrystallization of intracellular ice is responsible for the death of cells cooled at supraoptimal rates. There is less understanding of the cause of injury in cells cooled at suboptimal rates. One favored hypothesis is that injury results from the concentration of electrolytes

during freezing. A second hypothesis ascribes injury to the shrinkage of cells during freezing. Additives such as glycerol and DMSO are hypothesized to protect by reducing the electrolyte concentration or by reducing cell shrinkage. As discussed previously,[1] data on the survival of slowly frozen Chinese hamster tissue-culture cells vs temperature do not appear consistent with these proposed mechanisms of protection. The present experiments were designed to determine whether survival of the cells could be correlated with the extent to which the extracellular medium was solidified. The latter was estimated by measurements of the ac electrical resistivity of solutions during slow freezing. The solutions used were 0 to 1 *M* concentrations of DMSO, glycerol, and sucrose in Hanks' balanced salt solution (HBSS). Between the freezing point and the temperature of total solidification, the resistivity increases by some 5 to 6 orders of magnitude. Plots of the survival of hamster cells vs the resistivity of the solution showed the following:

1. Injury after slow freezing in HBSS occurred before the medium completely solidified.
2. In the presence of partly protective additives, injury correlated better with solidification of the medium than with either temperature or the concentration of electrolytes.

It appears that, during slow freezing, hamster cells are subjected to at least two sequential injurious events. Cells in HBSS are injured by the concentration of solutes or by cell shrinkage. Glycerol, sucrose, and DMSO protect against this source of injury but often do not protect against the second injurious event – total solidification of the medium.

1. Peter Mazur and S. P. Leibo, *Cryobiology* 8, 382 (1971).

EFFECT OF ELECTROLYTE COMPOSITION OF SUSPENDING MEDIA ON SURVIVAL OF SLOWLY FROZEN HAMSTER TISSUE-CULTURE CELLS

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

The mechanism responsible for injury of mammalian cells when they are frozen at less than optimum rates remains unknown. We have ascribed this injury to "solution effects," a purposefully ambiguous phrase intended to include those physical and chemical properties that are known to undergo drastic changes during freezing and thawing of multicomponent solutions. This concomitancy of changes includes solute concentration, pH, osmotic pressure, hydration, and phase state. Each of these has been suggested as the primary change responsible for cell damage during slow freezing.

A beginning attempt to distinguish between the relative roles of these changes is being made by determining the temperature ranges over which hamster tissue-culture cells are inactivated when slowly frozen in solutions of glycerol, dimethyl sulfoxide, or sucrose dissolved in either (1) Hanks' balanced salt solution (HBSS, a solution of seven salts plus glucose) or (2) sodium chloride alone. Each of the components of HBSS will affect the properties of the solution as a whole and the temperatures at which changes occur. The changes of the two-component system, NaCl plus additive, can be much more precisely defined. For example, the unbuffered NaCl-glycerol solution will not exhibit the pH change during freezing that occurs in the buffered HBSS-glycerol solution.

Comparison of the survival of hamster cells slowly frozen in HBSS plus each of the additives as a function of temperature with that of cells frozen in NaCl plus additive indicates that inactivation in the sodium chloride solutions occurs over a much narrower temperature range and, in general, at significantly higher temperatures. When completed, these survival measurements will then be examined in the light of the temperature ranges over which the solutions undergo complete solidification, as estimated by measurement of their resistivity during slow freezing.[1]

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FACTORS AFFECTING THE SURVIVAL OF FROZEN-THAWED MOUSE EMBRYOS

S. P. Leibo, Peter Mazur, and Suzanne C. Jackowski*

A central issue in low-temperature biology is the mechanism by which certain compounds are able to protect cells against the potentially damaging events that occur when cells are slowly frozen to subzero temperatures. The preimplantation stages of mouse embryos offer some advantages in studying this question: no embryos survive freezing in the absence of a protective compound, whereas almost 90% of two- and eight-cell embryos survive freezing to −196°C when cooled at 0.2°C/min in 1 *M* DMSO. This means that the embryos are protected by DMSO against high electrolyte concentrations and altered pH during the 5 hr required to cool them to below −60°C. (Above that temperature, the suspensions are not completely frozen.)

Present evidence argues that protection of the embryos does not require permeation by DMSO since

51%, 71%, 67%, 72%, and 71% of eight-cell embryos survive freezing to −196°C after suspension in 1 *M* DMSO at 0°C for 30 sec, 5 min, 15 min, 30 min, and 60 min, respectively. Preliminary studies indicate that little DMSO can have permeated these cells at 0° in less than 20 min.

One of the more striking initial findings for these embryos, confirming theoretical calculations of the likelihood of intracellular ice as a function of cooling rate, was that cooling rates of 6°C/min or faster killed all the embryos. We have now found that the deleterious consequences of rapid cooling occur between −4° and −30°C.

An equally striking initial finding was that survival required warming frozen embryos from −196°C at 4° or 25°C/min. This observation has now been extended, demonstrating that the ultimate survival of frozen two- and eight-cell embryos depends on warming rate as critically as on cooling rate. The injurious consequences of rapid warming have been shown to occur below −35°C; that is, frozen mouse embryos must be warmed slowly to above that temperature if they are to survive.

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

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

The ability to freeze cow embryos would be an important step in increasing the productivity of beef and dairy cattle. It would enhance the practicality of using superovulation to amplify the number of calves normally produced by a cow, and it would facilitate the transport and importation of exotic breeds. The successful freezing of mouse embryos reported last fall[1] increased the likelihood that a successful procedure can be found for bovine embryos and made it desirable to make the attempt.

Injections of follicle-stimulating hormone (FSH) and human chorionic gonadotropin into sexually mature Hereford cows at doses comparable to those in the literature have yielded as many as 20 embryos per animal. However, the results have been more erratic with sexually immature animals. The embryos (usually two- or four-cell) undergo two to three cleavages when cultured *in vitro* in a 5% CO_2–5% O_2–90% N_2 environment. Exposure to 1.2 *M* DMSO at 0°C for at least 30 min is not damaging. About 20 embryos have been frozen to date using conditions comparable to those that yielded optimal recoveries of mouse embryos. Five of the thawed embryos have undergone one or two cleavages but generally have not cleaved to the extent of unfrozen controls.

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PHYSICAL STRUCTURE OF ISOLATED EUCARYOTIC NUCLEI*

D. E. Olins, Ada L. Olins,† Marilyn B. Senior,
Everline B. Wright, and Mayphoon Hsie-Hsu

The purpose of the present studies is to describe the macromolecular structure of chromatin at its various levels of organization. Previous investigations from this laboratory have demonstrated that 4° structure (i.e., chromatin condensations) can be disrupted by removal of divalent metal ions and decreased solvent ionic strength, without concomitant destruction of 2° structure (i.e., periodic folding of a single nucleohistone fiber).

In the present investigations, isolated eucaryotic nuclei (chicken erythrocyte, rat thymus, and rat liver) were allowed to swell in distilled water, fixed with 1% formalin, centrifuged onto electron microscope grids, and positively or negatively stained. Such swollen nuclei revealed chromatin spilling through the ruptured envelope. Long (up to 8 μm) parallel and unbranched fibers were observed. Closer examination revealed a structure like "particles on a string"; spherical particles (~70 Å diameter), denoted ν bodies, were seen frequently connected by a thin strand (~15 Å diameter) throughout the spread chromatin. The three types of nuclei examined possessed ν bodies of very similar dimensions. We are currently collecting low-angle x-ray diffraction data on whole isolated nuclei in order to test models of 2° structure which involve close packing of spherical particles into folded or helical arrays.

The use of bifunctional chemicals to cross-link nuclear proteins in close proximity within nuclei has also been very productive. Isolated chicken erythrocyte nuclei treated with dilute glutaraldehyde (4 m*M*) at 0°C very rapidly (~10 min) exhibit the formation of histone polymers. These acid-soluble polymers have been isolated and characterized by amino acid analysis. Data are consistent with the view that the polymers consist largely of one histone type, F2C, cross-linked into structures containing, perhaps, six to eight molecules. It

is not yet possible to relate these apparent F2C clusters to any particular level of chromatin organization. Current speculation favors the view that F2C is on the surface of ν particles and that their susceptibility to cross-linking (i.e., their apparent proximity) is a consequence of close packing of the spheroid chromatin particles.

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†Consultant..

Cs_2SO_4 GRADIENTS CONTAINING BOTH Hg^{2+} AND Ag^+ EFFECT THE COMPLETE SEPARATION OF SATELLITE DNA'S HAVING IDENTICAL DENSITIES IN NEUTRAL CsCl GRADIENTS

D. M. Skinner and Wanda G. Beattie

Density gradients of cesium salts have long been used to separate and characterize DNA's of various organisms. Differences in density of two DNA species can be increased by binding mercury to T-rich DNA's or silver to G-rich DNA's in Cs_2SO_4 gradients. These techniques are particularly useful for separation of those satellite DNA's that have very marked base compositional bias. We report here experiments using both metals simultaneously to separate satellite DNA's that have identical densities in neutral CsCl gradients. We refer to these satellites as "isopycnic twins." To our knowledge, there has been no previous use of both metals concomitantly.

The DNA of the hermit crab, *Pagurus pollicaris*, forms a main band and a single, sharp satellite band of density = 1.725 g/cm^3 when centrifuged in neutral CsCl. The 1.725-g/cm^3 component, comprising less than 1% of the total DNA, consists in reality of isopycnic twins, both of which have interstrand bias in base composition; centrifugation of these satellites in alkaline CsCl gives rise to four distinct bands.[1]

Some experiments require the two satellites in the native state rather than as single-stranded molecules from preparative alkaline CsCl gradients. However, neither mercury nor silver alone effected a usable separation. The buoyant density and melting behavior of one of the two satellites are similar to those of the synthetic polymer poly d(T-G:A-C).[2] This suggested that the introduction of both mercury and silver into Cs_2SO_4 gradients might effect the separation of this satellite from the other.

The addition of mercury at r_f = 0.1 and silver at r_f = 0.64 leads to complete separation of the isopycnic twins in Cs_2SO_4 gradients. Appropriate fractions from preparative gradients were pooled, dialyzed to remove the heavy metals, and analyzed in the analytical ultracentrifuge. In neutral CsCl, both had densities of 1.725 g/cm^3, as they had before separation, indicating no cooperative interaction between the two in the native state. In alkaline CsCl, specific fractions of the preparative mercury plus silver–Cs_2SO_4 gradient separated into the two components of intermediate densities while other fractions contained the least and the most dense strands.

The separation of the two satellites in mercury plus silver–Cs_2SO_4 gradients is dependent on the concentration of DNA. When DNA is present at less than 20 μg/ml, centrifugation in the presence of the same molar ratios of silver and mercury is ineffective. Use of one or both of the heavy metals with total DNA did not effect the separation of the satellites; perhaps this is because of the small amount of satellite relative to the total DNA.

Because of their behavior in alkaline CsCl gradients, we previously concluded that lobster satellite DNA's (ρ = 1.715 g/cm^3 in neutral CsCl) were composed of two components; one appeared to be of unbiased base composition in the two strands, and the other appeared to have one strand richer in guanosine and/or thymine than its partner.[1] The mercury plus silver–Cs_2SO_4 method separates native (purified as a single band in neutral CsCl) lobster satellite(s) into two components. However, in alkaline CsCl each forms multiple bands, indicative of multiple components.

These experiments reemphasize the fact that the buoyant densities of DNA cannot be simply accounted for by overall base compositions,[3,4] nor can their metal binding properties; they are also dependent on the sequence of the bases in the polymer. If the satellites described here are composed of a large number of reiterated short sequences, the immediate neighborhood of any one repeating base might not be randomized to the extent expected in a large polymer of nonreiterated sequences; this nonrandom microenvironment may importantly influence the physical properties of the DNA.

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CHARACTERIZATION OF TWO ISOPYCNIC TWIN CRUSTACEAN SATELLITE DNA'S

D. M. Skinner and Wanda G. Beattie

One percent of the DNA in all tissues examined (gonads, hemocytes, epidermis, nerve) of the hermit crab, *Pagurus pollicaris,* is comprised of two GC-rich satellites of identical densities in neutral CsCl; we refer to these as "isopycnic twins." In the presence of both Ag^+ and Hg^{2+} in Cs_2SO_4 density gradients, the isopycnic twins may be separated;[1] some of their properties have been described previously.[2] We have separated the isopycnic twins and determined their T_m's individually in SSC/10. The data are summarized in Table 3. We have also purified the individual strands of the two satellites by centrifugation of *Satellites I* and *II* separately in alkaline CsCl preparative gradients and determined the densities of the single strands. The density determinations indicated that the strands are strongly biased in base composition (unlike the GC-rich satellite of the land crab, *Gecarcinus,* whose partner strands do not show interstrand bias).

Analyses of DNAse digests (nucleosides) of the separated strands of the isopycnic twins by ion exclusion chromatography on Aminex 6 (data to be published) reveal the following: *Satellite I, H* strand, contains 50% G, 3% C, 24% A, and 23% T residues; *Satellite I, L* strand, contains the complementary proportions of the bases. A similarly low content of C and G residues has also been reported in the opposite strands of the α-satellite of the guinea pig.[3] *Satellite II, H* strand, contains 37% G, 27% C, 9% A, and 27% T residues, while its partner again contains the complementary bases.

It should be noted that the densities of *Satellite I, H* strand, in both neutral and alkaline CsCl are the same as those of a synthetic polymer of alternating G-T residues,[4] yet the satellite contains only 23% T residues (in addition to the other residues listed above).

Table 3. Characteristics of the isopycnic twin satellites of hermit crab DNA

Satellite	T_m (°C)	Strand	Density (g/cm^3)	
			Neutral	Alkaline
I	72	H	1.771	1.828
		L	1.718	1.725
II	83	H	1.751	1.803
		L	1.720	1.762

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NUCLEOTIDE SEQUENCE OF A CRUSTACEAN SATELLITE DNA

D. M. Skinner, F. R. Blattner,* and J. E. Dahlberg†

With the present available methods a nucleic acid must possess two properties to permit its sequencing: (1) the molecule must be small or the sequence simple and (2) it must be single stranded, or, if double stranded, the strands must be physically separable from each other. Some, but not all, satellite DNA's meet these requirements. Their dissociation and reassociation characteristics indicate that they are simple in sequence and that the base composition of the partner strands differ sufficiently to permit their separation in alkaline CsCl density gradients.

We have transcribed the separated strands of the hermit crab *Satellite I* with *E. coli* RNA polymerase and determined the sequence of the RNA products. The product of the C-rich strand is a tetramer of UpApGpGp; happily, the product of the G-rich strand is complementary. There are also a few oligonucleotides which occur only 1 to 5% as often as the major oligonucleotides. We are determining whether these are opposite-strand contaminants or an additional satellite.

The crab tetramer is a sequence which occurs in the repeating hexamer proposed for the guinea pig α-satellite from depurination products.[1]

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CIRCULAR DICHROISM STUDIES ON ALTERNATING AND NONALTERNATING AT-RICH SATELLITE DNA'S

D. M. Gray* and D. M. Skinner

The particular characteristics of CD spectra of synthetic oligonucleotides are dependent upon the nucleotide sequence, a fact which raises the possibility of sequencing simple naturally occurring DNA's by this technique. Few such studies have been done on

naturally occurring DNA's, partly because of their complexity. We have initiated studies on some satellite DNA's whose dissociation and reassociation characteristics indicate that they contain simple and highly repetitive sequences. Two of the satellites are poly d(A-T)'s of the land crab, *Gecarcinus lateralis,* and the Jonah crab, *Cancer borealis;* the other is the A+T-rich satellite of the edible blue crab, *Callinectes sapidus.*[1,2]

The CD spectra of both the *Cancer* and *Gecarcinus* satellites very closely resemble the spectrum of synthetic poly d(A-T), although the CD band at 262 nm in the *Gecarcinus* spectrum is less in magnitude than the 262 band in the *Cancer* spectrum and is thus not quite as close to the spectrum of synthetic poly d(A-T). This difference between the *Cancer* and *Gecarcinus* satellites indicates that the latter contains more AA:TT neighbors.

After denaturation, the *Gecarcinus* poly d(A-T) does not anneal as perfectly as does the *Cancer* poly d(A-T). The annealed *Gecarcinus* satellite has a 262-nm band which is larger than that in the native DNA spectrum. This possibly means that the alternating A-T regions of the *Gecarcinus* satellite have been preferentially annealed. In any case, the *Gecarcinus* satellite does not have as perfectly repeating a sequence as does the *Cancer* DNA.

The *Callinectes* satellite CD spectrum is unlike that of the spectrum of poly d(A-T). It is more like the spectrum for an alternating trimer sequence, poly d(ATT):d(AAT). The comparison is not exact, especially for the 220-nm band, due to the presence of 13% G,C pairs. Denatured, partially annealed *Callinectes* satellite again approaches the native DNA in its CD spectrum, an unlikely result if the sequence contained tandem regions of varying lengths containing either AT:AT or AA:TT neighbors, as opposed to a repeating AAT:TTA basic sequence.

The CD spectra will be subjected to a computer program which provides consistent first-neighbor frequency distributions.

In summary, although the *Cancer* and *Gecarcinus* satellites have some optical similarities, consistent with their large alternating AT contents, there are also definite differences. The *Callinectes* satellite does not appear to contain a significant proportion of alternating AT sequences.

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INTERACTING CONTROLS OF CRUSTACEAN MOLTING AND REGENERATION

Christie A. Holland* and D. M. Skinner

The processes of limb regeneration and molting are intimately, though complexly, interrelated in crustacea. For example, in the land crab *Gecarcinus lateralis,* the loss of limbs during the intermolt period is a highly effective stimulus to initiate preparations for ecdysis (i.e., precocious onset of premolt period) including regeneration of the missing appendages. During the early growth phase of these regenerates in the premolt period, their loss is an effective inhibitor to preparations for ecdysis (e.g., synthesis of new exoskeleton; further growth of remaining regenerate limbs) until re-regeneration of the lost limbs restores the coordinated developmental phases of pre-ecdysis.[1,2] This inhibition, or premolt pause, may result in an ecdysis delayed as much as double the usual time (i.e., 50 days).

We have initiated studies extending these morphological observations, made on whole animals, to the molecular level; our first concern has been with DNA synthesis as determined by the rates of incorporation of [^{3}H] thymidine into limb buds removed from the animal and cultured *in vitro* to allow better control of the assay conditions. Specific activities were determined on acid-insoluble material banding at appropriate densities in CsCl preparative gradients. Our findings to date are: (1) During a normal uninterrupted premolt period (ca. 55 days), the rate of DNA synthesis in a regenerating limb bud is initially high and falls progressively as the limb approaches its final dimensions. (2) If in early phases of regeneration, four limb buds are taken for assay, they show the characteristic rate of DNA synthesis. If the remaining four limb buds are assayed 48 hr later, they show a near-complete inhibition of DNA synthesis subsequent to the loss of the first four regenerates. (3) If the same experiment is done late in the premolt period, the inhibition subsequent to the loss of the first four limbs is not observed.[3] In parallel observations on the whole animal, there is no premolt pause and ecdysis is not delayed.[2]

These results confirm and extend our earlier conclusion that the preparations for ecdysis are not simply a chain of events triggered by a single stimulus, like the cessation of molt-inhibitory hormone secretion, as has been proposed. On the contrary, the preparations for ecdysis are a complex of interrelated, parallel morphogenetic and biochemical events that suggest multiple interlocking controls rather than a single trigger.

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

L. H. Yamaoka and D. M. Skinner

We have selected as a model system for studies on muscle degeneration and resynthesis under physiological regulation, the land crab, *Gecarcinus lateralis,* an animal in which the claw muscle undergoes degeneration and regeneration during normal growth (i.e., the intermolt cycle).[1] Up to 40% of the muscle protein is lost prior to ecdysis and is rapidly synthesized after the molt.[1] Although our ultimate goal is to determine the controls of this system, at the outset it is necessary to characterize some of the basic biochemical aspects of the turnover of muscle protein. For this purpose, we needed (1) to be able to initiate the degradation-regeneration (molt) cycle *ad libitum;* (2) to define the phase of degradation at the enzyme level; and (3) to define the synthetic phase of muscle protein.

1. Procedures have been developed in this laboratory to induce precocious molting throughout the year without significant mortality.[2]

2. We have assayed the activities of two lysosomal enzymes in muscle homogenates during the period of tissue degradation. Elevated levels of both enzymes – acid phosphatase, assayed with *p*-nitrophenyl-phosphate, and cathespin D, assayed with denatured hemoglobin – were found during the premolt and early postmolt period when the muscle mass is reduced to its lowest levels.

The magnitude of the muscle lost implies that the major structural proteins are involved. Therefore, we are preparing to measure the turnover of one of these proteins, myosin. Whether or not the observed loss of muscle protein represents accelerated degradation cannot be known without information on the other half of the turnover question, namely, changes in the rates of synthesis. Determination of the uptake and loss of an isotope from a protein requires isolating and clearly identifying the protein by a rapid and reproducible method.

3. We have initiated studies on the synthetic phase of the muscle protein, myosin. As a first step we have devised appropriate modifications of existing procedures for the isolation of crustacean myosin, which is being characterized by DEAE-Sephadex chromatography and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

After identification of the components of crustacean myosin analytically, muscle proteins will be labeled *in vivo,* and myosin will be extracted and sulfonated to prevent aggregation of the heavy subunits and to permit their migration as a single band.[3] A continuous flow acrylamide gel electrophoresis apparatus will be used to isolate the individual subunits.

Meaningful measurements of the uptake and loss of label from a protein also require information on the free pool of amino acids, as dilution effects can significantly affect the resultant rate of uptake observed. Measurements of the free amino acid levels in hemolymph and muscle are in progress.

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APPARENT DISSOCIATION OF THE DNA–CELL MEMBRANE COMPLEX IN DIVIDING BACTERIAL CELLS

W. D. Fisher and D. R. Stallions

Both biochemical[1] and electron microscopic evidence[2] suggest that the bacterial chromosome is attached to the cell membrane. In addition to a possible role in DNA synthesis, the DNA–cell membrane complex has also been assigned a direct physical role in chromosome segregation.[3] We examined DNA-membrane association in dividing cells by the sarcosinate M-band technique[1] and found a marked transient decrease in dividing cells.

Escherichia coli (Bug 6) is a temperature-sensitive mutant which forms typical rod-shaped cells at 32°C but grows as long multinucleoid filaments at 42°C. Under both growth conditions, the DNA is largely (>90%) membrane associated. When the temperature-induced filaments are shifted back to 32°C, they divide rapidly into unit cell lengths; and, just prior to separation of the filament into daughter cells, the extent of DNA-membrane association decreases markedly (Fig. 3). A culture of *E. coli* B/r synchronized

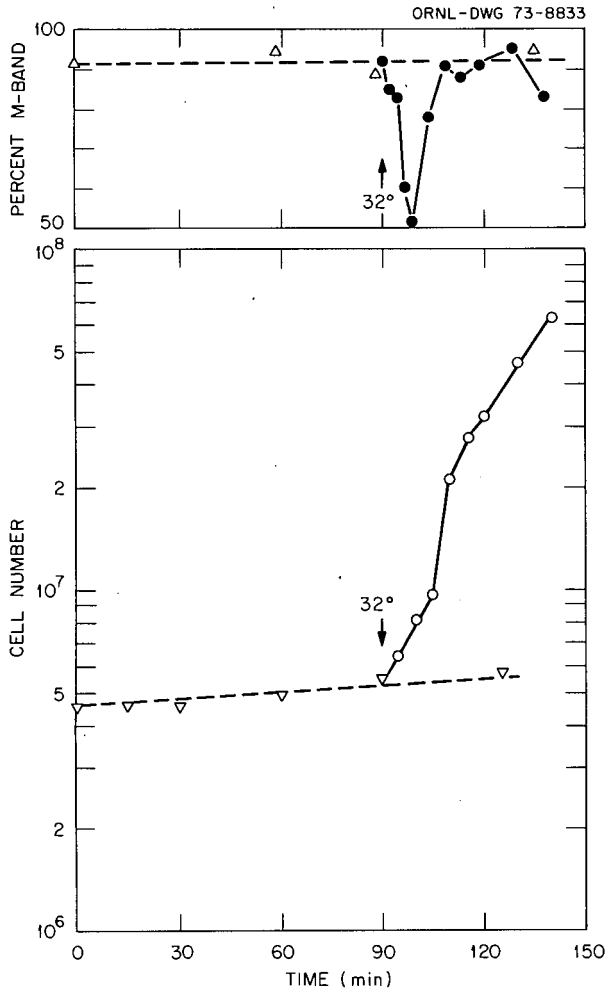

Fig. 3. DNA-membrane association in *E. coli* Bug 6. At T = 0, cells prelabeled at 32°C with [^{3}H]thymidine were shifted to 42°C. After 90 min a portion of the cells was returned to 32°. △ percent of ^{3}H counts in membrane fraction in cells at 42°C, • percent of ^{3}H counts in membrane fraction of cells shifted to 32°C, ▽ cell number at 42°C, ○ cell number after shift to 32°C.

by the sucrose gradient techniques gave similar results. Again there was a rapid and transient decrease in the amount of DNA in the membrane fraction just prior to separation of the daughter cells. Analyses of cells of *E. coli* strains Bug 6, B/r, and 1899NM, which had been separated by size (and therefore "age") on sucrose gradients, are consistent with the idea of a dissociation of the DNA from the membrane just prior to cell division: the large cells showed a decreased DNA-membrane association.

There appears to be an alteration of the cell-membrane–DNA complex during cell division. These data conflict with the proposal[3] that the DNA attachment sites effect the separation of daughter chromosomes unless one assumes that segregation has occurred prior to detachment of the DNA. It is also possible that the decrease in DNA-membrane association results from an alteration in the affinity of the membrane for the sarcosinate crystals which is the basis of the assay, rather than from a detachment of the DNA. This is under investigation.

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CELL DIVISION AND MINICELL FORMATION IN *E. COLI*: A COMMON GENETIC CONTROL

G. G. Khachatourians,* D. J. Clark,† H. I. Adler, and Alice A. Hardigree

Cell division and minicell formation in *E. coli* are the end result of biochemical reactions and regulatory networks that begin at a specific time in the life cycle of the cell and are coordinated with the chromosome replication cycle. These reactions normally proceed in an integrated manner and culminate in the physical separation of the two daughter cells. To understand the nature of the genetic control of cell division in the central vs polar region of the cell, we have studied a conditional-lethal cell-division mutant constructed in this lab.

E. coli Div 124(ts) is a conditional-lethal cell-division mutant formed from a cross between a mutant that produces polar anucleated minicells[1] and a temperature-sensitive cell-division mutant affected in a stage in cross-wall synthesis.[2] Under permissive growth temperature (30°C), Div 124(ts) grows and produces normal progeny cells and anucleated minicells from its polar ends. When transferred to nonpermissive growth temperature (42°C), growth and macromolecular synthesis continue, but cell division and minicell formation are inhibited. Growth at 42°C results in formation of filamentous cells showing some constrictions along the length of the filaments. Return of the filaments from 42° to 30°C results in cell division and minicell formation in association with the constrictions and other areas along the length of the filaments. This gives rise to a "necklace-type" array of cells and minicells. Recovery of cell division is observed after a lag and is followed by a burst in cell division and finally by a return to the normal growth characteristic of 30°C cultures. Recovery division takes place in the presence of inhibitors of protein or DNA synthesis when these are added at the time of shift from 42° to 30°C and indicates that a division potential for filament fragmentation is accumulated while the cells are at 42°C.

We conclude that minicell production and normal cell division share at least one common biochemical step. Furthermore, the evidence suggests that the temperature-sensitive division protein known to accumulate in Bug 6[2] is at least one of the intermediates controlling both normal and minicell-yielding divisions. The manner in which the division potential is used, for example, at the catalytical or structural level in the control of cell division in minicell formation is still unknown.

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DEGRADATION OF CONJUGALLY TRANSFERRED DNA IN MINICELLS FROM RECOMBINATION AND DNA REPLICATION DEFICIENT STRAINS OF *E. COLI* K12

G. G. Khachatourians* and R. J. Sheehy†

We have previously shown that minicells from wild-type F^- parental cells can degrade conjugally transferred Hfr DNA.[1] This suggested the presence of nucleases in minicells. We have examined the ability of minicells from various strains bearing known mutations which alter the levels and types of nucleases and DNA recombination-repair proficiency and DNA replication in *E. coli.* We have examined minicells from *rec* A^-, *rec* B^-, *end* I^-, *dna* A (TS), and *dna* B (TS) mutants and have shown that they are not affected in their recipient ability for DNA transferred by Hfr donor cells. The weight-average molecular weight of DNA transferred into these minicells varies and is in the range of 25–32 $\times 10^6$ daltons. Our studies indicate that single-strand specific endonuclease(s) and exonuclease(s) are present in the minicells. Two of these activities have been identified by genetic means as the energy-dependent exo-endonuclease complexes coded by the *rec* B–*rec* C genes and the inhibitor protein *rec* A gene product. Single-stranded DNA degradation is maximal in minicells from *rec* A^- strains and is reduced by 60% in *rec* B^- strains. The endonuclease I and exonuclease activities associated with DNA replication in *dna* B (TS) mutant[2] and *dna* A (TS) minicells have identical DNase activity for single-stranded Hfr DNA and resemble wild-type F^- strain.

Since considerably more single-stranded DNA is preserved in nuclease-deficient minicells, we think these are powerful tools for isolation and study of bacterial chromosomal genes and molecular mechanism of genetic recombination.

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MOLECULAR EVENTS ASSOCIATED WITH COLICINE E_2 INDUCED DNA BREAKDOWN

G. G. Khachatourians* and J. C. Riddle†

It has been established that covalently closed circular (CCC) forms of various plasmids can segregate into minicells of *E. coli* K12. This system is particularly suitable for the study of the molecular events associated with the bacteriocine (colicine E_2)-induced chromosome breakdown, because of our earlier observation[1] that minicells contain DNases.

CCC forms of the plasmid R64-11 (MW 70 $\times 10^6$ daltons) were used in this study. Effect of colicine E_2 (CET) on chromosomal DNA and CCC in cells and CCC in minicells has been examined. Nicking of CCC and conversion to linear molecules is initiated at 2–3 min in minicells and in cells and occurs with the same kinetics in both. In 3–5 min after the addition of CET to cells, chromosomal DNA and CCC begin to be degraded to trichloroacetic acid (TCA) soluble material and low molecular weight polynucleotides as judged by chromatography and sedimentation through alkaline sucrose gradients. CET-induced DNA degradation activity results in loss of 80–100% of TCA insoluble material in cells and only 50–60% in minicells in 3 hr at 37°C. Various kinetic and physiological aspects of this process have been examined and show a parallel relationship between DNA breakdown in cells and minicells. Only nicking of CCC into linear DNA molecules takes place in the presence of 2,4-dinitrophenol, indicating that unlike the DNA degradation to TCA soluble material, endonucleolytic scission is energy independent.

This system is very sensitive for the study of the individual steps involved with colicine E_2 action. Our results indicate that cellular growth and division are not prerequisites for colicine E_2 action. It appears probable that addition of colicine E_2 to a sensitive host activates a nuclease complex system by its interaction with the

cell surface, and the nuclease complex is present throughout the cell and polar minicells.

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A NEW METHOD FOR THE PREPARATION OF MINICELLS FOR PHYSIOLOGICAL STUDIES

G. G. Khachatourians* and C. A. Saunders†

Minicells are small bodies, approximately one-tenth to one-twentieth the size of their progenitor cells, that arise as a result of displaced septation and fission near the polar ends in a mutant strain of *E. coli.* We have used plasmid-containing minicells of *E. coli* for studies of the development of bacteriophages, in the course of which we have found that minicells purified by rate-zone centrifugation on sucrose gradients[1] show reduced biosynthetic activities because of some physiological shock induced by sucrose and centrifugation at 4°C. The new procedure involves centrifugation of whole cultures of minicell-producing strains for 5 min at 3500 rpm in an SS34 rotor of a Sorvall centrifuge at 24°C to remove the bulk of the cells. The supernatant with minicells and some contaminating cells is then concentrated four- to tenfold and is incubated in the presence of 10 units/ml penicillin which specifically inhibits cell division but not longitudinal growth of *E. coli* cells. The supernatant is then subjected to continuous sonic treatment at a setting of 4 on a Bronson Sonifier. Sonic oscillation kills whole cells by rupturing the bacterial cell envelope but does not affect minicells.[2] An additional low-speed centrifugation (2000 *g* for 5 min) yields purified minicells with less than one contaminating cell per ten minicells. Minicells prepared by this method are very active in DNA, RNA, and protein synthesis and readily support multiplication of bacteriophage T4.

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THE SEPARATION OF AN ULTRAVIOLET-SPECIFIC ENDONUCLEASE FROM A γ-ENDONUCLEASE OF *M. LUTEUS*

W. L. Carrier and R. B. Setlow

There are numerous reports concerning the ability of an endonuclease from *M. luteus* to make breaks in modified DNA. Most notable is the action of a dimer-specific endonuclease on uv-irradiated DNA. An extract of *M. luteus* containing uv-specific endonuclease also exhibits endonucleolytic cleavage of γ-irradiated DNA.[1,2] Heavily uv-irradiated DNA used as a competitor significantly inhibits both activities, suggesting that the same enzyme acts on both dimers and on base damage resulting from gamma rays. In recent experiments we have separated the major portion of the uv-specific endonuclease from the γ-endonuclease. Purified *E. coli* DNA, labeled with ^{3}H or ^{14}C dThd, dissolved in 0.1 *M* sodium phosphate buffer, pH 7.0, and 0.01 *M* Tris buffer, pH 7.5, was irradiated with 20 kilorads of gamma rays under anoxic conditions or with 800 ergs/mm^2 of uv at 280 nm. Sedimentation in alkaline sucrose showed that gamma rays made ~1 single-strand break in DNA of 5,000,000 daltons and also produced ~2 endonuclease susceptible sites. The uv irradiation produced no breaks, ~4 dimers, and ~4 endonuclease sites. The major uv-specific activity (fraction A) does not stick to DEAE cellulose. It contains no activity toward gamma-irradiated DNA. Another activity (fraction B) is eluted with buffered 0.3 *M* NaCl. This fraction makes single-strand breaks in both uv-irradiated and gamma-irradiated DNA. The activities in fraction B cosediment. Neither requires the addition of divalent metal ions. Both uv-endonuclease and the γ-endonuclease have a broad pH optimum between pH 7.0 and 8.0.

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ACTION OF AN ENDONUCLEASE ON ULTRAVIOLET-IRRADIATED BROMOURACIL-CONTAINING DNA: SPECIFICITY FOR URACIL RESIDUES

W. L. Carrier and R. B. Setlow

When BrUra-containing DNA is irradiated by uv, large numbers of alkali-labile bonds result. If the irradiation is carried out in the presence of cysteamine, there are relatively few alkali-labile bonds but large numbers of

bonds susceptible to the action of partially purified endonuclease from *M. luteus.*[1] The endonuclease also attacks gamma-irradiated DNA and is purified in terms of its activity on DNA containing pyrimidine dimers. Two lines of evidence indicate that the phosphodiester bonds in BrUra DNA that are attacked by the nuclease are those near the Ura-residues that result from uv-induced debromination of BrUra. Uracil can be detected in acid hydrolysates of irradiated BrUra DNA, and the amount of uracil, as a function of 313-nm fluence, is within 5% of the number of endonuclease-sensitive sites. (The endonuclease is not attacking dimer-containing sequences because 313 nm makes a negligible number of dimers.) A more critical experiment is the action of the endonuclease on a naturally occurring DNA – the DNA of phage PBS2. This DNA contains dUrd instead of dThd. It is degraded to oligonucleotides by both fractions A and B of the purified endonuclease, and there is a negligible amount of mononucleotides in the hydrolysate. Thus the enzyme preparations containing repair endonuclease activities also contain a restriction-type activity that could be the first step in the rapid repair of uv damage to BrUra DNA *in vivo.*[2]

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GAMMA-RADIATION-INDUCED DAMAGE TO DNA: DEPENDENCE ON BASE COMPOSITION

R. J. Brake* and R. B. Setlow

The sensitivity of several strains of bacteria to ionizing radiation is directly proportional to the GC content of their cellular DNA.[1] This correlation might be explained by an increase in total damage yield or a decrease in the repairable fraction with increasing GC content. In order to test these possibilities, we isolated DNA's from organisms with various base compositions (38–72% GC) and exposed them to ^{60}Co gamma radiation under well-defined conditions in aqueous solution. The yield of gamma-ray-induced single-strand breaks was determined by sedimentation in alkaline sucrose. The number of strand breaks increased linearly with dose but was independent of the base composition of the DNA. Irradiated samples were also treated with an enzyme extract of *Micrococcus luteus* which contained endonuclease activity toward gamma-irradiated DNA. The number of strand breaks introduced by the extract (presumably an indication of repairable base damage) also increased linearly with dose, but again was independent of base composition. Under both anoxic (nitrogen) and aerated conditions, and in the presence of several protective solutes (ethanol, cysteamine, potassium iodide, yeast extract), the yields of strand breaks and endonuclease-susceptible sites were still independent of the GC content of the irradiated DNA. From this we conclude that the observed dependence of the *in vivo* radiosensitivity on base composition is due to damage which was either not produced under our conditions or not detected by our methods.

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GAMMA-RAY LESIONS IN BACTERIAL SPORES

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In recent years, techniques have been perfected to quantitate and measure the repair of several of the lesions produced by exposing cells to various physical and chemical insults. Thus ultraviolet (uv)-induced dimers may be studied directly by acid hydrolysis and paper chromatography while lesions produced by ionizing radiation (strand breaks or alkali-labile regions in DNA) may be observed in alkaline sucrose gradients. Recently a second lesion in DNA has been observed following *in vivo* or *in vitro* exposure to ionizing radiation. This lesion, while its nature is yet unknown, can be observed by treating DNA extracted from irradiated cells with an enzyme isolated from *M. luteus.*[1] When DNA so treated is sedimented in alkali, it exhibits more strand breaks than samples not treated with the enzyme. This lesion also appears to be repaired, as it decreases if the cells are allowed to grow before extraction of the DNA.

Bacterial spores are extremely resistant to most chemical and physical treatments. We have shown, in the case of uv, that the resistance can be explained, in part, by a much altered photochemistry. Thus the usual cyclobutane dimer is not produced in spore DNA; instead, the spore photoproduct [5-thyminyl-(5-6-dihydro)thymine] appears in its place. We have attempted to show that the structural change producing the altered photochemistry also leads to a change in the production of the gamma-ray lesion sensitive to the enzyme discussed above.

Cells and spores of *Bacillus megaterium* suitably labeled with radioactive thymidine were exposed to appropriate doses of ^{60}Co gamma radiation and their

DNA extracted. The samples were combined, treated with enzyme, and analyzed by alkaline sucrose centrifugation. Unlike the complete change observed after uv irradiation, gamma-irradiated spores appear to behave in a similar qualitative but not quantitative fashion to that of vegetative cells. A fivefold increase in the amount of radiation required to produce a single-strand break (or alkali-labile region) in spores as compared to vegetative cells was observed, while we measured a fivefold decrease in the number of enzyme-sensitive sites. The energy per break and the number of enzyme-sensitive sites observed were higher and lower respectively in our vegetative cells as compared to other systems studied. This latter fact suggests these lesions are being repaired during the time required to extract the DNA. It is felt that repair was not occurring in spores, as they remained dormant during the procedure. Thus, in this case, the unique spore structure conveys resistance to the organism not by causing a change in lesions produced but by reducing their number.

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AN ENZYMATIC ASSAY FOR DNA DAMAGE IN BACTERIAL AND MAMMALIAN CELLS

R. J. Wilkins*

There is a paucity of sensitive and rapid assays for damage in cellular DNA. In order to correct this situation, an enzymatic assay has been developed which is sensitive enough to detect, with ease, one lesion per 10^8 daltons of DNA and which should have fairly universal application. In fact, one would expect it to detect any kind of damage that is normally removed from DNA by an excision repair mechanism. In this assay, cellular DNA is exposed to "repair" endonucleases contained in a crude *Micrococcus luteus* extract. Lesions in the DNA are converted to single-strand breaks by this specific endonuclease action, and these breaks are, in turn, detected by sedimentation of the DNA through alkali. Simplicity in the assay is achieved with bacteria by incubating lysates of the cells with the *M. luteus* extract and with mammalian cells by using osmotic shock to render the cells porous to the extract. Thus there is no need to laboriously isolate the DNA by methods which preserve its high molecular weight. The assay has been used successfully with uv-irradiated cells and also in gamma-irradiated bacteria. There is every reason to believe that other types of chemical damage to DNA can be assayed in the same way, and it is possible that the action of other enzymes (e.g., photoreactivating enzyme) could be used instead of the *M. luteus* enzymes.

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EVIDENCE FOR EXCISION-TYPE REPAIR IN GAMMA-IRRADIATED *ESCHERICHIA COLI*

R. J. Wilkins*

As described elsewhere in this report, a sensitive assay has been developed for lesions induced in bacterial DNA by agents such as ultraviolet light and gamma rays. In the case of gamma rays, these endonuclease-sensitive sites disappear from the DNA very rapidly. In fact, within $2\frac{1}{2}$ min after irradiation with 50 kilorads of gamma rays *in anoxia, Escherichia coli* remove one-half of these sites from their DNA. By 10 min all have gone. Surprisingly, these sites disappear just as rapidly from a uvr^- strain (uvrA-6) as from the wild type, which is proficient in the excision of pyrimidine dimers. Thus, it is possible that the uvr^- strain, which cannot excise pyrimidine dimers nor remove the endonuclease-sensitive sites produced by uv, nevertheless has an excision-repair-type repair mechanism which deals with some (base ?) damage produced by gamma rays.

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STRAND-REJOINING REPAIR OF GAMMA-RAY-DAMAGED PLASMID DNA IN A *rec A*-MINICELL-PRODUCING STRAIN OF *ESCHERICHIA COLI*

M. C. Paterson,* G. G. Khachatourians,† and T. E. Worthy‡

In *Escherichia coli* K-12 a functional product of the *rec A* gene is involved in repairing single-strand breaks induced in chromosomal DNA by ionizing radiation. We have measured strand-rejoining repair of the plasmid λ*dv* in χ1256, a *rec* A^- minicell-producing mutant of *E. coli* (λ*dv* is a multiply depleted portion of the bacteriophage λ genome recoverable as superhelical covalently closed circular DNA molecules). In contrast to the well-known deficiency for the repair of bacterial chromosomal DNA, strand breaks in gamma-ray-damaged plasmid DNA of χ1256 are restituted with kinetics indistinguishable from those found in a *rec* A^+

strain. Although this proficiency in rejoining "relaxed" circular plasmids is best quantitated using chromosomeless minicells as a plasmic source, λ*dv* molecules obtained from parental cells yield similar results. Thus, at least for λ*dv*, the *rec A* gene product is unnecessary for strand restitution.

The source of information mediating strand rejoining in λ*dv* DNA remains obscure. If bacterial enzymes are involved, they must not be derived from the *rec A* locus. Alternately, the plasmid may provide its own instructions. In the latter case, a potential candidate, the *red* system, is absent from λ*dv*.

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PREFERENTIAL DNA REPAIR IN HUMAN CELLS

R. J. Wilkins* and R. W. Hart

For several years it has been known that mammalian cells irradiated with ultraviolet light contain some pyrimidine dimers in their DNA which are refractory to excision repair. In human cells, for instance, anywhere from 20% to 50% of the dimers induced by low uv doses persist in the DNA for many days. We have modified the enzymatic assay for DNA damage described elsewhere in this report to show that there is a portion of chromosomal DNA covered with protein, probably histones, in which excision repair seems to proceed very slowly. We osmotically shocked human fibroblasts, which had been previously uv irradiated and then incubated for various times, and took two samples of these cells, one of which was exposed to 2 *M* NaCl for 5 min. Both cell samples were then assayed for endonuclease-sensitive sites in their DNA. Cells which had been exposed to high salt were found to initially contain about 65% more uv-induced sites in their DNA than those treated only with low salt. Furthermore, whereas all of the sites detected under low salt conditions disappeared from the cellular DNA by 44 hr of post-uv incubation, the additional sites persisted in the DNA for much longer. Indeed, 90 hr after 100 ergs/mm^2 of uv, 13% of these additional sites were still found in the DNA. We believe that all the sites we detected resulted from pyrimidine dimers, and furthermore, it is only after high salt removes protein from about 40% of the DNA that all these sites are acted on *in vitro* by exogenous repair endonucleases. Probably, under *in vivo* conditions, this protein also impedes the excision repair of about 40% of the DNA.

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EXCISION REPAIR OF HUMAN DNA DAMAGED BY GAMMA RAYS*

R. B. Setlow and James D. Regan

Irradiation of DNA results in strand breaks and in base damage. Strand breaks, detected by sedimentation of DNA in alkaline gradients, are repaired rapidly (~60 min) in fibroblasts from normal and from xeroderma pigmentosum individuals. If repair takes place in BrdUrd-containing medium, we have shown by use of the BrUra-photolysis technique that ~1 BrUra residue is inserted per initial strand break.[1,2] Base damage in DNA may be detected by the additional strand breaks introduced by endonuclease treatment of irradiated DNA. DNA irradiated anoxically has relatively more base damage than that irradiated oxically.[3,4]

Xeroderma pigmentosum (XP) cells are defective in the endonuclease function involved in excision repair of pyrimidine dimers and in the repair of chemical damages that mimic uv damage in normal cells.[2] Since repair endonucleases attack gamma-irradiated DNA, we looked for evidence of endonuclease-mediated repair of gamma-ray damage in human cells. Such repair is characterized by the incorporation of many BrUra residues per repaired region and should be absent in XP cells. Cells were irradiated oxically (10 kilorads) or anoxically (30 kilorads), incubated for 20 hr in BrdUrd-containing medium so as to permit any excision repair to go to completion, and analyzed by the BrUra-photolysis technique so as to estimate numbers and sizes of repaired regions.

For cells irradiated oxically, both normal and XP cells show evidence for some large repaired regions. Xeroderma pigmentosum cells can repair some damage by inserting large numbers of BrUra into parental DNA. In cells irradiated anoxically (relatively large amounts of base damage), there is clear evidence for large repaired regions in normal fibroblasts but none in XP cells. The latter data are consistent with the notion that normal cells have an excision system that operates on some types of base damage whereas XP cells do not, but the situation is more complicated than indicated by our original naive notions.

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A CORRELATION BETWEEN DNA REPAIR AND LONGEVITY IN PLACENTAL MAMMALS

R. W. Hart and R. B. Setlow

It is a reasonable assumption that the ability of an organism to repair DNA damage induced by environmental agents may influence its ability to survive. Within the placental mammals there exists a wide range of life expectancies, and excision repair data on uv-irradiated cells from three species (mouse, hamster, man)[1] indicated a correlation between the extent of repair and longevity. It was therefore of interest to determine whether there existed a correlation, among a large number of species, between longevity and repair by measuring the amount of unscheduled DNA synthesis in cells exposed to several fluences of 254-nm radiation.

Skin fibroblasts from various mammals (shrew, mouse, rat, hamster, cow, chimpanzee, and man) were obtained, suspended in complete media, and incubated. At the completion of the first passage, cells were exposed to fluences of 5, 10, and 20 J/m^2 in the presence of 2×10^{-3} *M* hydroxyurea and [^{3}H]-thymidine. The incorporation of ^{3}H into cells was determined autoradiographically at several times after irradiation. The average background, after discarding those few cells undergoing scheduled DNA synthesis, was less than 6 grains/nucleus after 48 hr incubation in [^{3}H] thymidine (20 Ci/millimole)-containing media. The number of grains per nucleus in irradiated cells was two- to tenfold greater than the average background. The results showed that: (1) for all species and incubation times, the net incorporation was approximately proportional to fluence and (2) at a given fluence and incubation time, the net incorporation was species dependent. The species with the greater longevity incorporated more.

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EVIDENCE FOR CHANGES IN DNA REPAIR WITH AGE

R. W. Hart and R. B. Setlow

Damage to DNA results in changes in physiological processes such as growth and division and alterations in transcription, cell death, and mutation.[1] The sensitivity of any organism to these effects depends to a large extent on its ability to repair damage induced by either chemical or physical agents. Hence damage to DNA may be magnified if repair genes themselves are altered so as to lower the ability of a cell to repair DNA damage. A decrease in repair ability may result in the accelerated accumulation of damage in old cells. Price et al.[2] and Modak et al.[3] have suggested that DNA from old cells contains relatively large numbers of breaks, and Epstein et al.[4] showed that progeria cells were defective in strand-break repair. On the other hand, Goldstein[5] observed no differences in unscheduled DNA synthesis in uv-irradiated fibroblasts isolated from patients of differing ages. If some aspects of DNA repair did decrease with the age of cells in culture, such a finding would implicate the change of DNA repair with the aging process, although it would not prove a causal relationship. A useful experimental system for such studies is the human fibroblast line WI-38. In older cultures the fraction of cells that can be labeled with ^{3}HdThd decreases as does the fraction of cells that goes through division. Autoradiographic analysis of the cells that do label in old cultures indicated that they label with the same kinetics as do young ones. After the supralethal gamma-ray doses (see below) employed in these experiments, there was little change in the fractions of cells that could be heavily labeled, but there was an appreciable lag time in labeling.

We have measured unscheduled DNA synthesis in cultures of different ages (passage numbers 17, 30, 42, 50) that were exposed to gamma rays (10 and 20 kilorads), uv (10 and 20 J/m^2), or the carcinogenic compound N-acetoxyacetylaminofluorene (10^{-5} *M*). The measurements are made by incubating the treated cells in ^{3}HdThd plus hydroxyurea (to suppress normal synthesis) for 2 to 72 hr, making autoradiographs, and determining the distribution curve relating numbers of cells to grains per cell.

The three treatments gave rise to similar results. The fraction of cells doing unscheduled DNA synthesis was slightly less for passages 42 and 50 than for passages 17 and 30 (85% compared to 95%). A more significant change was in the distribution of autoradiographic grains among the repairing cells. For example, for a particular treatment and incubation time, early passage cells might show ~50 grains/cell as a result of unscheduled synthesis. However, late passage cells would show a bimodal distribution – one group at ~50 and the other at ~25 grains/cell. The fraction of cells in the first group decreased with increasing passage number and was the same as the fraction of cells that could do normal synthesis in untreated cultures.

These data strongly suggest that, on the average, older cells do not repair as much DNA damage as do younger ones. Therefore such cells can be expected to accumulate more random damage from the environment. Those cells in older cultures that are active in DNA synthesis seem to be as proficient as young cells in repairing damage.

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THERMAL ENHANCEMENT OF DAMAGE TO DNA IN MAMMALIAN CELLS

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

We have earlier reported[1] on the enhancement of DNA breakage which occurs when both Chinese hamster and human cells are treated with methyl methanesulfonate at 42°C instead of 37°C. One hypothesis for this enhanced DNA breakage is that an essential step in DNA repair is inactivated.[2] We have since looked at the ability of cells to repair other types of DNA damage during incubation at 42°C. In human cells, rejoining of single-strand breaks induced by gamma rays proceeds normally at 42°C, and repair of uv-induced damage, as measured by the 5-bromodeoxyuridine photolysis method, is also normal. In fact, the only abnormal effect to DNA that we have observed to result from exposure to 42°C is in Chinese hamster cells that have been labeled with ^{32}P. After several hours at elevated temperature, breaks appear in the DNA, presumably due to a synergistic effect between ^{32}P disintegrations and the high temperature. Thus, the detailed mechanisms which sensitize cells to DNA damage at high temperatures are not clear.

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SYNTHESIS AND FUNCTIONING OF CHICKEN-SPECIFIC REPAIR ENZYMES IN MAMMALIAN CELLS FOLLOWING CELL FUSION

J. S. Cook and K. L. Beattie*

The aim of this project is to introduce into mammalian cells, via *de novo* synthesis directed by a (foreign) nucleus, a repair enzyme that is normally lacking but that once introduced is capable of carrying out repair of the ultraviolet-irradiated mammalian nucleus. The outline of the project is: (1) chicken erythrocytes are fused by the Sendai-virus technique to Chinese hamster cells (CHO or V79, kindly provided by A. Hsie) or to human HeLa cells. (2) In time, the dormant erythrocyte nuclei are activated in the mammalian cytoplasm to RNA synthesis and synthesis of chicken-specific proteins, including photoreactivating enzyme (PRE). We have previously demonstrated that PRE is one of the basic "housekeeping" enzymes found in undifferentiated chick fibroblasts but is absent from chicken erythrocytes and from all mammalian cells. The erythrocyte nucleus presumably carries the genetic information for the enzyme. (3) The PRE so formed will be available for the light-dependent repair of the mammalian nucleus. PRE-activity may be assayed by the reduction of unscheduled synthesis in ultraviolet-irradiated mammalian nuclei. Since unscheduled synthesis is an autoradiographic measurement of the repair-associated incorporation of [^3H] thymidine in non-S nuclei of irradiated cells, the extent of such incorporation may be reduced after irradiation if a substantial fraction of the radiation lesions are first repaired by photoreactivation. The assay further permits scoring only those cells in which swollen chicken nuclei are cytologically identifiable within the non-S mammalian cell cytoplasm. To date, we have demonstrated the feasibility of the assay in nonhybridized fibroblasts, where photoreactivation is capable of repairing approximately 50% of the ultraviolet lesions detected by unscheduled synthesis, and our preliminary results are also positive in chick-HeLa hybrids. The project will be continued with this and other hybrids until statistically more certain results are obtained. If the final results are positive, this system will provide a handle to the question of whether a nucleus, activated in a foreign cytoplasm, may transcribe genes whose product has no counterpart in the host cell.

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INVESTIGATIONS INTO THE FUNCTIONAL DICHOTOMY OF MACROPHAGE MEMBRANE

R. L. Krogsrud* and J. S. Cook

Phagocytosis of latex beads by mouse peritoneal macrophages was characterized and quantitated. Adenosine transport was also characterized by kinetic analysis, and the metabolic products of the transported 8-^{3}H-adenosine were identified chromatographically as a function of time. (Values approach 62% AMP, 15% ADP, and 12% ATP within 5 min.) Unphosphorylated adenosine was always sufficiently small so that back transport was negligible.

Preliminary experiments indicate that extensive phagocytosis of nonmetabolizable latex beads with concomitant internalization of surface membrane does not impair adenosine transport as characterized in mouse peritoneal macrophages. This indicates a functional dichotomy in the surface membrane. Proposed experiments will probe the possibility of internalizing membrane-bound enzymes by attaching substrates to phagocytizable particles.

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THE CONTROL OF RESPIRATION IN IRRADIATED BACTERIA AND ITS SIGNIFICANCE IN RECOVERY FROM RADIATION DAMAGE

Introduction

P. A. Swenson

Irradiation of glycerol-grown *E. coli* B/r cells with uv or ionizing radiations causes almost complete inhibition of respiration about an hour after irradiation. The cessation of respiration is dependent upon RNA and protein synthesis and is associated with the disappearance of pyridine nucleotides from irradiated cells. We hypothesize that irradiation damage to DNA interferes with a metabolic control system and that a fraction of the cells die because their respiration ceases. This hypothesis is supported by the observation that post-uv treatment of cells with 5-fluorouracil maintains respiration, promotes repair of DNA, and greatly increases viability.

I. Respiration, Growth, and Viability of Repair-Deficient Mutants of *E. coli* after Ultraviolet Irradiation

P. A. Swenson and R. L. Schenley

Escherichia coli WP2 (a tryptophan-requiring strain of B/r) and three mutants deficient in DNA repair processes were used for a comparative study of the effects of ultraviolet radiations on respiration, growth, and viability. All cultures were grown on minimal medium containing glycerol plus tryptophan and irradiated at 254 nm in the midexponential phase of growth. An *hcr*$^-$ strain behaved in a manner similar to WP2 *hcr*$^+$; respiration and growth ceased about an hour after uv. The doses used for stopping respiration were 55 and 520 ergs/mm^2, respectively; these doses reduced survival in each case to about 0.5%. Growth and respiration of both *hcr*$^+$ and *hcr*$^-$ strains were closely coupled after low doses which allowed resumption of respiration; at doses that normally cause these processes to cease, they were maintained by postirradiation thermal (42°C) or 5-fluorouracil (FU) treatment. WP2 *rec*$^-$ (Rec A) and WP2 *exr*$^-$ strains did not cease respiring after receiving a wide range of doses up to 520 ergs/mm^2, but growth did cease at a time dependent on dose. Following irradiation, WP2 *hcr*$^+$ and WP2 *hcr*$^-$ cells degraded their DNA slightly, if at all, but degradation was extensive in the *rec*$^-$ and *exr*$^-$ strains at doses that reduced survival to 1.0% (33 and 150 ergs/mm^2 respectively). The results are consistent with our hypothesis that uv interferes with a normal respiratory control system and that cessation of respiration requires synthesis of RNA and protein. We conclude that cessation of respiration of irradiated *Escherichia coli* is dependent on the integrity of their DNA and not on the radiation sensitivity.

II. Membrane Changes in UV-Irradiated *E. coli* B/r Cells That Are Thought to Die of Respiratory Failure

P. A. Swenson and R. L. Schenley

Ionic and nonionic detergents have little effect on respiring bacteria, but in cultures poisoned with KCN, rapid dissolution of the plasma membrane, as indicated by turbidity losses, takes place. Ultraviolet (uv) radiations cause *Escherichia coli* cells grown in minimal medium with glycerol as a carbon source to cease respiring and growing about 1 hr after irradiation. We tested the effect of the nonionic detergent Triton X-100 on growth and plasma membrane dissolution (both measured by turbidity changes), respiration, and viability of unirradiated and irradiated *E. coli* B/r cells. When the detergent was added to cells immediately after irradiation, a decrease in turbidity occurred only when respiration was about to cease; when it was added after cessation of respiration, the turbidity loss was immediate. In both cases the turbidity loss was about 60%, and cell disintegration did not take place.

5-Fluorouracil (FU) and thermal (42°C) treatments cause respiration of irradiated cells to be maintained and also cause viability increases. Irradiated cells treated with FU and detergent show no turbidity loss just prior to the time respiration normally ceases, but a loss does occur in irradiated cells incubated with detergent at 42°C. We conclude that FU maintains respiration for all of the cells but that thermal treatment maintains respiration for only part of the cells. In all cases the detergent had only a negligible effect on the respiration and viability of irradiated cells. We conclude that Triton X-100 causes dissolution of plasma membranes only of nonrespiring cells, that is, those cells destined not to survive. These results support our hypothesis that uv irradiation causes death through respiratory failure of a fraction of the irradiated population.

III. Preparative Isolation of Nonrespiring, Nonviable Cells from a UV-Irradiated Culture of *E. coli* B/r

G. G. Khachatourians,* R. L. Schenley, and P. A. Swenson

Cultures of *Escherichia coli* B/r cease respiration and growth within an hour after ultraviolet irradiation (52 J/m^2 at 254 nm). Treatment of such cells (~1% survival) with the nonionic detergent Triton X-100 causes dissolution of cell membranes of nonrespiring cells resulting in a loss of turbidity; two distinct types of cells, normal and small, are observed in these cultures when examined microscopically. Respiration and viability of irradiated cells are not affected by detergent, and only negligible effects on these processes and on turbidity of unirradiated respiring cells can be observed. Using velocity sedimentation through neutral sucrose (10 to 20%, w/v), we have obtained two widely separated bands of cells from an irradiated detergent-treated culture. The upper band contains only small cells; however, the lower band contains a mixture of normal and small cells after a single separation. The viability of the cells in the top band is 10^{-3} to 10^{-4} times that of the cells in the bottom band. Both types of cells contain DNA (of the same size) which degrades in response to colicin E2; however, only the cells in the bottom band synthesize DNA, RNA, and protein. Electron microscopic observations revealed the absence of plasma membranes in the top band cells but not in the normal size bottom band cells. Moreover, biochemical analysis of these cells showed that the membrane localized enzymes; NADH oxidase and succinic dehydrogenase were present only in the bottom band cells.

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RADIATION RESISTANCE IN A BACTERIUM HARBORING A COLICINE FACTOR

R. J. Wilkins,* P. A. Swenson, and R. L. Schenley

Several colicine factors are known to confer radiation resistance on the bacteria in which they are harbored. We have made a preliminary study of strains of *S. typhimurium* and *E. coli* which harbor col Ib-P9, Ib-1995, and Ib-1420. These colicine factors confer, in order, high, moderate, and no additional uv resistance to the host. Most studies were made with the col Ib-P9 factor. As yet, we have not detected any significant difference in the col± strains with respect to the rate at which dimers are excised, DNA broken, and DNA degraded after uv irradiation. The uv irradiation does not stimulate the production of col factor DNA at the expense of chromosomal DNA synthesis. In fact, as might be expected, both DNA synthesis and growth are considerably slower in the col$^-$ than the col$^+$ strain. We do find, however, that after uv the col$^-$ cells of *S. typhimurium* grow into long filaments, whereas the col$^+$ strains do not. Furthermore, the degree to which the respiration is turned off after uv irradiation is inversely related to the degree of resistance that a colicine factor confers on the bacterium. We are investigating the possibility that some, as yet undetected, DNA repair differences exist in col± strains or that some other metabolic factor plays a role in survival after uv damage.

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ISOLATION, CHARACTERIZATION, CRYSTALLIZATION, AND PRELIMINARY X-RAY STUDIES OF TWO MAJOR TOXO CONSTITUENTS FROM *ABRUS PRECATORIUS*

C. H. Wei

Our interest in obtaining crystals of abrin, present in the seeds of *Abrus precatorius,* has been stimulated by recent work concerning its antitumor activity (as well as that of ricin from *Ricinus communis*) against various sarcomas in mice.[1,2] Although purification procedures for abrin have been described by several authors,[3-5] crystallization to usable size for x-ray diffraction studies has not yet been reported. Our continued effort to seek a proper purification method, however, has now provided not only suitable single crystals but also evidences that abrin preparations previously reported

by two independent groups[3,4] are actually different from each other.

A 5% acetic acid extract of the kernels of *Abrus precatorius* was first partially purified by stepwise ammonium sulfate fractionation followed by chromatography on a column of DEAE–Sephadex A-50 with 0.005 *M* sodium acetate buffer (pH 5.8) as elutant. Nontoxic viscous impurities were removed by CM-cellulose chromatography using 0.01 *M* sodium phosphate buffer (pH 5.5) as elutant. The column was then eluted with a linear gradient of sodium chloride (0.05 to 0.5 *M*) in the phosphate buffer. Further purification by DE-cellulose chromatography with a gradient elution using 0.01 to 0.28 *M* potassium phosphate buffer (pH 7.7) yielded two major constituents, abrin A and abrin C. Both toxins appear homogeneous as judged by ultracentrifuge sedimentation patterns and by the results of disc-gel electrophoresis.

Abrin A, the more positively charged protein obtained from DE-cellulose chromatography, can be crystallized as needles (up to 1 mm long) from 65% ammonium sulfate solution by the interface diffusion technique; under various similar conditions, the other fraction, abrin C, failed to yield crystals. X-ray precession photographs[6] have indicated the crystals of abrin A to be orthorhombic, with unit cell dimensions a = 74.9, b = 269.8, and c = 70.3 Å. Systematic absences indicate a probable space group to be $P2_12_12_1$. Calculations show that there are two molecules per asymmetric unit.

The amino acid composition (determined by F. C. Hartman, Protein Chemistry Group) of abrin A is similar to that of the abrin preparation reported by Lin et al.[7] and is different from that found for abrin C. Molecular weights determined by sedimentation equilibrium (by A. P. Pfuderer, Biochemistry Section) are 60,100 for abrin A as compared to 62,300 for abrin C. After abrin A has been treated with 2-mercaptoethanol, polyacrylamide-gel electrophoresis in the presence of 2% sodium dodecyl sulfate gives rise to one strong and two much weaker bands (corresponding to subunit molecular weights of 32,100, 29,500, and 27,900), in sharp contrast with the pattern for abrin C: two approximately equal-intensity bands, corresponding to subunit molecular weights of 31,500 and 27,000, in good agreement with the results reported by Olsnes and Pihl for their abrin preparation.[4] Abrin C exhibits more toxic effect on mice than abrin A (as determined by W. K. Yang, Carcinogenesis Section), in accord with the observations of previous authors.[4] It seems safe to conclude, therefore, that abrin A corresponds to Lin et al.'s preparation,[7] while abrin C corresponds to the preparation reported by Olsnes and Pihl.[4]

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AUTOMATED MICRODENSITOMETRY OF X-RAY DIFFRACTION PHOTOGRAPHS IN A TIME-SHARING ENVIRONMENT

J. R. Einstein and B. W. McClelland

An Optronics scanning microdensitometer has previously been interfaced to the Biology Division's PDP-11 computer and operated with the use of FORTRAN programs under the DOS monitor. A handler for the scanner has now been written in the PAL-11 language and incorporated into the RSTS monitor, under which the computer is now constantly operated for general time-sharing usage. Collection and reduction of intensity data from x-ray diffraction photographs by our BASIC-language "user" programs can thereby take place simultaneously with the other data-collection and -reduction functions of this PDP-11 system. The disk-control segments of the RSTS monitor have been altered to dedicate part of the disk to the scanner. Temporary storage of portions of the scanner data (up to 20,000,000 points per film) in "staggered" records on disk (a technique that is applicable with other computers) and double buffering in both disk and core allow reflections to be measured from standard precession photographs at maximum rates of about 30 reflections per second. The "user" programs have been written to allow considerable on-line control of the several steps in the data-collection and -reduction procedure.

X-RAY STUDIES OF CRYSTALS OF THE VARIANT HALF OF BENCE-JONES PROTEIN (LEN)

J. R. Einstein, S. W. Hawkinson, and B. W. McClelland

We have continued our efforts to prepare a series of heavy-atom derivative crystals isomorphous with the

monoclinic crystal form of this protein. We have also examined the effects of various solutes (salts, 2-methyl-2,4-pentanediol) on the crystal solubility. Diffusion of K_2PtCl_4 into the crystals does not significantly change the unit-cell dimensions but causes intensity changes in the diffraction pattern. These crystals may possibly be useful derivatives if their resolution can be improved and if the heavy-atom positions can be found.

DETERMINATION OF A LARGE ACENTRIC CRYSTAL STRUCTURE BY X-RAY DIFFRACTION

B. W. McClelland

Because of their large unit cell and high stability, crystals of the γ form of aluminum acetylacetonate were chosen for comparison of diffraction intensity measurements by diffractometer and film-scanner methods. The diffractometer data have been employed in a successful test of direct methods of phase determination for a large (88 independent nonhydrogen atoms) acentric structure containing no exceptionally "heavy" atom, as is the case for many biological materials. A. C. Larson's program TANFORM (a modification of M. Drew's program PHASEM) was used to develop 32 different sets of phases for 500 reflections, and the most consistent set yielded positions for 16 atoms. The remaining atoms were found from two subsequent Fourier syntheses. At the present stage of refinement (nonhydrogen atoms only, with isotropic thermal parameters), the conventional R-factor is 0.102.

PARTIAL CHARACTERIZATION OF THE ACTIVE SITE OF HUMAN TRIOSE PHOSPHATE ISOMERASE

F. C. Hartman and R. W. Gracy*

Triose phosphate isomerase from human red blood cells was recently isolated, and a number of its physical, chemical, and enzymatic properties were described.[1] A thorough characterization of this enzyme appears warranted in order to provide a basis for the elucidation of the molecular defect in a mutant enzyme present in patients with the genetic disease known as triose phosphate isomerase deficiency.[2] To partially characterize the active site of the human enzyme, we have studied its reaction with chloroacetol phosphate, a reactive analog of the substrate dihydroxyacetone phosphate. Chloroacetol phosphate totally inactivates human triose phosphate isomerase by the selective modification of a single residue per catalytic subunit. The stability of the protein-reagent bond and the analogies of this active-site modification to those described previously for isomerases from other species[3] indicate that inactivation results from the esterification of an essential glutamyl γ-carboxylate. From peptide maps and their autoradiograms, we conclude that the primary structure adjacent to the glutamyl residue is the same as or similar to that found in triose phosphate isomerases from rabbit and chicken muscle and from yeast.

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3-BROMO-2-BUTANONE 1,4-BISPHOSPHATE AS AN AFFINITY LABEL FOR RIBULOSE BISPHOSPHATE CARBOXYLASE

F. C. Hartman, Mary H. Welch, and I. Lucille Norton

3-Bromo-2-butanone 1,4-bisphosphate has been synthesized in an attempt to find a reactive analog of ribulose 1,5-bisphosphate for labeling the active site of ribulose bisphosphate carboxylase. The reagent irreversibly inactivates the carboxylase from spinach, and several observations suggest that the inactivation results from modification of an active-site residue: (1) Ribulose 1,5-bisphosphate protects against inactivation. (2) The extent of reagent incorporation shows that modification of one residue per catalytic site can account for the inactivation. (3) Comparisons of autoradiograms of peptide maps prepared from carboxylase treated with the ^{32}P-labeled reagent in the absence and presence of substrate indicate that inactivation results from a fairly selective modification. (4) Although the reagent's greatest inherent reactivity is toward sulfhydryl groups, inactivation of the enzyme is due to alteration of some other amino acid residue.

INACTIVATION OF CLASS I FRUCTOSE BISPHOSPHATE ALDOLASES BY THE SUBSTRATE ANALOG N-BROMOACETYLETHANOLAMINE PHOSPHATE

F. C. Hartman, B. Suh,* R. Barker,* and Mary H. Welch

N-Bromoacetylethanolamine phosphate, prepared by the bromoacetylation of ethanolamine phosphate, has

been tested as an active-site-specific reagent for rabbit and rat muscle fructose diphosphate aldolases. The reagent inactivates both enzymes, and inactivation is prevented by substrates or competitive inhibitors. Loss of activity is pseudo first-order until the later stages of inactivation, and a rate-saturation effect is observed as the reagent concentration is increased. The stoichiometry of the reaction has been determined with ^{14}C-labeled reagent. Inactivated enzyme contains 2 to 2.5 molar equivalents of reagent per mole of aldolase subunit, whereas the enzyme modified in the presence of a competitive inhibitor contains only 1 to 1.5 equivalents of reagent. Thus, alterations in the catalytic properties of aldolase that accompany its modification apparently reflect the alkylation of a single essential residue. Characterization of the protein derivatives reveals that the preservation of enzymatic activity is due primarily to the protection of a histidyl residue. This observation supports earlier evidence for the involvement of a histidyl residue in aldolase activity.[1]

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DETECTION OF ALKALI-LABILE BONDS IN DMSO-SUCROSE GRADIENTS

R. O. Rahn

Alkaline sucrose gradients have been widely used to determine the reduction in molecular weight of single-stranded DNA due to radiation-induced chain breaks. Considerable evidence indicates that a significant portion of the breaks observed under alkaline conditions represent alkali-labile bonds rather than "clean" breaks. We have measured chain breaks following irradiation (313 nm) of DNA in the presence of the triplet sensitizers acetone, acetophenone, and benzophenone. To test the extent to which breaks measured in an alkaline sucrose gradient represent alkali-labile bonds, we sedimented the DNA in a 90% DMSO, 0 to 10% sucrose gradient. DNA denatures in this solvent, and strand separation occurs without exposure to high pH. To test for alkali-labile bonds, samples were exposed to pH 13 before neutralization and sedimentation. The results indicate that nearly all of the breaks observed upon acetophenone sensitization represent alkali-labile bonds, while with acetone and benzophenone nearly all the breaks are "clean" breaks.

Benzophenone is known to form ketyl radicals plus hydroxyl radicals (OH·) when photolyzed in aqueous solution. Since uv irradiation of H_2O_2 generates OH·, it was of interest to determine the nature of the chain breaks obtained following uv irradiation of DNA in the presence of H_2O_2. Since nearly all of the chain breaks obtained by photolysis of H_2O_2 were detected in a DMSO-sucrose gradient without alkaline exposure of the DNA, we conclude that OH·-induced breaks are not alkali-labile bonds and that OH· radicals are most likely responsible for the "clean" chain breaks obtained with benzophenone sensitization. However, OH· must not contribute significantly to chain breaking during acetophenone sensitization, since most of the breaks observed were alkali-labile bonds.

Evidence has been obtained by others that the alkali-labile bonds induced by ionizing radiation are due to hydroxyl radicals attacking the DNA. These radicals create three times as many alkali-labile bonds as clean chain breaks. Our results using the photolysis of hydrogen peroxide to generate hydroxyl radicals are in conflict with these results in that we find that nearly all of the breaks are clean breaks. One possibility is that the alkali-labile bonds formed by hydroxyl radicals are also labile to DMSO.

FORMATION OF CHAIN BREAKS IN BU-LABELED DNA

R. O. Rahn and L. C. Landry

Photolysis of BU-labeled DNA results in chain breaks as determined by sedimentation of the irradiated DNA on an alkaline sucrose gradient. There exists some conflict in the literature as to whether these breaks are alkali-labile bonds or not. To test for alkali-labile bonds in irradiated BU-labeled DNA, we sedimented the DNA in a 90% DMSO, 0–10% sucrose gradient. No change in the molecular weight was noted. Hence the breaks observed in an alkaline sucrose gradient are alkali-labile bonds and are expressed in the DMSO sucrose gradient only upon prior exposure to pH 13.

In order to understand more about the mechanism of formation of uv-induced lesions in irradiated BU-labeled DNA, the irradiation was done in the presence of Ag^+ which binds to the bases and increases the intersystem crossing between singlet and triplet levels. Such a heavy atom effect would be expected to quench photoreactions which proceed from the singlet level but not the triplet level. We found that stoichiometric amounts of Ag^+ added to BU-labeled DNA quenched the formation of chain breaks as determined by sedimentation in an

alkaline sucrose gradient. This result suggests that debromination, which is the initial photochemical event, takes place from the singlet state.

Irradiation in the presence of excess Ag^+ resulted in photochemical cross-linking as evidenced by the appearance in an alkaline sucrose gradient of material which sedimented faster than the unirradiated DNA. The appearance of this faster-sedimenting material was dose dependent and not due to Ag^+ cross-linking the strands since Ag^+ was removed prior to sedimentation.

DETECTION OF DEFECTS IN DNA BY MEANS OF THE KINETIC FORMALDEHYDE METHOD

R. O. Rahn and R. S. Stafford

Formaldehyde reacts considerably faster with denatured DNA than with native DNA. DNA containing defects in the form of either pyrimidine dimers or single-strand chain breaks has localized denatured regions associated with these defects. Hence, the rate of reaction of DNA with formaldehyde is greatest at these defect sites. Of interest is the rate of reaction of formaldehyde at the dimer site as compared with the rate at a single-strand break. Lazurkin and co-workers[1] have assumed that the rate of reaction of formaldehyde with a dimer defect is the same as with a chain-break defect. However, when a small number of dimers are made, the resulting number of defects as determined by the kinetic formaldehyde method is one-sixth of the expected value. Lazurkin and co-workers explain this result by assuming there is clustering of the dimers due to long-range energy transfer. However, we question their assumption that the rate of unwinding of DNA by formaldehyde at a dimer site is the same as at a chain break and feel that unwinding is more likely six times slower at a dimer than at a chain break. The intact phosphodiester linkages on both strands at a dimer site should inhibit unwinding.

We have done preliminary experiments comparing the rate of formaldehyde reaction at the site of a single-strand break with that at a dimer site. These experiments were carried out using DNA containing either breaks made by photolysis of hydrogen peroxide or thymine dimers made by irradiation in the presence of Ag^+ which binds to the DNA and enhances dimerization. Samples were allowed to react with 2.6% formaldehyde at 52°C for several hours, and the increase in absorbance was plotted as function of t, the time of reaction. For a given concentration of defects, chain breaks reacted 6 to 10 times faster than dimers. Thymine dimers in DNA were then converted into chain breaks, using the uv-endonuclease, an enzyme which nicks DNA at the site of a thymine dimer. The rate of formaldehyde reaction with the DNA was then measured before and after treatment with the enzyme. Enzyme-treated DNA reacted about six times faster than untreated DNA, confirming our hypothesis that chain break defects unwind considerably faster than dimer defects.

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ELECTRON MICROSCOPY OF IRRADIATED DNA: VISUALIZATION OF DENATURED REGIONS

R. S. Stafford, D. P. Allison, and R. O. Rahn

DNA was subjected to 5000 ergs/mm^2 of 254-nm radiation in the presence of sufficient Ag^+ to saturate all the base binding sites. Over 15% of the thymine was converted into cyclobutane thymine dimers. This amount of thymine dimer disrupts a sufficient number of hydrogen bonds, particularly in certain thymine-rich regions, so as to make possible the visualization with the electron microscope of certain denatured regions in the DNA. Presumably, these regions represent thymine-rich sequences. Conventional Kleinschmidt[1] techniques with formamide fixing were used to prepare the DNA for examination with the electron microscope. In contrast to the Inman[2,3] procedure for locating thymine-rich regions, prior treatment of the irradiated DNA with formaldehyde at elevated temperatures or pH was not necessary to obtain observable single-stranded regions. This technique, using photodimerization for locating thymine-rich regions, should be an improvement over existing methods for mapping thymine-rich regions in DNA.

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THE INTERACTION OF DNA WITH Pt^{2+} AND Cu^{2+}

R. O. Rahn, Linda Munchausen, and L. C. Landry

A. **Platinum.** The binding of Pt^{2+} in the form of *cis*-Cl_2 Pt(II)$(NH_3)_2$ to DNA was followed spectrophotometrically, and the photochemical and luminescence properties of the completely complexed DNA were examined. The effect of Pt^{2+} binding on these properties was minimal since little change in the thymine dimer yield was observed and only a twofold enhancement in the phosphorescence intensity occurred. Further experiments are being conducted using

poly A as model system for studying the heavy atom effect caused by Pt^{2+} binding. It appears that Pt^{2+} shows a much smaller heavy atom effect than either Ag^{+} or Hg^{2+} bound to DNA.

B. Copper. The photochemical properties of single- and double-stranded DNA complexed with Cu^{2+} have been studied as a function of pH. Our results are in partial agreement with those of Sutherland and Sutherland.[1] We find that Cu^{2+} enhances dimerization in native DNA, but we did not find as much quenching of dimerization by Cu^{2+} in denatured DNA as the Sutherlands. In the course of these studies, we observed that Cu^{2+} reacts with native DNA at 25°C provided the relative amount of Cu^{2+} is high enough ($r = 2$) and that the pH is greater than 6.5. It has been generally assumed that DNA binds Cu^{2+} at the base sites only upon heating and partial denaturation of the DNA. However, previous studies have usually been done at pH 5.5. Hence, if proton release accompanies Cu^{2+} binding, then binding should be facilitated by raising the pH.

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VISCOSITY STUDIES ON DNA

V. L. Koenig*

For approximately six months, the viscosity of DNA was studied. The emphasis was upon the viscosity behavior of single-stranded DNA under various conditions of pH and ionic strength. Single-stranded DNA is produced by subjecting double-stranded DNA to alkaline pH and low ionic strengths. The change is irreversible at pH 11.00 or above. The viscosity is influenced more by ionic strength than it is by pH. At low ionic strengths, less than 0.001, the intrinsic viscosity of single-stranded DNA is greater than that of double-stranded DNA. At ionic strengths of 0.001 or above, the intrinsic viscosity is less for single-stranded DNA than it is for double-stranded DNA. Exposure of double-stranded DNA to DMSO irreversibly produces single-stranded DNA. The fraction is temperature dependent and also dependent upon the concentration of DMSO – the higher the temperature, the lower the concentration of DMSO needed to produce single-stranded DNA.

Data were collected on the viscosity of DNA in 40% DMSO and subsequently of single-stranded DNA after heating the DNA in 40% DMSO to 60°C or above. Viscosity measurements demonstrated the production of double-chain breaks in DNA by sonic irradiation and of single-strand breaks in DNA by uv irradiation in the presence of H_2O_2. Some viscosity determinations were made on PBS-2 DNA, a DNA having deoxyuridylic acid residues instead of thymidylic acid. In collaboration with W. L. Carrier, some values of molecular weights for DNA, single-stranded DNA, and PSB-2 DNA were determined and correlated with the respective intrinsic viscosities.

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MOLECULAR WEIGHT AVERAGES OF DNA FOLLOWING RANDOM STRAND BREAKS

M. L. Randolph

Molecular biologists frequently estimate the average number of randomly distributed breaks, μ, in DNA strands from measurements of average molecular weights. Four commonly used approximate expressions are:

$$\mu_N = \lambda/M_N \tag{1}$$

$$\mu_W = 2\lambda/M_W \tag{2}$$

$$\mu'_N = (\lambda/M_N) - 1 \tag{3}$$

$$\mu'_W = (2\lambda/M_W) - 1 \tag{4}$$

where λ is the molecular weight of a maximum length of a DNA strand and M_N and M_W are the number- and weight-average molecular weights. When μ is large, all the approximations are good. When μ is small, the precision of the approximations depends on the initial configuration and distribution of molecular weights before the introduction of the breaks. We have considered all DNA from stationary-phase cells as either linear or circular with finite length λ. For DNA from log-phase cells, we consider the replication rate to be constant throughout the cell cycle and the initial length distribution to contain two linear strands of length λ per cell plus two partial-length linear strands with lengths distributed as cell "ages" are distributed, or as a full circle plus a partial-length linear strand, or (using the rolling circle model of replication) as a full-length circle, a full linear length, and two partial linear lengths. Using Poisson statistics and these various conditions, we find the ratios of μ to the listed approximations to vary with μ as shown in Fig. 4. If from *a priori* information one knows the initial conditions, one can obtain precise estimates of μ from measurements of either M_N or M_W and use of one of the approximations and the figure.

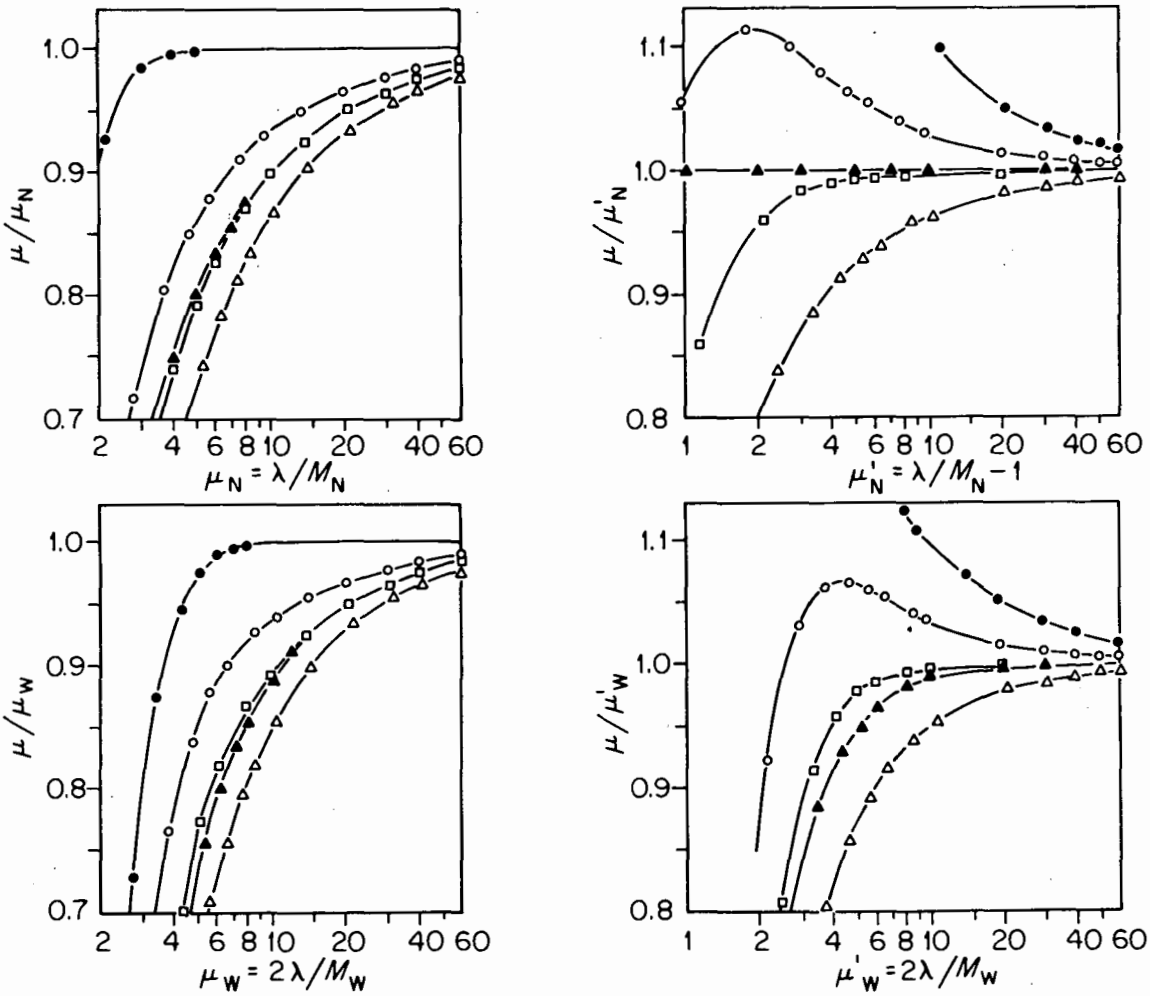

Fig. 4. Plots of numbers of strand breaks vs estimators of breaks per strand for various initial conditions. Stationary cells, circular DNA ●, linear DNA ▲; log-phase cells, circular DNA ○, linear DNA △; rolling circle replication □.

Charlesby[1] has treated idealized cases in a somewhat different way and reached similar results.

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SEDIMENTATION OF DNA IN SUCROSE GRADIENTS IN "SWINGING BUCKET" ULTRACENTRIFUGE ROTORS

M. L. Randolph

Previous studies[1] on the rate of sedimentation of DNA in 5–20% sucrose gradients in commercially available ultracentrifuge rotors have been extended to include the effects of hydrostatic pressure (hence rotational speed) and salt concentration. In most of the rotors considered, the rate of sedimentation from 20 to 80% of the length of the sucrose column is found constant to within less than 5% for 15–20°C temperatures, 0.2–2 *M* NaCl, and 0 to 60 krpm. The sedimentation rate increases 2.5%/°C, 2–6%/(krps)^2, and 2–12% over the column length; it decreases 18% per mole of NaCl present. A plot of the average fractional velocity vs a dimensionless ratio of radii,

$$(r_{max} + r_0)/(r_{max} - r_0)\,,$$

is found to be linear. From an understanding of these factors one can readily compare or predict calibrations

and experimental results for many combinations of rotor dimensions and experimental conditions.

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HETEROGENEITY OF TRYPTOPHAN PHOSPHORESCENCE FROM CYCLIC PEPTIDES

C. F. Beyer* and J. W. Longworth

The tryptophan (trp) phosphorescence of the cyclic decapeptides tyrocidine B (TyB, which contains one trp) and tyrocidine C (TyC, two trp), and of a linear derivative of TyB, has been studied at liquid nitrogen temperature. TyC exhibits two overlapping trp emissions whose 0-0 transitions are centered at about 408 nm (blue trp) and 415 nm (red trp). The relative intensities of these two emissions are solvent dependent (solvents used were ethanol, trifluoroethanol, and 50% aqueous glycerol), and their phosphorescence lifetimes are different, the blue trp having the longer lifetime in all solvents. Also, the absorption spectrum of the red trp of TyC is shifted to longer wavelengths relative to the absorption spectrum of the blue trp. TyB shows only one trp phosphorescence emission in ethanol (0-0 transition at 408 nm) and 50% aqueous glycerol (0-0 transition at 415 nm) but shows two emissions of about equal intensity in trifluoroethanol. The lifetime of the trp phosphorescence of TyB in each solvent is similar to the lifetime of the corresponding trp emission of TyC. In contrast, neither the wavelength position nor the lifetime of the trp phosphorescence of a linear derivative of TyB is dependent upon solvent, indicating the importance of the cyclic structure for heterogeneous trp phosphorescence. Our data suggest that these cyclic peptides may exist in at least two distinct, solvent-dependent conformational forms in which the trp residues are in markedly different environments.

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EVIDENCE FOR TRIPLET-TRIPLET ENERGY TRANSFER IN α-TRYPSIN

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Energy transfer between triplet states requires a close approach of the donor and acceptor groups, since the transfer occurs by an electron exchange interaction. When the singlet state of the triplet acceptor is at a higher energy than the singlet state of the triplet donor, the triplet donor can then be excited by light of a wavelength that is not absorbed by the triplet acceptor. Under these conditions, phosphorescence of the triplet acceptor is observed only if there is triplet-triplet energy transfer.

α-Trypsin was dissolved in a 1:1 mixture of glycerol-H_2O and adjusted to pH 11.5. The phosphorescence emission spectrum of this solution (at 77°K) upon 280-nm excitation was characterized by the broad structureless emission of tyrosinate anion superimposed by that of tryptophanyl, with its sharp peaks at 412 nm and 440 nm. These peaks were still detectable even when the solution was excited with 309-nm light where the tyrosinate is the predominant absorbing residue. When trypsin was unfolded in 4.5 *M* guanidine·HCl, the phosphorescence emission upon 280-nm excitation was qualitatively the same as for the native enzyme; with 309-nm excitation, the tryptophanyl phosphorescence peaks were not detectable and only the tyrosinate phosphorescence could be demonstrated. These observations may be interpreted as evidence that triplet-triplet energy transfer occurs from tyrosinate to a tryptophanyl residue in the native but not the unfolded form of α-trypsin.

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A MODEL FOR THE LUMINESCENCE OF LYSOZYMES AND LACTALBUMINS

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Human leukemia (HLL) and hen egg-white lysozyme (HEL) are believed to share a common conformation with bovine a-lactalbumin (BAL). It is possible to consider the detailed environment of the individual tryptophan residues of these three proteins using the atomic structure for HEL and the three primary sequences. For example, BAL shares four W residues in common with the six of HEL. In HEL, W62 and W108 fluoresce, and only W108 does in HLL; there are two fluorescence components of HEL and one for HLL, judged by spectra and lifetime. In HEL, W111 is quenched by K116, and W28 transfers to W111. W63 interacts with disulfide 64-80 and becomes the source of phosphorescence, probably through triplet sensitization from W62 and W108; it also acts as a quencher for W108 through transfer. W123 is quenched by K33. HEL lacks W62 and W123; hence only W108 fluoresces; hence the blue short-lived fluorescence. The additional W34 is quenched by K33. BAL lacks W111, so W28 contributes to fluorescence as does W123 since K is replaced by F33. Hence the greater intensity and long-lived phosphorescence. Fluorescence anisotropy spectra of all three proteins show no depolarization,

consistent with either no transfers between the fluorescent tryptophan residues or a parallel orientation (e.g., W62 and W108 of HEL). This result is in agreement with the molecular structure.

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FLUORESCENCE SPECTRA AND LIFETIMES OF BENCE-JONES PROTEINS AND LIGHT CHAINS OF IMMUNOGLOBULINS*

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

Fluorescence of tyrosyl (tyr) and tryptophyl (trp) residues in type kappa (κ) and lambda (λ) Bence-Jones proteins and light chains were measured. No two proteins gave identical spectra in comparative studies on over 50 proteins belonging to the three basic κ-chain groups and four λ-chain groups. Fluorescence yield of tyr and trp varied 100-fold, and the Stokes shift of trp varied between 330 and 360 nm. The monomeric and dimeric forms of a κ chain gave similar spectra. The ability to cleave selectively light chains into their respective variant and constant halves permitted comparative studies which revealed that an intact light chain and its variant half have similar spectra. KI proteins Roy, Ag, and Au which have extensive variant half sequence homologies possess distinguishable spectra. Low trp yield was found among certain KI, KII, and KIII proteins as well as among certain λ chains of different groups; the fluorescence of these proteins is attributable largely to tyr. The results of phosphorescence spectra and lifetime measurements suggest that a special interaction with trp accounts for the low yield. Proteins could be grouped in accordance with similarities in fluorescence lifetimes and fluorescence yields.

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MEASUREMENT OF THE LIFETIME OF PROTEIN FLUORESCENCE BY DELAYED COINCIDENCE TIME SPECTROSCOPY

J. W. Longworth and S. S. Stevens

Our intention is to measure the decay of protein fluorescence, a process which occurs in several nanoseconds, and to define whether the decay is comprised of one or more exponential components. This requires a detailed definition of the measuring systems' time response. A hydrogen gas spark discharge provides the required quartz uv continuum light and is adequately fast in its relaxation after excitation. Use of zirconium electrodes allowed 10^9 discharges. The system response for single photoelectron processes is dominated by the photomultiplier at early times and the lamp beyond 25 nsec. Full width at half maximum (FWHM) of 1.5 nsec is routinely achieved; late pulse frequencies of 1.7×10^{-2} and 2.9×10^{-4} at 12 and 24 nsec with FWHM's of 3 and 6 nsec were observed. The lamp discharge possesses a long-lived component, 0.35% with 14-nsec lifetime. Lifetimes between 2 and 5 nsec can be observed for 2–2.5 decades of intensity decay, even though this time interval encompasses the first late pulse output. Asparaginase with its single tryptophan decays with a single exponent, as do several proteins with multiple tryptophan residues (elastase, subtilisin BPN′, pepsinogen, BSA, trypsin, triose phosphate isomerase). Hen lysozyme possesses two components; human leukemia lysozyme has only a single component. Human serum albumin with its single tryptophan decays with two lifetimes, suggesting multiple environments.

DATA ACQUISITION FACILITY

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

Following installation of the basic equipment for a common data acquisition facility for the Biology Division,[1] demonstrations[2] of the improved precision, convenience, and reliability inherent in the system are leading to an increasing number of applications. These applications include both (1) passive reception at times dictated by a clock or an external interrupt, storage, and simple processing of analog or digital data from anywhere in the Biology Division complex and (2) interactive response whereby the central processor on receipt of data from a remote instrument analyzes the current situation and under program control returns a control signal to the remote instrument telling it what to do next. For low data rates (e.g., 30 points per minute), many such operations may proceed seemingly simultaneously without interference and even leave the central data processor free part time to do some calculations on a minicomputer scale. The overall goals include facilitating the acquisition of quantities of simple or sophisticated experimental data thus enabling the performance of otherwise impossible experiments or the performance of refined experiments, the data processing for which could otherwise only be done crudely. At present there are about 40 active users of

the system with nine distinct achievements in interfacing or system upgrading, nine projects in progress, and an additional half dozen proposed.

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EXCITED-STATE ENERGY TRANSFER. GENERAL THEORY AND MODEL SYSTEMS

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

We continue to be interested in the kinetic behavior of excitons, that is, mobile excited states, in photosynthetic systems and in biopolymers. Last year we reported[1] our results on the kinetics of hopping excitons in two- and three-dimensional lattices subject to quenching (or trapping) by impurities that have arbitrary kinetic constants, including the case of partially reversible quenching. We also reported our treatment of the kinetics with randomly located quenchers or with anisotropic hopping and our further consideration of the quenching kinetics of the doped crystalline polyacenes as a kinetic model of excitation transfer in photosynthetic units.

During the past year, we have concentrated on a problem that has important ramifications in the organic solid state generally, as well as in the biological systems of interest. This is the problem of how the motion of excitons is affected by molecular vibrations (sometimes called "phonons"). We understand rather well the limiting situation in which the phonon interaction completely dominates – exciton motion is then entirely hopping. Our understanding is fair in the opposite limit of wavelike excitons, when the phonon interaction is negligible. However, the important general case of arbitrary exciton-phonon interaction, which is likely to apply especially to photosynthetic systems, has only just begun to yield to our theoretical approaches.

In our initial attack on this very difficult problem we have independently developed a mathematical formalism with which we have verified the results of other workers on the behavior of the exciton diffusion constant in the presence of the exciton-phonon interaction. The most striking result so far is that wavelike (coherence) properties determine the magnitude of the exciton diffusion constant until the exciton splitting energy diminishes to the size of the exciton-phonon interaction parameter or less. (This result is striking because it previously had been supposed that once the exciton splitting energy became small enough for diffusion to set in at all, all effects of coherence were lost.) We hope to extend our presently meager understanding of exciton transport in the presence of the exciton-phonon interaction to situations in which exciton quenching occurs.

Since the completion of our work on the quenching of purely hopping (i.e., randomly walking) excitons on lattices, a theory has appeared in the literature[2] that imputes what we believe to be spurious properties to random walks and thereby impugns our published explanation of anomalous exciton quenching kinetics in doped crystalline polyacenes. To settle this question regarding random walks, and hopefully to reinstate our model of exciton quenching, we have undertaken a computer study of lattice random walks with trapping. We have already established that one of the alleged properties is indeed spurious, namely, that the introduction of long steps (further than nearest neighbor) causes a deviation from simple first-order quenching kinetics. We find no deviation due to long steps.

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LIGHT SCATTERING THEORY AND FLUORESCENCE DEPOLARIZATION THEORY

R. P. Hemenger

Two contributions to the theory of light scattering by cell suspensions have been completed. In the first, formulas have been developed that describe the influence on scattered light of an irregular distribution of absorbing pigments in the individual cells. This is very useful for studies, for example, of laser light scattered from chloroplasts. However, this work was proximally motivated by a need of the Chromosome Chemistry Group to interpret data on uv light scattering by cell nuclei.

The other contribution is a theoretical explanation of the "backscattering turbidimeter," a remarkable instrument recently developed in the Protein Synthesis Group. (The instrument measures cell concentration as a linear function of light transmitted through a fiber optical system over many times the concentration range previously possible.) This work involves a new contribution to the theory of light transmission by strongly scattering cell suspensions, namely, the development of

a forward-scattering approximation to the transport equation which describes light scattering. The solutions of this "forward-scattering equation" (FS equation), which are derived, are much simpler than those of the full transport equation, which have never been obtained in full. Moreover, these approximate solutions provide an accurate description of the significant phenomena involved in the backscattering turbidimeter.

As a bonus, the FS equation is mathematically identical to the equation that describes the spatial distribution of segments of randomly coiled polymers. Thus, the solutions of the FS equation, properly reinterpreted, are also solutions of the latter problem, which had not previously been solved. The theory of the backscattering turbidimeter and of the FS equation will each be published, separately.

In a separate but related activity, an explicit method has been developed for determining the distribution of end-to-end distances of randomly coiled polymers (e.g., polypeptides). If identical chromophores, for example, aromatic amino acid residues or organic dyes, occur at or are attached to the ends of the polymer, excited-state energy transfer between these chromophores causes depolarization of the chromophore fluorescence. The time dependence of the fluorescence depolarization subsequent to flash excitation can be measured with suitable fast electronics. By numerically performing an integral transform (Laplace transform) on the data thus obtained, one converts that time-dependent data to the spatial distribution of end-to-end distances.

Last year we reported[1] our preliminary results on the theory of time-dependent concentration depolarization of fluorescence and indicated its usefulness in elucidating the mechanisms of excitation energy transfer between identical molecules of biological importance, such as chlorophyll. This work has now been completed and accepted for publication. An error contained in last year's report has been corrected – the time-dependent relative fluorescence anisotropy (a quantity closely related to depolarization) itself, rather than the logarithm of this quantity, can be expressed as a series in powers of the square root of time following flash excitation. In addition to the results reported last year, which are otherwise correct, we have also been able to obtain, through the Laplace transform relation, a series expansion of the steady-state fluorescence anisotropy in powers of the solute concentration. Finally, we treated one correction to the point-dipole model used, namely, the effect of finite molecular volume, which slows the early decay of the time-dependent fluorescence anisotropy.

1. R. M. Pearlstein and R. P. Hemenger, *Biol. Div. Annu. Progr. Rep. June 30, 1972*, ORNL-4817, p. 57.

NANOSECOND FLUORESCENCE POLARIZATION CHRONOMETER

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

We are constructing an instrument to measure simultaneously the two perpendicular components of linear fluorescence polarization as a function of time in the nanosecond range. This polarization chronometer (PC) works on the principle of single-photon timing (SPT), as does the Excited-State Biophysics Group's currently operational SPT instrument. However, the latter determines only total fluorescence intensity as a function of time and is not sufficiently red-sensitive for photosynthetic work. For these reasons, the PC will have a wider range of applicability than the current SPT instrument. We expect the PC to be used, for example, for protein conformational studies as well as for studies of energy transfer in nucleic acids and in photosynthetic systems. Most major components of the PC are on hand; a few are on order. The completed instrument will be hooked on-line to the Biology Division's PDP-11 computer.

This sophisticated instrument will be of little use unless we can reliably reduce the data it supplies to the computer. The analysis of transient response data has been perennially plagued by the "deconvolution" problem: how to separate or deconvolute the instrument's response to some initial impulse from that of the sample under study. While this is a widespread and long-standing problem in experimental science, we are particularly interested in obtaining its solution in order to deconvolute the fluorescent response of biomolecules to a flash – necessary, for example, in reducing the data from the PC and other single-photon timing instruments. During the past year, we have developed a new approach to this problem that explicitly accounts for (and indeed takes advantage of) the fact that data are not collected as a continuous function of time but rather in discrete chunks or "channels." The approach is an algebraic one, involving the transformation of polynomials. Algorithms suitable for electronic data processing have been derived, and are currently being tested. Assistance with the development of computer programs for this problem has been provided by T. J. Mitchell of the Mathematics Division's Statistics Department.

PHOTOSYNTHESIS

W. A. Arnold, J. R. Azzi, and N. A. Nugent

We conducted three studies of light emission from green plants. These are: (1) new studies on glow curves,

the light emitted by frozen algae or chloroplasts as they are heated; (2) remeasurement of the steady-state fluorescence polarization of chlorophyll in unaligned *Chlorella* in order to check on recently published anomalous experiments from the University of Illinois; (3) completion of studies characterizing swollen chloroplast membranes, or "blebs," as delayed light amplifiers in an electric field.

We also continued our experiments on electrodes clad with MnO_2, as a model of photosynthetic oxygen production. We have used such electrodes to produce oxygen electrolytically at a substantially lower voltage than is required for the bare electrode. In addition, we have found that a medium containing suspended MnO_2 particles will produce oxygen in the dark upon the addition of an exogenous oxidant such as ionic cerium (Ce^{4+}).

We have also performed experiments with chloroplasts electrolytically adhered to glass surfaces. We found that such chloroplasts will produce oxygen from the Hill reaction (illuminated chloroplasts reduce added electron acceptor and oxidize water to oxygen) for up to two days – fifty or a hundred times longer than suspended chloroplasts. This finding could have great significance for photosynthetic conversion of solar energy to hydrogen (see next report).

Finally, we report a modification of the Hill reaction so sensitive that one can easily measure the photochemistry done by a single chloroplast. The chloroplasts are imbedded in nuclear track emulsion and, while still wet, are exposed to red light. (Red light by itself does not affect the emulsion.) Illumination of the chloroplasts reduces some of the silver ions (perhaps three to ten) in nearby grains to form a "latent image." The grains, about 0.25 μm in diameter, contain about 3×10^8 silver ions. The small amount of silver reduced by a chloroplast is thus amplified some millions of times by the photographic process (exposure and development) to form black grains that can be seen with the light microscope. We estimate the process to be sensitive enough to form one visible grain for approximately 3000 absorbed light quanta.

SOLAR ENERGY

R. M. Pearlstein

During the past year, we undertook a study on the possibility of developing photosynthetic technologies to convert the terrestrial solar flux directly to hydrogen. All present concepts of this possibility involve the construction of a "SPEC reactor." Sunlight absorbed by photosynthetic organisms, or parts thereof, in the SPEC reactor provides the energy to photolyze water. The organisms produce oxygen gas directly as a by-product, but the hydrogen is bound chemically to an "electron carrier." Hydrogen-liberating enzymes (hydrogenases), also in the SPEC reactor, release hydrogen gas from the reduced electron carrier. Only sunlight and water are consumed in the SPEC reactor. The photosynthetic organisms, hydrogenases, and electron carriers function catalytically and are not consumed. Approaches to the construction of a SPEC reactor vary, dependent on whether alive, intact organisms or just "active fractions" of organisms are used. Full technical feasibility has yet to be demonstrated for any approach.

From an economic point of view, the most promising ideas at present probably fall in the second category of approaches, that is, the development of engineered catalytic surfaces containing immobilized proteins (e.g., ferredoxin, hydrogenase, and photochemically active chlorophyll-protein). We have estimated the maximum absolute efficiency of such catalytic surfaces and also the land requirements to meet the energy equivalent of the projected U.S. gasoline demand in the year 2000. These are, respectively, 10% (which corresponds to a yield of about 9 moles of hydrogen per square meter per day for an average day in the southwestern U.S. desert) and 20,000 square miles, or about one-tenth the combined area of Arizona and New Mexico. While this is a vast area, it should be contemplated with three points in mind: (1) Other solar power schemes (e.g., solar thermal energy conversion) cannot likely supply more *hydrogen* per unit collector area; (2) a portion, at least, of the hydrogen-generating capacity might be sited at sea; and (3) all solar energy conversion, including SPEC, is unlikely to supply any large fraction of the U.S. energy demand by the year 2000 nor more than 30% of it ultimately.

DISTINGUISHING BETWEEN CHEMICAL REGULATION OF SENESCENCE AND CHEMICALLY INDUCED LETHALITY IN EXCISED WHEAT LEAF TISSUE: LETHALITY RESULTING FROM LOW CONCENTRATIONS OF CYCLOHEXIMIDE

A. H. Haber* and L. L. Triplett

In the apical section of the wheat leaf, chlorophyll loss is a specific reflection of loss of chloroplast ultrastructure as well as a general reflection of senescence, since it is accompanied by protein loss and by loss of ultrastructural integrity of all cell organelles. In this system, benzyladenine, a true senescence retarder, decreases chlorophyll loss and protein loss both in light

and in darkness. Conversely, abscisic acid, a true senescence promoter, increases chlorophyll loss and protein loss both in light and in darkness. These two chemicals influence death only indirectly, by retarding or accelerating, respectively, the physiological processes of senescence that ultimately result in death. By contrast, cycloheximide, at concentrations equal to or greater than 10 μg/ml, produces lethality according to the four criteria of death established in studies of chlorophyll loss in this system after lethal gamma irradiation.[1] These criteria are (1) promotion of chlorophyll loss in the light, (2) inhibition of chlorophyll loss in darkness, (3) abolition of chemical regulation of chlorophyll loss, and (4) abolition of a normal temperature optimum for chlorophyll loss in darkness. In darkness, lethal concentrations of cycloheximide retard protein loss as well as chlorophyll loss. In the light, lethal concentrations of cycloheximide similarly retard protein loss even though chlorophyll loss is accelerated. Lesser, sublethal concentrations of cycloheximide appear to accelerate senescence, since they accelerate chlorophyll loss in both darkness and light.

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CYTOKININS IN RELATION TO MITOTIC INHIBITION IN "GAMMA-PLANTLETS"

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

The cytokinin content of wheat seedlings germinating without cell division following irradiation of the dry grain with 500 kilorads of gamma rays has been measured by extraction and tobacco callus bioassay. Detectable cytokinin rose from a barely detectable quantity in dry grains to a tissue concentration similar to that in seedlings from unirradiated controls during four days of germination. Since the cytokinin content per seedling was no greater for seedlings with endosperm attached than for embryos germinated free of endosperm in nutrient solution, the cytokinins of the germinating grain appear to be localized in the embryo. Chromatographic mobility of the active material on Sephadex LH-20 columns eluted with 35% ethanol suggests that the cytokinins from the two sources are identical. Treatment of the "gamma-plantlets" with cytokinins and several combinations of other growth substances failed to induce cell division.[1]

We conclude that accumulation of extractable cytokinins can be uncoupled from cell division and that failure of cell division in gamma plantlets is not due to a gross deficiency of extractable cytokinins.

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GREENING OF ETIOLATED LEAF TISSUE IN ABSENCE OF THYMIDINE INCORPORATION INTO DNA

Paula J. Thompson,* A. H. Haber,† L. L. Triplett, and O. J. Schwarz‡

Apical sections of the first leaf of week-old seedlings, whether dark- or light-grown, take up [^{14}C] thymidine but fail to incorporate label into DNA. Etiolated apical leaf tissue exposed to light does show a low level of label in either perchloric acid-insoluble or diethyl pyrocarbonate-extractable material after incubation in [^{14}C] thymidine for extended periods (4–24 hr). Sufficient amounts of this ^{14}C-labeled material extracted by diethyl pyrocarbonate were run on CsCl density gradients. As a marker on the same gradients, we used ^{3}H-labeled DNA from dark-grown leaf bases, which had been incubated in the dark with [^{3}H] thymidine while undergoing extensive nuclear DNA synthesis. The ^{3}H profile on the gradients showed a sharp peak corresponding to nuclear DNA. However, the ^{14}C profile, from the material from dark-grown apical tissue incubated in the light for 16 or 24 hr, failed to peak anywhere in the gradients, including the positions corresponding to nuclear and plastid DNA.

We conclude that during the extensive metabolic syntheses that occur during greening, there is no incorporation of labeled thymidine into either nuclear or plastid DNA. From studies of nucleoside phosphotransferase activity, both before and after illumination of the dark-grown apical leaf sections, we conclude that the absence of thymidine incorporation into DNA cannot be attributed to absence of thymidine phosphorylating enzyme activity.

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ABSENCE OF UNSCHEDULED INCORPORATION OF THYMIDINE INTO DNA AFTER GAMMA IRRADIATION OF APICAL WHEAT LEAF TISSUE

Beth C. Mullin, A. H. Haber,* and L. L. Triplett

The lack of incorporation of labeled thymidine into DNA of apical wheat leaf tissue[1] provides a favorable system for looking for possible repair synthesis of DNA, because any unscheduled DNA synthesis would occur without a background of normal, semiconservative nuclear DNA synthesis. In this study we have confirmed the inability of apical tissue to incorporate [^{14}C] thymidine into DNA despite the presence of normal levels of thymidine-phosphorylating enzyme activity and in contrast to the very great incorporation into DNA in basal tissue from the same leaves. After irradiation with about 3, 30, and 300 kilorads of gamma rays, all of which are sublethal doses in this system,[2] there is still no significant incorporation of labeled thymidine into the DNA of the apical wheat leaf tissue.

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THYMIDINE-PHOSPHORYLATING ACTIVITY IN RELATION TO AGING IN WHEAT

O. J. Schwarz,* Paula J. Thompson,† and A. H. Haber‡

The thymidine-phosphorylating enzyme activity of an organism is frequently correlated with the capacity to initiate or maintain DNA synthesis and cell division. We studied the phosphorylation of thymidine to thymidine monophosphate as a function of age within different regions of the same organ. Thymidine-phosphorylating enzyme activity was determined in parts of the first foliage leaf of seven-day-old wheat seedlings. In this same organ there are young, dividing cells at the base and older, nondividing cells at the apex. DNA synthesis occurs in basal segments but is absent in apical segments, as shown by studies of [^{14}C] thymidine incorporation into NDA. By contrast, thymidine-phosphorylating enzyme activity, in the form of a nucleoside phosphotransferase, was found in all segments cut from the leaf. Nucleotide phosphatase activities were also determined because of their possible competing effects on thymidylate production *in vitro*.

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CHEMICAL MUTAGENESIS OF SOYBEANS: INDUCTION OF CHANGES IN SEED STORAGE PROTEIN

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

The amino-acid analysis of the storage protein of the soybean has shown a uniformly low concentration of the essential amino acid methionine. This level has been consistent at 0.0022 to 0.0033 micromole/ml. To increase the methionine level, the germinating soybean embryos have been exposed to various concentrations of N-methyl-N′-nitro-N-nitrosoguanidine at different time intervals. The F2 generation will be examined for possible mutations that increase the essential amino acid levels and specifically the methionine level. Screening can be accomplished through an automated Amino Acid Analyzer. High-speed, accurate analyses can be carried out using a modified procedure for rapid methionine detection.

Soybean tissue callus cultures have been isolated from soybean cotyledonary tissue on 2,4-D supplemented media. These cultures will be used to isolate a line of soybeans having a high methionine content in the storage protein. The approach to be used will be a mutagen treatment of the callus followed by regeneration of plantlets from the callus. Storage proteins of the cotyledons from the next generation (F2) of these plantlets will then be examined. Initial studies are presently concerned with conditions necessary for the production of plantlets from the callus.

ELECTROPHORETIC METHODS FOR DETECTING DIFFERENCES IN SEED PROTEINS AMONG SOYBEAN VARIETIES AND INDUCED MUTANTS

D. E. Foard, J. E. Caton,* and Kathleen L. Lowry†

Of eight solvents compared, a Tris-glycine buffer (pH 8.6) containing 2-mercaptoethanol extracted the most protein for electrophoretic analysis of the flours of soybean varieties Lee, Pickett, and Harosoy and three radiation-induced morphological mutants of Harosoy. Sonication extracted more protein than agitation. The band patterns of the extracted proteins were not

affected by any of the solvents or methods of extraction tested. Electrophoresis on gradient polyacrylamide slab gels revealed previously unreported differences in band patterns between Lee and Pickett varieties and showed the proteins from two of the mutants to be similar to each other and different from the third, which had a pattern similar to the parent Harosoy. Following treatment with sodium dodecylsulfate and 2-mercaptoethanol before electrophoresis on gels containing sodium dodecylsulfate, the patterns of the two previously undistinguishable mutants differed from each other while the three varieties and the third mutant had identical band patterns which differed only in the relative intensity of several of the bands.

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GENETIC DISSECTION OF PHOTOMORPHOGENESIS: ISOLATION OF PRESUMPTIVE MORPHOLOGICAL MUTANTS IN FERN GAMETOPHYTES

G. P. Howland, E. L. Boyd,* and Margaret L. Yette

Light plays a crucial role in the development of plants, controlling such varied processes as germination, elongation, and flowering. We are attempting to elucidate the genetic sequences which are involved from the perception of the light stimulus to the final morphological manifestation and to provide a series of mutant types for future biochemical studies.[1]

In the absence of blue-light irradiation (e.g., darkness or red light), the gametophytes of many ferns have only tip growth, resulting in a uniseriate filament. Upon irradiation with blue light, growth in two dimensions is induced,[2] resulting in a plate of cells or prothallus. Haploid spores of *Pteridium aquilinum* were subjected to mutagenic treatments (ethyl methanesulfonate or gamma irradiation) resulting in approximately 50% reduction in survival. After germination of the spores in axenic culture, followed by a period of growth in darkness, the cultures were screened for the presence of prothallial gametophytes. From the 300 presumptive mutants thus isolated and propagated, 25 actively growing isolates have retained the mutant phenotype upon regrowth in darkness, and the phenotype has remained stable through repeated testing. These isolates were all derived from gamma-ray-treated spores.

Further evaluation of these presumptive photomorphogenetic mutants will include backcrossing to the wild-type gametophyte and selfing to establish dominant/recessive characteristics. (Gametophytes of homosporous ferns are hermaphroditic, and diploid gametophytes can be regenerated from the sporophyte leaf tissue for evaluation of the photomorphogenetic response.) Finally, pairwise matings of recessive mutants, followed by testing of diploid gametophytes, will allow assignment of mutants into genetic complementation groups. From this analysis we propose to estimate the genetic complexity of the photomorphogenetic response.

*ORAU Undergraduate Summer Research Participant, 1972.

1. G. P. Howland and E. L. Boyd, *Amer. J. Bot.* **60** (Suppl.) 31 (1973).

2. G. P. Howland, *Physiol. Plant.* **26**, 264 (1972).

PLATING OF CULTURED PLANT CELLS: *HAPLOPAPPUS GRACILIS*

G. P. Howland and Margaret L. Yette

Suspension cultures of *Haplopappus gracilis* are being used to quantitatively evaluate the sensitivity of isolated cells to various physiological insults (e.g., chemical mutagen, ionizing radiation, freezing). Experiments have been carried out to evaluate several parameters of the plating assay for survival. A minimum plated density of 2500 "units" (i.e., single cells or small groups obtained as the 53–102 μm filter fraction) per milliliter is required for maximum plating efficiency (50–80%). Is the survival of otherwise viable cells affected by the presence of killed cells? When untreated cells are plated in various proportions with cells killed by massive gamma-ray doses, the observed plating efficiencies faithfully reflect the proportions of untreated cells plated. (Results of a preliminary experiment with ethyl methanesulfonate-treated cells suggest that this point should be tested with regard to the particular treatment under investigation.) Variable growth of plated cells can be greatly reduced by (1) rinsing the cells in sterile distilled water before plating and (2) plating in 25% conditioned medium. Under these conditions we can reliably plate cells of *Haplopappus gracilis*, and evaluate growth after about two weeks. We are interested in eliminating the conditioned medium from our plating experiments and are examining some variations in our culture procedures and medium.

DOSAGE EFFECT ON ENZYME LEVELS IN HAPLOID AND DIPLOID TOBACCO TISSUE CULTURES

G. P. Howland, Margaret L. Yette, and Jane A. Smith

Diploid and (anther culture-derived) haploid tissues of *Nicotiana tabacum* var. Wisconsin-38 are being quan-

titatively assayed for levels of enzyme activity. Activities (units per milligram dry weight of tissue) of glucose-6-phosphate dehydrogenase, acid phosphatase, and malate dehydrogenase are correlated with gene dosage; the diploid tissue has about twice the activity of the haploid. We are currently evaluating our finding that diploid tissue cultured on medium containing *p*-fluorophenylalanine exhibits the haploid levels of malate dehydrogenase and acid phosphatase and the diploid levels of glucose-6-phosphate dehydrogenase. Peroxidase activity does not appear to correlate with dosage.

CELLULAR RADIOBIOLOGY GROUP

Alexander Hollaender

International activities. The Proceedings of the Eleventh International Symposium, held at the University of La Plata, Argentina, November 28–December 3, 1971, appeared under the title of *Gene Expression and Its Regulation.* It was edited by F. T. Kenney, B. A. Hamkalo, G. Favelukes, and J. T. August. This is an impressive volume that has found wide acceptance. The 1972 symposium held in Cali, Colombia, dealt with the subject of *Fundamental Approaches to Plant and Animal Improvement.* Several members of the Biology Division attended this timely symposium. The Proceedings were edited by Dr. Adrian Srb of Cornell University, and the volume is scheduled to appear this year.

A new series of symposia has been started in India following the same pattern as the Latin American symposia. The first was held in Calcutta, India, in February of this year. The subject was *Control of Transcription*, and it was organized under the direction of Dr. B. B. Biswas of the Bose Institute. Five members of the Biology Division participated in this symposium. The Proceedings have been edited by Dr. B. B. Biswas, Dr. R. K. Mandal, Dr. Audrey Stevens, and Dr. W. E. Cohn, and the book is scheduled for publication early in 1974.

Preparations for the 1973 symposium on Reproductive Physiology have developed well, and the subject has elicited widespread interest. This symposium will be held in Salvador da Bahia, Brazil, on December 2–7, 1973, and is being organized by Dr. Elsimar Coutinho in Bahia and Dr. Fritz Fuchs of the Cornell Medical Center in New York. The subject is of such strong appeal that it appears there will be considerably more money available than for the previous year. The program for the Bahia symposium has stimulated sufficient interest to provide the impetus for a follow-up meeting with a similar but more specific topic dealing with reproductive physiology of the female. This symposium is planned for 1975 under the direction of Dr. G. P. Talwar of the All India Institute of Medical Sciences in New Delhi. The Population Council of New York is cooperating in the organization of this symposium.

Cooperative activities. The third volume of *Chemical Mutagens: Principles and Methods for Their Detection* has gone to press and will appear by August of this year. The International Congress of Genetics and the Environmental Mutagen Societies have coordinated the dates and locations for their meetings. The First International Conference on Environmental Mutagens will take place in Asilomar, California, on August 29–September 1, immediately following the Genetics Congress in Berkeley.

Alexander Hollaender has been active in helping the newly formed United Nations Environmental Programme (UNEP) to develop an international registry of potentially toxic compounds. An effort is being made to include the Oak Ridge Information Centers in this undertaking.

Attempts to develop a workshop to formulate methods for detecting teratogens were unsuccessful in getting support from the United States, but the Institut de la Vie in Paris will support a workshop on this subject. It will be held January 25–28, 1974.

We continue our efforts to assist the University of Tennessee with the Area Health Education Center, The Archival Center, and other projects and the UT–Oak Ridge Graduate School of Biomedical Sciences with the Black Colleges Program.

GENETICS AND DEVELOPMENTAL BIOLOGY

W. E. Barnett

Mutagenesis and Cytochemistry

R. F. Kimball

Mammalian Cytogenetics

J. G. Brewen
R. J. Preston
Diana B. Smith[a]

Human Genetic Biochemical Defects Analysis

Genetics and Cell Culture of Human Variants and DNA Repair

James D. Regan

Biochemical Analysis of Human Variants

J. L. Epler
C. R. Fisher[a]
J. X. Khym

Enzymology of Human Genetic Defects

W. E. Barnett
L. I. Hecker[b]
C. G. Mead
C. E. Nix[c]

Somatic Cell Genetics and Biochemistry

A. W. Hsie
Kohtaro Kawashima[b]
J. P. O'Neill[b]
C. H. Schröder[b]

Mammalian Biochemical Genetics

R. A. Popp
G. P. Hirsch[b]
Diana M. Popp

Yeast Genetics

J. F. Lemontt

Drosophila Biochemical Genetics

E. H. Grell
N. E. Hewitt[b]
J. E. Tobler

Drosophila Chromosomal Behavior

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

Structure and Function of Multienzyme Complexes

F. H. Gaertner
D. A. Casciano[b]
A. S. Shetty[d]

Cell Growth and Differentiation

Tuneo Yamada
J. N. Dumont
V. P. Idoyaga-Vargas[b]
Christian Michel[e]

Chromosome Ultrastructure

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

Reproductive Biology

R. A. Wallace
E. W. Bergink[b]

Hymenopteran Genetics and Genetic Methods for Insect Control

Roger H. Smith
Anna R. Whiting[c]
P. W. Whiting[c]

Molecular Basis of Recombination and Repair of DNA

Jane K. Setlow
M. E. Boling
Amir Muhammed[e]
Linda L. Munchausen[b]

[a]Temporary appointment.
[b]Postdoctoral investigator.
[c]Consultant.
[d]Visiting investigator.
[e]Visiting investigator from abroad.

MUTATION FIXATION AND DNA SYNTHESIS IN *HAEMOPHILUS INFLUENZAE*

R. F. Kimball and Jane K. Setlow

As reported earlier,[1] very few mutations are produced if cells treated with MNNG are lysed immediately, the lysate is used to transform bacteria, and mutations to novobiocin resistance are looked for in the transformants. If the treated cells are incubated for 30 or more minutes before lysing, many mutants are found in the transformants. Thus, transformation can be used as an assay for mutation fixation.

Contrary to earlier evidence from experiments[2] with ^{32}P, the time course of this mutation fixation coincides well with the time course of semiconservative DNA synthesis as judged by uptake studies and by isopycnic centrifugation of the DNA from density-labeled cells. One round of DNA replication occurs immediately after MNNG treatment, but then the cells cease multiplying for a time. The mutations are fixed during this initial round of synthesis. Since fixation continues through most of this round of replication, it is unlikely that most mutations arise only from initial lesions formed at the replication fork.[3] Rather, initial lesions distant from the fork must have a finite probability of being converted to mutations. This probability might decrease with distance from the fork if repair can remove part of the lesions.

The mutations that are fixed during this period are initially more sensitive to inactivation by irradiation of the lysates with 313-nm light than they are after they have undergone several rounds of replication. Thus the newly fixed (i.e., transformable) mutations are in some intermediate state not identical with the final mutations.

Incubation with 5-bromodeoxyuridine (BrdUrd) during the initial DNA synthesis decreases by nearly half the number of mutations that are fixed and also slows down DNA synthesis. Somewhat surprisingly, the mutations that are fixed in the presence of BrdUrd are not more sensitive to 313-nm light than those in the group incubated in thymidine. This suggests that the fraction of the mutants fixed in the presence of BrdUrd may be in regions with very little DNA synthesis, possibly in regions of repair synthesis.

1. R. F. Kimball and Jane K. Setlow, *Mutat. Res.* 14, 137 (1972).
2. R. F. Kimball and Jane K. Setlow, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, p. 105.
3. E. Cerdá-Olmeda, P. C. Hanawalt, and N. Guerola, *J. Mol. Biol.* 33, 705 (1968).

REPAIR OF PREMUTATIONAL LESIONS IN *HAEMOPHILUS INFLUENZAE*

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

Treatment of cells with mutagenic alkylating agents leads to a variety of lesions in the DNA. Several investigators have reported the disappearance of certain of these lesions during post-treatment incubation, suggesting that certain of the lesions can be repaired. However, since it is not known which of the lesions can lead to mutations, these findings do not necessarily indicate repair of premutational lesions. We have devised a method which, instead of chemically detecting the disappearance of a particular product, biologically detects the disappearance of premutational lesions in *Haemophilus influenzae.* The rationale behind the method is as follows. A chemically induced lesion in DNA is not a mutation until it has been converted into a heritable change in the nucleotide sequence. This process is known as mutation fixation. There is strong evidence that DNA replication is required for at least a majority of the mutation fixation. If a potentially mutational lesion is repaired before the replication fork reaches it, it will not give rise to a mutation. Assuming that repair of premutational lesions occurs, if mutation fixation were delayed by stopping DNA replication for a time, then a greater proportion of the premutational lesions would be repaired before the replication fork reached them, and the frequency of mutation in the population of cells would be decreased.

A temperature sensitive DNA synthesis mutant, *dna* 9, was treated at 36°C in Tris-maleate buffer, pH 6, with either N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) or nitrosocarbaryl. The cells were then washed and resuspended in growth medium at 41°C. DNA replication ceases almost immediately at this temperature. At various times aliquots were moved back to 36°C and grown for at least three generations before measuring the frequency of mutation to novobiocin resistance. An estimate of the extent of disappearance of premutational lesions in the absence of DNA replication was obtained by plotting the mutation frequency vs the length of time that the mutagenized cells were held at 41°C. With both mutagens, approximately 75% of the premutational lesions disappeared during incubation for 40 min in the absence of DNA replication, presumably by a repair mechanism.

The kinetics of disappearance of premutational lesions suggested that a fraction of the premutational lesions may not be repaired in the absence of DNA replication. This fraction could represent fixation of mutations by processes other than replication, such as repair or recombination. To determine whether some mutation fixation does occur in the absence of DNA replication, we used a method based on the fact that DNA extracted from a mutagenized culture will transform a second culture to mutant phenotype (such as novobiocin resistance) only after mutation fixation has occurred. By growing mutagenized cells and making cell lysates at various times, and measuring the ability of the lysates to transform a second culture to novobiocin resistance, we can follow the course of fixation of mutations to novobiocin resistance. This experiment was done using the *dna* 9 mutant treated with nitrosocarbaryl, and mutation fixation was followed at both 36° and 41°C. The results indicated that incubation of the mutagenized culture in the absence of DNA replication resulted in a sharp reduction in the extent of mutation fixation, as expected. Some mutation fixation occurred in the absence of DNA replication, however, suggesting that some other process may also be involved in fixation of mutations.

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

CHANGES IN CELL CYCLE PARAMETERS AND CELL SIZE DURING GROWTH OF CHINESE HAMSTER OVARY CELLS IN THE PRESENCE AND ABSENCE OF DIBUTYRYL CYCLIC ADENOSINE 3′-5′-MONOPHOSPHATE

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

It was shown previously[1] that treatment of Chinese hamster ovary (CHO) cells *in vitro* with dibutyryl cyclic AMP converts the culture from one of compact, randomly oriented cells that grow in multilayers to a monolayer of elongated-fibroblast-like cells growing parallel to one another. The present work was designed to find out what if any influence this major transformation in growth form has on cell cycle and cell size parameters. For this purpose, a combined microspectrophotometric and autoradiographic procedure, which had been used earlier[2] to study the growth of Chinese hamster lung (V79) cells, was employed. The results are still incomplete, but certain conclusions can be drawn.

The procedure was as follows. A number of cultures of CHO cells were set up, some with and some without dibutyryl cyclic AMP. The culture medium, including the cyclic AMP when present, was renewed daily, and at the same time two or three cultures of each kind were pulse-labeled with tritiated thymidine, harvested by trypsinization, counted for cell number, and then fixed and prepared for autoradiography and microspectrophotometry. The DNA content of each cell was determined microspectrophotometrically by Feulgen staining; the protein content, by dinitrofluorobenzene staining.

There were no significant differences in the growth curves of the two kinds of cultures. The cultures remained in exponential growth for the first three to four days, reached maximum numbers at day 6, and remained at this level through day 8. Somewhat surprisingly, in view of the great difference in cell form, the size of the cells, as measured by the protein content of mitotic cells, was identical in the two cultures. In both, the mitotic cells were appreciably larger on day 2 than on day 6. Both kinds of cultures showed an increase in the fraction of the cycle occupied by G_1 and a decrease in the fraction occupied by S between day 2 and day 6. Even after the culture reached maximum numbers there were still appreciable numbers of labeled cells and of mitotic cells, though less than in the earlier days of culture. In all these respects the cultures behaved much like the V79 cells we had studied earlier, but the changes in cell cycle parameters and in cell size were less marked in the CHO than in the V79 cells.

There were several differences, however, between cultures with and without dibutyryl cyclic AMP. Thus the decline in the frequency of labeled cells with age of the culture was less in cultures with the cyclic AMP than in ones without it. There were very few G_2 cells in either the two- or six-day cultures with the cyclic AMP but an appreciable number of such cells in cultures without it. There were more pycnotic cells in cultures with the cyclic AMP but more abnormally large and polyploid cells in cultures without it.

No overall conclusions will be drawn at this time except to say that despite the overall similarity in the growth curves and cell sizes in the two kinds of cultures there were distinct, though relatively small, effects of dibutyryl cyclic AMP on cell cycle parameters during culture growth. There was no evidence that the cyclic AMP had any major effect on the ability of cells in late culture stages to pass through the cell cycle even though with it the cells form a monolayer whereas without it they form multilayers.

1. A. W. Hsie and T. T. Puck, *Proc. Nat. Acad. Sci.* **68**, 358 (1971).

2. R. F. Kimball, Stella W. Perdue, E. H. Y. Chu, and J. R. Ortiz, *Exp. Cell Res.* **66**, 17 (1971).

THE STUDY OF RECIPROCAL TRANSLOCATIONS INDUCED IN HUMAN SPERMATOGONIA BY X RAYS*

J. G. Brewen and R. J. Preston

Before the great wealth of data on the genetic effects of ionizing radiation on mice can be extrapolated to man, information on comparisons between the two species must exist. We have reported one cytogenetic comparison[1] between the two species in a study on the kinetics of asymmetrical translocation production in peripheral leukocytes. Other studies[2] have shown that the yield of asymmetrical translocations produced in the leukocytes must be corrected by a factor of 0.25 to arrive at an estimate of reciprocal translocation yield in the primary spermatocytes for at least two other species; therefore, it seemed imperative that some quantitative data be obtained on human germ cells to determine if leukocyte data might be used as a predictive tool.

There existed a group of male volunteers that had been subjected to various doses of testicular irradiation. These volunteers were being biopsied at regular intervals in conjunction with studies on human reproductive physiology. We arranged to receive a portion of the biopsy material and made slide preparations in order to analyze the diplotene–metaphase I figures of primary spermatocytes for reciprocal translocations.

Table 4 summarizes the irradiation protocols that were followed, the numbers of individuals in each protocol, and the observed and expected data. The expected values were calculated by multiplying the known leukocyte aberration yields at each dose by 0.25, except in the case of the 600-R dose where no expected yield was calculated because the translocation dose response curve in the mouse is known to bend over at high doses. Not knowing the dose at which this occurs in human primary spermatocytes, we assumed that it had done so by 600 R.

A typical normal and a translocation bearing cell are shown in Figs. 5 and 6. The chiasmata frequency in the human male is high, and this presents a difficulty in analyzing the cells for translocations due to the complex appearance of normal bivalents. Consequently, many cells with only 22 "bivalents" were photographed and subsequently karyotyped. Yet there were cells that could not definitely be classified as containing a translocation and were excluded from the translocation class. If these "questionable" are included, the frequency of translocation is slightly higher at each dose.

Table 4. Observed and expected reciprocal translocation frequencies in human primary spermatocytes following testicular irradiation

Dose (R)	Number of individuals	Number of cells	Translocations (%)	
			Observed	Expected
0	2	200	0	0
78	2	371	4.0 ± 1.0	2.0
200	2	300	5.0 ± 1.3	8.4
600	2	180	6.1 ± 1.8	*a*
100 + 600	2	150	4.7 ± 1.8	9.4[b]
300 + 200	1	125	4.8 ± 2.0	25.0[b]

[a] See text for explanation.

[b] These values were derived by assuming an additive effect of the two doses, based on combining leukocyte and primary spermatocyte data.

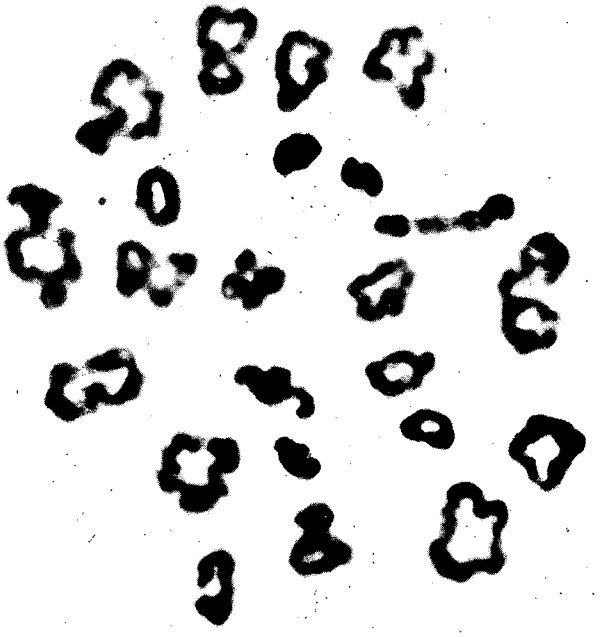

Fig. 5. Normal diakinesis of human primary spermatocyte.

The data, although presently meager, do show that reciprocal translocations can be recovered in human primary spermatocytes following spermatogonial stem cell irradiation and that the yields at the lower doses are close to expectation based on mouse and human leukocyte and mouse primary spermatocyte data.

*In cooperation with Dr. C. G. Heller, Pacific Northwest Research Foundation, Seattle, Washington.

1. J. G. Brewen et al., *Mutat. Res.* 17, 245–54 (1973).

2. J. G. Brewen and R. J. Preston, *Nature*, in press.

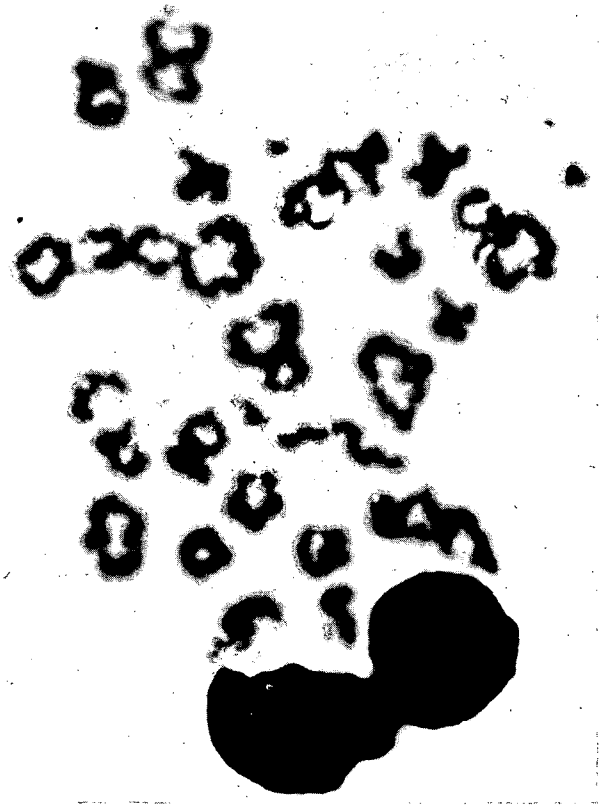

Fig. 6. Human primary spermatocyte containing a cVI.

X-RAY-INDUCED TRANSLOCATIONS IN FETAL AND NEWBORN MOUSE SPERMATOGONIA

J. G. Brewen, Nelwyn T. Christie, and H. E. Luippold

The radiation sensitivity of adult mouse spermatogonia to the induction of reciprocal translocations and specific locus mutations is well established. There have been recent reports that claim newborn mice are approximately one-half as sensitive as adults to mutation induction[1] and fetal mice (13½ days) are insensitive to translocation induction.[2] We have undertaken experiments to compare the sensitivity of fetal, newborn, adolescent, and adult mice to the production, by acute x rays, of reciprocal translocations in spermatogonial stem cells.

C3H female mice were mated with 101 males. Thirteen and one-half days after a vaginal plug was detected, one group of the females was irradiated with acute x-ray doses of 25 to 400 R. The rest of the females were held until parturition. The newborn mice were then irradiated with acute x-ray doses of 50 to 400 R. After weaning, the male offspring were held until sexual maturity, at which time preparations of diplotene-diakinesis figures of meiosis I were analyzed for reciprocal translocations. The yields of translocations observed at each dose are summarized in Table 5. For the purpose of comparison, previously published[3] data from adult C3H X 101 male mice are also included in Table 5.

Table 5. Frequencies of reciprocal translocations observed in the primary spermatocytes of irradiated 13½-day fetal, newborn, and adult mice

Dose (R)	Frequency of reciprocal translocations		
	Fetal mice	Newborn mice	Adult mice
0	0.0	0.0	0.0
25	0.0		
50	0.25 ± 0.18	0.67 ± 0.33	0.67 ± 0.33
100	1.55 ± 0.37	4.29 ± 1.11	1.50 ± 0.50
200	2.91 ± 0.51	4.71 ± 1.05	4.33 ± 0.85
300		5.78 ± 0.93	7.83 ± 1.14
400			13.50 ± 1.30

Unfortunately, the animals that received the higher doses had no meiotic activity at the time of writing, and, consequently, there are data for only four doses. In the instance of the newborn mice, only a few hundred cells have been analyzed and the data are subject to a large error.

Although incomplete, the data indicate two conclusions might be drawn. First, unlike the previous study mentioned,[2] fetal mice are sensitive to the production of reciprocal translocations in the germinal stem cells. Second, although newborn mice are not as sensitive as adult mice to specific locus mutation induction, they appear to be equally sensitive to reciprocal translocation induction.

These studies are currently being expanded to include more dose points and more individual mice at all the dose points for the 13½-day fetal and newborn mice, as well as studying the effect of two doses (200 and 400 R) at later fetal stages and up to ten days postpartum.

1. P. B. Selby, *Mutat. Res.* 18, 63–75 (1973).
2. B. Ivanov et al., *Mutat. Res.* 18, 89–92 (1973).
3. R. J. Preston and J. G. Brewen, *Mutat. Res.* 19 (in press).

SOMATIC AND GERM CELL CHROMOSOME ABERRATIONS AS AN INDEX OF GENETIC DAMAGE*

J. G. Brewen, R. J. Preston, and H. E. Luippold

During the preceding five to ten years, a large amount of data has been accumulated on the analysis of

chromosome aberrations in human somatic cells and mouse germ cells. The ultimate goal of these diverse studies has been to use the chromosome aberration data as an index for estimating man's genetic hazard from ionizing radiations. A cursory glance at the data shows that in the low- to intermediate-dose ranges, human somatic cells appear to be almost an order of magnitude more sensitive to chromosome exchange production than mouse spermatogonia. In addition to this apparent large discrepancy in sensitivity, the basic dose response characteristics are different. The aberration yields in human cells increase exponentially with dose up to doses of 5000 R, whereas the yields in mouse spermatogonia reach a peak at 500–600 R and decline with increasing dose.

We have recently published a study in which radiation-induced chromosome exchanges have been studied in six mammalian species.[1] The results showed that exchange frequency was linearly related to the number of chromosome arms available for exchange, and consequently, human leukocytes are twice as sensitive as mouse leukocytes. Further investigation on the mouse and Chinese hamster has allowed us to conclude that the exchange yield observed in leukocytes can be used to predict exchange yields in germ cells.[2] The data show that, for whole body x-ray doses up to 400 R, the exchange yields observed in leukocytes immediately after exposure need to be corrected by 0.25 to arrive at the frequency of exchanges produced in spermatogonial stem cells and analyzed in primary spermatocytes (Fig. 7).

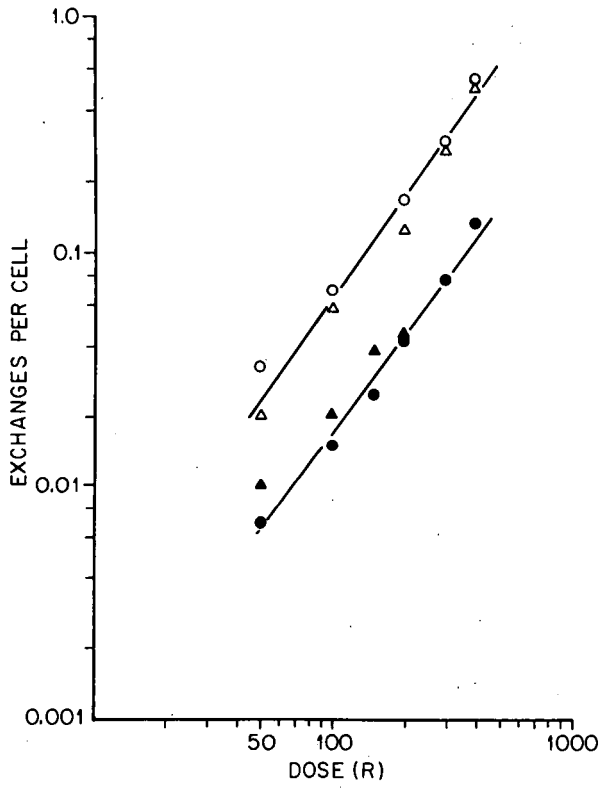

Fig. 7. Yields of dicentric chromosomes and reciprocal translocations in peripheral leukocytes and primary spermatocytes, respectively, of the Chinese hamster and mouse. Chinese hamster and mouse leukocytes, ○ and △; Chinese hamster and mouse primary spermatocytes, ● and ▲.

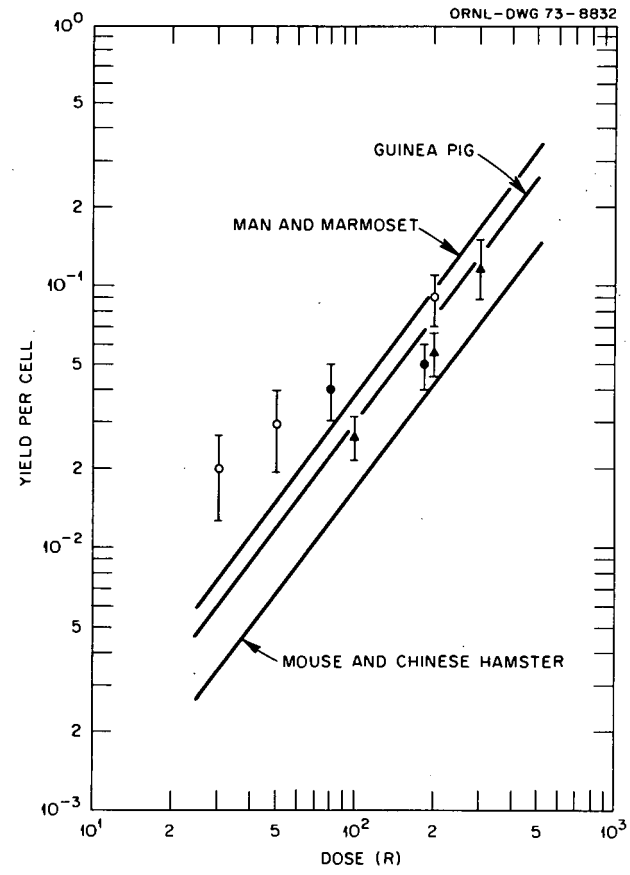

Fig. 8. Predicted and observed yields of reciprocal translocations in the primary spermatocytes of five mammalian species. ○ Marmoset, ● man, ▲ guinea pig.

By using a combination of the correction factors for chromosome arm number and leukocyte vs primary spermatocyte exchange yields, coupled with actual experimental data on exchange yields in mouse primary spermatocytes, it is possible to construct expected exchange yield vs dose curves for any mammalian species. We have done this for man, the marmoset, and the guinea pig (Fig. 8). Actual data obtained, at the time of writing, on these three species are plotted for comparison. It can be seen that the actual data obtained at this time agree well with expectation.

Since it will be impossible to obtain any more data of this type on man, it will be necessary to estimate hazards to man from mouse and other mammalian studies. We are confident that these estimations can now be made with a high degree of accuracy. Thus we postulate that accurate estimates of chromosome exchange yields in human germ cells can be made from data obtained on experimental organisms.

*The experiments with marmosets are being performed in collaboration with Dr. N. Gengozian, Oak Ridge Associated Universities, Oak Ridge, Tennessee.

1. J. G. Brewen, R. J. Preston, K. P. Jones, and D. G. Gosslee, *Mutat. Res.* 17, 245–54 (1973).

2. J. G. Brewen and R. J. Preston, *Nature New Biol.* 244, 111–13 (1973).

ISOLATION OF AUXOTROPHIC MUTANTS IN CHINESE HAMSTER CELLS

R. J. Preston, A. W. Hsie, Nelwyn T. Christie, and J. G. Brewen

In order to instigate a program in somatic cell genetics, it was necessary to isolate and characterize auxotrophic mutants in a Chinese hamster cell line. The methods used were basically those of Kao and Puck.[1]

The line used was a CHO-K_1 (pro^-) obtained from T. T. Puck's laboratory. This line was used because it has 20 chromosomes, and we thought that there was a reasonable chance that it would be haploid for several loci. Cells were exposed either to EMS at a concentration of 400 μg/ml for 16 hr or 800 R of x rays. The survivors were allowed to grow for five days in F12 medium supplemented with 10% fetal calf serum, after which time they were subcultured into 40 petri dishes, with 2.5×10^4 cells per plate, in F12 deficient medium supplemented with 10% dialyzed serum. The medium was deficient for alanine, glycine, glutamic acid, aspartic acid, thymidine, hypoxanthine, inositol, vitamin B_{12}, and lipoic acid. Under these growth conditions, only wild-type cells will grow, and any mutant cells induced by EMS or x rays will not grow. After 24 hr growth in deficient medium, 1×10^{-5} *M* BudR was added to the plates and left for a further 24 hr. The wild-type cells will incorporate BudR, but mutant cells will not. The cells were then exposed to white light for 1 hr so that cells which had incorporated BudR would be killed but any mutant cells induced would survive. The medium was changed to F12 complete, and the surviving cells allowed to grow into colonies, which takes about six days. Individual colonies were isolated and tested in F12 complete and F12 deficient media. Any colony which grew in complete medium but not in deficient medium was provisionally categorized as a mutant. In the EMS experiment, 150 colonies were isolated, and in the x-ray experiment, 40 colonies; 8 were mutant in the former and 2 in the latter. The components lacking in the deficient medium were added back one at a time to determine the nature of the mutation. All the mutants isolated in these experiments required glycine for growth, but we are still in the process of determining whether or not they are all mutant for the same step in the pathway of glycine metabolism.

Three of the mutant lines have been tested for spontaneous reversion frequency, and in all three it is in the range of $2–5 \times 10^{-8}$.

We intend to characterize these mutants biochemically and genetically by the use of cell hybridization and then to study reversion by radiation and chemicals. We also are attempting to make hamster-human hybrids (using our mutant hamster cells) and, using the fact that human chromosomes are preferentially lost from such hybrids, to select for lines containing the hamster chromosome complement, plus, ideally, the one human chromosome conferring a wild-type phenotype to the cell. It should then be possible to study forward and reverse mutation for the human locus.

1. F. T. Kao and T. T. Puck, *Proc. Nat. Acad. Sci.* **60**, 1275 (1968).

THE DISTRIBUTION OF THE CAPACITY FOR DNA REPAIR IN NORMAL AND REPAIR-VARIANT HUMANS

James D. Regan and R. B. Setlow

Using the 5-bromodexoyuridine photolysis assay, we have examined an array of normal and repair-variant humans for their capacity for DNA repair.

The assay has been applied to skin fibroblasts *in vitro* from these individuals. The capacity for DNA repair is expressed as the change in the reciprocal of the weight-average molecular weight, $\Delta(1/M_w)$, with increasing fluences of 313-nm irradiation. The details of this assay have been described previously.[1,2] The distribution of repair capacity (Fig. 9) is essentially binomial. Normal individuals fall between 2.7 and 5.6, with a marked peak at 3.7 to 4.6. Individuals with the inherited disease xeroderma pigmentosum fall between 0 and 0.6. One individual showed a remarkable capacity for DNA repair, $\Delta(1/M_w) = 5.7$, and this result was found with skin fibroblasts cultured from a patient with systemic lupus.

There has been one report[3] of reduced DNA capacity in lymphocytes from patients with systemic lupus.

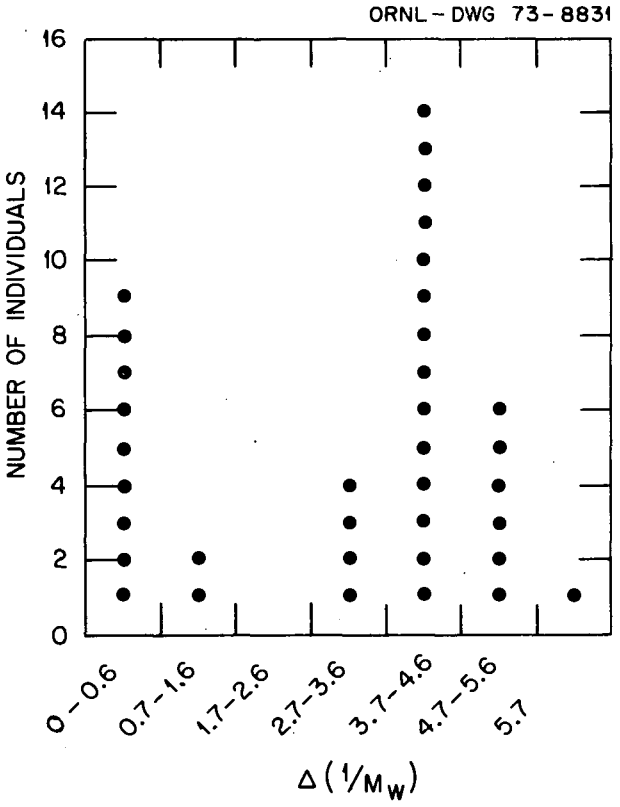

Fig. 9. Distribution of capacity for DNA repair in normal and repair-variant humans.

Thus, it appears that skin fibroblasts from such patients do not show reduced repair and, in this instance, showed an expansive capacity for DNA repair.

1. Regan et al., *Proc. Nat. Acad. Sci.* 68, 708 (1971).
2. Regan et al., *Science* 174, 147 (1971).
3. Altman et al., *1st Eur. Congr. Biophys.*, vol. II, p. 143 (1971).

DNA REPAIR IN THE HUMAN GENETIC DISEASE PROGERIA

James D. Regan and R. B. Setlow

It has been reported by Epstein et al.[1] that cells derived from a patient with the Hutchinson-Gilford syndrome of progeria are defective in their capacity to rejoin DNA strand breaks induced by ionizing radiation. We have examined cells from a patient having this syndrome for their capacity to rejoin DNA strand breaks induced by ionizing radiation (^{60}Co gamma rays). The repair profiles of these cells, immediately after gamma irradiation and examination on alkaline sucrose gradients, show a slight delay in rejoining DNA strand breaks. However, 90 min after irradiation these cells showed a complete rejoining of the radiation-induced DNA strand breaks. Thus it appears, in the case of the progeria patient whose cells we examined, there is no impressive defect in the ability to repair the DNA after radiation damage.

1. Epstein et al., *Proc. Nat. Acad. Sci.* 70, 977 (1973).

RECOVERY OF THE ABILITY TO SYNTHESIZE DNA IN SEGMENTS OF NORMAL SIZE AT LONG TIMES AFTER ULTRAVIOLET IRRADIATION OF HUMAN CELLS

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

DNA synthesized in human cells within the first hour after ultraviolet irradiation is made in segments of lower molecular weight than in nonirradiated cells. The size of these segments approximates the average distance between pyrimidine dimers in the parental DNA. This suggests that the dimers interrupt normal DNA synthesis and result in gaps in the newly synthesized DNA. However, DNA synthesized in human cells at long times after irradiation is made in segments equal or nearly equal to those synthesized by nonirradiated cells. The recovery of the ability to synthesize DNA in segments of normal size occurs in normal human cells, where the dimers are excised, and also in cells of the human mutant xeroderma pigmentosum, where the dimers remain in the DNA. This observation implies that the pyrimidine dimer may not be the lesion that causes DNA to be synthesized in smaller than normal segments.

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DNA REPAIR OF ULTRAVIOLET IRRADIATION DAMAGE IN *POTOROUS TRIDACTYLIS*

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

Potorous tridactylis cells grown in tissue culture have the following DNA repair systems: DNA chain elongation and joining (post replication repair), recovery of the ability to synthesize DNA in normal size segments at long times after uv irradiation, excision repair, and photoreactivation. DNA synthesized immediately after irradiation is made in smaller than normal size segments equal approximately to the average distance between dimers. The lesions interrupting DNA replication are photoreactivable, and their disappearance can be measured by a decrease in repair endonuclease sensitive sites and a loss of dimers from the DNA. Recovery of the

ability to synthesize DNA in normal size segments at long times after irradiation occurs slowly, requiring approximately 24 hr for full recovery. However, recovery can be facilitated by photoreactivation. Excision repair occurs minimally in Ptk cells which excise approximately 10% of the dimers in 24 hr. Photoreactivation is the most prominent repair system for removing the pyrimidine dimers formed by ultraviolet irradiation. Approximately 25% of the dimers are monomerized during the first hour of photoreactivation.

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THE EFFECTS OF CAFFEINE ON DNA REPLICATION AND POST-REPLICATION REPAIR IN HUMAN CELLS

S. N. Buhl* and James D. Regan

DNA synthesized shortly after ultraviolet irradiation of human cells is made in smaller than normal size segments, while DNA synthesized at long times after irradiation is made in normal size segments. Upon incubation the shorter segments as well as the normal size segments are elongated and joined by the insertion of exogenous nucleotides to form high-molecular-weight DNA as in nonirradiated cells. These processes occur in normal human cells where the pyrimidine dimers are excised and in xeroderma pigmentosum (XP) cells where the dimers are not excised. The effect of caffeine on these processes was determined for both normal human and XP cells. Caffeine, which binds to denatured regions of DNA, inhibited DNA chain elongation and joining in irradiated XP cells but not in irradiated normal or nonirradiated cells. Caffeine also caused a regression in the ability to recover synthesis of DNA in normal size at long times after irradiation in XP cells but not in normal cells.

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FURTHER STUDIES ON INHIBITION OF DNA AND RNA SYNTHESIS, BUT MINIMALLY OF PROTEIN SYNTHESIS, IN HUMAN LEUKEMIC CELLS BY A SERINE ANTIMETABOLITE

N. H. Pazmiño, D. G. Doherty, and James D. Regan

The synthesis and experimental testing of a cyclic serine antimetabolite, 1-(hydroxylamino) cyclohexane carboxylic acid, or cyclohexylserine, are presented. Action of the drug at 10^{-3} *M* against human chronic granulocytic leukemic cells is to completely inhibit DNA and RNA synthesis while having minimal and probably indirect effect on protein synthesis. Experimental evidence (Table 6) suggests that the mode of action of the drug is to inhibit one-carbon transfers from serine to the synthesis of nuclei acid precursors.

The inhibition of DNA and RNA synthesis in CGL cells by cyclohexylserine (cHxSer) at high concentration (10^{-3} *M*) and at lower serine concentration (10^{-5} *M*) is essentially complete. Protein synthesis is reduced but only by about 50%. This result implicates not inhibition of incorporation of serine into protein in these cells but rather the role of serine in the synthesis of DNA and RNA precursors. The effect of cHxSer (at 10^{-3} *M*) on HeLa cell DNA synthesis is minimal, even at 10^{-4} *M* serine, probably due to the capacity of these cells to synthesize serine. Inhibition of nucleic acid synthesis in HeLa cells would probably occur only at extremely high (i.e., 0.1 *M*) concentrations of cHxSer.[1]

A serine antimetabolite that can interfere with nucleic acid synthesis (and thus, by implication, synthesis of nucleic acid precursors) and yet show only partial inhibition of protein synthesis (the major effect of this inhibition, seen only at later times in the experiment) should not inhibit serine incorporation into protein. The results indicate this is the case. We suggest this lack of inhibition by cHxSer of [^{14}C] serine into protein is due to lack of affinity of cHxSer for seryl-tRNA synthetase by cHxSer; this is probably the basis for lack of inhibition of protein synthesis by this antimetabolite.

One-carbon donations in purine synthesis and the methylation of uridylic acid can be made by 5,10-methylenetetrahydrofolate or 10-formyltetrahydrofolate; depending on which folic acid derivative is in excess, the two are readily interconvertible by the 5,10-methylenetetrahydrofolate cyclohydrolase. Thus, if cHxSer blocks one-carbon donation from serine, excess tetrahydrofolate plus formate could compensate for this one-carbon deficiency. Our results are consistent with this suggestion: incorporation of serine into DNA is inhibited by cHxSer. Purine nucleosides relieve inhibition of DNA synthesis in serine-requiring CGL cells; pyrimidine nucleosides do not.

Table 6. Cyclohexylserine administered to CGL cells over 48 hr

Serine concentration (*M*)	Percent DNA synthesis at cyclohexylserine concentration of –		
	0	10^{-4} *M*	10^{-3} *M*
10^{-3}	100	88	4
10^{-4}	100	45	40

From these results we suggest that cHxSer interferes with one-carbon donations to purine synthesis. Inhibition of methylation of uridylate to thymidylate is also a possible source of DNA inhibition and is now under study. If cyclohexylserine, in fact, inhibits one-carbon donations from serine to purine synthesis and uridylate methylation, this may be due to binding of the drug to serine transhydroxymethylase and blocking of the action of this enzyme. Experiments on inhibition of serine transhydroxymethylase by cHxSer are in progress.

1. Pazmiño et al., *Journal of the National Cancer Institute,* in press.

INHIBITION OF NUCLEIC ACID SYNTHESIS IN HUMAN LEUKEMIA CELLS BY THE CARBOXAMIDE DERIVATIVE OF CYCLOHEXYLSERINE

N. H. Pazmiño, D. G. Doherty, and James D. Regan

The carboxamide derivative of cyclohexylserine seems to have a more drastic effect on the synthesis of DNA in human chronic granulocytic leukemia (CGL) cells than cyclohexylserine.

The cyclohexylserine reduced DNA synthesis to 20% of the control values in 24 hr and completely abolished it by 48 hr.[1] However, the carboxamide derivative causes 100% reduction of DNA synthesis in CGL cells within 24 hr (Table 7). We are currently investigating the possible toxic effects on cells that do not require serine.

1. Pazmiño et al., *Journal of the National Cancer Institute,* in press.

Table 7. Reduction of DNA synthesis in CGL cells within 24 hr

Serine concentration (M)	Percent DNA synthesis at carboxamide concentration of –		
	0	10^{-3} M	10^{-4} M
10^{-4}	100	0	97
10^{-5}	100	0	65

EFFECT OF SOME POSSIBLE SERINE ANALOGS ON DNA SYNTHESIS OF HUMAN CHRONIC GRANULOCYTIC LEUKEMIA CELLS

N. H. Pazmiño, D. G. Doherty, and James D. Regan

The effect of some putative serine analogs on DNA synthesis in serine-requiring human chronic granulocytic leukemia cells was studied. Some of the results are shown in Table 8.

Of all the compounds tested only ethyl 2 hydroxy hexahydrobenzoate had some inhibitory effect, but it was still a small one when compared with hydroxyl amino cyclohexane carboxylic acid (cHxSer) or with the carboxamide derivative of cHxSer. Therefore no further studies are being made with these compounds.

Table 8. Results of serine analogs on DNA synthesis in human CGL cells

Analog – 10^{-3} M	Percent DNA synthesis at 10^{-4} M serine	
	24 hr	48 hr
None	100	100
3-Hydroxy hexahydrobenzoic acid	106	80
4-Hydroxy hexahydrobenzoic acid	110	95
Glucosaminic acid	148	140
Ethyl 2 hydroxy hexahydrobenzoate	41	50
Diamino D.L. succinic acid	110	115
1 Cyclo pentane D.L. alanine	106	97
3 Cyclo hexane D.L. alanine	100	100

BIOCHEMICAL ANALYSIS OF GENETIC VARIANTS

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

The primary objectives of this study in human genetics are to detect and characterize previously undescribed inborn errors of metabolism. The design of the program is to use automated analytical instrumentation to survey the low-molecular-weight components present in the blood, urine, saliva, etc., of individuals (preferably children, and in most instances with affected siblings) with possible genetic abnormalities. These analyses would permit the detection and isolation of metabolites whose presence is associated with a particular genetic defect.

In the initial phase of the program, the instrumentation has consisted of ultraviolet analyzers, amino acid

analyzers, gas chromatography, and mass spectroscopy. Samples are also channeled through a series of preliminary screening tests when appropriate. The uv system utilizes the high-pressure–high-resolution ion-exchange chromatographic system developed by the Body Fluids Program at Oak Ridge. Thus, our approach is the initial attempt to use this instrument in a study committed to genetic screening.

In the initial months of screening, a large number (over 300 complete analyses) of body fluid samples have shown potentially interesting results in the form of massive urinary accumulates. These results fall into roughly three cases: (1) not reproducible, (2) unexplained differences from normal needing further controls – drugs, diet, organic disease, etc. – and (3) reproducible variations from normal, channeled to gas chromatography and mass spectroscopy for identification.

The main problem, then, in this initial phase has not been one of obtaining samples and analyzing them but one of obtaining the proper high-genetic-risk samples that will lead into the further stages in the study – cell culture and enzymology. In an attempt to resolve this issue, liaisons between the Biology Division and a number of university medical centers have been set up. It is hoped that (1) analyses of the highly selected samples from this contact will lead to concrete cases to explore in depth and that (2) the medical personnel will supply the medical knowledge and input to make the program workable.

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

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

The available uv-sensitive mutants of *Neurospora crassa* were examined for their ability to excise and photoreactivate cytosine-containing dimers *in vivo*. All strains exhibited *in vivo* photoreactivation, including *upr-1*, which was originally thought to be deficient in photoreactivation. Two strains, *uvs-2* and *upr-1*, were shown to be deficient in excision repair; *uvs-3* was shown to contain a residual amount of excision capability. The remaining strains, *uvs-1*, *uvs-5*, and *uvs-6*, were normal in their ability to excise dimers. Based on these results, tentative analogies were drawn between the *Neurospora* mutants and the known classes of uv-sensitive mutants in *E. coli*. Accordingly, the *N. crassa* mutants were classified as *uvs-1, -lon; uvs-2, -uvr; uvs-3, -rec; uvs-5, -lon; uvs-6, -rec;* and *upr-1, uvr*.

*Predoctoral investigator, Institute of Radiation Biology, University of Tennessee, and Graduate Laboratory Participant under appointment from Oak Ridge Associated Universities.

THE ROLE OF N^6,$O^{2'}$-DIBUTYRYL ADENOSINE CYCLIC 3′:5′-MONOPHOSPHATE IN THE REVERSE TRANSFORMATION OF CHINESE HAMSTER OVARY CELLS

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

Previously, we have demonstrated that Chinese hamster ovary (CHO) cells, growing randomly *in vitro* in a compact, multilayered colony, transform into a monolayer of elongated fibroblast-like cells when treated with N^6,$O^{2'}$-dibutyryl adenosine cyclic 3′:5′-monophosphate (But_2 cAMP) and hormones, such as testosterone and prostaglandin E_1.[1,2] This morphological conversion, which we termed "reverse transformation,"[3] is accompanied by the appearance of certain properties characteristic of a mammalian fibroblastic state.[1–3] In studying reverse transformation of CHO cells, But_2 cAMP was used in the expectation that this lipophilic derivative would penetrate cells more effectively than the unsubstituted cAMP and perhaps might also be less susceptible to the hydrolytic action of phosphodiesterase inside the cell, as suggested by many investigators.

The present study demonstrates that contrary to what has been generally believed, cAMP is in fact more readily being taken up by CHO cells than its dibutyryl derivative. Furthermore, both cAMP and But_2 cAMP are rapidly metabolized. Homogenates of CHO cells were found to contain two cAMP phosphodiesterase activities with distinct K_m with respect to cAMP: a high K_m(1 to 3 m*M*) and a low K_m(2 to 5 μM) enzyme. N^6-Monobutyryl adenosine cyclic 3′:5′-monophosphate (But_1 cAMP) was found to be a better competitive inhibitor for the cAMP phosphodiesterase activity than But_2cAMP; the K_i for But_1 cAMP is 40 to 60 μM, whereas for But_2cAMP it is 0.3 to 0.4 m*M*.

Addition of 1 m*M* But_2cAMP to the growth medium of monolayered CHO cells causes recognizable morphologic changes within 1 hr, and almost all cells have clearly become highly elongated within 6 hr.[3] After the addition of 1 m*M* But_2cAMP, the intracellular concentrations of both But_1cAMP and But_2cAMP rose gradually and reached 40 to 80 and 20 to 30 μM respectively 3 hr later. The intracellular concentrations of both of these butyryl cyclic nucleotides remain constant thereafter. The intracellular cAMP concentration has risen from basal level, 1 μM, to approximately

15 μM 3 hr after. It is worthy to note that under this condition the accumulated intracellular But_1cAMP (40 to 80 μM) is approximately equal to the K_i value (40 to 60 μM) of the low K_m cAMP phosphodiesterase using cAMP (0.3 to 2.0 μM) as substrate, whereas the intracellular But_2cAMP concentration (20 to 30 μM) is far below its K_m (0.3 to 0.4 mM) to cAMP phosphodiesterase. Such studies led us to conclude that after uptake of But_2cAMP by CHO cells, the majority of But_2cAMP was rapidly metabolized and in part converted to But_1cAMP; the accumulated But_1cAMP concentration within the cell is sufficient enough to inhibit cAMP phosphodiesterase activity competitively, which, in turn, caused the increase of intracellular cyclic AMP level. Compatible with the foregoing suggestions, we have obtained evidence to show that the increased level of cAMP is formed *de novo* rather than as a result of deacylation of the But_2cAMP being taken up into the cells.

It appears highly likely that the "induced" intracellular level of cAMP by exogenous addition of But_2cAMP is responsible for the subsequent reverse transformation phenomenon in CHO cells.

*Deutsche Forschungsgemeinschaft Postdoctoral Fellow.

1. A. W. Hsie and T. T. Puck, *Proc. Nat. Acad. Sci. U.S.A.* 68, 358–61 (1971).

2. A. W. Hsie, C. Jones, and T. T. Puck, *Proc. Nat. Acad. Sci. U.S.A.* 68, 1648–52 (1971).

3. T. T. Puck, C. A. Waldren, and A. W. Hsie, *Proc. Nat. Acad. Sci. U.S.A.* 69, 1943–47 (1972).

MECHANISMS OF HORMONE ACTION IN REVERSE TRANSFORMATION OF CHINESE HAMSTER OVARY CELLS

Kohtaro Kawashima and A. W. Hsie

Testosterone or prostaglandin E_1 alone, in any concentration tested, or low concentrations of dibutyryl cyclic AMP, produce no immediate effect in causing morphological transformation. However, simultaneous addition of 15 to 30 μM testosterone or 20 to 60 μM prostaglandin compound and 0.1 to 0.2 mM dibutyryl cyclic AMP produces a synergistic effect. Experimental results demonstrated that addition of prostaglandin E_1 to CHO cells causes an approximately 20-fold increase in the intracellular concentration of cyclic AMP within 1 min, whereas no significant change in cyclic AMP concentration was caused by testosterone for as long as 24 hr. Thus the synergistic effects of testosterone and prostaglandin E_1 appear to be acting through different mechanisms. We have also shown that among several prostaglandins other prostaglandins are less effective in effecting synergistic action, and the degree of morphological change parallels the ability of the compounds to stimulate cyclic AMP synthesis *in vivo*, for example, $E_1 > E_2 = A_1, A_2 \gg F_2\alpha$.

PRODUCTION OF VARIANTS WITH RESPECT TO REVERSE TRANSFORMATION IN CHINESE HAMSTER OVARY CELLS

A. W. Hsie

After standard treatment of CHO cells with ethyl methanesulfonate (EMS), several classes of variants with respect to reverse transformation have been produced: class I, insensitive epithelial variants which no longer respond to reverse transformation agents; class II, hypersensitive epithelial variants which become highly elongated and almost assume an extended mature neuron-like appearance in the presence of reverse transformation agents; and class III, fibroblast-like variants – those variants which display highly elongated morphology even when grown in the basal growth medium without the addition of the hormonal agents; however, the cells became further elongated when reverse transformation agents were added. The growth characteristics, the cyclic AMP level, and the enzyme systems for cyclic AMP metabolism (adenyl cyclase, cyclic AMP phosphodiesterase, and cyclic AMP dependent protein kinase) are being examined. Cell fusion studies demonstrate that the alterations in these variants toward reverse transformation agents behave in a dominant fashion over the same property in normal cells.

PRODUCTION OF FIBROBLAST-LIKE VARIANTS FROM THE EPITHELIAL-LIKE CULTURED CHINESE HAMSTER LUNG CELLS

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

Treatment of epithelial-like Chinese hamster lung (CHL) cells that have lost contact inhibition of growth with EMS produces variants which display fibroblast-like morphology as well as rigorous contact inhibition even when grown on basal medium without addition of the hormonal agents. The frequency of the mutagen-induced morphological conversion is approximately 0.1%. The fibroblast-like variants so produced are stable since no reversion was observed over 1000 generations of growth. Physiological and biochemical studies showed that, compared to its parental lung cell, one fibroblast-like CHL variant is less agglutinable by wheat germ agglutinin, is capable of synthesizing more

collagen, has lower t-RNA methylase activity and capacity, and has an altered esterase electrophoretic pattern and total activity. Study of the cyclic AMP system of these two cell types is in progress. These experiments provide a genetic basis for study of mechanisms involved in control of mammalian cell transformation.

STRUCTURAL MODIFICATIONS OF REVERSE TRANSFORMATION IN CHINESE HAMSTER OVARY CELLS

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

The morphological aspects of reverse transformation in CHO cells are being studied in greater detail. To summarize: (1) phase-contrast microscopy of the control epithelial-like CHO cells reveals pseudopod-like knob structures with a high optical density extending from the cell periphery out into the external medium; under reverse transformation conditions, such knob structures are not visible except occasionally at the two pointed tips of the elongated fibroblast-like cells; (2) histological staining demonstrated that knobs are rich in RNA, but there is no detectable DNA; (3) electron microscopic studies have shown that there are abundant polysomes but not other visible subcellular organelles in these knobs; (4) under reverse transformation conditions, we observe an increase in the number of microtubules and an organization into parallel arrays; and (5) one variant that displays fibroblast-like morphology even when grown in the absence of reverse transformation agents was found to have smooth membrane structure and to possess more microtubules per unit cytoplasm.

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ANTAGONISM OF REVERSE TRANSFORMATION IN CHINESE HAMSTER OVARY CELLS

A. W. Hsie, Kohtaro Kawashima, and J. P. O'Neill

Inhibitors of either microtubule aggregation such as colcemid and vinblastine, or of microfilament formation such as cytochalasin B, prevent reverse transformation. Estrogens such as 17β-estradiol and diethylstilbestrol also antagonize the actions of reverse transformation agents. When equal molar concentrations are used, diethylstilbestrol is more effective than 17β-estradiol in preventing reverse transformation. It is noteworthy that recent studies with experimental animals reveal that diethylstilbestrol is potentially carcinogenic.

INDUCTION OF TEMPERATURE-SENSITIVE AND TEMPERATURE-SENSITIVE NUTRITIONAL-DEPENDENT MUTANTS OF CHINESE HAMSTER OVARY CELLS

C. H. Schröder* and A. W. Hsie

Conditional lethal temperature-sensitive mutations have been used in a number of studies on microorganisms and should prove to be valuable tools in studies with mammalian somatic cell genetics, physiology, macromolecular syntheses, and possibly for the elucidation of cellular functions required for viral multiplication or mammalian cell neoplastic transformation.

Employing the conventional "BUdR-light" technique for somatic mammalian cell mutagenesis, we have isolated over 60 temperature-sensitive (Ts) and temperature-sensitive nutritional-dependent (TsNd) mutants of CHO cells after EMS treatment. The Ts mutants will grow at 34°C but not at 40°C in all media. The TsNd mutants grow well at 34°C and 40°C in an enriched medium but fail to grow at 40°C in basal medium unless supplemented by small molecules such as certain amino acids and nucleotide precursors. Characterization of these mutants is in progress.

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SOMATIC BASE SUBSTITUTION MUTAGENESIS IN PRIMATES

G. P. Hirsch and R. A. Popp

The purification scheme previously applied to the preparation of highly purified hemoglobin from sheep has been utilized to obtain alpha and beta chains from marmoset normal and anemic peripheral blood. After one molecular-sieve chromatography and three separations of the hemoglobin forms by carboxymethylcellulose chromatography, the alpha and beta chains contain the majority of isotopic isoleucine (as shown in Fig. 10). Amino acid substitution data are obtained for individual amino acids by sequencing each chain through 20 amino acids. Somatic base substitution mutagenesis can be estimated by subtracting the substitution frequencies for a given amino acid coded by a codon which cannot mutate to isoleucine codons from the substitution frequency for that same amino acid coded by a codon which can mutate to an isoleucine codon. The substitution frequency of isoleucine for threonine at position 4

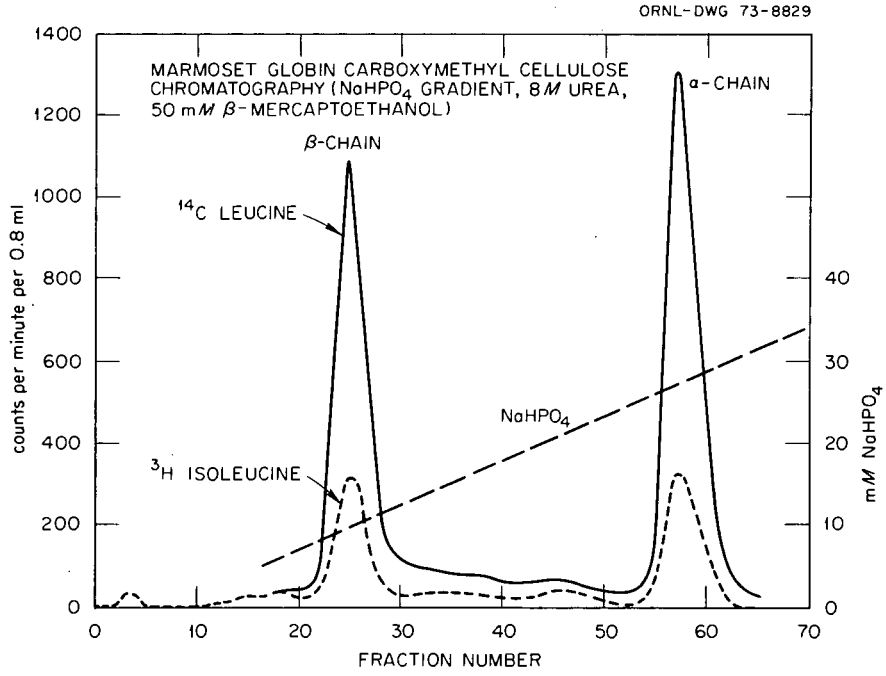

Fig. 10. Separation of alpha and beta chains of marmoset globin.

in the marmoset beta chain is three times the substitution frequency of isoleucine for threonine at position 8 in the alpha chain. Mutagen feeding experiments have been initiated to determine if the substitution frequency at position 4 will be selectively elevated by known mutagens.

Amino acid analyses of the major portions of the alpha and beta chains reveal that some of the tritium is in fact present in amino acids other than isoleucine. Thus, final determination of substitution frequencies will depend upon isotope recovery in isoleucine after sequencing. Based on the specific activity of isoleucine incorporated into nonhemoglobin proteins, the average substitution frequency for a 10-year-old marmoset is fourfold higher than for a 1.5 year old.

AMINO ACID SUBSTITUTION FREQUENCY IN HUMAN HEMOGLOBIN

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

Methods developed for the detection of errors and somatic mutations in the synthesis of sheep and marmoset hemoglobins have been modified to determine the amino acid substitution frequency in human peripheral blood. Human hemoglobin can be recovered in different regions of a single chromatography system, depending upon the oxidation state of the heme ligand. Substitution of isoleucine for other amino acids in the highly purified alpha and beta chains is then measured by chemical analysis. Since human adult hemoglobin does not contain coded isoleucine, its presence must originate by errors in translation, transcription, and/or somatic mutation. The average substitution of isoleucine for other amino acids in the hemoglobin of a 34-year-old male is 1 in 10,000. Similar studies are being done on hemoglobins from younger and older humans and from organic chemists who might have been exposed to larger than normal amounts of potential mutagens.

THE DETERMINATION OF SOMATIC MUTATIONS BY AUTORADIOGRAPHY

G. P. Hirsch and R. A. Popp

Somatic mutations in either the alpha or beta chain genes of red cell precursors should cause these cells to produce an abnormal hemoglobin. To date, methods have not been developed to detect the low frequency of cells containing mutant hemoglobin which might appear in the peripheral blood. However, in species that do not contain coded isoleucine in their hemoglobin, for example, some breeds of sheep, marmosets, and man, mutations which result in the production of isoleucine codons should be detectable by isotopic methods. If

reticulocytes of these species are incubated *in vitro* with tritiated isoleucine of high specific activity, the mutant cells should incorporate large amounts of coded isoleucine into their hemoglobin relative to the low levels of isoleucine incorporated into the hemoglobin of normal reticulocytes by errors in transcription or translation.

Sheep seemed to be a good species in which to investigate the possibility that reticulocytes synthesizing hemoglobin with a coded isoleucine could be identified by autoradiographic methods. When sheep with A-type hemoglobin are made anemic, they synthesize a C-type hemoglobin. C-type hemoglobin has two coded isoleucine residues per molecule of hemoglobin, whereas A-type hemoglobin has no coded isoleucine. Sheep with B-type hemoglobin are unable to synthesize C-type hemoglobin even when they are made severely anemic. Without prior knowledge of the hemoglobin types of the sheep, autoradiographs were prepared of four samples of *in vitro*-labeled sheep reticulocytes; two of the four samples show about 0.3% labeled red cells while the other two show less than 1 in 10,000 labeled cells. If the labeled cells are in fact making C-type hemoglobin, which will be verified by chemical analysis, then it will be very easy to detect mutant cells by autoradiographic methods in systems in which somatic mutations would be the only origin of isoleucine-labeled red cells.

EFFECT OF AGE ON ANTIGEN-SENSITIVE CELLS

Diana M. Popp and R. A. Popp

The response of antigen-sensitive cells to alloantigens can be visualized by histologic examination of splenic tissue following the transfer of lymph node lymphocytes into lethally irradiated allogeneic mice. Donor lymphocytes respond to antigens of the hosts by transforming into large pyroninophilic cells (LPC) followed by an increase in the mitotic index. If congenic crossover lines of mice are used, subpopulations of lymphocytes can be distinguished since antigens determined by the D-end of the H-2 region induce transformation and proliferation later than the antigens of the K-end of the H-2 region. These populations are believed to be responsible for cellular and humoral immunity, respectively. Studies indicate that the immune capacity of old animals is deficient. If the deficiency is at the level of the antigen-sensitive cell, the use of old mice as lymphocyte donors should result in a deviation from what is seen with young donors. In addition, the congenic crossover lines of mice might yield information as to which population of lymphocytes is affected in old mice. When lymphocytes of 24- to 30-month-old mice were injected into lethally irradiated recipients differing at H-2, H-2K, and H-2D, there was no difference in the number of LPC or the MI observed from that seen when 3- to 4-month-old donors are used. Thus, the deficiency in the immunological capacity of the old mouse is not at the level of the antigen-sensitive cell.

DIFFERENTIAL EFFECT OF CORTISONE ACETATE ON THE RESPONSE OF LYMPHOCYTES TO H-2D AND H-2K ANTIGENS

Diana M. Popp

Lymphocyte transformation induced by H-2K antigens occurs earlier than transformation induced by H-2D antigens. The time and site of transformation suggest a correlation with T and B cell populations. Further correlation was obtained by using lymph node lymphocytes from donors previously injected with cortisone acetate. The lymphocytes were washed and filtered; 10×10^6 cells each were injected into lethally irradiated recipients which differed from the donors at H-2, H-2K, or H-2D. Spleens were removed daily and used for histologic evaluation and enumeration of large pyroninophilic cells and mitotic figures. The LPC or mitotic index of cortisone acetate resistant lymph node lymphocytes responding to H-2 antigens shows no significant difference from normal lymph node lymphocytes. Likewise, the cellular activity of cortisone acetate resistant lymph node lymphocytes responding to H-2K antigens is indistinguishable from that of normal lymphocytes. However, the response of lymph node lymphocytes to H-2D antigens is abolished if the donors are treated with cortisone acetate 48 hr before the donor cells are removed. Studies in the literature suggest that the cortisone-sensitive cell is the T helper cell and that cortisone-resistant cell types are T killer and B cells. These observations confirm the interpretation that the early peak responding to H-2K antigenic differences represents activation of cells responsible for cell-mediated immunity, which are thymus-dependent cells. The late response to H-2D antigens represents activation of cells responsible for humoral immunity, which are T helper or T helper plus B cells.

CHARACTERISTICS OF MOUSE ANTIBODIES SPECIFIC FOR TWO H-2 ISOANTIGENS

Mary W. Francis and R. A. Popp

Antibodies to mouse H-2 isoantigens have never been purified sufficiently to determine in which class of

immunoglobulins they belong. A combination of specific absorptions of isoantibody and column chromatography is being employed to isolate two specific isoantibodies (anti-H-2.4 and anti-H-2.11) from mouse serum which will be used in subsequent studies to determine whether they belong to more than one immunoglobulin class. The structure of the antigen-antibody combining sites in the light chains of each of these antibodies will be determined to learn what amino acid sequences are associated with the specificities of these antibodies to their respective isoantigens.

Quantitatively, the antibody to specific H-2 isoantigens is a small amount of the total immunoglobulin even in a hyperimmunized mouse. The isoantibody can be selectively separated from other immunoglobulins by absorption onto washed red cell stroma containing the specific isoantigens. The antibody can be readily recovered following treatment of the red cell stroma with pH 2.5 glycine-sulfate buffer. The antibodies specific for each isoantigen have multiple chromatographic properties of DEAE–Sephadex A-50, but its significance regarding the physical and chemical differences of these antibodies remains to be studied.

PARTIAL HYBRIDS BETWEEN *DROSOPHILA MELANOGASTER* AND *DROSOPHILA SIMULANS*

E. H. Grell

The species most closely related to *D. melanogaster* is *D. simulans.* The two species cross to yield F_1 hybrid flies, but the F_1's are sterile and therefore an ordinary genetic analysis of differences between the two species is not possible. Muller and Pontecorvo[1] showed that by mating triploid *melanogaster* females to x-irradiated *simulans* males some diploid offspring could be obtained which contained two *melanogaster* members of one of the autosomes and no *simulans* chromosomes of that type. The remainder of the chromosome complement was hybrid; that is, one *melanogaster* and one *simulans* member of each pair. The difficulty of this method is that the yield of these partial hybrids is very low, one or two partial hybrids per culture bottle, and each attempt to produce them involves the irradiation procedure.

A method was devised which gives yields of up to 100 partial hybrids per culture bottle, eliminates the irradiation, and gives resolution down to the chromosome arm rather than the whole chromosome. The method involves using *melanogaster* females with one autosome pair represented by a compound chromosome with two identical chromosome arms attached to one centromere and the other arm present as two free arms. These females are crossed to *simulans* carrying a Y-autosome translocation with the site of rearrangement near the centromere. Euploid zygotes are those with the compound chromosome and a free arm chromosome from the *melanogaster* parent and the part of the translocations homologous to the free arm from the *simulans* parent.

The four possible partial hybrids with one homozygous major *melanogaster* arm in an otherwise hybrid background have been synthesized. These partial hybrids are fairly normal in external appearance. The absence of some bristles is common, and abnormal tergites are often observed. Internal organs appear to be normal except for gonads. In every partial hybrid as well as in the regular hybrids, ovaries and testes are greatly underdeveloped.

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1. H. I. Muller and G. Pontecorvo, *Nature (London)* **146**, 199–200 (1940).

GENETICS OF ASPARTYL AMINOTRANSFERASES IN *DROSOPHILA MELANOGASTER*

E. H. Grell

Drosophila, like most animals, have two distinct aspartyl aminotransferase enzymes. The form which migrates more rapidly to the anode in pH 8.6 gel electrophoresis is designated GOT1, and the slower, GOT2. GOT1 is the soluble enzyme, and GOT2 is the mitochondrial enzyme. Gel electrophoresis of samples of flies from approximately 150 laboratory stocks and wild population of *D. melanogaster* did not uncover a single natural variant of either enzyme; however, both GOT1 and GOT2 of *D. simulans* are electrophoretically distinguishable from corresponding enzymes of *D. melanogaster.* F_1 hybrids of *melanogaster* and *simulans* have enzyme triplets consisting of the parental enzymes plus one hybrid enzyme of both GOT1 and GOT2. Since these hybrids are completely sterile, no ordinary analysis of the genetic differences between the two species can be done.

Partial hybrids having one *melanogaster* chromosome arm homozygous in an otherwise *melanogaster-simulans* hybrid background were synthesized. Partial hybrids with each of the four major autosomal arms in turn homozygous for *melanogaster* genes were tested by electrophoresis. If only the *melanogaster* form was present, it was concluded that the gene responsible for the difference between the species is located on the chromosome arm which is homozygous *melanogaster.* Using this method, GOT1 was assigned to 2R, and GOT2 was assigned to 2L.

After the chromosome arm was known, male flies with compound chromosomes of the appropriate arms were treated with EMS, and F_2 progeny were examined by electrophoresis. Each fly represents two mutagen-treated genes per locus. Mutations of both enzyme loci were selected. GOT1 is located at a map position between 70 and 80 on the second chromosome. GOT2 is located at a genetic map position of 3.0 on the second chromosome, and cytogenetic analysis places it in section 22B of the salivary chromosome map.

BETA-HYDROXY ACID DEHYDROGENASE: GENETICS AND THE EFFECTS OF A MUTATION

E. H. Grell

Beta-hydroxy acid dehydrogenase catalyzes the interconversion between large numbers of aldonic acids and their 3-keto derivatives. β-L-hydroxy butyrate is also a substrate. In this work gluconic acid was used as a substrate for the enzyme. Since there are so many possible substrates, the most common natural substrate is not known. The enzyme is widely distributed in *Drosophila* tissues. It is strongly present in the alimentary canal and fat bodies but is present to a lesser extent in muscle and Malpighian tubules.

No genetic variants of the enzyme were discovered in *Drosophila melanogaster,* but an electrophoretic difference exists between the enzymes of *D. melanogaster* and *D. simulans.* F_1 female hybrids have the parental enzymes and a hybrid form with an electrophoretic migration between the parental forms.

Male hybrids between *D. simulans* and *D. melanogaster,* which contain a *simulans* X chromosome and a *melanogaster* Y chromosome, have only the *simulans* form of the enzyme. This indicates that the locus which specifies the electrophoretic behavior of the enzyme is located on the X chromosome. A series of chromosomes which duplicate regions of the *melanogaster* X chromosome were synthesized. Hybrids with a duplication of the region between 13B8-9 and 16A1 have more of the *melanogaster* form of the enzyme than other hybrids. The locus for the enzyme is located in that salivary chromosome map interval.

A mutant was selected which has no detectable activity of this enzyme. It was induced by EMS treatment and detected by a spot plate test in which a colored product was coupled with the oxidation of gluconic acid. Flies without the enzyme appear morphologically normal, but males are only slightly fertile.

The testes of these sterile males have normal morphology, but no sperm motility has been observed. Normal-appearing sperm are produced, but they remain in sperm bundles instead of moving apart. The low level of fertility indicates that a few sperm manage to function in spite of the failure to observe motility.

The need for beta-hydroxy acid dehydrogenase in sperm motility is not understood. The *Drosophila* testes have low levels of this enzyme, but the accessory organs which secrete seminal fluid are very rich in this enzyme. Perhaps the enzyme normally is involved in the synthesis of a metabolite necessary for sperm motility or in preventing a substance which inhibits sperm motility.

DNA REPLICATION AND RECOMBINATION IN THE *DROSOPHILA* OOCYTE

Rhoda F. Grell

Enhancement of crossing-over by heat has been applied to a variety of eucaryotes to mark the time of meiotic exchange. Comparison of this time with that of DNA replication should reveal if the two events are coincident. In *Drosophila,* genetic and autoradiographic studies have delimited each event.[1] Both begin in early premeiotic interphase and are coincident over much, if not all, of their length.

The temperature studies have shown that response is not uniform throughout the genome. Instead it varies in time and magnitude in different regions. Thus far, 21 regions, averaging ten crossover units in length and comprising approximately 75% of the genome, have been examined for their initiation point, their termination point, and their degree of response. Increases are limited to an ~36-hr period beginning close to oocyte formation. The characteristic responses of different regions have generated a thermal recombination map of the *Drosophila* genome. The temporal specificity that is observed is a constant and reproducible property of a region and may reflect the sequences of crossing-over in the genome.

The results from genetic and autoradiographic studies to date are summarized in Fig. 11. The two curves, one including single and the other multiple crossovers for the proximal region of chromosome 3, represent crossover response of the 75% of the genome examined to a 12-hr heat treatment of 35°C initiated at the times indicated on the abscissa. Maximal increase occurs between 138 and 150 hr and coincides with the middle of the S period.

Temporal localization of the two periods under study has led to the conclusion[1] that synaptinemal complex formation must be concurrent with DNA replication and the temperature responsive interval. This conclusion has recently been confirmed by an electron

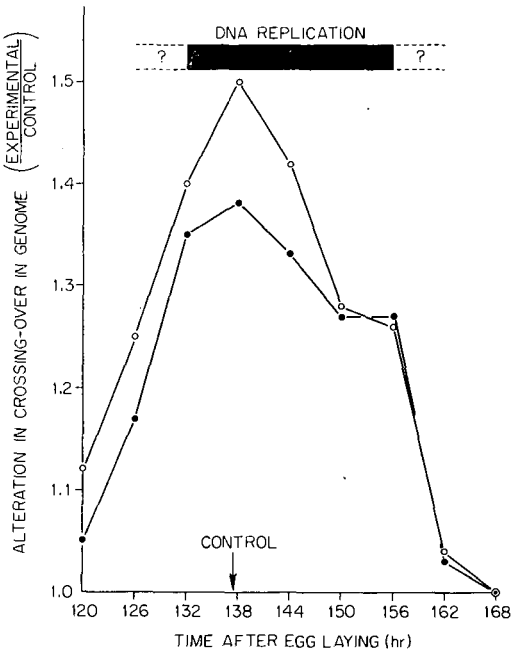

Fig. 11. Crossing-over response of 72% of the genome to 12-hr heat treatments at 35°C initiated at times shown on abscissa. ○, measured to include multiple crossovers in proximal region of chromosome *3* between *st* (44) and *Sb* (58); ●, measured to include only single crossovers between *st* and *Sb*.

microscopic determination of the time of complex formation.[2]

1. R. F. Grell, *Genetics* 73, 87 (1973).
2. J. W. Day, "Synaptinemal Complexes in *Drosophila* Oocytes," this report.

HEAT AND THE INTERCHROMOSOMAL EFFECT ON RECOMBINATION

Rhoda F. Grell

The "interchromosomal effect" is a well-documented but poorly understood recombination phenomenon. It refers to the ability of structural alterations in one part of the genome to enhance crossing-over elsewhere in the genome. Within a chromosome, the pattern of response is nonuniform; the greatest increases occur at the tips and centromeric regions. An apparent exception has been the tips of chromosome 3, where, according to Schultz and Redfield,[1] increases are not observed.

One goal of our temperature studies has been to determine if the pattern of recombination increase bears any resemblance to the pattern induced by the interchromosomal effect. If so, a common mechanism could be involved. Studies of the X chromosome have now shown that heat increases crossing-over distally and proximally and has a minimal effect in the medial region. The pattern is identical to that observed for the interchromosomal effect (Fig. 12*a*). Similarly, recombi-

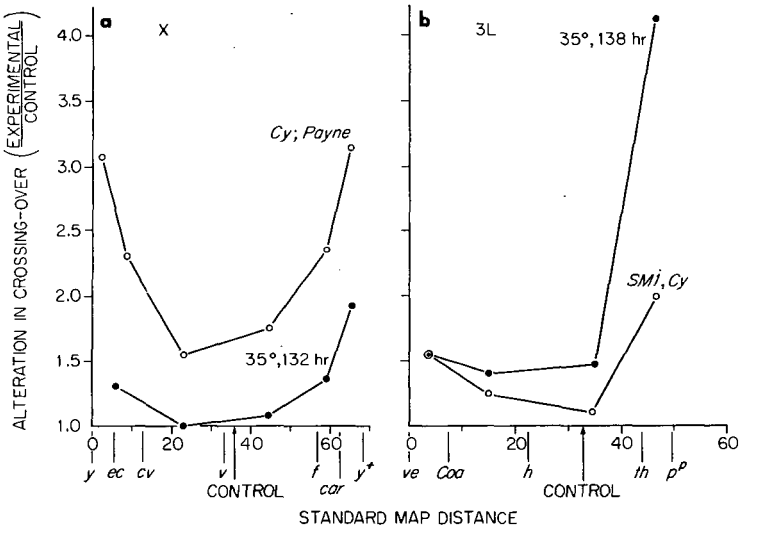

Fig. 12. Comparison of interchromosomal effects and heat effects on crossing-over in (*a*) *X* chromosome and (*b*) left arm of chromosome *3*. (*a*) ○, interchromosomal effect of Cy and Payne inversions; ●, effect of 12-hr heat treatment of 35°C initiated at 132 hr. (*b*) ○, interchromosomal effect of SM1, Cy inversions; ●, effect of 12-hr heat treatment of 35°C initiated at 138 hr.

nation in the proximal region of chromosome 2 (the only portion studied thus far) is markedly enhanced by heat, again resembling the interchromosomal effect. In the case of chromosome 3, however, a departure from the pattern reported by Schultz and Redfield is encountered since heat enhances distal as well as proximal crossing-over.

The possibility that previous failures to observe interchromosomal effects at the tips of chromosome 3 stemmed from the absence of, or the failure to use, properly placed markers for monitoring these regions has led to a reinvestigation of them. A new dominant marker, *Coa*, located at approximately 7 crossover units from the tip of 3L, is well suited for this purpose. The response of the region between the 3L tip (marked with *ru*) and *Coa* to the presence of a multiply inverted second chromosome, SM1, has been measured. Contrary to the earlier report, an approximately 50% increase in crossing-over is observed. A comparison of the interchromosomal effect and that of heat on the left arm of chromosome 3 (Fig. 12*b*) again demonstrates a striking similarity. A reexamination of the interchromosomal effect on the tip of 3R is now in progress.

The results obtained thus far indicate that the thermal recombination map and the interchromosomal recombination map are alike. Each effect extends the genetic map of the chromosome both distally and proximally so as to reduce the tendency of markers to cluster in these regions. As a result, the genetic map comes to resemble the cytological map more closely.

1. J. Schultz and H. Redfield, *Cold Spring Harbor Symp. Quant. Biol.* **16**, 175 (1951).

REC$^-$ MUTANTS IN *DROSOPHILA*

Rhoda F. Grell and Estela E. Generoso

Recombinationless (*rec*$^-$) mutants have been useful tools for recombination studies in microbial systems. Of particular value are temperature-sensitive (*ts*) *rec*$^-$ mutants which permit recombination at control temperatures but almost eliminate it at higher temperatures. In *Drosophila*, c(3)G is the only known mutant to virtually eliminate meiotic exchange throughout the genome. Neither of the two known alleles is temperature sensitive. The availability of a *ts rec*$^-$ mutant in *Drosophila* coupled with the extensive information concerning asynchronous thermal responses of different chromosomal regions would offer the possibility of selectively controlling recombination in different parts of the genome. At the same time it would provide an independent criterion of the time of meiotic exchange.

A program to recover and characterize EMS-induced *rec*$^-$ mutants at or in the vicinity of c(3)G as defined by the limits of the deficiency (3R)sbd^{105} was begun several years ago. The methodology involves a two-generation test of EMS-treated third chromosomes. The first series of experiments examined approximately 1000 treated chromosomes and produced a new allele of c(3)G. This allele lacked temperature sensitivity and was similar in its genetic properties to the original mutant. In a second series of experiments, 3475 treated third chromosomes were examined. One *rec*$^-$ mutant was recovered which shows complementation with c(3)G and on this basis is considered a separate locus. This mutant reduces meiotic exchange to about 8% of normal at 25°C and greatly increases the number of triploids and intersexes. It has the desirable feature of temperature sensitivity in that an additional 50% decrease in exchange accompanies a 5°C rise in temperature. Electron microscopic examinations indicate that unlike c(3)G, which eliminates the synaptinemal complex, oocytes hemizygous for this mutant possess the complex. Genetic studies are under way to precisely locate the mutant with respect to c(3)G and to determine its effect on forward and reverse mutation. The program to isolate additional *ts rec*$^-$ mutants is continuing.

SYNAPTINEMAL COMPLEXES IN *DROSOPHILA* OOCYTES

J. W. Day

Ovaries from immature *Drosophila* females ranging in age from 132 to 156 hr post egg laying were studied in order to determine the time of appearance of synaptinemal complexes in developing oocytes. Ovaries were fixed in glutaraldehyde–osmium tetroxide, embedded in Epon, and sectioned with an LKB ultratome III. Sections were stained with uranyl acetate–lead citrate and examined in a Hitachi HU-11C electron microscope.

Each ovary was sectioned in such a way as to provide 10 to 20 600–700 Å thin sections at each of several different levels through the ovary, each level separated by at least 6 μm. The sections were scanned at low magnifications in order to locate presumptive oocytes and at higher magnifications to determine the presence or absence of synaptinemal complexes in their nuclei. The data obtained are summarized in Table 9.

The frequencies listed in the last column are a function both of the probability of sectioning through an oocyte nucleus at a given level of the ovary and the proportion of oocytes of a particular age in which

Table 9. Occurrence of *Drosophila* oocytes with synaptinemal complexes

Age of ovary (hr)	Number of ovaries examined	Total levels examined	Number of nuclei with synaptinemal complexes	Frequency
132	4	27	0	0
138	8	55	13	0.24
144	4	21	13	0.62
150	3	22	21	0.95
156	3	22	22	1.00

synaptinemal complexes have formed. The former probability is assumed to be constant for all age groups studied. The frequencies, therefore, reflect the increase with age in the proportion of oocytes which have formed synaptinemal complexes.

The data indicate that synaptinemal complexes form as early as 138 hr of development and that all oocytes may contain complexes by 150 hr. This time period coincides with that for the premeiotic DNA synthetic period determined previously.[1]

1. R. F. Grell, *Genetics* 73, 87–108 (1973).

CYTOLOGY OF *DROSOPHILA* EGGS

Estela E. Generoso and Rhoda F. Grell

Distributive pairing in the *Drosophila* oocyte is a size-dependent, postexchange process that occurs between univalent homologs or between univalent nonhomologs and determines their segregation behavior. Recognition and analysis of this pairing have come entirely from genetic studies since the chromosomes of the oocyte are fairly refractory to cytological study. Recently, high frequencies of nonhomologous metaphase pairing have been observed cytologically in the oogonia and brain cells of *Drosophila*.[1,2] Like distributive pairing in the oocyte, the frequencies of nonhomologous pairing at mitotic metaphase are positively correlated with the similarity in length of the participating chromosomes. These findings have prompted renewed efforts to penetrate the cytology of the oocyte in order to obtain a cytological demonstration of distributive pairing.

Females carrying two nonhomologs of approximately equal length, one the X duplication 1144 and the other a single free fourth chromosome, are being employed. Previous genetic studies have shown that meiotic pairing between the two small chromosomes is highly regular, occurring with a frequency of 0.998. A method has been designed to obtain large numbers of eggs of this genotype arrested at metaphase of the first meiotic division. The method employs XO sterile males which stimulate egg deposition but which, lacking motile sperm, fail to trigger resumption of meiosis beyond the metaphase stage. Use of hypotonic solution to spread the extremely minute chromosomes coupled with proper staining procedures will, hopefully, permit visualization of distributively paired chromosomes.

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2. C. M. Moore, *Genetica* 42, 445 (1971).

PURIFICATION OF TWO MULTIENZYME COMPLEXES IN THE AROMATIC/TRYPTOPHAN PATHWAY OF *NEUROSPORA CRASSA*

F. H. Gaertner

A procedure originally designed to isolate the anthranilate synthetase complex of *Neurospora crassa* also provided the aromatic complex in homogeneous form. The ionic and physical properties of the two complexes were shown to be similar. Through the use of parallel sedimentation-equilibrium analyses in H_2O and $D_2{}^{18}O$, the aromatic complex was shown to have a partial specific volume of ~0.72 and a molecular weight of ~290,000. Disk-gel electrophoresis in sodium dodecyl sulfate appeared to show that the complex dissociates in the detergent into four unequal components with molecular weights totaling ~290,000. Apparent differences in physical properties were found between the aromatic complex isolated here and the aromatic complex isolated from *N. crassa* by other investigators.

DISTINCT KYNURENINASE AND HYDROXYKYNURENINASE ACTIVITIES IN MICROORGANISMS: OCCURRENCE AND PROPERTIES OF A SINGLE PHYSIOLOGICALLY DISCRETE ENZYME IN YEAST

A. S. Shetty* and F. H. Gaertner

Saccharomyces cerevisiae grown in the presence of 1.0 m*M* L-tryptophan slowly excreted fluorescent

material that was chromatographically identifiable as 3-hydroxyanthranilate but did not excrete detectable amounts of anthranilate nor rapidly deplete the medium of L-tryptophan. Under similar growth conditions, *Neurospora crassa* rapidly excretes anthranilate and rapidly depletes the medium of L-tryptophan.

Chromatographic analysis of crude extracts from yeast revealed a single kynureninase-type enzyme whose synthesis was not measurably affected by the presence of tryptophan in the medium. Previous studies have provided evidence for two kynureninase-type enzymes in *N. crassa,* an inducible kynureninase and a constitutive hydroxykynureninase.

Kinetic analysis of the partially purified yeast enzyme provided Michaelis constants for L-3-hydroxykynurenine and L-kynurenine of 6.7×10^{-6} and 5.4×10^{-4} *M,* respectively. This and other kinetic properties of the yeast enzyme are comparable to those reported for the constitutive enzyme from *N. crassa.*

These findings suggest that *S. cerevisiae* has in common with *N. crassa* the biosynthetic enzyme hydroxykynureninase but lacks the catabolic enzyme kynureninase. Therefore, it can be predicted that, unlike *N. crassa, S. cerevisiae* does not carry out the tryptophan-anthranilate cycle. Distinct kynureninase-type enzymes may exist in other microorganisms and in mammals.

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ISOLATION AND CHARACTERIZATION OF A HYDROXYKYNURENINASE FROM HOMOGENATES OF ADULT MOUSE LIVER

Christine E. McDermott,* D. A. Casciano, and F. H. Gaertner

A kynureninase-type enzyme was isolated from adult mouse liver. With kynurenine as the substrate, this enzyme has a K_m of 300 μM; when the substrate is hydroxykynurenine, the K_m is 6 μM. We conclude that this enzyme is a hydroxykynureninase. No enzyme which we could characterize as a kynureninase was found in this preparation. This suggests that tryptophan metabolism in the mouse occurs primarily through pathways that use hydroxykynurenine rather than kynurenine. Preliminary studies indicate that the enzyme is inhibited by its reaction product, hydroxyanthranilate, which is an intermediate in the synthesis of nicotinamide adenine dinucleotide (NAD). Such control of the hydroxykynureninase reaction may be of physiological importance in regulating the synthesis of NAD and/or in preventing the accumulation of hydroxyanthranilate, a putative carcinogen.

*Supported by an NDEA Title IV Fellowship.

A SPECIFIC AND SENSITIVE FLUOROMETRIC ASSAY FOR TRYPTOPHAN OXYGENASE

D. A. Casciano and F. H. Gaertner

A spectrophotofluorometric assay was used to measure tryptophan oxygenase activity in several species. The fluorescent assay depends on the conversion of the product of the reaction, N-formyl-L-kynurenine, to anthranilate by means of the coupling enzymes kynurenine formamidase and kynureninase. These enzymes are easily obtained from L-tryptophan-induced *N. crassa*; and the product, anthranilate, is readily separated by organic extraction from other tryptophan catabolites and easily identified fluorometrically. With this assay, tryptophan oxygenase has been demonstrated *in vitro* for the first time in *N. crassa.*

NEUROSPORA CRASSA CHORISMATE SYNTHASE: A FLAVOPROTEIN

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

Chorismate synthase catalyzes the formation of an important branch-point intermediate in the biosynthesis of the aromatic amino acids and certain coenzymes from by-products of glycolysis. Gaertner and Cole[1,2] reported the isolation and some properties of the enzyme in *Neurospora crassa.* In their investigations with partially purified chorismate synthase, chromatography via diethylaminoethyl- (DEAE)-cellulose yielded multiple enzymic forms all of which exhibited the following characteristics: (1) a requisite need for NADPH as cofactor; (2) over tenfold stimulation of activity by a commercial diaphorase preparation; and (3) activation by the substrate, 3-enolpyruvylshikimate-5-phosphate (ES-5-P), in concert with diaphorase.

The investigations of the previous authors[1,2] were extended with the purpose of ascertaining a possible catalytic and/or regulatory role for a flavin moiety as a cofactor in chorismate synthase function. Spectrophotofluorometric analysis indicated the presence of endogenous flavin in partially purified fractions. Upon activation of the enzyme by ES-5-P, the characteristic flavin fluorescence was quenched by 50–60%. Furthermore, in assay supplementation studies, flavin mononucleotide (FMN) specifically was shown to stimulate activity, providing a tenfold stimulatory effect in the absence of exogenous diaphorase.

In an effort to more clearly define the effect of flavin on enzyme activity, *N. crassa* chorismate synthase was purified to >90% homogeneity (based upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis). Studies with purified enzyme have revealed the following: (1) the purified enzyme preparation, completely devoid of endogenous flavin, was totally inactive in the absence of NADPH and exogenous flavin (or diaphorase); (2) FMN elicited optimal enzymic activity; (3) dithionite could substitute for the NADPH-FMN requirement; (4) the enzyme was shown to be cooperatively activated by ES-5-P and FMN; and (5) in the presence of ES-5-P, FMN, and NADPH, the enzyme preparation exhibited diaphorase activity with 2,6-dichlorophenolindophenol (DCPIP) as electron acceptor.

Consonant with findings in *Escherichia coli,*[3] work reported herein confirms the flavoprotein character of chorismate synthase. Ostensibly, no net electron transfer is a result of the overall reaction process. Consequently, with regard to the coupling of biosynthesis and cellular reducing power, a requirement for flavin in conjunction with reduced pyridine nucleotide poses interesting regulatory feasibilities. Further studies will be aimed at the elucidation of the precise catalytic and/or regulatory function of the flavin cofactor in the enzymic reaction mechanism.

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

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N-ACETYLGLUCOSAMINIDASE AND DEDIFFERENTIATION

V. P. Idoyaga-Vargas and Tuneo Yamada

Lentectomy is followed by dedifferentiation of the epithelial cells from the dorsal iris in the adult newt *Triturus viridiscens.* Alterations in the surface properties of these cells are one of the conspicuous changes occurring during dedifferentiation. Zalik and Scott[1] demonstrated that marked shifts in electrophoretic mobility (E.P.M.) can be detected in the cells of the dorsal iris. Further, they had shown that exogenous glycosidases could affect the E.P.M. of these cells.[2] Thus alterations in cell surface carbohydrates could be involved in the changes of E.P.M. occurring during dedifferentiation. One of the possible mechanisms whereby this carbohydrate change may come about is by the action of endogenous glycosidases. This possibility was approached in the present investigation by determining the level of activity of glucosaminidase, a lysosomal glycosidase, during dedifferentiation.

The first step of the investigation was to determine whether or not any alteration in enzyme activity occurs in the dorsal and ventral iris during dedifferentiation. Although the ventral iris does not participate in the formation of a new lens, it undergoes induced proliferation and partial depigmentation. Enzyme assays were performed according to a modification of the Sellinger and Hiatt technique.[3] Conditions of linearity were established and used for the enzyme assay. The ultramicrotechnique of Keck[4] was used for DNA determinations.

There was a progressive increase in the total activity during day 4 and day 17, followed by a decline toward control levels. This pattern was confirmed when the activity was determined in relation to the DNA content of the irises. Thus, profound alterations in glucosaminidase activity are associated with the dedifferentiation of iris cells. During the period of highest intracellular activity, the values obtained are 4.4 times as high as that of the normal dorsal iris. In the ventral iris the increase in activity over the same period is less.

The next step of the investigation was designed to test whether the increase in the enzyme activity was associated with the iris epithelium or with the iris stroma. Recent evidence[5] demonstrated that the iris epithelial cells are transformed into lens cells while the iris stroma cells are not involved in lens formation. The epithelium and stroma were separated by microdissection. It was found that on day 0 and day 4 the activity was equally distributed between stroma and epithelium. However, a dramatic increase occurred within 17 days in the epithelium. This increment in activity was statistically highly significant, while the stroma increase was not.

If the enzyme were to act at the cell surface on the carbohydrate moieties, it could be possible to detect its activity outside the cells. To test the possibility that glucosaminidase is present in the extracellular milieu, the following experiments were performed. Dorsal irises were dissected, washed, and then stored in PBS for 30 min. They were then spun down at a relatively low speed, and the supernatant was tested for enzyme activity. A consistent increase of glucosaminidase activity in the medium was observed throughout dedifferentiation. This finding supports the idea that glucosaminidase was present outside the cells. If indeed this was the case, glucosaminidase activity will be detectable in the natural medium bathing the cells (i.e., the

aqueous and vitreous humors). Thus aliquots of optic chamber fluid were obtained with specially modified Pasteur pipettes from newts undergoing regeneration as well as from unoperated animals. A fixed amount of fluid (0.1 ml from about 50 animals) was assayed for glucosaminidase activity. It was found that the enzyme activity increases progressively after lentectomy, remains high during the dedifferentiation phase, and declines toward control levels after the completion of dedifferentiation. During the peak of activity, values as high as 4 times the control were obtained.

These results are consistent with the concept that glucosaminidase is actively involved in the control mechanisms of dedifferentiation by altering cell surface carbohydrates, presumably by the hydrolysis of carbohydrate moieties liberating acetylglucosamine, as shown by Mahadevan[6] with the subsequent acquisition of new adhesive properties, or by releasing cytoplasmic components of the dedifferentiating cells. Much, however, remains to be demonstrated to see whether or not this is the case. Attempts will be made to localize the site of enzyme activity at the ultrastructural level using the cytochemical technique of Pugh.[7] This approach should yield information regarding the cellular type in which the enzyme is localized as well as whether any activity could be associated with the surface of iris epithelial cells.

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THE CELL CYCLE OF DEDIFFERENTIATING IRIS EPITHELIAL CELLS

Tuneo Yamada

During Wolffian lens regeneration in situ, the iris epithelial cells undergo dedifferentiation during days 6 to 13. It is known that during this period those cells are in proliferation triggered by lentectomy. For further analysis of dedifferentiation it is important to know how the cells are proceeding in the cell cycle and to correlate the dedifferentiative events with cell cycle parameters. Our earlier data on cell cycle parameters of this phase are not accurate enough, because they are indirect data based on a number of assumptions.[1] In the present work, attempts are being made to obtain direct data on cell cycle parameters of this phase. Methyl-^{3}H-thymidine was injected into the peritoneum of adult newts at day 4 and day 6 after lentectomy. At various time intervals after injection, the heads were fixed; the eyes were sectioned at 5 nm and bleached; autoradiographs were prepared. The total number of labeled and unlabeled mitoses in the dorsal and ventral iris epithelia were counted in each series, and the curves of absolute numbers of labeled and unlabeled mitoses, as well as that of percent labeled mitoses, were prepared. Considering the situation that day 4 is the starting time of the first S period initiated by lentectomy and that at day 6 a fraction of iris epithelial cells have already completed their first induced S period, the curves were evaluated for cell cycle parameters. In the day 4 series the preliminary value of the duration of $S + G_2 + \frac{1}{2}M$ was found to be 38 hr. The preliminary values from the day 6 series are: $G_2 + \frac{1}{2}M = 8$ hr, $S = 28$ hr, $G_1 = 64$ hr. It is planned to have cooperation from the Statistics Department on this subject, and the data will be subjected to statistical examinations.

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INDUCTION OF THE STELLATE CONFIGURATION BY ADENOSINE AND THE CELL CYCLE

J. R. Ortiz and Tuneo Yamada

Induction of the stellate configuration by compounds related to cyclic AMP in the cultured iris epithelial cells reported elsewhere[1] occurs always in a fraction of the cell population tested. The question was raised whether the cells are reactive to those compounds only in a specific cell cycle period. To approach this problem, adenosine treatment was combined with autoradiography of chased cell labeling. In the first series a culture of iris epithelial cells was treated with 1 μCi methyl-^{3}H-thymidine and 1 m*M* adenosine for 80 min. In the following series the culture was treated with 1 μCi methyl-^{3}H-thymidine for 80 min, washed, and fixed after culturing for 4 hr (series 2), 8 hr (series 3), and 10 hr (series 4). Before fixation, each culture was treated for 80 min with 1 m*M* adenosine. From the autoradiographs the percentages of total labeled cells, labeled and stellate cells, and the total stellate cells were obtained and plotted.

According to S. E. Zalik's preliminary data, the cultured iris epithelial cells have $G_1 = 26$ hr, $S = 36$ hr, $G_2 + \frac{1}{2}M = 7$ hr. Assuming the data are applicable to our case, the following predictions can be made: If the cells are reactive in S period alone, in series 1 all labeled

cells should be stellate, and no stellate cells should be nonlabeled. If cells are reactive in G_2 period alone, no labeled cells should be stellate in series 1, but from series 2 onward there should occur a rise in the percentage of labeled stellate cells. If the cells are reactive in G_2 period alone, no labeled cells should be stellate in series 1, but from series 3 to 4 there should occur an increase in the percentage of labeled stellate cells. The results obtained did not support any of those possibilities and could not be interpreted by a combination of those three possibilities. Further, the direct observation of the stellate cells shows that the mitotic period is not reactive. Thus the data demonstrate that the cell cycle is not involved in the control of the reactivity of cells toward adenosine.

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GALACTOSYLTRANSFERASE AND DEDIFFERENTIATION

V. P. Idoyaga-Vargas, Tuneo Yamada, and Christian Michel

Galactosyltransferase has been shown to be involved with the control of specific adhesion of embryonic retina.[1] Further evidence was presented in support of the cell surface localization of the enzyme. It is possible therefore that galactosyltransferase could be regulating the changes in cell surface carbohydrates that seem to occur during dedifferentiation of iris cells. To investigate this possibility the following approach was taken.

Dorsal irises were dissected out at 4, 8, 17, and 21 days after lentectomy. The control group was composed of dorsal irises from unoperated animals. The material was sonicated, and the endogenous activity of galactosyltransferase was determined according to a modification of the technique of Roth and White.[1] The incubation mixture contained Hepes buffer at pH 7.2, containing 20 m*M* of $MnCl_2$, 10 m*M* NaN_3, 0.1% Triton X-100 and 19.7 μM of uridine diphosphate galactose ([^{14}C] galactose). The method of choice for DNA determinations is the one of Keck. Preliminary results indicated that a peak appears at day 4 with a value 3.3 times higher than 0 day. This was followed by a decline in the activity from 1.6 times the control level at 8 days to 1.3 times at 21 days. It is noteworthy that the peak of activity correlates quite well in time of appearance with the initial changes in cell electrophoretic mobilities previously reported.[2] Thus it appears that galactosyltransferase may be involved in changes of cell surface carbohydrates occurring very early during dedifferentiation.

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THE EFFECT OF X IRRADIATION ON WOLFFIAN LENS REGENERATION

Christian Michel and Tuneo Yamada

Recent experiments have demonstrated that lens regeneration was inhibited in adult *Triturus viridescens* after local irradiation of the eye with 1500 R of x radiation.[1] Our results after total body irradiation with the same and smaller radiation doses (1000 R of 250-kV x rays) at different days after lentectomy confirmed this report. In these preliminary experiments we were especially interested in the response of macrophages to radiation, because macrophages are involved in depigmentation of iris epithelial cells.[2] It appeared probable that the inhibition of lens regeneration is due to inactivation of macrophages by irradiation. The result obtained did not confirm the speculation. It was not possible to inhibit infiltration of the iris and phagocytic uptake of melanosomes by macrophages even after irradiation with 4000 R. Thus it now appears that the effect of radiation on macrophage activity is not responsible for inhibition of lens regeneration. As a result, the cellular events occurring in iris epithelial cells have become the candidate for the main target of irradiation. Autoradiographic studies of labeling of iris epithelial cells with [^{3}H] thymidine injected intraperitoneally at various time intervals from lentectomy are currently in progress.

Parallel to the *in vivo* experiments, cultures from dorsal iris cells and iris epithelial cells (IE) were irradiated with 1500 R of 250-kV x rays. The culture medium used was 50% L-15 medium, 40% water, 10% fetal calf serum, and 2 ml of antibiotic-antimycotic solution per 100 ml. Irradiation of cultures from dorsal iris cells at the same day or 4 days after putting in culture resulted in a significant loss of cells after two to three weeks. While untreated cultures showed large colonies after 60 to 70 days, the irradiated cultures contained only very few cells. The radiation-induced decrease in the cell number could be attributed to cell killing, the failure of attachment of the cells, and/or to deficient proliferation.

If primary cultures of dorsal iris epithelial cells were irradiated at a later stage, 7 days after culture started, the cell loss was reduced, but there was still a distinct

difference in cell number per culture between the experimental and control series. Cells of both series showed characteristic process formation and cytoplasmic shedding. Many cells became less pigmented or in some cases even unpigmented during the culture period. However, the degree of depigmentation is significantly less in irradiated cells than in unirradiated cells. The possibility of inhibition of cell proliferation by irradiation *in vitro* is being studied by autoradiography. Experiments will be designed to investigate the sensitivity of morphogenetic reactions of iris epithelial cells toward adenosine and dibutyryl cyclic AMP in connection with the work included in this report.[3]

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EFFECTS OF COMPOUNDS RELATED TO CYCLIC AMP ON THE STRUCTURE OF IRIS EPITHELIAL CELLS IN CULTURE

J. R. Ortiz and Tuneo Yamada

Earlier observations indicate that intensive structural alterations precede dedifferentiation of newt iris epithelial cells engaged in lens regeneration. Since there are some similarities between those alterations and alterations of melanophores which are known to be controlled by cyclic AMP, the question was raised whether cyclic AMP may be involved in the control of the dedifferentiation process in iris epithelial cells. As a first step of approach to this problem, a number of compounds structurally and functionally related to cyclic AMP were tested on iris epithelial cells in culture for their possible effects on cell structure. The test was made on the sparse culture of those cells derived from the adult newt, in which the cytoplasm forms a broad undulating membrane except during mitosis. Dibutyryl cyclic AMP (But_2 c AMP) and monobutyryl cyclic AMP (But c AMP) added to culture at 1–5 m*M* alters the membrane into fine branching strands radiating from the cell center within 80 min. The stellate configuration of cells is transient, and the cells revert to the membranous configuration within 160 min to 12 hr. The same effect is obtained with c AMP but only at a higher concentration (5 m*M*). The derivatives of other cyclic nucleotides like But_2 c GMP, But c UMP, and But c IMP are found negative. So are Na-butyrate and ribose. Theophylline, an inhibitor of phosphodiesterase which degrades c AMP, is effective at 5 m*M*. While guanosine, cytidine, and uridine are ineffective, adenosine is effective even at 0.01 m*M*. Together with 5′ AMP, adenosine is the most effective compound so far tested. Those two compounds are known to be most effective in raising the intracellular level of c AMP. Thus it is possible that the positive effect of the two compounds is due to their effect on the cellular c AMP level. This possibility is supported by the positive effects of combination of theophylline with adenosine at concentrations of each compound ineffective when administered separately. This synergistic effect can be readily understood if both compounds raise the level of intracellular c AMP. Synergistic effects were obtained also by combining theophylline either with c AMP or But_2 c AMP.

Vinblastine, which disrupts microtubules, disturbs appearance of the stellate configuration when given in conjunction with adenosine. This suggests the possibility that microtubules are involved in induction of the stellate configuration. That microtubules become prominent during dedifferentiation of iris epithelial cells *in vivo* has been observed earlier.

CHANGES IN THE NUMBER OF MITOCHONDRIAL MATRIX GRANULES DURING OOCYTE DEVELOPMENT IN *XENOPUS*

J. N. Dumont

The matrix of mitochondria of many cells contains electron-dense deposits of a material that has been identified as calcium. Since changes in calcium content, translocation, and permeability occur during oocyte development, we have attempted to quantitate the number of mitochondrial profiles showing dense granules at various stages of oocyte development from chorionic gonadotrophin-stimulated and unstimulated frogs. Our results to date indicate that in the most immature, stage I oocytes from stimulated frogs, 72% of the mitochondrial profiles show one or more dense calcium phosphate deposits. During stage II of development, this percentage declines to 63% of the profiles with deposits, and during stage III declines still further to 30%. The values for stages III through VI (mature oocytes) remain fairly constant. These values are based on counts of nearly 1000 mitochondrial profiles from at least two oocytes from each of four different animals. The values are essentially the same for comparable stages of oocytes from unstimulated animals.

There is a dramatic decrease in the number of mitochondrial profiles showing dense calcium deposits in the ovulated (colonic) egg. Only 5% of the mitochondrial profiles show deposits. Therefore, there appears to

be a release or translocation of calcium during early phases of development and growth and later when the mature oocyte is ovulated. This may be correlated with (1) the possible requirement of calcium during micropinocytotic uptake of yolk (stages III and IV) and (2) the changes in membrane potential following ovulation and germinal vesicle breakdown in preparation for meiosis and fertilization.

THE EFFECTS OF HORMONES AND STARVATION ON OOCYTE DEVELOPMENT IN *XENOPUS LAEVIS*

Christie A. Holland* and J. N. Dumont

It is known that hormones [estrogen and human chorionic gonadotrophin (HCG)] administered to female frogs, *Xenopus laevis*, induce the hepatic synthesis of yolk protein (vitellogenin). HCG also promotes the uptake of vitellogenin by developing oocytes through the process of micropinocytosis. Estrogen, on the other hand, does not promote uptake, and consequently vitellogenin accumulates in the serum. It has also been suggested that food availability plays an important role in promoting oogenesis. The relationship of estrogen, HCG, and food availability to micropinocytotic uptake of vitellogenin by developing oocytes was studied.

When only estrogen is administered to an animal, there is no evidence of micropinocytotic activity at the oocyte surface, although it induces hepatic synthesis and secretion of large amounts of vitellogenin. However, after subsequent treatment of the animal with HCG, uptake is initiated and many micropinocytotic vesicles and pits appear on the cell membrane of the oocyte. If, on the other hand, a frog is treated with HCG, inducing micropinocytosis, and is then given estrogen, micropinocytotic activity diminishes. Therefore, estrogen can inhibit uptake even after it has been induced by HCG.

To investigate the relationship of food supply and vitellogenic activity of the developing oocytes, animals were given HCG and then starved for three weeks. At the end of this time the surface of developing oocytes showed little micropinocytotic activity, suggesting food is essential for a normal reproductive cycle. Although food supply exerts an influence on oogenesis, the intermediate processes relating food availability to micropinocytosis are unknown.

Animals were starved for three weeks prior to the administration of estrogen or HCG to test the effects of starvation on the hormonal response of the developing oocytes. Oocytes from starved animals show little micropinocytosis, but after treatment with HCG the surface is highly convoluted and contoured, indicating that HCG is capable of inducing activity and promoting the development of immature oocytes even when an animal is starved. On the other hand, estrogen does not have this ability. Oocytes from starved estrogen-treated animals do not show micropinocytotic activity; they remain small and do not develop.

Therefore, HCG acts to stimulate vitellogenesis and oocyte development while estrogen and starvation inhibit oocyte development.

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THE EFFECTS OF CYTOCHALASIN B AND COLD (0°C) ON MICROPINOCYTOSIS AND YOLK UPTAKE BY OOCYTES *IN VITRO*

J. N. Dumont

It has been previously established in this laboratory (1) that micropinocytosis is the mechanism by which specific yolk protein (vitellogenin) is incorporated into developing oocytes and (2) that vinblastine, which disrupts microtubules, inhibits uptake by blocking micropinocytosis and, in addition, alters the surface architecture of the oocytes. Since it is also known from other work that cold (0°C) temperatures disrupt microtubules, experiments were conducted to determine if this condition may also alter the oocyte surface architecture and prevent micropinocytotic uptake. Furthermore, microfilaments have been implicated in playing an active role in micropinocytosis. Thus, cytochalasin B, an agent known to disrupt the orientation and function of microfilaments, was also tested under *in vitro* conditions of oocyte uptake. Normally, the oocyte surface is highly sculptured with many microvilli and deep crypts or unfoldings of the cell membrane which penetrate into the cytoplasm. Both 0°C and cytochalasin B disrupt the surface contours of the oocyte in such a way that the crypts disappear after only a short exposure period (1 to 4 hr). Accompanying this change in surface architecture is the increasing loss of micropinocytotic activity of the cell membrane. As exposure time is increased (to 20 hr), the surface becomes increasingly flattened, micropinocytosis ceases, and eventually, in the case of cytochalasin B, the microvilli swell and gradually disappear. These cytologic changes have been correlated with the actual uptake of radioactive yolk protein (vitellogenin) with the following results. The uptake is indicated as percent of

control, and the hours of exposure to either cytochalasin B or 0°C are given. The total culture period was 24 hr.

	Hours of exposure	Uptake (% of control)
Cytochalasin B (10 μg/ml)	4	64
	8	34
DMSO	24	188
	24	24
0°C	8	28
	24	9

These data show that both cytochalasin B and 0°C inhibit vitellogenin uptake and substantiate the cytological finding that micropinocytotic activity is also inhibited. DMSO (1% in culture medium) serves as a solvent for cytochalasin B; the results indicate that uptake is greatly enhanced. Cytological examination of DMSO-cultured oocytes reveals also an increased amount of micropinocytotic activity. The significance of this induced activity and the mechanism of its induction are not understood. However, (1) the disruption of microfilament function by cytochalasin B, (2) the alterations in surface architecture induced by both cytochalasin B and cold, and (3) the loss of micropinocytosis with both agents suggest that microfilaments and microtubules play an important role in the micropinocytotic incorporation of macromolecules into this cell and that the maintenance of the configuration of the cell surface is an important aspect of this phenomenon.

ULTRASTRUCTURE OF ACTIVE EUCARYOTIC GENOMES

Barbara A. Hamkalo,* O. L. Miller, Jr.,† and Aimée H. Bakken‡

Using simple preparative procedures and electron microscopic techniques, it is possible to visualize transcription of both ribosomal precursor RNA (rpRNA) genes and nonribosomal deoxyribonucleoprotein (DNP) in various eucaryotes. Nuclear contents of *Drosophila* embryos are being studied as representative of a differentiating system. Structures analogous to active rpRNA genes of amphibian oocytes are visible in these preparations as clusters of closely spaced ribonucleoprotein (RNP) fibril gradients along the DNA separated by matrix-free segments of varying lengths. A typical matrix measures 2.6 μm, slightly shorter than the length of an rpRNA gene (2.8 μm) based on a molecular weight of 2.8×10^6 for the *Drosophila* precursor, and the shortest intergene spacer measures 0.5 μm. As in oocyte rpRNA genes, dense granules are seen at the free ends of nascent RNP's. Other active sites show fewer RNP fibrils per length of DNP, indicating less frequent initiation of transcription than for rpRNA genes, although there are regions where initiation frequency is sufficiently high to generate short to long fibril gradients. In contrast, nuclear material of HeLa cells exhibits no obvious fibril gradients at sites of nonribosomal transcription. However, putative active rpRNA genes of HeLa show the same basic features seen in all eucaryotes where such genes have been identified: clusters of densely packed RNP fibril matrices, each close to the length expected from precursor molecular weight; adjacent matrices separated by spacers; and dense granules at the free ends of nascent RNP's. Study of nuclear contents from yeast shows that similar structural conformations of active chromosome segments occur in lower eucaryotes.

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DISTRIBUTION OF INCORPORATED AND SYNTHESIZED PROTEIN AMONG CELL FRACTIONS OF *XENOPUS* OOCYTES

R. A. Wallace, D. W. Jared, and J. N. Dumont

When developing oocytes from *Xenopus laevis* are isolated and cultured for 1 hr in the presence of labeled vitellogenin, approximately 0.73 μg is incorporated by each oocyte. One to four hours after the oocytes are transferred to unlabeled medium, most of the labeled vitellogenin is directly transformed into the yolk proteins lipovitellin and phosvitin. A similar culture procedure with leucine as the labeled component revealed that these yolk proteins are not synthesized by the oocyte to any discernible extent. The analytical procedure used for these studies was chromatography of a strong salt extract of the incubated oocytes.[1]

We have now performed similar experiments, but we have developed a sucrose gradient system to discern the cell fractions involved in the uptake, conversion, and synthetic processes. Developing oocytes of *X. laevis* were isolated, pulsed for 10 min with either [^{3}H, ^{32}P] vitellogenin or a mixture of L-[^{3}H] leucine and $^{32}P_i$, and subsequently incubated for various lengths of time in unlabeled medium. Homogenates were then

prepared and centrifuged on 20 to 60% sucrose gradients.

[^{3}H, ^{32}P] Vitellogenin was found to associate initially with the membranous material, but within 45 min more than half the label was associated with the yolk platelets. Since it takes at least 60 to 120 min for vitellogenin to be converted into lipovitellin and phosvitin, this transformation must occur within the platelets rather than at the oocyte surface or within pinosomes.

Eighteen hours after a pulse with L-[^{3}H] leucine and $^{32}P_i$, neither the yolk nor the mitochondria fraction became labeled, indicating that the macromolecular components of these structures were not synthesized or phosphorylated by the oocyte during vitellogenesis. Instead, various components within the membranous and "soluble" regions became labeled. Further work with an appropriate gradient and sufficient centrifugation to resolve specifically the components of these regions should prove rewarding.

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CHARACTERIZATION OF THE ESTRADIOL-INDUCED AMPHIBIAN SERUM PROTEIN VITELLOGENIN AND THE PRECURSOR-PRODUCT RELATIONSHIP OF THIS PROTEIN WITH THE YOLK PROTEINS LIPOVITELLOGENIN AND PHOSVITIN

E. W. Bergink and R. A. Wallace

After addition of estradiol to a male or female frog, vitellogenin is synthesized in the liver and secreted into the blood. During oogenesis in the female frog, vitellogenin is taken up into growing oocytes and is transformed into the yolk proteins lipovitellin and phosvitin.[1] In order to analyze this conversion of serum protein into yolk protein, we determined the molecular weights of the peptide chains in both serum and yolk proteins by SDS–polyacrylamide gel electrophoresis and by gel filtration in 6 *M* guanidinium chloride (GuHCl) on Bio-Gel A-15 m. For the vitellogenin peptide chain, these methods resulted in the following molecular weights: 208,000 (3.3% acrylamide gel), 190,000 (5.0% acrylamide gel), and 185,000 (gel filtration). The accuracy of these determinations is about 10%. No smaller subunits could be observed if, during the isolation procedure of the protein, extreme care was taken to prevent proteolytic breakdown. We conclude that the native protein (molecular weight of about 500,000 and a lipid content of 13%) consists of two polypeptide chains of equal size.

Lipovitellin consists of two different peptide chains, the molecular weights of which were found to be 110,000 and 31,000 (on 3.3% and 5.0% acrylamide gels). The smaller subunit appears to be a phosphoprotein with a phosphate content of about 2%. The larger subunit is not an aggregate of the smaller peptide chain since (1) the larger subunit contains no phosphate, (2) the amino acid composition of the two peptide chains is different (the smaller subunit has a 50% higher serine, 30% higher glycine, and 50% lower leucine content), and (3) the molar ratio of both components is 1:1. Unlike the results with the polyacrylamide gel electrophoresis, only one component was observed after gel filtration of ^{32}P-labeled, delipidated lipovitellin in 6 *M* GuHCl. The molecular weight of this component was 140,000. The incomplete dissociation in 6 *M* GuHCl was not due to incomplete reduction of thiol groups, since complete dissociation occurred when the "140,000" component was further denatured in SDS without reducing agents.

The molecular weight of the phosvitin peptide chain was found to be 35,000 (5% acrylamide gel). The phosphate content (10%) is four to five times higher than the phosphate content of the smallest lipovitellin subunit. During electrophoresis of ^{32}P-labeled phosvitin, about 20% of the phosphate label migrates with the dye front, indicating the existence of phosphopeptides with a molecular weight less than 10,000. The origin of this material is uncertain. The total molecular weight of lipovitellin and phosvitin peptide chains is thus about 175,000. This result is in good agreement with the molecular weight of the yolk protein precursor (vitellogenin) and the observation that, during uptake into the growing oocyte, about 10% of the amino acid material of the precursor is converted into nonlipovitellin, nonphosvitin material.[2]

We also incubated ^{32}P, ^{3}H-labeled vitellogenin with trypsin or chymotrypsin in order to obtain information about the susceptibility of the precursor protein to proteolytic attack. Dissociation into specific peptides occurred after 5 min of incubation at 37°C with very low concentrations of trypsin or chymotrypsin. Trypsin ($1:10^4$ enzyme:substrate ratio) degraded about 95% of the precursor. The ^{3}H label (associated with leucine) was primarily present in a nonphosphopeptide with the same electrophoretic mobility as the large lipovitellin subunit, whereas the ^{32}P label appeared in peptide chains with molecular weights of about 70,000 and 35,000. In the presence of the same low concentration of chymotrypsin (plus trypsin inhibitor), about 40% of the ^{3}H label was converted into a peptide indistinguishable from the large tryptic product.

The evidence thus supports the view that the vitellogenin precursor is a continuous peptide chain and that degradation into specific yolk proteins occurs by proteolytic splitting in regions which are also sensitive to proteolytic attack by trypsin and chymotrypsin.

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SEQUESTERED AND INJECTED VITELLOGENIN: ALTERNATE ROUTES OF PROTEIN PROCESSING IN *XENOPUS* OOCYTES

R. A. Wallace and Paula F. Dehn*

The available evidence indicates that vitellogenin is incorporated by a micropinocytotic mechanism and that the derived pinosomes subsequently fuse within the cortex of the oocyte and give rise to yolk platelet primordia.[1] The conversion to lipovitellin and phosvitin involves a macromolecular restructuring within yolk platelet primordia rather than breakdown and resynthesis,[2] but the conversion mechanism remains unknown. Protein synthesized by the developing oocyte appears to undergo some degree of turnover whereas sequestered vitellogenin does not.[2] This would imply that vitellogenin injected into the cytoplasm of the oocyte might eventually undergo breakdown rather than conversion unless it could traverse the membranes of the pinosomes and primordial yolk platelets. We have tested this assumption and found that indeed vitellogenin injected into, rather than sequestered by, oocytes is not converted to lipovitellin and phosvitin, but instead appears to be simply catabolized.

Oocytes were incubated in medium containing [^{3}H, ^{32}P]vitellogenin for 30 min, after which they were washed and, as a control procedure, injected with saline. After an additional 11-hr incubation in unlabeled medium, the labeled oocytes were extracted with strong salt, and the dialyzed extract was subsequently chromatographed on TEAE-cellulose. The chromatograph (Fig. 13*a*) indicated that the principal components present were lipovitellin and phosvitin, eluting at about positions 0.42 and 0.72 respectively. Labeling in the eluent fractions (Fig. 13*b*) indicated that macromolecular conversion had taken place, with the ^{3}H label and ^{32}P label primarily associated with lipovitellin and phosvitin, respectively.

Equivalent amounts of vitellogenin were injected into oocytes, and these were also incubated for 11 hr, extracted, and chromatographed. Although the absorbance profile remained the same (Fig. 13*a*), labeling in the eluent fractions (Fig. 13*c*) indicated that no conversion had taken place. Rather, a residual amount of doubly labeled vitellogenin was found at position 0.64, and small amounts of heterogeneously labeled components appeared throughout the chromatograph.

These results indicate that vitellogenin is taken up by oocytes and converted into the yolk proteins lipovitellin and phosvitin within a compartment different from and inaccessible to vitellogenin injected into the oocyte

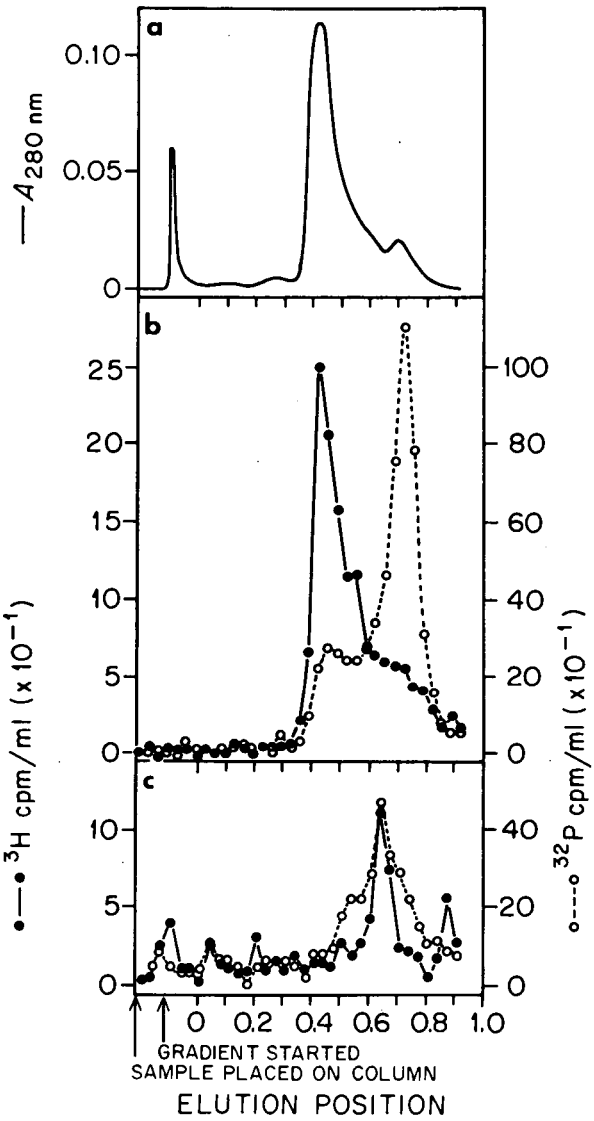

Fig. 13. TEAE-cellulose chromatography of oocyte extracts containing about 5 mg of protein from 60 oocytes. (*a*) Absorbance trace. (*b*) Labeling in extract from oocytes cultured in the presence of [^{3}H,^{32}P]vitellogenin. (*c*) Labeling in extract from oocytes injected with [^{3}H,^{32}P]vitellogenin.

cytoplasm. The latter appears to be hydrolyzed into small molecular weight products, some of which are then incorporated into other macromolecules.

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THE ROLE OF FOLLICLE CELLS AND CALCIUM DURING PROTEIN UPTAKE BY ISOLATED AMPHIBIAN OOCYTES

R. A. Wallace, Ti Ho, and D. W. Salter*

We have previously observed that treatment of vitellogenic oocytes with calcium-free medium containing EDTA resulted in both a loss of follicle cells and the subsequent ability to sequester protein.[1] Our conclusion at the time, therefore, was that the presence of follicle cells was necessary during protein uptake by amphibian oocytes. We have now made some additional observations which tend to cast doubt on this initial conclusion. These observations include:

1. The follicular epithelium frequently tears on isolated oocytes and subsequently forms strands or sheets of cells. After 48 to 72 hr in culture these are frequently lost to the oocyte, yet we have generally observed the process of protein uptake to be essentially linear over several days.

2. Autoradiographs revealed that incorporation of protein occurs rather informally over the surface of the oocyte, whether or not follicle cells are present. Sometimes follicle cells appear closely attached to the surface of the oocyte and if anything seem to occlude the passage of protein into the oocyte.

3. Oocytes treated with EDTA appear to lose their follicle cells more rapidly than they lose their ability to sequester protein.

4. EDTA-treated oocytes, which have lost most if not all of their follicle cells, show a substantial recovery of their ability to sequester protein if they are simply washed and incubated in a balanced salt solution for 26 to 36 hr. During this time additional follicle cells are not recovered by the oocyte.

From these observations we have concluded that (1) follicle cells do not seem to play a role during protein corporation by *Xenopus* oocytes *in vitro,* (2) the lace of external calcium inactivates or promotes the loss of protein-binding sites on the oocyte surface, and (3) these sites can be regenerated in calcium-containing media.

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LACK OF MUTAGENICITY OF HYCANTHONE IN *HABROBRACON SERINOPAE* FEMALES

Roger H. Smith

Hycanthone, an antischistosomiasis agent, has been shown to be mutagenic in some biological systems but not in others. In order to determine whether or not dominant or recessive lethal mutations were induced in germ cells of insects, *Habrobracon serinopae* females were fed hycanthone mixed in sucrose. Virgin females produce haploid progeny parthenogenetically, so the entire genome can be screened for mutations in the generation following the treatment.

Virgin females of the *p* (plum eye color) stock were starved at 28°C for approximately 3 days, at which time the abdomens showed dorsal-ventral flattening. These females were divided into five groups of 24 females each: (1) fed 40% sucrose, (2) fed 40% sucrose and x-irradiated (3 kR, 501 R/min, 250 kVp at 30 mA with 3 mm aluminum), (3) fed 0.1 *M* hycanthone in 40% sucrose, (4) fed 0.05 *M* hycanthone in 40% sucrose, and (5) fed 0.01 *M* hycanthone in 40% sucrose. The females were observed while they fed to verify that each female did indeed eat the mixture. As the wasp feeds, one can see the crop expand to full capacity. Only the females exposed to the 0.1 *M* hycanthone did not feed to full capacity. They rubbed their mouth parts with their front tibiae, indicating an aversion to the mixture.

The females were placed individually with host larvae, *Anagasta kuhniella,* for oviposition immediately after feeding. Oviposition was allowed continuously for ten days, with each female being transferred twice daily at approximately 9 AM and 3 PM to a new Stender dish containing a fresh host. Egg counts were made after each transfer. Hatchability was scored 36 to 48 hr later. Adult survival of the F_1 generation was recorded eight to ten days after oviposition. The entire experiment was carried out at 28 ± 0.5°C and 50 to 60% relative humidity.

A total of 28,319 eggs were deposited by the females over the ten-day egg-laying period. In the pattern of egg production, there was no significant difference between

females fed sucrose and those fed sucrose mixed with the three concentrations of hycanthone. The pattern produced by females irradiated with 3 kR is typical for cells going through oogenesis. The valley indicates the cells that were transitional oogonia at the time of exposure to x rays. The recovery 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. There are no differences in results between the control group and the females fed the different concentrations of hycanthone.

In summary, 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 indicates that no change was induced in the general physiology of this insect. The absence of the typical depression in the egg production curve, like that seen after x irradiation of virgin females, and the high hatchability of haploid eggs show that the feeding of hycanthone does not induce dominant or recessive lethal mutations in meiotically or mitotically active cells.

THE RELATIONSHIP BETWEEN PROPHAGE INDUCTION AND TRANSFORMATION IN *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow, D. P. Allison, M. E. Boling, and K. L. Beattie

The interaction between transformation and prophages of HPIcl, S2, and a defective phage of *H. influenzae* has been investigated by measurement of (1) the effect of prophage on transformation frequency and (2) the effect of transformation on phage induction. The presence of any of the prophages does not appreciably alter transformation frequencies in various Rec^+ and Rec^- strains. However, exposure of competent lysogens to transforming DNA may induce phage, but only in Rec^+ strains, which are able to integrate transforming DNA into their genome.

Wild-type cells contain a defective prophage, which can be induced by transformation with *H. influenzae* DNA, and a small fraction of the cells is killed by such transformation. Defective phage in wild-type cells are also induced by DNA from the related strain *H. parainfluenzae,* and up to 70% of the cells are killed. Quantitative electron microscopic measurements of the phage produced following transformation with these two types of DNA show a good correlation between the number of phage produced and the amount of killing, suggesting that the lethal effect of transformation results from the induction of defective phage. Further evidence for this hypothesis is that a strain of *H. influenzae* that is highly transformable but is not inducible for defective phage is not killed by exposure to *H. parainfluenzae* DNA or *H. influenzae* DNA. Since transformation by *H. parainfluenzae* DNA is as low in this strain as it is in the wild type, we conclude that the lethal effect of the heterospecific DNA cannot account for the low frequency of heterospecific transformation.

ISOLATION AND PROPERTIES OF A MINICELL-PRODUCING STRAIN OF *HAEMOPHILUS INFLUENZAE*

Jane K. Setlow, K. L. Beattie, Karen M. Hart, and D. P. Allison

Among a series of 12 uv-sensitive mutants isolated following nitrosoguanidine treatment of a thymine-requiring strain of *H. influenzae,* one was a minicell-producing cell. Electron microscopy has confirmed that small cells sometimes bud off from the end of the normal-sized ones. The density of the minicells is similar to that of the parent cells, as determined by density gradient centrifugation, but they are separable from the parent cells by sedimentation velocity. The membrane mutation in the minicell strain results in the DNA being relatively inseparable from other cell components, so that most of the DNA sediments extremely rapidly, even on alkaline sucrose gradients, which normally allow cell DNA to sediment freely as a single component. Similarly, relatively low yields of DNA are obtained with the usual chloroform-octanol separation of DNA from other cell components. The mutant has the interesting property that as much as 80% of a competent culture is killed by the introduction of transforming DNA. Observation of lysis of these cells exposed to transforming DNA and then grown for about 90 min led to the hypothesis that transformation of the mutant causes induction of the defective prophage carried by almost all of our strains of *H. influenzae.* This hypothesis has been confirmed by electron microscopic observation, the only method available for measurement of this phage, which we have shown cannot attach to cells, probably because of a defective tail plate. Thus the phage cannot infect, eliminating the possibility of a biological plaque assay. The phage is induced to some extent when the mutant is made competent. Many more are induced when the cells are transformed, which can account for the large fraction of the population killed by transforming DNA.

The mutant lysogenized with the infective phages S2 or HPIcl and made competent is also supersensitive to

induction of these phages, indicating that the mutation responsible for superinducibility is in the cell genome rather than in the defective prophage itself. The mutant strain is extremely sensitive to heat when it is competent, but not in exponential growth, probably because heat causes extra induction of defective phage.

Transformation of the minicell strain with wild-type DNA carrying three drug markers widely spaced on the genetic map causes suppression of the minicell property. Transformation with another drug marker, nalidixic acid resistance, does not alter this property. There is some evidence in other microbial systems that resistance to the first three drugs alters the transcription or translation machinery of the cell, whereas nalidixic acid seems to affect DNA synthesis. Thus it is possible that the minicell property is suppressed in strains with altered ribosomes or RNA polymerase.

ISOLATION AND CHARACTERIZATION OF TEMPERATURE-SENSITIVE DNA SYNTHESIS MUTANTS OF *HAEMOPHILUS INFLUENZAE*

J. E. LeClerc,* K. L. Beattie, D. P. Allison, and Audrey M. Nicholson*

For studies on the role of DNA replication in recombination and repair, it is essential to have mutants whose DNA synthesis can be readily turned on and off. About 100 temperature-sensitive DNA synthesis mutants have been obtained by mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) of a spontaneous thymine-requiring mutant previously isolated. Temperature-sensitive DNA synthesis mutants in the mutated culture were selected by killing cells which had incorporated 5-bromodeoxyuridine at 42°C with irradiation at 313 nm, a wavelength that preferentially photolyzes DNA containing bromouracil in place of thymine. Since mutants were wanted that were deficient only in DNA synthesis at the high temperature, individual surviving clones were tested for the desired phenotype by measurement of incorporation of labeled protein and nucleic acid precursors. Two types of mutants were obtained: those that apparently fail to initiate DNA synthesis at the higher temperature and those that cannot carry out any synthesis at 42°C. Some of the mutants show DNA degradation at the restrictive temperature.

Two of the mutants which do not undergo degradation at the restrictive temperature were selected for studies of recombination and repair. Protein synthesis in these mutants continues normally for about 40 min, whereas DNA synthesis is shut off within a few minutes after shifting to the restrictive temperature, and DNA synthesis resumes at the normal rate when cells at the restrictive temperature are shifted back to 37°C. Because MNNG often makes multiple mutations in a single cell, the mutations in the two selected strains were transferred into the wild-type strain by transformation, followed by a selection procedure in which colonies grown at the permissive temperature (37°C) were replica-plated onto two plates, one incubated at the permissive and one at the restrictive temperature.

Both the transformed strains form filaments at the restrictive temperature. Electron micrographs of the filaments show the nuclear regions usually in about two discrete parts of the filaments. Preliminary mapping experiments with one of the mutations indicate that it is near the dalacin marker.

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THE ROLE OF DNA SYNTHESIS IN TRANSFORMATION OF *HAEMOPHILUS INFLUENZAE*

J. E. LeClerc,* Jane K. Setlow, and K. L. Beattie

Three methods have been used to investigate the relationship of DNA synthesis and recombination: (1) measurement of recombination in the presence of 6-(*p*-hydroxyphenylazo)-uracil, which inhibits semiconservative DNA synthesis but not repair synthesis; (2) comparison of recombination in temperature-sensitive DNA synthesis mutants at the permissive and restrictive temperatures; and (3) pulse labeling of highly competent cells with or without prior exposure to transforming DNA and examination of the sedimentation pattern of the labeled DNA on alkaline sucrose gradients.

At a concentration of the inhibiting compound such that uptake of tritiated thymidine was essentially abolished, linkage of a genetic marker from donor DNA with another marker in competent recipient cells was completely normal. Thus it is clear that semiconservative DNA synthesis is not required for the recombination events in transformation, although some type of repair synthesis may be.

The formation of recombinant molecules in a temperature-sensitive DNA synthesis mutant at the restrictive temperature is also normal, and the amount of radioactive label from transforming DNA containing heavy isotopes that is associated with light recipient DNA is approximately the same at the restrictive and permissive temperatures. These data eliminate an earlier hypothesis that about half the associated donor label results from

degradation of donor DNA followed by incorporation of the degradation products into recipient DNA.

After a brief exposure of competent wild-type cells to transforming DNA, followed by a pulse of tritiated thymidine in growth medium, the sedimentation pattern of labeled DNA on alkaline sucrose is more heterogeneous than for competent cells not exposed to transforming DNA, and the average molecular weight is smaller. A recombination-defective strain shows the same pattern with and without exposure to transforming DNA, suggesting that the shift in pattern observed in the wild type reflects a special type of DNA synthesis associated with the recombination event, possibly a failure to synthesize DNA past a region of the chromosome containing associated donor DNA which is not completely integrated.

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ABERRANT DNA SYNTHESIS IN COMPETENT *HAEMOPHILUS INFLUENZAE* CELLS

J. E. LeClerc*

Competence may be defined as a physiological state of bacteria such that the cells bind nucleic acid in a form resistant to nuclease. Since transformation is carried out with competent cells, the question may be asked whether there is also any condition peculiar to competent cells that favors recombination of DNA. One possibility is that some of the DNA in such cells may be in a configuration that makes pairing with transforming DNA more probable.

Competent wild-type cells labeled with tritiated thymidine for 10 min in a nongrowth medium favoring competence development and then lysed on alkaline sucrose gradients show a sedimentation pattern of the labeled material that is different from that of similarly labeled exponentially growing cells, in that most of the labeled material in the competent cells is about one-third the size of that in the growing cells. With incubation in nonradioactive growth medium, this material is chased into larger DNA. These data suggest that newly synthesized DNA in competent cells contains nicks or gaps. Experiments with two recombination-defective mutants support the hypothesis that such structures affect the probability of pairing of recipient DNA and donor DNA. The *rec1* mutant behaves like the wild type with respect to pulse-labeled DNA in competent cells, but the competent *rec2* mutant does not show appreciable small single-strand DNA. Since it was previously shown in the laboratory that the *rec2* mutation prevents association of donor and recipient DNA's, whereas the *rec1* mutation permits such association, although integration is not completed, it is concluded that the aberrant DNA synthesis in competent wild-type and *rec1* cells favors association of donor and recipient DNA in transformation.

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INTEGRATION AND REPAIR OF ULTRAVIOLET-IRRADIATED TRANSFORMING DNA IN *HAEMOPHILUS INFLUENZAE*

Amir Muhammed* and Jane K. Setlow

We had previously found that repair of uv-irradiated transforming DNA takes place only after integration of the damaged DNA into the genome of the recipient cells which are proficient in excision of pyrimidine dimers. The decrease of integration induced by uv irradiation of transforming DNA was earlier measured by an indirect method, in which the amount of labeled recipient DNA specifically released into the medium by exposure of competent cells to uv-irradiated transforming DNA was considered to be equivalent to the amount of integration. A more direct measurement of repair and integration has been obtained by exposing cells of varying repair capacities and containing tritium-labeled DNA to irradiated transforming DNA labeled with ^{32}P and heavy isotopes. After various times of incubation of cells containing transforming DNA, they were lysed with digitonin and the mixture centrifuged at low speed. The pellet contained almost all of the resident label and associated donor DNA, and the supernatant contained unassociated donor DNA. The pellet material was subjected to equilibrium centrifugation in CsCl, and fractions from the gradients were analyzed for radioactivity and for biological activity of genetic markers from the original donor DNA as assayed on excision-defective mutants.

The relative insensitivity of transforming DNA to uv-induced loss of integration observed by the indirect method was confirmed by the isopycnic analyses. The biological assays showed considerable repair of the uv-resistant novobiocin marker on donor DNA associated with resident DNA in the wild-type host with increasing incubation times, but little or no repair of the much more uv-sensitive streptomycin marker. Furthermore, the streptomycin donor activity appeared at a slightly heavier position on the gradients than the novobiocin marker, suggesting that a larger piece of

irradiated transforming DNA must be associated with resident DNA to produce a donor-recipient complex with transforming activity for the donor streptomycin marker.

Two excision-defective strains were used as recipients for the heavy transforming DNA. One is unable to carry out the first incision step, and the other (DB116) is defective in a later step in excision. A small amount of repair was observed in DB116 when gradient fractions were assayed on the incision-defective strain, but no repair was detected in the incision-defective strain with fractions assayed on strain DB116. Thus the data confirm the order in which the steps in excision are presumed to occur.

The fact that there was little or no detectable loss of associated ^{32}P counts during repair of transforming DNA in wild-type cells indicates that the excision repair of integrated donor DNA is not accompanied by much loss of donor DNA in the regions surrounding pyrimidine dimers.

*Visiting investigator from Pakistan supported by the Agency for International Development.

ORGANIC-STRUCTURE RELATIONSHIPS IN MUTATION OF *HAEMOPHILUS INFLUENZAE* BY NITROSO COMPOUNDS*

Rosalie K. Elespuru,† William Lijinsky, and Jane K. Setlow

One approach to the problem of the molecular basis of chemical mutagenesis is to attempt to correlate particular organic structures with mutagenic efficiency. A series of structurally related compounds has been synthesized, purified, and tested for lethal and mutagenic effects, as well as for stability in aqueous and alcohol solution. These are: N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), N-ethyl-N′-nitro-N-nitrosoguanidine, N-methyl-N′-nitroguanidine, N-methyl-N-nitrosoguanidine, N-methyl-nitrosourea, nitrosotrimethylurea, nitrosomethylurethane, nitrosocarbaryl, dimethylnitrosamine, and nitrosomorpholine. All these compounds are stable in ethanol, and all but nitrosomethylurea are relatively stable in the Tris-maleate buffer, pH 6, used for treatment of bacteria. The principles derived from this study are: (1) The nitroso group is a necessary but not sufficient condition for mutagenicity and toxicity (dimethylnitrosamine, N-methyl-N′-nitroguanidine, and nitrosomorpholine being inactive). (2) The ethyl group renders nitrosoguanidine less toxic and less mutagenic. (3) The acid esters of nitrosomethyl compounds are considerably more mutagenic and toxic than the corresponding guanidines. (4) Mutagenicity is usually correlated with toxicity, except for nitrosomethylurethane, which is less toxic and more mutagenic than MNNG. (5) The addition of alkyl groups to nitrosomethylurea stabilizes the compound in aqueous solution, but does not affect mutagenicity. (6) The nitrosoureas are much less mutagenic and toxic than MNNG. One of the compounds, nitrosocarbaryl, is almost two orders of magnitude more mutagenic than MNNG, as tested in *Escherichia coli* as well as in *H. influenzae.* This compound is also of interest because of the possibility that it may be formed in the acidic environment of the stomach from two ingested compounds, sodium nitrite, a food preservative, and the commonly used pesticide carbaryl.

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THE TRANSFECTION EFFICIENCY OF MULTIPLE FORMS OF PHAGE DNA IN *HAEMOPHILUS INFLUENZAE*

Karen M. Hart and Jane K. Setlow

It was shown previously in this laboratory that preparations of DNA from purified *H. influenzae* phages contain some linear multiple forms of the phage DNA, which are the most efficient molecules for transfection of the wild-type cell, and that the *rec1* mutant can make little or no use of dimers and trimers for transfection. Since phage DNA put into the competent cell is degraded in this mutant, but not in the *rec2* mutant, it was desirable to measure the relative transfection efficiency of dimers and trimers of phage DNA in the *rec2* mutant.

Tritium-labeled phage DNA was sedimented in neutral sucrose gradients, and fractions were collected for assay of radioactivity and transfection on the wild type and the *rec2* mutant. In the wild-type cells the most efficient transfection was with the heavier DNA molecules, but transfection in the *rec2* mutant was only observed with molecules of monomer length. It is concluded that fragmentation of transfecting DNA in *rec1* cells has nothing to do with the inability of these cells to make use of multiple forms of phage DNA in transfection, and that a functional recombination system is required for transfection by dimer and trimer phage DNA molecules.

MARKER RESCUE IN PHAGE OF *HAEMOPHILUS INFLUENZAE*

M. E. Boling and Jane K. Setlow

When wild-type transfecting phage DNA is put into a competent cell, followed by superinfection with a temperature-sensitive mutant phage, there may be recombination between the wild-type transfecting DNA and the injected mutant DNA, resulting in infected bacteria that can produce phage which grow at the restrictive temperature. There are several orders of magnitude more infected wild-type cells which produce wild-type phage from this recombination than result from the transfecting DNA alone. However, in some, but not all, recombination-defective mutants there is little or no rescue of the wild-type phage markers. Since we previously showed that *H. influenzae* phages depend on host genes for recombination, measurement of marker rescue in various types of mutants provides information on the different pathways or kinetics for host-controlled recombination of phage DNA, which enters the competent cell by injection or as naked DNA. Cells carrying the *rec1* mutation, which allow association between donor and recipient DNA's in transformation but do not complete the integration process, show a very low level of marker rescue. In the *rec2* mutant, which does not exhibit DNA-DNA association in transformation, there is no measurable marker rescue. In neither of these strains is there detectable recombination between injected phage DNA's. The transformation-defective KB mutants, in which there is normal recombination between injected phage DNA's, also show no marker rescue. These data suggest that marker rescue can be blocked either because of a defect in some part of the recombination pathway common to transforming DNA, transfecting DNA, and injected phage DNA; or because of some phenomenon, such as a membrane mutation, which prevents recombination of DNA that enters the competent cell as naked DNA.

As a function of time between the entrance of transfecting DNA into the wild-type cell and the superinfection, the number of infected cells which produce wild-type phage decreases, reflecting the intracellular fragmentation of transfecting DNA observed previously by the sedimentation velocity technique. The amount of marker rescue, which does not vary appreciably with the particular marker being rescued, is linearly related to the concentration of transfecting DNA, unlike transfection itself, where the efficiency increases markedly with increasing concentration of phage DNA. This may indicate that the injected phage DNA to some extent protects the transfecting phage DNA against degradation.

MAMMALIAN GENETICS

W. L. Russell

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X-CHROMOSOME LOSS IN THE OFFSPRING OF IRRADIATED FEMALE MICE

W. L. Russell, Patricia R. Hunsicker,
Elizabeth M. Kelly, Carolyn M. Vaughan,
and Georgia M. Guinn

The series of experiments[1-5] on X-chromosome loss in the offspring of irradiated female mice is now almost finished. The major objectives of this project were, first, to determine the radiation-induced frequency of this type of chromosomal abnormality, which is the cause of Turner's syndrome in women, and, second, to find out how this representative of one of the two major classes of mutational damage responds to dose, dose rate, and other factors that might affect its frequency. Specifically, is the risk of induction less at low dose rates, etc., as it is with the entirely different biological end point – specific-locus mutations – already tested extensively by us?

The last experiment in this series is now nearing completion. The frequency of X-chromosome loss has been determined in over 10,000 progeny of female mice exposed to 400 R of gamma rays at the low dose rate of 0.006 R/min and in a similar number of controls. The induced mutation frequency is significantly less than that obtained earlier at 0.6 R/min, which, in turn, was less than that observed at 80 R/min. This continuous reduction in frequency with lowered dose rate parallels the results obtained with specific-locus mutations. There is a further important parallel with those results in that the frequency of X-chromosome loss in conceptions occurring later than a few weeks after irradiation is not significantly higher than the control frequency, despite the high total dose of 400 R.

Thus, for the offspring of irradiated females, these results, combined with those reported earlier, establish the important conclusion that X-chromosome loss, a representative of one of the two major classes of mutational damage, namely chromosome breakage, responds in the same way as specific-locus mutations: the risk per roentgen is less at small radiation doses, fractionated doses, low dose rates, and for longer, vs short, intervals between irradiation and conception.

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TRITIUM-INDUCED MUTATION RATE IN POSTSPERMATOGONIAL STAGES OF GERM CELLS IN THE MOUSE

R. B. Cumming, W. L. Russell,
Elizabeth M. Kelly, and Marva F. Walton

Since last year's report,[1] our information has doubled on specific-locus mutations scored in offspring obtained from mouse germ cells that were in postspermatogonial stages throughout their exposure to radiation from the tritium. The new data came from 15 groups, each containing 18 male mice, which have passed through the specially designed isolators following injection of 0.50 mCi of tritiated water per gram of body weight. Six presumed specific-locus mutations have been observed in the 4101 offspring obtained from cells irradiated in postspermatogonial stages. Added to the results reported earlier,[1] this provides a total frequency of 11 presumed specific-locus mutations in 7943 offspring from germ cells exposed in postspermatogonial stages to tritium.

The earlier data came from 2 groups of male mice injected with 0.75 mCi and 12 groups injected with 0.50 mCi of tritiated water per gram of body weight. For the total 7943 offspring obtained from the earlier and recent experiments combined, the mean radiation dose per germ cell is estimated, by the method described elsewhere,[2] to be approximately 430 rads. On the assumption that this dose estimate is not greatly in error, the mutation rate induced by tritium in the postspermatogonial germ cell stages is well within the statistical limits of what would have been expected from a comparable dose of externally applied x or gamma radiation.

1. R. B. Cumming, W. L. Russell, Elizabeth M. Kelly, and Marva F. Walton, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, p. 111.
2. R. B. Cumming et al., "Radiation Dose to the Mouse Testis from Injected Tritiated Water," this report.

TRITIUM-INDUCED MUTATION RATE IN SPERMATOGONIA OF THE MOUSE

W. L. Russell, R. B. Cumming,
Elizabeth M. Kelly, and Marva F. Walton

The information now obtained on specific-locus mutations induced in mouse germ cells that received almost all of their tritium radiation exposure in spermatogonial stages is almost twice as extensive as that reported last year.[1] Here the increase in data came, not from a new experiment, but from continued breeding of the 14 groups of males described earlier,[1] of which 2 groups were injected with 0.75 mCi and 12 groups with 0.50 mCi of tritiated water per gram of body weight. The number of offspring from germ cells irradiated predominantly as spermatogonia has now reached 19,112, and the number of presumed mutations at the seven specific loci scored is 14.

Although the spermatogonial stage is the stage of prime importance so far as genetic risk from irradiation of human males is concerned, many difficulties, unfortunately, are encountered in estimating the accumulated absorbed radiation dose which the genetic material of the spermatogonia received from the tritiated water injected in our experiments.[2] If our present estimated dose to the testis could be taken as a reliable guideline, then the mutation frequency observed would represent approximately twice that expected from external gamma radiation delivered at low radiation dose rate. However, in view of the various uncertain factors and the sample size, a relative biological efficiency (RBE) of 1.0 for the tritium radiation relative to external gamma radiation is not excluded. A very high RBE does already appear to be excluded.

The distribution of the mutations among the seven loci shows a possible departure from that expected. Only one of the 14 mutations was at the *S* locus. The number expected at this locus, according to the results from external radiation, was between 5 and 6. The difference is significant on the basis of some statistical assumptions, but perhaps not on others. More data are needed to answer this question. If it is shown that tritium produces an array of mutations that is qualitatively different from that obtained with external radiation sources, this could be important in the estimation of human hazards.

The results given here, together with those in the concurrent report on postspermatogonial stages, represent the first body of data ever obtained on gene mutation induction from tritium in any mammal. The importance of this kind of information is obvious, in view of the concern about the possible biological hazards of man-made tritium in the environment.

1. R. B. Cumming, W. L. Russell, Elizabeth M. Kelly, and Marva F. Walton, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, p. 111.
2. R. B. Cumming et al., "Radiation Dose to the Mouse Testis from Injected Tritiated Water," this report.

MUTATION AT THE HEMOGLOBIN LOCUS IN THE MOUSE

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

We have continued the work described earlier[1] in which offspring of irradiated mice are scored for mutations at the hemoglobin locus. The two main purposes are, first, to measure frequency at a biochemically well-characterized and vital locus, and, second, if enough mutants can be obtained, to determine the relative frequencies of deficiencies and point mutations. The experiments consist of irradiating either 101 or SEC strain mice, mating them with nonirradiated mice of the other strain, and screening the F_1 progeny for hemoglobin variants. The two parent strains have distinctly different electrophoretic banding patterns, and the normal pattern for the offspring has been studied along with various blood mixes to test the system. The improvements in electrophoretic technique and in streamlining of procedures[2] have greatly facilitated the collection of data. Screening for hemoglobin variants is also being done with a solubility technique.

From experiments at various radiation doses, a combined total of 3355 F_1 offspring has now been screened by electrophoresis. One spontaneous mutation has been found, but, so far, none induced by radiation. The absence of any induced mutations in this many offspring does, of course, permit the calculation of an estimated upper limit to the induced mutation rate. The upper 95% confidence limit of the zero frequency of induced hemoglobin mutations would not include a mutation rate per roentgen three times as high as that for the most mutable locus (*S*) among the seven used in our specific-locus tests. The same confidence limit would also exclude a mutation rate as high as seven times that of the mean rate per locus of the seven specific loci.

1. W. L. Russell, Carolyn Vaughan, and K. B. Jacobson, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, p. 113.
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THE NATURE OF RADIATION-INDUCED MUTATIONS: CLASSIFICATION OF *c*-LOCUS MUTANTS

Liane B. Russell, W. L. Russell,
and Elizabeth M. Kelly

In order to estimate the magnitude of risk of any mutagen to human populations, it is necessary to determine not only the rate of induction of mutations but also the nature and effect of the mutations induced under various physical and biological conditions. Such information would also carry over to the calculation of genetic hazards from radiation emitters that have not yet been directly studied.

We have therefore embarked on an extensive program of determining the nature and effect of the numerous mutations recovered during many years of specific-locus mutation-rate experiments. At the basis of complementation analysis and heterozygous fitness experiments (described in other reports) lie determinations of homozygous effects of mutations obtained from irradiation of various cell stages under various physical conditions. We have this year completed homozygosity tests and tabulations on most of the mutations at the albino (*c*) locus.

All new mutations were detected in the heterozygous state with the c^{ch} allele carried by our test stock. Since all but one of 103 completely and 12 partially tested mutants were distinguishable in color from c^{ch}, testing consisted of crossing the original mutant to c^{ch}/c^{ch} and then intercrossing heterozygotes. Where homozygotes were viable, they were characterized for phenotype and tested for fertility. Crosses involving apparently lethal mutants were carefully checked from the time of birth to distinguish between postnatal, neonatal, and prenatal lethals. In the case of the one mutant that was indistinguishable from c^{ch}, outcrossing and backcrossing were used to establish 12 descendant lines that had to be homozygous either for the mutant- or the stock-c^{ch}. All of these lines were viable.

Classifications based on complete testing of 103 *c*-locus mutants, and partial testing of another 12, are shown in Table 10. In 6 cases in the controls and in 11 in the irradiated groups (where somewhat larger totals were scored), the original mutant animal was a mosaic. This confirms earlier results[1] which indicated that mosaic mutations may be assumed to be of spontaneous origin. It would, in any case, be difficult to explain how irradiation of spermatogonia (which are several divisions, mitotic and meiotic, removed from fertilization) could produce mosaicism.

Among irradiated spermatogonia, there was no significant effect of dose rate of x or gamma irradiation on distribution of mutations into the various classes, nor was there any clear change with fractionation. All x and gamma results for irradiated spermatogonia have therefore been lumped in Table 11, which shows distributions in terms of percentage of completely tested mutants.

The x- or gamma-irradiated spermatogonial group is closest in distribution to controls. (The absence of lethals in the controls does not differ significantly from the incidence of lethals in the x- or gamma-irradiated gonial groups, $P > 0.2$, and spontaneous lethals are not infrequent at other loci used in our specific-locus test.) Neutron irradiation, when compared with x or gamma, produces a shift from the viable to the lethal category.

Table 10. Classification of *c*-locus mutants by homozygous effect and mode of origin

Cell stage	Treatment	Viable[a]		Subvital	Lethal		Incomplete test
		Albino	Intermediate		Neonatal	Prenatal	
Control		$2 + 3^m$	$2 + 1^m$	1^m			1^m
Spermatogonia	X or γ, >10 R/min	9	$2 + 1^m$	1	3	2	7 (5 alb. + 2 inter.)
	X or γ, 9 R/min	2				1	
	X or γ, <1 R/min	$4 + 1^m$	$2 + 2^m$			1	1
	X or γ, fractionated, exc. 24-hr	$1 + 1^m$	2	1		2	
	X or γ, 24-hr fractions	8		1		3	
	Neutrons	$7 + 3^m$	1^m	1	3	4	
Postspermatogonia	X or γ	2				2	
	Neutrons	1				2	1
Oocytes	X or γ	$5 + 1^m$			1	1	1
	Neutrons	$2 + 1^m$				7	1

[a] m indicates that the original mutant animal was mosaic.

Table 11. Distribution of tested *c*-locus mutants

Cell stage and treatment	Number of mutants tested	Viable (%)	Subvital (%)	Lethal (%)
Control	9	89	11	
Spermatogonia, x or γ	50	70	6	24
Spermatogonia, neutrons	19	58	5	37
Postspermatogonia, x or γ	4	50		50
Postspermatogonia, neutrons	3	33		68
Oocytes, x or γ	8	75		25
Oocytes, neutrons	10	30		70

This is true at all cell stages, although the numbers in postspermatogonial and oocyte groups are small. Taken as a whole, irradiation of postspermatogonial stages or oocytes produces relatively more lethals than does irradiation of spermatogonia. In these various general aspects, the present data for the *c* locus resemble earlier results for *d* and *se*.[2]

Embryological experiments are now in progress to determine the time of death of prenatal lethals.

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ANALYSIS OF THE *c*-LOCUS REGION BY MEANS OF COMPLEMENTATION TESTING AND BIOCHEMICAL STUDIES

Liane B. Russell, Dale L. DeHamer,*
and Clyde S. Montgomery

Specific-locus mutation-rate experiments carried out at this laboratory during the past two decades have yielded a wealth of genetic material. We have embarked on a program to obtain more information about the genetic nature and action of these mutations, this program being directed toward two main fields of inquiry: (1) We hope to illuminate possible relationships between the various physical and biological variables of mutation induction on the one hand and the type and effect of mutations induced on the other. These results obviously have a strong bearing on calculations of genetic risks. (2) We hope to shed light on genetic organization in a mammal by the exploration of small chromosomal regions.

In the past, we have reported on rather extensive analyses of mutants at the *d* and *se* loci.[1] More recently our detailed studies have extended to *c*-locus mutants. In other reports,[2,3] we discuss determinations of homozygous effects and measurements of heterozygous fitness for mutants classified according to mode of induction. This report deals with complementation studies on 30 albino lethals. The derivation of these mutants is shown in Table 10.

In complementation- and deficiency-mapping in the *c* region we do not have the advantage of the two extremely closely linked specific loci that we had in the *d-se* region. Markers available in the vicinity of *c* are arranged as follows, according to recent maps: *sh-1*–(3)–*Mod-2*–(1)–*c*–(2)-*tp* (the numbers in parentheses representing map distance). We have tested most of the *c* lethals against *tp* (*taupe*) and against *sh-1* (*shaker-1*), and none of these combinations have been anything but wild type, indicating that any deficiencies that are present do not extend to these markers. Testing against *Mod-2* (*malic enzyme, mitochondrial*) will shortly get under way.

The number of possible combinations of 30 independent mutants is 435. To date, we have made 359 of these and have raised over 7000 young. On the basis of the viability of the *c*-lethal compounds, the 30 mutants group themselves into five complementation groups (Fig. 14). Seven functional units must be postulated to

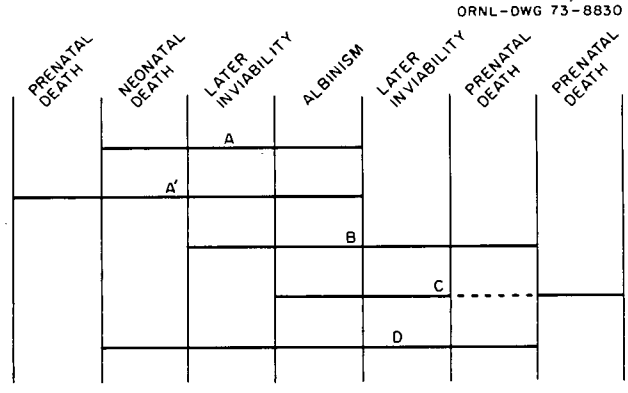

Fig. 14. Complementation map in the region of the *c*-locus, LG1, of the mouse. The map has been derived from intercrosses of 30 independent *c*-locus lethals.

account for all of the interaction effects on a linear basis.

It must be stressed that the map in Fig. 14 is purely an attempt to fit to a linear order the results based on complementation for lethal effects: it is not a recombination map. Therefore, the C-group mutant here indicated in two portions does not necessarily represent two mutations separable by crossing-over, and we have not obtained any such recombination. Our data, so far, are, however, sufficient only to rule out a genetic map distance greater than 0.4.

We are now in process of investigating at what stage of embryonic or fetal life the prenatal death occurs in the various albino lethals. Our preliminary results indicate that certainly the B group, and possibly the D group, can be further subdivided and that one of the "prenatal-death" functional units, depicted here to the right of *c*, will be split into at least two new functional units.

The number of mutants that fall into each of these various complementation groups, and their distribution according to mode of origin, is shown in Table 12. In brackets following each number is the number expected if the mutants of a given complementation group were distributed in the ratio of the total mutants in the various classes. It appears that any given treatment is not preferentially causing a particular type of mutation – except for the B-group mutants, which may be more often induced by neutrons and in oocytes than is expected on a random distribution.

Three albino lethals (probably representing only two independent mutations) sent by us to Dr. Gluecksohn-Waelsch several years ago have now been shown by us to belong to group A. These mutants were used by her and Cori and several coworkers in a number of studies that indicated that homozygotes were hypoglycemic and deficient in glucose-6-phosphatase activity.[4] We have this year checked glucose-6-phosphatase activity in a large number of crosses involving albino lethals, using a modified Fiske-Subbarow method (which measures the inorganic phosphate produced along with the glucose, when glucose-6-phosphatase acts on G6P as a substrate). Of necessity, one is restricted to combinations that survive at least until the time of birth (since the enzyme activity does not normally appear until then), which means that the litters examined have excluded those from crosses of mutants within or between the B and D groups. Although we have not yet studied all possible remaining combinations, we find, thus far, no exception to a complete correlation between neonatal death and glucose-6-phosphatase deficiency. All combinations that complement for newborn lethality, regardless of their later degree of viability, also have normal G6Pase activity. We are thus unable, to date, to split the functional unit for neonatal death (Fig. 14) any further.

Working with a considerably larger number of mutants and combinations than the Waelsch-Cori group, we, like they, find no evidence for a gene-dosage effect in measurements of G6Pase activity. Because of this absence of a gene-dosage effect, the fact that there were, in some neonatal mutants, also morphological abnormalities whose genesis must have occurred prior to the normal time of action of G6Pase, and other reasons, Gluecksohn-Waelsch and Cori have speculated[5] that the albino lethals are not structural genes for G6Pase but that the basic effect of these mutants is to cause an abnormality of membrane structure through an effect on membrane proteins. More recently, Thorndike et al.[6] have demonstrated another biochemical abnormality in some of their mutants (though it is not clear in which ones), namely, a deficiency in tyrosine aminotransferase (TAT). Again, no gene-dosage effect was found. The authors feel that the new results strengthen their earlier conclusion that the albino lethals do not represent mutations of the structural

Table 12. Distribution of mutants by complementation group and mode of origin

Mode of origin	A	A′	B	C	D	?
Spermatogonia	5 [4.4]	2 [1.5]	3 [8.8]	0 [0.7]	6 [6.6]	2 pre + 1 neo
Postspermatogonia	0 [0.5]	0 [0.1]	2 [0.9]	0 [0.1]	2 [0.7]	
Oocytes	1 [1.1]	0 [0.4]	7 [2.3]	1 [0.2]	1 [1.7]	
Total	6	2	12	1	9	3
X or γ	4 [4.0]	2 [1.3]	3 [7.9]	1 [0.7]	6 [5.9]	1 pre
Neutrons	2 [2.0]	0 [0.7]	9 [4.1]	0 [0.3]	3 [3.1]	1 pre + 1 neo
Total	6	2	12	1	9	3

genes for G6Pase and for TAT. They suggest that the rough endoplasmic reticulum, or a step in the induction pathway, may be the target of the mutant effect. We feel that it will be very important to determine whether there can be complementation for the TAT activity separate from complementation for G6Pase activity, and we have initiated experiments to explore this.

In view of the demonstration we have already provided, that complementation for neonatal death can occur without complementation for albinism and separate from complementation for various kinds of prenatal death, we are inclined to suggest that many, if not all, the albino lethals, including those studied by Waelsch and Cori's groups, may be very small deficiencies involving several cistrons. It is therefore important to exert caution in drawing conclusions about gene action on the basis of multiple effects. Our continuing complementation tests, using biochemical as well as morphological characters, should be most helpful in the analysis of genetic organization and gene action.

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INITIATION OF A PROGRAM TO STUDY HETEROZYGOUS EFFECTS OF INDUCED MUTATIONS ON FITNESS

Liane B. Russell and W. L. Russell

It is quite possible that induced mutations may exert most of their effects on human populations while in the heterozygous state. Empirical experiments have been reported by many authors[1] on the effects on mammalian populations of radiation administered to preceding generations, but most of the conclusions were equivocal, and nothing was known in such experiments about the number, location, or nature of new mutant genes actually in the population. On the other side, while specific-locus mutation-rate experiments and the subsequent analysis of mutants have provided considerable data about the types of mutations induced following treatments delivered under various physical and biological conditions, more information should be added to what is known[2] about the heterozygous effects of these mutations. We feel that it is of paramount importance for the estimation of genetic effects in human populations to utilize the well-characterized genetic material provided by the specific-locus mutation-rate experiments for studies of heterozygous effects, and we have therefore embarked on such a program, even though it will take several years for completion.

Last year[3] we reported on relative survival to weaning age (as measured in segregating progeny) of animals heterozygous for a lethal at either the *d* or *se* locus, using for this purpose 12 d^{pl} and 5 se^{l} mutants which had been characterized in earlier complementation studies.[4] Although these results could detect effects of large magnitude, it was obvious that, for a more critical study, one would need contemporary control matings segregating for nonlethal alleles or "standard" variants at the same loci and that the genetic background for these various matings would have to be uniform. With such a setup one could then measure other parameters as well (e.g., longevity and reproductive capacity).

We have embarked on a long-term program that will achieve these ends, utilizing a number of mutants at each of the *d, se,* and *c* loci. These mutants have been chosen not only because they have been characterized in complementation studies but also because the close linkage of *d* and *se* and the existence of intermediate alleles at *c* facilitate the process of achieving coisogenic populations. The various mutants, as well as the "standard" variant at a given locus, are being made coisogenic with an available inbred strain. After 15 generations of repeated backcrossing, which we hope to achieve after three to four years by use of a special breeding program, we shall expand the populations and compare the segregant ratios in the mutant stocks with the segregant ratios in the standard-variant stock on the same inbred background. For example, after 15 generations of crossing + se^{l}/d + × *d* +/*d* + (inbred) and + *se*/*d* + × *d* +/*d* + (inbred) — to make both se^{l} and *se* coisogenic with the same *d* +/*d* + inbred strain — we shall compare + se^{l}/d +:*d* +/*d* + ratios with + *se*/*d* +:*d* +/*d* + ratios at various ages to determine the heterozygous effect of se^{l} on survival. At the same time, both coisogenic populations will be crossed to another inbred strain to measure the same parameters under conditions of hybrid vigor (along with genetic uniformity).

To date we are only in the first generation of the various crosses. In the case of a few other specific-locus mutants, however, breeding for coisogenicity was initiated some time ago. Thus, 18 independently induced mutations at the *a* locus, some of which are homo-

zygous lethal but all of which, in combination with *a*, produce a coat color distinguishable from *a/a* or *A/a* (generic symbol, a^x), are being made coisogenic with our C57Self inbred strain, which is *a/a*. In all of the stocks, backcrossing has proceeded past F4; and in three of them, it is well past F15. In the latter group there is no clear evidence for heterozygous effect on survival to weaning age. Other measurements of fitness have, however, not yet been made.

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INDUCTION OF MUTATIONS IN A CHROMOSOMAL SEGMENT: A NEW METHOD

Liane B. Russell and Clyde S. Montgomery

The characterization in the course of our complementation analyses of certain deficiencies spanning specific loci made it possible to develop a method for the detection of new mutations within certain chromosome segments. It was hoped that such a method would yield new kinds of mutation-rate information, provide additional data on the number of functional units per recombinational length, and lead to the recovery of useful genetic material. Last year, the stocks were produced, some of the irradiations carried out, and initial matings made. This year saw the beginning of the experiment proper.

Mice homozygous for the standard markers are irradiated and mated to wild type. The F_1 animals 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 at least two and no more than ten map units. Each tested F_1 represents one irradiated chromosome, the test cross being *d se* (irrad. chrom)/++ × Df(d)+/+ Df(*se sv*). If a lethal has been induced in one of the marked segments, either *d* or *se* progeny will be lacking except for the relatively rare possibility of a crossover between the lethal and the nearest marker. (Since neither deficiency can be longer than 6 centimorgans, the maximum probability of progeny with marker phenotype – despite presence of a lethal in the segment – is 2%). Similarly, if a grossly visible mutation has been induced within the marked segment, it will show up in the phenotypically *d* or *se* progeny.

Since the doubly deficient mates for the tested F_1 are relatively inviable and thus at a premium, a mating setup has been developed where a given male is moved at weekly intervals through 7 tiers of 4 females each, returning in time for the first 4 females to be ready to mate again after having raised their litters. At any one time, 1736 F_1 females are in the test setup (about half of them being controls), but because of the poor productivity of the doubly deficient mates, only a fraction of these conceive.

As soon as an F_1 female has produced two or more each of normal-appearing *d* and *se* progeny, she is discarded as nonmutant. If she fails to produce a *d* or *se* offspring in her first 12 or more young (expectation, $\frac{1}{4}$ of each) or has given only a single such offspring in her first 18 or more, she is classified "possible exceptional," and some of her offspring (*d*, if *se* is lacking; and vice versa) are saved for further testing.

To date, results are available for an experiment in which a dose of 600 rads of x rays was given to spermatogonia. (Another series with irradiation to postgonial stages has only recently been initiated.) The total number of chromosomes tested as nonmutant is 885 in the irradiated series (12,414 progeny classified) and 850 in the control series (11,513 progeny classified). In addition, approximately 500 chromosomes are partially tested nonmutant (i.e., at least one normal *d* and one normal *se* young produced to date). The number of "possible exceptional" F_1 females was 101; 28 and 73 of these showed absence or deficiency of *d* and *se* progeny, respectively. Further accumulation of progeny from 26 of these 101 females subsequently removed them from the "possible exceptional" category. Test matings of offspring have been made in the case of an additional 35. Presumably virtually all of these 35 and the remaining 40 females will turn out, on further testing, to be in the "possible exceptional" category by virtue of representing one tail of the distribution.

An estimate of the number of lethal mutants to be expected has been calculated on the assumptions that the mutation rate in the segment is similar to that for the seven specific loci and that the number of functional lethal units between *d* and *se* that has been found in complementation studies is representative of the distribution of such units over the whole segment. If these assumptions are correct, one should detect a new lethal in each 350 to 560 tested chromosomes per crossover unit being screened. Since the deficiencies used measure at least 2 centimorgans, the irradiated group tested to date should contain at least 4 or 5 lethal mutants, if the above assumptions are correct.

In addition to possible mutations in the screened segment, the experiment to date has yielded two other dominant mutants elsewhere in the genome: a *Ta*-like and a cataract-microphthalmia mutant.

It should be pointed out that while this method undoubtedly will turn out to have merit for the purposes for which it is being used, it would be quite inefficient for experiments in which the main purpose is to compare the effects of various treatments on induction of mutations. The number of test animals that can be screened in a few minutes in a specific-locus experiment would take a week to screen with this method.

ELUCIDATION OF X-INACTIVATION MECHANISMS FROM AUTORADIOGRAPHIC STUDIES OF X-AUTOSOME TRANSLOCATIONS IN THE MOUSE

N. L. Cacheiro, Liane B. Russell, and Margaret S. Swartout

X-autosome translocations [T(X;A)'s] constitute uniquely suitable material for elucidation of problems of X-chromosome inactivation. Since, in reciprocal T(X;A)'s, the genetic material of one of the X's is partitioned to two chromosomes which are cytologically identifiable, the translocations can be used to determine whether inactivation affects both portions of the X, to estimate the extent of inactivation of the autosomal segment, and to shed light on questions of randomness of inactivation. It is generally accepted that late replication of one X is a concomitant of inactivity. Labeling early in S period leaves the late-replicating X selectively unlabeled.[1] This method has been used in the present investigation, in conjunction with quinacrine-mustard (QM) staining which facilitates identification of chromosomes by their banding patterns.

Six reciprocal translocations, originally discovered and extensively studied at this laboratory, were used, as well as the *flecked* insertion (both balanced, type I, and duplication, type II, animals). Of the reciprocal translocations, four (R2, R3, R5, R6) are T(X;1)'s, and two (R1 and R7) are T(X;8)'s. Tissue culture cells from two young adult females of each of the types enumerated were labeled early in S period with [^{3}H] thymidine, and QM staining was used to identify chromosomes by their banding patterns. Cells were first examined to see whether or not they had sufficient [^{3}H] thymidine incorporation. Subsequently, those that were adequately labeled were analyzed as to the amount and distribution of label in the X and in the translocation product(s). The long and short translocation products will be referred to as X^t and *t*, respectively (regardless of whether the centromere is in the X or autosomal portion), while the intact X will be referred to as X^n.

Of 2937 cells in the preliminary screening, 1048, or 35.7%, had adequate incorporation of [^{3}H] thymidine. Among these 1048 were 112 cells in which all chromosomes had substantial numbers of grains (presumably labeled mid rather than early S), leaving 936 that exhibited some sort of asynchrony. In about 94.9% of these, either the X^n or a major portion of the X^t was unlabeled (presumed inactive). The distribution for the various translocations is shown in Table 13.

The last two columns of Table 13 show the distribution, among differentially labeled cells only, of cells in which X^n or X^t, respectively, is unlabeled (presumably inactive). Very marked departures from equal proportions are found for the *fd* insertion and for R6 and R5, with a great preponderance of cells having X^n unlabeled. A lesser inequality in the same direction is found in the other T(X;1)'s; but in the two T(X;8)'s there is either equality or actually a slight inequality in the opposite direction.

The present results for the *fd* translocation differ from earlier ones of Nesbitt and Gartler,[1] who found random inactivation in cells derived from fetal tissue cultures. It may therefore be suggested that, in kidney cells, selection occurs against cells in which X^t is inactive. Such selection could be explained on the basis of a locus in LG1 whose activity is vital to survival of kidney cells: in cells where X^t is inactive, this locus would be inactivated some or all of the time, and the cell would then be selected against. This hypothesis is being tested in recently initiated experiments with T(X;1)'s, using tissue cultures derived from 18-day fetuses.

Table 14 shows average grain counts in the small translocation product in those cells in which X^n is unlabeled and in those in which X^t is unlabeled. The two values are approximately equal in the four translocations already studied, indicating that inactivation occurs only in the long translocation product, where it is an alternative to inactivation in the intact X. This supports the hypothesis advanced some time ago[2] of an inactivation center or region in the X: the portion of the X that has become separated through translocation is apparently no longer subject to inactivation. Cytological work reported last year,[3] as well as earlier genetic findings,[2,4] show clearly that X^t can have either an X centromere (R1, R5, R6) or an autosomal centromere

Table 13. Distribution of cells according to X-chromosome labeling in seven X-autosome translocations

Translocation	Total number of labeled cells	No. of cells[a] with –				Percent of differentially labeled cells	
		All chromosomes labeled	$X^n = 0$ $X^t = 0$	$X^n = 0$	$X^t = 0$	$X^n = 0$	$X^t = 0$
R1	199	16	11	78	94	45.4	54.6
R7	76	8	8	27	33	45.0	55.0
R2	103	9	1	59	34	63.4	36.6
R3(212G)	279	27	12	189	51	78.8	21.2
R3(MWA)	79	20	3	40	16	71.4	28.6
R5	84	6	8	62	8	88.6	11.4
R6	67	8	3	55	1	98.2	1.8
fd-type I	31	2	1	27	1	96.4	3.6
fd-type II	130	16	1	106	7	93.8	6.2
Total	1048	112	48	643	245		

[a] $X^n = 0$: cells with less than 3 grains over the intact X. $X^t = 0$: cells with no grains over X^t or with grains only over the autosomal region.

Table 14. Average grain counts in small translocation product of various T(X;A)'s

Translocation	Cells with $X^n = 0$[a]		Cells with $X^t = 0$[b]	
	Number of cells	Av grains over *t*	Number of cells	Av grains over *t*
T(X;8) R1	54	5.2	53	4.8
T(X;8) R7	13	5.7	16	6.6
T(X;1) R3	53	9.0	20	9.1
T(X;1) R5	62	3.4	8	3.0

[a] $X^n = 0$: cells with less than 3 grains over the intact X.
[b] $X^t = 0$: cells with no grains over X^t or with grains only over the autosomal region.

(R7, R2, R3), indicating that the inactivation center is not the centromere.

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TIMING OF OOCYTE DEVELOPMENT IN THE ADULT MOUSE

E. F. Oakberg and Patricia D. Tyrrell

The ability to relate specific oocyte (and follicular) stages to specific postirradiation litters is a primary step in understanding the dramatic change in radiation-induced mutation rate that Russell[1] observed for different oocyte stages in the adult. The dynamics of the concurrent processes of oocyte and follicle development in the adult female and the effect of pregnancy and radiation on these processes, however, are not known with the precision required for investigation of the biochemical, metabolic, and morphological factors involved in the mutation response.

Recently Pedersen[2] estimated that an oocyte in a follicle of 20 to 30 cells required about 19 days to reach maturity and be ovulated. However, 50 R reduces the number of oocytes in follicles of less than 20 cells to less than 1% (0.13%) of control, whereas oocytes in follicles with an antrum are not killed. We had assumed that the 4+ litters borne by these females came from these late follicle stages. The 19-day development time given by Pedersen[2] would lead to exhaustion of the oocyte supply before four litters could be conceived. This is especially true since loss of oocytes in irradiated

females occurs at a more rapid rate than in controls,[3] and oocyte loss is the same in pregnant and virgin females.[3]

Adult female mice (101 X C3H), eight weeks old, were given two injections of 50 μCi of N-(acetyl-^{3}H)-D-glucosamine 16 hr apart (total of 100 μCi per female). Mice were killed at intervals from 1 hr to ten weeks after injection, and the ovaries were fixed in Zenker-Formol. Tissues and slides were treated by the technique of Kopriwa and Huckins,[4] which allows both PAS staining of the mucopolysaccharide of the zona pellucida and autoradiography.

Positive labeling was observed in the zonae of all oocytes in follicle stages from late 3 (a single layer of cuboidal cells) through stage 8 (mature Graafian follicles). By five weeks all oocytes with labeled zonae had disappeared from the ovary, a result of both atresia and ovulation. (Labeled zonae from degenerating oocytes were observed ten weeks after treatment.) The progressive appearance of unlabeled oocytes and concurrently developing follicles was used to obtain an estimate of about 35 days for a follicle of 20 to 30 cells to reach maturity and ovulation. This is almost twice the 19 days estimated by Pedersen,[2] but still not long enough to account for the number of litters conceived after 50 R. We are planning experiments to determine the rate of development of older follicle stages in the irradiated pregnant female in hope of resolving this problem.

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SPERMATOGONIAL SURVIVAL AFTER INTRAPERITONEAL INJECTION OF TRITIATED WATER (THO)

E. F. Oakberg and Patricia D. Tyrrell

The assessment of killing of A_S (stem) spermatogonia in mice by THO was undertaken in order to provide an estimate of the severity of spermatogonial selection, to estimate the x-ray dose required for comparable effect, and to estimate a probable x-ray–tritium RBE.

Male hybrid mice were given an intraperitoneal injection of 0.5 mCi THO per gram of body weight and killed at 3, 5, 12, 19, 33, and 47 days after injection. Testes were fixed in Zenker-Formol, and 5-μm sections were stained by the PAS-hematoxylin technique. Surviving spermatogonia and preleptotene spermatocytes were counted in a sample of 99 tubule cross sections, distributed among the 12 stages of the cycle of the seminiferous epithelium on the basis of tubule frequency in controls, for each mouse. Spermatogonia were classified according to the description of Oakberg.[1] Testis weight loss was severe, and Sertoli cells were counted in order to correct for tubule shrinkage. All counts were converted to spermatogonia/Sertoli cell ratios, and the experimental values expressed as fraction of control.

The x-ray doses estimated from stem cell survival were somewhat higher than tritium doses estimated from scintillation counting.[2] The data are not complete and have not been corrected for wasted irradiation (i.e., the dose was taken as that estimated at the time of killing, yet a portion of this dose would not have had time to affect cell survival). The discrepancy was greater at the longer time intervals owing to the effect of the continuous tritium irradiation on the repopulation process. The 3.57 and 3.14% survival observed at 5 days for two groups of mice is comparable to an x-ray dose of ~600 R, and the estimated tritium dose is 483 R. The 0.5% survival at 12 days is comparable to a 1000-R dose divided into two 500-R fractions 24 hr apart. The estimated tritium dose is 602 R. On the basis of these results, an RBE somewhere between 1 and 2 would be estimated for the tritium/x-ray comparison. This figure, however, must be considered as only a crude estimate. In view of the different nature of the radiations and their different distribution in space and time, it may be more meaningful to strive for sufficient tritium data to reach hazard estimates independent from the RBE concept.

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2. Gary Sega, personal communication.

THE CYTOTOXIC ACTION OF HYCANTHONE ON MOUSE SPERMATOGONIA AND A COMPARISON WITH RADIATION

E. F. Oakberg and Patricia D. Tyrrell

The present experiments were prompted by the failure to detect mutations in mouse germ cells after an intraperitoneal injection of 150 mg/kg of hycanthone free base.[1] Demonstration of a cytotoxic action on spermatogonia would indicate that failure to reach the germ cells could not be invoked as an explanation of the lack of mutagenic activity of hycanthone.

F_1 hybrid male mice (101 X C3H), 12 weeks old, were given either intraperitoneal (IP) or intramuscular

(IM) injection of 24, 37.5, 75, 118, and 150 mg/kg of hycanthone free base suspended in cold balanced Hanks' solution. Controls were given cold balanced Hanks' solution. Animals were killed in groups of four at 72 and 120 hr after injection, and the testes fixed in Zenker-Formol. Five-micrometer paraffin sections were stained by the PAS technique and counterstained with hematoxylin. Numbers of spermatogonia and preleptotene spermatocytes were scored in a comparable sample of tubules for each mouse, and final values were expressed as percentage of control.

Survival 72 hr after IP injection revealed a progressive increase in sensitivity which paralleled spermatogonial differentiation. A_s (stem) cells were the most resistant; A_4-In the most sensitive. Whereas a comparable result was observed at 150 mg/kg after IM injection, a lesser response at 75 and 118 mg/kg was obtained for A_3, A_3-A_4, and A_4-In spermatogonia. The data suggest that generalized defense mechanisms, including detoxification, excretion, and repair of the host, were equally effective for either injection route at low doses but were overridden at 150 mg/kg. The lesser response of A_3-In spermatogonia at intermediate doses after IM injection can be considered as a dose rate phenomenon resulting from slower absorption. That this is a cell-specific effect is demonstrated by no difference between IM and IP groups for A_1 and A_2 spermatogonia and by the consistently lower response of A_s cells at all doses after IM injection.

Recovery after hycanthone was more rapid than after irradiation, and the A_{al}, A_{pr}, and A_1 spermatogonia already had returned to control levels by five days. Thus, even at the highest doses, hycanthone should have no effect on fertility.

Survival curves for A_3, A_3-A_4, and A_4-In spermatogonia for both hycanthone and ^{60}Co gamma rays giving comparable survival were compared. Although the curves were closely similar, the drug was consistently less effective at low doses and more effective at high doses. (That is, the shoulder of the curve was greater, but the remainder of the curve had a steeper slope after hycanthone.) Using the same scale, survival of A_s spermatogonia after hycanthone was consistently higher after all doses, demonstrating the greater resistance of A_s spermatogonia. Also, the difference in sensitivity between A_s and A_3-In spermatogonia is greater for hycanthone than for gamma rays.

These data demonstrate that dose, route of injection (IP or IM), time of observation, end point scored, and cell type all are important variables in evaluating the total response of spermatogonia. The complexity of the response emphasizes the difficulty in estimating possible hazards and the pitfalls that await those who base conclusions on a single dose, cell type, or observation time.

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1. W. L. Russell and E. M. Kelly, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, pp. 113–14.

DOSE EFFECTS OF MUTAGENIC CHEMICALS IN THE INDUCTION OF CHROMOSOMAL ABERRATIONS

W. M. Generoso, W. L. Russell, Sandra W. Huff, and Katherine T. Cain

Dose is a very important consideration in the evaluation of mutagenicity of chemicals. Yet, information on the dose effect of chemicals in the induction of genetic damage to mammalian germ cells is meager. For a proper evaluation of the genetic hazards of chemicals to humans, it is necessary to study in mammals the extent to which a variety of mutagenic chemicals differ in their efficacy at low as well as at high doses. In other words, what are the shapes of the dose-effect curves for a variety of chemicals? We are studying this problem in depth, and this report is on the progress of this study.

Initial experiments now in progress involve the compounds ethyl methanesulfonate (EMS) and triethylenemelamine (TEM). Dose effects of these two compounds are being studied in spermatozoa and spermatids, using dominant lethal mutations and heritable translocations as end points (for details of the heritable translocation procedure, see ref. 1). This study allows us to make two comparisons: (1) between chemicals in their effectiveness in inducing chromosome breakage at high and low doses and (2) between dominant lethal mutations and heritable translocations as indicators of chemically induced chromosome breakage in male germ cells. EMS was tested from 50 to 300 mg/kg and TEM from 0.0125 to 0.4 mg/kg. The EMS experiments are now completed. For TEM only the dominant-lethal dose-effect study is completed, but some information on heritable translocations at low doses is already available.

EMS and TEM differ markedly in the shapes of the dominant-lethal dose-effect curves at doses below the saturation point. For EMS, the dominant-lethal dose-response curve is clearly not linear – it is markedly concave upward. Similarly, the EMS translocation dose-response curve showed that there is a more rapid increase in the number of translocations with dose than would be expected on the basis of dose-response kinetics. These results clearly indicate that the effectiveness of EMS in inducing chromosome breakage to

postmeiotic male germ cells is much lower at low doses than at high doses. The TEM dose-effect curve for dominant lethal mutations, on the other hand, approaches linearity and shows that this compound is proportionately more effective at low doses than EMS.

Although the dose-effect study of TEM-induced heritable translocations is not yet completed, it is already possible to compare the relative efficiency of dominant lethal mutations and heritable translocations as end points for measuring chromosome breakage induced by the two compounds. In the EMS and TEM studies, the lowest doses at which significant increases in induced dominant lethal mutations were detected are 150 and 0.05 mg/kg, respectively, whereas significant increases in heritable translocations were already detectable at 50 and 0.0125 mg/kg, respectively. In the EMS 50-mg/kg and TEM 0.0125-mg/kg doses, the translocation induction rates are 6 in 853 and 6 in 914 F_1 males tested, respectively. These rates are significantly higher than the spontaneous rate of 3 translocations in 2633 F_1 males tested with p values equal to 0.009 and 0.012, respectively. It is likely that for these two compounds, dominant lethal mutations were induced at doses lower than 150 and 0.05 mg/kg (for EMS and TEM, respectively) but were not detected because of the relative insensitivity of the dominant-lethal procedure. In addition to the higher sensitivity of the translocation procedure, translocations are a much more reliable end point in terms of human hazard than dominant lethal mutations, because unlike the latter they represent transmissible genetic damage. Thus, for the detection of low levels of induced chromosome breakage, translocations appear to be a more reliable end point than dominant lethal mutations. From the practical standpoint, it is important to find out if this relationship between heritable translocations and dominant lethal mutations is also true for a wide variety of compounds.

Another interesting comparison between EMS and TEM that has importance from the practical point of view lies in the ratio of the lowest genetically effective dose (as measured by heritable translocations) to lethal dose (Table 15). This ratio for TEM is 1/400, while for EMS it is only 1/8.5. Thus it is clear that TEM, in contrast to EMS, is mutagenic far below the toxic level. The finding that TEM is mutagenic at extremely low doses strengthens the belief that chemicals in the human environment constitute a potential genetic hazard and that chromosome breakage and its consequences are important components of this hazard.

1. W. M. Generoso, D. G. Gosslee, and W. L. Russell, "A Sequential Procedure for the Detection of Translocation Heterozygotes in Male Mice," this report.

Table 15. Relative effectiveness of EMS and TEM in inducing heritable translocations

Chemical compound	Lowest genetically effective dose, A (mg/kg)	Lowest lethal dose, B (mg/kg)	A/B
EMS	50	425	1/8.5
TEM	0.0125	5	1/400

INDUCTION OF CHROMOSOMAL ABERRATIONS IN MOUSE SPERMATOGONIA WITH CHEMICALS AND X RAYS

W. M. Generoso, Katherine T. Cain, and Sandra W. Huff

A thorough understanding of chemical effects in spermatogonia stem cells is necessary because this germ cell stage is the most important in terms of genetic hazard to males. Cytological analysis done by a number of workers, including us, at the diakinesis–metaphase I spermatocytes showed that while x rays are effective in inducing reciprocal translocations on treated spermatogonia stem cells, alkylating chemicals are not. This is a paradox since these chemicals, like x rays, are potent chromosome breakers in the postmeiotic stages. It is well known, however, that very often a reciprocal translocation does not manifest itself in diakinesis–metaphase I stage. Thus there remains the possibility that translocations not detectable cytologically can be passed through some of the progeny. To check on this possibility and to have a thorough understanding of the chemical and radiation hazards to the spermatogonia, we are studying extensively the induction of heritable reciprocal translocations at this cell stage with chemicals and x rays. It should be emphasized that with radiation the amount of data on the rates of induced heritable translocations in spermatogonia stem cells is also still very limited.

Twelve-week-old (101 × C3H)F_1 male mice were either injected intraperitoneally with various concentrations of TEM, TEPA, or cyclophosphamide (doses, in mg/kg, are 3.0 and 4.0; 20, 25, and 30; and 350 and 400, respectively) or exposed (partial body) to acute doses of 150 R, 300 R, 600 R, and 1200 R. Each male was caged with a female of the (SEC × C57BL)F_1 or (C3H × C57BL)F_1 strain 42 days after treatment. Several litters were weaned from each breeding pair.

Data on effects of the three chemicals and x rays on cell killing and repopulation – accomplished indirectly by observing the induction of sterility and the length of time required for the recovery of fertility – and on the

frequency of translocations as observed in the diakinesis–metaphase I spermatocytes have already been reported.[1,2] At the present time, all F_1 males needed in the study are already weaned. In the x-ray experiment, the numbers of F_1 males weaned are 1177, 1109, 1126, and 1094 for the doses of 150 R, 300 R, 600 R, and 1200 R, respectively. A total of 38 completely and partially sterile F_1 males have been found so far from all doses, but the actual rates for each dose and the dose-effect curve cannot be made at the present time because the testing is not complete yet. In the chemical study, the numbers of F_1 males weaned for each chemical are (all doses combined) 1161 for cyclophosphamide, 1057 for TEPA, and 1669 for TEM. The testing for translocation heterozygosity of the F_1 males has just begun.

1. W. M. Generoso, R. J. Preston, and Katherine T. Cain, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, pp. 124–25.
2. R. J. Preston, J. G. Brewen, and W. M. Generoso, *Biol. Div. Annu. Progr. Rep. June 30, 1972,* ORNL-4817, p. 69.

A SEQUENTIAL PROCEDURE FOR THE DETECTION OF TRANSLOCATION HETEROZYGOTES IN MALE MICE

W. M. Generoso, D. G. Gosslee,* and W. L. Russell

As we gain better understanding of the efficacy of certain mutagenic chemicals in inducing chromosome breakage in mammalian germ cells, it becomes increasingly clear that this class of genetic damage represents an important hazard to the human population. For instance, in male mice, TEM induced a significant increase in heritable reciprocal translocations at the dose of 0.0125 mg/kg, which is about 1/400 of the lethal dose.[1] Reciprocal translocation is an important consequence of chromosome breakage from the point of view of human hazard. It is well known that reciprocal translocations and the secondary aberrations arising from them constitute a significant proportion of chromosomal anomalies in man.

So far, evaluation of chromosomal aberrations as genetic hazards of chemicals has been made primarily by using results of cytogenetic studies of certain types of somatic and germinal cells. These two test systems do not measure transmissible genetic effects, which are the most important mutagenic effects. Our findings, which show that heritable translocations are a more accurate and sensitive end point for measurement of low levels of TEM- or EMS-induced chromosome breakage in male mice than dominant lethal mutations,[1] clearly point to a more extensive use of the translocation procedure in the evaluation of the ability of chemicals to induce chromosome breakage. To this end, we are developing the translocation procedure to become more suitable for wide-scale screening and experimentation.

This procedure is the result of our extensive study on the fertility of EMS-induced partially sterile translocations. The procedure as it stands now is as follows. The key to this screening procedure is the exceptional fertility of (SEC × C57BL)F_1 females. Each F_1 male to be tested is caged with an (SEC × C57BL)F_1 female. The females are used for the first time when they are 10 to 12 weeks old. Breeding pens are checked for newly born mice when they are expected (i.e., pens are examined daily during weekdays beginning 18 days after pairing and 18 days after appearance of a litter). Young are discarded immediately after they are scored. For each male, if the size of the first litter is 10 or more, the male is declared fertile and discarded immediately after scoring the litter. If the first litter is less than 10, a second litter is scored. If the second litter is 10 or more, the male is declared fertile and discarded; otherwise the male is a suspect and is tested further by mating him to three virgin females which are killed during pregnancy. In either case, another male is placed with the female one week after the litter is born, and the same procedure is followed until the last male is added no later than the tenth litter. The lapse of one week is required so that parentage of litters will not be confused. From our study on the fertility of partially sterile translocation heterozygotes, the probability that a partially sterile male will sire a litter of size 10 or more is estimated as 0.02.

Analysis of the fertility data of the (SEC × C57BL)F_1 females mated with fully fertile males revealed that, if litter size of 10 or more in the first or second litter sired by a male is used as the indicator of full fertility and if each female is allowed to produce 12 consecutive litters, an average of 5.27 males from a sample free of translocations may be tested per female. Out of these, the number of test males per female that can be declared fully fertile on the basis of one or two litters sired is estimated as 4.20. Thus it is now possible to test several F_1 males per female instead of killing three or more females per F_1 male as in the old procedure. The new procedure is now routinely used in our laboratory for screening male translocation heterozygotes in chemical and radiation experiments.

At the present time the spontaneous frequency of heritable translocations, pooled from the controls of the EMS and TEM experiments, is 0.001139 (3 trans-

locations in 2633 progeny tested). Using this spontaneous rate and specified probability levels, the number of F_1 males needed to be tested for each given rate of induction can be calculated (Table 16). The exact probabilities for selected sample sizes in Table 16 were calculated using the binomial distribution directly. The probability of falsely declaring an induced translocation rate (C/N) to be greater than the spontaneous rate is α. Due to the discrete nature of the binomial distribution, the actual level of significance cannot be constant for all the sample sizes (N). In all cases, the sample sizes were chosen so that the probability level for a significant difference from the spontaneous rate is close to 0.05 and so that the probability of detecting rates higher than the spontaneous rate is 0.95 or higher.

For example, in the EMS comparative dominant-lethal and translocation studies, it was found that the lowest dose at which a significant increase in dominant lethal mutations was detected was 150 mg/kg. EMS doses of 50 or 100 mg/kg, on the other hand, induced significant increases in heritable translocations at the rates of 0.7 and 1.78%. From Table 16, the number of 500 F_1 males was calculated as the minimum number needed to be tested in order to detect, with a probability of 0.95, a translocation rate of $\Pi = 1.5\%$, a rate which is lower than what the EMS dose of 100 mg/kg induced. As Π decreases, N increases, but the sample size of 500 F_1 males is certainly not too large for the purposes of large-scale screening. Improvement of the translocation procedure, especially in the determination of statistical limits and levels of significance, is expected as more data become available (1) on the comparative dominant lethals and translocation dose-effect studies with a number of mutagenic chemicals and (2) on spontaneous level of translocations.

Table 16. Minimum number (N) of F_1 males needed to detect with a probability of at least 0.95 a translocation rate of Π

N	C^a	α^b	Π
300	2	0.047	0.020
500	3	0.020	0.015
700	3	0.047	0.010
1800	5	0.057	0.005
3000	7	0.059	0.004

[a]Minimum number of translocations needed to declare that the observed rate is significantly larger than the spontaneous rate of 0.001139.

[b]Actual level of significance for a nominal level of 0.05.

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1. W. M. Generoso, W. L. Russell, Sandra W. Huff, and Katherine T. Cain, "Dose Effects of Mutagenic Chemicals on the Induction of Chromosomal Aberrations," this report.

CHEMICAL INDUCTION OF SEX-CHROMOSOME LOSS IN FEMALE MICE

W. M. Generoso, Katherine T. Cain, and Sandra W. Huff

The X-chromosome-loss method has already been proven to be a reliable genetic system in the studies of Liane B. Russell and W. L. Russell for measuring radiation-induced chromosomal aberrations in the mouse oocytes. One of the objectives of the present study is to explore the possibility of using this genetic system as an efficient test for measuring chemically induced chromosomal aberrations in female mice. Such a genetic system is much needed in case of chemical studies because all the evidences available so far, although strongly suggestive of the effectiveness of chemicals in inducing chromosomal aberrations, are of the nontransmissible kind, thus by no means unequivocal. For instance, all chemicals we have studied so far that induce dominant lethals in males also induce embryonic mortality among treated females. Unfortunately, since the females themselves received the treatment, embryonic mortality in females is not conclusive evidence of induced chromosomal aberrations. Another important point in regard to this objective is the fact that with some chemicals, such as hycanthone and IMS, we have found that females are more sensitive than males in the induction of embryonic mortality. In fact, in the case of hycanthone, embryonic mortality was induced in females but not in males. Thus, we hope to study the relationship between presumed dominant lethal mutations and X-chromosome loss in females for a number of chemicals.

We have started some experiments to score X-chromosome loss in female mice injected with IMS. X^+/X^+ females of the (SEC × C57BL)F_1 or (C3H × C57BL)F_1 strain were injected intraperitoneally with either 75 or 50 mg/kg of isopropyl methanesulfonate (IMS). 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}/O). Presumed X^{gs}/O were tested by mating them to *sparse fur* males ($X^{spf}Y$). The appearance of *sparse fur* female progeny ($X^{spf}O$) is taken as the confirmation.

The study is still in progress, but data from early litters (born within 43 days post treatment) are already

adequate for comparison with controls. In IMS-treated (SEC X C57BL)F_1 females, 4 *Greasy* female progeny (confirmed XO) were found out of 1473 scored, and in (C3H X C57BL)F_1 females, 4 *Greasy* female progeny (1 confirmed XO, the other 3 being tested) were found out of 2860. In the control groups, so far, no XO individual was found out of 2318 and 2037 female progeny scored in the (SEC X C57BL)F_1 and (C3H X C57BL)F_1 strains, respectively. Combining data for the two strains, the minimum of 7 and the maximum of 8 XO females out of 4333 are significantly higher than the spontaneous level with p values equal to 0.007 and 0.004, respectively. This clearly indicates that IMS induces sex-chromosome loss in female mice and is the first case in which a chemical has been shown to induce a heritable chromosomal aberration in the mouse oocyte.

CHEMICAL INDUCTION OF X-CHROMOSOME NONDISJUNCTION AND LOSS IN MOUSE OOCYTES

K. E. Suter

Chromosomal aberrations fall into two major classifications – those that result from chromosome breakage and those that arise through chromosomal nondisjunction. In the past, chromosomal aberration studies with chemicals on mammalian germ cells have concentrated on the problems related to chromosome breakage, and virtually no work has been done on the effects of chemicals on chromosomal nondisjunction. In addition to chromosome breakage, there is great concern for the possible chromosomal nondisjunction effect of chemicals in germ cells. There are two main reasons for this concern. First, aneuploidy from nondisjunction in the sex chromosomes and certain autosomes constitutes the most common class of chromosomal anomalies in man. Second, there is a wide variety of chemical actions, and it is well known that certain chemicals such as certain heavy metal compounds interfere with normal meiotic or mitotic divisions. For instance, genetic investigation in *Drosophila*[1,2] showed that methylmercury and triethyllead increased the incidence of meiotic nondisjunction. It is believed that the disturbance in chromosome segregation was brought about by the interaction of these metal compounds with the spindle fiber mechanism. At present there is a dearth of information on chemical effects on chromosomal segregation in mammalian germ cells. Thus, the importance of this research is emphasized in the evaluation of genetic hazards of chemicals to the human population.

Preliminary studies that are necessary before the X-chromosome nondisjunction and loss experiment in female mice can be carried out have been started. These include (1) the toxicity effects of single intraperitoneal injections of monomethylmercury, mercuric chloride, and cadmium chloride (Table 17) to establish the doses

Table 17. Toxicity effects of monomethylmercury, mercuric chloride, and cadmium chloride on 101 x C3H females

Chemical	Dose (mg/kg)	Number of injected animals	Number of dead animals[a]	Number of mated females within 5 days after treatment[b]	Number of fertile matings
Monomethylmercury	0.0	12		12	9
	10.0	12		11	11
	12.5	12		6	2
	15.0	11	2	2	
	17.5	12	10	1	1
Mercuric chloride	0.0	12		11	11
	2.0	12		10	9
	3.0	12	1	5	2
	4.0	12	3	4	
	7.5	12	11	2	
Cadmium chloride	0.0	12		10	8
	1.0	12		10	9
	2.0	12	1	11	9
	3.0	12	11	1	1
	4.0	11	9	2	1

[a]Animals that died within 30 days post injection.

[b]Females were caged immediately after injection with (101 x C3H)F_1 males and were checked for presence of vaginal plugs for 5 days. Females that showed plugs were separated from the males.

that will be used in the genetic study and (2) effects of these metal compounds and IMS on dominant-lethal induction and on total reproductive capacity which are now under way.

IMS will be used in the X-chromosome nondisjunction and loss studies because this compound was shown to be effective in inducing X-chromosome loss in treated females (Generoso et al., this annual report). However, whether this effect is due to chromosome breakage or nondisjunction is not known. It is hoped that the present study will resolve this problem. Only one of the heavy metals will be tested initially, and the choice will be based on the results of the preliminary studies.

The genetic procedure, developed by Liane B. Russell and W. L. Russell, takes advantage of the X-chromosome inactivation phenomenon. Two X-linked markers, *Greasy* (*Gs*) and *sparse-fur* (*spf*), which affect the coat will be used. With this method, products of X-chromosome nondisjunction and loss are phenotypically detectable among some of the F_1 progeny. Exceptional animals will be verified by means of genetic and cytological tests. The stocks of mice needed in this study are now being built up.

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METABOLIC ACTIVATION OF NITROSAMINES

R. B. Cumming, Mavis Shure,
and Marva F. Walton

Many nitrosamines have been shown to be highly carcinogenic in a number of mammalian species but appear to be mutagenic in *in vitro* tests only when activated by mammalian liver microsomes or a reaction mixture which mimics the action of a microsomal mixed-function oxidase.[1] Feeding of mice with butylated hydroxytoluene (BHT) dramatically protects them from toxicity from a subsequent treatment with diethylnitrosamine (DEN),[2] but it gives them no protection from dimethylnitrosamine (DMN) toxicity.[3] It has been reported that DMN demethylase activity in rat liver microsomes correlates with DMN-induced hepatic tumor incidence[4] and that DEN deethylase activity of mouse liver microsomes is correlated with DEN hepatocarcinogenesis.[5] It was previously shown that pretreatment of mice with BHT increases the capacity of their liver microsomes to metabolize DMN to mutagenic compounds *in vitro*.[6]

In the present experiments we have shown that both DMN demethylase and DEN deethylase are inducible activities largely limited to the microsome fraction of the liver and requiring NADPH and O_2. The assay of these activities, particularly in crude postmitochondrial supernatant fractions, is complicated by aldehyde oxidase activities which are also BHT inducible and requiring NADPH and O_2. When the microsomes are pelleted by centrifuging at 105,000 × *g* for 1 hr, almost all of the nitrosamine dealkylase activity is in the microsomal pellet, while almost all of the aldehyde oxidase activity is in the supernate. Prefeeding the animals with BHT results in higher levels of both DMN demethylase and DEN deethylase in their liver microsomes.

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RADIATION DOSE TO THE MOUSE TESTIS FROM INJECTED TRITIATED WATER

R. B. Cumming, G. A. Sega, and
Marva F. Walton

In experiments carried out in this laboratory to gain information on the genetic effects of low-energy beta radiation from injected tritiated water in male mice,[1–4] it has been necessary to obtain the best radiation estimates possible for the experimental conditions used. In previous studies of radiation dose from injected THO,[2,5] the tritium was given in tracer amounts, and radioactivity was monitored in wet and dry components of the testis as a function of time post injection. It became apparent that many factors could influence water turnover and metabolic incorporation rates and that the dosimetry experiments needed to be carried out under conditions identical to those used in the genetic experiments. Some factors which proved to be important were age of the animals, injected dose, conditions of caging, and number of animals per pen. Thus dosimetry experiments were set up under conditions identical to those used in previous genetic experiments,[1,2] and the injected males were serially sacrificed to provide radioactivity measurements at various time intervals after injection. Nine animals were sacrificed at each time point to give a measurement of individual variability. Males, (C3Hf × 101)F_1, were

injected with 0.5 mCi/g of THO and placed individually in pens with five T-stock females each, the same dose and caging arrangement used in most of our specific-locus experiments. As in genetic experiments, the pens were placed in plastic isolators designed to prevent atmospheric contamination. At weekly intervals, each injected male was placed in a fresh pen with five new females. After the second week the pens were removed from the isolators and maintained in mouse rooms reserved for this purpose. The offspring which resulted from these matings were scored for additional specific-locus data, mostly from irradiated postspermatogonial germ cell stages.[3] The postinjection (p.i.) serial sacrifice time points for this dosimetry study were 1 hr, 12 hr, 1 to 10 days (1-day increments), 10 to 100 days (5-day increments), and 605 days.

The testes of each treated animal were homogenized in distilled water and brought to a constant volume (10 cm^3). Aliquots of these homogenates were solubilized wet or dried under a vacuum on filter paper and counted for radioactivity in a liquid scintillation spectrometer. Dried aliquots were oxidized by combustion prior to counting to obtain optimum samples for the determination of absolute activity.

THO becomes rapidly distributed in body water after injection, and most of it is eliminated without having undergone any chemical transformation. However, water enters into many metabolic reactions which incorporate some fraction of the tritium into cellular macromolecules such as DNA and protein. At early time periods following injection, the overwhelming majority of the radioactivity is in the form of water and is evenly distributed in cell water. While some parts of the cell are more hydrated than others, the assumption of uniformity of distribution of radioactivity is probably valid. This assumption is less justified at longer time periods (several weeks to years) after injection because higher percentages of the total radioactivity present are incorporated into biological molecules. The percentage of radioactivity in the dry material from the testis on day 1 p.i. is 0.6%, on day 7 it is 3.2%, and on day 605 it is 95%. Thus at long time periods following injection, most of the radioactivity is incorporated into materials which could have a very nonrandom distribution within the cell.

One can estimate the average dose to the testis by integrating the area under the time-radioactivity curve arrived at by the above procedures. Of the total dose which will be received by the mouse testis from a single injection of 0.5 mCi/g of tritiated water, 98% has been received by 25 days post injection. Present estimates of the total dose to the testis 605 days after injection of 0.5 mCi/g THO are approximately 690 rads using an exponential biological decay extrapolation between time points which are presently analyzed and about 790 rads using a linear extrapolation between these points. As more data are analyzed, the range of uncertainty should become smaller.

The dose rate on day 1 post injection for this experiment is 157 rads/day. By day 25 p.i., the dose rate is down to 5 rads/day.

Testis weights reach a minimum value of 25% of the normal weight by day 30 p.i. and remain at this level through day 50. The testis weights then begin to gradually increase in value, but the variabilities in these weights from day 50 to day 100 p.i. are much greater than the weight variabilities from day 0 to day 50 p.i.

The homogenates of testes from these experiments will also be used for determining the patterns of radioactivity in various cellular macromolecules, especially DNA, as a function of time after injection. At present, while average radiation dose to the entire testis can be determined, the meaning of this value in terms of energy delivered to genetically significant targets is not clear. The uncertainty is greater for spermatogonia than for postspermatogonial germ cell stages. It is hoped that studies of incorporation of tritium from injected THO into specific biological macromolecules may help to reduce this uncertainty.

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STUDIES OF THE ETHYLATION PATTERNS IN SATELLITE AND MAIN BAND DNA FROM THE MOUSE TESTES AND MATURE SPERMATOZOA*

G. A. Sega

The major site of attack by ethyl methanesulfonate in DNA has been shown to be the N-7 position of guanine,[1] resulting in the formation of 7-ethylguanine. Mouse DNA can be subdivided into a main band DNA with a GC content of about 40% and a satellite DNA

with a GC content of about 35%.[2] Now that we have obtained purified DNA from mouse spermatozoa and testes exposed to ethyl methanesulfonate,[3] we have begun work on fractionating these ethylated DNA's into main band and satellite components using CsCl and Ag^+-Cs_2SO_4 gradients.

As an example, 80 μg of testes DNA exposed to a 400-mg/kg [^{3}H]EMS dose for 4 hr produces about 200 dis/min. After fractionating this DNA on a gradient, about 10% of the DNA is in the satellite component. The radioactivity of the satellite is thus about 20 dis/min, which is readily measurable. For the lowest [^{3}H]EMS dose of 5 mg/kg the radioactivity reaches a minimum of about 80 dis/min for 80 μg of DNA, so pooling of several satellite bands will be required here. If the major sites of ethylation in the DNA are the guanine moieties, then for equivalent amounts of DNA from the satellite and main band fractions the ratio of ^{3}H activities should be ~35/40 = 0.88. Determinations of such ratios are currently in progress.

In studying the dosimetry of chemical mutagens in DNA, it will be important not only to determine the total number of chemical events that have occurred in the DNA but also how these chemical events are distributed throughout the DNA. This current work on the study of the relative ethylation of satellite and main band DNA in the mouse is a first step in this direction.

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

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THE DEGREE OF ETHYLATION IN THE SPERM AND TESTES OF THE MOUSE AS A FUNCTION OF THE ADMINISTERED DOSE OF ETHYL METHANESULFONATE*

G. A. Sega, R. B. Cumming,
and Marva Walton

Tritium-labeled ethyl methanesulfonate ([^{3}H]EMS) of high specific activity (~320 mCi per millimole) is being used to study the degree of ethylation of mouse sperm, sperm DNA, and testicular DNA as a function of [^{3}H]EMS dose administered intraperitoneally. The high specific activity of the [^{3}H]EMS has allowed the administered dose vs ethylation curves to be extended down to a low dose of 5 mg/kg.

After a 4-hr exposure to the labeled EMS, the sperm cells from the vasa deferentia and epididymides are recovered, and the amount of whole sperm ethylation is determined from the ^{3}H activity. For whole sperm, the administered dose vs ethylation curve appears linear from 400 mg/kg down to 50 mg/kg but may be nonlinear from 50 mg/kg to 5 mg/kg. At a dose of 200 mg/kg the average number of ethylations per sperm cell found in repeated experiments has been about 10^6.

The dosimetry for the ethylations in sperm DNA has proven more difficult because of the recovery of small amounts of ethylated protamine along with the DNA and a possible nonrandom recovery of the sperm DNA.[1] Revised techniques using enzymatic digestions have now removed all measurable protamine from the DNA and facilitated the recovery of a random sample of the sperm DNA. The protamine has been shown to be removed by labeling the sperm cell nuclear contents with [^{3}H]arginine and [^{3}H]lysine, which together make up about 50% of the mouse sperm protamine,[2] and then sampling the purified DNA for ^{3}H labeling. No tritium counts above background are found associated with the DNA after the enzymatic digestions.

To check for random recovery of DNA fragments, mouse sperm is doubly labeled with [^{3}H]deoxyguanosine and [^{14}C]thymidine or any other appropriate pair of labeled deoxynucleosides. When the label is incorporated into the DNA of mature sperm, one group of the labeled mice is held as controls and a second group is injected with unlabeled EMS. No change in the ^{3}H/^{14}C ratio between whole sperm and the recovered sperm DNA was found in either the control or the EMS-treated group of mice, indicating that our new procedures result in the recovery of what appears to be a random sampling of the sperm DNA. It will soon be possible, therefore, to measure the ethylations that have occurred specifically in the mouse sperm DNA.

The testicular DNA has been easier to purify, and an administered dose vs ethylation curve for EMS has been determined following exposure of mouse testes to [^{3}H]EMS for 4 hr. The ethylations of the testicular DNA range from ~10^{-4} ethylations per nucleotide at a dose of 400 mg/kg to ~4 × 10^{-7} ethylations per nucleotide at a dose of 5 mg/kg. The dose vs ethylation curve in this case appears to be distinctly nonlinear but is well fitted by a curve in which the ethylations per nucleotide are proportional to the 4/3 power of the dose.

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

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RECOVERY OF MOUSE SPERM CELLS FROM INSEMINATED FEMALES FOR DOSIMETRIC STUDIES OF CHEMICAL MUTAGENS*

G. A. Sega and Mary Hall

When the genetic effects of a chemical agent are studied in the mouse a common procedure is to inject one or several doses of the chemical into a group of males and then mate the treated males with females at various times after injection. The biological end points usually observed are dominant lethal mutations, chromosome aberrations, and specific-locus mutations. Dosage-response and time-response curves for the chemical agent under study are then made and often present a bewildering array of shapes, which are sometimes hard to interpret.

Using ethyl methanesulfonate as an example, it would be most useful to determine ethylations per nucleotide in the mouse sperm found in inseminated females so that these ethylations per nucleotide could then be directly related to the genetic effects produced by EMS. In this particular case, the postmeiotic germ cell stages at the time of EMS treatment should show the greatest effect. With high specific activity [^{3}H]EMS and the techniques now available for purifying mouse sperm DNA,[1] it should theoretically be possible to make such a study if enough mouse sperm can be recovered from the inseminated females.

We have been successful in recovering sperm cells from inseminated females. Following matings, the uterine contents of the females are flushed with saline, and the recovered sperm cells are washed several times. Each female yields $\sim 1.3 \times 10^5$ sperm cells. Since $\sim 10^6$ sperm are needed to get a good measure of the [^{3}H]ethyl activity present, this requires that the sperm cells from about eight inseminated females be pooled.

If females with unsynchronized estrus cycles are paired with the treated males, the percentage of matings on a given day is relatively low and variable. To increase the percentage of matings, we have injected females with 5 I.U. of pregnant mare's serum (PMS) on day 0 at 4:00 PM and 41 hr later given 2.5 I.U. of human chorionic gonadotropin (HCG). The females are then placed with males and checked for plugs the following morning. Approximately 75% of the females have mated by this time.

These results indicate that 11 or 12 males treated with [^{3}H]EMS will be needed for each dosage point. This number of males can be processed in a reasonable amount of time. Thus, determinations of the ethylations per nucleotide in the DNA of treated mouse sperm recovered from inseminated females are being pursued.

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

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PATHOLOGY AND IMMUNOLOGY

J. B. Storer

Mammalian Radiation Recovery

C. C Congdon
R. E. Toya

Radiation Recovery of Hemopoietic Cells

L. H. Smith

Experimental Hematology

Joan Wright Goodman[a]

Animal Facility and Diagnostic Laboratory

C. B. Richter

Cellular Immunology

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Effects of High LET Radiations

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SPLEEN WHITE PULP BLURRING IN THYMECTOMIZED RADIATION CHIMERAS*

C. C Congdon, Helen S. Payne, Nazareth Gengozian,† and Paul Urso†

Diffuse proliferation of large basophilic cells throughout the spleen white pulp within 24 to 48 hr after intravenous injection of antigen has been called white pulp blurring. The process quickly subsides in the following 24-hr period, and many germinal centers form in the white pulp. Young adult mice were thymectomized, then given 950 R whole-body irradiation and 5,000,000 syngeneic bone marrow cells. Challenge three months later with sheep or rat red blood cells revealed a marked defect in white pulp blurring and in germinal center formation compared with the several control groups (Fig. 15). The data indicate the importance of the thymus for these parameters of immunologic function in the radiation chimera. One suggestion has been that the white pulp blurring represents T-B cell collaboration. Another possibility is that it plays a role in lymphokine production.

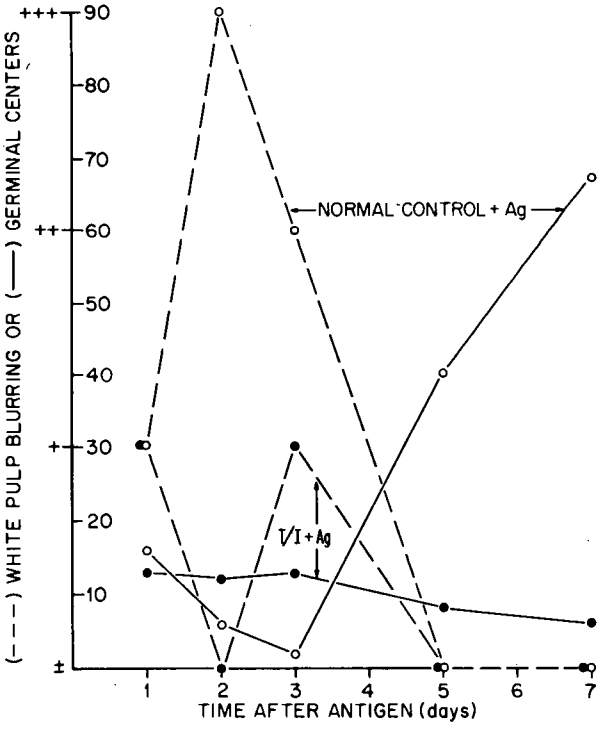

Fig. 15. White pulp blurring and germinal centers as a function of time after antigen injection.

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†Medical Division, Oak Ridge Associated Universities.

THYMECTOMIZED RADIATION CHIMERAS

C. C Congdon and Helen S. Payne

The primary purpose of the work under this interagency agreement is to provide scientists studying leprosy in the U.S.-Japan Program with mice that have been thymectomized, irradiated, and kept alive by bone marrow transplantation. Animals treated in this manner have a markedly depressed immune function and may be unusually susceptible to inoculation with human leprosy organisms. A second purpose of the work is to study a variety of immune responses in the same type of animal for information about its reactions to other types of antigens. Data from the latter study are to be correlated with the work of leprosy investigators.

In keeping with the first goal, 1395 thymectomized and irradiated mice and 980 normal mice were shipped to investigators designated by the U.S.-Japan Program Officer during FY 1973. During FY 1972, Levy (LSM, Memo L 310/2) had found a tenfold greater multiplication of *M. leprae* in Oak Ridge experimental mice compared to normal mice. The growth of *M. leprae*, however, did not resemble that seen in lepromatous leprosy in man.

Further evaluation of Oak Ridge experimental mice (thymectomized and irradiated) by leprosy investigators indicates difficulty in deciding the usefulness of this type of animal for *M. leprae* studies. However, it was found that the Oak Ridge experimental mice have increased susceptibility to *L. monocytogenes* and a decreased antibody and foot pad response to *M. leprae* antigen. BCG vaccination has been used to enhance resistance to leprosy in man. Study of Oak Ridge experimental mice showed them to be ideal animals for producing a disseminated BCG infection when compared to normal animals.[1,2]

Immune status studies at Oak Ridge on the experimental mice revealed a markedly shortened total life span for these animals and probably an unusual sensitivity to mouse hepatitis virus. The experimental mice failed to grow in a normal manner, and a few died from an unexplained wasting syndrome. Antibody production and foreign skin grafting at 18 months after thymectomy revealed defects in the mice.

In another study on immune status, the Oak Ridge experimental mice had an unusual cellular defect in their response to antigen,[3] which may represent a lesion in T-B cell collaboration or a defect in lymphokine production.

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ACUTE GRAFT-VERSUS-HOST DISEASE

R. E. Toya

The problem of acute graft-vs-host disease (GvH) was studied in the parent to F_1 foreign spleen model. In this system, irradiated F_1 hybrid mice injected with the proper dose of parental spleen cells die prior to radiation-induced death. This death has been termed an "immunological death" with little descriptive information available as to its etiology. The work summarized here has had as its directive to either eliminate or prevent the disease. To accomplish this directive, more information was needed in terms of the basic mechanisms involved in GvH disease. It has been proposed that the disease is an expression of a series of detrimental effects on various organs. If this were the case, then the effects would have to be additive, since no one particular organ has been implicated to date. The second directive, that is, prevention, has been studied through the use of an erythropoietic stimulating agent, phenylhydrazine. By treating the donor with this agent we have been able to compare the effects in the recipient mice which are possibly associated with life or death.

We have shown that pretreatment of parental donor mice with phenylhydrazine ($PhNHNH_2$) and transplanting the spleen cells from these animals to lethally irradiated F_1 mice prevents the acute GvH disease. One of the queries is whether $PhNHNH_2$ was correcting an anemia in the F_1 recipient. In other words, was there an anemia associated with acute GvH disease and was this anemia corrected by the $PhNHNH_2$-treated spleen cells? We found that there is not an anemia in the parent to F_1 foreign spleen reaction and therefore the $PhNHNH_2$ is not correcting a preexisting anemia. Spleen-weight data suggested that hemopoiesis (probably erythroid) was more active in F_1 mice given $PhNHNH_2$-treated spleen cells. A series of ^{59}Fe uptake studies confirmed this suggestion. Thus, reduced GvH deaths may result from an erythroid hyperplasia that physically crowds out or sharply reduces growth of immunocompetent cells that are responsible for GvH death. The basis for erythroid hyperplasia appears to be related to the fact that in the parent donor given $PhNHNH_2$, the erythropoietic base is greatly expanded in the spleen, perhaps at the expense of lymphopoiesis.

DISTRIBUTION OF HEMOPOIETIC COLONY-FORMING CELLS IN THE SPLEEN*

K. L. Mossman† and A. L. Kretchmar‡

We previously found that the results of a Till and McCulloch assay for hemopoietic stem cells do not fit a Poisson distribution as expected for an assay procedure.[1] Instead, our data are consistent with a binomial distribution, the parameters of which suggest that the controlling factors in the assay are local in the spleen of the assay mice. We have tentatively interpreted our data to mean that the spleen has a number, N, of niches (hemopoietic inductive microenvironments) that are competent to support stem cell replication sufficient to give rise to gross splenic nodules at eight to ten days when the assay mice are killed and colonies on their spleens counted. The statistics of the assay procedure can be fully accounted for by this model. The complicated array of variance levels that are encountered in experiments with various strains of mice and varying treatment of donor and recipient animals can be accounted for within the context of the niche hypothesis as arising from three sources. The first is the variance arising from the process of seeding the niches with stem cells; second is the variance arising from the possibility that not all niches are "competent"; and third is the variance arising from limitation or failure of stem cell replication (e.g., when cells from irradiated donors are injected).

Current results indicate that cell-cell interaction at the level of the niches in the spleen of the assay mice is necessary for, or at least influences, the production of gross nodules. The preliminary evidence suggests that there is inhibition of replication in splenic niches as well as stimulation of replication. Markedly increased colony production from 500-R-irradiated donor mice is observed, for example, if the assay mice are given the cells three days after lethal irradiation as compared with 1 ml of the same cell suspension injected on the day of lethal irradiation.

*Work performed in collaboration with the Mammalian Recovery Group.

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‡Research Professor at the University of Tennessee Memorial Research Center.

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RADIOPROTECTION BY PHENYLHYDRAZINE: EVALUATION OF STEM CELL RADIOSENSITIVITY AND POTENTIAL STIMULATION OF THE RETICULOENDOTHELIAL SYSTEM

L. H. Smith and T. W. McKinley, Jr.

Phenylhydrazine ($PhNHNH_2$) is radioprotective when administered to mice about one week before x irradiation. There are indications that radioprotection by this drug is associated with changes in the number and distribution of hemopoietic stem cells,[1,2] but the possibility that other mechanisms are involved has not been ruled out. We examined two factors which conceivably could enhance survival of irradiated mice given $PhNHNH_2$ before exposure: (1) increased *in vivo* radioresistance of hemopoietic stem cells and (2) stimulation of the reticuloendothelial system (RES) by drug-damaged red blood cells (RBC). The first factor would result in survival of a larger number of cells, and the second factor might improve defenses against bacterial infection, thereby providing sufficient time for surviving hemopoietic cells to regenerate a blood-forming system.

Results show that radioprotection by $PhNHNH_2$ cannot be explained by increased *in vivo* radioresistance of hemopoietic stem cells because the D_{37} for endogenous CFU in drug-treated mice was almost the same as that for vehicle-treated animals (98 R vs 113 R). These results corroborate our previous observations on the *in vitro* radiosensitivity of exogenous CFU from $PhNHNH_2$-treated mice.[2]

In terms of endogenous colonies arising on the spleen, $PhNHNH_2$ increased radioresistance about 200 R, which is equivalent to an enhancement ratio of 1.3. From previous work[2] the increase in $LD_{50/30}$ for $PhNHNH_2$-treated mice exposed to whole-body x rays was about 140 R, which is equivalent to an enhancement ratio of 1.2. Thus, compared to the survival rate of protected mice, the endogenous CFU method slightly overestimated radioprotection by $PhNHNH_2$. The basis for the discrepancy in ratio for the two assays may be related to the fact that more than half of the endogenous spleen colonies are erythropoietic, and thus the number of spleen colonies may not accurately reflect the state of leukopoiesis and thrombopoiesis, restitution of which is critical for survival.

We considered stimulation of the RES to be a possible contributor to radioprotection by $PhNHNH_2$ solely

Table 18. Effect of graded doses of isogenic RBC on radioprotection by $PhNHNH_2$

Treatment[a]			30-day survival (%)	Number of mice treated	Hematocrit[c] (± standard error)
Initial injection	Subsequent intravenous injections[b]				
	Day 1	Day 2			
V	V	V	0	58	47.4 ± 0.9
$PhNHNH_2$	V	V	93	45	29.1 ± 0.7
$PhNHNH_2$	RBC(0.5)	None	75	20	33.8 ± 0.6
$PhNHNH_2$	RBC(0.5)	RBC(0.5)	50	24	38.9 ± 1.2
$PhNHNH_2$	RBC(0.7)	RBC(0.7)	12	25	44.4 ± 1.1
V	RBC(0.5)	None	0	20	52.8 ± 1.4
V	RBC(0.5)	RBC(0.5)	8	24	58.7 ± 1.4
V	RBC(0.7)	RBC(0.7)	0	15	68.9 ± 1.1

[a]Mice exposed to 850 R seven days after initial intraperitoneal injection of 0.5 ml of vehicle (V) or 3.0 mg of $PhNHNH_2$.

[b]Numbers in parentheses indicate volume (ml) of washed packed RBC from normal mice.

[c]Determined on day 4 after $PhNHNH_2$ or vehicle (V) injection for about half of the mice in each group.

because altered RBC are known to activate this system. The observation that 30-day survival was not enhanced in irradiated mice given blood from $PhNHNH_2$-treated donors implies that the RES is not involved in radioprotection by the drug. This implication is strengthened by the fact that the degree to which erythropoiesis is spoiled by injection of normal RBC correlates inversely with radioprotection (Table 18), and there is no reason to believe that transfusion of normal RBC would prevent $PhNHNH_2$-induced RES stimulation. The data, however, do not discount the possibility that radioprotection by $PhNHNH_2$ involves synergism between RES stimulation and hypererythropoiesis.

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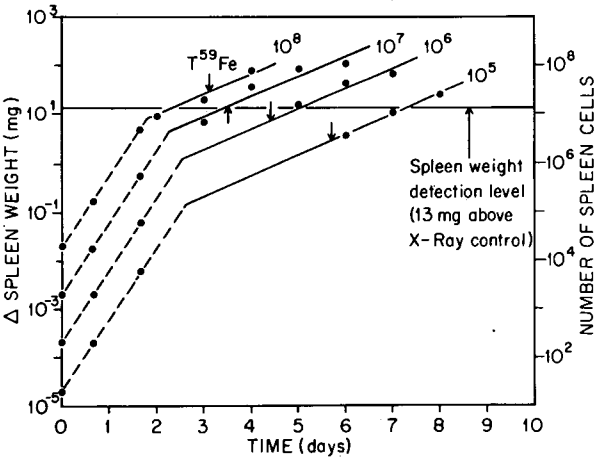

Fig. 16. Spleen weight (mg above x-ray control) as a function of time after injection of 10^5 to 10^8 syngeneic bone marrow cells. Solid lines are least-squares fits to the data; dashed lines are hypothetical growth curves based on a T_D of 0.2 days. Right ordinate is estimate of number of cells per spleen. $T^{59}Fe$ arrow is time when RBC ^{59}Fe reached detectable level of 1%.

EFFECTS OF ISOGENIC BONE MARROW CELL DOSE ON ERYTHROKINETICS OF LETHALLY X-IRRADIATED MICE

L. H. Smith, Joan Wright Goodman, and G. S. Hodgson*

The effect of isogenic bone marrow cell dose on several parameters of hemopoietic regeneration in lethally x-irradiated mice (900 R) was studied. The appearance of ^{59}Fe in red blood cells (RBC) and spleen, the hematocrits, and the spleen weights were measured at various times after irradiation and the injection of 10^5, 10^6, 10^7, or 10^8 marrow cells. Results show that with increasing cell dose, regeneration of erythropoiesis was detectable earlier, proceeded at a faster rate, reached a peak more quickly, and subsided to normal levels sooner. The initial spleen-weight growth rate was independent of cell dose, although the onset of growth was earlier the higher the dose. The maximum rate of RBC production was attained earlier the larger the cell dose and was a decreasing function of the hematocrit at that time, which in itself was an increasing function of cell dose. These data support the concept of a proportional model for control of erythropoiesis as described by Hodgson.[1]

The present data are interpretable in terms of an A- and B-cell model for erythropoiesis as suggested by Lajtha et al.[2] This model postulates a compartment of undifferentiated multipotent A cells and a compartment of differentiating erythropoietic B cells capable of amplification according to the need for RBC. Applying the A-B cell model to our data shown in Fig. 16 and making the auxiliary assumptions that (1) 10^6 cells = 1 mg of spleen and (2) for every 10^5 cells injected, 20 A cells lodge in the spleen and initiate the regeneration process, the corresponding initial curves for B cells in the spleen can be constructed. Then, B-cell amplification of the system can be estimated from the point at which the hypothetical initial portion of the B curve intersects the experimentally derived curve which depicts a population growing with an A-type T_D. As can be observed (Fig. 16), amplification of approximately 10^4 was similar for 10^5 and 10^6 cells, decreased slightly for 10^7, and dropped sharply for 10^8 cell dose. Existence of variable controlled amplification as suggested by the present experiments would explain how RBC production can be maintained in the face of depleted hemopoietic stem cells produced, for example, by continuous irradiation, and would complement the idea that a major function of erythropoietin is to control the number of cell divisions in the pre-erythropoietin-responsive cell stage of the B population.

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FUNCTIONAL PANCREATIC ISLET TRANSPLANTATION IN DIABETIC MICE OR IN DIABETIC, ISOGENEIC, OR ALLOGENEIC RADIATION CHIMERAS

R. C. Kramp,* C. C Congdon, and L. H. Smith

Grafts of skin, tumors, ovaries, or endocrine glands have been used to study immunological aspects of tissue transplantation in normal animals or in animals made tolerant to foreign tissue by immunosuppressive agents. Transplants of pancreatic islets capable of functionally reversing a diabetic state would provide a transplant system in which the fate of grafts can be assessed by measuring changes in blood or urinary glucose in diabetic hosts.

Limited success with islet transplants capable of providing functional remission from experimentally induced diabetes in rats has been reported.[1,2] We have developed a method of pancreatic transplantation that consistently showed substantial reduction of hyperglycemia in mice made diabetic with streptozotocin (a specific B cell cytotoxic agent) and transplanted with previously ligated pancreases from isogeneic donors. Ligation of pancreatic ducts six to eight weeks before transplantation caused acinar tissue to degenerate, while islets remained intact and possibly differentiated from ductular epithelium. Isogeneic transplantation has been performed in three different strains of mice with variable results. In $BC3F_1$ or $B6C3F_1$ diabetic hosts transplanted with ligated pancreases from isogeneic donors, a return to normoglycemia occurred within five to nine weeks after transplantation. In isografted $CD2F_1$ hosts a return to normoglycemia did not occur by 11 weeks, but a significant reduction of blood glucose below that of sham-transplanted controls ($P < 0.001$) was evident. It is not clear if the failure of grafted $CD2F_1$ hosts to attain complete remission from hyperglycemia was due to a more severe diabetic state existing in this strain compared to other strains injected with streptozotocin, or if there is an inherent difference in the capacity of ligated pancreases from different donor strains to bring about normoglycemia in grafted hosts. In $B6C3F_1$ or $CD2F_1$ hosts allografted with ligated pancreases from $CD2F_1$ or $B6C3F_1$ donors, remission of hyperglycemia or a significant reduction of blood glucose did not occur, indicating that ligated pancreas cannot be transplanted across a major histocompatibility barrier.

A group of $B6C3F_1$ mice were lethally irradiated (950 R) and injected with 5×10^6 bone marrow cells from $B6C3F_1$ donors. Twenty weeks later these isogeneic radiation chimeras were made diabetic with streptozotocin and transplanted with ligated pancreases from either $B6C3F_1$ or $CD2F_1$ donors. Five to nine weeks after pancreatic transplantation the majority (13 of 16) of isografted recipients showed normal or slightly elevated blood glucose levels, whereas allografted or sham-transplanted controls remained severely hyperglycemic, indicating that lethally irradiated mice reconstituted with isogeneic bone marrow were fully capable of rejecting foreign islet tissue.

A group of $B6C3F_1$ mice were irradiated and injected with 10×10^6 bone marrow cells from $CD2F_1$ donors. A mild graft-vs-host reaction subsequently ensued with a cumulative mortality of 16% (9 of 55) at 19 weeks; all mice showed 100% red blood cell chimerism. These allogeneic radiation chimeras were then made diabetic and grafted with ligated pancreases from either $B6C3F_1$ or $CD2F_1$ donors. Nine weeks after pancreatic transplantation, normal or slightly elevated blood glucose levels were noted in 7 of 13 hosts receiving $B6C3F_1$ grafts and in 3 of 13 hosts receiving $CD2F_1$ grafts. The animals in each of these groups that did not show normoglycemia showed a reduction of blood glucose after transplantation. All five sham-transplanted mice remained severely hyperglycemic. These results indicate that allogeneic radiation chimeras tolerated ligated pancreatic tissue from the host strain ($B6C3F_1$) or from the strain that served as a source of bone marrow ($CD2F_1$).

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DECLINE IN PHYTOHEMAGGLUTININ RESPONSIVENESS OF SPLEEN CELLS FROM AGING MICE

Yasuharu Hori* and E. H. Perkins

Many studies have demonstrated that there is a dramatic decline in humoral immune competence during senescence. Systematic studies to determine whether a comparable decline in cell-mediated immunity (CMI) occurs are needed, as previous studies are not in agreement.[1] It has been postulated, and considerable interest has been generated in the concept, that age-related failure of immune surveillance mechanisms plays a prominent role in carcinogenesis and in other age-dependent disease processes. Demonstration of an age-related decline in CMI is essential if this concept is to remain tenable.

Thymus-dependent lymphocytes (T cells) are an absolute requirement of CMI. It is generally accepted that phytohemagglutinin (PHA) selectively stimulates T cells, and therefore PHA stimulation of lymphocytes is commonly used as a measure of CMI. Thus, it seems apparent that the *in vitro* blastogenic response of mouse spleen cells to PHA should be useful in identifying age-related immunodeficiencies that might exist in the T-cell population of the spleen, although it is recognized that PHA responsiveness at best measures only one parameter of CMI. Accordingly, after determining optimal experimental conditions (concentration of PHA, length of culture, and serum requirements for mouse spleen cells), age-dependent changes in T-cell stimulation with PHA were assessed with spleen cells from long-lived $BC3F_1$ mice ranging in age from 2 weeks to 36 months and in medium-lived BALB/c mice of 4, 12, 19, and 24 months of age. As judged from ^{3}H-Tdr incorporation, the growth of a PHA-responsive T-cell population is remarkable from birth to one month of age. Functional maturity appears maximal at eight months of age in $BC3F_1$ mice, decreases gradually, and then falls more dramatically during senescence, reaching only 2.5% of maximum at 36 months of age. In BALB/c mice a similar pattern is seen, although only four age groups were tested. The peak value is at four months, and a marked decrease is seen at 19 and 24 months of age (to 11.5 and 8.5% respectively). This reflects a marked age-dependent decrease in T-cell activity in both strains, as measured by *in vitro* ^{3}H-Tdr incorporation by PHA-stimulated mouse spleen cells. Concurrently with these studies, Goodman and Makinodan[1] observed a marked decrease in CMI responsiveness assessed by *in vivo* allogeneic tumor resistance and *in vitro* cytolytic activity. Peterson[2] noted that spleen cells from aged animals are less effective in producing acute mortality in the classical graft-vs-host reaction. The present and aforementioned studies were all carried out in long-lived $BC3F_1$ mice, and although each study assessed CMI by a different immunologic parameter, all noted a significant decline. Combined they provide strong evidence that CMI declines with age and lend credence to the concept that an age-related decline in CMI may play an exacting role in carcinogenesis and other age-related diseases.

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DECLINING CELL-MEDIATED IMMUNOCOMPETENCE WITH AGE – NATURE OF THE DEFECT, T-CELL PROLIFERATION

E. H. Perkins, Sarah A. Goodman,* Mary K. Halsall, and Yasuharu Hori†

Our current studies,[1–3] each using a different parameter for measuring cell-mediated immunity (CMI) (decline in resistance to allogeneic tumor cells, decreased *in vitro* cytolytic activity, impaired GvH reactions, and decreased responsiveness to PHA stimulation), provide strong evidence that CMI declines with age in long-lived mice. This decline in T-cell activity can be attributed to (1) a decrease in functional activity of individual cells (e.g., decreased cytolytic activity of individual killer cells), (2) a reduction in the absolute number of cells responding, or (3) reduced proliferative activity of antigen or PHA-stimulated precursor cells which give rise to a functional progeny.

The first possibility seems unlikely, because cytolysis of target cells elicited by optimally immunized spleen cells from young and old animals was the same at spleen-cell–target-cell ratios ranging from 1:1 to 200:1. Analysis of this cytolysis data indicates that a single killer cell from an old animal can destroy a target cell as efficiently as a killer cell from a young animal. Limiting dilution analysis demonstrated an approximate twofold difference in the frequency of functional precursor cells between spleen cells from old and young mice. Therefore, a significant portion of the observed fourfold difference in cytolytic activity of old and young mice must be ascribed to a decreased proliferative capacity of precursor cells from old animals; that is, they produce fewer functional progeny.

Similarly, studies have been performed to determine if the decrease in PHA responsiveness with age was due to a reduction in the relative number or a decreased proliferative activity of T cells. To estimate the number of T cells present in the spleens of young and old animals, the percentage of θ positive cells (all thymus-derived cells bear the θ antigen) in the spleen of $BC3F_1$ and BALB/c mice was determined. There was no age-related decrease in the relative number of θ positive cells of the spleen. The total numbers of nucleated cells in the spleens of young and aged mice were the same, and differential counts of spleen cell smears demonstrated only a marginal decrease in the percentage of lymphoid cells. Therefore, the observed decrease in CMI could not be correlated with a decrease in the number of T cells in the spleen.

These current findings of a decreased proliferative capacity (as measured by ^{3}H-Tdr incorporation and

cytolytic activity) of T cells responsible for CMI is reminiscent of our earlier finding of a proliferative defect in the B lymphocytes from aged mice to a thymus-dependent antigen.[4] While those experiments demonstrated that the basic defect was the inability of B lymphocytes to generate the maximum number of antibody-forming progeny, it was not ruled out that defective helper T-cell function or interaction was responsible for the observed results. Indeed, a tenfold reduction in the ability of T cells to proliferate was detected. Therefore, the proliferative defect in the T-cell population responsible for CMI may provide a basic defect (T-cell proliferation) common to both CMI and humoral immunity in aged mice. This may be an oversimplification, because heterogeneity of the T-cell population has been observed; that is, there are different subpopulations of T cells. Furthermore, it is not known if the same T-cell populations respond to PHA and tumor antigens, and it remains to be established whether the helper T cell in the thymus-dependent humoral immune response is the same or different from the effector T cell of CMI.

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THE LATE EFFECT OF SELECTED IMMUNOSUPPRESSANTS ON IMMUNOCOMPETENCE, DISEASE INCIDENCE, AND MEAN LIFE-SPAN. I. HUMORAL IMMUNE ACTIVITY

E. H. Perkins, W. J. Peterson, Takashi Makinodan,* C. F. Gottlieb, and Lucia H. Cacheiro

It is well known that the incidence of certain types of cancer, autoimmune disease, and infection increases with age, and, in mice, at least, the increase in the susceptibility to these diseases is correlated with a decrease in the activity of the immune system. Proponents of the immune surveillance theory of Thomas (Walford, Burnet) suggest that the age-related deterioration of the immune system is responsible for these diseases. If so, immunosuppression, especially by long-lasting and irreversible insults, should increase the incidence and accelerate the appearance of these diseases. Accordingly, a long-term study was initiated with two objectives in mind: (1) to evaluate methods for inducing lasting immunodeficiency and (2) to determine effects of temporary or permanent immunosuppression on disease incidence and life expectancy. Young adult (2 to 3 months) and adult (12 to 15 months) $BC3F_1$ mice were subjected to surgical (thymectomy or splenectomy), chemical (cytoxan or cortisone), or radiation (400 R) insults, and humoral immune activity was assessed 18 to 22 months later. (Effects on cell-mediated immunity, disease incidence, and life-span are presented in a following report.) To test humoral immune activity of 30-month-old mice insulted as adults (12 to 15 months), 2.5×10^7 spleen cells and 2.5×10^8 sheep RBC were injected into lethally irradiated syngeneic recipients, and hemolysin titers and the number of plaque-forming cells (PFC) in recipient spleens were determined seven days later.

Spleen cells from cortisone- and cytoxan-treated donors did not respond differently from the age controls by either the hemolysin or PFC assay; in contrast, spleen cells from thymectomized or irradiated donors produced only one-sixth as great a response. Comparable results were obtained with 24-month-old donor mice insulted as young adults (2 to 3 months). Because the cell transfer system measured the immune activity of a fixed number of donor spleen cells in a constant environment (irradiated syngeneic young adult mouse), *in situ* studies were carried out to determine total immune capacity as judged by maximum hemolysin titers and the number of PFC in the spleen following antigen injection of 24-month-old mice. In these *in situ* studies, the mean peak hemolysin titers for cortisone- and cytoxan-treated mice were not different from the age control animals, while thymectomy or splenectomy at two to three months resulted in eight- to tenfold reduced titers. Both 19S and 7S plaque-forming cells closely paralleled the response as measured by hemolysin titers. Irradiated animals were not available for this study.

These findings demonstrate that the reduced humoral immune competence of aged mice is further compromised when permanent insults (surgical) are administered early in life. It should be noted that even with these severe insults, residual immune activity was still present. The finding of complete recovery from treatment with cortisone and cytoxan is not surprising, as the immune system is characterized as a rapidly turning over self-renewal system. However, the late effects of

sublethal (400 R) irradiation were clearly evident, and, although temporary recovery may have occurred, such an insult to the immune system is accentuated late in life, and in conjunction with damage accrued to other organ systems has lasting consequences.

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THE LATE EFFECT OF SELECTED IMMUNOSUPPRESSANTS ON IMMUNOCOMPETENCE, DISEASE INCIDENCE, AND MEAN LIFE-SPAN. II. CELL-MEDIATED IMMUNITY

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Characterization of the late effect of immunosuppressants on the immune response was extended by studying the cell-mediated immunologic activity. As no single index fully evaluates cell-mediated immune activity, a variety of indices were used to measure persisting immunodeficiency.

Allogeneic tumor cells (P815-mastocytoma) were used to assess the *in vitro* cytolytic activity of spleen cells of treated mice and to test the resistance of these mice to tumor growth *in situ.* For the *in vitro* test, ^{51}Cr-labeled target tumor cells were mixed with sensitized spleen cells from donors of each treatment group. Only spleen cells taken from thymectomized animals showed a decrease in cytolytic activity as compared with controls. In regard to tumor resistance, the 30-day cumulative percent mortality of each treatment group was determined following the injection of 1×10^6 and 1×10^7 tumor cells. The percent mortality obtained in the 400-R-treated animals and the splenectomized animals was no different from that seen in the control groups given the low and high tumor-cell dose (20 and 40% respectively). In contrast, increased mortality was seen in the thymectomized group and decreased mortality in the cortisone and cytoxan groups.

Using the blastogenic index as a measure of cell-mediated immunity, the relative activity of PHA-stimulated cultures was significantly suppressed in the thymectomized and the 400-R-treated animals, whereas the activity of the cortisone- and cytoxan-treated animals was similar to the PHA-stimulated controls. In thymectomized animals the activity of the stimulated spleen cells was at the level of the nonstimulated controls, whereas the 400-R-treated animals were only moderately suppressed; blastogenic activity was about 70% of that of the PHA-stimulated control.

Using the graft-vs-host reaction (GVHR) as a measure of cell-mediated immunity, 10×10^6 bone marrow cells and 50×10^6 spleen cells from the various treatment groups were injected into 850-R- and 450-R-treated histoincompatible BALB/c mice. The 120-day cumulative percent mortality of bone marrow recipients was not different from the controls. On the other hand, considerable difference was seen in the acute 30-day cumulative percent mortality of recipients transfused with spleen cells. The GVHR activity of spleen cells from thymectomized and 400-R-treated donors was suppressed. In contrast to these results, a moderate enhancing effect was seen in the GVHR activity of spleen cells from donors treated with cortisone and cytoxan.

These results emphasize the unequal late effect of various immunosuppressants on the cell-mediated immune response. Complete recovery and in some cases moderate enhancement occurred with chemical immunosuppressants when administered early in life while thymectomy and 400-R x irradiation had a lasting effect. Although 400 R reduced the activity of isolated spleen cells as judged by the *in vitro* blastogenic index, *in vitro* cytolytic test, and GVHR, no effect was seen *in situ* when resistance to tumor growth was assessed. In this respect, only thymectomy was effective in potentiating tumor growth. These findings reflect the presence of an immunologic reserve and suggest that a reduction in immune capacity is best achieved through the use of permanent insults affecting central immune mechanisms.

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EFFECT OF SELECTED IMMUNOSUPPRESSANTS ON IMMUNOCOMPETENCE, DISEASE INCIDENCE, AND MEAN LIFE-SPAN. III. DISEASE INCIDENCE AND MEAN LIFE-SPAN

C. P. Peter,* W. J. Peterson, H. E. Walburg, Jr.,† and Takashi Makinodan‡

Our objective of this preliminary study was to test the proposition that the decay of the immune system is the cause and not the result of many of the diseases associated with senescence. If this view is true, immuno-

suppressive treatments should shorten life expectancy, increase the infectious and neoplastic disease incidence, and possibly accelerate the aging process. However, this is a gross oversimplification since nothing is known about the significance of the degree, duration, and recovery from early immunosuppression as it relates to late effects which are manifest as disease incidence and how they alter mean life-span. Therefore, a series of studies were undertaken to evaluate the late effects of immunosuppression induced by irradiation (600 R), cortisone acetate, cytoxan, splenectomy, and thymectomy on survival and cause of death in long-living $BC3F_1$ mice.

Tentative conclusions follow. Only x irradiation and cytoxan (a radiomimetic chemical) altered the mean life-span. With cytoxan, critical damage to organ systems in addition to the immune system is implicated because both cellular and humoral immunity 18 to 22 months after drug treatment were equal or superior to age-control animals. In contrast, x irradiation sharply reduced cellular and humoral immune competence. This life shortening by x irradiation was due in part to early-occurring lymphoid malignancies (lymphosarcoma and thymic lymphoma). This finding supports previous observations. Thymectomy, before three months of age, produces a slight but not significant life shortening. As the life-shortening effect of neonatal thymectomy is well known, this marginal life shortening was unexpected, particularly in light of the impaired humoral and cell-mediated immunity measured 18 to 22 months after thymectomy. Splenectomy and cortisone did not alter survival. Although splenectomy reduces humoral immunity, other lymphoid organs apparently provide sufficient immunocompetent reserve. Cortisone, as administered, failed to yield late immunodepression, with early transient immunosuppressive effects followed by apparently complete recovery.

It was clear from the causes of death in the immunosuppressed animals that all treatments except splenectomy had an enhancing effect on nonneoplastic disease processes. The effect on various neoplastic diseases was variable. For example, only thymectomy affected the incidence of reticulum sarcoma, whereas the incidence of thymic lymphoma and endocrine tumors was higher in irradiated mice. This is in agreement with all previous observations. The incidence of other tumors was not affected by any of the treatments. It should be noted that immunological suppression as a consequence of these treatments may have enhanced progression of various tumors, which are otherwise localized, resulting in early mortality. These findings suggest that immunosuppressive treatments may indeed alter the pathology and cause of death, and some treatments can reduce mean life-span.

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APPLICATION OF TRANSFORMATIONS TO NORMALIZE THE DISTRIBUTION OF PLAQUE-FORMING CELLS

C. F. Gottlieb

The hemolysin-in-agar technique of Jerne[1] has been extensively used by numerous laboratories since its discovery in 1963. The plaque-forming cell (PFC) responses resulting from a variety of experimental treatments are generally used to make a statistical comparison among the treatment groups. Because such statistical comparisons are most often based on the normal (Gaussian) distribution, the distribution of the data must, therefore, be approximately normal for the statistics to be meaningful. I have approached this question of the normality of PFC distributions using data generated in three types of systems: *in situ,* using the immunized mouse; *in vivo,* using both the spleen cell transfer and diffusion chamber systems; and *in vitro,* using both the Marbrook and Mishell and Dutton-type systems.

Three statistical distributions seem likely candidates in the context of our knowledge of the immune system, and were therefore tested. These are the Gaussian distribution, the exponential distribution (suggested by the proliferative nature of the PFC response), and the Poisson distribution, because the immune response is the result of the stimulation of a small number of responding units (T, B, and accessory cells). The data transformations required to "normalize" these distributions are none, logarithmic, and square root, respectively.

Examination of the theoretical properties of these distributions and comparing them with those of the experimental data provides a means to judge the best-fitting distribution. The first four moments – mean, variance, skewness, and kurtosis (a measure of peakedness) – of each of the experimental distributions were evaluated for the untransformed (Gaussian), logarithmically (exponential), and square root (Poisson) transformed observations. The logarithmic transformation appears the most satisfactory for all the systems

tested (*in situ, in vivo, in vitro*). The two major reasons are: (1) the variance was independent of the mean and (2) the least amount of skewness was found for this transformation. I conclude therefore that all PFC data should be transformed by logarithms before making statistical computations.

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ANALYSIS OF THE LIMITING DILUTION ASSAY USED FOR ESTIMATING FREQUENCIES OF IMMUNOCOMPETENT UNITS

Mary K. Halsall and Takashi Makinodan*

A decreased frequency of immunocompetent units (IU) in aged $BC3F_1$ mouse spleen cells has been shown using limiting dilution analysis. When the data are plotted in the customary form of proportion nonresponse vs lymphoid cell dose, a straight line occurs as expected. However, frequently a "shoulder" is detected at the 100% nonresponse level, indicating a lack of responsiveness at low lymphoid cell doses. Since this "shoulder" is not mathematically predicted by the Poisson distribution, ambiguity can be introduced into the interpretation of the assay. Therefore, varying numbers of cells were cultured in *in vitro* Marbrook chambers of varying membrane areas to determine if the "shoulder" is caused by the failure of the cells to grow when cultured in small numbers and what effect this "shoulder" has on interpretation of results.

The data show that the frequency of response by low numbers of spleen cells can be elevated by culturing cells at optimum concentration. Specifically, the frequency of response is highest at concentrations of 3×10^5 spleen cells per square millimeter of membrane. When cells are cultured at this optimum concentration, the "shoulder" often seen in limiting dilution analyses under standard conditions is eliminated, and the slope of the resultant regression line remains unaltered. Moreover, dose-response analyses at optimum and nonoptimum cell concentrations show that the maximum PFC response (for a given number of cultured cells) occurs at the optimum cell concentration, indicating that the cellular events necessary to promote growth and differentiation can be spacially inhibited by both dilution and crowding.

The slope of 1.0 derived from the dose-response curve of cells cultured at optimum cell concentration is interpreted as one limiting cell being assayed, with other interacting cells present in excess. A slope of approximately 2 is found when varying nonoptimum cell concentrations are cultured, indicating the presence of two or more events for a response to occur. Slopes greater than unity would be expected at these varying concentrations (i.e., a constant membrane area for all cell doses) because the altered spatial separation between interacting cells prevents maximum interaction. Thus, at varying nonoptimum cell concentrations, the slope becomes a direct measure of the interaction potential of the cells involved in the IU, while at optimum cell concentrations interaction is maximal and the potential response is expressed. Clearly, caution must be taken of kinetic interpretations based on statistical evaluation of slopes of dose-response curves derived from cells cultured under nonoptimum conditions, especially in the cell dose range where the IU is limiting.

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HOST RANGE RESTRICTION FUNCTIONS OF MAMMALIAN CELLS FOR MOUSE RNA TUMOR VIRUSES

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Our previously reported studies on the restriction of Moloney leukemia virus in human cells (W1-38) showed that the nonpermissive state was dominant. The results also suggested the restriction was due to an intracellular function. Rowe and his colleagues have shown that the FV-1 mouse gene locus functions as a host range restriction in cell culture. We have attempted to analyze the nature of the FV-1 restriction using the experimental approaches applied in the study of the human cell restriction. The mouse cells fall into two host range classes, N or B, and we have analyzed the restriction by following the fate of N or B tropic viruses in heterokaryons formed by Sendai virus-induced fusion. Both heterokaryons and cells synthesizing viral protein can be detected by simultaneous autoradiography and fluorescence microscopy. We have found that both N and B tropic viruses are restricted in heterokaryons of N and B cells, showing that the restriction is dominant in cultured cells. It does not appear that the restriction is at the level of virus adsorption since [^{3}H] leucine-labeled N tropic virus adsorbs at the same rate to N, B, or human cells. Therefore, we infected N cells with N tropic virus and fused them with nonpermissive B-type cells at various intervals to determine the relative step

restricted. Results to date suggest that the restriction functions within the first 12 hr of infection and, therefore, is a postpenetration effect. Experiments are now in progress to determine that the restriction represents a cell transcriptional or translational event.

AN ASPECT OF MEGAKARYOCYTOPOIESIS IN RATS

T. T. Odell, Jr., J. P. Long, Jean R. Murphy, and Diane K. Beeman

Earlier experiments describing the labeling index of megakaryocytes at intervals after injection of tritiated thymidine (^{3}HTdR) have suggested that the rate of entry of labeled cells into the recognized population varies during the approximately 20-hr time period taken to reach maximum labeling of the immature recognized megakaryocytes. Because more detailed information about fluctuations in entry rate might help to clarify the nature of cycling of cells just prior to their entry into the recognized population, the labeling index of megakaryocytes was examined at frequent intervals during the first 28 hr after injection of ^{3}HTdR. It was confirmed that a dip in the rising labeling index curve occurs at 12 hr after injection of label. The halt in entry of labeled cells during the period between approximately 11 and 13 hr can be interpreted as indicating that the cells entering at that time were in a G_2-M-G_1 period of their generation cycles at the time when the ^{3}HTdR was injected. Earlier studies of the generation cycle time of recognized megakaryocytes indicated that the non-DNA-synthesis duration in those cells was also about 2 hr. Therefore it appears that megakaryocyte precursors are not in continuous DNA synthesis, as was once suggested to explain the near 100% labeling that the population eventually reaches. There still remains an unexplained inconsistency between these results and the generation cycle time established for immature megakaryocytes of the recognized population, because entry of labeled cells into the recognized population continued for 12 hr after injection of ^{3}HTdR in this study, whereas the duration of the S period in the studies on recognized megakaryocytes was about 7.6 hr.

SHORT-TERM SUSPENSION CULTURES OF RAT BONE MARROW

Jean R. Murphy, T. T. Odell, Jr., and Diane K. Beeman

A reproducible *in vitro* system for bone marrow culture could facilitate investigations of hemopoiesis and of humoral agents such as erythropoietin and thrombopoietin. It might also promote studies of altered systems such as that existing in acute myeloid leukemia. The major types of marrow cells have been maintained and identified in human, mouse, and marmoset marrow cultures. Proliferation and maturation of granulocytes, monocytes, and macrophages have been examined in rat marrow cultures but not the megakaryocyte-platelet line. Accordingly, the establishment of rat bone marrow suspension cultures for short-term *in vitro* studies was undertaken. Various culture media, CO_2 proportions, and media pH's were evaluated. Culture term, cell growth, and cell variety were examined. As a result of this information, tibial and femoral marrow were cultured at 37°C, 5% CO_2, in RPMI 1640 (GIBCO) supplemented with 15% fetal calf serum. This liquid culture system permits regular cell counts and easy cell retrieval for morphology and possibly biochemical information without the use of an enzyme such as agarase. Thus cell kinetics and functional maturation can be studied. Preliminary data strongly suggest a correlation between the pH of the cultures and the spontaneous proliferation and maturation of the megakaryocytes. This seems to be the case whether the pH is altered by decreasing the CO_2 level or by altering the buffer system by adding Hepes or additional $NaHCO_3$ to the medium. At present, the most conducive pH to sustain viable cells in this rat marrow system lies between 7.8 and 8.0. In more acid environments, fibroblasts appear earlier. At a pH of 7.6, fibroblasts were seen at three days while at 7.84 they appeared at eight days. When $NaHCO_3$ was used to maintain a pH of 8.0, there were still no fibroblasts at 13 days, but the cells looked altered, having characteristics similar to macrophages.

It also appears to be advantageous to begin primary cultures with a marrow plug of approximately 4×10^7 cells per milliliter of medium rather than a suspension of the same number of cells or a plug containing fewer cells. The use of a suitable plug size may help to maintain a more normal microenvironment and cell density.

Experiments utilizing tritiated thymidine and autoradiography are being undertaken to investigate the dynamics of megakaryocyte proliferation in this system. Nucleated cell numbers are also being used as an index of cell proliferation. Cells are being collected on cover glasses and stained with Giemsa to study morphology and to obtain information about the kinetics of these cells *in vitro*.

PROGRAMMING FOR INCREASED THROMBOCYTOPOIESIS IN THROMBOCYTOPENIC RATS

B. G. Haik,* Jean R. Murphy, Diane K. Beeman, and T. T. Odell, Jr.

It has been reported previously that the number of megakaryocytes, the proportion of immature megakaryocytes, and the average ploidy level of megakaryocytes all increase in rats recovering from severe, acute thrombocytopenia. The latter two changes were first noted at 18 hr after induction of thrombocytopenia with platelet-specific antiserum (APS), while changes in platelet number were seen at 24 hr. These findings raise questions about what cells in the megakaryocyte line receive the stimuli that lead to these changes. Since recognized megakaryocytes do not divide, the programming for increased numbers must occur in early megakaryocyte precursor cells. However, the appearance of normally absent 64*N* cells at 18 hr suggests that programming for additional DNA replication occurs in cells that have already advanced part way through the maturation process. The generation cycle time of megakaryocytes in untreated rats is about 9 hr. Eighteen hours is therefore approximately two generation cycles, implying that the stimulus is received by a 16*N* cell. To examine the appearance of changes in DNA synthesis by another technique, the 30-min labeling index of megakaryocytes with tritiated thymidine (^{3}HTdR) was examined at intervals after induction of thrombocytopenia. The number of megakaryocytes per high-power field and the proportion of immature megakaryocytes were also determined in the same rats. No difference in any of the measured parameters was seen between controls and experimentals at ½ hr. At 6 hr neither the number of megakaryocytes nor the proportion of immature megakaryocytes was significantly altered, but the labeling index was approximately 50% greater in the thrombocytopenic rats than in controls. By 24 hr all experimental values were greater than those of controls. These results support the hypothesis that the stimulus of thrombocytopenia acts on megakaryocytes that are undergoing their last replications of DNA before becoming fully mature, platelet-producing cells. The thrombocytopenic stimulus thus appears to have two points of action on thrombocytopoiesis: one is on late immature cells to program an additional replication of DNA and another is on early precursors to provide a larger population of megakaryocytes. Additional experiments have been initiated to provide further evaluation of the time period between 0 and 18 hr with these techniques.

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EFFECT OF DEGREE OF THROMBOCYTOPENIA ON THROMBOCYTOPOIETIC RESPONSE

T. T. Odell, Jr., Jean R. Murphy, and Diane K. Beeman

Although it has been shown that the production of blood platelets is stimulated by acute, severe thrombocytopenia, the thrombocytopoietic response to more moderate thrombocytopenia has received little attention. Some results have suggested the possibility that the nature of the response may differ depending on the degree to which the circulating platelet mass is depleted. Because information about responses after varying degrees of thrombocytopenia may be valuable in understanding mechanisms of recovery from thrombocytopenia, experiments were undertaken in which rats were exchange transfused with platelet-poor blood to reduce the platelet count to various percentages of the normal circulating number, and platelet recovery was observed by carrying out peripheral platelet counts at frequent intervals during an eight- to ten-day period. Exchanges of 5–50 ml reduced the circulating platelet count 15 min later to 71–13% of the preexchange count. In rats exchanged with 5, 10, or 15 ml (71, 54, and 46%), the rates of platelet increase in the peripheral blood during the first 96 hr could be fitted to single regressions having slopes of 0.52 to 0.66. After exchanges of 30 and 50 ml (28 and 13%), the data between 15 min and 96 hr were fitted to two regressions rather than one. The rates of increase between 15 min and about 45 hr were similar to the single rates of the 5-, 10-, and 15-ml groups, having slopes between 0.66 and 1.02. The rates were greater, however, after about 45 hr, having slopes of 1.45 to 1.94. The maximum platelet count was reached at about 125 hr in all groups, regardless of the degree of thrombocytopenia. These results clearly demonstrate a marked difference in the thrombopoietic response of moderately and severely thrombocytopenic rats, and show that rats exposed to different degrees of platelet depletion restore the circulating platelet level within about the same length of time by adjusting thrombocytopoiesis. The question arises whether the adjustment to provide different rates of platelet production is a quantitative one or whether different mechanisms may

be involved. It has been shown that at least two changes in megakaryocytopoiesis are stimulated by severe thrombocytopenia, one being an increase in the number of cells entering the recognized megakaryocyte population and another being an increase in the average ploidy of megakaryocytes. In addition, there may be an increase in the rate of maturation of megakaryocytes. The way in which the changes in rates of platelet production and in maturation of megakaryocytes relate to each other remains to be clarified.

PATHOLOGICAL LESIONS IN MICE GIVEN BUTYLATED HYDROXYTOLUENE AND DIETHYLNITROSAMINE

N. K. Clapp, R. L. Tyndall,* and R. B. Cumming

The addition of antioxidants to foodstuffs for human consumption is a common practice to reduce spoilage. Butylated hydroxytoluene (BHT) is used extensively as a preservative for unsaturated lipids. These compounds have been considered to be harmless as used,[1] although some hepatic enzyme changes have been detected. Previously, Cumming and Walton[2] reported a marked protective effect upon acute lethality by diethylnitrosamine (DEN) when mice were prefed BHT in the diet. We sought to determine whether similar effects would be observed relative to the later effects of DEN, namely, on its carcinogenic activity.

We treated male BALB/c's with 0.75% BHT in the feed for their lifetime, and in addition gave DEN for seven weeks (330 mg per kilogram of body weight); other mice were given BHT and DEN alone, and some mice received no treatment. Mice receiving DEN began to die and a majority of the mice were killed as scheduled at 12 months, with the remainder being killed at 18 months.

BHT mice developed increased incidences of lung tumors (63.6%) above controls (24.0%) at 18 months, and those mice receiving BHT and DEN had more tumors per tumor-bearing mouse than either BHT alone, DEN alone, or controls (4.0 to 1.4–2.2). The incidence of reticulum cell sarcoma (RCS) in mice killed at 12 months of age in the BHT plus DEN treatment group exceeded control values (52.6% vs 22.7%), while incidences in groups receiving DEN alone or BHT alone were not different from controls. At 18 months, RCS incidences in both groups given BHT were significantly less than in those groups not receiving BHT. At 12 months of age, a higher percentage of squamous cell carcinomas was seen in the forestomachs of mice after BHT plus DEN than in mice receiving DEN alone; the production of cysts within the liver parenchyma after DEN also appears to be potentiated by BHT treatment. In addition, a marked bile duct hyperplasia was observed at 12 months in 6 of 18 mice receiving BHT alone but not in other groups with or without DEN; no such lesion was seen at 18 months, suggesting possible population selection or regression. The fate of this lesion is uncertain at this time.[3]

While little is known about the interactions of BHT and DEN and of the severity of these changes upon the human population, the report of these various tumorigenic effects and liver lesions suggests the need for caution in utilizing this compound as an antioxidant in foodstuffs commonly consumed by human beings on a long-term basis. Further studies are in progress to determine the etiology and mechanism of these changes.

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2. R. B. Cumming and M. F. Walton, *Food and Cosmetics Toxicology*, in press.

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ONCOGENICITY OF NITROSAMINES AND METHANESULFONATE ESTERS GIVEN INTRAPERITONEALLY IN RF MICE

N. K. Clapp

The sensitivity of different organs to cancer induction by dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) has been reported in a number of animal species and several strains of mice. By giving these alkylating agents orally in the drinking water, long-term exposures at low dose rates were obtained and organ specificities were observed. After finding squamous cell carcinomas in the forestomachs after DEN, we were interested in determining the effect that change in the route of administration would have upon the organ sensitivity of DMN, DEN, methyl methanesulfonate (MMS), and ethyl methanesulfonate (EMS).

A total of 221 male mice were given three dose levels of DMN (5, 10, and 15 mg/kg), two dose levels of DEN (150 and 175 mg/kg), and one dose level each of MMS (150 mg/kg) and EMS (175 mg/kg) intraperitoneally in single injections in physiological saline solution. Mice lived out their life-spans and were examined grossly and histologically for tumors.

$LD_{50/30\ days}$ for DMN, DEN, and MMS were suggested at 12 to 13, 190 to 200, and 175 to 200 mg/kg, respectively; no figure was obtained for EMS in this

study. DEN induced lung adenomas, liver hepatomas, and forestomach tumors (squamous cell carcinomas and papillomas) at all doses and Leydig-cell tumors of the testis at the highest dose given. DMN, while increasing lung-tumor incidence, failed to induce liver tumors when given intraperitoneally, in contrast with high incidences after oral administration; the reason for this difference is not obvious at this time. With this exception, single injections of DMN and DEN were nearly as effective in causing tumors as long-term oral treatments with much higher doses. The susceptibility of the testis may relate in part to physical accessibility through the inguinal canal but also indicates some peculiar organ sensitivity. MMS and EMS were not carcinogenic as used in this study, in contrast with lung tumor induction by MMS at high oral doses. None of these alkylating agents were leukemogenic.

RELATIVE BIOLOGICAL EFFECTS OF WHOLE-BODY IRRADIATION WITH 60-MeV PROTONS AND 300-kVp X RAYS IN RF MICE

N. K. Clapp, E. B. Darden, Jr., and M. C. Jernigan

Over 3,100 young adult female RF/Un mice were given whole-body exposure to 60-MeV protons or 300-kVp x rays (0 to 400 rads) and were observed for life-shortening effects and neoplastic and nonneoplastic diseases. Complete pathological examinations were done on all animals, and 81% were examined histologically.

Similar dose-response curves were seen for most diseases, but with the exception of ovarian tumor induction, x rays were slightly more effective in inducing a specific disease than were protons. For life shortening, x rays were more effective at all doses (0.34 to 0.80 day per rad), with the greatest difference in effect between x rays and protons at the two lowest doses, 50 and 100 rads. X rays increased the combined incidences of thymic lymphoma and myeloid leukemia, but these leukemias did not account for all life shortening at higher doses (100 to 400 rads).

X rays were more effective than protons in inducing myeloid leukemias at 50 to 200 rads and thymic lymphoma at 300 to 400 rads, with no differences at other doses. Maximum incidences were reached with x rays at 300 and 400 rads for thymic lymphoma and at 200 rads for myeloid leukemia, while there was no difference in effect between radiations on reticulum cell sarcoma and nonthymic lymphoma frequency. The shape of the dose-response curves of corrected incidences for thymic lymphoma and myeloid leukemia remained the same, but incidences were increased while corrected incidences of reticulum cell sarcoma showed no dose effect, contrasting with a negative dose-response in observed incidences. Combined observed incidences of all leukemias were not different for control and irradiated groups at 50 rads, nor at 100 rads for protons, but increased at higher doses.

Lung tumor incidence showed a negative dose-response due to the failure of animals to survive long enough to develop this usually nonfatal disease. Ovarian tumors were increased by all doses of both radiations, and this was the only instance where protons were more effective than x rays; maximum response was seen at 100 rads for both radiations. Pituitary gland and Harderian gland tumors may have been increased by radiation ($p < 0.10$ and < 0.05, respectively), but the effect was not dose dependent. Nonneoplastic diseases showed little effect from radiation.

Relative biological effectiveness (RBE) for 60-MeV protons was estimated at 0.63 for life shortening and at generally slightly less than 1.0 for all parameters, excluding ovarian tumors. Only thymic lymphoma, myeloid leukemia, and ovarian tumors were significantly increased by radiation, with suggested increases in Harderian gland and pituitary gland tumors. Other pathological alterations in incidence and severity of disease would appear to be minimal when compared to nonirradiated populations.

TRANSPLANTABLE MALIGNANT MURINE LUNG CANCERS

J. M. Yuhas, Judith O. Proctor, R. E. Toya, Anita E. Walker, and N. H. Pazmiño

In the course of our investigations on the use of radioprotective drugs in the radiotherapy of lung cancer,[1] it became apparent that the lung cancer models available in the mouse were ill suited since the cancers in question either changed histologic type and/or metastasized only rarely. We therefore attempted to adapt the malignant lung carcinomas we were observing in the BALB/c mouse[2] to transplantable and tissue-culture-maintained lines. More than 30 lines have now been isolated, expanded, and frozen. In each case, the cancers transplanted readily, and we report below our experience with our oldest line, line No. 1.

This cancer was isolated from an unirradiated 12-month-old BALB/c female mouse and has been maintained in transplantable form for more than 30 generations without loss of its original histologic character. At the 12th transplant generation, the cancer was adapted

to tissue culture and has been maintained in culture for 20 generations. Reinoculation of the tissue-culture-maintained line into BALB/c mice results in tumors that are histologically indistinguishable from the primary tumor or the *in vivo* transplanted line.

The line No. 1 cancer grows rapidly following inoculation (T^2 = 3 days) and kills the hosts by the 30th to 60th day after inoculation. Metastasis from the subcutaneous injection site to the lungs is first detectable at four weeks after transplant. From an analysis of the growth rate of intravenously injected cancer cells, it would appear that these metastases had seeded to the lungs by the second week after inoculation.

The syngeneic BALB/c hosts in which this cancer is transplanted do not appear to mount an effective immunologic defense against any tumor-specific transplantation antigens this cancer may express. Less than 100 cells are required to transplant the cancer either subcutaneously or intravenously. This interpretation is further supported by our inability to immunize mice against the tumor with radiation-killed tumor cells and by direct testing of the cell-mediated and humoral immune responses to the tumor.

We are continuing these studies in an attempt to account for the metastatic properties of these cancers and to use these cancers in therapy studies.

1. G. Kollman, B. Shapiro, S. Leone, and J. M. Yuhas, "Distribution and Metabolism of WR-2721 in the Normal Tissues and Lung Tumors of Mice," this report.

2. J. M. Yuhas, C. C. Lushbaugh, D. C. Swartzendruber, N. H. Pazmino, A. E. Walker, and J. O. Proctor, "Induction of Malignant or Benign Lung Tumors in the BALB/c Mouse," this report.

LOCALIZED RADIATION INDUCTION OF BENIGN PULMONARY ADENOMAS IN THE RFM MOUSE

J. M. Yuhas and Anita E. Walker

In the more than 20 years that various sublines of the RF mouse have been used in radiation carcinogenesis studies in the Biology Division, there has been no demonstration of radiation-induced lung cancer. These data can be interpreted in two ways: the total body exposures used, although approaching the maximum tolerated, were insufficient to induce the tumor, or the wide spectrum of other radiation-induced diseases killed the mice before sufficient time had elapsed for the tumors in the lung to appear. In order to test these possibilities, we exposed the chest of RFM male mice to graded doses of x rays (750 R to 3000 R) and followed them for 11 months. During this time, only a few of the animals died, and essentially all exposed mice were available for analysis at 11 months. The incidence of benign pulmonary adenomas rose from 33% in the control group to 86% in the 1500 R group and then declined at higher doses. The decline cannot, in this case, be attributed to premature death of the animal and must therefore result from the dose-dependent interaction of transformation and cytotoxicity. Back extrapolation of the linear region of the curve data (750 R through 1500 R) predicts that elevated lung tumor incidences should be observable in the total body tolerated dose range (i.e., a 10% increase in risk per 100 R). We are currently investigating the basis of this difference between prediction and observation by comparing the lung carcinogenic effects of localized and total body exposures of 100 through 400 R.

DISTRIBUTION AND METABOLISM OF WR-2721 IN THE NORMAL TISSUES AND LUNG TUMORS OF MICE

G. Kollman,* B. Shapiro,* S. Leone,* and J. M. Yuhas

We have previously observed that injection of RFM mice bearing urethane-induced lung adenomas with WR-2721 (S-2-3-aminopropylaminoethylphosphorothioic acid) immediately before radiotherapeutic exposures provided protection to the normal tissues but offered no protection to the tumor. If the time between injection and exposure was extended to 90 min, however, a moderate protection of the tumor tissue was also observed. This time dependence for tumor protection offered a means of determining the basis of the differential protection of normal and tumor tissue and of gaining an insight into which metabolite of the drug was actually effecting protection.

Analysis of the distribution and metabolism of ^{35}S-labeled WR-2721 demonstrated a fourfold greater concentration of the drug in the normal tissues (spleen, liver, kidney, etc.) than in the tumor during the period in which only the normal tissues were protected from irradiation. The normal tissue concentration dropped only minimally by 90 min after injection, while the tumor concentration rose significantly. This differential rate of absorption of the radioprotective drug apparently accounts for the differential chemoprotection against radiation injury. Further, the only metabolite of WR-2721 which increased appreciably at 90 min after injection was the mixed disulfide of the drug, suggesting that this is the protective form.

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CARCINOGENIC EFFECTS OF 8 RADS PER DAY DELIVERED DURING THE PRENATAL PERIOD

J. M. Yuhas and Judith O. Proctor

Retrospective studies conducted on human populations exposed, as fetuses, to diagnostic x rays have demonstrated a direct correlation between the number of these small exposures received and the risk of childhood cancer. No such grossly elevated risk has been demonstrated in experimental animals, however, possibly due to the use of high doses which imposes a strong selective force or which "miss" the critical period at which sensitivity to cancer induction is highest. In order to circumvent these problems and to determine whether the mammalian fetus in general is highly sensitive to the carcinogenic effects of repeated small exposures, we exposed 200 BALB/c fetuses of either sex to 0 or 8 rads/day throughout fetal life. Each pair of parents also received a variable but known amount of preconception exposure at the same rate.

Each of the four experimental groups (control and irradiated males and females) is now two years old, and each has reached its 50% survival time. Although no significant differences have appeared in average survival, irradiation has disturbed the death distributions. Irradiation is associated with fewer deaths in the 100-to-300-day period but a more rapid mortality rate after 400 days. This late effect of irradiation is not associated with any specific lesion in the males, but is due, at least in part, to an increased incidence of ovarian tumors and associated hemorrhagic cysts (4% vs 35%) in irradiated females. Neither survival time nor cancer incidence bears a direct relation to the amount of preconception exposure received by the parents.

LOW DOSE AND LOW DOSE RATE INTERACTIONS IN RADIATION CARCINOGENESIS

J. M. Yuhas and Anita E. Walker

In order to determine the carcinogenic effects of low total doses of gamma rays when given at low dose rates, we have exposed a total of 7496 BALB/c female mice to total doses of 49, 98, 196, and 392 rads at dose rates of 1.75, 3.5, 7, 14, 28, 56, 112, or 6×10^4 rads/day. Also included are mice given the acute exposures at advanced ages and groups given fractionated exposures. Upon completion, this study will allow quantitative description of the importance of total dose and dose rate and their interaction in determining carcinogenic hazards. Further, due to the use of twofold increases in dose and dose rate, the effects of exposure time can also be assayed. Short-term studies are also being conducted on the importance of aging, immunocompetence, and hormonal factors in the overall dose rate effect.

Of the original number of mice included in the experiment, 2308 had died as of July 1, 1973. The remaining mice are all at least 550 days old and are entering or are within the period of significant mortality. In order to avoid excessive thymic lymphoma incidences, the mice were exposed at 16 weeks of age. This cancer has accounted for less than 5% of the total deaths in all experimental groups. Since all mice in the experiment have passed the critical period for this leukemia's development, this should represent a final estimate.

While the study is not yet complete, it is already apparent that a number of solid tumors have been induced by the exposures, and further certain preliminary estimates of the effects of dose rate are apparent. The solid tumors which have been shown to be radiation induced are: malignant lung carcinomas but not the typical adenomas,[1] hepatomas, fibrosarcomas, ovarian tumors of varied histologic type, mammary carcinomas, and salivary carcinomas. The most interesting aspect of these preliminary estimates is that the dose rate effect does not appear the same for all tumor types, and further the dose rate effect varies as a function of the total dose studied.

If life shortening is analyzed as a function of both dose and dose rate, one observes the classically expected patterns (i.e., larger doses and higher dose rates induce more life shortening).

1. J. M. Yuhas, C. C. Lushbaugh, D. C. Swartzendruber, N. H. Pazmino, A. E. Walker, and J. O. Proctor, "Induction of Malignant or Benign Lung Tumors in the BALB/c Mouse," this report.

INDUCTION OF MALIGNANT OR BENIGN LUNG TUMORS IN THE BALB/C MOUSE

J. M. Yuhas, C. C. Lushbaugh,* D. C. Swartzendruber,*
N. H. Pazmiño, Anita E. Walker,
and Judith O. Proctor

In addition to the typical papillary lung adenomas which are observed in most mouse strains, the BALB/c mice in our laboratory develop a highly malignant alveolar cell carcinoma of the lung. In contrast to the adenoma, this tumor is highly invasive and metastasizes in about half of the cases observed therefor. Preliminary data indicate that both types of tumors, the adenoma and the carcinoma, arise from the same type II alveolar

cell in the mouse lung. The simplest proposal to account for the presence of both of these tumor types in the same animal is that the carcinoma is merely an extreme form of the adenoma and chance determines whether the tumor will be highly malignant or benign.

If this proposal is correct, then agents which induce adenomas in other strains should induce both types of tumors in the BALB/c and vice versa. On the contrary, we have observed only adenomas induced in BALB/c given 1 g of urethane per kilogram of body weight. These studies are not yet complete, but the incidence of adenomas has reached 100% at 9 months after injection, and the mean number of adenomas per mouse has reached a plateau at 2.3. We are continuing to follow these animals and are exposing some to radiation in order to determine whether the adenoma can develop into a carcinoma. Total body irradiation studies have demonstrated an effect which is the reverse of urethane; carcinomas are induced while adenomas are not. Further localized exposures of more than 1000 rads induce only carcinomas in the BALB/c, while they induce only adenomas in the RFM.[1]

From these data we conclude that the carcinoma is not merely a chance variant of the adenoma, but that each requires a specific set of conditions for its induction. Whether this variability resides within the nominally homogeneous type II alveolar cell or in some factor extrinsic to the transformed cell is presently under study.

The most interesting aspect of the radiation induction of these cancers is the effect of dose rate on inductive efficiency. Total body doses of 49 to 392 rads have been given at dose rates of 1.75 through 6×10^4 rads/day. At the highest dose tested, 392 rads, maximum inductive efficiency occurs at the 14 to 28 rad/day level. At a lower dose, 196 rads, maximum efficiency occurs at the 56 rad/day rate. Preliminary conclusions from these data are that the highest dose rates are not necessarily the most efficient inducers of these lung cancers and that the dose rate at which efficiency is maximum may increase as total dose decreases.

Direct testing of a recovery component in lung carcinoma induction is presently in progress. Split equal doses of x rays are less efficient inducers of lung carcinomas than is the same total dose given as a single exposure. Injection of a DNA repair inhibitor, chloroquine, immediately after the first of two exposures increases the carcinogenic effects of the split exposure regimen. As an example, 104 BALB/c mice were given localized lung exposures of 500 to 5000 rads in two fractions separated by 5 hr, and 102 were given similar exposures but with chloroquine injection (30 mg/kg) immediately after the first exposure. We have observed 13 cases of malignant lung carcinoma in the irradiation-only group and 20 cases in the irradiation and chloroquine group. This and other studies are being continued in an effort to understand the dose rate effects involved in the induction of lung carcinomas.

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*Oak Ridge Associated Universities.

1. J. M. Yuhas and A. E. Walker, "Localized Radiation Induction of Benign Pulmonary Adenomas in the RFM Mouse," this report.

EFFECTS OF A DNA REPAIR INHIBITOR ON ONCOGENIC VIRUSES *IN VIVO* AND *IN VITRO*

N. H. Pazmiño, R. W. Tennant, and J. M. Yuhas

The ability of the antimalarial drug chloroquine, CHL, to inhibit the repair of DNA in mammalian cells may make this drug either a promoter or an inhibitor of carcinogenesis. The promoter action would be exerted by inhibition of the repair of genetic alterations leading to transformation in irradiated or chemically treated cells. Analyses of this possibility are presented elsewhere in this report.[1] Alternatively, CHL might interfere with the replication of RNA oncogenic viruses, since there is evidence suggesting the involvement of a DNA intermediate.

In order to test this possible interference of CHL with RNA tumor virus replication, two *in vivo* model systems were used: the induction of rhabdomyosarcomas by the Moloney strain of the murine sarcoma virus (MSV-M) and the induction of leukemias by the Rauscher leukemia virus (RLV). In both systems, injection of CHL at a dose far below toxic levels, 30 mg/kg, inhibited the development of the tumors. Since *in vivo* systems do not lend themselves to precise mechanism studies, we conducted our further experiments in tissue culture. A CHL concentration of 1 μg/ml was nontoxic to Swiss mouse embryo cells in culture as evidenced by growth rate, DNA synthesis, and RNA synthesis. However, this same concentration inhibited the replication of Moloney murine leukemia virus as evidenced by the number of cells which expressed the specific antigens following virus exposure. If CHL is added 3 or more hours after the virus, no inhibition of antigen expression is observed, suggesting that CHL affects one or more of the early steps in virus infection and replication. These studies demonstrate that the inhibitory effect is expressed *in vitro* just as it

is *in vivo*, although it is too early to conclude identity of mechanism.

Preliminary data on the mechanism of inhibition *in vitro* have indicated that at least two factors are involved. CHL inhibits the cellular absorption of the virus by 40 to 50%, and it also inhibits the activation of endogenous virus by IUdr. We are presently expanding these preliminary data and determining the effects of CHL administration *in vivo*.

1. J. M. Yuhas, C. C. Lushbaugh, D. C. Swartzendruber, N. H. Pazmino, A. E. Walker, and J. O. Proctor, "Induction of Malignant or Benign Lung Tumors in the BALB/c Mouse," this report.

DNA REPAIR INHIBITION AND RADIATION THERAPY

N. H. Pazmiño and J. M. Yuhas

The postirradiation injection of DNA repair inhibitors, most especially chloroquine (CHL), has been suggested as a possible means of increasing the efficiency of radiotherapy. Data to support this proposal have been restricted to analyses of tumor response, and therefore no evaluation could be made. Since selective or at least preferential sensitization of the tumor, as opposed to the normal tissues, is required for therapy, we evaluated this hypothesis by studying a number of normal mouse tissues as well as the tumor following radiation exposure and chloroquine treatment.

BALB/c mice were given two equal x-ray exposures separated by 5 hr. CHL was injected (30 mg/kg) at varying times between the two exposures. Our studies on the line No. 1 alveolar cell carcinoma[1] confirm the ability of CHL to increase net tumor injury when it is given immediately after the first exposure. However, the increase in radiation injury in the skin, bone marrow, and those tissues which determine survival following total body irradiation was at least equal to that observed in the tumor. Injection of the drug 2 or more hours after exposure fails to increase injury in the normal tissue, but it appears that the increase in tumor injury is also lost. We conclude therefore that postirradiation injection of CHL is not likely to increase the efficiency of radiotherapy.

1. J. M. Yuhas, J. O. Proctor, R. E. Toya, A. E. Walker, and N. H. Pazmino, "Transplantable Malignant Murine Lung Cancers," this report.

SENESCENT LOSS OF RESISTANCE TO MURINE SARCOMA VIRUS (MOLONEY) IN THE MOUSE

N. H. Pazmiño and J. M. Yuhas

Sensitivity to the effects of many RNA oncogenic viruses, including murine sarcoma virus Moloney strain (MSV-M), is maximal at birth and declines rapidly as the mouse approaches young adulthood. This decline in sensitivity is a reflection of the maturation of the immune system, which, through a complex interaction of cell-mediated and humoral factors, controls tumor appearance and progression. The resistance of the young adult is not an absolute one, however, since mice which resisted tumor development and progression as young adults eventually show an elevated leukemia and sarcoma risk late in life. We have proposed and tested the hypothesis that aged animals will demonstrate a return of the sensitivity they manifested as newborns due to the decline of immunocompetence with advancing age.

Our analysis of the oncogenic effects of MSV-M in CD_2F_1 mice ranging in age from newborns through three years has confirmed the rapid decline in sensitivity to the virus as the mouse matures and has demonstrated a return of this sensitivity in mice more than one year old (Fig. 17). The sensitivity becomes progressively more severe as the animal ages, and at the oldest ages tested (2.5 to 3 years), the sensitivity is equal to that of neonates.

Serum antibodies, which react specifically with MSV-M antigens, were assayed in newborn, six-month-, two-year-, and three-year-old mice for the first eight weeks following MSV-M injection. None of the mice that were sensitive to tumor appearance (newborns, two

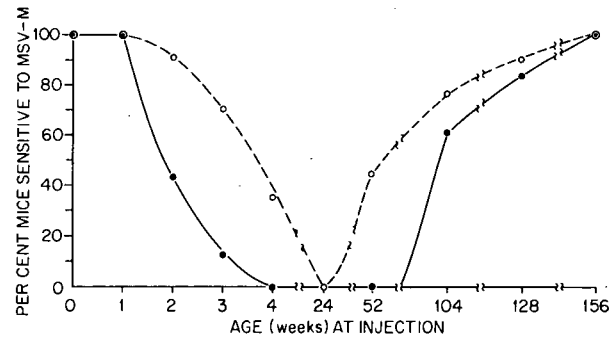

Fig. 17. Change in sensitivity to MSV-M of $CD2F_1$ mice as a function of age. •, percentage of mice dying with tumors during the period of the experiment (160 days); ○, percentage of mice developing tumors at any time during the experiment.

years, and three years) demonstrated detectable serum antibody levels during this period, while the six-month-old mice which are resistant to the oncogenic effects of MSV-M demonstrated high titers of an antibody which reacted specifically with MSV-M and which neutralized the infectivity of the virus.

Our studies are continuing on the cell-mediated immunity in these animals and its importance in determining the fate of the tumors following appearance.

LIFE-SPAN AND DISEASE INCIDENCE IN MICE IRRADIATED WITH FISSION NEUTRONS DURING THE PRONUCLEAR-ZYGOTE STAGE

G. E. Cosgrove, W. Friedberg,* G. D. Hanneman,*
E. B. Darden, Jr., and D. N. Faulkner*

We have been investigating effects of ionizing radiation given at times in the early postconception period when pregnancy in humans is ordinarily not recognized.[1] In the present experiment, CD-1 mouse embryos in the pronuclear-zygote stage were irradiated *in utero* with a dose of 15 rads of fission spectrum neutrons or were sham irradiated. Approximately 55% of the irradiated embryos died *in utero*. Mice that survived at least until 30 days after birth were used in the study. They were allowed to live out their normal life span, and at death, necropsies were performed and pathological findings recorded. Table 19 shows median life span and incidence of principal diseases at death. Results for irradiated and control groups of the same sex were tested for significant difference at the 5% probability level. Differences in median life span (30-day survivors) were not significant. A higher percentage of irradiated than control females had liver tumors, the only disease in which a difference between groups is significant. The fact that this difference is significant is likely a chance occurrence because of the number of comparisons made. The difference is not significant at the 1% level.

*Civil Aeromedical Institute, Federal Aviation Administration, Oklahoma City, Oklahoma.

1. W. Friedberg, G. D. Hanneman, D. N. Faulkner, E. B. Darden, Jr., and R. B. Deal, Jr., *Int. J. Radiat. Biol.* (in press).

Table 19. Median life span and principal diseases at death

	Control		Irradiated	
	Male	Female	Male	Female
Median life span (days)	660	657	647	715
Number of mice	192	170	102	94
Diseases				
Number of mice	186	163	98	90
Neoplasms	67%[a]	72%	58%	77%
Leukemia/lymphoma	28%	39%	26%	31%
Reticular cell	24%	33%	17%	23%
Thymic	5%	5%	7%	7%
Tumor				
Lung	26%	21%	23%	26%
Ovary		20%		22%
Liver	19%	4%	12%	11%
Breast		9%		9%
Nonneoplastic				
Pyelonephritis	23%	18%	27%	24%
Severe dermatitis	10%	1%	9%	2%
Pneumonia	4%	2%	3%	1%
Hematopoietic hyperplasia	15%	12%	8%	16%
Amyloidosis	5%	5%	3%	4%
Auricular thrombosis	18%	9%	18%	16%
Abscesses	5%	4%	6%	1%

[a]Percent of mice afflicted.

EFFECTS OF FISSION NEUTRONS OR X RAYS ON THE PREIMPLANTATION MOUSE EMBRYO

E. B. Darden, Jr., W. Friedberg,*
G. D. Hanneman,* and D. N. Faulkner*

This project arose because of the scarcity of adequate data concerning the effects in mammals of prenatal exposure to densely ionizing radiation. Information was particularly sparse for very early postconception exposure when, in the human, pregnancy may be unsuspected. From the practical standpoint there was concern about the possible risk associated with the high-LET component of space radiation for passenger aircraft at high altitudes. Now similar risks, resulting from casual or occupational exposure to fast neutrons from small man-made sources becoming available on earth, may assume greater significance.[1]

The principal end point has been relative embryo survival. As described previously, young adult female mice, following overnight mating, are irradiated with fission neutrons (Health Physics Research Reactor) or with x rays. Just before term, the uteri are examined for live and dead embryos. We reported previously on mice irradiated at the pronuclear zygote stage (day 0 after mating). The findings, including RBE data and effects of split and protracted exposures, have been summarized in a paper in press.[2] The results suggest that embryo death following irradiation with either neutrons or x rays at the one-cell embryo stage is probably the consequence of a single irrepairable injury event. Work now in progress is concerned with effects of irradiation at later preimplantation stages (day 1 after irradiation). Preliminary results of neutron irradiation at the two-cell stage point to a more complex prenatal survival curve (than the simple exponential type found for the pronuclear zygote) with a shoulder of more than 60 rads. This finding suggests that both blastomeres have to be lethally irradiated to kill the embryo.

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EFFECT OF LYMPHOCYTES ON THE PATTERN OF HEMOPOIETIC DIFFERENTIATION IN CHIMERIC SPLEENS

Nancy L. Basford and Joan Wright Goodman

When it was first reported that thymocytes augmented hemopoiesis in donor-host combinations showing poor growth, the possibility was raised that augmentation was restricted to one type of differentiation or that erythropoiesis had been increased at the expense of other cell lines. To investigate such possibilities, we undertook to study chimeric spleens histologically a week after cells had been transplanted. The first results, from experiments using a single marrow dose and a fixed thymic lymphocyte:bone marrow (L:B) ratio, will be published.[1] Results presented here extend the previous findings to other marrow doses and a variety of L:B ratios. They also include data from recipients of lymph node lymphocytes (LNC) and of lymphocytes, either thymic or LNC, from donors exposed to 900 R.

F_1 hybrid recipients were exposed to 900 R whole-body radiation; 18 to 24 hr later, parental cells were injected intravenously. Chimeras were killed and their spleens were removed seven or eight days after cells had been transplanted. Spleens were fixed in Tellyesniczky's fluid, and after a count was made of surface colonies, they were prepared for histologic study. Hemopoietic foci were counted and classified as to the type of differentiation they represented. One midline section from each recipient was scored, and each group within an experiment contained 20 to 30 mice. Occasionally mice died before the end of the experiment in groups that might be expected to develop graft-vs-host (GVH) disease (i.e., recipients of LNC or large numbers of thymocytes).

In confirmation of earlier work[2] it was found that thymocytes increased the number and size of colonies in recipients of marrow. A shift of differentiation toward granulopoiesis occurred when thymocytes were given, although erythropoietic colonies were the most frequently seen type except at very high L:B ratios. Lymph node lymphocytes shifted the pattern more markedly toward granulopoiesis, even at low L:B ratios. When lymphocytes from either source were given without marrow, very few colonies could be found in recipients, and if differentiated, they were almost exclusively granulopoietic. This study of differentiation patterns indicates that the presence in the spleen of lymphocytes, either from lymph nodes or thymus, influences the direction in which marrow stem cells will

develop. It is clear from control groups receiving no marrow that there is a true change in pattern and not simply the appearance of additional, lymphocyte-derived granulopoietic colonies.

Irradiation (900 R) of lymphocyte donors reversed the shift so that a normal pattern of differentiation, like that resulting from marrow alone, was seen. Irradiated lymphocytes (900 R) were nonetheless capable of augmenting the size and total number of hemopoietic colonies. Probably the most interesting findings came from these experiments because they help delineate different kinds of effects: (1) an increase in colony number which occurs even after donor irradiation, (2) a shift toward granulopoiesis which is completely destroyed by 900 R, and (3) an increase in colony size which appears not to require division of injected lymphocytes.

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ORIGIN OF SPLENIC COLONIES IN IRRADIATED MICE GIVEN BONE MARROW AND THYMOCYTES

Dale L. DeHamer* and Joan Wright Goodman

It has been adequately documented that thymocytes isogenic with marrow transplanted to irradiated mice can augment hemopoiesis in donor-host combinations that show poor growth.[1] Although the origin of dividing splenic cells in thymus-marrow chimeras has been presumed to be the donated marrow, the problem has not been definitely solved. There are three obvious possibilities: (1) cells are donor-marrow derived, thymocytes augmenting their growth in an as yet undefined way; (2) thymocytes, although unable when transplanted alone, are capable of giving rise to blood formation under the influence of rapidly dividing marrow cells; and (3) host cells, damaged by heavy irradiation, are induced by thymocytes to repair rapidly to support hemopoietic growth. The study described here was done to determine which of these possibilities was most likely.

Lethally irradiated hybrid mice were injected with male rat bone marrow and female rat thymocytes (or female marrow and male thymocytes). Appropriate marrow-only, thymocytes-only, and radiation control recipient groups were prepared in parallel. Eight days after transplantation of cells, mice received an injection of colchicine, and 2 hr later their spleens were removed.

Table 20. Origin of dividing spleen cells

Inoculants	Percent derived from –		
	Female rat	Male rat	Mouse
10^7 female rat bone marrow cells plus 10^8 male rat thymocytes	93.8	4.9	1.3
10^7 male rat bone marrow cells plus 10^8 female rat thymocytes	6.9	92.3	0.7

Cells were prepared for scoring and identification of metaphase chromosomes by suspension in hypotonic saline, fixation in methanol and glacial acetic acid, spreading on a wet slide, air drying, and staining with Giemsa.

At least 1000 cells were analyzed for each value appearing in Table 20. The results were very clear: more than 92% of the dividing cells were of donor-marrow origin; fewer than 7% were derived from the thymocytes injected. This experimental confirmation of our long-held impression was nonetheless disturbing in view of the histologic picture of these chimeric spleens which often appear to be 30 to 40% filled with lymphoid cells, many of them dividing. Inasmuch as recipients of thymocytes only show similar white pulp proliferation, it is surprising to find that marrow-plus-thymocyte chimeras seem to have repopulated these areas from transplanted marrow, not thymocytes. It is possible, of course, that the method of preparing cells preferentially destroys lymphoid rather than hemopoietic elements. The other possibility is that the chromosomal preparation fairly represents all elements and that lymphoid cells are showing augmented marrow-derived proliferation in the presence of thymocytes.

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RATE OF APPEARANCE OF B LYMPHOCYTES IN CHIMERIC MICE*

Joan Wright Goodman and J. J. Cohen†

Those elements responsible for humoral antibody production – B lymphocytes – are generally believed to differentiate directly from bone marrow stem cells, uninfluenced by the thymus. We have observed in histologic examination of spleens of F_1 hybrid mice that have been irradiated and given parental (P) marrow transplants that lymphocytes, both lymph node and

thymic, not only augment hemopoiesis but also lead to an early regeneration of white pulp areas. Control mice that had received lymphocytes only also showed repopulation of lymphoid areas, and the simple deduction was made that in both groups – marrow-only and marrow-plus-lymphocytes – the dividing cells were derived from donor lymphocytes. Recent studies[1,2] using chromosomal analysis cast doubt on this interpretation, as they show well over 90% of cells in mitosis in marrow-plus-lymphocyte chimeras are of donor-marrow origin. If the assumption is made that lymphoid cells are not preferentially destroyed by the technique used in preparation of the cells, both studies appear to show that transplanted lymphocytes are aiding the development of *marrow-derived* B lymphocytes. We decided to examine splenic cells from chimeras seven or eight days after transplantation and to determine the relative numbers carrying surface immunoglobulin and thus being identifiable as B lymphocytes. Suspensions were made from spleens of several mice in each group, and cell concentrations were adjusted to be comparable in all cases. Polyvalent rabbit anti-mouse-immunoglobulin serum was reacted with the cells, which after appropriate washing were exposed to fluorescein-labeled goat anti-rabbit immunoglobulin. Lymphocytes were identified under phase, and the relative number stained was determined by use of fluorescent light.

The results of one experiment are shown in Table 21. Very few lymphocytes of any kind could be found in marrow-only recipients, and no viable lymphocytes appeared in preparations of radiation control spleens. Lymphocytes were relatively plentiful in lymph node cell recipients, but they were not fluorescein-labeled. When both lymph node cells and marrow had been transplanted, half of all lymphocytes identified were stained. We are very much interested in these results as they indicate a second cell type is involved in the maturation of B lymphocytes from marrow stem cells. Currently, we are repeating and expanding this study with thymic as well as lymph node lymphocytes as donor material.

Table 21. Relative numbers of B lymphocytes in spleens of radiation chimeras (B6 → B6D2F$_1$) one week after transplantation

5×10^5 bone marrow	2×10^7 lymph node cells	Ratio of B lymphocytes to total lymphocytes
+	0	0/5
0	+	1/43
+	+	15/30
0	0	0

*This work performed while first author was an American Cancer Society Scholar in the Division of Allergy and Immunology, Department of Medicine, University of Colorado Medical Center, Denver.

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POOR GROWTH OF PARENTAL MARROW IN F$_1$ HYBRIDS: EFFECT OF THYMUS CELLS FROM LETHALLY IRRADIATED DONORS

Linda L. Pritchard*

Cells of F$_1$ hybrids between two inbred mouse strains generally carry all the transplantation antigens from both parent strains. Hence parental tissue grafts should grow well in F$_1$ offspring, but in some mouse strain combinations, marrow cells from one parent grow poorly in the F$_1$. Thymus cells isogenic with donor marrow augment marrow growth.[1,2] One measure of marrow growth is the gross splenic nodule assay.[3] If enough thymus cells are transplanted along with the marrow, parental marrow can be induced to form as many splenic nodules in F$_1$ recipients as in isogenic recipients.[4]

In order to determine whether thymus cells must be able to divide in order to exert their effect, thymus cells from lethally irradiated donors were tested in this system. (C57BL/6 × DBA/2)F$_1$ male or female mice or (C57BL × C3H)F$_1$ female mice were exposed to 900 R of x rays. The next day they received intravenous injections of thymus cell suspensions from donors (C57BL/6 or C57BL, depending on recipient) exposed to 900 R immediately before removal of their thymuses. Within 4 hr, the recipients received a low dose of marrow cells isogenic with the thymus cell transplant. Eight days later, their spleens were removed and fixed in Tellyesniczky's solution. Gross nodules[3] were counted under a dissecting microscope. If large enough numbers of these irradiated thymus cells are administered, marrow growth is significantly enhanced. However, there is a lag in the dose-response curve, and thymus cells from lethally irradiated donors are not so effective as unirradiated thymus cells in augmenting marrow growth.

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LATE SOMATIC EFFECTS IN MICE RECEIVING ACUTE FRACTIONATED OR PROTRACTED EXPOSURE TO FISSION NEUTRONS OR GAMMA RAYS

E. B. Darden, Jr., M. C. Jernigan, J. B. Storer, and L. J. Serrano

The major purpose of the neutron study is to determine the effect of dose rate of densely ionizing radiation on induction of late somatic effects, particularly neoplasia. With opportunities for casual or occupational exposure, especially to fast neutrons, continuing to increase, the available mammalian data on low-intensity exposure in particular are inadequate to evaluate the long-term significance to the individual, especially in regard to carcinogenesis.

As described in more detail previously,[1] approximately 8000 mice of both BALB/c and RF strains (to provide a broad spectrum of diseases) are being exposed to acute and fractionated doses of fission neutrons in the Health Physics Research Reactor at high dose rates (25 rads/min) or to protracted radiation by living in a 1-rad/day ^{252}Cf radiation field. Determination of RBE will be made with mice of similar age and strain receiving acute and protracted doses of ^{137}Cs gamma radiation in a coordinated study (low level experiment) here.

The ^{252}Cf irradiations have been completed; however, a recent prolonged reactor shutdown has extended the acute and fractionated exposure schedule into the fall of 1973. The only data as yet available are from the earliest-treated dose groups. In mice dying up to 600 days of age, acute and fractionated modes of exposure appear to be equally effective in reducing longevity. Radiation leukemias accounted for most of these deaths in the RF strain, as would be expected; in the BALB/c strain, solid tumors of accessory gland (largely mammary) origin have been the principal cause of lethality.

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INDUCTION OF NEOPLASIAS IN MICE IRRADIATED UNDER HYPOXIC CONDITIONS OR IN AIR

E. B. Darden, Jr., G. E. Cosgrove, and M. C. Jernigan

Some time ago we reported that in female RF mice given single x-ray exposures up to 1000 R, the mean survival time of 30-day survivors was substantially increased for mice irradiated in a mixture of 5 to 6% oxygen in helium compared to those irradiated in a mixture of 21% oxygen in helium or in air.[1] The pathologic findings suggested that the protective effect was due largely to an increased resistance to thymic lymphoma with the resistance to the other radiation-induced leukemia, myeloid, remaining unaffected. Incidence of the two major solid neoplasms observed, tumors of the lung and ovary, was, on the other hand, generally higher in the mice irradiated in the hypoxic mixture (hypoxia groups). Comparative data were, however, inadequate to draw firm conclusions.

A second experiment was then set up with six treatment groups, also of ~100 mice each, receiving 0 (sham irradiation), 150, or 300 R either in 5% oxygen in helium or in air. The conventionally reared RF 100% mice originally used being no longer available, RFM barrier-bred ("clean") mice were substituted. Disease incidence in these mice, even in normal controls, is not the same as in the conventional ("dirty") RF mice. Both thymic lymphomas and reticulum cell sarcomas occurred more frequently, whereas the incidence of myeloid leukemia was very low ($\leq$3%) in all groups. Similar differences in "clean" and "dirty" RF mice have been observed here in various other studies.

In the second experiment, histological examination has been completed, but only a few preliminary results are as yet available. At the 150-R level, the hypoxic mice show a slight but probably nonsignificant increase in mean survival time (491 compared to 466 days in air) despite a higher incidence of thymic lymphoma (36% vs 30% in air). At the 300-R level, the protective effect of hypoxia is more pronounced (490 days as compared to 395 days in air) and the incidence of thymic lymphoma is relatively less (39% vs 51%). In the first experiment, at the 450-R level the difference was even greater, 17% vs 38%, in the hypoxic and air groups, respectively.

In regard to tumors of the lung and ovary, the results also supported those of the first experiment showing that hypoxia enhanced the frequency of these diseases. In the groups receiving 450 R, lung tumors were three times as prevalent in the hypoxic group (26% as compared to 8% in air). In the second experiment, the difference was greatest at the 150-R level (39% vs 17%

in air) but still present at 300 R (30% vs 24% in air). Tumors of the lung are not usually radiation induced, yet this effect of hypoxia was observed only in the irradiated groups. In its effect on ovarian tumors, which, unlike those of the lung, are strongly induced by radiation, hypoxia appeared to be protecting the tumor cells from overkill at high doses. At the 150-rad level, tumor incidence was not affected by hypoxia (26% vs 25%), but at the 300-R and 450-R levels, it was elevated in the hypoxic groups, 36% vs 17% and 52% vs 35%, respectively. The total incidence of other types of tumors was about 10% in all groups with the incidence of any one kind too small for comparison. The major nonneoplastic disease, glomerulosclerosis, was observed in severe form in about half of the animals in all groups. In the second experiment the incidence of auricular thrombosis was 15 to 18%, which is nearly twice that found in the air groups.

It is clear from these observations that temporary exposure in a hypoxic atmosphere with or without radiation being present, depending upon the system or organ observed, may give rise to complex delayed effects of only partially known significance. Further clarification should come with completion of the pathologic analysis.

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LATE SOMATIC EFFECTS OF LOW DOSES OF GAMMA RAYS

J. B. Storer, G. E. Cosgrove, E. B. Darden, Jr., M. C. Jernigan, W. C. Klima, Lou C. Satterfield, H. C. Swann, and J. M. Yuhas

A total of 16,200 female mice and 2000 male mice of the RFMf/Up strain were exposed to gamma rays from a ^{137}Cs source at a dose rate of 40 rads/min. Total doses given were 0 (controls), 10, 25, 50, 100, 150, and 300 rads. The mice were irradiated when they were ten weeks old and were maintained under rigid barrier (pathogen-free) conditions throughout their lives. All the mice are now dead, and autopsy information was obtained on more than 97%. As of July 1973, the autopsy records have been reviewed on 12,000 of the female mice and all the male mice. Data on longevity and tumor incidence have been encoded (after considerable delay) on an on-line computer. Preliminary analyses show that at 10 rads there is not a statistically significant effect on longevity or on the incidence of any type of neoplasm. The minimum dose required to increase significantly the incidence of tumors depends on tumor type. In the female mice, thymic lymphomas and ovarian tumors were increased in the 25-rad group. At 50 rads, pituitary tumors and uterine tumors were increased. Myeloid leukemia and Harderian gland tumors were increased at 100 rads, while lung tumors and reticulum cell sarcomas apparently are not increased at doses as high as 150 rads. In fact, reticulum cell sarcomas show a decreased incidence (corrected) at doses of 50 rads and above. Qualitatively similar results were found with the male mice.

In the second phase of the experiment, 7000 BALB/c and 7000 RFM female mice have been exposed to gamma rays delivered at 40 rads/min or 10 rads/day to determine how much less effective the protracted radiation is when compared to high-dose-rate exposures. Autopsy data are accumulating, but we have made no analyses.

THE EFFECTS OF PREPUBERTAL OVARIECTOMY ON SURVIVAL AND CAUSE OF DEATH IN IRRADIATED RFM MICE

J. M. Holland

It has been suggested that most, if not all, of the life-shortening effects of radiation in mice, delivered at low to moderate doses, may be explained on the basis of induction or acceleration of lethal neoplastic disease.[1] It has also been a frequent observation that the female is more sensitive than the male to the life-shortening effects of whole body radiation.[2] If both of these observations are valid, then one would expect the total effect of neoplastic disease to be greater in the female than in the male. This prediction is even more attractive in view of the known coleukemogenic effects of estrogens and the extreme sensitivity of the mouse ovary to tumorigenesis following irradiation.[3]

In an effort to define more critically the role played by the ovary in controlling the pattern and intensity of those diseases influenced by whole body radiation, the following experiment was begun. Inbred female RFM mice were ovariectomized at from three to four weeks of age. Both ovariectomized and intact mice were given 300 R of x rays at from five to six weeks of age and were permitted to survive, along with a parallel group of intact and ovariectomized unirradiated controls. The group designation, mean survival time (and standard error), and longevity of each treatment group are shown in Table 22. Each animal was permitted to die or was killed when moribund and subjected to detailed gross and microscopic pathologic analysis. A positive diagnosis was established for more than 80% of the animals from each of the four treatment groups. Specific disease

Table 22. Design and results of experiment with inbred female RFM mice

Group	Treatment	Number	Mean survival time (days)	Longevity (days)
A	Control	109	639 ± 16	899
B	X ray	160	232 ± 10	830
C	Ovariectomy	103	631 ± 17	997
D	Ovariectomy + x ray	152	277 ± 13	819

incidence and cause-of-death data were compiled using methods which correct for intercurrent mortality.[4] Such adjusted data were used to estimate the significance of between-group differences for specific disease categories. Age-specific mortality curves were derived for each disease or pooling, and the area defined by these curves was used as an overall estimate of the effects of radiation on a given disease or group of diseases.

In both ovariectomized and intact groups, radiation induced significant life shortening and corresponding changes in the survival curves for all causes of death. Thymic and myeloid leukemias were both clearly enhanced by radiation in groups B and D ($P < 0.001$). Ovariectomy significantly reduced the background incidence of thymic leukemia ($0.1 > P > 0.05$) in ovariectomized controls. Since no myeloid leukemia was diagnosed in either intact or ovariectomized controls, no comment may be made on the effects of ovariectomy on this disease. Nonthymic lymphosarcoma did not appear to be affected by either x radiation or ovariectomy. The incidence of reticulum cell sarcomas was significantly reduced by radiation in intact but not in ovariectomized groups ($0.1 > P > 0.05$).

Of the solid tumors, only pituitary tumors occurred with sufficient frequency for analysis. These tumors were significantly enhanced by radiation in intact ($P < 0.001$) and ovariectomized groups ($0.01 > P > 0.002$).

Septic metritis occurred more frequently in intact irradiated animals than in control animals ($P < 0.001$), an effect that was reduced but not eliminated by ovariectomy ($0.1 > P > 0.05$). Definite conclusions with respect to the effect of radiation on enhancement of septic metritis and the apparent suppressing influence of ovariectomy must be deferred pending repetition of this experiment in order to ensure that chance variation in microbial environment did not affect this finding.

Nephrosclerosis is a disease of complex pathogenesis influenced both by radiation and by ovariectomy. The disease is induced by radiation ($0.01 > P > 0.002$) and to a lesser extent by ovariectomy ($0.1 > P > 0.05$). Radiation superimposed on ovariectomy had no effect on this disease, but since nephrosclerosis tends to occur late in the life span, too few animals may have been left alive in the ovariectomized irradiated group to detect a difference, had there been a synergistic effect between x radiation and ovariectomy.

To summarize, x radiation caused equal life-span shortening in both intact and ovariectomized groups. Septic metritis and nephrosclerosis, both nonneoplastic diseases, were clearly enhanced by radiation, more so in intact than in ovariectomized animals. This would suggest that at least some of the sex difference in response to x radiation previously reported in this strain may be due to ovarian influences on infectious and degenerative diseases.

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AN ATLAS OF RF MOUSE PATHOLOGY: DISEASE DESCRIPTIONS AND INCIDENCES*

N. K. Clapp

Despite the extensive pathological studies performed on many inbred mouse strains, there is no single place where one can find the various diseases described with the incidences. The information in this report was derived from gross necropsies together with histological examination of 80% of the mice from a large 3000-mouse radiation experiment. Diseases are described along with pictorial examples of the major diseases and common variants; incidences in nontreated controls are listed. Some helps in differential diagnosis of the leukemias found in the RF mouse (thymic lymphoma, myeloid leukemia, reticulum cell sarcoma, and nonthymic lymphoma) are included, along with a leukemoid reaction with which they may be confused. Nonneoplastic diseases, including glomerulosclerosis, arterial hyalinization, auricular thrombosis, plus several other inflammatory and degenerative diseases, are described and illustrated. The compilation of this information demonstrates for pathologist and nonpathologist alike the diseases that comprise most of the pathological entities routinely observed in mice.

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CARCINOGENESIS PROGRAM*

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Respiratory Carcinogenesis

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Mechanisms of Chemical Carcinogenesis

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INHALATION COCARCINOGENESIS STUDIES WITH NITROGEN DIOXIDE, FORMALDEHYDE, AND ACROLEIN

D. A. Creasia and Paul Nettesheim

This study is a continuation of the inhalation cocarcinogenesis study initiated in early 1972. The objectives of this study and the techniques by which these objectives are to be reached have been previously described.[1] Briefly, hamsters are treated with a carcinogen at dose levels that will produce few, if any, respiratory tract tumors. The carcinogen is administered by intratracheal injection. The hamsters are also exposed to *either* nitrogen dioxide, formaldehyde, or acrolein by inhalation techniques. With each gas, some groups of animals are exposed to the gas simultaneously with the carcinogen application, while others are exposed to the gas after carcinogen treatment in a typical promotion-type scheme.

The series of experiments shown in Table 23, with 88 hamsters each, has been started during the past year. The entire experiment will include 33 groups and a total of approximately 3000 animals.

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PROLIFERATIVE RESPONSE OF RESPIRATORY TRACT EPITHELIUM OF RATS AND HAMSTERS TO FERRIC OXIDE, BENZO[*a*]PYRENE, AND MIXTURES OF BENZO[*a*]PYRENE WITH FERRIC OXIDE

Hans Schreiber, D. H. Martin, and N. H. Pazmiño*

In an earlier study we have shown that rats and hamsters reveal large differences in the site of early metaplastic and neoplastic response to intratracheally (i.tr.) injected polycyclic hydrocarbons (PCH). Rats develop tumors of peripheral bronchiolo-alveolar origin,

Table 23. Exposure protocol for inhalation cocarcinogenesis studies

Exposure	Group	Treatment and frequency
Formaldehyde	1H1	HCHO, 10.0 ppm, 5 times per week for life; BaP, none
	1H2	HCHO, 20.0 ppm, 5 times per week for 17 weeks; BaP, 5.0 mg, once per week for 15 weeks
	1H3	HCHO, 10.0 ppm, 5 times per week for life after BaP; BaP, 5.0 mg, once per week for 15 weeks
	1H7	HCHO, 20.0 ppm, once per week for 17 weeks; BaP, 5.0 mg, once per week for 15 weeks
	1H8	HCHO, 50.0 ppm, once per week for 17 weeks; BaP, 5.0 mg, once per week for 15 weeks
	1H9	HCHO, 50.0 ppm, once per week for 17 weeks; BaP, none
	2H1	HCHO, 20.0 ppm, once per week for 17 weeks; BaP:Fe_2O_3, 1.4 mg, once per week for 15 weeks
	2H2	HCHO, 20.0 ppm, 5 times per week for 17 weeks; BaP:Fe_2O_3, 1.4 mg, once per week for 15 weeks
	2H3	HCHO, 50.0 ppm, once per week for 17 weeks; BaP:Fe_2O_3, 1.4 mg, once per week for 15 weeks
	3H1	HCHO, 50.0 ppm, once per week for 17 weeks; MCA, 5 mg, twice, 24 hr apart
Acrolein	1A1	C_3H_4O, 2.5 ppm, 5 times per week for life; BaP, none
	2A1	C_3H_4O, 2.5 ppm, once per week for life; BaP:Fe_2O_3, 1.4 mg, once per week for 15 weeks
Nitrogen dioxide	1N1	NO_2, 10.0 ppm, once per week for life; BaP, none
	1N2	NO_2, 10.0 ppm, once per week for life after BaP; BaP, 5.0 mg, once per week for 15 weeks
	1N3	NO_2, 10.0 ppm, once per week for 17 weeks; BaP, 5.0 mg, once per week for 15 weeks
	1N4	NO_2, 10.0 ppm, once per week for life; BaP, 5.0 mg, once per week for 15 weeks
	1N5	NO_2, 10.0 ppm, 5 times per week for life; BaP, 5.0 mg, once per week for 15 weeks
	2N1	NO_2, 10.0 ppm, once per week for life; BaP:Fe_2O_3, 1.4 mg, once per week for 15 weeks
Controls	1B1	BaP, 5.0 mg, once per week for 15 weeks
	1B2	BaP, 5.0 mg, once per week for 15 weeks
	1B3	BaP, 5.0 mg, once per week for 15 weeks
	1B4	BaP, 5.0 mg, once per week for 15 weeks
	2B1	BaP:Fe_2O_3, 1.4 mg, once per week for 15 weeks
	101, 102, 103, 104	Room controls

while hamsters mainly develop tumors in the upper tracheal bronchial tract. The objectives of this study were: (1) to investigate if differences in site of tumor origin correlate with differences in the early proliferative response to i.tr. injected benzo[*a*] pyrene (BP) and (2) to determine the proliferative response of the various segments of the respiratory tract to i.tr. injected ferric oxide (Fe_2O_3), BP, BP with Fe_2O_3 added (BP + Fe_2O_3), and BP physically attached to equal parts of Fe_2O_3 by grinding (BP–Fe_2O_3). The doses for rats and hamsters were adjusted for the differences in lung size. Rats received a single i.tr. injection of either 10 mg BP, Fe_2O_3, or both; hamsters received 6 mg of the substances. Animals were injected with [^{3}H] thymidine at various times from 1 to 21 days after i.tr. injection and were sacrificed 1 hr later. By autoradiography the number of cells in S phase (NS) was determined in the epithelium of the various segments of the respiratory tract. Marked differences in the site of proliferative response of rats and hamsters were found, which correlated with the different sites of tumor origin in both species. Hamsters showed a much higher NS in the tracheal epithelium than rats did, and while NS returned to normal in rat tracheas after 4 days, it still was considerably elevated in hamster tracheas at 21 days. The opposite was found in areas of carcinogen deposition in the lung periphery. Only rats showed a continuous high increase of labeled cells at this site.

The NS in tracheal epithelium of hamsters was highest following BP–Fe_2O_3 injection, intermediate for BP + Fe_2O_3, and lowest for BP. These results correlate with differences in the elimination of these suspensions, as observed in another study in this laboratory. Alone, Fe_2O_3 had only an early, slight, and transitory proliferative effect on the tracheal epithelium. This suggests that the main significance of Fe_2O_3 in BP–Fe_2O_3-induced respiratory carcinogenesis is its ability to increase the retention of carcinogen.

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COMPARISON OF CARCINOGEN- AND NONCARCINOGEN-INDUCED MORPHOLOGIC CHANGES OF THE HAMSTER RESPIRATORY TRACT

Hans Schreiber, Paul Nettesheim, C. B. Richter, W. A. Clyde,* D. H. Martin, and M. Virginia Cone

Several chemical, physical, and infectious agents, as well as mechanical irritation and vitamin A deficiency, can produce striking proliferative, sometimes tumor-like, reactions of the respiratory tracts of animals. These lesions may easily give rise to difficulties in the cytologic or histologic diagnosis of lung cancer or premalignant lesions of the respiratory tract. Therefore, we initiated a cytologic and histologic (light and electron microscopic) study to determine which differences may be found in the morphologic changes induced in the respiratory tract of hamsters by benzo[*a*] pyrene–ferric oxide injection, formaldehyde inhalation, mycoplasma pneumoniae and PR8 virus infection, and vitamin A deficiency. No single parameter, such as large nucleoli, increased nuclear cytoplasmic ratio, orangophilia, coarse nuclear material, cellular and nuclear pleomorphism, breakage of the basement membrane with cells protruding into subepithelial tissue, etc., sufficiently differentiated carcinogen- from noncarcinogen-induced changes in exfoliative cytology or at the light microscopic level. Correct differentiation could always be made, however, by taking into account all of the observed morphologic parameters. The electron-microscopic investigation is in progress.

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RETENTION OF CARCINOGEN IN LUNGS AND TRACHEAS OF RATS AND HAMSTERS AFTER INTRATRACHEAL APPLICATION OF BENZO[*a*]PYRENE WITH OR WITHOUT FERRIC OXIDE

Hans Schreiber, N. H. Pazmiño,* and D. H. Martin

The objectives of this study were: (1) to determine whether the differences in neoplastic response of respiratory tissues of rats and hamsters to intratracheally (i.tr.) injected polycyclic hydrocarbons (PCH) can be explained by differences in carcinogen retention of the two species and (2) to determine whether ferric oxide (Fe_2O_3) added or attached to PCH can retard the carcinogen elimination from the respiratory tract. Three different carcinogen preparations were used: benzo[*a*] pyrene (BP) alone, BP with equal parts of Fe_2O_3 added, and BP physically attached to equal parts of Fe_2O_3 by grinding. The carcinogen doses for rats and hamsters were adjusted for the differences in lung size. Rats received a single i.tr. injection of 0.2 ml suspension containing 10 mg BP in saline; hamsters received 0.12 ml suspension containing 6 mg BP. Animals were killed at various times from 0 to 21 days,

and the amount of BP was determined, separately, in lungs and in tracheas. The extracted BP was determined by ultraviolet spectrophotometry at 385 nm wavelength. There was no interference with tissue blanks at this wavelength. The carcinogen elimination was retarded considerably in both species when BP was physically attached to Fe_2O_3. It may be even more important that simple addition of Fe_2O_3 to the BP caused a slight but significant increase in carcinogen retention, since addition of both substances may occur under environmental conditions.

Between 0 and 2 days, BP was eliminated from the lungs of rats relatively faster than from hamster lungs, but between 2 and 21 days the elimination rate was slower in rats. In tracheas, relatively more carcinogen was retained in rats than in hamsters at all time points. This demonstrates that the high tumor incidence in hamster tracheas and the absence of tracheal tumors in rats after i.tr. BP injections cannot be explained through differences in carcinogen elimination. Differences in carcinogen susceptibility on a cellular level or differences in penetrability of the mucociliary barrier for carcinogens may be responsible for this dissimilarity in tumor origin of the two species.

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EFFECT OF VITAMIN A ON 3-METHYLCHOLANTHRENE-INDUCED SQUAMOUS METAPLASIAS AND EARLY TUMORS IN THE RESPIRATORY TRACTS OF RATS

M. Virginia Cone and Paul Nettesheim

It has been recognized for many years that vitamin A is required to maintain cellular differentiation of certain epithelial tissues. Numerous studies have demonstrated the relationship between vitamin A deficiency and keratinization of normally nonkeratinizing epithelia. Studies of *in vitro* systems have shown that vitamin A prevents keratinization of epithelial tissues. Of particular interest to us is the induction, by polycyclic hydrocarbons, of squamous metaplasias in the respiratory tract and their progression to squamous cell carcinomas. It has been shown that *in vitro* induction of hyperplasias and metaplasias in hamster tracheas by benzo[*a*]pyrene can be inhibited by vitamin A.

We tested the influence of high levels of vitamin A on the susceptibility of respiratory tract epithelium to the metaplastogenic and tumorigenic effects of 3-methylcholanthrene (3-MCA). F-344 rats were maintained on either 1740 μg or 87 μg of retinyl acetate given twice per week intragastrically. At nine weeks of age they received two intratracheal injections of 5 mg of 3-MCA each. The development of squamous metaplasias and squamous cell tumors was significantly reduced in rats receiving a high dose of retinyl acetate (Table 24). Our

Table 24. Mean numbers and volumes of tumors

	5 weeks after 3-MCA		8 weeks after 3-MCA		12 weeks after 3-MCA	
	Group 1[a]	Group 2[a]	Group 1	Group 2	Group 1	Group 2
Total tumors per group	1.0	7.0	19.0	54.0	54.0	88.0
Mean number of tumors per animal	0.25 (±0.25)	1.8 (±0.63)	4.8 (±2.2)	13.5 (±3.8)	10.8 (±3.2)	17.6 (±3.9)
Total tumor volume (mm^3) per group	0.52	33.6	230.7	600.3	1177.3	2420.6
Mean tumor volume (mm^3) per animal	0.13 (±0.13)	8.4 (±3.0)	57.7 (±40.9)	150.1 (±54.0)	235.5 (±96.9)	484.1 (±96.9)

[a]Dosage: Group 1 received 1740 μg RA twice per week; Group 2 received 87 μg RA twice per week.

Table 25. Growth of tumor stem cell compartment as a function of time after transplantation

	Average tumor weight (mg)	Number of viable cells per tumor	Number of tumors per 200,000 viable cells (mean ± S.E.)	Number of stem cells per tumor
Time of secondary transfer				
3 weeks	4.2	3.2×10^5	37.0 ± 4.0	59.2
4 weeks	25.7	14.0×10^5	51.2 ± 6.5	357
5 weeks	53.5	26.0×10^5	60.5 ± 4.2	787
Relative increase between 3 and 5 weeks	5.2	8.1	1.6	13.3

experiments confirm earlier findings suggesting an inhibitory effect of vitamin A on the development of respiratory tract tumors in hamsters.

LUNG COLONY ASSAY WITH A SQUAMOUS CELL CARCINOMA DERIVED FROM THE RESPIRATORY TRACT OF MICE

Mary L. Williams and Paul Nettesheim

A lung colony assay with a respiratory tract carcinoma has been developed. This transplantation model provides a method for determining the relative size of the stem cell pool of a tumor grown in its natural environment. Experimental evidence indicates that this well-differentiated squamous cell carcinoma consists of heterogeneous cell populations and that the stem cell pool is the most rapidly expanding cell compartment during the logarithmic growth phase of the tumor. The average tumor weight increased by a factor of 5.2 between three and five weeks after intravenous inoculation of 2×10^5 tumor cells. In contrast, the number of tumor stem cells (i.e., transplantable tumor cells) increased by a factor of 13.3 during the same time interval as measured by a "double transfer" experiment (Table 25). This indicates that tumor mass is not a good indicator to study growth potential of malignant cells in differentiating epithelial tumors.

URETHAN-INDUCED PULMONARY ADENOMA AS A TOOL FOR THE STUDY OF SURFACTANT BIOSYNTHESIS*

Catherine M. Snyder, Boyd Malone,† Paul Nettesheim, and Fred Snyder†

The biosynthesis of lung surfactant lipid, which is mainly 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine, is still poorly understood. Interpretation of earlier investigations has been difficult because the biological preparations (lung lavage and whole lungs) used were from mixed cell populations. We have used mouse pulmonary adenomas induced by urethan to provide a single population of alveolar type II cells and have found that it contains significant quantities of disaturated phosphatidylcholine, the main constituent of lung surfactant. Data obtained after phospholipase C hydrolysis, acetate derivatization of the resulting diacylglycerols, and argentation thin-layer chromatography enabled us to determine that the disaturated molecular species are about 28% of the phosphatidylcholine present in the adenoma cells and that at least 60% of this fraction is 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine.

Homogenate preparations, in the presence of added ATP, CoA, and Mg^{2+}, incorporated palmitate at the 1 and 2 positions of the glycerol moiety, suggesting that the alveolar type II epithelial cells that make up the pulmonary adenoma contain an enzymic system capable of producing disaturated phosphatidylcholine. Adenoma homogenates also produce labeled alkyl glycerolipids from hexadecanol-1-^{3}H. This is characteristic of most tumors, including the lung squamous cell carcinoma that we tested. The carcinoma also actively incorporated palmitic acid into phosphatidylcholine but not to the same extent that the adenoma did.

The addition of Ca^{2+} did not inhibit the incorporation of palmitic acid-1-^{14}C into phosphatidylcholine but decreased its total incorporation into lipids. In contrast, the formation of O-alkyl phospholipids from hexadecanol-1-^{3}H was completely blocked by Ca^{2+}. Since Ca^{2+} is known to inhibit cytidine diphosphate-base transferase activity, these data demonstrate that the major incorporation of palmitic acid-1-^{14}C into phosphatidylcholine in the adenoma homogenate does not take place via the cytidine diphosphate choline pathway

or by methylation of phosphatidylethanolamine. From our experimental observations we propose that the efficient incorporation of palmitic acid-1-^{14}C at both the 1 and 2 positions of phosphatidylcholine by homogenates of mouse pulmonary adenomas cannot be incidental and that it does provide additional evidence for the role of alveolar type II epithelial cells as the enzyme source of lung surfactant.[1] Although the adenoma is a tumor, the facts that its individual cells are similar to normal type II epithelial cells and that its incorporation of palmitic acid-1-^{14}C into phosphatidylcholine is much slower than that of the squamous cell carcinoma, a faster-growing tumor, indicate that it should be a useful system for elucidating the biochemical mechanisms responsible for the biosynthesis of lung surfactant in both normal and cancerous pulmonary tissues.

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†Medical Division, Oak Ridge Associated Universities.

1. C. Snyder, B. Malone, P. Nettesheim, and F. Snyder, *Cancer Res.* (in press).

COMPARISON OF CIGARETTE SMOKE TOXICITY IN SEVERAL HAMSTER STRAINS

Joseph Kendrick, Lora H. Phipps, D. W. Parsons, and Paul Nettesheim

The hamster is generally considered to be the test animal of choice for studies designed to test the carcinogenicity of tobacco smoke. Spontaneous tumor incidence in the respiratory tract of hamsters is practically nonexistent; thus tumor formation in smoke-exposed animals would reliably be a measure of cigarette smoke carcinogenicity. Unlike the rat, the hamster shows little predisposition to respiratory tract infection. In addition, the bronchial epithelium of hamsters is quite similar to that of man, and cancers induced by chemical carcinogens are generally the same type seen in man, namely, squamous cell carcinoma.

As with most rodents, the disadvantage in using the hamster as a test animal is its susceptibility to the acute toxic effects (carbon monoxide and nicotine poisoning) of cigarette smoke. Since any successful cigarette smoke carcinogenic bioassay will entail delivering maximum smoke aerosol into the lungs while minimizing toxicity, a study was made of several hamster strains to determine their relative tolerance to acute cigarette smoke toxicity.

Table 26 compares the survival time of five inbred and one noninbred hamster strains. All animals were exposed continuously to a 10% smoke aerosol according to the following scheme: 30 sec of smoke, 30 sec of fresh air. LD_{50} time (survival time) is reported as the total *smoke* exposure time (in minutes) required to kill 50% of the animals of each group on the smoke exposure device.

Table 26. LD_{50} in hamsters exposed to a 10% smoke aerosol

Hamster strain	LD_{50}[a] (min)
LSH/SSLAK	21
PD_4/SSLAK	69
LHC/LAK	54
MHA/SSLAK	40
87.20	84
RGH/ARS	50

[a]Survival times are the means from five animals.

The survival time ranged from a low of 21 min in the LSH/SSLAK inbred strain to a high of 84 min in the inbred strain 87.20. The strain used most frequently in this laboratory has been the noninbred strain RGH/ARS–Sprague Dawley, which has a survival time of 50 min. Studies such as this are useful in aiding selection of the strain best suited for chronic testing. A chronic study, designed to determine if strain 87.20 is also more resistant to chronic smoke exposure toxicity than strain RGH/ARS, has recently been initiated. Preliminary studies of respiratory function indicate that animals of the 87.20 strain have a lower minute volume than animals of the RGH/ARS strain. The apparent greater tolerance of 87.20 hamsters to smoke toxicity may therefore be mostly due to the decreased toxic dose rather than to an increased resistance to smoke toxicity.

TRACHEAL GRAFTS: A NOVEL TECHNIQUE FOR STUDYING RESPIRATORY TRACT CARCINOGENESIS

Joseph Kendrick, Anna S. Hammons, and Paul Nettesheim

The use of tracheal explants, grafted in the muscle fascia of the shoulder region, has been explored as a model for studying respiratory tract carcinogenesis. Isogenic rat, hamster, and mice grafts maintain normal morphology and have thus far remained functional (in terms of mucus secretion) up to 11 months. One-month-old rat grafts treated with single 5-mg doses of benzo[*a*]pyrene (BP) and methylcholanthrene (MCA) develop various degrees of basal cell hyperplasia, dys-

plasia, and squamous cell metaplasia, as early as four days after treatment.

Hyperplastic and dysplastic eipthelium have been detected in grafts two months after treatment with 7.25 mg of cigarette smoke condensate (CSC) embedded in beeswax. Applications of 25 and 50 mg of CSC without a vehicle resulted in epithelial hyperplasia in the 25-mg-treated graft and squamous metaplasia in the 50-mg-treated graft after three weeks.

In one chronic rat study now in progress, 60% of MCA-treated and 40% of BP-Fe_2O_3-treated grafts have developed invasive squamous cell carcinoma six to seven months after treatment with a single 5-mg dose.

Studies are to be initiated soon to determine carcinogen dose-response curves for several polycyclic hydrocarbons and for cigarette smoke condensate from different types of cigarettes.

MAXIMIZATION OF CIGARETTE SMOKE EXPOSURE ON AN INTERMITTENT SMOKE DELIVERY DEVICE

Joseph Kendrick, Ira B. Rubin,*
B. E. Gill,* and Paul Nettesheim

The ORNL intermittent smoke delivery device[1] has been studied in terms of toxicity of smoke offered and total particulate matter (TPM) deposited into the lungs of exposed hamsters. All efforts have been aimed at maximizing TPM deposition while minimizing smoke toxicity.

Basically, the instrument takes a 35-cm^3 puff of 2-sec duration and delivers it into a 350-cm^3 exposure chamber to effect a 10% smoke aerosol dilution. The standard procedure has been to expose hamsters to 30 sec of smoke and then purge the chamber with air for 30 sec before another puff is introduced. The instrument can be adjusted so that smoke exposure time per minute is less than or more than 30 sec.

Recent LD_{50} (survival time) studies have been useful in determining the effect of varying the smoke exposure time per 60-sec cycle on toxicity of smoke offered to hamsters. Adjusting the smoke exposure time in the intermittent device from the usual 30 sec to 20 and 40 sec results in virtually no change in the LD_{50}.

An analysis of the CO content of the exposure chamber indicated that the level of this lethal component of cigarette smoke rapidly decreases to similar levels whether smoke stands for 20, 30, or 40 sec. This may mean that exposed animals absorb a similar quantity of CO, and thus no great difference in survival time is seen.

The detection of radiolabeled particles in the lung ([^{14}C] dotriacontane) has been used as a tool for estimating TPM deposited during exposure on the intermittent device. Deposition studies were made with the machine set to vary the residence time of smoke in the chamber (same as in LD_{50} study). If smoke is held 20, 30, or 40 sec before the fresh air purge, the corresponding TPM deposition in the lungs is 0.10, 0.25, and 0.55 mg, respectively, or more than a doubling for each 10-sec increase in smoke exposure time. These observations are particularly important since animals in acute toxicity studies survive as well receiving 40 sec of smoke and 20 sec of air as those receiving 30 sec of smoke and 30 sec of air. This suggests that a 40-sec–20-sec smoke–air cycle may prove more effective in increasing the total smoke dose.

A chronic smoke exposure study is under way in which survival of hamsters exposed to 30-sec–30-sec and 40-sec–20-sec smoke–air cycles is being compared.

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*Analytical Chemistry Division, ORNL.

1. M. Guerin and P. Nettesheim, *Tobacco Smoke Research Program Progr. Rep.*, ORNL-4879, April 1973, pp. 59–60.

CHEMICAL STRUCTURE AND CARCINOGENIC ACTIVITY IN N-NITROSO COMPOUNDS

William Lijinsky and H. W. Taylor

Little is known of the mechanism of carcinogenic action of N-nitroso compounds, especially of cyclic nitrosamines. As in the case of other groups of carcinogens, indications of the chemical characteristics needed for carcinogenic activity might be obtained from differences in tumor response to nitrosamines with slightly different structures, for example, with substituents at various positions in the molecule.

A number of derivatives of nitrosopiperidine with methyl, hydroxyl, or carboxyl groups at several positions in the molecule have been prepared and given to rats at the same concentration in drinking water for the same length of time. Results so far show that all three monomethyl derivatives produce the same type of tumor as the parent compound. However, while the 4-methyl derivative was equally potent with nitrosopiperidine, the 3-methyl derivative was weaker and the 2-methyl derivative much weaker. The 2,6-dimethyl and 2,2,6,6-tetramethyl compounds have not yet given rise to tumors.

A similar study with homologs of dinitrosopiperazine and nitrosopyrrolidine is in progress.

The results so far of equivalent tests of four trialkylnitrosoureas show significant differences in their car-

cinogenic activities. It is not possible to draw any firm conclusions, but the most reactive compound, nitrosotrimethylurea, is the only one of the four which has not yet given rise to tumors.

Because dimethylnitrosamine fully labeled with deuterium (DMN-d_6) is significantly less carcinogenic than DMN, indicating that loss of an α-hydrogen atom might be a rate-limiting step in carcinogenesis by this compound, a similar study is in progress with nitrosomorpholine 3,3,5,5-d_4. If this compound proves to be less carcinogenic than nitrosomorpholine, loss of an α-hydrogen atom would seem to be a likely first step in carcinogenesis by nitrosamines in general.

MECHANISM OF NITROSATION OF SECONDARY AND TERTIARY AMINES

A. R. Jones, William Lijinsky, and G. M. Singer

A study of the kinetics of formation of nitrosamines from piperidine or methyl-substituted piperidines and nitrous acid has shown that the presence of methyl substituents on the carbon atoms α to the nitrogen atom reduces the rate of reaction, presumably due to a steric effect. The rates of reaction of piperidine, 2-methylpiperidine, 2,6-dimethylpiperidine, and 2,2,6,6-tetramethylpiperidine were in the ratio of 100: 50:10:1. The rate of formation of nitrosopiperidine from N-methylpiperidine was somewhat less than 1% of that for formation from piperidine.

The mechanism of formation of nitrosamines from tertiary amines is not well understood. Since such reactions might be an important source of carcinogens in the environment, we are studying their kinetics with several tertiary amines under various conditions of temperature and concentration and in the presence of catalysts. The reactions of trimethylamine and trimethylamine oxide with nitrous acid seem to follow different courses, the oxide reacting more readily at ratios of nitrous acid to amine above 1:1. At ratios below 1:1, the amine reacts more readily than the oxide. Aldehydes act catalytically to increase the yield of nitrosamine from secondary amines but have little or no effect on nitrosamine formation from tertiary amines. The formation of nitrosamines from tertiary amine oxides has a quite different pH profile from the reaction of secondary amines and tertiary amines with nitrous acid. The products of reaction of tertiary amines and their N-oxides with nitrous acid – a nitrosamine, a carbonyl compound, and nitric oxide – are being quantified in experiments with trimethylamine, triethylamine, tripropylamine, tributylamine, and triethanolamine. The reaction takes place more readily as the molecular size of the amine increases.

THE FORMATION OF CARCINOGENIC NITROSAMINES FROM AMINES AND NITRITE

William Lijinsky

Recent studies have indicated that the formation of carcinogenic N-nitroso compounds by interaction of secondary or tertiary amino compounds with nitrite in the stomach could be related to human cancer. The formation of nitrosamines from a variety of tertiary amines that are commonly used drugs and from some agricultural chemicals that are dialkylureas or dialkyl carbamates has been examined under various conditions of temperature and concentration. Although the yield of nitrosamine varied considerably between one chemical structure and another, in every case the expected nitrosamine was formed in detectable amounts, even at 37°C and at concentrations of a few mg/ml of reactants. Among the compounds studied were aminopyrine, oxytetracycline, chlorpromazine, trimethylamine, tolazamide, lucanthone, dimethylphenylurea, thiram, and disulfiram.

To examine the possibility that these reactions occurring *in vivo* could be significant, several secondary and tertiary amines are being given to rats together with nitrite in drinking water for a year, and the animals will be observed for development of tumors induced by the nitrosamines. Aminopyrine with nitrite and heptamethyleneimine with nitrite have both given rise to a high incidence (more than 50% of the animals) of tumors of the liver and lung, the same tumors as those induced by feeding the respective nitrosamine products of the interactions. Neither amine nor nitrite has induced tumors when given to rats alone. Tests of the other compounds with nitrite are incomplete. Carbaryl has been given together with nitrite to pregnant rats to study the possibility that nitrosocarbaryl is formed in sufficient amount to act transplacentally.

To aid in the identification of N-nitroso compounds formed by reaction of amines with nitrous acid, a catalog of the mass spectra of almost 100 nitroso compounds has been compiled in collaboration with W. T. Rainey and W. H. Christie of the ORNL Analytical Chemistry Division. It is expected that analysis of the mass spectra and of the fragmentation patterns of the compounds will lead to easy identification of an isolated unknown nitroso compound.

DEVELOPMENT OF ANALYTICAL METHODS FOR DETERMINATION OF NITROSATABLE AMINES IN FOOD AND OTHER MATERIALS

G. M. Singer

A number of reports have demonstrated the presence of significant quantities of carcinogenic nitrosamines in nitrite-treated food. Other studies, at ORNL and elsewhere, have clearly shown the carcinogenic effects of feeding nitrosatable amines and sodium nitrite to experimental animals. There are only a few scattered determinations of variable reliability in the literature, however, of nitrosatable amines in foods and other natural mixtures, for example, tobacco smoke.

We have essentially completed development of a procedure for the determination of these amines in natural products. The product is pureed in dilute sulfuric acid. The puree is made basic and steam distilled into dilute acid. Overnight ether extraction removes most of the steam-volatile neutral compounds. The extracted distillate is subjected to Hinsberg derivatization and separation to provide separate fractions of primary tosylamines, secondary tosylamines, and tertiary amine hydrochlorides for gas chromatographic analysis.

Gas chromatography–mass spectrometry is carried out (in conjunction with W. T. Rainey, Jr., of the ORNL Analytical Chemistry Division) for identification purposes. Quantification is done by gas chromatography using either a flame ionization detector and/or a Coulson conductivity nitrogen-specific detector.

Preliminary results using [^{14}C]dimethylamine as a tracer indicate that recoveries are high. Ocean perch frozen fillets purchased locally, for example, appear to contain between 120 and 600 ppm of dimethylamine.

To aid in the quantification of the complex mixtures anticipated from some materials, for example, tobacco smoke condensate, work is in progress to connect the gas chromatograph directly to the Biology Division's PDP-11 computer. The computer will then be programmed to carry out the tedious and difficult quantification of the mixtures.

STUDIES OF THE HISTOPATHOLOGY OF TUMORS INDUCED BY ADMINISTRATION OF NITROSAMINES OR AMINES PLUS NITRITE TO RATS

H. W. Taylor

Nitrosoheptamethyleneimine (NHMI), when administered to Sprague-Dawley (SD) rats in drinking water, induces tumors of the tongue, pharynx, esophagus, trachea, and lung. Experiments employing the use of NHMI in Fischer-344 rats have been limited to administration via subcutaneous injection, and no tumors were observed.

Experiments now in progress are designed to (1) determine whether the lack of response in F-344 rats that received subcutaneous injections of NHMI was due to the strain of rat or to the route of administration, (2) establish a dosage schedule that produces a high incidence of tumors but is below acute toxic levels, and (3) determine the origin and histogenesis of tumors produced by NHMI in SD rats. Information gained from the first two objectives would make possible the development of a tumor system that could be standardized to lend itself to future studies employing cocarcinogens or carcinogenic cofactors. Also, such a system of known dose-response would be valuable in F-344 rats because of their syngeneity and use in tumor transplantation studies. The third objective would determine the exact origin of squamous cell carcinomas that are produced in SD rats by administration of NHMI orally. There is now doubt about the site of origin and histogenesis of these tumors.

At present, 50% of the F-344 rats that were dosed with NHMI for 20 weeks via gavage have died, and the expected tumors were present in almost 100% of the dead animals. Attempts at transplantation of the tumors are now under way.

Carbon tetrachloride, aminopyrine, and nitrite studies in Sprague-Dawley rats. Previous studies have shown that aminopyrine and nitrite given in drinking water to SD rats produces a very high incidence of hemangioendothelial sarcomas in the livers. No other types of tumors were produced, and no other primary location was observed. These results raised some questions: why these tumors of endothelial origin occurred only in the liver when endothelium is present in all tissues of the body, and why no hepatocellular tumors were observed when the liver seemingly must play some role in the genesis of these sarcomas. It appeared reasonable that liver-cell tumors might be induced if the mitotic activity of hepatocytes were increased while the aminopyrine and nitrite were being administered.

Carbon tetrachloride provides a means by which hepatocellular mitosis can be increased and has not been shown to produce hepatocellular tumors in rats when given alone. Present experiments are employing the concurrent administration of CCl_4, aminopyrine, and nitrite to determine if hepatocellular tumors will

be induced by the combination of the three, or if tumor induction will be suppressed due to reduction of carcinogen activation in the liver, or whether no change in tumor type or induction will be seen. The latter result would indicate a minor importance of hepatocellular participation. Tumors induced by the above combination will be compared with dimethylnitrosamine-produced tumors in the same strain of rat.

EVIDENCE FOR A ROLE OF ULTRAVIOLET-INDUCED PYRIMIDINE DIMERS IN TUMOR INDUCTION

R. W. Hart and R. B. Setlow

In man there is a very strong association between exposure to the ultraviolet component of sunlight and skin cancer. The fact that individuals with xeroderma pigmentosum (a genetically recessive transmitted condition characterized by a high incidence of light-induced cancer at early ages) are defective in the ability to excise pyrimidine dimers from their DNA is evidence for the hypothesis that DNA damage may result in malignant transformation. Thus there is a good possibility "that the accumulation of pyrimidine dimers and other nonexcised products in the DNA of a skin cell either directly or indirectly causes or predisposes to neoplastic transformation of that cell."[1] The useful experimental systems that can evaluate this possibility are few; man is not an acceptable one. Mice are good animals to use for dose-response relations, but, since their level of excision repair is very low, they do not seem suitable for DNA studies. Certain species of fish are ideal systems because they may be grown as clones and because they contain photoreactivating enzyme.[2,3] The enzyme monomerizes dimers but has no other known activity. If uv-induced tumor induction in fish is photoreversible, such a finding would indicate that pyrimidine dimers in DNA are responsible for neoplastic transformation.

Cell suspensions of tissue from various organs of the gynogenetic (nonsexually reproducing) fish *Poecelia formosa* were exposed to several fluences of 254-nm radiation. Portions of the suspensions containing ~5 × 10^5 cells were injected into the middorsal region of 100 to 200 members of the clone from which the tissue originated. The fraction of fish with tumors was determined three to four months afterward. Tumor incidence was <0.2% for fish that received unirradiated cells and rose directly proportionately to the incident fluence at a rate dependent on the tissue. For liver an incident fluence of 1000 ergs/mm^2 of 254 nm (representing an average fluence through the sample of ~100 ergs/mm^2) yielded tumors in 15% of the fish. If the irradiated liver-cell suspension was illuminated with photoreactivating (PR) light (320 to 450 nm) before injection, the yield of fish with tumors was reduced by an amount that increased with the illumination time (0.5 to 30 min). Cytological examination showed that the PR light had not killed the cells, and preliminary experiments indicate that PR illumination *before* 254-nm exposure did not appreciably reduce the numbers of tumors. Thus we seem to be observing true enzymic PR with a sector of >0.8. These experiments indicate that pyrimidine dimers in DNA can lead to neoplastic transformation.

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DNA REPAIR IN TISSUES FROM HUMAN NEOPLASMS

R. W. Hart and R. B. Setlow

It is of interest to determine if defects in DNA repair are associated with spontaneous neoplasias in man. We have measured unscheduled DNA synthesis (an indication of excision repair) after uv irradiation of tissue samples from 80 individual patients having four histologically defined types of tumors. Tissue obtained as surgical specimens from Ohio State University School of Medicine was dispersed and suspended in a complete medium. Cells were seeded onto cover slips exposed to 10–20 J/m^2 of 254-nm radiation and incubated in ^{3}HdThd for various times. Autoradiographs were prepared and the average number of grains per cell determined from counts on 400 cells from each sample (1000 cells from colon carcinoma samples).

Typical data are shown in Table 27. The initial rate of repair was the same for all tissues, but there seemed to be some differences – on the verge of significance – in the extent of unscheduled synthesis. Most of the colon carcinomas were low and the fibrosarcomas (radioresistant tissues) were high compared with normal tissues.

Table 27. Repair after uv irradiation of cells from neoplastic tissues

Tissue	Number of individuals	Initial rate of repair (grains per nucleus in 4 hr)	Extent of repair (grains per nucleus in 48 hr)
Normal tissue	30	29	54
Mammary carcinoma	20	25	55
Fibrosarcoma	20	30	72
Endometrial carcinoma	20	28	50
Colon carcinoma	11[a]	20	30
	9	28	40
	Standard deviation	±5	±7–10

[a]Hereditary polyposis.

REPAIR ENDONUCLEASE SENSITIVE SITES IN DAUGHTER DNA OF ULTRAVIOLET-IRRADIATED HUMAN CELLS

S. N. Buhl* and James D. Regan

We have used xeroderma pigmentosum (XP) cells derived from individuals who lack excision repair[1] to demonstrate the appearance of ultraviolet (uv) lesions in the DNA made after uv irradiation.

Normal human and XP cells were uv irradiated and labeled with either [^{3}H]thymidine or $^{32}PO_4$ for 6 to 8 hr. The label was removed and the cells were further incubated for 18 hr to allow for DNA chain elongation and joining of the short DNA segments synthesized after irradiation. Then the cells were osmotically shocked and treated with a repair endonuclease contained in *Micrococcus luteus* extracts and sedimented in 5 to 20% alkaline sucrose gradients. The enzymatic assay[1] and the sedimentation procedure[2] are described elsewhere. The daughter DNA from either irradiated or nonirradiated normal human cells does not contain repair endonuclease sensitive sites. This is expected because normal human cells excise and repair most of the pyrimidine dimers after uv irradiation.[3] DNA synthesized after irradiation of XP cells contains repair endonuclease sensitive sites. The number of sensitive sites appears to be small. Under our experimental conditions, there is approximately one sensitive site for every 15 to 35 dimers in the parental DNA.

We conclude that the finding of repair endonuclease sensitive sites could result from either DNA exchanges between parental and daughter strands or from the insertion of some abnormal base into the DNA made from irradiated templates and the recognition of these abnormal bases by the repair endonuclease.

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THE EFFECT OF THE NITROSO DERIVATIVE OF CARBARYL ON HUMAN DNA

James D. Regan, R. B. Setlow, and William Lijinsky

Elespuru and Lijinsky[1] have synthesized the nitroso derivative of carbaryl. Since nitrosocarbaryl is structurally similar to several well-known carcinogens, notably nitrosomethylurethane, we tested the effects of nitrosocarbaryl on the DNA of human skin fibroblasts cultured *in vitro*. The results of these experiments, as visualized on alkaline sucrose gradients, show that there is apparently some kind of long-lasting association between the nitrosocarbaryl and the DNA of human cells, such that when the treated cells (60 min at 10^{-7} *M*) are placed on alkaline sucrose gradients even 20 hr after treatment, a large number of alkaline-sensitive bonds are present in the DNA, resulting in a greatly decreased molecular weight, which essentially amounts to fragmentation of this macromolecule. Experiments with phage (pm2) DNA on neutral sucrose gradients treated with nitrosocarbaryl showed that the effect was due to the formation of alkaline-sensitive bonds rather than direct strand breakage of the DNA by this compound. Experiments were performed with human cells using nitrosomethylurethane, which is the methyl analog of the nitrosocarbaryl. There was essentially no difference between the effects of nitrosomethylurethane

and nitrosocarbaryl on the DNA of human skin fibroblasts *in vitro.*

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STUDIES ON GLUCOSE TRANSPORT SYSTEMS IN MAMMALIAN CELLS

D. W. Salter,* F. C. Hartman, and J. S. Cook

Martineau et al.[1] recently reported that chick embryo fibroblasts show a 30-fold enhancement of glucose transport after being starved for glucose for 24 hr. We have confirmed this observation in mouse 3T3 cells and in the human diploid strain HSWP (kindly provided by J. D. Regan) and have found that 2-deoxyglucose transport is also enhanced. The starvation effect can be mimicked to some extent in the presence of glucose by inhibiting transport with cytochalasin B. Restoration of glucose to the medium on starved cells reduces glucose transport to control levels within 2 hr. In a series of carbohydrates tested, those effective in reversing glucose transport only partially coincide with those effective in reversing 2-deoxyglucose transport, suggesting that glucose and 2-deoxyglucose are transported by separate systems under separate controls. This conclusion is strengthened by observations of the action of SH reagents on control (unstarved) cells: (1) bromoacetic acid, iodoacetic acid, and dinitrofluorobenzene stimulate glucose transport but do not affect 2-deoxyglucose transport; (2) mercuric nitrate and N-ethyl maleimide inactivate 2-deoxyglucose transport but do not affect glucose transport; and (3) *p*-chloromercuribenzoate inactivates both systems.

Several aminoglucoses have been bromoacetylated and are being further purified for testing as possible affinity labeling reagents for purifying transport proteins and for studying their turnover in response to various physiological stimuli.

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MEMBRANE TURNOVER AND MAINTENANCE OF ALKALI CATIONS IN HeLa CELLS

J. S. Cook, Emily T. Best, G. L. Vaughan,* and W. R. Proctor†

HeLa cells contain K^+ at a concentration of 160 meq per liter of cell water and Na^+ at approximately 40 meq per liter of cell water. Intracellular K^+ leaks from the cell as a single compartment with a rate constant of 1 hr^{-1}, and, in the steady state, K^+ is transported into the cell at the same rate. Transport is inhibited by the cardiac glycoside ouabain and is completely blocked when the cells have bound about 1.4×10^6 molecules of the inhibitor per cell. From these data it may be calculated that each site normally transports a minimum of 40 K ions per second. During the cell cycle, transport capacity per cell (presumably a linear function of the number of Na-K ATPases on the cell surface) increases at a rate indistinguishable from the rate of increase in area of the surface membrane, but the turnover of ouabain-binding sites is four to six times as rapid. If transport sites are partially inhibited by low levels of ouabain, the cells lose a small amount of K^+ and gain a small amount of Na^+. The increased intracellular Na^+ stimulates the Na-K transport ATPase, which may now carry as many as 90 ions per second until, via membrane turnover, the initial surface concentration of active ATPases is restored. Turnover may be slowed but not prevented by cytochalasin B, colcemid, and vinblastine. For direct determinations of turnover of membrane Na-K ATPase, we are developing methods for purification of labeled enzymes using affinity chromatographic techniques.

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HISTOPATHOLOGY OF TUMOR REGRESSION AFTER INTRALESIONAL INJECTION OF *MYCOBACTERIUM BOVIS*: DEVELOPMENT OF IMMUNITY TO TUMOR CELLS AND TO BCG

M. G. Hanna, Jr., M. J. Snodgrass,* B. Zbar,† and H. J. Rapp†

Recent results of our investigation of the mechanism of BCG-mediated therapy of established tumors with regional metastases in inbred guinea pigs suggest that activated cells of the macrophage-histiocyte compartment play a primary and essential role in the destruction of tumor cells. In this immunotherapy model which utilizes a transplanted syngeneic hepatocarcinoma and BCG, it is reasonable that tumor inhibition may not require the development of specific tumor immunity; there is a lack of evidence to substantiate that the activated histiocytes are specifically sensitized to the tumor cells. The destruction of

the established tumor, both in the intradermal transplantation site and in the regional lymph nodes, could in part be attributed to the detrimental environment created by the BCG-mediated granulomatous response, since this reaction is qualitatively and quantitatively similar in tumor-bearing and tumor-free animals. Also, studies in mice suggest that BCG-mediated tumor regression does not require development of specific tumor immunity, but no definite conclusions can be drawn on this point in the guinea pig immunotherapy model.

We performed studies to evaluate specific immunity to tumor cells and to BCG in this therapy model. The results demonstrate that the process of BCG-mediated regression of established tumors has both local and systemic features, both of which involve host-specific immunity. A requirement for early sensitization to BCG antigens was demonstrated in guinea pigs whose peripheral and central lymphocyte compartments were depleted as a result of pretreatment with antilymphocyte serum. A correlation was shown between the ability to develop delayed sensitivity to a purified protein derivative of BCG and tumor regression after injection of BCG into established tumors. The development of specific tumor immunity was shown to be an aspect of this experimental model. Guinea pigs with established tumors developed humoral and cell-mediated immunity during the course of BCG-mediated tumor regression. Antibody with specificity for line 10 tumor cells was demonstrated by the positive immunofluorescence and C1 fixation and transfer test of plasma from BCG-treated tumor-bearing guinea pigs. Systemic cell-mediated immunity was demonstrated by the classic lymphocyte-mediated rejection of line 10 tumor challenge in guinea pigs that had undergone BCG-mediated regression of line 10 tumors.

Thus it is not unlikely that during BCG-mediated regression of tumor in the skin, if metastasis does occur beyond the draining chain of lymph nodes, it could be controlled by means of the developing specific tumor immunity. Also, it is clear that an immediate requirement for BCG-mediated tumor regression, after intralesional injection of a critical number of viable organisms, is a specific reactivity of the host to BCG antigens. Both of these aspects of the BCG therapy model are relevant to clinical application of BCG in human cancer.

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HISTOPATHOLOGY OF TUMOR REGRESSION AFTER INTRALESIONAL INJECTION OF *MYCOBACTERIUM BOVIS:* ULTRASTRUCTURAL STUDIES OF HISTIOCYTE TUMOR CELL INTERACTIONS DURING TUMOR REGRESSION

M. J. Snodgrass* and M. G. Hanna, Jr.

We have presented histopathological evidence that suggests that a nonspecific granulomatous reaction, characterized by a proliferation of stromal reticuloendothelial components and infiltrating histiocytes, both at the tumor site and in the regional lymph nodes, is a necessary part of the antitumor response in BCG-treated animals. In contrast, induced nonspecific lymphoproliferative reactions (which are characteristic of developing delayed-type hypersensitivity) and nonspecific inflammatory reactions do not have detrimental effects on the tumor. Our earlier data further suggest that BCG-activated cells of the macrophage-histiocyte series are the effective killer cells in this experimental model and that phagocytosis is not the primary mechanism of the cytotoxic reaction. However, the level of resolution of these studies prevented elucidation of the cytological characteristics of the interaction between histiocytes and tumor cells.

The purpose of this study was to evaluate the ultrastructure of the histiocytes involved in the granulomatous reaction induced by BCG and to examine the interaction between histiocytes and tumor cells at the transplantation site and in the draining lymph nodes at various intervals after intralesional injection of BCG. Critical morphological similarities were found on the ultrastructural level among activated histiocytes in the various reactive regions: infiltrating at the tumor site, autochthonous to the draining lymph node, migrating from the tumor site to the subcapsular marginal sinus of the draining lymph node, and infiltrating into the medullary sinuses of the lymph node. This observation indicates that these are all forms of a specific class of cell within the macrophage-histiocyte series. A cytopathic effect of these activated histiocytes on tumor cells is mediated by cell surface contact; we observed sizable areas of apparent fusion of the cellular membranes of histiocytes and hepatocarcinoma cells. Thus, destruction of tumor cells by a nonphagocytic mechanism may be a phenomenon related to activated but not specifically sensitized cells of the macrophage-histiocyte series. This response of histiocytes and tumor cells is reminiscent of the interactions between "activated" histiocytes and tumor cells described by others in *in vitro* and *in vivo* systems and is similar to the cytoplasmic

continuity that has been shown between histiocytes and endothelial cells during renal homograft rejection. The cytopathic mechanism by which such "activated" histiocytes destroy tumor cells is not completely understood; however, others have suggested that protuberances of the target cells may be pinched off and engulfed by the histiocyte, resulting eventually in irrepairable membrane damage that leads to the degradation of the target cells. At present we have no evidence to indicate the involvement of a cytotoxin or of a cytophilic antibody, although each is necessary for contact of specifically immune macrophages with target cells.

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THE DIFFERENTIAL EFFECTS OF TWO STRAINS OF BCG ON TRANSPLANTATION IMMUNITY TO SV40-INDUCED TUMORS IN HAMSTERS

G. W. Fortner,* J. H. Coggin, Jr.,† and M. G. Hanna, Jr.

Variables which have been cited in the use of BCG to stimulate immunity against malignancies include sources, dose and number of viable organisms, and time and route of administration. A less apparent but in our experience critical variable is ability of the experimental animal to control the infectious process established by the live BCG. This is extremely relevant in developing reliable experimental models for establishing alternatives for therapy in man where BCG infection is limited.

In order to gain insight into the basis of protection or enhancement as a function of BCG treatment, we chose the transplantable hamster SV40 tumor system. This model is advantageous because it is well characterized with respect to tumor immunology, and the hamster has been used to characterize BCG strains for their virulence in relation to their use as live vaccines for tuberculosis. A correlation is made between immunoprophylactic and immunotherapeutic properties and growth-culture kinetics and morphology of a lyophilized and live frozen source of BCG.

The effect of BCG on the development of SV40-induced tumor transplantation immunity in the hamster was as follows: immunization with a live preparation caused a delay in tumor appearance, although final tumor incidence was only 13% lower than the controls. In combination with irradiated tumor cells, the live BCG preparation had a synergistic effect on transplantation immunity, resulting in a significant protection against tumor challenge. Immunization with a common lyophilized strain resulted in a significant enhancement of tumor incidence. The time of tumor appearance paralleled the controls until after the seventh week, when enhancement led to 100% tumors. This correlated with the time when systemic BCG infections became histologically apparent. The lyophilized BCG strain, mixed with irradiated tumor cells, afforded some protection by delaying tumor appearance and slightly decreasing tumor appearance, but these results were not statistically significant.

The present studies of immunoprophylaxis with BCG show protective effects as measured by delay of appearance and reduction of tumor incidence with the live BCG preparations, but also show actual enhancement of tumor incidence with a lyophilized strain. In combination with irradiated tumor cells, both had synergistic effects on the development of transplantation immunity in hamsters. However, the effect of the lyophilized strain was significantly lower than the live preparation. Further, our results indicate that there are differences in BCG strains which may have influenced their efficacy as immunoprophylactic or immunotherapeutic agents. These differences can be correlated with the kinetics of growth and the colonial morphology of solid media, as well as virulence for the hamster. These results are extremely critical to clinical application with BCG in cancer therapy and are being repeated using several other BCG sources.

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EVALUATION OF THE SUPPORTIVE ROLE OF IMMUNOSUPPRESSION IN CARCINOGENESIS INDUCED IN HAMSTERS BY TOPICAL APPLICATION OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE

A. K. Szakal and M. G. Hanna, Jr.

The hypothesis that carcinogen-induced immune suppression is required for successful establishment in the host of clones of induced neoplastic cells has received circumstantial support from studies based on the premise that all chemically induced short-latency tumors are highly antigenic. It has recently been shown that all early sarcomas induced by methylcholanthrene in mice are highly antigenic, and an inverse relationship between latency and antigenicity exists. Thus the results of experiments in which a

variety of non-tumor-related antigens have been used to assess immune competence during chemical carcinogenesis are difficult to evaluate, and although many studies have demonstrated the immunosuppressive properties of chemical carcinogens, the causal relationship between immunosuppression and tumor development in chemical carcinogenesis is still equivocal.

We conducted a series of experiments to examine the hypothesis that there is a causal relationship between immune suppression induced by chemical carcinogens and the early, successful establishment of tumors. The results demonstrated the following: (1) Continuous application of DMBA during the period of tumor latency results in early temporary suppression and then complete recovery of humoral immunity to an extraneous antigen. This is closely followed by a more persistent depression of the cell-mediated immunity to skin allografts. (2) After DMBA treatment for the minimum period necessary to suppress humoral immunity, cell-mediated immunity still becomes depressed after a period of latency. (3) The degree of depression of cell-mediated immunity, both in animals continuously treated with DMBA and in those given the minimum number of carcinogen treatments, is related to the extent of tumor development. (4) Histologic changes in the draining lymph node and in the spleen substantiate the immunosuppressive effect of DMBA. (5) DMBA-treated, immunogenetic papilloma-bearing skin grafts and antigenic primary tumor grafts have a higher percentage of survival and/or an increase in survival time on animals with DMBA-depressed cell-mediated immunity.

These studies suggest that in this system the dose-dependent DMBA-mediated systemic suppression of immunity to non-tumor-related antigens is also applicable to tumor-related antigens and that this hyporeactivity of the immune system, although not an essential aspect of chemical carcinogenesis, supports the successful establishment and growth of immunogenic autochthonous carcinomas.

AUTOGENOUS IMMUNITY TO ENDOGENOUS RNA TUMOR VIRUS: CHRONIC HUMORAL IMMUNE RESPONSE TO VIRUS ENVELOPE ANTIGENS IN B6C3F$_1$ MICE

B. L. Batzing, Michael Yurconic, Jr.,
R. W. Tennant, and M. G. Hanna, Jr.

Although successful persistence and vertical transmission of murine leukemia virus (MuLV) are observed in wild and in most, if not all, laboratory mouse strains, the incidence of lymphoma is highly variable. One important question concerning this variation is the role of host immunological mechanisms in the control and regulation of endogenous virus burden and leukemogenesis. This question is especially relevant if the leukemia virus must reach a critical concentration in the host before leukemogenesis begins or if virus transformation of target cells depends on some multiplicity of infection.

Previous studies of naturally acquired immunity to endogenous MuLV-related antigens have tended to indicate that the major detectable autogenous immune response is directed at virus-mediated leukemia cell surface antigens rather than at virion antigens. In an attempt to provide insight into the function of host immunological mechanisms in the regulation of endogenous MuLV, we have studied the hybrid mouse strain B6C3F$_1$ (C57BL/6 ♀ × C3H/Anf ♂). These mice have a mean survival time of approximately 884 days and a very low natural incidence of thymic lymphoma; a similar hybrid from a reciprocal mating has been shown to have a much lower Gross virus susceptibility than either parental strain. By the use of immunoelectron microscopic techniques, it was demonstrated that throughout life these mice possess significant levels of free serum antibody specific for murine leukemia virus envelope antigens. The functional aspects of this antibody were demonstrated by its ability to achieve some neutralization in cell culture of the infectivity of RNA tumor virus produced by spontaneously activated AKR mouse embryo cells. Comparable neutralization of Moloney leukemia virus also was shown. Immunoglobulin and endogenous murine leukemia

virus antigen(s), presumably in the form of immune complexes, were observed to become localized within kidney glomeruli with age, and this deposition correlated well with the chronic development of glomerulonephritis. Immunoglobulin eluted from kidneys of aged animals also was shown to neutralize AKR virus and to be specific for virus envelope antigens. Although the role of the anti-virus-envelope-antigen antibody in $B6C3F_1$ mice cannot be positively defined from these studies, it may be one critical component in the systemic regulation of levels of infectious endogenous leukemia virus.

AUTOGENOUS IMMUNITY TO ENDOGENOUS RNA TUMOR VIRUS: RADIOIMMUNE PRECIPITATION ASSAY OF MOUSE SERUM ANTIBODY LEVELS*

J. N. Ihle,† Michael Yurconic, Jr., and M. G. Hanna, Jr.

The expression of endogenous murine leukemia virus (MuLV) during the development and throughout life of normal mice has been demonstrated serologically and ultrastructurally. In addition, experiments have indicated that the consequence of this natural virus expression in the host is an autogenous humoral immune response. Antibodies specific for virion and nonvirion antigens – virus envelope antigens (VEA) and virus-mediated cell surface antigens (VCSA), respectively – have been detected in serum and kidney eluates by immunoelectron microscopy, immunofluorescence, and complement fixation. In some strains of mice this chronic humoral immune response has immunopathologic manifestations, as exemplified by glomerulonephritis.

The role of this autogenous immunity in the regulation of virus-mediated pathogenesis may be a function of the quality and/or quantity of the humoral response. The purpose of the present study was to evaluate the quantitative changes, with age, in the serum levels of free natural antibodies to MuLV antigen(s) in strains of mice with high and low natural incidences of lymphoma. To facilitate the detection and quantitation of antibodies directed against virion antigens, we have utilized a radioimmune precipitation assay with radioactively labeled MuLV. The radioimmune precipitation assay, using ^{3}H-labeled AKR leukemia virus, was applied to the detection and quantitation of natural serum antibodies directed against endogenous murine leukemia virus envelope antigens. $B6C3F_1$ and BALB/c mice, which have a low natural incidence of leukemia and lymphoma, and AKR mice, which have a high incidence, were used in this study. Sera from mice of various age groups were assayed. As shown in Table 28, a marked difference in age-associated levels of the autogenous immune response to endogenous murine leukemia virus was detected, and the quantitative differences among these strains were inversely related to the incidence of lymphoma. The radioimmune precipitation test, as applied, was 500 times more sensitive than the XC virus neutralization test. That the reactions we have observed are specific

Table 28. Age-associated changes in autogenous immunity to endogenous RNA tumor virus

Strain	Age	Radioimmune precipitation titer[a]	Incidence of leukemia and lymphoma	Mean survival time
$B6C3F_1$	5 weeks	128		
	12 weeks	845	<5%	884
	1 year	5020		
	1 1/2 years	8450		
	2 years	2160		
BALB/c	5 weeks	20		
	1 year	317	<5%	733
	1 1/2 years	1260		
AKR	8 weeks	25		
	8 months	40	>80%	402
	1 year	40		

[a]Titrations are defined as the reciprocal of the dilution of antiserum that gives 50% precipitation of labeled AKR virus.

is suggested by several lines of evidence, including the nonreactivity of normal hamster and absorbed serum, the positive reaction of absorbed rat anti-AKR serum, the inhibition of precipitation of labeled virus by purified unlabeled virus, and isopycnic gradient analysis of the reactive products.

The distinction between the functionality of the immune reaction and the genetic regulation of infectious virus is complicated, and generalizations regarding host functions in the regulation of RNA tumor virus pathogenesis cannot be applied to all strains of mice. However, the point remains that while a major consideration is presently made of the cellular control of endogenous virus expression, it is clear that there does exist a systemic mode of regulations exerted by the humoral immune systems.

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†American Cancer Society Postdoctoral Fellow (PF-848).

CROSS-REACTIVITY OF TUMOR-ASSOCIATED FETAL ANTIGENS*

F. A. Salinas† and M. G. Hanna, Jr.

Recently tumor-associated fetal antigens have been detected, using a quantitative *in vivo* assay. This assay utilizes the spleen colony-forming capacity of transplanted fetal liver cells in tumor- or fetal-cell-immunized, irradiated adult mice. Previous results show that immunization with either plasma-cell tumor (PCT) or fetal liver cells (FLC) caused a two- to threefold suppression of fetal colony-forming units (FCFU) in the spleen of irradiated recipients. Now by the use of this *in vivo* assay we have also demonstrated that association of fetal antigen(s) with PCT is not a restricted phenomenon but is common to a variety of tumors of different origins (i.e., plasma-cell tumor, EL-4 lymphoma cells, SV-40 transformed hamster fibroblast, and Ki-MSV-transformed BALB/3T3 cells). To increase the effectiveness of this assay, a modification was performed by mixing immune sera or lymphoid cells from tumor- or fetal-cell-immunized donors with fetal liver cells. Following an incubation period, the mixture was adoptively transferred into lethally irradiated normal or immunized recipients. This procedure resulted in a more marked suppression (more than tenfold) of fetal colonies. Finally, antifetal immunity in female mice was studied as a function of parity status. The number of FCFU was determined at different weeks after the last delivery. The results demonstrated that while there was a clear antifetal activity in females after the first pregnancy, this activity was less after the second pregnancy. The retired breeder females (five or six pregnancies) again showed strong antifetal activity, expressed as a reduction in the number of FCFU.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

†New York Cancer Research Institute Postdoctoral Fellow.

EFFECTS OF POLYADENYLIC ACIDS ON MOUSE RNA TUMOR VIRUSES *IN VIVO**

R. W. Tennant, J. G. Farrelly, and M. G. Hanna, Jr.

Based upon the inhibitory effects of polyadenylic acid [poly(A)] and 2′-O-methyl polyadenylic acid [poly(Am)] on RNA viruses in cell culture, we have tested the effects of these polymers on virus infection and tumorigenesis *in vivo*.

Studies are now in progress, but the following observations can be reported on experiments with Moloney sarcoma virus (MSV) and Rauscher leukemia virus (RLV). The MSV experiments tested the effect of poly(Am) at 10 and 50 μg/ml on tumor development in newborn BALB/c mice. In these experiments the polymer was given only within 4 hr prior to and one week after the virus. The results indicate that poly(Am) has at least two effects, depending on virus and poly(Am) doses. At the higher doses of poly(Am) (50 μg/ml), sarcomas developed in treated and untreated mice at about the same rate but regressed in the treated group. The regression was followed by remissions and death over the next 60 days. In another experiment in which mice were given a lower dose of virus and poly(Am) at only 10 μg/ml, no tumors developed in the treated group while all untreated mice died within 85 days.

The experiment with RLV was performed with two virus concentrations. In contrast to the results with MSV, the poly(Am)-treated mice infected with RLV showed an enhancement of splenomegaly, particularly at the lower virus concentration.

These results suggest, therefore, that the effects of poly(Am) *in vivo* are related principally to some host immunological function. If poly(Am) acted *in vivo* principally by competitively binding the viral polymerase, it would be expected that the result would be a delay or inhibition of tumorigenesis, which we observed in one experiment. However, the results seen in the other experiment suggest that the primary effect is on the host immune system which promoted rejection of the MSV-induced tumors in one case and enhancement in the other. Since RLV has been shown to replicate in

immunoblasts, it is possible that poly(Am) stimulates proliferation of some immunocompetent cells or precursors in which the virus replicates. In the case of MSV this nonspecific stimulation provides a response which is beneficial to the host. Experiments are now in progress to determine the specificity of poly(Am) on the pathogenesis of MSV and to determine if humoral or cell-mediated immune mechanisms can be identified. While certain double-stranded polynucleotides [e.g., poly(AU)] have been shown to affect the immune system, single-stranded polynucleotides have not been shown to be active. However, the nuclease resistance of poly(Am) may provide the property required for these effects.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

POLYNUCLEOTIDE INHIBITION OF VIRUS EXPRESSION*

J. G. Farrelly, R. W. Tennant, J. N. Ihle,†
B. C. Pal, and F. T. Kenney

We are continuing our study of polynucleotides as inhibitors of various functions of the murine oncogenic RNA tumor viruses. In an earlier report from this laboratory,[1] it was established that single-stranded polyribonucleotides are competitive inhibitors of murine leukemia virus RNA-dependent DNA polymerase (reverse transcriptase), using either the resident viral RNA, native calf thymus DNA, or poly[d(A-T)] as template. Subsequently it was demonstrated that 2′-O-methyl polyadenylic acid [poly(Am), also a competitive inhibitor of reverse transcriptase] was an efficient inhibitor of Moloney leukemia virus (MoLV) replication in mouse embryo cells.[2] Poly(Am) had no effect on cellular DNA synthesis or growth.

We next tested whether polyribonucleotides would interfere with uptake of virus rather than inhibiting viral reverse transcriptase in tissue culture. Therefore, [^{3}H]leucine- or [^{3}H]uridine-labeled virus was prepared. MoLV was prepared from chronically infected Swiss mouse embryo cells and AKR virus from spontaneously activated AKR cells. The effects of poly(A) and poly(Am) on uptake of both of these virus preparations were identical and can be summarized as follows: At 50 μg/ml of polynucleotide, there was complete inhibition of uptake of virus in tissue culture; intermediate inhibition occurred at 25 μg/ml; and no inhibition at 10 μg/ml. Similar experiments were carried out on [^{3}H]leucine-labeled Sindbis virus. Poly(A) at 100 μg/ml had no effect on uptake of this virus while poly(Am) at 100 μg/ml gave only 40% inhibition. At concentrations as high as 500 μg/ml, poly(A) had no effect on the replication of Sendai virus in $LLMK_2$ cells. It is possible that polyribonucleotides inhibit the uptake of the oncogenic RNA viruses only.

Poly(Am) was then tested for its ability to inhibit MoLV synthesis at polynucleotide concentrations which do not appreciably affect viral uptake. At 10 μg/ml this compound inhibited the synthesis of MoLV by 85%; at 20 μg/ml the inhibition was 95%. The effect of addition of poly(Am) on the MoLV replicating system was then examined as a function of time of addition of inhibitor. Maximum inhibition was seen when poly(Am) was added prior to or at the time of infection. When poly(Am) was added 1 hr past infection, the inhibition was 75%. A 40 to 50% inhibition was found when poly(Am) was added 2 hr after infection. Little inhibition was noted at 3 hr or later. These results suggest that a component of the inhibition involves an early event in the replication cycle. This event is probably the action of viral reverse transcriptase.

Next, we tested the ability of poly(Am) to inhibit the transformation of 3T3 cells by the Moloney pseudotype of mouse sarcoma virus (MSV). Even at concentrations as low as 5 μg/ml, poly(Am) inhibited MSV-induced transformation by 80%. Essentially complete inhibition was achieved at 10 μg/ml. These results remained unchanged whether the poly(Am) was added during or immediately after infection and suggest that reverse transcriptase function plays an important role in MSV transformation of mouse cells.

AKR mouse embryo cells can be activated to produce leukemia virus by treatment with IdUrd. We tested the ability of poly(A) at 100 μg/ml and poly(Am) at 10 μg/ml to inhibit this process. No inhibition of activation was observed using either compound. It appears, therefore, that reverse transcriptase plays little or no role in the activation of AKR cells by IdUrd.

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†American Cancer Society Postdoctoral Fellow.

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PHYSIOLOGICAL PARAMETERS AFFECTING INDUCTION OF MURINE LEUKEMIA VIRUS BY IdUrd*

J. N. Ihle,† R. W. Tennant, L. E. Roberson, and F. T. Kenney

Rowe et al.[1] initially observed that compounds such as IdUrd or BdUrd could induce leukemia virus synthesis in cultured fibroblasts of the AKR strain of mice. We have attempted to study some of the physiological requirements for activation by IdUrd to elucidate the mechanism of activation and to seek similarities in these requirements to those of virus infection that might reflect similar regulatory controls.

When AKR cells are treated with IdUrd at 100 μg/ml for 24 hr and are assayed 48 hr later for virus synthesis by fluorescent antibody techniques, 0.5% to 5% of the cells are "activated" for virus synthesis. (In general, the extent of activation within one experiment is fairly constant, but it will vary extensively from experiment to experiment depending upon cell line, number of passages, confluency at time of treatment, etc.) However, if the cells are deprived of serum 3 to 24 hr prior to IdUrd treatment and are subsequently maintained on medium lacking serum, no activation is observed. If IdUrd treatment is started at the time of serum removal, some activation is observed. If the cells, deprived of serum for 3 to 12 hr, are treated with IdUrd in the absence of serum *but* are allowed to recover in the presence of serum, activation of virus expression is observed. Since DNA synthesis and cell division are not completely stopped until 24 hr, and since the cell cycle time under these conditions is 12 hr, the above results can be interpreted to indicate that two cell cycles are required for activation of virus synthesis. More specifically, the results indicate that, during the initial cell cycle, activation is "fixed" and the second cell cycle is required for full virus expression.

The stability of the intermediate formed during the first cell cycle is demonstrated by the following results. When cells are serum-deprived 6 hr prior to starting IdUrd treatment, no activation of virus synthesis is observed at one, two, or three days after treatment, when the cells are allowed to recover in the absence of serum. If serum is added back at one, two, or three days after IdUrd treatment, a significant proportion of cells will initiate virus synthesis.

Further to examine the requirement for two cell cycles, we have examined the effect of thymidine (25 μg/ml) and cytosine arabinoside on activation. In these experiments, cells were serum-deprived 6 hr prior to and during IdUrd treatment; then the IdUrd was removed, and medium containing serum was added back. Thymidine, at the concentrations used, will inhibit normal activation of virus synthesis by IdUrd, but only if added at the same time as IdUrd. These results confirm the observation of Rowe[1] that IdUrd incorporation is required for activation. However, thymidine does not affect the second stage of virus expression, as it does not inhibit when added at the time of serum addition. These results suggest that during the initial cell cycle, IdUrd is incorporated and activates the virus to a stable nonexpressed intermediate, the subsequent expression of which requires a second cell cycle. Cytosine arabinoside, added at either time, inhibits virus expression, indicating that both events require DNA synthesis.

In summary, these results indicate that the initial event in virus activation by IdUrd is its incorporation into cellular DNA, an event that requires cell division. Once incorporated, the IdUrd may effect activation by causing breakdown of cellular DNA, as suggested by Rowe.[1] After IdUrd incorporation, a stable intermediate is formed, the subsequent expression of which is dependent upon a second cell cycle. It may be that this intermediate is the same as the "provirus" intermediate formed during exogenous viral infection.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

†American Cancer Society Postdoctoral Fellow.

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AFFINITY CHROMATOGRAPHY OF RNA-DEPENDENT DNA POLYMERASE*

D. R. Joseph,† J. G. Farrelly, and F. T. Kenney

Because polyribonucleotides are competitive inhibitors of RNA-dependent DNA polymerase (reverse transcriptase),[1] we have prepared poly(G)-Sepharose and poly(2′-O-methyl U)-Sepharose [poly(Um)-Sepharose] and have tested their ability to act as a support for affinity chromatography. At pH 6.0, 70% of the poly(Um) added to cyanogen bromide-activated Sepharose was bound to the support, while 52% of the poly(G) was bound. When poly(G)-Sepharose columns were used, both cellular DNA polymerases and reverse transcriptase adhered at low salt concentrations. DNA polymerase I from mouse spleen or *E. coli* could be eluted from the poly(G)-Sepharose column with 0.42 *M* NaCl. DNA polymerase II from a number of sources (including rat liver, human lymphocytes, NIH-3T3 cells, and mouse spleen) eluted between 0.32 *M* and 0.42 *M*

NaCl. However, reverse transcriptase eluted at a substantially higher salt molarity. Enzyme from Rauscher leukemia virus-infected spleen eluted at 0.60 *M* NaCl, while enzyme from avian myeloblastosis virus eluted at 0.80 *M* NaCl. In contrast to the poly(G)-Sepharose columns, poly(Um)-Sepharose columns had no affinity for the cellular DNA polymerases of mouse spleen. However, the reverse transcriptase of Rauscher leukemia virus isolated from JLS-V9 tissue culture did adhere to the column and eluted at 0.27 *M* NaCl. The reverse transcriptase from RLV-infected mouse spleens eluted at 0.26 *M* NaCl. These affinity chromatography methods should provide a fast and simple method to screen for the presence of reverse transcriptase in biological samples.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

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FRACTIONATION OF RNA TUMOR VIRUS 34S RNA SUBUNITS ON POLYURIDYLATE-SEPHAROSE COLUMNS*

J. N. Ihle,† Kai-Lin Lee, L. E. Roberson, and F. T. Kenney

The genome of murine leukemia viruses (NuLV) is composed of a 70S RNA complex, which when heat dissociated is resolved into three to four 34S RNA subunits. Recent reports have described the presence of polyadenylic acid regions in the viral RNA. The existence of subunits obviously presents the possibility that some distinction may exist among them; evidence presented by Duesberg and Vogt[1] suggests that, in the avian sarcoma viruses, the information required for transformation may be localized on a single subunit. We have analyzed purified viral RNA subunits from various viruses by chromatography on poly(U)-Sepharose columns, which resolve RNA molecules on the basis of the absence or presence of poly(A) and the length of the poly(A) regions.

To obtain intact viral RNA subunits, virus was isolated from 4-hr harvests of culture media from cells continuously labeled with [^{3}H]uridine or ^{32}P. The virus was subsequently purified by isopycnic gradient centrifugation and immediately phenol-extracted with Tris buffer at pH 9.0. The viral 70S RNA was subsequently isolated by sucrose gradients and heat dissociated, and the 34S RNA subunits were isolated by sucrose gradients. Under the conditions we have used, generally 80% or more of the radioactivity migrates as a single peak on SDS gel electrophoresis with an apparent S value of 33 to 35.

To chromatograph the viral subunits on poly(U)-Sepharose columns, the RNA was loaded on a 1.0-ml column of poly(U)-Sepharose previously equilibrated with 0.1 *M* NaCl, 0.01 *M* Tris buffer at pH 7.6 at 41°C. The temperature of the column was slowly lowered (1°C per 2 min) to 20°C and equilibrated at 20°C for 15 min. This procedure was chosen to obtain a uniform and optimal hybridization between the viral poly(A) regions and the poly(U) on the Sepharose. The unhybridized RNA is eluted, and the temperature of the column is then raised at 2°C intervals. The final fraction collected is at 44°C at which point all radioactivity has generally been recovered. The viral RNA from MuLV isolated from spontaneously activated AKR mouse cells elutes exclusively at 20°C and 44 to 46°C. Based on the elution of poly(A) standards, the fraction eluting at 20°C probably has no poly(A) regions longer than 20 residues while the fraction eluting at 44 to 46°C has poly(A) regions of 150 to 200 nucleotides. SDS gel electrophoresis of each fraction reveals that they are predominantly 34S RNA. These data show that the MuLV 34S RNA subunits are composed of two chromatographically distinct fractions, consisting of poly(A)-containing subunits representing approximately 60 to 70% of the viral RNA and non-poly(A)-containing subunits comprising 30 to 40% of the total RNA.

To determine if comparable heterogeneity exists in the RNA of other viruses, we examined two additional viral genomes by chromatography on poly(U)-Sepharose. When 34S RNA isolated from a Moloney leukemia virus was examined, exactly analogous results were obtained to those above. However, when we examined the 34S RNA from the RD-114 virus, approximately 60% of the RD-114 34S RNA did not bind to the column and eluted at 20°C. This fraction presumably has no poly(A) regions greater than 20 nucleotides. Of the RNA that bound to the column, approximately 28% of the radioactivity eluted at 30 to 34°C. This corresponds to the elution position of RNA containing poly(A) stretches of 50 to 75 nucleotides. The remainder of the radioactivity, approximately 12%, eluted at 38 to 42°C. This elution position corresponds to RNA containing 100 to 150 nucleotides in the poly(A) region. In contrast to the murine viruses, no radioactivity eluted at 44 to 46°C. RNA acrylamide gel electrophoresis of each of these fractions results in profiles similar to the above profiles of the murine virus RNA's, each fraction being predominantly a single 34S RNA species.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

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TYPE C VIRUS IN HEPATOMA CELLS*

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Ching-Yuan Hung, Wen-Kuang Yang, and J. E. Fritz

In a beginning attempt to examine the possibility of a viral etiology of solid tumors of epithelial origin, we have analyzed H-35 cells for type C viruses and for known components of these viruses. The H-35 cell line is one in which specific enzyme synthesis is regulated by several hormones; it was derived from a chemically induced rat hepatoma. Electron microscopy revealed a few particles, both free and apparently budding, which could be interpreted as type C particles. To look for virus production by the cells, we collected 1500 ml of medium (15 roller flasks), precipitated with polyethylene glycol and NaCl, and centrifuged through 25% sucrose. The resulting pellet was assayed for reverse transcriptase activity with the following results: (1) no activity with $(rA)_n(rU)_n$ or $(rC)_n(dG)_6$ as templates and (2) small but definite activity with $(rA)_n(dT)_{10}$ as template. Direct assay of cellular extracts by poly(G)-Sepharose chromatography, using various templates, yielded no indication of reverse transcriptase in the cells.

Medium from ^{32}P-labeled cells was examined on sucrose gradients; a band with density slightly less than 1.16 g/cm^3 was observed. When RNA was extracted from peak tubes (under conditions yielding 70S RNA from known virus-producing cells), only small RNA's (about 5S) were found.

From these results, it is apparent that the amount of type C virus produced in H-35 cells, if any, is very small and that the secreted viral particles are very labile. In view of the facts that the production of viruses can be activated by treatment with agents such as halogenated pyrimidines in cultured mouse cells and that this activated production of virus can be enhanced with glucocorticoids, we are attempting to test the effects of these agents on the production of type C virus in H-35 cells.

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REEXAMINATION OF "SUPERINDUCTION" OF TYROSINE TRANSAMINASE IN CULTURED HEPATOMA CELLS*

Kai-Lin Lee, C. D. Stiles,† J. E. Fritz,
and F. T. Kenney

The "superinduction" of tyrosine transaminase has been studied extensively in cultured hepatoma cells. Based on ample evidence, we have concluded that "superinduction" of the transaminase by high concentrations of actinomycin D is unrelated to steroid action but is attributable to inhibition of degradation of the enzyme. Nevertheless, our inability to detect "superinduced" synthesis of the transaminase has been attributed by others to nutritional "step down" conditions,[1] since we grew the cells in enriched medium and conducted the experiments in nonenriched medium. In consideration of the importance attached to the "superinduction" phenomenon, we repeated our experiments under fully enriched conditions. We observed that actinomycin D at 5 μg/ml caused a marked decrease in the rate of transaminase degradation, while the rate of enzyme synthesis declined steadily with a half-life of about 2 hr. These results confirmed our earlier observations; thus the discrepancies in the results from Tomkins' laboratory and our laboratory cannot be attributed to different experimental conditions. Furthermore, cordycepin (3′-deoxyadenosine) effectively prevented the steroid-mediated transaminase induction by inhibiting posttranscriptional modification of transaminase mRNA, but it did not cause superinduction. These results reaffirm our earlier conclusion that "superinduction" is the result of inhibition of normal enzyme degradation by high concentrations of actinomycin and is not related to steroid hormone action in enzyme induction.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

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ISOLATION OF TYROSINE TRANSAMINASE mRNA*

Kai-Lin Lee, J. E. Fritz, and F. T. Kenney

Previously we have presented evidence in support of the view that glucocorticoids induce tyrosine transaminase by selectively promoting transcription of the

transaminase structural gene, resulting in increased synthesis of enzyme-specific mRNA. However, the critical and direct test of our interpretation requires isolation of the transaminase mRNA as a chemical entity and demonstration that the synthesis of this message is enhanced by steroid hormone.

We have initiated attempts to isolate the transaminase-specific mRNA, using a combination of immunological techniques and poly(U)-Sepharose chromatography. Selective precipitation of transaminase-specific polysomes from total H-35 cell polysomes could be achieved with specific anti-tyrosine transaminase $F(ab')_2$ fragments. The RNA species of the precipitable fraction (<2% of the total ^{32}P radioactivity) and that of the fraction not precipitated were obtained by SDS-phenol extraction and subsequently analyzed by acrylamide gel electrophoresis. The RNA not precipitated resembled the total RNA preparation and contained distinct 28, 18, and 4–5S species as well as a great deal of RNA with S values ranging from 5 to 20S. In contrast, the antibody-precipitable polysomes contained one distinctive RNA species (14–15S) in addition to ribosomal and transfer RNA's. This RNA can be further purified by hybridization to, and controlled elution from, columns of poly(U)-Sepharose. The isolated 14–15S RNA species is the size expected for mRNA coding for the tyrosine transaminase subunits (assuming four subunits, each of about 220 amino acids and containing in addition about 150 adenylate residues).

We are in the process of scaling our isolation procedures up to the level required to prepare the apparently specific mRNA species in quantities sufficient to determine if it does, indeed, code for tyrosine transaminase.

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GLUCOCORTICOID RECEPTORS IN MORRIS HEPATOMAS*

S. E. Lane† and F. T. Kenney

In studies using rat liver and H-35 cells, binding of glucocorticoids to cytoplasmic receptor proteins has been described. We have demonstrated also, *in vitro*, the nuclear uptake of steroid. In this process the steroid must be bound initially to a cytoplasmic receptor.

Several of the inducible enzymes of some Morris hepatomas appear to be insensitive to hormonal manipulation by glucocorticoids. One example is tumor 9618A. However, in tumor line 7800, the amount of tyrosine transaminase is significantly increased by the steroid hormone. This suggests that some of the tumors may not have the specific cytoplasmic receptors or that the process of nuclear uptake of steroid may be deficient.

Our studies reveal binding of dexamethasone (a synthetic glucocorticoid) to high-molecular-weight receptors from each of the tumors. Quantitatively, there are no significant differences in the amounts of receptor protein from the hepatomas (although much less than in normal rat liver), and each sediments as a 4S component in glycerol density gradients. Experiments using rat liver nuclei, as well as nuclei from a sensitive tumor line (7800), show an uptake of steroid previously bound to the cytoplasmic receptors of each of the tissues *in vitro*. Using nuclei from tumor 9618A, little or no uptake of steroid from 9618A supernatant was demonstrated, although a liver supernatant preparation did promote nuclear uptake. This may indicate that the cytoplasmic receptor from 9618A is defective or that some other component from the supernatant is needed to promote steroid uptake.

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PHOSPHORYLATION OF THE CYTOPLASMIC GLUCOCORTICOID RECEPTOR PROTEIN FROM RAT LIVER*

S. E. Lane† and F. T. Kenney

The initial events of glucocorticoid action in rat liver appear to be binding of the steroid to a cytoplasmic receptor protein, followed by an active process of nuclear uptake. In the nucleus, the steroid is bound to a receptor and is associated with the nuclear acidic proteins. Although many of these acidic proteins are phosphorylated, it is not known whether the steroid receptor is a phosphoprotein. We have demonstrated the acidic nature of the cytoplasmic receptor protein and have eliminated sialic acid as a contributing factor.

In order to determine whether the receptor contains phosphate, rats were injected with $^{32}PO_4$ 24 hr prior to death. A supernatant was subsequently obtained at high speed from the livers and incubated with [^{3}H] dexamethasone. After preincubation with DNase and RNase, free ^{32}P and ^3H labels were removed from the preparation by gel filtration. The protein was subjected to ammonium sulfate fractionation to 33% saturation, and the pellet resuspended. In 0.3 *M* NaCl the steroid receptor sediments as a 4S component, while in low salt

the receptor aggregates and appears as an 8S molecule. The receptor was then passed through a Sephadex G-200 column with high salt, and the labeled fractions corresponding to 4S were pooled and recycled through the same column with low salt. Both of the labels now eluted near the void volume. This fraction was applied to a DEAE-cellulose column and eluted using a shallow NaCl gradient. At 0.1 *M* NaCl the eluate contained both labels. Experiments are now in progress to demonstrate the *in vitro* nuclear uptake of the $^{3}H/^{32}P$ label.

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ISOLATION OF SPECIFIC TYROSINE TRANSAMINASE ANTIBODY*

Joanne M. Nichol,† J. E. Fritz, and Kai-Lin Lee

The immunochemical isolation of polysomes involved in the synthesis of a specific protein has been studied by several laboratories. Schimke et al.[1] have successfully isolated the specific mRNA coding for ovalbumin, using this immunochemical technique. However, many investigators have noted that this immunochemical precipitation has low specificity when whole antisera or gamma globulin is used. It seems reasonable to assume that using specific antibody rather than the entire gamma globulin fraction should increase the specificity. A series of experiments was carried out in order to develop a reliable method for the preparation of specific antibody. The method described below suggests a general procedure for isolating specific antibody.

When tyrosine transaminase antibody-antigen complex was hydrolyzed with pepsin at pH 4.0, the antigen was extensively broken down and the antibody was converted to $F(ab')_2$ fragment. The formation of $F(ab')_2$ fragment was confirmed by immunodouble diffusion and sucrose gradient analysis. This $F(ab')_2$ fragment could be further isolated and purified by $(NH_4)_2SO_4$ fractionation, gel filtration, and DEAE column chromatography. The yield of purified $F(ab')_2$ was about 25 to 40% of the original antibody, and it was more than 95% homogeneous with respect to reaction with tyrosine transaminase.

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RELATIVE TRANSLATION RATES OF A STABLE AND A LABILE MESSENGER RNA IN CULTURED MAMMALIAN CELLS*

C. D. Stiles,† Kai-Lin Lee, and F. T. Kenney

Previous kinetic experiments suggested that the normal turnover of tyrosine aminotransferase (TAT) mRNA is contingent upon translation; this was confirmed by experiments in which TAT mRNA activity is measured directly by immunoisotopic methods. Since turnover of TAT mRNA is contingent upon translation, we sought to determine whether the rates of translation of individual mRNA's correlated with their *in vivo* half-lives. TAT mRNA has a half-life of about 2 hr in H-35 cells, while alanine aminotransferase (AAT) mRNA turns over at the same rate as total mRNA, with a half-life of about 11 hr. The relative rates of peptide elongation on TAT and AAT mRNA were measured by adding [^{3}H]leucine to flasks of H-35 cells and, at intervals thereafter, precipitating the newly synthesized TAT and AAT with their respective homospecific antisera. Results show that the rates of peptide elongation on TAT and AAT mRNA's are the same. The basal rate of TAT synthesis is five times the rate of AAT synthesis, indicating either that there are more copies of TAT mRNA or that the process of initiation is more efficient for TAT mRNA. Our results suggest that the diversity of half-lives exhibited by individual mRNA's in mammalian cells is not attributable to differences in efficiency of translation.

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A HEAT-LABILE PROTEIN STABILIZING ACTIVE HOLO-TRYPTOPHAN OXYGENASE*

Joanne M. Nichol† and Kai-Lin Lee

It is known that tryptophan oxygenase is present in the liver cytoplasm in various forms. In order to assay total tryptophan oxygenase, several procedures have been developed to convert various forms of the enzyme into active holoenzyme. One of the methods which optimizes the expression of the total amount of tryptophan oxygenase present in liver homogenate is thermal activation in the presence of tryptophan and a heme source. We found that active holoenzyme generated by thermal activation (heating at 55°C for 5 min) could maintain a linear rate for only 6 to 12 min.

The rapid inactivation of the heat-activated enzyme is due to heat inactivation of some factor or factors since the initial rate could be maintained up to an hour in the presence of an unheated liver soluble fraction. A procedure was developed to quantitatively assay the amount of stabilizing factor in a given sample. Preliminary results indicated this factor is protein in nature and could be partially purified by conventional procedures.

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THE 4S RNA'S OF RNA TUMOR VIRUSES. I. ANALYSIS OF HETEROGENEITY BY REVERSED-PHASE COLUMN CHROMATOGRAPHY*

Wen-Kuang Yang, J. N. Ihle,† ChongKun Koh, Ti Ho, and L. C. Waters

Sedimentation analysis of RNA isolated from RNA tumor viruses generally demonstrates two major components, one 60–70S and the other 4–7S. Properties of tRNA, such as amino acid accepting activities and content of modified bases, have been detected in preparations of the 4S RNA (here designated as "free 4S RNA"). Upon denaturation, the 60–70S RNA dissociates into subunits of 30–40S and 4S molecules containing sequences (pGp and TψCG) generally found in tRNA molecules. Recently, these resident 4S RNA molecules (here designated as "derived 4S RNA") have attracted considerable interest because of their role in RNA-directed DNA synthesis (reverse transcription). The 30–40S subunits do not serve as templates for reverse transcription unless they are reannealed with a certain portion of the derived 4S RNA, which presumably act as primers for the viral DNA polymerase.[1] Thus, it has become obvious that the low-molecular-weight RNA components, especially the 4S RNA's, of the RNA tumor viruses are heterogeneous and that physical separation of the 4S RNA is desirable for further characterization. In the present study we employ the reversed-phase chromatographic system to separate the viral 4S RNA components into multiple peaks, and the chromatographic profiles are analyzed to understand the relationship of the derived 4S RNA and the free 4S RNA from RNA tumor viruses and cellular 4S RNA from virus-producing cells.

Three lines of cultured cells producing "C"-type virus particles were used: a spontaneously transformed and activated AKR mouse embryo fibroblast line, a continuous suspension culture from AKR mouse thymoma, and a human embryonic rhabdomyosarcoma line, RD-114. After exposure of the cells to radiolabeled precursors ([^{3}H] uridine or [^{32}P] phosphate), the culture medium was harvested at 4-hr intervals for isolating labeled viruses. The labeled RNA, obtained by phenol extraction, was separated into 60–70S and free 4S components by rate zonal centrifugation in a sucrose gradient. The derived 4S RNA was prepared by heat denaturation of the 60–70S component and subsequent rate zonal centrifugation. The cellular 4S RNA was prepared from the cells by phenol deproteination and rate zonal centrifugation. All the 4S RNA preparations were purified by DEAE-cellulose chromatography. Reversed-phase chromatography was performed in RPC-5 columns. Usually two samples containing different labels were cochromatographed. Results of the chromatographic analyses are summarized in the following:

1. The derived 4S RNA of both AKR virus and RD-114 virus can be resolved into multiple peaks at the salt concentrations characteristic of tRNA elutions. Analysis by a Dupont curve resolver demonstrated a peak number of about 30, of which 15 were major peaks. Cochromatography with cellular 4S RNA revealed chromatographic profiles which suggest that the derived 4S RNA of the RNA tumor viruses may contain selected species of cellular tRNA.

2. The chromatographic pattern of the free 4S RNA is best interpreted as a mixture of the derived 4S RNA and of cellular 4S RNA. Calculation showed that many, if not all, of the derived 4S RNA species are also present in large quantities in the free state within the virion.

3. Comparison of the 4S RNA preparations from the AKR murine virus and the RD-114 virus showed an apparent similarity in position but considerable differences in relative quantity of the eluted peaks. Both showed less complex and diffuse peak patterns than the respective cellular 4S RNA.

4. The RPC-5 system can better resolve the virus 4S RNA if certain chromatographic conditions are modified. For example, chromatography at higher temperature usually gives better resolution of peaks but requires higher salt concentration for elution. At 70°C, a considerably different profile with very sharp peaks is obtained.

These results suggest that the RPC system can be applied in the study of RNA tumor virus 4S RNA, especially the separation and purification of the RNA primer molecule involved in reverse transcription. Also, the presence of derived 4S RNA in the free state suggests that a large excess of the 4S RNA species may

be required in the assembly of 30–40S subunits into 60–70S molecules.

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THE 4S RNA'S OF RNA TUMOR VIRUSES. II. THERMODISSOCIATION OF 4S RNA FROM 60–70S RNA*

L. C. Waters, Shigemi I. Simms, L. G. Hardin, and Wen-Kuang Yang

It has been known for some time that denaturation conditions, for example, heat or DMSO, convert the 70S viral RNA into smaller subunits of about 36S. More recently, it was shown that in the conversion of 70S to 36S RNA a significant amount of 4S RNA is also produced. Of this at least a portion has base sequences characteristic of tRNA. Several laboratories have strongly implicated 4S RNA molecules as initiators in the reverse transcription of viral 70S RNA by reverse transcriptase. This finding implies a central and fundamental role for this RNA in the replication of and possibly transformation of normal cells by these viruses. For this reason and because of our familiarity with the techniques and methods used in tRNA studies, we have initiated attempts to isolate and characterize these "70S-derived" 4S RNA molecules. The present study concerns detailed RPC-5 chromatographic analyses of the 4S RNA components, [^{3}H]uridine and/or $^{32}PO_4$ labeled, of an AKR virus produced in tissue culture.

The RPC-5 profiles of the "free" 4S RNA are characterized by many peaks which are numerically similar to that observed with tRNA isolated from the cell which is producing the virus. A small number of peaks, three to four, do, however, appear to be more abundant in the "free" 4S than in the cellular tRNA preparation. The "70S-derived" 4S RNA obtained by direct heating of 70S RNA to 80°C, followed by quick cooling, is also characterized by the presence of several peaks. These are sharper and less diffuse than that seen with the "free" 4S and would suggest that a significantly less heterogeneous population of 4S RNA molecules are found associated with the 70S RNA component than are found in the "free" 4S RNA. Because of the number of peaks and the complexity of the profiles, it is impossible at this point to state that there are 70S-associated 4S RNA components which are not found either in the viral "free" 4S or the cellular 4S.

Canaani and Duesberg[1] reported that the 70S RNA of virus could be heated to 60°C and that up to 80% of the 4S RNA was dissociated before the template-primer activity was lost. Furthermore, addition of the residual 20% of the 4S RNA, obtained by further heating to 80°C, back to the resulting 36 RNA (inactive alone) restored template-primer activity. We have used similar conditions to heat dissociate and have examined the RPC-5 profiles of the 4S RNA released at different temperatures. At 55°C about 50 to 70% of the 4S RNA is released, but the RNA remains 70S. At 60°C a similar amount of 4S RNA is released, and RNA is converted partially to 36S (~40%). At 65°C, complete conversion to 36S RNA is observed. The 4S RNA obtained by heating to 55 or 60°C has several components and is not easily distinguished from that obtained by heating the 70S RNA directly to 80°C. When the 70S RNA, previously heated to 55 or 60°C, is further heated to 80°C, the resulting 4S RNA has a characteristic and unique chromatographic profile. This profile is characterized by only three to four prominent peaks. It is of considerable interest that these peaks correspond to those seen in the "free" 4S RNA preparation which appeared in excess when compared with the cellular tRNA. Furthermore, these peaks have half-lives unlike the bulk of the 4S RNA but very similar to that of the 36S viral RNA.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

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THE 4S RNA'S OF RNA TUMOR VIRUSES. III. METABOLIC STUDIES*

Wen-Kuang Yang, L. C. Waters, J. N. Ihle,† and Shigemi I. Simms

We have demonstrated that 4S RNA derived from heat-dissociated 70S RNA of RNA tumor viruses can be resolved into multiple peaks by reversed-phase column chromatography and also that, of these multiple chromatographic peaks, a few are dissociated at higher temperatures than the others. In this report, we describe another approach for characterization of the multiple 4S RNA species derived from virus 70S RNA, namely, by determining their turnover rate within the host cells. Mammalian tRNA in general is metabolically rather stable, with an estimated half-life of 90 to 100 hr within the cell. This may make it possible for one to differentiate cellular tRNA's from virus-specific 4S RNA, which may have a faster turnover rate within the host cell.

Virus-producing cells are exposed to [^{3}H]uridine in culture medium for 16 hr. The medium is then changed to contain excess cold uridine and sometimes [^{32}P]phosphate, and thereafter collected by complete medium change at 4-hr intervals for three times. The viruses in the medium are subsequently isolated by isopycnic centrifugation. The free and the derived 4S RNA from the isolated virus preparations are chromatographed in the reversed-phase column to determine the quantity of radioactivities in individual peaks. Under "chasing" conditions, tritium radioactivity in a chromatographic peak will decrease with time after labeling, and the degree of the decrease will follow its respective rate of turnover. If the turnover of 4S RNA species follows first-order kinetics, as turnover of most macromolecules within the cell does, then a formula can be derived:

$$T_{1/2} = \frac{t \ln 2}{\ln (S_1/S_2)},$$

where $T_{1/2}$ is the half-life of a peak, S_1 is the radioactivity of this peak, determined by using a virus sample of the culture medium collected from time 0 to time t following the chase, and S_2, that from time t to time $2t$.

Application of such experimental procedures and kinetic analyses to the AKR murine RNA tumor virus reveals the following: (1) Peaks 4, 8, 9, 10, and 26 of the RPC-5 chromatogram have half-lives of 3 to 4 hr, similar to that of the 36S subunits of 70S RNA. Interestingly, peaks 4, 8, 9, and 10 are the species found to have higher melting temperatures from the 70S RNA than the other 4S RNA species. (2) Half-lives of the other 4S RNA peaks are much longer than 4 hr. The tritium radioactivity in these peaks declined only slightly in the 12-hr chasing period, and an approximate estimation showed a half-life of 70 to 100 hr. (3) The 4S chromatographic peaks of the virus showed an increase in ^{32}P radioactivity after the cells were exposed to this precursor. As expected, ^{32}P radioactivity appeared faster and to a greater extent in those peaks having rapid turnover rates. However, kinetics of ^{32}P appearance did not follow quantitatively what can be predicted by the turnover data using tritiated uridine. This may be due to different precursor pool properties of phosphate for RNA synthesis and also to 3′ hydroxyl end (–CCA) turnover of cellular tRNA.

The results suggest the following: (1) Two populations of RNA species are present in the derived 4S RNA preparations from virus 70S RNA. Those having very slow turnover rates constitute about 80% of the radioactivity and are presumably cellular tRNA's. The others, about 20%, are presumably authentic viral components. (2) Peak 9 of the AKR murine tumor virus is the major viral 4S RNA component. Whether it has primer property for reverse transcription remains to be determined. (3) Since the relative quantity of radioactive precursor incorporation into a macromolecule depends on its turnover rate and size of pool within the cell, an experiment can be designed to enrich the label content of the virus 4S RNA components by employing the fact that they have rapid turnover rates within the cell.

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†American Cancer Society Postdoctoral Fellow.

EFFECTS OF VARIOUS PROTEIN FRACTIONS EXTRACTED FROM SEEDS OF *RICINUS COMMUNIS* ON TUMOR CELLS IN MICE AND IN CULTURES*

Wen-Kuang Yang, Kelly Gregg,† C. W. Wei, and Ti Ho

Recently several reports described antitumor activities of phytotoxin proteins extracted from seeds of *Ricinus communis* (ricin) and of *Abrus precatorius* (abrin). A possible value of these toxic proteins for cancer treatment has been emphasized. However, these phytotoxin proteins are usually isolated and purified on the basis of LD_{50} for normal animals and thus are toxic to normal cells. The undesirable toxic side effect may disqualify them for clinical application. Nevertheless, since it is known that certain plant proteins such as concanavalin A and wheat germ agglutinin show an apparent selective effect on neoplastic cells, it is possible that plant proteins with relatively specific toxicity to tumor cells may exist. In view of this, we made a survey on various protein fractions separated from extract of *R. communis* seeds for toxicity to both normal cells and tumor cells. The results demonstrated that selection of a phytotoxin protein based merely on LD_{50} may exclude certain protein fractions having more antitumor activity.

Crude extracts were prepared from crushed and defatted *R. communis* seeds by acetic acid extraction, ammonium sulfate precipitation, and heat treatment as previously described.[1] Chromatography of the extract in a carboxymethyl-cellulose column separated it into eight protein fractions which we designate as ricins A, B, C, D, E, F, G, and H. Results of comparative studies are summarized as follows:

1. Toxicity for BALB/c mice, by LD_{50} determinations, is in the order of H, G, E, C, F, D, B, and A. Ricin H with an LD_{50} of 0.2 μg per 20-g mouse is the protein studied for antitumor activity by most investigators. Two types of toxicity were noted: an acute type causing death of the mice in 48 hr and chronic type showing death in seven days following cachexia of the mice. Ricin H has both types of toxicity, but ricin C has only the acute type of toxicity.

2. Agglutination activity determined using sheep red blood cells was found mainly in the ricin B fraction (positive at 0.2 μg/ml). Other fractions also showed hemagglutination properties at levels of 5 to 50 μg/ml.

3. Antitumor activity was determined in BALB/c mice carrying transplantable Ehrlich ascites tumor cells and plasma cell tumor MOPC 31C. Except ricin A and B, every ricin fraction at half the LD_{50} amount caused the Ehrlich ascites tumor to regress in a week. However, only mice receiving ricin C showed an apparent cure, while mice receiving other ricin fractions showed recurrence of the tumor in three months. Only ricin C was found to be effective against plasma cell tumors, but the effectiveness lasted only for three weeks. Other ricin fractions, especially ricin H, even decreased the survival time of mice carrying the plasma cell tumors.

4. Inhibitory effect of the ricin fractions on protein and DNA synthesis of cultured cells was examined by using two pairs of normal and tumor cells: HT-1 (MSV-transformed) with golden hamster embryo primary cultures and SV40 transformed BALB 3T3 with untransformed BALB 3T3 (or BALB/c embryo primary cultures). All ricin fractions except ricin C showed more inhibitory activity to embryo primary cultures than to the established cell lines. Ricin C showed higher toxicity to the transformed cells than to the normal cells, but the other ricin fractions appeared to have equal toxicity to both kinds of cells.

5. Comparison was made between morphological alternation of culture cells as a result of ricin C and ricin H administration. Ricin H treatment caused marked abnormal cell morphology such as multinucleated cells and intracellular granule accumulation, whereas ricin C caused only occasional pycnosis of nuclei.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

†Student trainee, Oak Ridge Associated Universities.

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AGE-RELATED DIFFERENCES IN DNA POLYMERASE ACTIVITIES IN SPLEENS OF BALB/c MICE*

R. W. Barton† and Wen-Kuang Yang

The DNA polymerase activities in the spleens of young adult (4-month-old) and aged (30-month-old) BALB/c mice have been examined. Comparisons were made directly, using extracts from cytoplasmic and nuclear fractions. Further resolution of the polymerase activities was obtained by analysis of the multiple enzyme forms from each subcellular fraction by rate zonal sedimentation in sucrose gradients. Both activated calf thymus DNA and poly(rA):poly(dT) were used as template primers for determining DNA polymerase activity in terms of incorporation of [^{3}H]dTMP. The large-molecular-weight DNA polymerase activity (6 to 7S) was equivalent in both young and old spleen homogenates. The small-molecular-weight DNA polymerase activity (3 to 4S) was decreased two- to fivefold in the aged spleens. This decreased activity was reflected in both the cytoplasmic and the nuclear fractions but most markedly in the nuclear fraction. Mixing experiments did not indicate the presence of inhibitors in the polymerase extract from the spleens of the old mice. Extraction of nuclear fractions at various salt concentrations also did not reveal any poor extraction of DNA polymerase activity from the spleen material of the old mice. No apparent alteration in template-primer specificity or in enzyme kinetics (such as K_m of the substrate) was found with the DNA polymerase preparations from the old spleens. Preliminary results indicated that this decrease of small-molecular-weight DNA polymerase activity occurs only in the spleen but not in the liver or kidneys of the old BALB/c mice.

*Research supported by the Virus Cancer Program of the National Cancer Institute.

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ELECTRON MICROSCOPIC STUDIES OF THERMAL DENATURATION OF ISOLATED AND *IN SITU* LIVER NUCLEI FROM BALB/c MICE OF DIFFERENT AGES*

Ching-Yuan Hung† and Wen-Kuang Yang

Isolated and *in situ* hepatic nuclei from livers of 4- and 28-month-old BALB/c mice were subjected to thermal denaturation in the range of 40° to 85°C, with

increments of 5°C, and fixed for electron-microscopic studies of the chromatin structure. Untreated nuclei were also fixed as a control.

The results revealed that there is a striking difference between 40° and 85°C treatments. The granular structures of heterochromatin in the 40°C treatment are essentially similar to the untreated one; the differences in granular size are statistically insignificant. After 85°C treatment, the granular structure totally disappeared and was replaced by 30 to 35 Å fibrous structures. However, it is generally difficult to distinguish the difference between samples treated at adjacent temperatures, that is, between 40° and 45°C, 45° and 50°C, etc. It is apparent that the change is a gradual and slow process. There are no detectable, distinct age-related differences in this transition.

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†Postdoctoral Fellow, NICHD Training Grant for Research in Aging.

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