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Printed in the United States of America. Available from
the Energy Research and Development Administration,
Technical Information Center
P.O. Box 62, Oak Ridge, Tennessee 37830
Price: Printed Copy $8.00 ; Microfiche $3.00

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ORNL-5195
Distribution Category UC-48

Contract No. W-7405-eng-26

BIOLOGY DIVISION
ANNUAL PROGRESS REPORT
For Period Ending June 30, 1976

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

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Date Published: November 1976

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INTRODUCTION

THIS REPORT provides summaries of recent research achievements of the Biology Division's scientific staff. Since our primary sponsor is the U.S. Energy Research and Development Administration (formerly the U.S. Atomic Energy Commission), much of our research is concerned with the biological impact of energy production. These studies include assessments of the carcinogenic and mutagenic effects of radiation and chemical byproducts (or related compounds) from fossil fuels; they also include efforts to characterize cellular repair processes that mitigate damage induced by potential carcinogens and mutagens. Other research in the areas of virology, growth and development, cellular transformation and differentiation, and molecular biology provides insights into mechanisms of regulation of gene expression that in turn further our understanding of carcinogenic and mutagenic events. A variety of basic studies in the areas of genetics, physiology, biochemistry, and biophysics are also supported in the Division and these, reciprocally, support the intellectual framework within which the more applied work is carried out.

A strong program in basic research must be maintained to provide the foundations not only to solve the applied problems of the future but also to provide the flexibility necessary for adaptation to the changing scientific needs of society as we and our funding agencies see them. Whether our research is applied or basic, we attempt to ensure that it is scientifically meritorious so that we may fulfill our obligations to our sponsors, the taxpayers, and humanity.

John B. Storer
Director, Biology Division

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INTRODUCTION

THE MUTAGENESIS AND TERATOGENESIS SECTION consists of the Mammalian Genetics and Development, Comparative Mutagenesis, Mammalian Cellular and Molecular Studies, Genetics of Microorganisms and *Drosophila*, and Development and Reproductive Physiology Groups.

Mammalian Genetics and Development.—The primary concern of this Group is study of the effects of radiation and of chemical mutagens on germ cells, with the aim of not only providing specific risk assessments but also elucidating basic mechanisms of mutagenesis. Other important aspects of the work include investigation of the properties and kinetics of the cell systems in which mutations are being induced, detailed studies of various kinds of mutations and chromosome aberrations that result from the mutagenesis studies, and method development.

This year, the Group made effective use of a battery of tests it had developed or standardized in the past (specific locus, dominant lethal, reproductive capacity, etc.) to determine the possible mutagenic effects of compounds related to energy production (polycyclic hydrocarbons; sodium bisulfite, a metabolite of SO_2) and of compounds to which humans are widely exposed (pyrrolizidine alkaloids; butylated hydroxytoluene). The specific-locus test was also successfully used in a large-scale radiation experiment, the results of which refute Lyon's suggestion that at very low dose rates of gamma irradiation, there may be an upturn in mutation rate per roentgen.

A quick *in vivo* method for scoring presumed somatic mutations, which we had earlier developed for radiation, was explored extensively this year with regard to its value in predicting the induction of germinal point mutations by chemicals. Results indicate the potential usefulness of the method as a quick and cheap prescreen, provided care is taken to avoid possible pitfalls in the scoring of positive events.

Method development also included modification of the specific-locus method to encompass two loci of the mouse that control hemoglobin. It is hoped that comparison of mutant and normal gene product will, in the case of this thoroughly known protein, permit conclusions concerning the nature of the genetic damage. Some of the mutants resulting from this project, as well as a mutant from an unrelated mutagenesis experiment, are already providing important animal models for human genetic disease.

The Group is also continuing its study of the cytogenetics of male sterility. In addition, detailed cytogenetic studies of unusual chromosome aberrations discovered in the course of various experiments (tandem duplication, small deficiencies, X-autosome translocations) are contributing to a basic understanding of the mammalian genome.

A line of research we began a few years ago, and reactivated this year, concerns the phenotypic effects of mutations induced anywhere in the genome. Using the skeleton as the system for study, a number of dominants with pleiotropic effects were characterized and are now being further investigated. This research into the nature of the actual damage expressed in descendants of irradiated populations shows promise for filling what had heretofore been the most serious gap in the information necessary for adequate estimation of genetic hazards of radiation in man.

Unscheduled DNA synthesis (UDS) in germ cells, taken to be DNA repair, has been extensively explored this year. The capacity of a compound to reach and damage the DNA of mammalian germ cells *in vivo* can be measured by this technique. UDS, which has now been induced by a number of chemicals, was shown to be affected by strain and to be damaged by X-ray treatment. UDS was found not to take place in stages following the midspermatid, even in the male pronucleus, and there is no evidence that its occurrence affects induced dominant-lethal or specific-locus frequencies.

The importance to the interpretation of mutagenic mechanisms of understanding the nature of cell populations within which genetic changes are being induced has been recognized by us for some time. This year, studies on females showed that the point at which a dramatic shift in mutational response of the oocyte occurs probably coincides with initiation of zona pellucida formation. Studies on males showed that cell-selection effects on mutation frequency are not drastically greater at 600 R than at 500 R. Both of these findings are in conflict with alternative interpretations of our mutation-rate data that have been made in the past year by Abrahamson and Wolff. —Another important study on cell populations carried out this year concerns the timing of events between mating and the first cleavage in normally ovulated eggs. This is basic to an understanding of stages we have shown in the past to be particularly sensitive to chromosome loss, which are now being demonstrated by our Group to have significance in the "fixation" of genetic events initiated much earlier.

Comparative Mutagenesis.—The major goal is the development and use of a tier-system approach to testing a variety of materials, especially those resulting from fossil-fuel technology, for mutagenic activity. Comparisons between test systems are an important part of the work in order to check the generality of the results and the validity of the tier-system approach. Information obtained will be used to help other groups in the Section set priorities for testing materials.

Mammalian Cellular and Molecular Studies.—Several research programs are included which employ mammalian cells to study chromosomal aberration induction, mutation induction, and repair processes. These investigations are developing mammalian cellular test systems for potential mutagens and providing basic information on the cellular and molecular processes involved in the interaction of such cells with potentially hazardous agents. Cooperation exists not only with other units within the Section but also with the two Sections in the Division.

Genetics of Microorganisms and Drosophila.—This Group makes use of genetically favorable lower organisms to study basic processes involved in the behavior and segregation of chromosomes at meiosis, in mutagenesis, and in DNA repair. Such processes are fundamental to an understanding of how potentially hazardous agents may cause various kinds of genetic damage. They can frequently be studied most effectively in these genetically well-defined systems.

Development and Reproductive Physiology.—This Group presently concentrates most of its effort on the earliest stages in the development of the amphibian, namely, the formation and early development of the oocyte. This is a model case for the important early stages in development and for the ways in which various environmental agents can influence these stages. It therefore provides basic information and concepts for the teratogenesis part of the program.

SPECIFIC-LOCUS MUTATION FREQUENCIES IN MOUSE SPERMATOGONIA EXPOSED TO GAMMA RADIATION AT VERY LOW DOSE RATES

W. L. Russell and E. M. Kelly

One purpose of these experiments was to increase the reliability of measures of mutation frequency at the lowest dose rate practicable and at a level which causes no spermatogonial stem cell killing, thereby removing the possibility of an effect on the observed mutation rate through differential cell killing. Another purpose was to help resolve divergent views on the mutational response at low radiation levels. At one extreme, Lyon *et al.*,[1] citing our data and theirs, have suggested that at dose rates below about 0.03 R/min there may be an actual increase in mouse spermatogonial mutation frequency as dose rate is lowered. At the other extreme, Newcombe[2] suggests a possible "beneficial" effect of low levels of radiation in trout and *Drosophila*, supporting the view,

attributed to Abrahamson, that lower doses of radiation stimulate repair of part of the naturally occurring genetic damage.

The results from the current experiments support neither of these extreme views but are in agreement with our original interpretation that, although mutation frequency decreases with lowered dose rate down to about 0.8 R/min, further reduction in dose rate gives no further change in mutation frequency. To date, a 300-R dose (^{137}Cs source) has produced, at a dose rate of 0.0007 R/min, 11 presumed mutations in 42,020 offspring, and, at 0.005 R/min, 19 presumed mutations in 74,842 offspring. The frequencies are highly significantly above control, thus negating the possibility of a "beneficial" effect, and they differ by less than one mutation from the frequencies expected on the null hypothesis of no difference between the two groups.

The most important application to human hazards is that the results give no support to the view of Lyon *et al.*[1] that the genetic risks at very low dose rates may be much higher than had been estimated on the basis of our earlier work. Other details of the experiment relevant to this point have been discussed in an earlier report.[3]

Some of the presumed mutations have not yet been confirmed by breeding tests, but no major change is expected in the total mutation frequencies given here.

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MOUSE SPECIFIC-LOCUS TEST OF POSSIBLE GENETIC HAZARD FROM POLYCYCLIC HYDROCARBONS ASSOCIATED WITH NONNUCLEAR ENERGY TECHNOLOGIES

W. L. Russell

A specific-locus test has been started on benzo-[*a*]pyrene (BP) as part of the Mammalian Genetics Group's coordinated investigation of the possible mutagenicity of polycyclic hydrocarbons that might be a human hazard as the result of coal liquefaction and gasification processes. BP has already been found by Generoso *et al.*[1] to cause dominant-lethal mutations in mice. It has also been shown by Russell[2] and by Davidson and Dawson[3] to produce somatic mutations in the mouse test developed by Russell.[4]

It was, therefore, obviously important to find out if gene mutations and small deficiencies could be detected in treated germ-cell stages. Our standard specific-locus test was used to score for mutations at seven loci in the offspring of male mice given a single intraperitoneal (ip) injection of 500 mg/kg of BP. To date, no mutations have been found in 1387 offspring from germ cells exposed in postspermatogonial stages, while in 5461 offspring from treated spermatogonial stages, one mutation, at the *p* locus, has been observed. The dose used is, therefore, clearly not as mutagenic as a dose of 600 R of X rays, but more data are obviously needed for quantification.

A pilot specific-locus experiment has been started on another polycyclic hydrocarbon, 7,12-dimethylbenz[*a*]anthracene (DMBA), using an injected dose of 10 mg/kg. Too few offspring have been obtained so far for any meaningful conclusions.

1. W. M. Generoso, K. T. Cain, and S. W. Huff, this report.
2. L. B. Russell, this report.
3. G. E. Davidson and G. W. P. Dawson, *Mutation Res.* **38**, 151–154 (1976).
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SPECIFIC-LOCUS TEST OF THE MUTAGENICITY OF SULFUR DIOXIDE IN MICE

W. L. Russell and E. M. Kelly

The rationale for this project and the details of the experimental procedure were described in last year's annual progress report.[1]

The number of offspring now scored, in our standard specific-locus test, from males that had received a total of 8000 mg/kg of sodium bisulfite injected over 20 days, is 17,899. No mutations have been observed. Thus there is no evidence of mutagenicity, and there is further confirmation of our earlier conclusion, based on the upper 95% confidence limit of the observed zero mutation frequency, that if there is any genetic hazard at all in man from sulfur dioxide in the atmosphere, it is almost certainly not large.

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A TEST FOR MUTAGENICITY OF DIETHYLNITROSAMINE USING THE MOUSE SPECIFIC-LOCUS METHOD

W. L. Russell

In investigating the basic nature of chemical mutagenesis in mice, diethylnitrosamine (DEN) is a compound that presents several interesting features, perhaps the most cogent of which is that in *Drosophila* it fails to break chromosomes, giving no dominant lethals, for example, but is a potent mutagen in the recessive-lethal test for gene mutations. Since it also fails to induce dominant lethals in mice, it is important to find out if it induces gene mutations in the mouse specific-locus test.

Two experiments have been run, the first with 150 mg/kg and the second with 100 mg/kg, injected intraperitoneally. The offspring obtained to date are all from treated postspermatogonial stages, primarily spermatozoa and spermatids, the stages most sensitive in *Drosophila*. In a total of 1702 offspring, 1105 from the first dose and 597 from the second, no mutations have been observed. More data are being collected.

RELATIVE INEFFECTIVENESS OF INTRAMUSCULAR COMPARED WITH INTRAPERITONEAL INJECTION OF HYCANTHONE ON X-CHROMOSOME LOSS IN FEMALE MICE

W. L. Russell, P. R. Hunsicker, E. M. Kelly, C. M. Vaughan, and G. M. Guinn

In a preliminary comparison of the effect of different routes of administration of hycanthone on litter size in offspring conceived in the first week after treatment of female mice, it was found that only ip injection gave a marked reduction in litter size.[1] It was obviously important to find out whether similar results would be obtained with a more definitive measure of genetic damage, namely, the X-chromosome-loss test. Since the drug is administered intramuscularly in humans, this route of administration was tried in the mouse for comparison with earlier results on X-chromosome loss induced by ip injection.[2]

The test procedures and dose of hycanthone (150 mg/kg) were identical with those used for ip injection.[2] In a total of 1928 offspring conceived in the first week after intramuscular (im) injection of females, only seven X0s have been observed. Although this is clearly greater than the control spontaneous frequency of 19 X0s in 32,642 offspring found in our total series of experiments, it is much less than the 19 in 1351 observed after ip injection.[2] When the control frequency is subtracted, the ratio of the induced rates for im and ip injection is 0.23.

These results, in combination with our finding that X-chromosome losses occur only in offspring conceived shortly after treatment,[2] tend to confirm our earlier conclusion[1] that the mature oocytes located in follicles on the surface of the ovary may be especially subject to damage from a compound injected directly into the peritoneum. This provides a particularly sensitive response that may be of general use in mutagenicity testing.

With regard to the application of the results to human risk, it should be emphasized that the genetic risks estimated even on the basis of ip injection are very small, if not zero, at the therapeutic dose and, therefore, are presumably acceptable for an effective antischistosomal drug. Thus the findings on im injection, the route of administration used in humans, simply reveal an extra safety factor.

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1. P. R. Hunsicker and W. L. Russell, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, p. 125.
2. W. L. Russell, P. R. Hunsicker, E. M. Kelly, C. M. Vaughan, and G. M. Guinn, *Biol. Div. Annu Prog. Rep., June 30, 1975*, ORNL-5072, pp. 123–125.

THE IMPORTANCE, IN CHEMICAL MUTAGENESIS RESEARCH, OF MEASURING TRANSMITTED MUTATIONS INDUCED IN MAMMALIAN GERM CELLS

W. L. Russell

A review of our recent chemical mutagenesis research in mice reveals that a prominent feature of it is the unpredictability of the results. A chemical that produces only one type of mutational lesion, and only in dividing cells, in bacteria, causes another type of lesion, and only in nondividing cells, in mice. The high ratio of gene mutations to chromosomal aberrations induced by some chemicals in *Drosophila* is the opposite of what is found for the same chemicals in mice. Within the mouse itself, results in one sex or cell stage are often of little or no predictive value as to what will happen in the other sex or other cell stages. The effect of one chemical may be quite different from that of a very closely

related compound, and neither of them may parallel the effects of radiation. These findings indicate that attempts to predict mammalian results on the basis of such simplistic concepts as the DNA content relative to that in lower organisms are doomed to failure. Similarly, the view that the rem-equivalent-chemical (REC) determined for a particular chemical in a lower organism will be generally predictive of the REC in mammals, and therefore useful in risk estimation, is already shown to be erroneous. All of these findings indicate that for some time to come there will be no substitute for the direct measurement of transmitted mutations induced in mammalian germ cells.

VALIDATION OF THE "SPOT TEST" FOR THE DETECTION OF POINT MUTATIONS IN THE MOUSE

L. B. Russell, C. S. Montgomery, and M. G. Hayes

The induction of somatic mutations involving specific loci in the mouse was first demonstrated by us in 1957.[1] In a somatic mutation experiment *in vivo*, each individual observed represents a large number of cells in which a mutation could have occurred, instead of a single cell as is the case in a germinal mutation study. Such an experiment thus, theoretically, combines some of the advantages of a cell-culture system with those of an *in vivo* system. The major disadvantages are that the genetic nature of the presumed mutational events cannot be clearly established and that, for various reasons, the mutation rate cannot be accurately determined. In our 1957 X-ray study, we developed a method that could serve as a useful qualitative tool in the detection of mutational and other events induced in embryonic precursors of hair pigment cells. Such events result in spots of altered color in the fur, and the method has subsequently been named the "spot test."[2]

The spot test can be useful in prescreening for mutation induction in mammals to the extent to which it is predictive of germinal "point" mutations (gene mutations and small deficiencies), particularly those occurring in germ-cell stages of interest for risk considerations. We have therefore embarked on a spot-test study of those chemicals with which large-scale specific-locus tests in germ cells have been conducted in the past or are now being conducted at our laboratory, with a view to determining whether or not parallelisms can be demonstrated. The loci chosen for our study are the same ones that have been and are being used in the germinal experiments.

Materials and methods.—Embryos heterozygous for readily detectable markers are treated *in utero* with the putative mutagen. They are raised to full fur growth and examined for mosaic patches. Since one of the stocks used in our earlier work[1] has become extinct, the heterozygous embryos are now derived from a cross of C57BL females with T males. The C57BL subline used in these experiments was chosen, after preliminary study,[3] on the basis of giving the lowest spontaneous frequency of white belly spots. Following the technique established earlier,[1] mutagen treatment is applied on day $10^1/_4$ after conception. The coat is examined on postnatal day 12 and again at weaning.

The high sensitivity of the $10^1/_4$-day mouse embryo to environmental insults limits the dose of chemical that can be used. As we began our study, little or nothing was known concerning the embryo toxicity of the compounds we had decided to test, and in some cases our initial doses were too high. The results presented here are limited to those doses that allowed sufficient survival for the scoring for spots. Doses range from $^1/_7$ to $^1/_2$ of those used in germinal mutation-rate experiments. All compounds were dissolved in Hanks' balanced salt solution (HBSS) except for hycanthone methanesulfonate, which was dissolved in water, and BP in corn oil. The mode of administration was by ip injection in all cases, except for a portion of the hycanthone experiment which included an im series. Abbreviations will be used as follows: EMS, ethyl methanesulfonate; MMS, methyl methanesulfonate; TEM, triethylenemelamine; MC, mitomycin C; BP, benzo[*a*]pyrene; HC, hycanthone methanesulfonate; DEN, diethylnitrosamine.

Results.—Altogether, 1579 offspring have been born in the chemical spot-test experiments to date. Results are only briefly summarized in Table 1. A more detailed report for one of the chemicals appears elsewhere,[4] and others will be reported separately as they are completed.

It may be noted that for the doses here reported, average litter sizes and survival to 12 days have been normal, or near-normal, everywhere except for the higher dose of TEM, and possibly for MC. Teratogenic effects were observed from TEM, MC, and HC; and these same compounds give clearly increased incidence of white belly spots. With regard to presumed somatic mutations ("other spots"), three of the compounds (TEM, MC, BP) are clearly positive; another one, DEN, probably so; and two, HC and EMS, are questionable.

Conclusions.—(*a*) Comparison between presumed somatic mutations and spermatogonial mutation

Table 1. Effects of several compounds injected at day 10¼ after conception in matings of C57BL ♀♀ × T ♂

Agent	Dose (mg/kg)	Number born	Average litter size	Surviving to 12 days (%)	Abnormal morphology at birth (%)	White belly spot (%)	Other spot (%)
EMS	50	93	5.2	88.2	0	3.7	(1.2)?
MMS	50	69	6.9	87.0	0	0	0
TEM	0.8	126	6.3	32.5	91.1	22.0	2.4
TEM	0.5	149	6.5	80.5	47.7	17.5	3.3
MC	2	91	5.7	72.5	5.5	15.2	3.0
BP	150	126	6.3	77.8	0	5.1	5.1
BP	100	180	7.5	77.2	0	5.0	2.2
HC	30	228	6.5	91.2	0.4	13.5	0.5
HC	20	57	5.7	98.2	0	8.9	(1.8)?
DEN	30	148	6.2	89.9	0	2.1	1.5
DEN	20	79	5.6	88.6	0	2.9	0
Control	-	84	6.5	84.5	0	2.8	0
Continuous mating	-	121	4.3	97.6	0	1.7	0

rates at the same loci is beginning to show evidence of correlation, although data on one side or other of the comparison, or on both, obviously need to be augmented. Germinal mutation-rate studies give clearly positive results for spermatogonia in the case of TEM[5] and MC[6] and clearly negative results in the case of EMS,[7] MMS,[7] and HC.[8] BP and DEN are now in the process of being tested, and the former has already given some indication that the mutation rate will be above control frequency.[9] Presumed somatic mutations thus show good parallelism with spermatogonial results in the positive cases of TEM, MC, and probably BP, and in the negative cases of EMS and MMS (although Fahrig[2] has reported a positive spot test for these compounds). The HC spot-test results are equivocal, and the DEN comparison awaits the spermatogonial data.

(*b*) It appears that those compounds that have teratogenic effects also yield a clearly increased incidence of white belly spots. No clear increase is found for compounds that do not produce teratogenic effects. We suggested earlier[1] that the white belly spots were the result of pigment-cell depletion, presumably caused by cell killing. This appears to be confirmed by the finding of correlation with teratogenic effects, since the latter probably also result from cell killing.

(*c*) There is no very clear correlation between increase in white belly spots and positive results for presumed somatic mutations. For example, BP produces the highest incidence of the latter but only a very questionable increase, at best, in the former; while HC, which yields a very clear increase in white-belly-spot incidence, may or may not produce presumed somatic mutations.

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PRESUMED SOMATIC MUTATIONS INDUCED IN THE MOUSE BY AN AROMATIC HYDROCARBON

L. B. Russell and M. G. Hayes

It is generally predicted that aromatic polycyclic hydrocarbons will be prominent among products of various methods of energy production, including those that involve coal liquefaction and gasification. One such hydrocarbon, BP, is currently being tested in a number of genetic systems in the mouse[1,2] and we have now explored its effectiveness in the "spot test" developed by us earlier[3] and briefly described elsewhere.[4] This test detects presumed somatic mutations *in vivo*.

C57BL and C57BL/self females were mated to T males and injected on the morning of the tenth day following observation of a vaginal plug with BP in Mazola corn oil. Offspring were checked at birth for survival and external morphology. At approximately 12 days of age, the fur was carefully scrutinized for the possible presence of nonblack patches or white belly spots. The former may be indicative of somatic mutations, and an increased incidence of the latter probably indicates cell killing.[3,4]

The first BP dosage level we tried, 200 mg/kg, produced very discouraging results: from 50 presumably pregnant females injected, only four scorable offspring were recovered (Table 2). Several of the females were experiencing obvious difficulties with parturition, and some, in fact, died in the process of giving birth. Dissection of a subset of these females on the day after the expected delivery date indicated that neither the number of uteri containing full-sized placentae nor the average number of placentae per uterus were affected (Table 2). Several dead, full-grown fetuses were still present; others had obviously been recently expelled and eaten.

In a small pilot series, five females each were injected with 200, 150, or 100 mg/kg of BP in a smaller volume of oil than used in the first run. At 200 mg/kg, survival per female treated was almost ten times better (Table 2), indicating that the volume of oil may have played a large part in the parturition problems. However, survival was still far from good at 200 mg/kg, even with the smaller volume of oil, and we therefore expanded the two lower dose series, where litter size turned out to be normal and survival to 12 days nearly so.

Somewhat to our surprise, no malformations were observed in the newborns. This confirms our results (unpublished) from BP treatment of other embryonic stages. The incidence of white belly spots was not increased (control frequency, 4.7%). Both of these findings make it unlikely that BP is as effective in cell killing as are certain other compounds.[4] On the other hand, the results were clearly positive with respect to induction of other spots that are presumed to have resulted from somatic mutation (or somatic crossing-over, or chromosome loss).[3] It may therefore be concluded that BP is mutagenically active in mammals and that it may induce point mutations.

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EXPLORATION OF OPTIMUM EMBRYONIC STAGE FOR TREATMENT IN THE "SPOT TEST" FOR THE DETECTION OF SOMATIC MUTATIONS *IN VIVO*

L. B. Russell, C. S. Montgomery, and M. G. Hayes

Using X irradiation, we had earlier developed a method for the detection of somatic mutations *in vivo*[1] which is presently receiving extensive use in our laboratory[2] and elsewhere[3] in the testing of putative chemical mutagens. One of the parent stocks, NB, used in the original X-ray experiment has become extinct and has been replaced by the noninbred T stock; and the other, C57BL, may have undergone some genetic change in the past two decades. It was therefore deemed necessary to reexplore with X rays the matter of embryonic stage(s) most optimum for treatment. It is obviously of advantage to treat embryos at a time when the number of pigment precursor cells is already large. However, if this number is too large, the spot resulting in the adult fur will be too small to be perceptible in every case.

Table 2. Effects of BP injection on day 10 after conception in matings of C57BL ♀♀ × T ♂♂

Dose (mg/kg)	Milliliters per female (approx)	No. of females treated	No. of litters	No. born	Average litter size	Surviving to 12 days	Abnormal morphology at birth	White belly spot	Other spot
200	0.35	50	9	22	2.4	4(18.2%)	0?	—	
		[incl. 11 dissected, 9 with 61 impl. 6.8]							
200	0.2	5	3	9	3.0	3 (33.3%)	0	0	0
150	0.2	34	20	126	6.3	98 (77.8%)	0	5 (5.1%)	5(5.1%)
100	0.2	34	24	180	7.5	139 (77.2%)	0	7 (5.0%)	3(2.2%)

Since the stage found in earlier work to be optimal was day $10^1/_4$ after conception, we include it and the two adjacent stages, days $9^1/_4$ and $11^1/_4$, in the present experiment. Further, since the maternal genotype may exert some influence over time relations in intrauterine development, all three of these stages were explored in each of the two reciprocal crosses. Results for 100-R irradiation are shown in Table 3.

With regard to nonblack patches (other than white belly spots), which may be indicative of induced somatic mutations,[1,2] irradiation on day $9^1/_4$ gave negative results. Day $10^1/_4$ was positive, as in earlier work with X rays[1] and current work with chemicals;[2] but day $11^1/_4$ turned out to be equally suitable. The increase in white belly spots, thought to be indicative of cell killing, was clearly greater on day $11^1/_4$ than on day $10^1/_4$. Since previous results[1] from day $11^1/_4$ were extremely fragmentary, it is not known whether the present data represent a change due to different genetic constitution of the embryos. The sensitivity pattern is the same in the two reciprocal crosses. One surprising finding is that the incidence of presumed somatic mutations from irradiation on day $10^1/_4$ is apparently lower now than it was in 1957.

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3. R. Fahrig, *Mol. Gen. Genet.* **138**, 309–314 (1975); G. E. Davidson and W. P. Dawson, *Mutat. Res.* **38**, 151–154 (1976).

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RADIATION-INDUCED MUTATIONS AT THE MOUSE HEMOGLOBIN LOCI

L. B. Russell, W. L. Russell, R. A. Popp, C. M. Vaughan, and K. B. Jacobson

Last year we reported on an experiment designed to detect new radiation-induced mutations at the hemoglobin loci.[1] Since that time, further testing of some of the mutants has resulted in some alterations of the original diagnosis of their nature. The now complete results[2] are presented in Table 4.

All hemoglobin variants found were detected on the basis of electrophoretic pattern, phosphate solubility, and crystal pattern. Results obtained by electrophoretic analysis are illustrated in Fig. 1. Blood that had shown abnormality by these criteria was used for further chemical analysis of hemoglobin.

Five hemoglobin variants were found among the mutants. In three, the genetic contribution from the irradiated father was not expressed with regard to the α chain; one carried a tandem duplication (the first such aberration reported in the mouse)[3] involving *Hbb*; and one probably resulted from double nondisjunction of chromosome 7. This last was originally [1] reported to carry a mutation that causes β^{SEC} inactivation or deletion. Subsequent work indicates that the variant F_1 (♀ 732) appears, instead, to carry two chromosome 7's derived from its 101

Table 3. The effects of X rays applied at various embryonic stages in the production of coat color spots

Dose (R)	Stage at irradiation (day)	Number born	Average litter size	Surviving to 12 days (%)	Abnormal morphology at birth (%)	White belly spot (%)	Other spot (%)
C57BL♀ × T♂							
100	$9^1/_4$	68	5.7	92.6	0	6.3	0
100	$10^1/_4$	62	5.2	98.4	0	3.3	1.6
100	$11^1/_4$	66	6.6	87.9	0	13.8	1.7
T♀ × C57BL♂							
100	$9^1/_4$	121	6.4	89.3	0	20.4	0
100	$10^1/_4$	85	5.3	96.5	0	4.9	2.4–3.7
100	$11^1/_4$	90	6.9	96.7	1.1	17.2	2.3
C57BL♀ × NB♂ (1957 data)							
100	$10^1/_4$	255	7.5	92.2		0	11.1
150	$10^1/_4$	34	8.5	61.8		0	4.8

Table 4. Summary of variants recovered in offspring of 101 × SEC or SEC × 101 crosses in which one parent has been irradiated

Proband	Irradiated parent	Irradiated germ-cell stage	Variant phenotype				Heritability	Probable cause
Blood character variants								
A. Hemoglobin variants			Electrophoretic pattern	Solubility[a] (*A*)	Crystal pattern	Reticulo-cyte %		
♀ 27	SEC ♂	Spermatids	Diminished fast band	~0.55	Abnormal	6	Yes	Mutation or deficiency causing nonexpression of α chain
♂ 72	SEC ♂	Spermatids	Diminished fast band	~0.55	Abnormal	3	?[b]	
♂ 352	SEC ♂	Spermatogonia	Diminished fast band	~0.55	Abnormal	2	Yes	
♀ 86	SEC ♀	Mature oocytes	Diminished fast band	0.15	Abnormal	1	Yes	Tandem duplication involving *Hbb*
♀ 732	SEC ♂	Early spermatocytes	Diminished slow band	0.30	Abnormal	4	No	Double nondisjunction of chromosome 7
B. Anemic variants								
♂ 81	SEC ♂	Spermatogonia	Normal	Normal	Normal(?)	10, 6	No	Environmental
♂ 122	SEC ♂	Spermatogonia	Normal	Normal	Normal	11, 14	No	Environmental
♀ 585	101 ♂	Spermatogonia	Normal	Normal	Normal	N.t.	?[b]	?
♂ 779	101 ♂	Spermatogonia	Normal	Normal	Normal	N.t.	No	Environmental
Specific-locus and miscellaneous variants								
♂ 152	101 ♂	Spermatozoa or spermatids	Coat color				Yes	*b*-Locus allele, viable
♀ 258	101 ♂	Spermatogonia	Coat color				Yes	*d*-Locus allele, prenatally lethal
♂ 281	101 ♂	Spermatogonia	Coat color				Yes	*c*-Locus allele, viable
♂ 1015	101 ♂	Spermatogonia	Coat quality				Yes	*Ca*-Locus, dominant
♂ 59	101 ♂	Spermatozoa	Scattered white hairs				Yes	New (?) dominant
♂ 98	101 ♂	Spermatozoa or spermatids	Dark coat, lop-ear				?[b]	?

[a]Measured as absorbance at 575 nm. *A* is 0.01 for SEC, 0.30 for 101, and 0.45 for the F_1.
[b]Proband sterile.

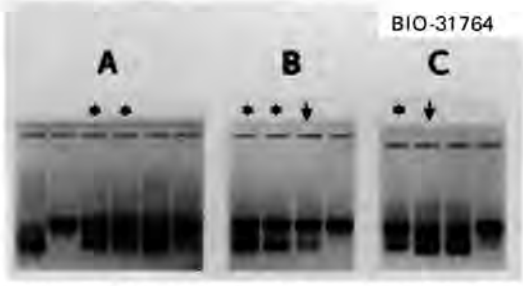

Fig. 1. Electrophoretic patterns of mouse hemoglobins. Normal F_1's from the cross between 101 and SEC strains are indicated by asterisks (*), presumed mutants by arrows. (A) Patterns characteristic of parental and F_1 hemoglobins and simulated deletion mutants. From left to right: 101, SEC, F_1, F_1, F_1-101 mixture (equal parts), F_1-SEC mixture (equal parts). (B) Pattern from mutant hemoglobin characterized by diminished fast band. From left to right: normal F_1, normal F_1, mutant ♂72, SEC. (C) Hemoglobin variant, ♀732, giving diminished slow band and probably resulting from double nondisjunction. From left to right: normal F_1, ♀732, 101, SEC.

mother and no chromosome 7 from its irradiated SEC father.

The finding that major chromosome aberrations (tandem duplication and double nondisjunction) can mimic hemoglobin mutations indicates the need, in similar experiments, to follow F_1 screening with thorough cytogenetic analysis.

The variants in which there is nonexpression of the α chain may be the result of small deficiencies or of faults in transcription or translation and may provide extremely valuable animal models for human thalassemia. They are being analyzed further to determine their nature. Mutation rates based on these three are not out of line with earlier specific-locus results, although confidence limits are still wide.

No cases of base-pair substitution were detected in the present series. However, it should be noted that only one of the mutants was derived from the

irradiation of spermatogonia, the cell stage that yields the lowest frequency of chromosomal aberrations. Expansion of the experimental series should provide more critical information in the future.

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GENETIC AND CYTOLOGICAL STUDIES WITH THE FIRST TANDEM DUPLICATION IN THE MOUSE

L. B. Russell and N. L. A. Cacheiro

The first and thus far the only tandem duplication in the mouse[1] was discovered in the course of a search for radiation-induced hemoglobin variants.[2] This duplication, which includes at least the *Hbb*, the *Mod-2*, and the *c* loci of chromosome 7, has this year been propagated in a stock, and some of its properties have been studied.

Animals heterozygous for the duplication are of considerably reduced body size. Weaning-age comparisons carried out for 71 males and 54 females indicate that the weight of duplication-bearing mice is only 58.7% of that of like-sexed normal litter mates. There is no significant difference between males and females in the relative degree of weight reduction. Even in the absence of other markers, duplication-bearing segregants can thus be recognized at weaning with a fair degree of reliability.

Data from crosses of $Dp(c^{ch})/c \times c/c$ or $Dp(c^{ch})/+ \times c/c$ indicate that only 29%, instead of the expected 50%, of the progeny carry the duplication by the time coat color can be diagnosed, indicating a survival level to that age of only about 40%. Duplication-bearing females probably also make relatively poor mothers, as judged by the fact that their average litter size in crosses to c/c is only 3.5, as compared with 5.5 for similar crosses of duplication-bearing males. Thus far, attempts to recover viable duplication homozygotes have failed.

Crossing-over within one of the duplicated segments occurs with low, but measurable, frequency. Crossovers occurring between the two duplicated *c* loci have been observed with a frequency of about 1.5% in 400 segregants. Recombination between the two duplicated *Hbb* loci has also been found. The occurrence of crossing-over makes it possible to introduce other markers into the duplication.

Crosses have been initiated to combine the duplication with two of the 30 *c*-locus deficiencies that we have characterized in complementation studies.[3] As a first step, we wish to determine whether the presence of a deficiency in one chromosome 7 will "rescue" the animal from the size reduction caused by the duplication in the other chromosome 7. Indications to date are that this will not be the case. We have also initiated crosses to combine the duplication with various T(X;7)'s, with the idea of determining whether the combination will rescue translocation males from their usual total sterility. Since offspring from such crosses must undergo complex testing, our results on this point are still equivocal.

Cytological analysis of the duplication by the use of quinacrine banding indicates that the duplicated portion extends from about the middle of the D band to about one-fourth of the way along the F band (Figs. 2 and 3). ▶

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2. L. B. Russell *et al.*, this report.
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DISCOVERY OF AN ANIMAL MODEL FOR A HUMAN GENETIC DISEASE, ORNITHINE CARBAMOYLTRANSFERASE DEFICIENCY

L. B. Russell and R. deMars*

Sparse-fur (*spf*), an X-linked mutation of spontaneous origin discovered at our laboratory in the course of the follow-up from a mutation-rate experiment,[1] is expressed externally in hemi- and homozygotes by the late, poor growth of fur. Expression in heterozygous females is rare.

In the course of linkage experiments involving X-autosome translocations and various X-linked mutations, we had occasion to dissect large numbers of males for the purpose of recording testis weights [low testis weight being diagnostic for the T(X;A) genotype]. During this procedure, we happened to notice the occasional occurrence of bladder stones in some males. Subsequently, the presence or absence of bladder stones was carefully recorded for one batch

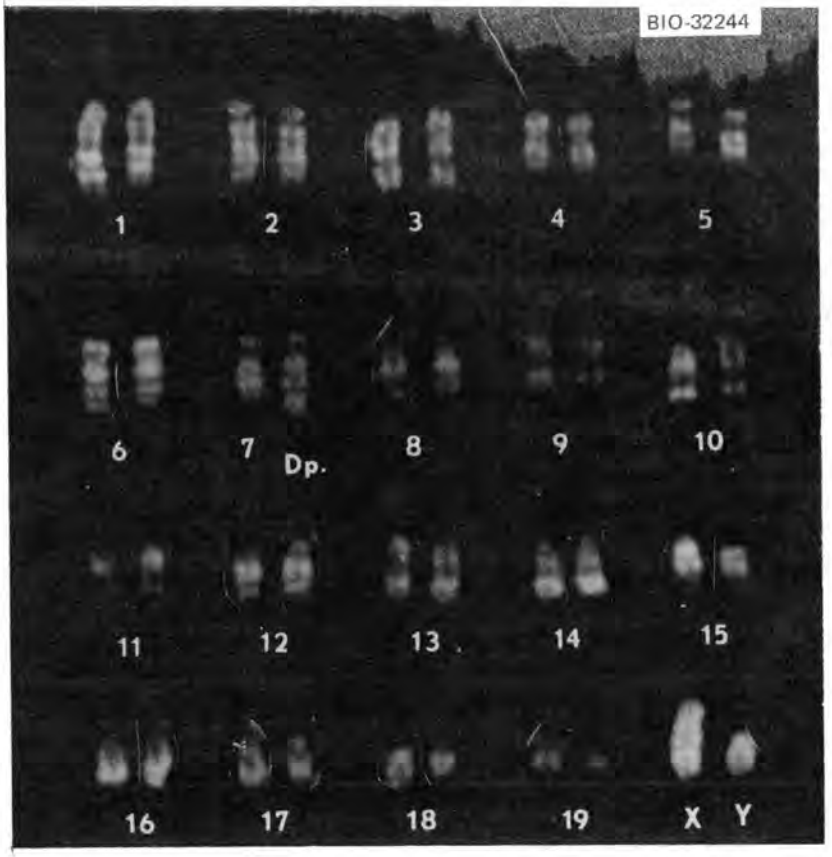

Fig. 2. Quinacrine-banding karyotype of a male heterozygous for a tandem duplication in chromosome 7.

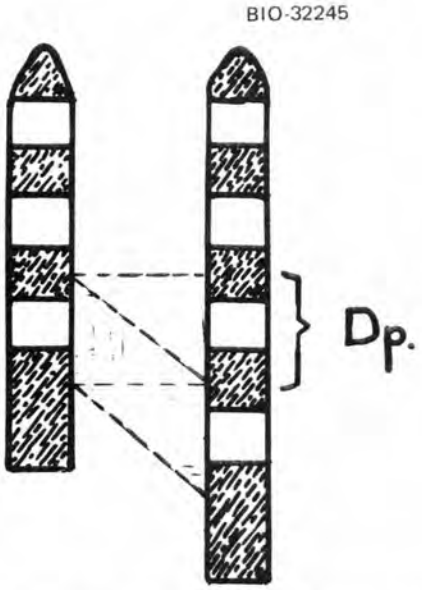

Fig. 3. Diagrammatic representation of chromosome 7 indicating the duplicated portion of the chromosome.

of males and was found to be distributed as shown in Table 5. Each of the groups of males shown was, except for its X-linked marker, of widely heterogeneous genetic background. It thus appeared highly probable that the *spf* gene itself was responsible for the presence of bladder stones.

The stones were shipped to Dr. R. deMars, whom we knew to be looking for an animal model for the Lesch-Nyhan syndrome of man, in which deposits of uric acid are found in various parts of the body. His studies, however, indicated that the bladder stones are composed mostly of orotic acid rather than uric acid.

A follow-up study on *spf*/Y, *spf*/*spf*, and *spf*/+ mice and their normal litter mates indicates that *spf*/Y and *spf*/*spf* animals have an abnormal form of liver ornithine carbamoyltransferase (OCT).[2] The apparent net deficiency of OCT in *spf*/Y males is about 90%, which may account for the accumulation of orotic acid and other pathological traits of these mice. Mixtures of normal and abnormal OCT

Table 5. Distribution of bladder stones in males of various genotypes

Genotype of male	No. of males		Percent with stones
	With bladder stone(s)	Without bladder stones	
spf/Y	32	11	74.4
Ta/Y	0	9	0
+/Y	0	118	0

▲

activities are found in the livers of *spf*/+ females, and the large variance in the proportion of OCT indicates that the gene is subject to single-active-X control.

Since mice having the *spf* mutation on certain genetic backgrounds have greatly reduced fitness, *spf* mice may provide information about alleviating the consequences of OCT deficiency in humans. Using *spf*/+ mice, it may also be possible to develop reliable methods for identifying heterozygous human females.

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CYTOLOGICAL STUDIES OF CHROMOSOME INACTIVATION IN THE MOUSE, USING A NEW X-AUTOSOME TRANSLOCATION

N. L. A. Cacheiro and L. B. Russell

In 1975 we presented a preliminary report[1] of a new T(X;A). This translocation, R13, involves the X chromosome and chromosome 12. Autoradiographic studies at that time, using the early pulse-labeling technique, indicated some sort of nonrandom inactivation, with 72% of the cells showing one of the two translocated chromosomes, 12^X, to be the unlabeled one.

Because only 25 cells were analyzed in this original study, and since some difficulties were encountered in the identification of the translocated chromosomes, we repeated the experiment, this time using kidney tissue cultures from an 18-day embryo. From a total of 58 cells studied, 68.9% showed the 12^X to be preferentially unlabeled. This departure from randomness is significant, with a P value <0.01, and is in an opposite direction from that observed for T(X;16)16H, in which both translocated chromosomes are almost always active, whereas the normal X is inactive in all cells.

In the case of T(X;16)16H, where nonrandomness is nearly complete, translocation females heterozygous for X-linked markers do not show the usual variegated phenotype but express only the allele present in the translocated X. In the case of T(X;12) R1 13, one would expect certain markers of the normal X to be preferentially expressed, namely, those that are carried in portions of the X corresponding to the X-chromosome segment of 12^X (as opposed to that of X^{12}). We have made T(X;12) heterozygous with normal X's bearing either *spf*, *Ie*, or *Gs*. The marker nearest to the centromere, *spf*, was not expressed at all, indicating, as might be expected, that that locus is located in X^{12}. *Ie* and *Gs* were expressed as in normal heterozygotes. Since it is likely that both of these loci, but at least *Gs*, are located in 12^X, this may indicate that the phenotypes are too insensitive to detect partial nonrandomness except in large populations.

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PULSE LABELING WITH [^{3}H]THYMIDINE IN THE STUDY OF CHROMOSOME INACTIVATION

N. L. A. Cacheiro

The use of [^{3}H]thymidine (dThd) in the study of chromosome inactivation in X-autosome translocations of the mouse was originally restricted because of the low frequency of cells showing asynchrony in DNA synthesis when [^{3}H]dThd was added during the last hours of culture (*in vitro*) or just a few hours before killing the animal (*in vivo*).

In a method subsequently developed,[1] a 30-min pulse label early during the synthetic period proved to be satisfactory for tissue cultures of mouse embryo fibroblast. Since we are using this early-labeling technique in cultured kidney cells from adult animals

in our studies of X-autosome translocations, we ran a pilot experiment to determine the optimum time of incubation following the pulse. A total of 1236 cells were scored in this study.

Table 6 shows the distribution of the cells according to their labeling pattern at various time intervals following a 30-min pulse. Cells with asynchronous label (last two columns) include those in which labeling occurred early in the synthetic period (one chromosome unlabeled) and those in which labeling occurred late (one chromosome heavily labeled). The former show a high peak (63.2%) at 11 hr after the pulse. Labeling during the late synthetic period yielded a maximum of only 8.4% of cells showing one chromosome heavily labeled at 6 hr after the pulse. A similarly low percentage is also observed in experiments that use continuous late labeling, leading to difficulty in the identification of a heavily labeled chromosome in a partially labeled cell.

The results have led to our adoption of [^{3}H]dThd pulse labeling during the early synthetic period for all of our *in vitro* studies of chromosome inactivation in X-autosome translocation stocks.

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▼

CYTOLOGICAL STUDIES OF STERILITY IN SONS OF MALE MICE TREATED WITH TRIETHYLENEMELAMINE IN POSTSPERMATOGONIAL STAGES

N. L. A. Cacheiro and M. H. Schultz*

In earlier work,[1] the sterility of F_1 males derived from X irradiation of either postspermatogonial or spermatogonial stages was shown to be associated with chromosome abnormalities much more frequently in the former case (69%) than in the latter (28%).

More recently, we have studied F_1 sterility following the use of mutagenic chemicals.[2] The present report concerns a detailed analysis of 19 of 40 F_1 steriles resulting from TEM treatment of spermatids in experiments of W. M. Generoso. Table 7 shows the morphological, histological, and cytological findings in the analysis of these 19 F_1 sterile males. With the exception of three, all the others showed testis weights below normal. Histological sections of the testis revealed the presence of spermatids in 13 males. In two males, diakinesis was the latest stage present; in four males, pachytene was the latest stage present. Ten out of 19 had epididymal sperm but in reduced quantities.

Table 6. Distribution of cells according to their label pattern after 30-min pulse label with [^{3}H]thymidine

Incubation after pulse (hr)	Total no. of cells scored	Percent of cells with all chromosomes labeled or unlabeled	Percent of cells with asynchronous label	
			One chromosome unlabeled	One chromosome heavily labeled
5	108	89.7	2.9	7.4
6	107	86.9	4.7	8.4
7	83	92.8	3.6	3.6
8	125	80.8	12.8	6.4
9	114	50.8	48.2	0.9
10	103	39.8	60.2	0.0
11	209	36.3	63.2[a]	0.5
12	82	39.0	61.0	0.0
13	91	46.2	53.8	0.0
14	76	59.2	40.8	0.0
15	52	75.0	25.0	0.0
16	76	90.8	7.9	1.3

[a]High peak.

Table 7. Results of morphological, histological, and cytological analysis of testis and spermatozoa in sterile sons of males treated in the spermatid stage with TEM

F_1 male number	Testes weight (mg)	Most advanced stage in testis	Presence of epididymal sperm	Chromosome abnormalities	Break location[a]
654	220	Spermatid	+		—
658	200	Spermatid	+	—	—
659	260	Spermatid	+	—	—
672	50	Spermatid	+	—	—
651	90	Spermatid	+	6^{Dl}	*m,m*
638	70	Spermatid	+	T(13;15)	*c/t*
639	95	Spermatid	+	T(1;7) and T(4;8)	*c/t* and *t/c*
644	90	Spermatid	+	T(6;16)	*t/c*
653	120	Spermatid	+	T(6;12)	*t/t*
645	60	Spermatid	+	T(Y;15) and T(8;9)	*m/t* and *t/t*
643	55	Spermatid	—	T(13;15)	*c/t*
650	70	Spermatid	—	T(11;19)	*c/t*
646	40	Spermatid	—	T(Y;10)	*m/c*
648	60	Diakinesis	—	T(7;10)	*t/c*
660	60	Diakinesis	—	T(7;12)	*c/t*
649	30	Pachytene	—	38 + X + T(X;Y)	*c/t*
652	60	Pachytene	—	T(Y;17)	*m/c*
655	50	Pachytene	—	T(Y;7)	*m/c*
656	60	Pachytene	—	T(7;10) and T(Y;?)	*t/m; m/?*

[a]*m* = break in middle of chromosome; *t* = break close to the centromere; *c* = break close to the telomere.

▲

Chromosome abnormalities, scored in the metaphase analysis of kidney cultures, were found in 15 males. Banding techniques were used to determine the approximate location of break points. The banding analysis shows that the majority of the abnormalities consist of reciprocal translocations, with three animals carrying two translocations each.

Of the total translocations analyzed, six (35%) are Y-autosome translocations; the balance are autosome-autosome translocations, virtually all of which have one or both break points close to one end of a chromosome. The results thus resemble those obtained with X rays for the same stages.[1] We have proposed[3] that this kind of male sterility may be caused by some type of position effect resulting from such rearrangements.

*ORAU undergraduate research participant, Summer 1975, from the University of Wisconsin-River Falls, River Falls 54022.

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STIMULATION OF SPERMATOGENESIS BY REPEATED LOW DOSES OF CLOMIPHENE CITRATE

N. L. A. Cacheiro

As part of our effort to analyze the mechanisms of genetically determined male sterility, we initiated a pilot experiment on attempted therapy using the drug clomiphene, which often stimulates spermatogenesis in humans.[1] Sterile mouse X-autosome translocation males treated with clomiphene citrate did not produce offspring.

We subsequently carried out a histological analysis of the testes of 27 sterile males heterozygous for one of four different X-autosome translocations and of six normal segregants from the same stocks, all of which had received repeated injections of clomiphene. Neither group was affected by the drug with regard to the distribution of germ-cell stages present in the testis.

Sixty daily doses of clomiphene were also administered to another group of sterile males, namely, seven F_1's derived from cyclophosphamide-treated postmeiotic stages. One testis of each male was

removed prior to treatment (control); the other was removed at the end of the series of injections. In these males, the cytology of the testis showed only spermatagonia in two, up to late pachytene in four, and up to diakinesis in one. Treatment with the drug was found to have no effect on the distribution of germ-cell stages in the testis.

Apparently, as in humans,[2] cases of azoospermia do not respond to the drug clomiphene.

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TESTING OF DOMINANT SKELETAL MUTATIONS TO DETERMINE IF THEY ARE RECESSIVE LETHALS

P. B. Selby and V. S. Mierzejewski

Earlier attempts to determine whether recessive lethals have deleterious effects in the heterozygote in mice have been largely negative;[1,2] however, it is likely that some of the end points looked for were too high in normal variation for detection (for example, breeding performance). The end point studied in greatest detail, namely, death *in utero*, was probably less relevant to human genetic disease than certain less extreme deleterious effects that could have been missed (for example, morphological damage). Furthermore, the mutations studied earlier, coming from late generations in population experiments, may have been atypical of recessive lethals in general as the result of selection. As the dominant skeletal mutations appear similar to the types of mutations which account for much of man's large burden of genetic disease,[3] there is much incentive for testing them to determine the proportion that are recessive lethals.

The mutations now being tested for recessive lethality are being given an extensive test involving the following parts: (*a*) intercrossing of carriers to reveal any excess of preimplantation or intrauterine death and (*b*) breeding of progeny from intercrossed carriers to determine if any of the progeny are homozygous for the mutation in question. Furthermore, progeny from the intercross will be examined to see if any of them show more extreme phenotypes than those found in heterozygotes. By doing these two parts of the test on a large scale, considerable additional information about the effects of these mutations on the fertility of heterozygotes will be obtained, in addition to answering the main question.

At present there are only sufficient data to mention for the first part of the test for the first two mutations being tested. Although this part of the test is far from complete for both mutations, the data already suggest that both mutations are recessive lethals. The procedure used is to outcross males heterozygous for a mutation and collect their progeny, which are held until about 10 weeks of age, at which time they are paired at random with other mice from the same generation, but avoiding full-sib matings. The females are observed for the presence of a vaginal plug indicating that copulation has occurred. The uterine contents are examined and the corpora lutea are counted 16 to 18 days after conception. The skeletons of both parents are then examined to determine their genotypes. Approximately one-fourth of the total number of matings are of each of the following types: (*a*) carrier female × carrier male, (*b*) carrier female × noncarrier male, (*c*) noncarrier female × carrier male, and (*d*) noncarrier female × noncarrier male.

This part of the experiment on mutation $565F_1$, which causes all heterozygotes to have at least eight specific extra bones,[4] is presently about one-fifth complete. There is a statistically significant excess of intrauterine death in the intercross compared with the other crosses ($P < 0.01$), and there is no statistically significant dominant-lethal effect; P is about 0.42. On the assumptions that the combined data for all crosses other than the intercross (*i.e.*, crosses of types *b*, *c*, and *d*) represent the spontaneous death rate and that any deaths due to homozygous lethality occur between implantation and birth, it can be calculated that the expected death rate in the intercross is 33.8% if mutation $565F_1$ is a recessive lethal. The observed intrauterine death rate, combining resorbing moles and late deaths, is 18/60, or 30%, and is not statistically significantly different from the expected value. The estimate of spontaneous death is 17/144, or 12%. On the same assumptions, it can be concluded from the data that between one-third and all homozygotes die from a recessive-lethal effect ($P < 0.04$).

The first part of the experiment on mutation $320F_1$, which causes effects similar to the genetic disease in man known as cleidocranial dysplasia (partial absence of clavicles plus skull defects),[3,4] is presently about one-tenth complete. There is a statistically significant excess of intrauterine death in the intercross compared with the other crosses ($P < 0.04$), and

there is no statistically significant dominant-lethal effect; P is about 0.71. On the same assumptions used for mutation $565F_1$, the expected death rate in the intercross is 32.4%. The observed death rate of 6/21, or 29%, is not statistically significantly different from this. The estimate of spontaneous death is 6/61, or 10%. On the same assumptions, it can be concluded that between one-sixth and all homozygotes die from a recessive-lethal effect ($P < 0.05$).

In the above comparisons, a total of two living fetuses were treated as if they had been dead because their gross abnormalities would almost certainly have been lethal before or soon after birth. One of these fetuses, a possible heterozygote for mutation $320F_1$, had spina bifida; the other, a possible heterozygote for mutation $565F_1$, had pseudencephaly. An additional possible heterozygote or homozygote for mutation $565F_1$ was dead with pseudencephaly. The data are too meager to demonstrate that these rare gross abnormalities are caused by these skeletal mutations; however, if such a relationship can be shown, these mutations will provide additional excellent illustrations[1] of dominant mutations having low penetrance for severe effects. Since in man there is no proved case in which an irregularly inherited disease results from a dominant mutation with incomplete penetrance,[5] our ability to show such effects in the mouse that are only hypothesized in man is of considerable importance.

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4. P. B. Selby and P. R. Selby, submitted to *Mutat. Res.*, Part II.
5. Personal communication from human geneticists C. O. Carter and F. Vogel.

ATTEMPTS TO FIND SYNERGISTIC INTERACTIONS OF DOMINANT SKELETAL MUTATIONS IN MICE

P. B. Selby and V. S. Mierzejewski

Mutant lines have been established for 17 of the 31 proved mutations causing abnormalities of the skeleton that were found in a mutation-rate experiment performed in Germany.[1] Many of these mutations can be characterized as models of irregularly inherited conditions in man.[1] Detailed genetical studies of these mutations should provide much knowledge about the types of mutations responsible for a major portion of man's burden of genetic disease.

The earlier experiment showed that most dominant mutations have incomplete penetrance for some or all of the abnormalities that they can cause. This finding, together with other evidence,[1] suggests that there are thresholds[2] for most malformations and that the dominant mutations change the likelihood that the thresholds for certain abnormalities will be exceeded. As many of the mutations cause a large number of abnormalities, it is clear that they must influence many developmental pathways severely enough to cause abnormal development, and it is likely that they influence still other developmental pathways to a lesser degree. Should two different skeletal mutations be present in the same individual, it is conceivable that together they might cause some types of genetic damage that neither one could effect individually. We are, therefore, studying the skeletons of double heterozygotes for evidence of synergistic interactions. Initially, most emphasis is being put on combining mutations having some effects in common, since it seems more likely that they may influence some of the same developmental pathways. Should synergistic interactions be found to be common, they could be an important cause of birth defects in man, since the nature of these mutations makes it likely that many mutations of this type are at low, but appreciable, frequencies in the human population.

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2. S. Wright, *Genetics* **19**, 537–551 (1934).

THE STAGE OF THE MOUSE OOCYTE AT WHICH THE CHANGE IN SENSITIVITY TO RADIATION-INDUCED MUTATIONS OCCURS

E. F. Oakberg and D. T. Palatinus

The observation of Russell[1] that all mutations induced by irradiation occur in litters conceived less than 7 weeks after exposure raised two important questions: (1) What is the basis of this dramatic shift in radiation response of the mouse oocyte, and (2) are data on the mouse relevant to other species, especially to the human female? The primary step in attacking these problems is the identification of the oocyte stage at which the change in sensitivity occurs.

Our first success in timing oocyte development in the adult mouse was achieved by labeling the zona

pellucida with N-[^{3}H]acetyl-D-glucosamine.[2] The initiation of zona formation occurs in follicles with a single layer of cuboidal cells (stage 3b of Pedersen and Peters).[3] All oocytes in 3b and later follicle stages were labeled. The development of oocytes with unlabeled oocytes from earlier stages (1 to 3a) allowed an estimate of 35 days for a stage 3b oocyte to reach ovulation. This estimate is about twice the interval of 19 days reported by Pedersen.[4]

It was important, therefore, to determine if the N-[^{3}H]acetyl-D-glucosamine had affected the rate of oocyte development. Young adult (8-week-old) 101 × C3H hybrid female mice were randomized into control, N-[^{3}H]acetyl-D-glucosamine, N-[^{3}H]acetyl-D-glucosamine + 50 R, and 50 R treatments. A single intraperitoneal injection of 65 μCi of N-[^{3}H]acetyl-D-glucosamine was given 24 hr before irradiation. Half of each group was mated 24 hr after irradiation; the remaining females served as virgin controls for determining the effects of pregnancy on oocyte development. Mice from each group were killed at intervals of 4 days and 2, 3, 4, 4$^1/_2$, 5, and 6 weeks after irradiation. Mated females in the 4- to 6-week groups had given birth to one litter, and many in the 4$^1/_2$-, 5-, and 6-week groups were pregnant with a second litter when killed. The numbers of young born, implants, and live fetuses were recorded. Ovaries were fixed in Zenker-formol, and autoradiographs of serial sections were scored for the stage of oocyte development and for labeling of the zona pellucida.

The data suggest that N-[^{3}H]acetyl-D-glucosamine had no effect on the number of eggs ovulated, the time of ovulation, or the mean litter size (control, 6.8 young per female; 50 R, 6.4; N-[^{3}H]acetyl-D-glucosamine, 7.2; 50 R + N-[^{3}H]acetyl-D-glucosamine, 6.2). Likewise, no effect on the percentage of living embryos was observed. There also was no effect of 50 R, N-[^{3}H]acetyl-D-glucosamine, or pregnancy on the rate of follicular development. The technique, therefore, appears to be a reliable one for studying the dynamics of follicular growth and for determining the stage at which the change in oocyte sensitivity to mutation induction occurs.

Two additional interesting observations were made. First, labeling was less than would have been expected on the basis of two 50-μCi doses given 24 hr apart, and second, some labeled stage 3b oocytes still were present 2 weeks after injection. In order to further explore these results, females were given three injections of 25 μCi of N-[^{3}H]acetyl-D-glucosamine 9 hr apart and were killed 6 hr and 4, 10, and 25 days after the last injection. The data still are not complete; however, labeling was heavier than observed with two 50-μCi injections 24 hr apart, and labeled 3b oocytes still were present at 10 days.

There appears to be a limit to the amount of N-[^{3}H]acetyl-D-glucosamine that can be incorporated from a single injection. Several small doses give better labeling than a single large dose. Of more importance for the timing of oocyte growth was the observation that many labeled 3b oocytes persist for 10 days. The frequency of labeled 3b stages suggests that they can persist for 2 weeks. Actually, we observed a few labeled 3b oocytes after 2 weeks in our initial experiment but discounted this observation because the frequency was low. However, it is now clear that 2 weeks for development of stage 3b into stage 4 must be added to the 4 weeks needed for stage 4 to reach ovulation. This gives an estimate of 6 weeks from the initiation of stage 3b to ovulation instead of the 5 weeks reported earlier.[2]

The selection of the 7th week as the point at which radiation-induced mutation frequency drops was conservative;[1] and 6 weeks would be a more realistic value for the period of high mutational sensitivity (W. L. Russell, personal communication). This estimate coincides precisely with the present 6-week estimate for stage 3b oocytes and follicles to reach ovulation. The time of initiation of zona pellucida formation therefore closely approximates the point at which a dramatic shift in the mutational response of the mouse oocyte occurs.

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SPERMATOGONIAL STEM CELL SURVIVAL AFTER ACUTE X-RAY EXPOSURE

E. F. Oakberg and D. T. Palatinus

Identification of the spermatogonial stem cell has made possible an analysis of the possible effects of cell killing and differential cell survival on radiation-induced spermatogonial mutation rates in the mouse.

Twelve-week-old 101 × C3H hybrid male mice were randomized into groups receiving 0, 300, 500, 600, 1000, and 500 plus 500 R of X rays at 92 R/min. The two 500-plus-500-R exposures were separated by a 24-hr interval. A duplicate series received 12.5 μCi of [^{3}H]dThd 1 hr before irradiation in order to check for differential cell survival. Mice were killed at 5 days and at 207 hr after irradiation, the testes were

fixed in Zenker-formol, and autoradiographs of 5-μm sections were prepared. Cells were counted in 198 tubule cross sections per mouse (Table 8).

The significant effect of 12.5 μCi of [^{3}H]dThd on stem-cell numbers was unexpected ($t = 2.43$; $t_{0.05}$ for 14 degrees of freedom is 1.761 for a one-tailed test). Therefore, the data of Table 8 are expressed as the number of cells counted instead of experimental/control ratios. Variance analysis indicated a significant effect at the 0.01 level of significance for [^{3}H]dThd at both 5 days and 207 hr. The interaction of radiation with [^{3}H]dThd was significant at the 5% level at 5 days but not at 207 hr. Recovery had begun by 207 hr for 300, 500, and 600 R but not for the two 1000-R exposures.

Data on the frequency of labeled cells are not complete, but they suggest comparable values for 300, 500, and 600 R, a very low value for the single 1000-R exposure, and a labeling frequency for the 500-plus-500-R exposure which is about twice that observed for the 300-, 500-, and 600-R groups. These data agree with our earlier observations.[1]

Thus, cell populations surviving the two 1000-R exposures are indicated as qualitatively different from 300, 500, and 600 R both by failure to initiate recovery by 207 hr and by percentages of labeled cells. The two 1000-R groups differ from each other in that cell number declined from 5 days to 207 hr after the single 1000-R exposure but remained constant after the fractionated 500-plus-500-R dose. These groups likewise differ significantly in the frequency of labeled cells 207 hr after irradiation. Therefore, the effects of cell selection on mutation frequency should be greatest for the single 1000-R and the fractionated 500-plus-500-R exposures. Experiments at 300, 500, and 600 R appear to involve cell populations which differ quantitatively but not qualitatively.

Table 8. Mean number of spermatogonial stem cells observed per mouse at 5 days and at 207 hr after irradiation

Dose (R)	Mean No. of cells counted per mouse			
	No [^{3}H]dThd		12.5 μCi of [^{3}H]dThd	
	5 days	207 hr	5 days	207 hr
0	147.5	147.5	133.25	133.25
300	39.5	69	24.0	65.5
500	16.5	33.5	13.8	25.5
600	13.8	23.8	12.3	16.8
1000	5.3	2.0	2.8	1.3
500 + 500	5.0	5.0	8.5	2.0

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INDUCTION OF SPECIFIC-LOCUS MUTATIONS IN MOUSE SPERMATOGONIA BY TRITIATED WATER

R. B. Cumming and W. L. Russell

This experiment, reported earlier,[1] is continuing. To date, 11 presumed mutational events have been scored, but breeding tests on the presumed mutations have not yet been completed. One of the two groups of males exposed appears to be giving an unusually high frequency of clusters. Additional data and analysis to check this are required, along with breeding tests of the mutants, before a mutation frequency and a relative biological effectiveness based on the new results can be reported.

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THE LACK OF INDUCTION OF SPECIFIC-LOCUS MUTATIONS IN MICE BY LONG-TERM EXPOSURE TO HIGH LEVELS OF BUTYLATED HYDROXYTOLUENE

R. B. Cumming, M. F. Walton, E. M. Kelley, and W. L. Russell

Butylated hydroxytoluene (BHT, 2,6-di-*tert*-butyl-*p*-cresol) is an antioxidant commonly added to human food to retard spoilage due to auto-oxidation of unsaturated lipids. Consequently, the populations of the United States and other industrialized countries receive chronic lifetime exposures to low concentrations of this compound.

When BHT is fed to laboratory animals at higher levels, it produces a variety of effects. These have been summarized previously.[1]

The question of potential genetic effects of BHT is one of some importance. The exposed human population is very large, and the exposure is continuous throughout each individual's reproductive life. Previous genetic studies using the dominant-lethal assay[2] have been negative, but the test as it was performed would have been expected to detect only

very large effects. In addition, gene mutations would not have been detected in the dominant-lethal assay. Teratogenicity tests have also been negative,[3] but for such a widely used food additive, it would still be better to have test results which allow a quantitative estimate of the reliability of negative results and a calculation of the level of genetic risk excluded at human exposure levels. The present paper provides the data for such a calculation. In addition, since BHT is used in this laboratory to modify the genetic effects of mutagens in mice,[4] it is important to know whether or not it, in itself, is mutagenic.

Male (C3H × 101)F_1 mice were kept throughout their lives on a diet containing 0.75% BHT. During this time they were mated to T-stock females, and the offspring were checked for specific-locus mutations at the seven marked loci.

In the 206 matings for the specific-locus experiment, offspring were conceived from the 1st day of mating and for 119 weeks thereafter. The mean interval between the start of feeding of the parents to conception of the offspring was 368 days. This figure was obtained by averaging the individual parental exposure periods from the beginning of treatment to conception for all of the offspring scored in this experiment. There were 3928 litters produced, and from these, 20,402 offspring were weaned. The mean litter size at weaning of 5.2 is within the normal range for control groups in the specific-locus test as performed in this laboratory. One mutation was obtained at the *b* locus, and this mutant was fully genetically tested. It was verified as a *b* locus mutant and was homozygous viable. Thus, the observed mutation frequency in this experiment is actually, but not of course significantly, lower than our estimate of the spontaneous frequency for these seven loci under similar experimental conditions based on the observation of more than a half million offspring (28 mutations in 531,500 offspring). BHT is not shown to be mutagenic even at high exposure levels over long periods of time in this experiment, and these results are consistent with the negative findings obtained recently for butylated hydroxyanisole in *Drosophila melanogaster*.[5]

In addition to simply failing to show a mutagenic effect, the data produced in this experiment permit following the procedure described by Russell[6,7] for the estimation of maximum levels of human risk relative to the spontaneous mutation frequency. The calculation follows: The mice in this experiment were exposed to 0.75% w/w (7500 ppm) BHT for an average time period of 368 days. It can be calculated that 5 ppm in human food is a conservative overestimate of the human exposure, and for genetic purposes the human exposure is taken to be 30 years (10,957 days).

From these figures we can calculate the relative exposure factor for the experimental mouse population and the human population as follows:

$$\frac{7500 \text{ ppm}/5 \text{ ppm}}{10{,}957 \text{ days}/368 \text{ days}} = 50.28$$

There is no indication from the present data, or any others that we know of, that BHT is mutagenic. But if we wish to quantify the upper limit of human genetic risk from BHT exposure, it can be done by the technique previously described by Russell.[6,7] The upper 95% confidence limit for the one observed mutation in this experiment is 5.323. The upper 95% confidence limit of the induced frequency is, therefore, obtained by taking this number (5.323/20,402) and subtracting the spontaneous frequency (28/531,500). To obtain the upper limit of the induced frequency at human exposure levels, we divide by the previously obtained exposure factor of 50, and then a ratio is made with the spontaneous frequency for the mouse to change our figures from absolute to relative terms. The calculation follows:

$$\frac{(5.323/20{,}402 - 28/531{,}500)/50}{28/531{,}500} = 0.079$$

We can therefore be 95% confident that current human exposure levels do not increase the spontaneous mutation frequency by more than 8%.

This calculation was made using the point estimate of the spontaneous mutation frequency. The spontaneous mutation data collected in this laboratory over many years have shown no evidence of any nonbinomial source of variability. Upper and lower 95% confidence limits can therefore be used to determine what effect an error in estimation of the spontaneous frequency would have on this calculation of risk. If the spontaneous frequency is higher than estimated from our data, the corresponding risk estimate would be lower than calculated, and vice versa. Recalculating the risk estimate using the upper and lower 95% confidence limits for the spontaneous frequency leads, respectively, to estimates of the maximum risk of 5.0% and 12.6%. Therefore variations in estimates of the spontaneous frequency do not lead to conclusions greatly different from those expressed above.

Several assumptions are made in the above calculation, but we believe all of them to be conservative.

The 5-ppm estimate of the human exposure level is probably higher than the actual exposure level. The assumption of linearity of effect over a large (1200-fold) exposure range is probably conservative because we know that for most biological effects produced by chemical exposure, the dose-response curve is concave upward. For example, 0.50% BHT in the diet gives less than half the enzyme-induction effect of 0.75% in our mice, and similar dose-response relationships have been observed by others.[8,9] We have assumed an absolute time scale in comparing exposure of the mouse and man, but a more proper comparison would probably be the number of spermatogonial stem-cell generations in the presence of the compound. The spermatogonial stem-cell generation time is much longer in man than in the mouse, perhaps as much as an order of magnitude longer.[10] If this were taken into consideration, it would make the exposure factor large and the calculated maximum risk smaller. All of the potential errors in this type of calculation appear to maximize the calculated potential hazard and, therefore, to lead to conservative and safe estimates.

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A SMALL TEST FOR DOMINANT-LETHAL MUTATIONS IN MICE FROM PYRROLIZIDINE ALKALOIDS

R. B. Cumming, T. J. Dunnigan,* and M. F. Walton

Pyrrolizidine alkaloids are compounds of widespread occurrence in many unrelated plant species throughout the world. They are particularly abundant in the plant genera *Senecio*, *Crotalaria*, *Heleobropium*, and *Cynoglossum*; also in the family *Boraginaceae*. Irreversible liver damage and other pathological effects due to the consumption of plants containing one or more of these compounds have resulted in heavy losses of livestock in many countries over the years (first described in 1787). Humans have also been subject to acute poisoning, and, in addition, there is considerable evidence that adventitious ingestion of several of these compounds may be responsible for veno-occlusive disease and cirrhosis and possibly liver carcinogenesis in man.[1–5]

The pyrrolizidine alkaloids are esters of the aminoalcohols derived from the heterocyclic pyrrolizidine nucleus (I).

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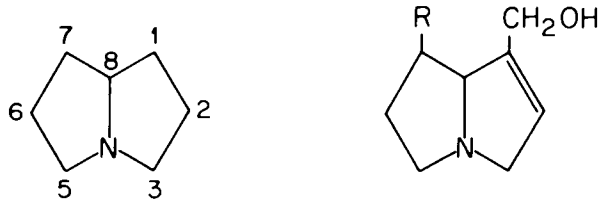

I II

Not all of the pyrrolizidine alkaloids are toxic, but those that are have generally been shown to be mono- or diesters of 1-hydroxymethyl-1,2-dehydropyrrolizidine (II) with various substituted butyric, glutaric, and adipic acids. The pyrrolizidine alkaloids do not appear to be toxic in their native state, but when consumed and subsequently metabolized by susceptible animals they form highly toxic short-lived metabolites called pyrroles.[6] These may function as bifunctional alkylating agents and react with cellular macromolecules, including DNA.[7]

The pyrrolizidine alkaloids have been reported to break chromosomes in onion root tips and *Vicia faba*.[8] Some of them are also highly mutagenic in *Drosophila*.[9,10] They appear not to be mutagenic in bacteria with or without metabolic activation. No studies have appeared until now on potential transmitted genetic effects in mammals.

Three pyrrolizidine alkaloids—monocrotaline, seneciphylline, and jacobine—were injected at two dose levels each into (C3H × 101)F_1 male mice. Doses used and 30-day survival are shown in Table 9. Males were individually mated with three virgin T-stock females approximately 3 months old, which were then checked daily for the presence of vaginal plugs. Mated females were removed and replaced. Females were killed at 13 to 17 days of gestation, and the uterine contents were scored. No evidence for a

Table 9. Thirty-day survival of (C3H × 101)F_1 male mice following ip injection of pyrrolizidine alkaloids

Compound	Dose	Percent survival
Controls	0.5 ml saline	100
Seneciphylline	50 mg/kg	100
	75 mg/kg	33.3
Monocrotaline	150 mg/kg	100
	200 mg/kg	66.6
Jacobine	50 mg/kg	100
	75 mg/kg	0

dominant-lethal effect was found in this experiment except for an apparent effect in early spermatids at the highest dose of monocrotaline (200 mg/kg). This point is based on a few females, and the effect must be considered as only suggestive until a more definitive test can be performed.

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MONOCROTALINE-INDUCED MUTATIONS IN *DROSOPHILA MELANOGASTER*

R. B. Cumming and T. J. Dunnigan*

Several of the pyrrolizidine alkaloids have been shown to be mutagenic in *Drosophila* when injected intra-abdominally.[1,2] Because of the increasing concern about these compounds as potential mutagens or carcinogens for man and our tests of some of them in mammalian test systems,[3,4] we decided to reexamine the mutagenicity of one of the pyrrolizidine alkaloids, monocrotaline, in *Drosophila* after feeding in a sucrose solution.

Monocrotaline is the most highly mutagenic of the pyrrolizidine alkaloids known for *Drosophila* when given by injection.[1] We fed the compound to Oregon R males, 15 m*M* in 1% sucrose solution, using two techniques. In the first (experiment I), 150 μl of the solution was placed on a glass filter, which was then placed in the bottles containing the flies. The second method (experiment II) involved inserting a 10-mm-diam glass frit, porosity D or E, through the foam stopper of a bottle and pipetting 100 μl per day onto the top of the frit. This second method resulted in similar survival, better fertility, and much higher mutation rates (see Table 10).

The sex-linked recessive lethal frequencies are very high in the first 3-day broods when the monocrotaline is given on a frit. These results are in contrast to those found in mammals.[3,4]

*Southern College University Union student, Winter 1976, from Centenary College of Louisiana, Shreveport 71104.

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Table 10. Effects of ingested monocrotaline on *Drosophila melanogaster*

	Experiment I		Experiment II	
	Brood 1	Brood 2	Brood 1	Brood 2
Percent survival	52.0	30.0	40.0	40.0
Live males, percent fertile	Not determined	44	80	60
Percent sex-linked recessive lethals	9.4	None detected	35.9	53.3

A SPECIFIC-LOCUS MUTATION TEST IN MICE FOR POTENTIALLY MUTAGENIC EFFECTS OF MONOCROTALINE

R. B. Cumming, M. F. Walton, T. J. Dunnigan,* and L. R. Lewis

The pyrrolizidine alkaloids are of some concern because of widespread human exposure and the possibility of their having environmental carcinogenic or mutagenic effect.[1] Monocrotaline is highly mutagenic in *Drosophila*.[2] A small specific-locus test has been initiated to see whether the high levels of mutagenicity seen in *Drosophila* could also occur in mammals.

Thirty male (C3H × 101)F_1 mice were injected with 150 mg/kg of monocrotaline and mated with four T-stock females per week for 20 weeks. Data are now available for 19 of those weeks: 9006 offspring have

been scored at weaning; 3103 of these were from germ cells treated in postspermatogonial stages, and the other 5903 were from treated spermatogonia. No specific-locus mutations have been observed.

From these data one can conclude that monocrotaline is not a highly mutagenic compound in mammals, though the present results do not rule out small effects.

*Southern College University Union student, Winter 1976, from Centenary College of Louisiana, Shreveport 71104.

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HALOGENATED URACIL MUTAGENESIS IN *ESCHERICHIA COLI*

R. B. Cumming and M. F. Walton

Previous studies in this laboratory and elsewhere have shown that three 5-halogenated uracil (Ura) base analogues — 5-chlorouracil, 5-bromouracil (5-BrUra), and 5-iodouracil—are all highly mutagenic when incorporated into the DNA of *E. coli*.[1] These studies also showed that the mutation frequency per survivor is not a linear function of the amount of the base analogue incorporated into the DNA. In fact, at high levels of incorporation, achieved by exposure of the halogenated Ura's for periods of 1 hr or more, the muation frequency per survivor dropped.[1] In an attempt to explain these results we have done a series of experiments. Cultures in flasks are periodically diluted to maintain a relatively constant population density, and other experiments were performed in a chemostat. Both types of experiment suggest that the physiological condition of the culture at the time of sampling is a critical factor in the observed mutation frequency.

In the chemostat experiments with 5-BrUra, the level of incorporation of the base analogue is held constant with time by suitable mixtures of BrUra and thymine in the culture medium. The bacteria can then be held under conditions where the growth rate and number of bacteria per milliliter of culture medium remain constant with time. Under these conditions, the mutation frequency, when these bacteria are periodically sampled and plated on selective medium, does not increase with time but is a function of the number of bacteria per milliliter of medium. The bacteria are less able to express mutations on plates when sampled from cultures of high population density. These experiments also indicate that there may be a differential capacity of *E. coli* to form colonies on survival plates and mutant selection plates. Cultures under physiological stress, such as may occur when they are exposed to chemical mutagens, may have substantially lowered capacity to express whatever mutations they have relative to their capacity to form colonies on survival plates. These results lead to some doubts about bacterial "treat and plate" tests as quantitative instruments of mutation measurement.

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INVESTIGATION OF POSSIBLE DNA REPAIR OF CHEMICALLY TREATED MOUSE SPERM CELLS FOLLOWING FERTILIZATION

G. A. Sega and R. E. Sotomayor

When male mice are treated with mutagens such as X rays[1] or alkylating agents like ethyl methanesulfonate (EMS),[2] unscheduled DNA synthesis (UDS), taken to be DNA repair, is observed in all germ-cell stages from early meiotic stages through mid-spermatid stages. However, in all later germ-cell stages no UDS has been observed. It is known that dominant lethals, chromosomal aberrations, and specific-locus mutations are induced in these later germ-cell stages by these particular mutagens and, in the case of EMS, that the DNA of these late germ-cell stages is ethylated.[3] If DNA repair does not occur in these late germ-cell stages in the male, does it occur in the male pronucleus after fertilization of the oocyte?

In an attempt to answer the above question, 32 (C3Hf × 101)F_1 males, 10 to 14 weeks old, were given ip injections of 75 mg/kg of methyl methanesulfonate (MMS). (MMS was used since the level of UDS induced by this chemical is higher than for EMS.) Each treated male was placed with three or four (C3Hf × 101)F_1 females every morning for 16 days from 8:00 to 8:30 AM, 2 hr after the end of a 5-hr dark period, according to procedures developed by Krishna and Generoso[4] to partially synchronize ovulation. Females with plugs were removed and replaced with fresh females each morning. The mated females were killed at noon, and the fertilized oocytes were recovered from the ampulla and maintained in media containing 100 μCi/ml of [^{3}H]dThd. At 4:00 PM, prior to the time for the onset of the first

scheduled pronuclear DNA synthesis, the fertilized oocytes were removed from the labeled media and fixed overnight in neutral buffered formalin. The oocytes were then squashed on a glass slide and dipped in NTB_2 emulsion, and the autoradiographs were developed and stained with Giemsa 28 days later.

By mating the treated males daily for 16 days, the later germ-cell stages, which showed no UDS in the males, could be studied for UDS in the fertilized oocyte. If DNA repair was occurring in the male pronucleus after fertilization, this repair could conceivably be detected by the unscheduled uptake of [^{3}H]dThd into the male pronucleus, before the first scheduled DNA synthesis occurred.

Over 500 fertilized oocytes were studied for any sign of UDS in the male pronucleus, but none was seen. The results indicate that when male mice are treated with MMS, the most mature germ-cell stages (sampled in the vas up to 16 days after treatment) show no UDS between the time of fertilization and the first scheduled pronuclear DNA synthesis. The possibility remains that these treated male germ cells may undergo DNA repair either at the time of pronuclear DNA synthesis or subsequent to it.

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UNSCHEDULED DNA SYNTHESIS CAUSED BY HYCANTHONE IN EARLY-SPERMATID STAGES OF THE MOUSE

G. A. Sega and J. G. Owens

Hycanthone, a commonly used antischistosomal drug, has been studied in a variety of test systems for possible mutagenic properties. Although it has proven mutagenic in some microbial test systems, the only clear-cut genetic effect in mice is an increased frequency of X-chromosome loss in treated female mice.[1]

In an attempt to further assess the effects of hycanthone in mammals, (C3Hf × 101)F_1 male mice were given ip injections of hycanthone methanesulfonate (150 mg/kg of the free base). The mice were also given 39 μCi of [^{3}H]dThd injected directly into each testis either at the time of hycanthone treatment or 90 min after the treatment. Sixteen days later the caudal sperm were recovered from the animals, and the amount of unscheduled uptake of [^{3}H]dThd into the germ cells was measured. The caudal sperm sampled 16 days after treatment represented early spermatid stages at the time of treatment.

The results, shown in Table 11, indicate that the hycanthone was able to induce a significant level of UDS in early spermatids. Since the [^{3}H]dThd is only available for incorporation into germ-cell DNA for approximately 1 hr after injection,[2] reparable lesions are apparently produced in the germ-cell DNA within 1 hr after injection of hycanthone. The level of UDS is reduced by 90 min after treatment, suggesting that the hycanthone has its maximum effect on the germ-cell DNA within about 1 hr after treatment.

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COMBINED EFFECTS OF METHYL METHANESULFONATE AND X RAYS ON UNSCHEDULED DNA SYNTHESIS IN SPERMATIDS OF THE MOUSE

D. A. Carpenter* and G. A. Sega

Previous experiments in our laboratory have shown that both MMS and X rays are able to induce

Table 11. Induction of unscheduled DNA synthesis in early spermatids of (C3Hf × 101)F_1 males after ip injection of 150 mg/kg of hycanthone

	Average [^{3}H]dThd dpm per million sperm[a]
[^{3}H]dThd + hycanthone given simultaneously	33 ± 3
[^{3}H]dThd given 90 min after hycanthone	23 ± 4
Controls	10 ± 2

[a]Errors are ± standard deviation and were determined from individual measurements on three different animals for each of the treated groups and ten animals for the controls.

UDS in early spermatids of the mouse. While MMS is a strong inducer of UDS, X rays are a comparatively weak inducer of UDS. It was, therefore, of interest to study the effect of combined treatments of MMS and X rays in inducing UDS in spermatids of the mouse.

(C3Hf × 101)F_1 male mice, 10 to 14 weeks old, were given testicular injections of [^{3}H]dThd. The mice were then given acute X-ray doses ranging from 50 R to 1000 R. This was followed by an ip injection of 75 mg/kg of MMS at various times after the end of the radiation treatment. The mice were killed 16 days after treatment, and the sperm cells from the caudal epididymides were recovered and purified by procedures described elsewhere.[1] The sperm cells present in the caudal epididymides 16 days after treatment represented germ cells that were early to mid spermatids at the time of treatment. The unscheduled presence of [^{3}H]dThd in these caudal sperm was detected using liquid scintillation counting.

In our first experiment the males were given X-ray doses from 50 R to 1000 R followed either immediately or 4 hr later by the MMS treatment. At all X-ray doses from 200 R to 1000 R there was a significant reduction in UDS in the spermatids when the MMS was given 4 hr after the X rays as compared with what was seen when the MMS immediately followed the X rays (see Fig. 4).

In a second experiment, a constant X-ray dose of 600 R was used, and the MMS was given at times up to 20 hr following the radiation. A significant reduction in UDS was seen in the spermatids when the MMS was given 4 hr or more after the X rays. No return to normal UDS levels in the spermatids was seen up to 20 hr following the X rays (see Fig. 5).

Our present interpretation of these data is that the X-ray treatment is damaging or inhibiting some part of the DNA repair process in the germ cells. This damage does not occur immediately after the X-ray treatment but rather takes a few hours to manifest itself.

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

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THE RELATIONSHIP BETWEEN GENETIC EFFECTS AND UNSCHEDULED DNA SYNTHESIS INDUCED BY MUTAGENS IN THE GERM CELLS OF MALE MICE

G. A. Sega

When EMS was first found to induce UDS in specific germ-cell stages of male mice, it was noted that these stages showed no detectable dominant-

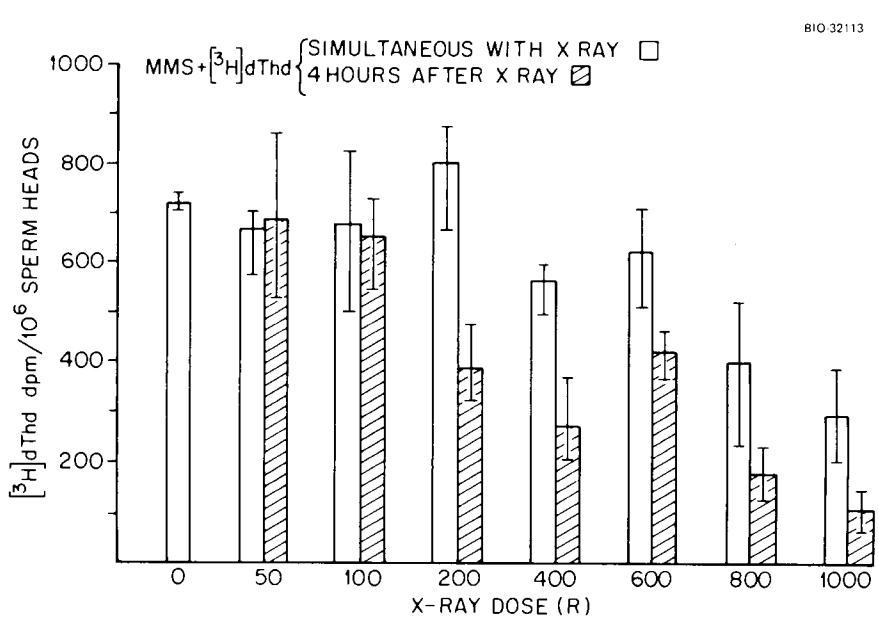

Fig. 4. Unscheduled DNA synthesis in early- to mid-spermatid stages of the mouse after combined X-ray and MMS treatment. The MMS exposure was 75 mg/kg given ip. Four animals were used for each of the treatments. The ranges in the values of unscheduled [^{3}H]dThd incorporation into the germ cells of the four animals used for each treatment are indicated.

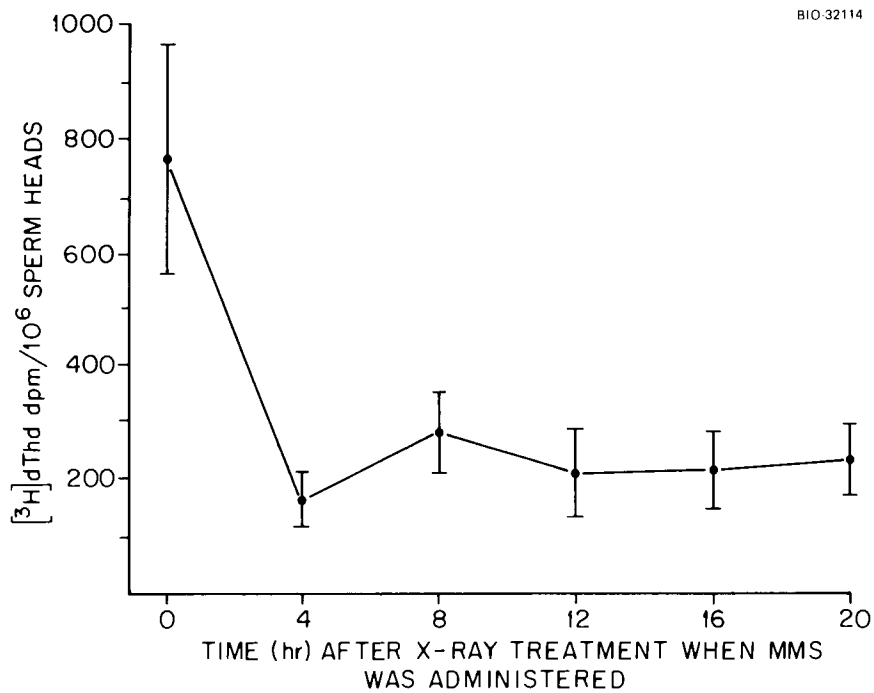

Fig. 5. Unscheduled DNA synthesis in early- to mid-spermatid stages of the mouse as a function of time after a 600-R acute X-ray dose when a 75-mg/kg dose of MMS was administered. Four animals were used for each of the time points, and ± 1 standard deviations are indicated.

lethal mutations.[1] This led to an initial hypothesis that UDS might be important in removing dominant-lethal mutations from the germ cells. However, other mutagens, including propyl methanesulfonate (PMS),[2] isopropyl methanesulfonate (IMS),[2] and X rays,[3] were then found that produced UDS in germ-cell stages that also showed dominant-lethal mutations.

Figure 6 shows the patterns of dominant-lethal frequencies seen after treatment of male mice with EMS[4,5] and X rays[6,7] and how they are related to UDS. While the germ-cell stages showing UDS after EMS treatment (and sampled as sperm in the vas during the 3d and 4th weeks after treatment) do not give rise to dominant-lethal mutations, these same germ-cell stages also undergo UDS after X-ray treatment but show nearly twice as high a dominant-lethal frequency as the germ cells sampled in the vas during the first 2 weeks of mating after treatment, where no UDS was detected. While it is possible that in the absence of UDS the dominant-lethal frequency might be still higher in the 3d and 4th weeks after X-ray treatment, there is no good evidence available at present to indicate that UDS has any effect on dominant-lethal frequencies in male germ cells.

Data on specific-locus mutation frequencies in each of the first 4 mating weeks following treatment with chemical mutagens is not extensive enough to determine if a difference in mutation frequency exists between germ-cell stages showing UDS (sampled in the 3d and 4th weeks of mating after treatment) and those not showing UDS (sampled in the first 2 weeks of mating). However, more data are available for X rays.[8] With an acute X-ray dose of 300 R, specific-locus mutation frequencies have been determined for each of the first 4 weeks of mating after treatment. The mutation frequency in each of the 4 weeks is at least an order of magnitude higher than the spontaneous frequency, and there is no reduction in the frequency in the 3d and 4th weeks as compared with the first 2 weeks. In fact, the mutation frequency is higher (at a 90% confidence level) in the 3d week than it is in the first 2 weeks. There is no indication that UDS results in a reduced specific-locus mutation frequency after X-ray treatment.

In summary, there is no good evidence to date that the occurrence of UDS in the germ cells of male mice affects either dominant-lethal or specific-locus mutation frequencies. The use of various mouse stocks that have been observed to yield high and low levels

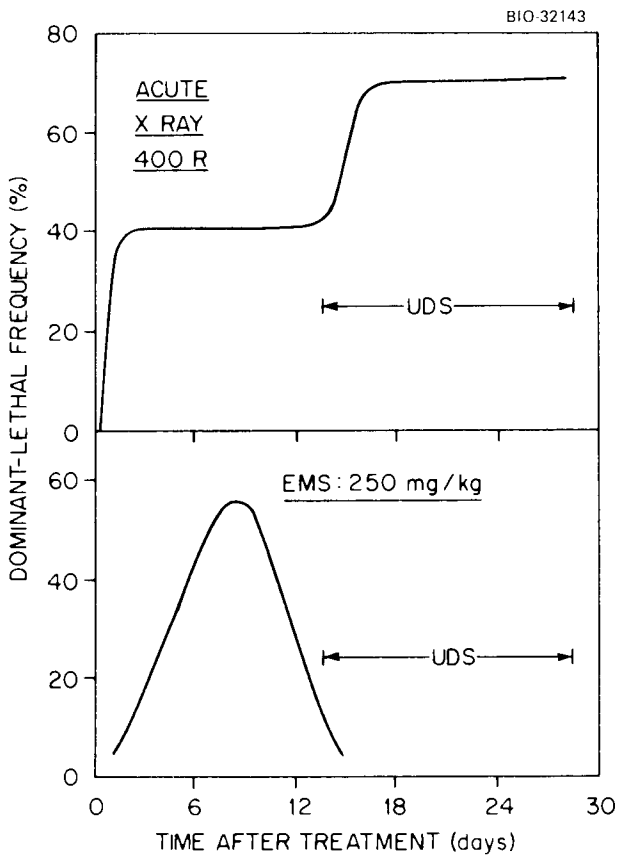

Fig. 6. Relationship between the induction of dominant-lethal mutations by EMS and X rays and the occurrence of UDS in the germ cells.

of UDS when exposed to chemical mutagens may be useful in future work to determine the genetic consequences of UDS. See also the following report.

1. G. A. Sega, *Proc. Natl. Acad. Sci. USA* **71**, 4955–4959 (1974).
2. G. A. Sega, J. G. Owens, and R. B. Cumming, *Mutat. Res.*, **36**, 193–212 (1976).
3. G. A. Sega, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, pp. 137–138.
4. U. H. Ehling, R. B. Cumming, and H. V. Malling, *Mutat. Res.* **5**, 417–428 (1968).
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8. W. L. Russell, unpublished data.

A COMPARISON OF THE LEVELS OF UNSCHEDULED DNA SYNTHESIS INDUCED BY METHYL METHANESULFONATE IN EARLY SPERMATIDS OF VARIOUS MOUSE STOCKS

G. A. Sega and J. G. Owens

(C3Hf × 101)F_1 hybrids have been used in most of our studies of the induction of UDS in the germ cells of male mice, primarily because this mouse stock has been commonly used for dominant-lethal and specific-locus mutation studies. However, it was also of interest to compare different mouse stocks for the relative abilities of the germ cells to undergo UDS when exposed to a chemical mutagen. The characterization of mouse stocks that show high or low levels of UDS in the germ cells in response to chemical mutagens will be useful in future work to determine the genetic significance of UDS. Such stocks will be important in establishing whether or not the occurrence of UDS in certain germ cells results in lower frequencies of dominant lethals, chromosomal aberrations, and specific-locus mutations.

A total of 22 mouse stocks have been screened in our preliminary study. The males used were all sexually mature and between 3 and 7 months old when treated. They received testicular injections of [^{3}H]dThd (38 μCi per testis), which were immediately followed by an ip injection of 75 mg/kg of MMS. This dose of MMS was known from previous work to induce a high level of UDS in several germ-cell stages of the (C3Hf × 101)F_1 male hybrids.[1]

The sperm cells were recovered from the caudal epididymides of the treated males 16 days after treatment, and the presence of [^{3}H]dThd was assayed by liquid scintillation counting. These sperm represented early-spermatid stages at the time of treatment, so the presence of [^{3}H]dThd in them indicated that an unscheduled DNA synthesis had occurred in the early spermatids following treatment. Since the [^{3}H]dThd was only available for incorporation into the "repaired" DNA of the early spermatids for about 1 hr after injection, the UDS measured represents only that DNA "repair" occurring within about 1 hr after chemical treatment. The results are shown in Fig. 7.

Within the experimental uncertainties, most of the 22 stocks showed about the same level of UDS in the

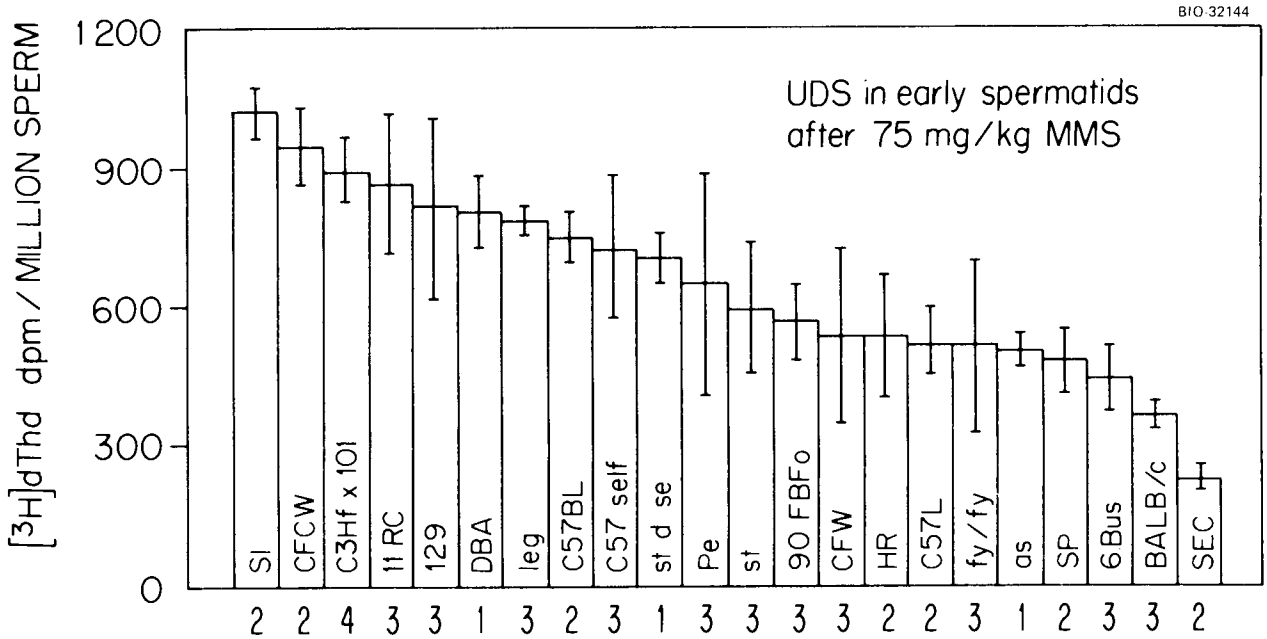

Fig. 7. Unscheduled DNA synthesis occurring in early spermatid stages of 22 different mouse stocks following exposure to 75 mg/kg of MMS. Caudal sperm were assayed for the unscheduled presence of [^{3}H]dThd 16 days after treatment. These sperm represent early-spermatid stages at the time of treatment. Each mouse was analyzed separately. The number below each bar is the number of males tested from each stock. The error bars represent ±1 standard deviation.

early spermatids. However, there was a significant difference between the stocks showing the highest UDS levels and those showing the lowest. The (C3Hf × 101)F_1 hybrids showed one of the highest UDS responses, while the SEC and BALB/c males were the two stocks showing the lowest UDS levels, only one-fourth to one-half of those for the (C3Hf × 101)F_1 males. A more detailed comparison of these stocks is continuing.

1. G. A. Sega, J. G. Owens, and R. B. Cumming, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, pp. 138–139.

AN ASSESSMENT OF TWO POLYCYCLIC AROMATIC HYDROCARBONS FOR THEIR ABILITY TO INDUCE AN UNSCHEDULED DNA SYNTHESIS IN MOUSE SPERMATIDS*

G. A. Sega, J. G. Owens, M. F. Walton, and R. B. Cumming

A large variety of chemicals found in coal and coal conversion products belong to a class of compounds known as polycyclic aromatic hydrocarbons (PAHs), many of which are already known to have carcinogenic properties. Because of the future role of coal and coal conversion products as a source of energy in this country, it was considered important to initiate a study into what genetic hazards there might be in the persistence of these PAHs in the environment.

For our study two classic PAHs have been used: BP and 7,12-dimethylbenzanthracene (DMBA). Both of these compounds have been studied for their carcinogenic properties, but until recently not much was known about the mutational hazard they posed. This situation is now beginning to change as genetic data on these compounds are being obtained in our Mammalian Genetics Group. (See also this year's annual reports of W. L. Russell, L. B. Russell, and W. M. Generoso.)

Previous studies had indicated that with simple alkylating agents the detection of UDS in the affected germ-cell stages of male mice was five to ten times as sensitive as the detection of dominant-lethal mutations or chromosomal aberrations.[1] It was, therefore, of interest to determine if UDS could be detected in the germ cells following treatment with BP and DMBA. (C3Hf × 101)F_1 males were given ip injections of 500 mg/kg of BP and 50 mg/kg of DMBA dissolved in corn oil. Testicular injections of [^{3}H]dThd were given either simultaneously with the chemicals or 90 min after the ip injections, and

sperm, representing early-spermatid stages at the time of treatment, were recovered from the caudal epididymides 16 days later. The unscheduled presence of [^{3}H]dThd in these sperm was assayed using liquid scintillation counting.

Many of the treated males became bloated with a milky fluid in the body cavity by 16 days following injection, and in some cases droplets of the corn oil used for the ip injections could still be seen. No UDS was detected in the germ cells of males treated with DMBA. There was a slight, though not a statistically significant, increase in incorporation of [^{3}H]dThd into the germ cells of BP-treated males over control values.

Since the [^{3}H]dThd is only available for incorporation into germ-cell DNA undergoing "repair" for about 1 hr after treatment, it is possible that the BP and DMBA penetrated too slowly to the germ cells to induce a measurable DNA repair response within 1 hr after the testicular injections of [^{3}H]dThd. Work is currently under way to try different methods and routes of administration of the PAHs. Carriers other than corn oil are being tried, and direct injection of the BP and DMBA into the testes is being attempted. These procedures may help to get the chemicals to the germ cells at a faster rate, so that if DNA lesions are being produced, their "repair" may then be detected by UDS.

*Research sponsored jointly by the Environmental Protection Agency and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

1. G. A. Sega, J. G. Owens, and R. B. Cumming, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, pp. 138-139.

UNSCHEDULED DNA SYNTHESIS IN THE GERM CELLS OF MALE MICE EXPOSED *IN VIVO* TO CYCLOPHOSPHAMIDE AND MITOMEN. A COMPARISON WITH ETHYL METHANESULFONATE-INDUCED UNSCHEDULED DNA SYNTHESIS*

R. E. Sotomayor, G. A. Sega, and R. B. Cumming

We have previously reported that cyclophosphamide and mitomen induced *in vivo* UDS in early-spermatid stages of mice.[1] The pattern of UDS induced by these compounds was quite different from that of EMS (Fig. 8). Mitomen and cyclophosphamide induced low levels of UDS at zero time (simultaneous injection of the mutagen and [^{3}H]dThd); they reached maxima at 0.5 hr after treatment, respectively, and then decreased rapidly. By 8 to 12 hr after treatment there was no detectable UDS. On the other hand, EMS induced a maximum level of UDS at zero time, and the level decreased steadily in an exponential fashion. By 12 hr after EMS treatment the level of UDS was still 15 times the control values. We have interpreted these results as indicating that the pattern of UDS induced by cyclophosphamide and mitomen, unlike that of EMS, may reflect the phenomenon of metabolic activation which these compounds undergo *in vivo*.

We have also studied various germ-cell stages of male mice with regard to their capacity to undergo UDS following *in vivo* treatment. From these experiments (Figs. 9 and 10) it is clear that both cyclophosphamide and mitomen induced UDS from mid- to early-spermatid back to meiotic stages. No UDS was observed in mature spermatozoa and late-spermatid stages. There are some differences in the pattern of UDS induced by both compounds in the various germ-cell stages studied. Mitomen, for instance, induced a level of UDS which, after reaching a plateau in mid- to early-spermatid stages, remained relatively constant through all meiotic stages. Cyclophosphamide, on the other hand, induced a level of UDS which reached a peak during early-spermatid stages and dropped thereafter to levels very close to those of the respective controls.

It is also clear from Figs. 9 and 10 that both cyclophosphamide and mitomen markedly inhibited the last scheduled DNA synthesis during the preleptotene-S spermatocyte stages. This is another difference from EMS, for which only a modest inhibition of the DNA synthesis in the preleptotene-S spermatocyte has been reported.[2] Even though there may be several explanations for this difference, we favor the idea that the *in vivo* inhibition of the last scheduled DNA synthesis in spermatocytes is due to the cross-linking of DNA strands, which has been reported for both mitomen and cyclophosphamide.

We would like to emphasize the point that the UDS technique may be a useful addition to existing *in vivo* tests for mutagenicity. The potential capacity of a chemical compound to reach and damage the DNA of mammalian germ cells *in vivo* can be measured by this technique.

*Research jointly sponsored by the National Center for Toxicological Research and by the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

1. R. E. Sotomayor, G. A. Sega, and R. B. Cumming, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, pp. 141-142.
2. G. A. Sega, *Proc. Natl. Acad. Sci. USA* **71**, 4955-4959 (1974).

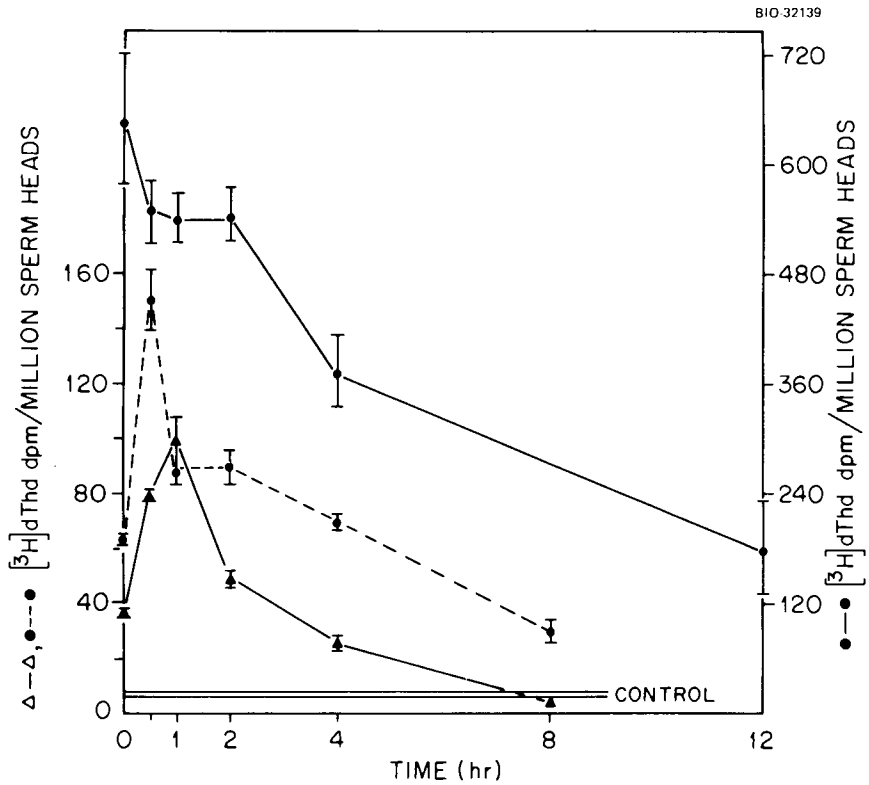

Fig. 8. Unscheduled DNA synthesis in early-spermatid stages occurring at various times after treatment of mice with 200 mg/kg of EMS (● — ●), 40 mg/kg of mitomen (● ---- ●), and 200 mg/kg of cyclophosphamide (▲ — ▲).

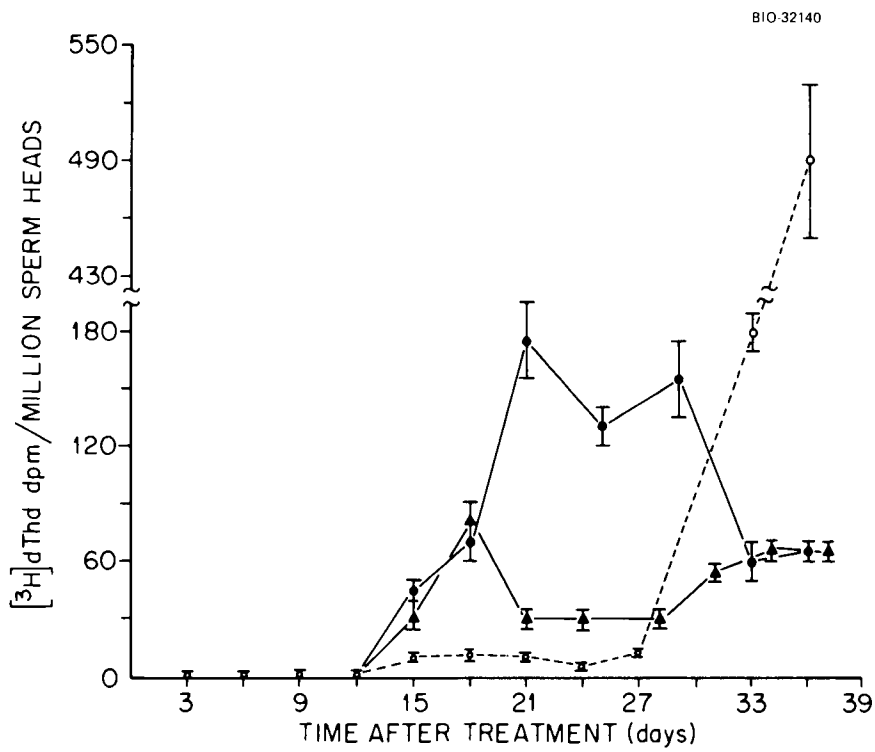

Fig. 9. Unscheduled DNA synthesis in various germ-cell stages of male mice treated *in vivo* with cyclophosphamide (▲), mitomen (●), and saline (control, (○). The presence of [^{3}H]dThd was measured in sperm heads obtained from the vasa deferentia.

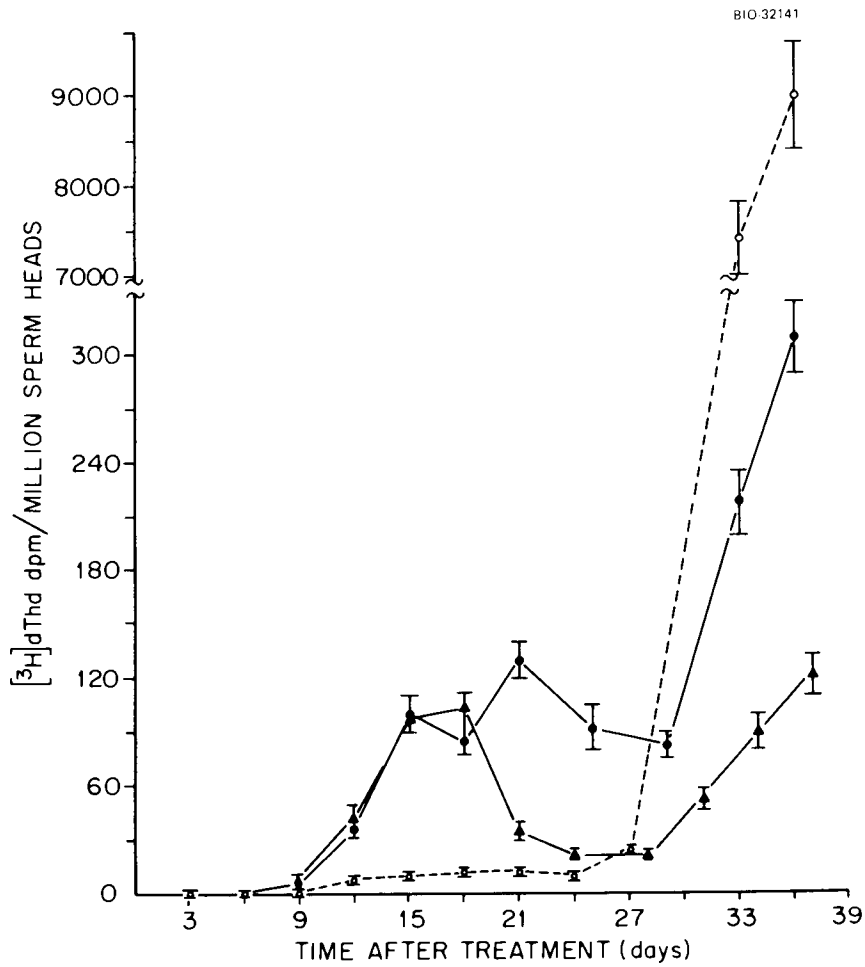

Fig. 10. Unscheduled DNA synthesis in various germ/cell stages of male mice treated *in vivo* with cyclophosphamide (▲), mitomen (●), and saline (control, ○). The presence of [^{3}H]dThd was measured in sperm heads obtained from the caudal epididymides.

AN AUTORADIOGRAPHIC STUDY OF UNSCHEDULED DNA SYNTHESIS IN VARIOUS GERM-CELL STAGES OF MALE MICE EXPOSED *IN VIVO* TO METHYL METHANESULFONATE AND X RAYS

R. E. Sotomayor, G. A. Sega, and R. B. Cumming

It has recently been shown that various germ-cell stages of male mice that normally do not exhibit the capacity of DNA synthesis do undergo UDS when treated *in vivo* with chemical mutagens.[1-3] We have undertaken a similar study, using autoradiography instead of liquid scintillation counting to measure the unscheduled incorporation of [^{3}H]dThd in the germ cells. Autoradiography has some advantages over liquid scintillation counting, such as: (*a*) a direct examination of various germ-cell stages undergoing UDS is possible; (*b*) analysis of the pattern of labeling of chromosomes at diakinesis may provide information about the randomness of the UDS with regard to the genome; (*c*) information about the UDS response of cells from the same germ-cell stage can be obtained.

Figures 11 and 12 show the grain counts per nucleus of different germ-cell stages from treated and untreated mice (control). A minimum of 100 cells were examined in each case. In Fig. 11 most of the treated germ-cell stages show unscheduled incorporation of [^{3}H]dThd well above control values. Midpachytene stages showed the greatest labeling, two to four times that of other treated spermatocyte stages and over 10 times that of the respective controls. A study of the distribution of the label on

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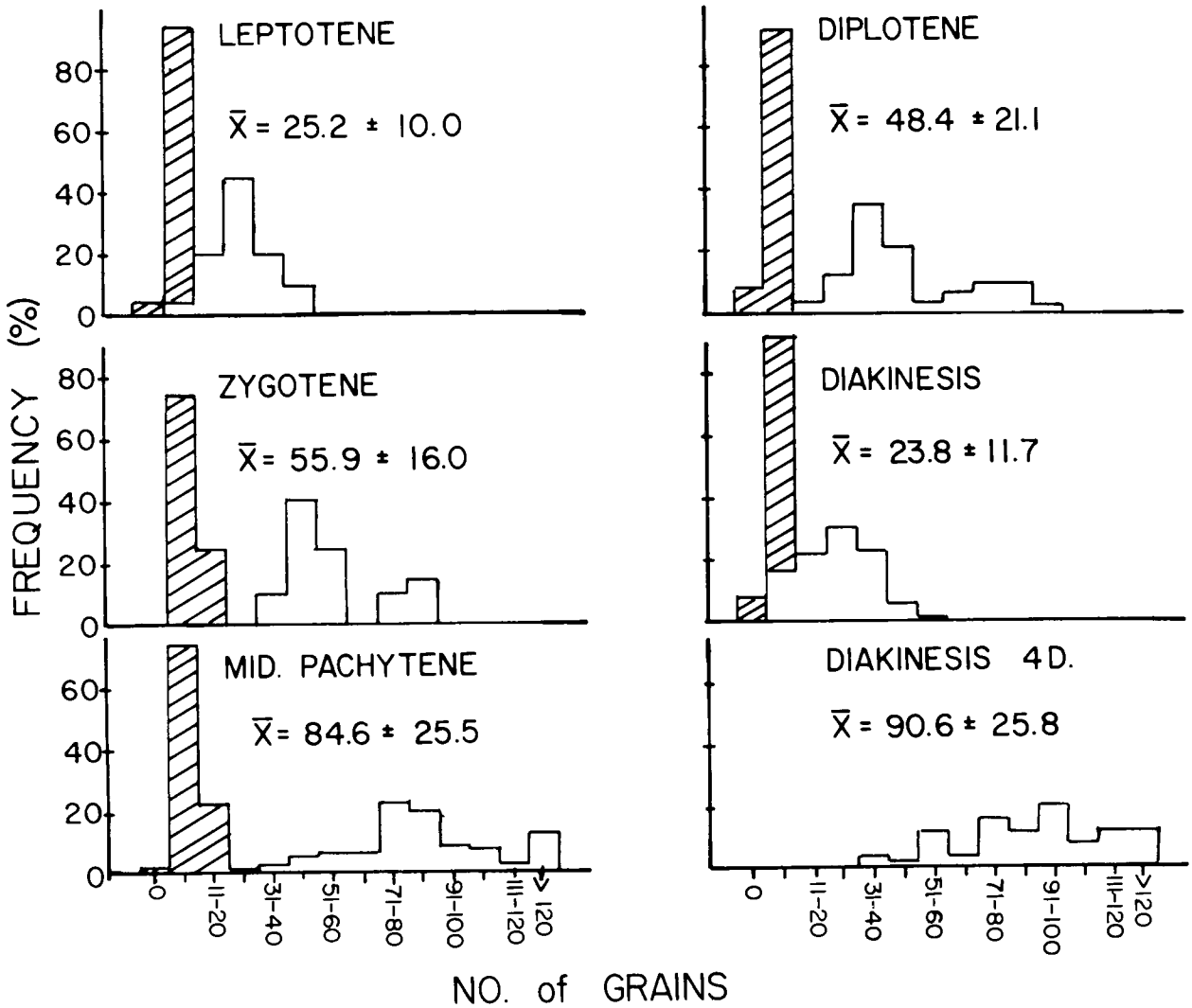

Fig. 11. Grain counts per nucleus in various stages of meiosis of male mice following *in vivo* treatment with 100 mg/kg of MMS (white bars) or with saline control (cross-hatched bars). Slides were exposed for 1 month.

individual bivalents at diakinesis 0 and 4 days after treatment indicates that UDS is randomly distributed with regard to the genome.

Individual nuclei from the same germ-cell stage showed high variability in the labeling. This phenomenon may have several explanations, one of which is that the mutagen is not equally distributed among the cells. Further investigation of this point is needed. In Fig. 12 it is shown that early spermatid stages (round and some elongating spermatids) undergo UDS, while most late and mature spermatid stages do not. The classification shown in Fig. 12 is arbitrary and is based on the nuclear morphology of various spermatid stages.

Preliminary results with X rays show a somewhat different picture, at least in some respects. Table 12 shows some of these results. In general, the same germ-cell stages that undergo UDS after MMS treatment also exhibit UDS with X rays. The main difference is that some chromosomes at diakinesis showed accumulation of grains, suggesting a nonrandom distribution of UDS. This experiment is being repeated to check these results.

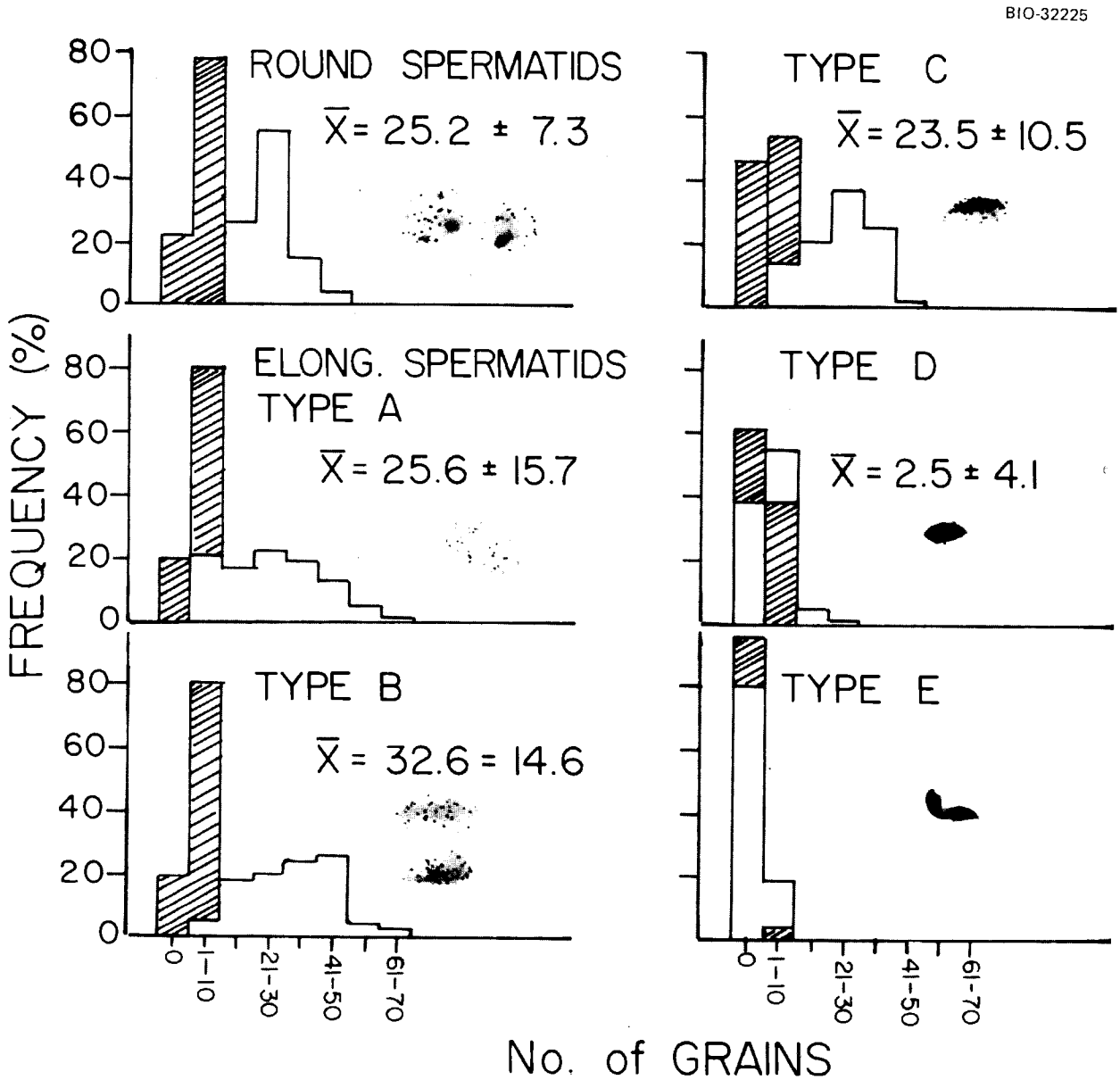

Fig. 12. Grain counts per nucleus in various stages of spermiogenesis after *in vivo* treatment of mice. MMS-treated (white bars), saline control (cross-hatched bars.)

Table 12. Distribution of grains over nuclei in various germ-cell stages of male mice exposed *in vivo* to 600 R of X rays
Slides were exposed for 3 months.

Grain distribution (grains per nucleus)	No. of cells			
	Pachytene	Diakinesis	Round spermatids	Testicular sperm
0	0	0	0	55
1-20	8	9	9	45
21-40	44	23	70	0
41-60	20	14	21	0
61-80	9	14	0	0
81-100	0	14	0	0
101-120	2	9	0	0
>120	17	17	0	0
Total No. of cells	100	100	100	100
Background grains (mean ± standard deviation)	15 ± 7	20 ± 7	3.5 ± 2	2 ± 2

So far the results obtained using autoradiography confirm previous ones using a different technique to measure UDS and different chemical mutagens.[1-3] Particularly it supports the finding that the most mature stages of mouse spermiogenesis lack the capacity of UDS or DNA repair.

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3. G. A. Sega, J. G. Owens, and R. B. Cumming, *Mutat. Res.*, in press.

TIMING OF SPERM PENETRATION, PRONUCLEAR FORMATION, PRONUCLEAR DNA SYNTHESIS, AND FIRST CLEAVAGE IN NATURALLY OVULATED MOUSE EGGS

M. Krishna* and W. M. Generoso

The early work of L. B. Russell[1-3] with X rays on induction of chromosomal aberration in postcopulation germ cells and zygotes of mice clearly demonstrated the importance of these studies in mammals and led to similar studies by Suter[4] and by Suter and Generoso[5] with alkylating chemicals. They found that the relative sensitivities of the various postcopulation stages differ depending upon the alkylating chemical used, and they inferred that these sensitivity differences can be related to the occurrence of pronuclear DNA synthesis. All published information on the timing of pronuclear DNA synthesis was obtained from eggs ovulated artificially through the use of hormone treatment.[6,7] The possibility exists that the timing of development of penetrated eggs is affected by such hormonal treatment. Our group is presently conducting fundamental research on the induction of chromosomal aberrations in mouse postcopulation stages. Obviously, in designing such studies and for proper interpretation of the genetic results, it is essential to have a full understanding of the timing of development of normally ovulated mouse eggs from fertilization to first cleavage, particularly with respect to the onset and duration of DNA synthesis.

Studies were conducted on virgin (C3H × C57BL)F_1 females, 10 to 12 weeks old, mated to (SEC × C57BL)F_1 males. Ovulation was partially synchronized by maintaining females on a short dark phase (2:00 to 7:00 AM daily) for at least 5 weeks prior to the start of an experiment. To limit the period of sperm entry, two females were caged with each male for only 20 min during an interval following the arrival of eggs in the ampulla. After this period, females were immediately separated from males and examined for presence of vaginal plugs. Sperm entry and pronuclear formation were studied in eggs that were collected 0.5 to 10.5 hr after mating. DNA synthesis was studied in pronuclei of eggs that were collected 7 to 16 hr after mating and incubated at 37°C for 1 hr in Whitten's mouse ova medium[8] containing 100 μCi/ml of [^{3}H]dThd (specific activity, 17 Ci/mmol). Uptake of [^{3}H]dThd was determined by autoradiography. Analysis of first cleavage was conducted on Fallopian tubes that were fixed 17 to 20 hr after mating and prepared histologically.

Totals of 819 eggs from 98 mated females, 879 eggs from 120 mated females, and 203 embryos from 21 females were analyzed for timing of sperm entry and pronuclear formation, pronuclear DNA synthesis, and first cleavage, respectively. Sequential development of mouse zygotes from sperm entry to first cleavage is shown in Fig. 13.

Evidence of sperm penetration (presence of one or more sperm in perivitelline space or inside the vitellus) was found beginning at 1.75 hr and peaking at about 4 hr after the end of the mating period. Pronuclei were first observed in eggs examined 4.5 hr after mating. The peak of pronuclear formation occurred more than 7.5 hr after mating. If one considers the time of first appearance as well as the time when the incidences reached the peak, it appears that sperm entry precedes pronuclear formation by 3 to 3.5 hr.

[^{3}H]dThd-labeled pronuclei were observed at all intervals studied, but at 8 and 16 hr after mating only 1.96 and 2.27% of eggs, respectively, were labeled. Thus, effectively, the pronuclear DNA synthesis began at 9 hr after mating, reached a peak at 12 hr, and terminated at 16 hr. It is estimated, by considering the time of first appearance as well as the time of peak incidence, that DNA synthesis follows pronuclear formation by 3 to 4 hr and sperm entry by about 6.5 to 8 hr.

At 17 hr after mating, 52% of the eggs were already undergoing their first mitotic division and were observed to be at either metaphase or anaphase stage. At 18 hr, 26% of the eggs had completed first cleavage, and by 20 hr almost all were two-cell embryos.

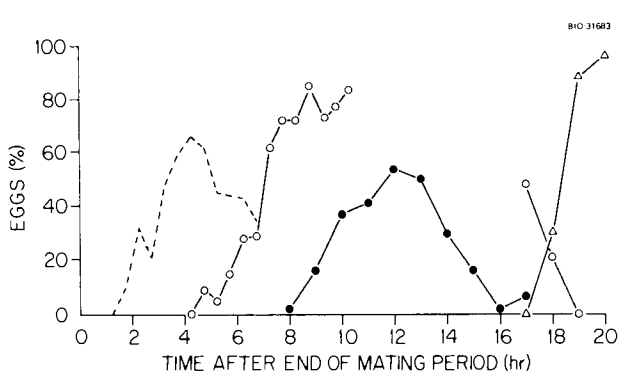

Fig. 13. Sequential development of mouse zygotes from sperm entry to first cleavage. Sperm entry and pronuclear eggs are indicated at the midpoint of the egg-collection interval, except at 17, 18, and 19 hr after mating. (--), sperm entry; (○), pronuclear eggs; (●), pronuclear DNA synthesis; (△), first cleavage completed.

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

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X-RAY INDUCTION OF HERITABLE TRANSLOCATIONS IN MOUSE DICTYATE OOCYTES*

M. Krishna† and W. M. Generoso

The rate at which heritable translocations are recovered among first-generation progeny following irradiation of dictyate oocytes has not been clearly established. On the one hand, Russell and Wickham[1] and Gilliavod and Leonard[2] did not find any increase in the frequency of translocation heterozygotes among male progeny of X-irradiated females (320 and 185 male progeny were scored, respectively). Searle and Beechey,[3] on the other hand, claimed that although they, too, did not detect transmissible translocations among male progeny from X-irradiated oocytes, induced translocations were recovered among female progeny (3 confirmed translocations in 294 female progeny tested). They did not find the same effect with fission neutrons.

We are now conducting an extensive study in female mice to settle the apparent difference described above and to determine the relative sensitivity of dictyate oocytes in various stages of follicular development. A total of 288 (SEC × C57BL)F_1 females, 11 to 17 weeks old at the time of irradiation, were used in the study. They were divided equally into four groups and exposed to 300 R of X rays (whole body) delivered at 68 rads/min (250 kV; half-value thickness, 0.75 mm of copper). Females from the first group were caged with males immediately after

irradiation, while females from the three other groups were caged with males on the 5th, 11th, and 15th day after irradiation. In all groups, each female was caged individually with a normal (101 × C3H)F_1 male. They were kept together until the female ceased to produce young. With the radiation dose used, 24 females from the first group and 12 females from the second group (caged on the 5th day after irradiation) produced two litters, while females in the two other groups produced only one litter. A total of 317 litters were born, and 884 male and 1005 female progeny were weaned. Testing for translocation heterozygosity of all male and female progeny is now in progress.

*Research sponsored jointly by the National Center for Toxicological Research and by the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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

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SENSITIVITY OF METAPHASE II OOCYTES OF MICE TO X-RAY INDUCTION OF DOMINANT-LETHAL MUTATIONS AND HERITABLE TRANSLOCATIONS

W. M. Generoso, S. W. Huff, and K. T. Cain

In mice, the rate at which reciprocal translocations induced in dictyate oocytes by X irradiation are recovered among the first-generation progeny has not yet been clearly established. For this reason, our group is currently conducting an extensive study to settle this point.[1] We are also studying the inducibility and recovery of reciprocal translocations in oocytes that are irradiated after ovulation but before sperm entry, the subject of this report. The oocyte is at metaphase II stage at the time of ovulation (the chromosomes are considerably more condensed than at dictyate) and is arrested at this stage until the time of sperm penetration. The early work of Russell[2-4] showed that the metaphase II oocytes have high sensitivity to X-ray induction of chromosome breakage which results in embryonic lethality and sex-chromosome loss.

All males, (SEC × C57BL)F_1, and females, (C3H × C57BL)F_1, that were used in the study were placed on a short dark phase (2:00 to 7:00 AM daily) for at least 5 weeks prior to the start of an experiment. Because the mouse has a 4-day estrous cycle, each experiment was conducted on females that were mated during a 4-day period. For the first 3 days (series I), two females per male were caged for 20 min beginning at 9:00 AM. At the end of this period, females were separated from males and checked for presence of vaginal plugs. Mated females were then exposed (partial body) to either 100 or 50 R of acute X irradiation at 9:50 AM. From our earlier work on (C3H × C57BL)F_1 females,[5] we determined that the ovulated eggs were already in the ampulla in the great majority of females at 9:00 AM and that at 9:50 AM, sperm penetration had not yet started. On the 4th day (series II), the remaining unmated females were irradiated with the same dose of X rays as before at 9:50 AM and then caged immediately with the males for 1 hr thereafter. Thus in series I both male and female genomes were irradiated, while in series II only the female genome (at metaphase II stage) was irradiated. The relative sensitivity of the male and female genome was compared for induction of dominant-lethal mutations and heritable translocations.

Results of the dominant-lethal studies are shown in Table 13. At the dose of 50 R there appears to be no difference between the results of series I and II, while at the dose of 100 R, slightly higher effects were found in series I. This suggests that the female genome is considerably more sensitive than the male genome to X-ray induction of dominant-lethal mutations when irradiated shortly after mating but before sperm penetration, when the oocytes are at metaphase II stage. Preliminary data on heritable translocations (obtained for 100 R) seem to show a diametrically opposite situation. In series I, out of 268 male progeny tested, 11 were partially sterile (4.1%) and 3 were sterile. In series II, out of 150 male progeny tested, 1 was partially sterile (0.7%) and 2 were sterile. All partially sterile males were confirmed cytologically as translocation heterozygotes, but cytological testing of complete steriles has not yet been completed. The heritable translocation study is continuing as more male, as well as female, progeny are being tested for translocation heterozygosity. It is the purpose of this study to compare the rate of recovery of heritable translocations induced by X radiation in dictyate and in metaphase II oocytes.

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Table 13. X-ray induction of dominant-lethal mutations in metaphase II oocytes of mice

Dose (R)	Experiment number	Series[a]	No. of mated females	No. of pregnant females	Average No. of implants among fertile females	Average No. of embryos among fertile females	Dead implants (%)
50	1	I	38	38	9.9	7.3	26
		II	28	25	10.7	8.8	18
	2	I	29	27	9.6	8.0	17
		II	23	21	9.4	7.5	20
	3	I	43	40	9.5	7.7	19
		II	24	23	8.4	7.0	17
100	4	I	52	49	9.5	4.8	49
		II	48	41	10.6	6.6	38
	5	I	37	33	9.2	4.9	47
		II	35	33	9.4	5.5	42
	6	I	29	29	9.4	5.2	45
		II	33	31	9.3	5.6	40

[a]Series I: females were irradiated shortly after mating and before sperm entry; series II: females were irradiated immediately prior to mating.

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CONTINUING STUDIES ON X-RAY INDUCTION OF HERITABLE TRANSLOCATIONS IN MOUSE SPERMATOGONIA

W. M. Generoso, K. T. Cain, and S. W. Huff

We have been studying the effects of various conditions of radiation exposure on the induction of heritable translocations in mouse spermatogonial stem cells. Our first report on this was on dose effects of acute X irradiation.[1] In this study, doses of 150, 300, 600, and 1200 R were used. We found that the frequencies of reciprocal translocations at all doses of X rays were significantly higher than the spontaneous level. The slope of the dose-effect curve is clearly "humped," as the highest frequency, which was observed at 600 R, was significantly higher than those observed at 300 or 1200 R. The results also brought up two points that need to be settled more firmly. First, although the frequency observed at 300 R (9 translocations in 1018 progeny tested) appears to be slightly higher than that observed at 150 R (6 translocations in 993 progeny tested), the difference is not significant. And second, with the exception of 150 R, for which the observed frequency is similar to that expected on the basis of cytological observation, the frequencies observed for all doses are significantly lower than those expected. Whether these two situations are correct or not can be settled only by testing more progeny for translocation heterozygosity at the 150- and 300-R doses. Accordingly, we are presently testing an additional 2159 male progeny for the 150-R dose and collecting additional male progeny for the 300-R dose.

The second condition of radiation exposure we are studying is that of dose fractionation. Although two 600-R exposures to X rays given 8 weeks apart to male mice had been used for induction of heritable translocations in spermatogonia,[2] there has been no serious attempt to study the effects of dose fractionation on induction of heritable translocations until now. Because we already have information on 600- and 1200-R single exposures, we started this study by using 600-plus-600-R doses given 8 weeks apart to

males. Testing for translocation heterozygosity is now under way on 1311 male progeny.

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CHROMOSOMAL ABERRATION EFFECTS OF POLYCYCLIC HYDROCARBON COMPOUNDS IN MALE AND FEMALE GERM CELLS*

W. M. Generoso, K. T. Cain, and S. W. Huff

Polycyclic hydrocarbons are a group of compounds that have to be dealt with in assessing the health hazard of large-scale coal liquefaction and gasification operations as well as in the use of various products from these processes. There are many reports which indicate that certain polycyclic hydrocarbon compounds are mutagenic in various submammalian test systems. On the other hand, there is very little information available on the mutagenic effectiveness of this class of compounds in mammalian germ cells. Accordingly, our group has embarked on a research program to correct this situation (see separate reports of W. L. Russell, L. B. Russell, and G. A. Sega). This report is on the progress of our study on chromosomal aberration effects in male and female germ cells of polycyclic hydrocarbon compounds. The first compound under investigation is BP.

BP was tested for dominant-lethal effects in (101 × C3H)F_1 males and for total reproductive capacity effects in (SEC × C57BL)F_1 females. In both studies the animals were used when they were about 12 weeks old. BP was dissolved in corn oil and administered intraperitoneally in a maximum of 0.5 ml volume. Control mice were given a comparable volume of corn oil. An acute toxicity study for BP was conducted in the beginning, and in males a single dose of 500 mg/kg was found to be the highest that could be given with few deaths occurring within 30 days after treatment. Of 32 males treated, only one died within this period.

The dose of 500 mg/kg of BP affected the mating ability of treated males, particularly in the 2d through the 4th week after treatment, as fewer matings occurred during this period. This dose of BP induced dominant-lethal mutations in treated spermatozoa (matings that occurred 4.5 to 7.5 days after treatment) as shown in Table 14. There is also a strong

Table 14. Dominant-lethal effects of BP in male mice

Treatment	Treatment-to-fertilization interval (days)	No. of mated females	No. of pregnant females	Average total No. of implants among fertile females	Average No. of living embryos among fertile females	Dead implants (%)
BP, 500 mg/kg	0.5–3.5	26	26	7.5	6.4	14.4
	4.5–7.5	16	14	6.4	4.7	25.8
	8.5–11.5	21	20	7.5	6.9	7.4
	12.5–15.5	30	25	8.0	7.5	6.5
	16.5–19.5	20	18	7.2	6.3	12.3
	20.5–23.5	37	29	7.0	6.6	6.4
	24.5–27.5	26	22	6.6	6.3	4.8
	28.5–31.5	21	17	6.8	6.5	4.3
	32.5–35.5	17	16	6.6	5.4	18.1
	36.5–39.5	25	22	6.2	5.7	8.8
	40.5–43.5	13	8	6.3	5.5	12.0
Control	0.5–3.5	37	37	8.1	7.5	6.7
	4.5–7.5	25	25	7.6	7.2	4.8
	8.5–11.5	40	38	7.5	6.8	9.1
	12.5–15.5	36	33	7.9	7.3	7.7
	16.5–19.5	33	32	7.6	7.2	6.1
	20.5–23.5	27	27	7.5	7.0	7.4
	24.5–27.5	27	27	7.2	6.4	11.3
	28.5–31.5	26	24	6.8	6.2	8.0
	32.5–35.5	30	27	6.6	6.1	7.8
	36.5–39.5	31	27	6.9	6.1	10.8
	40.5–43.5	11	11	6.2	5.9	13.2

indication that dominant lethals were induced in preleptotene spermatocytes and late differentiating spermatogonia (matings that occurred 32.5 to 35.5 days after treatment). Studies are now under way to verify this effect. Also, a heritable translocation experiment is now under way to see if dominant-lethal effects of BP (and polycyclic hydrocarbon compounds in general) are associated with transmissible exchanges. It is important to note in this respect that only alkylating chemicals have induced heritable translocations so far.

In the total reproductive capacity study in females, a single dose of 450 mg/kg was used. This study is not completed yet, but data obtained up to 195 days after treatment clearly showed that BP had a marked effect on the reproductive performance of treated females. Of the 30 females treated with BP, 27 produced young, while all 30 females in the control were fertile. The BP-treated females produced an average of 2.3 litters per female and 3.5 young per litter. The last litter was born within the interval 139 to 157 days after treatment. On the other hand, the control females produced an average of 8.4 litters per female and 10.9 young per litter. At present, these females are at the peak of reproduction. Studies on the nature of fertility reduction induced by BP are now under way. These constitute dominant-lethal experiments in females with lower doses and histological analysis of the ovary to look for evidence of oocyte killing.

*Research jointly sponsored by the Environmental Protection Agency and by the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

TESTS ON INDUCTION OF CHROMOSOME ABERRATIONS IN MOUSE GERM CELLS WITH SODIUM BISULFITE

W. M. Generoso, K. T. Cain, and S. W. Huff

Sodium bisulfite has been shown to be mutagenic in lambda phage,[1] *E. coli*,[2] *Saccharomyces cerevisiae*,[3] and *Micrococcus pyogenes*.[4] In both lambda phage and *E. coli*, production of mutants is thought to be by way of CG-to-TA transition. There have also been reports which suggest that chromosome aberrations were induced (*a*) by sodium sulfite in the oocytes of mice, ewe, and cow,[5] (*b*) by sulfur dioxide in human lymphocytes,[6] and (*c*) by sodium bisulfite in certain germ cells of male rats.[7] Because sulfur dioxide is a major pollutant of the air in the United States and in other industrialized countries, it is necessary to establish the degree of mutagenic effectiveness in mammalian germ cells of its tissue metabolites that were shown in various test systems to be mutagenic. We chose to test the effectiveness of sodium bisulfite in inducing chromosomal aberrations in male and female germ cells of mice.

In males, dominant-lethal and heritable-translocation studies were conducted on (101 × C3H)F_1 mice (about 12 weeks old) that were given repeated ip treatments with either 400 or 300 mg/kg of sodium bisulfite per day. The doses were given daily except on weekends and holidays. The higher dose was given 20 times during a 26-day period, while the lower dose was given 38 times during a 54-day period. Control mice were given a similar volume of distilled water. In the translocation study, each male was caged with two (SEC × C57BL)F_1 females immediately after the last injection. They were separated 1 week later, and each male was caged again with two fresh females for another week. Male progeny were weaned and tested for translocation heterozygosity. The dominant-lethal study was performed on 10- to 12-week-old (C3H × C57BL)F_1 females that were mated to treated males within $14^1/_2$ days after the last injection. In females, a dominant-lethal study was conducted on 10- to 12-week-old (C3H × 101)F_1 mice that were given a single injection of 550 mg/kg of sodium bisulfite and mated to untreated (101 × C3H)F_1 males within $4^1/_2$ days after treatment.

The doses used in the mutagenesis studies were based on results of acute toxicity studies conducted earlier. In both males and females, the highest dose that did not cause death after a single treatment was 550 mg/kg. (Doses of 500, 550, 600, 650, 700, 750, and 1000 mg/kg were tested with six mice per dose.) Of the 46 male mice that were given 400 mg/kg of sodium bisulfite per day, eight died before the start of mating, while of the 46 male mice that were given 300 mg/kg per day, five died.

Results of the dominant-lethal study in males and females are shown in Table 15. It is clear from the data that sodium bisulfite did not induce a detectable increase in dominant-lethal mutations in either male or female germ cells of mice. Similarly, there is no indication for induction of heritable translocations in the germ cells of males. No partially sterile animal was found among 858 male progeny tested (413 and 445 male progeny respectively from 400- and 300-mg/kg-per-day treatments). Thus, contrary to the reports that sulfur dioxide or its metabolites, sodium

Table 15. Dominant-lethal effects of sodium bisulfite in male and female mice

Treatment	Sex treated and stock of mice	Treatment-to-fertilization interval (days)[a]	No. of mated females	No. of pregnant females	No. of implantations (per pregnant female)	No. of living embryos (per pregnant female)	Percent dead implants
$NaHSO_3$, 400 mg/kg per day[b]	Male (101 × C3H)F_1	0.5–4.5	20	14	9.5	9.1	4.5
		5.5–9.5	27	21	10.4	10.0	4.1
		10.5–14.5	22	19	10.0	9.3	6.3
Control	Male (101 × C3H)F_1	0.5–4.5	40	32	9.6	9.1	4.6
		5.5–9.5	53	43	9.6	8.9	6.4
		10.5–14.5	42	36	10.5	9.9	5.6
$NaHSO_3$, 300 mg/kg per day[c]	Male (101 × C3H)F_1	0.5–4.5	20	14	9.9	9.3	6.5
		5.5–9.5	20	15	9.9	9.1	8.1
		10.5–14.5	20	18	9.8	9.4	4.5
Control	Male (101 × C3H)F_1	0.5–4.5	36	27	9.4	8.7	7.1
		5.5–9.5	20	16	10.4	10.1	3.0
		10.5–14.5	20	18	9.9	9.3	6.2
$NaHSO_3$, 500-mg/kg single dose	Female (C3H × 101)F_1	0.5–4.5	29	25	7.6	7.3	4.7
Control	Female (C3H × 101)F_1	0.5–4.5	32	28	7.8	7.4	5.5

[a]After the last injection.
[b]Given 20 times within 26 days.
[c]Given 38 times within 54 days.

sulfite and sodium bisulfite, induced chromosome aberrations in mammalian chromosomes, our results clearly indicate the lack of effectiveness of sodium bisulfite in inducing chromosome aberrations in mice germ cells.

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DELAYED FORMATION OF CHROMOSOME ABERRATIONS IN MOUSE PACHYTENE SPERMATOCYTES TREATED WITH TRIETHYLENEMELAMINE

W. M. Generoso, M. Krishna, R. E. Sotomayor, and N. L. A. Cacheiro

Qualitatively, there is generally no difference in measurable chromosome breakage effects in various germ-cell stages of mice between ionizing radiations and the many mutagenic alkylating chemicals. The question arises, then, as to the nature of events that lead to the formation of chemically induced chromosome aberrations in mouse germ cells and whether the mechanisms involved are similar to those for radiation. Both X radiation and TEM induce dominant-lethal mutations in pachytene spermatocytes of mice. In the case of X radiation, there is strong evidence that dominant-lethal mutations induced in this germ-cell stage arise from induced chromosome breakage.[1–4] No such information is available for TEM. Also, there is no information on the inducibility by any mutagen of heritable reciprocal translocations in meiotic spermatocytes of mice. Accordingly, the present study was conducted to determine the nature of TEM-induced dominant-lethal mutations in pachytene spermatocytes by cytological examination of diakinesis–metaphase I cells and by scoring for heritable translocations among first-generation progeny.

(101 × C3H)F_1 hybrid male mice, approximately 12 weeks old, were treated intraperitoneally with 2 mg/kg of TEM or exposed to 400 R of acute X rays (partial-body irradiation). Control mice were given comparable volumes of Hanks' balanced salt solution (HBSS). In the dominant-lethal study, analysis was

done on 10- to 12-week-old (C3H × C57BL)F_1 females mated on days 25 to 28 after treatment of males. In the heritable-translocation study, (SEC × C57BL)F_1 females mated to TEM-treated males during this posttreatment period were allowed to go to term. Female progeny were discarded, while the males were tested for translocation heterozygosity at maturity. Two groups of mice were killed for analysis of diakineses–metaphase I spermatocytes. The first group was composed only of TEM-treated males, while the second group included TEM- and X-ray-treated and control males. Two males per treatment were killed daily at intervals 3 to 7 days after treatment. The two testes of each male were prepared separately using the air-dry technique of Evans, Breckon, and Ford.[5] Twenty-five cells were scored per testis. It is assumed that TEM had no effect on the progression of spermatogenesis and that the cells that were analyzed at diakinesis–metaphase I stage are the same as those studied in the dominant-lethal and heritable-translocation experiments at the time of treatment.

Data presented in Table 16 show that the doses of TEM and X radiation used are generally comparable in causing reductions in pregnancy rate and number of living embryos. That dominant-lethal mutations were induced by the two agents is clearly demonstrated by the high frequencies of dead implantations. In the case of X rays, it is known that killing of spermatocytes, in addition to dominant lethality, contributes to litter-size reduction.[1] Similarly, the reductions in number of living embryos and pregnancy rate after treatment with 2 mg/kg of TEM is partially attributable to killing of pachytene spermatocytes. The rate of fertilization (as judged by the presence of at least one pronucleus) in females mated 25 to 28 days after TEM treatment of males was 42%, as compared with 84% for control (total eggs examined were 188 and 103, respectively).

Data on the inducibility of heritable translocations in pachytene spermatocytes by TEM clearly indicate a significantly higher frequency (13 translocations in 247 progeny tested, 5%) than the spontaneous frequency pool from previous experiments (four translocations in 4392 progeny tested, 0.091%). Of the 13 translocation heterozygotes, ten were partially sterile and three were completely sterile. All 13 were confirmed cytologically.

Results of the cytological analysis of diakinesis–metaphase I spermatocytes show that while a very high frequency of cells treated with X radiation at pachytene stage had fragments and/or interchanges (76.4%), only a small increase in aberrations was found with TEM (0.8 to 2.2%).

The results show that TEM and X radiation are alike in the sense that chromosome breakage resulted from treatment of pachytene spermatocytes. In the case of X radiation, chromosome breakage is manifested by dominant-lethal mutations and by the presence of chromosome aberrations in diakinesis–metaphase I spermatocytes. In the case of TEM, dominant-lethal mutations were also clearly induced, but analysis of diakinesis–metaphase I spermatocytes did not give proof that chromosome breakage was the underlying cause. The frequency of aberrations observed was much too low to account for all the lethals. The clear-cut proof that breakage was induced came from data on heritable translocations. The very low frequency of aberrations in diakinesis–metaphase I stage and the recovery of heritable translocations in the TEM study provide evidence for a chemical mutagen that formation of the bulk of breaks in pachytene spermatocytes of mice is delayed, a marked contrast to the more immediate formation of X-ray-induced breakage.

The present result shows that a reasonably high frequency of reciprocal translocations was recovered after treatment of meiotic spermatocytes with a chemical mutagen. This finding is significant in view of the observation from X-ray studies in mouse spermatogonia stem cells which strongly indicate that, for a hitherto unknown reason, the transmission of balanced exchanges is markedly lower than that expected on the basis of the frequency of multivalent

Table 16. Comparable dominant-lethal effects of X radiation and TEM in pachytene spermatocytes

Treatment	No. of females mated	Pregnancy rate (%)	Average No. of implants	Average No. of living embryos	Dead implants (%)
Control	76	92	10.2	9.5	6
TEM (2 mg/kg)	82	37	3.9	2.3	40
X ray (400 R)	31	29	4.0	1.3	67

association at diakinesis–metaphase I. Because of this and the fact that the interchanges induced by X radiation in pachytene spermatocytes are of the chromatid type, it is expected that, relative to dominant lethals, the frequency at which reciprocal translocations are recovered among progeny from treated pachytene spermatocytes is lower for X radiation than for TEM. On the other hand, it seems likely that the reason why heritable translocations were recovered at a frequency of 5% after TEM treatment of pachytene spermatocytes was the fact that the formation of aberrations was delayed—thus possibly similar in mechanism to TEM effects in postmeiotic stages. We are presently studying the transmission of X-ray-induced translocations in pachytene spermatocytes.

The question, which brings into focus the mechanism for aberration formation, is, When does formation of aberrations actually occur after TEM treatment of pachytene spermatocytes? Studies are now under way to obtain direct genetic evidence for the time of formation of aberrations after chemical treatment of various male germ-cell stages and to relate the genetic information to molecular mechanisms of aberration formation.

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ECO R1 DIGESTION OF MOUSE LIVER NUCLEI

E. G. Bernstine

Intact liver nuclei from male SEC mice were purified by sedimentation through 2.3 *M* sucrose followed by repeated washing in 0.25 *M* sucrose containing 50 m*M* Tris-HCl pH 7.5, 25 m*M* KCl, 10 m*M* $MgCl_2$, and 0.15 m*M* ethylene glycol bis(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid (EGTA). Digestion of these nuclei was carried out at 37° C in the same solution containing 25 m*M* NaCl and an amount of Eco R1 sufficient to completely digest four times the amount of phage λ DNA that was present in the nuclei under similar conditions. DNA was then extracted from treated and control nuclei by a very gentle procedure (devised by Berns and Thomas) involving treatment with pronase and sodium dodecyl sulfate (SDS) followed by gentle rolling with phenol. The results of neutral sucrose gradient analysis on these DNAs gave a weight-average molecular weight for control DNA of 1.4×10^8, while that of DNA isolated from treated nuclei is 4×10^6. Little difference is observed between the size distributions of DNA isolated from untreated nuclei before and after incubation at 37° C if the nuclei are sufficiently prewashed and kept in the presence of EGTA.

Extraction of digested nuclei in solutions of very low ionic strength (2×10^{-4} *M* EDTA) under a variety of conditions allows only 7 to 10% of the chromatin to be released into a low-speed supernatant fraction (15 min at 3000 *g*), as will be discussed below. Suspension of the digested nuclei in buffered 0.65 *M* NaCl, however, releases over 95% of the chromatin in soluble form (not pelleted at 12,000 *g* for 30 min). This procedure extracts the lysine-rich histone H1. When the supernatant fraction of the 12,000 *g* centrifugation is layered over a 5-to-20% sucrose gradient (containing 0.65 *M* NaCl) and centrifuged for 160 min at 25,000 rpm (SW 27 rotor), about 9% of the chromatin is recovered in a pellet. This fraction contains 24% satellite DNA, as compared with 7% in the unfractionated extract, determined by equilibrium banding in neutral CsCl in the analytical ultracentrifuge.

This result is certainly encouraging, but it does not achieve complete purification. Furthermore, most of the satellite-containing chromatin does not pellet. The reason for this is likely to be aggregation into complexes of very high frictional coefficient. Gel filtration on agarose beads has, in fact, provided evidence that there is extensive aggregation. This problem must be overcome if the yield, as well as the quality of the separation, is to be improved. It is also clear that changes should be made in the centrifugation procedure so that the formation of a pellet is avoided. This will increase the chances of getting a cleaner fraction.

As mentioned above, resuspension of digested nuclei in 0.2 m*M* EDTA will release 7 to 10% of the chromatin into a low-speed supernatant fraction. These fragments are excluded from columns of A-150M although the weight-average molecular weight of the DNA in them is 4.2×10^6 by analysis on sucrose gradients, thus implying aggregation. The amount of chromatin released into 0.2 m*M* EDTA as a function of time of incubation and Eco R1

concentration reaches a plateau (corresponding to about 10% of the total) in each case. The addition of fresh Eco R1 after 30 min of incubation results in no further release.

These fragments contain all five histones and a variety of nonhistone chromosomal proteins in a ratio of about 4:1 as determined on SDS polyacrylamide gels. DNA purified from this chromatin can be further digested with Eco R1, indicating that, as expected, many of the Eco R1 sites are not accessible to the enzyme during the *in situ* digestion. The released chromatin itself is not further digested by Eco R1.

It is of interest that no satellite DNA is detectable in this chromatin. First indications of this came from renaturation experiments and preparative Ag^+-C_2SO_4 gradients. In order to increase the sensitivity of detection, experiments were performed that preenriched for satellite DNA in fractions that were then examined in the analytical ultracentrifuge in neutral CsCl. In no case was satellite DNA observed.

I now have a fraction of chromatin that contains 24% satellite DNA and another that contains an undetectable amount (lower limit detectable is about 0.4%). While the actual definition of the protein composition of satellite-containing heterochromatin must await its complete purification, it should now be possible to determine whether there are proteins unique to this portion of the mouse genome by comparing the two fractions.

MUTAGENICITY ASSAY OF FRACTIONATED COAL-CONVERSION PRODUCTS*

J. L. Epler, A. A. Hardigree, J. A. Young, W. Winton, and T. K. Rao
with
M. R. Guerin[†], I. B. Rubin[†], C. H. Ho[†], and B. R. Clark[†]

We have extended the initial work on the crude product from a coal liquefaction (syncrude/COED) to subfractionation and have identified the potential genetic hazards with the Ames system. The most active fractions appear to be the neutrals and the basic (ether-soluble) components prepared by liquid-extraction procedures. Parallel identification work by the Analytical Chemistry Division has been carried out, and a selected group of polycyclic compounds involved has been assayed and evaluated for mutagenicity. The crude-product assays have been extended to the separator liquor components of the same process, again using the coupled analytical-biological assay approach.

Similarly, parallel studies with fractionated materials have been initiated with the Synthoil process (liquefaction), the Synthane process (gasification), and the shale oil process. Primary fractions of various steps or materials for these processes have been prepared and assayed for potential genetic damage.

Preliminary assays in "higher tier" systems have been attempted and extended to model compound studies. Major problems of toxicity and the proper route of administration of crude fractions have compounded the work in higher organisms. Concomitant toxicity and potential masking of genetically hazardous materials are also definite drawbacks to even the bacterial mutation assays.

Lower-tier assays have been initiated on all processes. Higher-tier assays are plagued with technical difficulties. Parallel analytical work is continuing. Work on purified compounds is on schedule but is hampered by enzymatic uncertainties in metabolic activation. The applicability of genetic results in the Ames system to higher organisms (and to carcinogenesis) is still in question.

*Research sponsored jointly by the Environmental Protection Agency and the Energy Research and Development Administration under contract with the Union Carbide Corporation.

[†]Analytical Chemistry Division.

IDENTIFICATION OF MUTAGENIC COMPOUNDS SUSPECTED TO OCCUR IN THE EFFLUENTS OF COAL CONVERSION PROCESSES*

T. K. Rao, J. L. Epler, and J. A. Young

In an effort to gather preliminary information on the potential genetic hazards of the existing or proposed energy generating or conversion systems, we have begun a correlated analytical and genetic analysis of the effluents from a number of processes. In a preliminary screen of the analytically fractionated samples from the coal conversion process, Rubin *et al.*[1] have reported a relatively high mutagenic activity in the basic and neutral fractions. It has been known for a long time that the tumorigenic nature of coal tars is due to the presence of a large quantity of polycyclic aromatic hydrocarbons, which are also suspected to be the main constituents of the basic and neutral fractions.

Two bacterial systems are currently being used to identify mutagenic compounds identified or suspected to occur in the fractionated effluents of the coal conversion processes.

Mutagenicity was assayed using a group of well-characterized histidine-requiring mutants of *Salmonella typhimurium* (TA-1537 and TA-98, frame-shift mutants; TA-100, base-substitution mutant, obtained from Dr. Bruce N. Ames[2]). The assay detects genetic damage induced by chemicals by reversion of these specific strains to a prototrophic condition. In order to incorporate mammalian metabolic activation of these compounds, homogenates of rat liver induced with Aroclor-1254 were applied along with the compound in the standard plate assay.

Another bacterial test system is being developed to reconfirm the observations made with *Salmonella* strains. A derivative of *E. coli* (K-12/$galR_{18}^{S}$/arg_{56}/nad_{113}), strain 343/113 (obtained from Dr. G. Mohn[3]) is being used in which mutations in several genes can be detected simultaneously by plating on various selective media. The mutation screening includes reversion of two auxotrophic markers for arginine or nicotinic acid and forward mutation leading to the resistance to 5-methyltryptophan and gal^{r} phenotype. Other types of damage to DNA such as deletions can be detected by selecting for gal^{+} lys^{-} mutants. Metabolic activation by mammalian organ extracts can also be incorporated into these assays.

Preliminary results implicate chemicals occurring in the basic (ether-soluble and -insoluble) and the neutral fractions as potential genetic hazards. The compounds that exhibited mutagenic activity include polyaromatic hydrocarbons such as benzopyrenes, benzanthracenes, benzofluorenes, chrysenes, and fluoranthene, along with aromatic amines such as α-naphthylamine, acridines, and aminoanthracenes (see Table 17).

*Research sponsored jointly by the Environmental Protection Agency and the Energy Research and Development Administration under contract with the Union Carbide Corporation.

Table 17. Mutagenic activity of coal fraction constituents (predicted or identified)

Compound	Fraction	Strain	Revertants/nmol[a]	Mutagen
Anthracene	Neutral	TA-100	—	—
Benz[*a*]anthracene	Neutral	TA-100	1.6	++
Benz[*b*]anthracene	Neutral	TA-100	3.7	++
7,12-Dimethylbenz[*a*]anthracene	Neutral	TA-100	1.4	++
1,2,3,4-Dibenzanthracene	Neutral	TA-100	53.4	++
1,2,5,6-Dibenzanthracene	Neutral	TA-100	3.1	++
Pyrene	Neutral	TA-1537	0.15	±
Benzo[*a*]pyrene	Neutral	TA-98	7.8	++
Chrysene	Neutral	TA-100	3.6	++
Fluoranthene	Neutral	TA-98	0.11	±
Phenanthrene	Neutral	TA-98	0.07	±
Fluorene	Neutral	TA-100	—	—
9-Methylfluorene	Neutral	TA-100	—	—
2,3-Benzofluorene	Neutral	TA-100	3.1	++
9-Methylanthracene	Neutral	TA-98	0.12	±
2-Aminoanthracene	Neutral	TA-100	23.1	++
Biphenyl	Neutral	TA-100	—	—
Diphenylamine	Neutral/base	TA-100	—	—
Isoquinoline	Base	TA-100	—	—
Acridine[b]	Base	TA-1537	0.11	+
Carbazole	Neutral/base	TA-100	—	—
α-Naphthylamine	Base	TA-100	0.57	+
2,6-Dimethylquinoxaline	Base	TA-100	—	—
2,3-Dimethylquinoxaline	Base	TA-100	—	—
Hydroquinone	Weak acids	TA-100	—	—

[a]Determined from plate assay, including metabolic activation with Aroclor-induced liver.

[b]Metabolic activation was not necessary.

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COMPARATIVE MUTAGENESIS OF QUINOLINE COMPOUNDS

J. L. Epler, F. W. Larimer, A. A. Hardigree, T. Ho, and T. K. Rao

In the study of a large group of heterocyclic quinoline derivatives, 8-aminoquinoline has been shown to be an effective frame-shift mutagen specific for *Salmonella* strain TA1537. For optimum effect, metabolic activation (rat liver, phenobarbital-induced) is required. Such treatment yields a mutagenic index of 7.2 revertants/nmol. Comparative studies have been carried out in yeast, human leukocytes, and *Drosophila*. Although effective in the bacterial reverse-mutation assay and also positive in the "repair assay," 8-aminoquinoline shows little effect on forward mutation at the *CAN1* locus of *Saccharomyces cerevisiae* or on mitotic gene conversion in the D4 yeast system. Only a twofold increase of chromosomal aberrations was observed in the leukocyte system. A slight increase (1.53% vs 0.19% control) in sex-linked recessive lethals was encountered in the *Drosophila* system. It was suggested that 8-aminoquinoline has diagnostic specificity for strain TA1537 of the *Salmonella* tester set devised by Ames.

COMPARATIVE MUTAGENESIS: YEAST SYSTEMS

F. W. Larimer and J. L. Epler

Work in yeast mutagenesis has focused on two areas, implementation of existing test systems and their verification and development of new testing methodology to improve sensitivity and overcome technical deficiencies.

We now have the capacity to monitor three genetic end points in *Saccharomyces cerevisiae*: mitotic recombination (strain D3), mitotic gene conversion (strain D4), and forward mutation at the *CAN1* locus (to canavanine resistance). These systems have been characterized using a number of known chemical mutagens and are routinely utilized. In addition, a highly sensitive assay of the induced-mutation *rate* has been established which is suitable for the study of chronic low-dose effects. These assay systems have been applied to testing quinoline derivatives in the general testing scheme.

Technological development has centered on improving the sensitivity of the existing systems by the inclusion of a DNA excision repair deficiency. This modification has been introduced into the mitotic recombination assay and the forward mutation and mutation rate schemes. The mitotic recombination assay has been further upgraded by basing it on the homozygosis of a canavanine-resistance marker, which allows the positive selection of recombinants rather than requiring visual inspection for sectored colonies using strain D3. In addition to these improvements, we hope to incorporate an improved metabolic activation scheme into the chemical testing protocol.

The yeast system acts as a comparative and validating assay for parallel bacterial testing, with the additional advantages of possessing the organized eukaryotic genome, where assays can encompass chromosomal effects and organelle effects as well as locus mutation.

MUTAGENICITY TESTING: EVALUATION OF SISTER-CHROMATID EXCHANGE TECHNIQUE

T. Ho, R. J. Preston, and J. L. Epler

In the past two years a technique has been developed for identifying sister-chromatid exchanges much more simply and efficiently than by the autoradiographic method.[1,2] The method utilizes the fact that a fluorescent strain, Hoechst 33258, binds to thymidine-containing DNA but not, or far less efficiently, to BrdUrd-substituted DNA. This means that the order of fluorescence would be brightest for DNA unreplicated in BrdUrd, intermediate for DNA after one round of replication in BrdUrd, and least for DNA following two rounds of replication in BrdUrd. Thus a sister-chromatid exchange can be seen as a switch of fluorescence pattern at the point of exchange. Perry and Wolff[2] combined Hoechst staining with Giemsa staining so that the brightly fluorescing regions stain darkly with Giemsa, and the dully fluorescent regions hardly stain at all.

Perry and Evans[3] utilized this method to show that analyzing the frequencies of sister-chromatid exchanges provides a very sensitive means of screening for effectiveness of chemical mutagens or potential mutagens in a Chinese hamster cell line.

Table 18. Number of sister-chromatid exchanges per cell in human and hamster cells following treatment with TEM or EMS

	Control	TEM ($1 \times 10^{-6} M$)	EMS ($1 \times 10^{-4} M$)	EMS ($1 \times 10^{-3} M$)
Chinese hamster cells	7.6	22.9	11.2	18.2
Human leukocytes	5.3	22.5	—	—

▼

We have recently begun evaluating the possible usefulness of this technique as an addition to the mutagenicity testing program. It is much simpler to analyze sister-chromatid exchanges than to score chromatid and chromosome aberration, and exchange analysis is more accurate in many ways because of the high frequencies of sister-chromatid exchanges even at low doses of unknown mutagens. Of course, this does not remove the need to conduct experiments to measure aberration frequencies, but it might suggest which compounds should be studied in such a way.

The results obtained to date are for known chemical mutagens, triethylenemelamine (TEM) and ethyl methanesulfonate (EMS). Table 18 shows a comparison of the frequency of sister-chromatid exchanges in control cells with that induced by $1 \times 10^{-6} M$ TEM in Chinese hamster cells (CHO) and human leukocytes and by $1 \times 10^{-4} M$ and $1 \times 10^{-5} M$ EMS in Chinese hamster cells. It is apparent that even at the low dose of TEM used, there is a considerable difference in the frequency of sister-chromatid exchanges from the controls for both human and hamster cells. The data with EMS are less convincing, but there is still a clear difference at $1 \times 10^{-3} M$.

We propose to extend this evaluation and also to begin to use the technique as part of the testing program, as even these preliminary results are encouraging.

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MUTAGENESIS IN *DROSOPHILA MELANOGASTER*

C. E. Nix, J. L. Epler, B. S. Brewen, and R. D. Wilkerson

The main research effort of this aspect of a comparative view of mutagenesis has been mutagenicity testing in the *Drosophila* system. The primary test has been the sex-linked recessive lethal since it has been shown by us and others to be the most sensitive. In addition to EMS and dimethylnitrosamine (DMN), which serve as positive controls, we have tested BP, DMBA, *N*-acetyl-2-aminofluorene (AAF), acrolein, 8-aminoquinoline, 8-nitroquinoline, and several coal-tar fractions. Most of these compounds require metabolic activation, and there is considerable evidence to suggest that many Diptera, and hence *Drosophila*, possess a microsomal system similar to the mammals.

DMN, a compound requiring metabolic activation, is a potent mutagen for *Drosophila*, as amply documented by others. It produces both completes and mosiacs (fractional or sectored mutants) in a manner very similar to EMS. On the other hand BP, DMBA, and AAF, which also require activation and are potent mutagens in the *Salmonella* system, are completely ineffective or very weakly positive in *D. melanogaster*. Of the other compounds tested, only 8-aminoquinoline was positive. This experiment is currently being repeated.

Previous investigators have reported that the polycyclic hydrocarbons are for the most part ineffective in producing sex-linked recessive lethals in *D. melanogaster* but that they act specifically to produce small deletions in heterochromatic loci such as "bobbed" and "Minute." In light of this and our preliminary results, we began a more exhaustive study of the effects of BP. Two different doses of BP were fed in a solution of 5% dimethyl sulfoxide (DMSO) and 2% sucrose, and approximately 10,000 to 15,000 F_1 flies per dose have been scored for "Minute." At neither 1 m*M* nor 5 m*M* BP was the incidence of "Minutes" different from the controls. Thus we conclude that BP, when administered by feeding, has no effect on specific loci such as the "Minutes." In the same experiment the frequency of sex-linked recessive lethals was not above background. Transport experiments are under way in order to ascertain whether BP reaches the target organ.

From these experiments we can also conclude that *Drosophila* are very efficient in activating some compounds, such as DMN, but they cannot activate others such as BP and AAF. Alternatively, they may have the ability to activate BP and AAF but not to transport these compounds to the target organ. As mentioned earlier, experiments are planned to resolve this question.

These results point out the need for using more than one test system in the evaluation of potential mutagens. DMN is a very effective mutagen in *Drosophila* in concentrations as low as 20–30 μg/ml, while in the *Salmonella* system the concentration required is much greater and the technical difficulties are considerable. On the other hand, BP and other polycyclic hydrocarbons are very effective mutagens in *Salmonella* but ineffective in *D. melanogaster*.

We feel no *one* test system is completely adequate for mutagenicity testing. For a valid evaluation of environmentally important chemicals, a comparative approach is necessary.

EXPRESSION TIME OF MUTATIONS IN QUANTITATIVE MUTAGENESIS OF THE HYPOXANTHINE–GUANINE PHOSPHORIBOSYL TRANSFERASE LOCUS IN CHINESE HAMSTER OVARY CELLS

J. P. O'Neill,* S. A. Brennan,† and A. W. Hsie

Recently, we have developed a mutational assay in Chinese hamster ovary (CHO) cells suitable for quantitating induced mutations at the hypoxanthine guanine phosphoribosyl transferase (HGPRT) locus by various physical and chemical agents.[1,2] In order to obtain maximum mutation frequency in this "CHO/HGPRT" system, the cells, after mutagen treatment, must be maintained in culture for several days before any cells resistant to 6-thioguanine can be detected. The expression time which yields the maximum number of mutants has been found to be 7 to 9 days for several mutagens and is independent of mutagen dose as well as initial cell survival. Our usual method had been to subculture the cells at a 1:5 dilution every 2 days in order to maintain conditions of logarithmic growth. Such a regimen is time-consuming as well as costly due to the amount of medium and petri dishes used. We now find that cell growth is not necessary during this expression time, at least when EMS, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), and ultraviolet light are the mutagenic agents. In these studies, cells were maintained in a nongrowing state in serum-free medium. Under these conditions, cell viability is maintained during the 7- to 9-day expression time, and the induced-mutation rate is similar to that found with cells which are growing during the expression time. These studies are presently being extended to other mutagens, and similar results are expected, as expression time *per se* does not appear to be directly involved in the mutation induction. Rather it is the time necessary for the loss of existing HGPRT and its mRNA due to normal turnover of macromolecules in the cells on which the gene locus has been mutated. These results suggest that the time course of this turnover is independent of cell growth but is more a function of time *per se*. The kinetics of the growth arrest which results when cells are shifted into serum-free medium is under study. Utilizing this method, the role of cell growth in mutation induction and fixation can be investigated.

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DOSE-RESPONSE RELATIONSHIPS OF CYTOTOXICITY AND MUTAGENICITY OF MONOFUNCTIONAL ALKYLATING AGENTS IN CHINESE HAMSTER OVARY CELLS*

D. B. Couch† and A. W. Hsie

It has been shown in certain microbes that alkylating agents which react through a unimolecular nucleophilic substitution (S_N1) mechanism produce relatively more miscoding alterations in DNA than those which react via a bimolecular (S_N2) mechanism, since the former react more extensively at the relatively weaker nucleophilic sites, including the O^6 atom of guanine. Since alkylation at this position produced mispairing, the mutagenicity of these compounds would be expected to correlate with tendency to react by the S_N1 mechanism.[1,2]

We have studied the biological effects of the alkylating agents methyl methanesulfonate (MMS), EMS, dimethyl sulfate (DMS), MNNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG), *N*-methyl-*N*-nitrosourea (NMU), *N*-ethyl-*N*-nitrosourea (ENU), and *N*-butyl-*N*-nitrosourea (BNU), in CHO cells.

Table 19. Cytotoxicity and mutagenicity of monofunctional alkylating agents in CHO cells

Mutagen	Dose for 90% killing (μM)	Mutants per 10^5 survivors	Mutants per 10^5 survivors per μM mutagen
DMS	89	9.2	0.10
MMS	95	14.0	0.13
EMS	3700	155	0.04
MNU	86	24.3	0.28
MNNG	0.54	32.0	59.3
ENU	1200	55.0	0.05
ENNG	6.1	52.5	8.6
BNU	2540	20.0	0.008

These compounds include typical S_N2 alkylating agents (DMS, MMS), those which have a greater S_N1 character (NMU, MNNG), and those which show intermediate properties (EMS). For each compound, single-cell survival and induced-mutation frequency at the HGPRT locus of CHO cells have been determined as a function of dose of alkylating agent. As shown in Table 19, the following order of increased mutagenicity (induced-mutation frequency per unit concentration of mutagen) was observed: BNU < EMS < ENU < DMS < MMS < MNU < ENNG < MNNG. Cytotoxicity followed approximately the same order: EMS < BNU < ENU < MMS < DMS,MNU < ENNG < MNNG. A positive correlation between mutagenicity and known chemical reactivity was not always found. Through the series of alkylating agents DMS to MNNG and MNU, both the tendency to react by the S_N1 mechanism and the mutagenicity increase.

However, the ethyl analog of each class of compound studied is known to have greater S_N1 character than the corresponding methyl derivative but was found to be less mutagenic.

*Presented in part at the Seventh Annual Meeting of the Environmental Mutagen Society, Atlanta, Georgia (1976).

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ANALYSIS OF THE CELL-CYCLE-PHASE SPECIFICITY OF MUTAGENESIS IN CHINESE HAMSTER OVARY CELLS

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

The susceptibility of cells in different phases of the cycle to the mutagenic action of both chemical and physical agents is being investigated by utilizing synchronized cells. Three approaches have been utilized in this study.

(1) CHO-K_1-BH_4 cells have been synchronized by the mitotic shake-off method, which yields populations of 90 to 100% mitotic cells. This method has been shown to cause minimal perturbation of cell-cycle parameters. The cells traverse the cell cycle with a generation time of 12 to 13 hr, which is divided into G1 (4.5–5.0 hr), S (4–4.5 hr), G2 (2–2.5 hr), and M (1–1.5 hr).

(2) Mutant lines of CHO cells which have longer cell-cycle times have been isolated by a modification of the 5-BrdUrd/visible light technique. We have utilized one line which has a generation time of 18 to 20 hr, which is divided into G1 (10–12 hr), S (4–5 hr), G2 (2–2.5 hr), and M (1–1.5 hr). The major alteration appears to be in the length of the G1 phase.

(3) CHO-K_1-BH_4 cells can also be synchronized by serum deprivation. Cells maintained in serum-free medium for up to 9 or 10 days retain high plating efficiency. Upon addition of 10% serum, the cells traverse the cycle with a generation time of 19 to 21 hr, which is divided into G0/G1 (11–12 hr), S (4–5 hr), G2 (2–2.5 hr), and M (1–1.5 hr). In this way, cells with a longer G1 phase can be utilized.

At present, our studies of mutagenesis over the cell cycle have been limited to MNNG treatment and UV irradiation. MNNG was chosen because it appears to act only on replicating DNA in microbial systems. However, we find by using synchronized CHO cells that MNNG is equally mutagenic during all phases of the cell cycle.

In contrast to these findings, when fluences of UV irradiation which yield a 5 to 15% survival rate are given at different time points during the cell cycle, fluctuations in the induced-mutation frequency are observed. Although achieving a high degree of reproducibility has been a problem, a general pattern has emerged. The mutation frequency is low during G1 and early S phases. This is followed by an increase during the first part of the S phase, with a decline towards the S/G2 boundary. However, when selection for resistance to ouabain is monitored, a shift of the major peak to the latter half of S phase is observed in a preliminary experiment.

An additional aspect of these studies has been the development of serum deprivation which yields populations of viable G0/G1-arrested cells. Utilizing this method, we are able to study mutagenesis in

nongrowing cells, thereby complementing our ongoing investigations with logarithmically growing cells. This will also enable us to study the role of DNA repair in mutagenesis much more easily, since the problem of DNA replication synthesis is virtually eliminated. Furthermore, cells in the arrested state can be treated with mutagen followed either (*a*) by addition of serum to initiate growth or (*b*) by maintenance of the arrested state to study the role of DNA replication on mutagenesis. Preliminary experiments of this type show that replication is necessary for mutagenesis with MNNG but not with EMS.

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FURTHER STUDIES ON MODIFICATION OF THE MICROTUBULE SYSTEM OF CHINESE HAMSTER OVARY CELLS BY DIBUTYRYLADENOSINE 3′:5′-PHOSPHATE

L. S. Borman* and A. W. Hsie

Previously, we have demonstrated that treatment of CHO cells with $N^6,O^{2'}$-dibutyryladenosine 3′:5′-phospate (Bt_2cAMP) causes the conversion of the randomly oriented epithelial-like cells to the contact-inhibited form.[1] This morphological transformation involves polymerization and reorientation of microtubules as well as changes in several physiologic–biochemical properties of the cells.[2]

The morphological response is presently being investigated through an extensive ultrastructural study to determine where within the cell the alteration of microtubule structure takes place and the exact nature of the alteration. Treated and untreated cells have been sectioned parallel to the substratum from the bottom of the cells to the top. It has been observed that both untreated and treated cells contain many microtubules in the bottom of the cell with fewer microtubules observed per unit cytoplasma as one progresses toward the top of the cell. Only treated cells have been observed to contain arrays of microtubules which are oriented parallel to the long axis of the cell. These are seen in the bottom half of the cells. Where cell-to-cell contact exists, both treated and untreated cells contain bundles of microtubules beneath the plasma membrane. These bundles are seen from bottom to top of the cell as long as cell-to-cell contact exists. Therefore only one alteration in microtubule structure is observed in treated cells—the production of parallel arrays of microtubules in the bottom half of the cell. A morphological variant (M7) which does not alter its cell shape after Bt_2cAMP treatment is also being studied in the above manner. Preliminary results show that, like the parental cells, variant M7 contains a stratum of microtubules from top to bottom of the cell and that microtubule bundles exist within cells that are contacted with other cells. However, treated M7 cells do not contain aligned microtubules in any level within the cell. These results are consistent with the hypothesis that Bt_2cAMP-induced cell elongation occurs through the production of aligned microtubules.

In an attempt to relate the observed alteration in microtubular structure to a biochemical property of tubulin, colchicine binding studies have been carried out with extracts from treated and untreated CHO-K_1 cells. Preliminary results show an alteration in the colchicine binding affinity of CHO tubulin assayed from treated cells. Therefore a specific alteration in microtubule organization has been observed for treated cells and concomitantly a modification in a biochemical property of CHO tubulin.

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ADENOSINE 3′:5′-PHOSPHATE-DEPENDENT PROTEIN KINASE ON THE OUTER SURFACE OF CHINESE HAMSTER OVARY CELLS

A. P. Li* and A. W. Hsie

Activation of cAMP-dependent protein kinases and the phosphorylation of protein substrates has been proposed to be the mechanism of cAMP-mediated physiological changes. Membrane-bound protein kinases have been described in erythrocytes, brain cells, adipocytes, and other systems. We have preliminary evidence demonstrating the occurrence of cAMP-dependent protein kinase activity on the outer surface of CHO cells.

Histone phosphorylation by the outer surface of CHO cells (measured by incubating the washed

monolayered cells with reaction mixture of protein kinase) increases linearly with time. The activity increases with increase in cell number per plate but plateaus at high cell density, probably due to the limitation of exposed cell surfaces caused by the contact among cells. The protein kinase activity is stimulated by cAMP, with a maximum stimulation by 10 μM cAMP. Control experiments show that the activity is not due to the leakage of cytoplasmic enzyme into the reaction mixture.

Our results demonstrated protein kinase activity on the outer surface of CHO cells. Ectoenzymes have been described in eukaryotic cells, including ATPase, 5′-nucleotidase, glyceraldehyde-3-phosphate dehydrogenase, and 3-phosphoglycerate kinase. The occurrence of ectoenzymes that can synthesize and utilize ATP makes it possible to speculate that they occur as an enzyme complex with activities regulated by one another.

The utilization of extracellular substrates by the ecto protein kinase makes it likely that phosphorylation of external membranal protein of one cell by the ecto protein kinase of another cell may occur. This transphosphorylation may be important in cell-to-cell communication, the understanding of which may unravel mechanisms of contact inhibition, a membranal function shown in some cases to be regulated by cAMP. Further characterization of the ecto protein kinase is now in progress.

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SUBCELLULAR DISTRIBUTION OF ADENOSINE 3′:5′-PHOSPHATE-DEPENDENT PROTEIN KINASE ACTIVITY IN CHINESE HAMSTER OVARY CELLS

A. P. Li,* D. P. Allison, and A. W. Hsie

Cyclic AMP-dependent protein kinase is believed to mediate most cAMP-induced physiological changes in mammalian cells. Association of the enzyme or its substrates with different subcellular elements may be one of the means by which the cAMP-induced changes are regulated. We fractionated CHO cell homogenate into the nuclear (500 *g* pellet), mitochondrial (500–5000 *g* pellet), plasma membranal (5000–7000 *g* pellet), microsomal (7000–27,000 *g* pellet), microsomal-ribosomal (27,000–100,000 *g* pellet), and cytosol (100,000 *g* supernatant) fractions using differential centrifugations. The content of each subcellular fraction obtained is confirmed by electron microscopy and by the association with marker enzyme. Subcellular fractions were assayed for cAMP binding as well as cAMP-dependent protein kinase activities. The distribution of the activities is shown in Table 20. Washing particulate fractions three times with phosphate-buffered isotonic saline failed to dissociate the cAMP binding activity and protein kinase activity from them, indicating the existence of tight association. The cAMP-binding activity is specific, as unlabeled cAMP competes readily with [^{3}H]cAMP for the binding while the other adenosine compounds do not. Cyclic AMP stimulates the protein kinase activities of the different fractions when exogenous histone is added as substrate. Protein kinase inhibitor, which inhibits specifically cAMP-dependent protein kinase, exhibits inhibitory effect on the activities of all subcellular fractions. There remain residual protein kinase activities even in the presence of the inhibitor, which indicates the presence of cAMP-independent protein kinase activities. The highest activities are found in the nuclear fractions. Triton X-100 (0.1%) stimulated the protein kinase activities of the subcellular fractions, probably through solubilization of the bound enzyme, thus facilitating interaction with the exogenous histone. Assay of protein kinase activities without the addition of exogenous substrate demonstrates the presence of endogenous protein kinase substrates. We could not, however, demonstrate significant stimulatory effect of cAMP on the phosphorylation of endogenous substrates. The physiological significance of the cAMP-dependent protein kinase and substrates associating with the different subcellular fractions is yet to be demonstrated.

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Table 20. Distribution of cAMP-binding[a] and protein kinase[b] activities in subcellular fractions of CHO cells

Fraction	Protein		cAMP binding		Protein kinase activity									
					− Histone					+ Histone				
					−cAMP		+cAMP		Activity Ratio	−cAMP		+cAMP		Activity Ratio
	Quanity (mg)	Distribution (%)	Specific activity	Distribution (%)	Specific activity	Distribution (%)	Specific activity	Distribution (%)	$\left(\frac{+cAMP}{-cAMP}\right)$	Specific activity	Distribution (%)	Specific activity	Distribution (%)	$\left(\frac{+cAMP}{-cAMP}\right)$
Nuclei (480 *g* pellet)	46.2	32.0	0.44	3.7	5.2	30.9	6.2	33.2	1.2	55.8	20.6	110.1	5.4	2.0
Crude mitochondria (5000 *g* pellet)	18.9	13.1	0.87	3.0	3.4	8.3	2.3	5.0	0.7	21.0	3.2	145.0	2.9	6.9
Plasma membrane (7000 *g* pellet)	2.8	1.9	1.36	0.6	3.5	1.3	3.7	1.2	1.1	138.7	3.1	490.8	1.5	3.5
Microsome (27,000 *g* pellet)	2.6	1.8	1.10	0.4	7.8	2.6	7.2	2.2	0.9	167.7	1.4	400.7	1.1	6.1
Microsome-ribosome (100,000 *g* pellet)	8.8	6.1	1.74	2.8	6.8	7.7	6.4	6.5	1.0	227.3	16.0	742.3	6.9	3.3
Cytosol (100,000 *g* supernatant)	65.0	45.1	7.64	89.6	5.9	49.3	6.9	51.9	1.2	107.7	55.9	1190.3	82.2	11.1

[a]Specific cAMP-binding activity is expressed as picomoles of [^{3}H]cAMP bound per milligram of protein in each fraction assayed.
[b]Protein kinase activity is assayed in the presence and absence of 1 μM cAMP, and the specific activity is expressed as picomoles of [^{32}P]phosphate incorporated per minute per milligram of protein.

CHARACTERIZATION OF TWO ADENOSINE 3′:5′-PHOSPHATE-DEPENDENT PROTEIN KINASES FROM CHINESE HAMSTER OVARY CELLS

A. P. Li* and A. W. Hsie

Adenosine 3′:5′-phosphate-dependent protein kinase in several mammalian tissues exists as isozymes separable by ion-exchange chromatography. We separated two species of cAMP-dependent protein kinase from the cytosol of CHO cells, through application of the cytosol onto a DEAE-cellulose column and elution with a 0 to 0.3 *M* NaCl linear gradient. One species, designated as PK I, eluted at the beginning of the gradient, and its activity is stimulated as much as tenfold by 1 μM cAMP. The other species, PK II, eluted later from the gradient and is stimulated only twofold by 1 μM of cAMP. Both enzyme species demonstrate a strict requirement for magnesium, with K_m values of 1.2 m*M* for PK I and 0.75 m*M* for PK II. Values of K_m for ATP in the absence and presence of 1 μM cAMP are 0.13 m*M* and 0.08 m*M* for PK I, and 0.17 m*M* and 0.08 m*M* for PK II. Binding affinities for cAMP are 5.6×10^{-9} for PK I and 4.5×10^{-9} for PK II. The molecular sizes of the two protein kinase species are determined using a 5 to 20% linear sucrose gradient. Using cytochrome C, lactate dehydrogenase, hemoglobin, chymotrypsinogen, and catalyase as molecular-weight markers, PK I is determined to have a sedimentation coefficient of 6.4 S, corresponding to a molecular weight of 105,000, while PK II has a sedimentation coefficient of 4.8 S and a molecular weight of 68,000. When sedimentation was done in sucrose containing 1 μM cAMP, the catalytic activities of both enzymes shifted to a sedimentation coefficient of 3.0 S, corresponding to a molecular weight of 32,000. The two enzymes also demonstrated a difference in substrate specificity toward exogenous protein. The order of decreasing preference is: PK I—histone, protamine, phosvitin; PK II—phosvitin, histone, protamine. The two species of cAMP-dependent protein kinase may play different roles in the mediation of cAMP-induced physiological changes in CHO cells.

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A RAPID SCREENING PROCEDURE OF LIPID METABOLISM IN CULTURED MAMMALIAN CELLS

E. A. Dosado,* A. W. Hsie, and F. Snyder†

We have developed a practical procedure[1] for monitoring lipid metabolism in monolayer cultures that is based on techniques that have been described for the extraction and chromatography of lipids from tissue slices and from microscopic sections of tissues directly on thin-layer chromatograms. We used CHO-K_1 cells cultured as monolayers on microscope coverslips. The slips are subsequently attached to silica gel layers for extraction and analysis of the labeled products formed. The technique not only provides information on the extent that labeled precursors are incorporated and their metabolic fate but also permits one to evaluate and select suitable solvents for quantitative extraction of the labeled products.

We have obtained labeling results with the coverslip technique when monolayers of CHO-K_1 cells were incubated with four different lipid precursors. As expected, [1,2-^{14}C]ethanolamine was mainly incorporated into phosphatidylethanolamine, whereas when [1,2-^{14}C]choline was the precursor, most of the activity was incorporated into phosphatidylcholine and sphingomyelin.

Our results indicate that the coverslip method, in conjunction with thin-layer chromatography, is applicable to screening lipid metabolism in cell cultures grown as monolayers. Useful preliminary results obtained with this method can then be confirmed and extended by the more time-consuming and expensive conventional analytical procedures.

*Postdoctoral investigator, Carcinogenesis Training Grant CA05287 from NCI to ORAU.

†ORAU.

1. E. A. Dosado, A. W. Hsie, and F. Snyder, *J. Lipid Res.* **17**, 285 288 (1976).

INDUCTION OF CHROMOSOME ABERRATIONS IN MOUSE OOCYTES BY METHYL METHANESULFONATE AND TRIETHYLENEMELAMINE

J. G. Brewen and H. S. Payne

Many chemical agents produce dominant-lethal effects when female mice are treated a few days prior to ovulation and conception. With one or two

exceptions, these effects disappear if the interval between treatment and conception exceeds 6 to 10 days. Furthermore, the great majority of chemical mutagens have been demonstrated to require replicative DNA synthesis in order to produce chromosome aberrations, the universally accepted cause of most dominant lethality. Since there is no DNA synthesis in the maturing oocyte (the first occurs after fertilization in the pronuclear stage), we undertook to investigate the qualitative and quantitative nature of the chromosome aberrations induced by MMS and TEM in mouse oocytes.

Young adult female mice, aged 10 to 12 weeks, were injected intravenously with either 50- or 100-mg/kg doses of MMS or a 1.6-mg/kg dose of TEM. The females were then mated to untreated males at intervals ranging from $^1/_2$ to $14^1/_2$ days after treatment. Two days prior to the scheduled mating, the females were injected with pregnant mare serum gonadotropin (PMS) followed, on the day they were caged with males, by an injection of human chorionic gonadotropin (HCG) in order to induce superovulation. The morning after mating, the plugged females were killed, and the fertilized ova were removed and cultured in the presence of colchicine to the first cleavage mitosis. Other groups of females were treated in a similar fashion except that colchicine was administered at the same time as the HCG and the females were not caged with males. The next day these females were killed, and preparations were made of metaphase I oocytes.

Both the first cleavage mitoses and the metaphase I oocytes were analyzed for structural chromosome aberrations. The results of the cytological analysis are summarized in Tables 21–23. In the analysis of the first cleavage division, chromatid-type aberrations were found after both the MMS and TEM treatments. In the case of MMS the most prevalent aberration type, excepting a shattering phenomenon, was the isochromatid deletion. After TEM, however, all classes of chromatid aberrations were observed at a high frequency (Figs. 14–17).

Studies on dominant-lethal induction[1,2] in females by these two chemicals indicate a very clear effect of

Table 22. Chromosome aberration yields in metaphase I oocytes following a 100-mg/kg dose of MMS

Interval (days)	No. of cells scored	No. of deletions	No. of exchanges
Control	200	0	0
$^1/_2$	100	1	0
$2^1/_2$	100	0	0
$14^1/_2$	100	0	0

Table 23. Chromosome aberrations in first cleavage mitosis after treatment of oocytes with 1.6 mg/kg of TEM

Interval (days)	No. of cells scored	No. of cells with aberration number–						
		0	1	2	3	4	>4	Shattered
$^1/_2$	50	2	4	2	3	3	27	9
$2^1/_2$	50	33	12	1	1	1	2	0
$4^1/_2$	90	77	11	0	0	0	2	0
$6^1/_2$	50	41	6	2	0	0	1	0

Table 21. Chromosome aberration yields at the first cleavage mitosis after treatment of dictyate oocytes with MMS

Dose (mg/kg)	Interval (days)	No. of cells scored	No. of cells with aberrations						Expected lethality (%)
			0	1	2	3	>3	Shattered	
Control	—	100	100	0	0	0	0	0	0
50	$2^1/_2$	75	70[a]	2	2	0	0	1	8.0[a]
50	$6^1/_2$	50	47	3	0	0	0	0	6.0
50	$10^1/_2$	50	49	1	0	0	0	0	2.0
50	$14^1/_2$	50	50	0	0	0	0	0	0
100	$^1/_2$	50	40	4	0	0	0	6	20.0
100	$1^1/_2$	50	43	3	1	0	1	2	14.0
100	$2^1/_2$	50	40	5	1	1	0	3	20.0
100	$6^1/_2$	50	43[a]	5	0	0	1	1	16.0[a]
100	$10^1/_2$	50	47	3	0	0	0	0	6.0
100	$14^1/_2$	50	50	0	0	0	0	0	0

[a]Includes a triploid zygote (see Fig. 14).

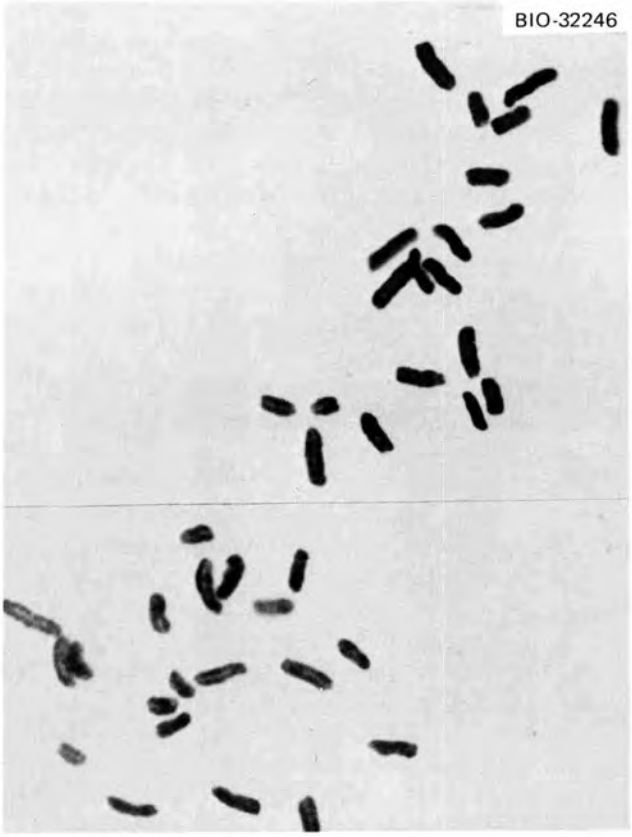

Fig. 14. Normal diploid first cleavage division.

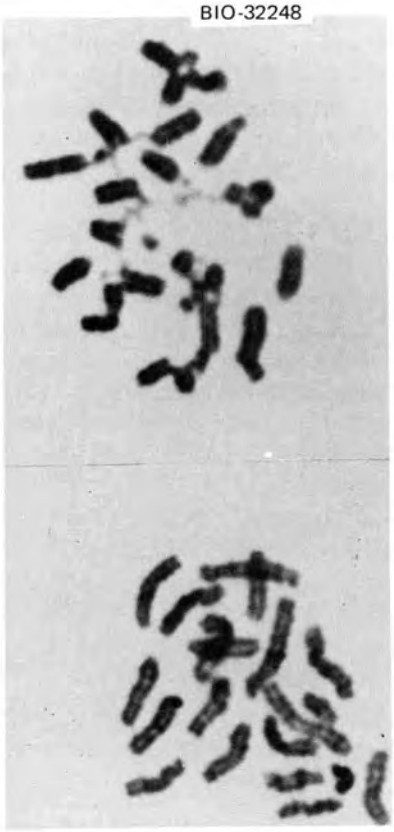

Fig. 16. First cleavage division with multiple chromatid aberrations in female pronucleus.

Fig. 15. Female first cleavage chromosomes with a chromatid interchange.

Fig. 17. First cleavage division with shattered female chromosomes.

the interval between treatment and mating, the highest frequency of dominant lethality occurring during the ½- to 4½-day interval between treatment and mating. The cytogenetic data derived from the first cleavage studies also indicate that the oocytes closest to ovulation are the most sensitive to the induction of chromosomal aberrations. In addition, the earliest interval examined (½ day with TEM and ½ to 2½ days with MMS) contained a significant number of cells with multiple aberrations and "shattered"chromosomes. This observation agrees with the high frequency of sterile matings and early embryonic (two-blastomere-stage) death observed by Generoso.[1] We propose that the heavily damaged cells would result in preimplantation death and apparent sterile matings.

In the analysis of metaphase I oocytes, no significant level of chromosome damage was observed. This observation is in accord with the proposition and experimental evidence that DNA replication is required for the chemically induced lesion to be translated into chromosomal damage.

1. W. M. Generoso, *Genetics* **61**, 461–470 (1969).
2. W. M. Generoso *et al.*, *Mutat. Res.* **11**, 411–420 (1971).

A COMPARISON OF THE ABERRATION FREQUENCIES IN MOUSE AND HUMAN LEUKOCYTES FOLLOWING ACUTE X-RAY EXPOSURES

R. J. Preston and J. G. Brewen

Because of the importance of the comparison of chromosome aberration frequencies in mouse and human leukocytes following irradiation in providing a "bridge" between the two for genetic risk estimating in man, it was decided to obtain considerably more data at low doses (less than 250 R). It was also important to add confidence to the comparison by using early fixation times to reduce the complication of second divisions, but also to correct for the few second divisions present, even at the early fixation times used.

The method for culturing mouse leukocytes that had been used previously[1] had become rather unreliable, and so changes were made in culture medium and growth conditions according to the method of Triman *et al.*[2] Using this method, it was possible to obtain large numbers of mitotic divisions at fixation times of 48 or 52 hr over the whole dose range used.

The results of the frequencies of dicentrics and acentric fragments induced in mouse and human leukocytes by acute X-ray exposure are given in Table 24, and the dose-response curve for dicentrics alone is shown in Fig. 18. The figure is plotted from the data, which have been corrected to account for aberration loss at the first mitotic division. Although the experiment is designed so that at the fixation times used, the cells should be at their first mitotic division, it is clear, from the fact that some dicentrics do not have an accompanying acentric fragment, that cells can reach their second mitotic division by 48 hr after stimulation by phytohemagglutinin (PHA) in both mouse and human cultures. It is apparent from Table 24 that the frequency of dicentrics not containing an accompanying acentric fragment is greater in the mouse than in man, which would mean that different correction factors should be applied to the two sets of data. The correction is calculated on the assumption that those cells containing dicentrics without a fragment are second-division ones and that the frequency of these is independent of dose. Thus the product of the yield at any dose and the proportion of dicentrics without fragments will be approximately the proportion of dicentrics lost at the first division. This is then added to the yield for the appropriate dose, giving the corrected yield. The same adjustment has also been made for the acentric-fragment data.

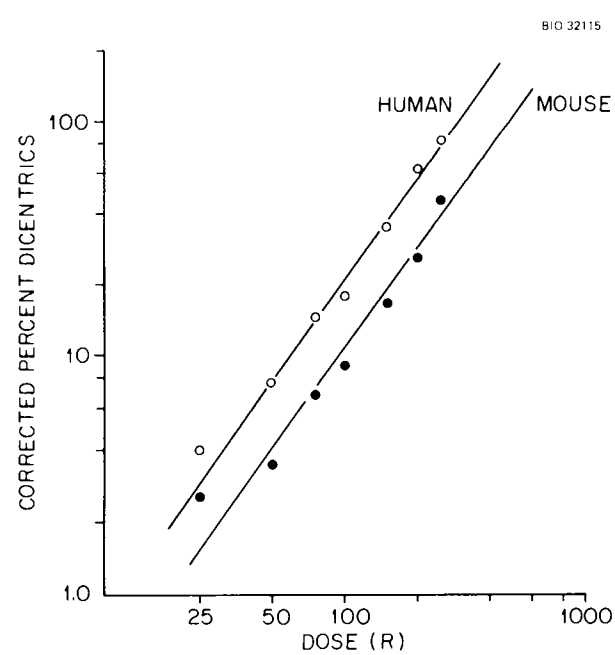

Fig. 18. Dose-response curve for dicentric aberrations (corrected) following acute X-ray exposure.

Table 24. Chromosome aberration frequencies following acute X-ray exposure of mouse or human leukocytes
Culture times of 48 to 52 hr

Dose (R)	No. of cells scored	Percent dicentrics	No. of dicentrics without accompanying fragment	Corrected percent dicentrics	Percent acentric fragments	Corrected percent acentric fragments
Human[a]						
0	500	0.0	—	0.0	0.8 ± 0.40	0.8
25	500	3.8 ± 0.8	2/19	4.0	1.6 ± 0.6	1.7
50	500	7.2 ± 1.2	2/36	7.6	5.8 ± 1.1	6.1
75	500	13.6 ± 1.6	7/68	14.4	6.8 ± 1.2	7.2
100	500	16.8 ± 1.8	6/84	17.8	11.8 ± 1.5	12.5
150	500	33.4 ± 2.6	13/167	35.3	19.2 ± 2.0	20.3
200	175	58.8 ± 2.0	6/103	62.2	31.4 ± 4.2	33.2
250	500	77.4 ± 3.9	13/387	81.8	46.4 ± 3.0	49.0
Mouse[b]						
0	500	0.0	—	0.0	0.0	—
25	500	2.2 ± 0.7	2/11	2.6	2.2 ± 0.7	2.6
50	400	3.0 ± 0.9	0/12	3.5	4.4 ± 1.2	5.1
75	500	5.8 ± 1.1	2/29	6.8	4.6 ± 1.0	5.4
100	500	7.8 ± 1.3	3/39	9.1	10.4 ± 1.4	12.1
150	500	14.4 ± 1.7	19/72	16.8	11.0 ± 1.5	12.8
200	400	22.5 ± 2.4	13/90	26.3	22.3 ± 2.4	26.0
250	500	38.8 ± 2.8	36/194	45.3	29.0 ± 2.4	33.9

[a]The corrected frequency is calculated by multiplying the observed frequency by the proportion of dicentrics without fragments (49/864) and adding this value to the observed frequency.

[b]The corrected frequency is calculated by multiplying the observed frequency by the proportion of dicentrics without fragments (75/447) and adding this value to the observed frequency, that is, observed plus 0.168 times observed.

From the corrected dicentric data it is apparent that the yield in human leukocytes is consistently twice that found in the mouse, whereas for the acentric fragments there is no significant difference between mouse and man. These data are very similar to those reported previously,[1] although the yields are slightly different because of the corrections applied.

1. J. G. Brewen, R. J. Preston, K. P. Jones, and D. G. Gosslee, *Mutat. Res.* **17**, 245–254 (1973).
2. K. L. Triman, M. T. Davisson, and T. H. Roderick, *Cytogenet. Cell Genet.* **15**, 166–176 (1975).

FRACTIONATION EFFECTS ON X-RAY-INDUCED CHROMOSOME ABERRATIONS IN MOUSE OOCYTES

I.-D. Adler,* H. S. Payne, and J. G. Brewen

In an earlier study[1] it was reported that the frequency of X-ray-induced chromosomal aberrations in maturing oocytes varied with the interval between irradiation and ovulation. The yield was lowest when the interval was 18 to 24 hr, and yield increased as the interval became longer. The peak yield occurs between 7 and 9 days and remains constant until day 21, when a slight decrease is observed. No data were obtained for longer intervals, as no oocytes could be obtained, indicating that the immature oocytes were killed. A dose-response study done at the 14-day interval clearly indicated that both chromatid interchanges and isochromatid plus chromatid deletions were produced by a combination of one- and two-track processes.

To better understand the mechanisms of the aberration induction in the maturing oocytes, a series of fractionation experiments were done. The first experiment consisted of fractionating a 400-R dose into two equal fractions separated by various intervals up to 3 hr. The metaphase 1 oocytes were collected and cytologically analyzed 14 days after irradiation. The second experiment consisted of splitting the 400-R dose into unequal fractions of 100 and 300 R and alternating the first dose so that various intervals were used for the 100-R-plus-300-R and 300-R-plus-100-R regimens. The results obtained in these experiments are summarized in Tables 25 and 26.

The reasoning in a fractionation experiment is to test the average length of time the X-ray-induced

Table 25. Frequency of expected and observed aberrations in metaphase I oocytes following 200-R, 400-R, and 400-R-fractionated doses of X rays

Dose (R)	Fractionation interval (min)	No. of cells scored	Percent interchanges	Expected with—		Percent deletions	Expected with—	
				Interaction	Additivity		Interaction	Additivity
0	—	300	0.0	—	—	0.3	—	—
200	—	225	13.3 ± 2.4	—	—	8.0 ± 1.9	—	—
400	—	225	55.6 ± 5.0	—	—	39.1 ± 4.2	—	—
200 + 200	90	200	54.0 ± 5.2[a]	55.6	26.6	31.5 ± 4.0[a]	39.1	16.0
200 + 200	135	200	43.7 ± 4.7[a]	55.6	26.6	19.5 ± 3.1[b]	39.1	16.0
200 + 200	180	150	30.0 ± 5.4[b]	55.6	26.6	23.3 ± 3.9[b]	39.1	16.0

[a]Significantly different from additivity.
[b]Significantly different from interaction.

Table 26. Frequency of expected and observed aberrations in metaphase I oocytes following 100-R, 300-R, 400-R, and 400-R-fractionated doses of X rays

Dose (R)	Fractionation interval (min)	No. of cells scored	Percent interchanges	Expected with—		Percent deletions	Expected with—	
				Interaction	Additivity		Interaction	Additivity
0	—	300	0.0	—	—	0.3	—	—
100	—	265	4.2 ± 1.3	—	—	4.2 ± 1.2	—	—
300	—	236	42.4 ± 3.2	—	—	23.3 ± 2.8	—	—
400	—	165	69.1 ± 6.4	—	—	36.7 ± 3.7	—	—
100 + 300	90	300	74.7 ± 5.0[a]	69.1	46.6	36.0 ± 3.5[a]	36.7	27.5
100 + 300	135	204	62.8 ± 5.6[a]	69.1	46.6	28.9 ± 3.8[b]	36.7	27.5
100 + 300	180	206	56.8 ± 5.3	69.1	46.6	36.9 ± 4.2[a]	36.7	27.5
300 + 100	90	—	—	69.1	46.6	—	36.7	27.5
300 + 100	135	239	61.5 ± 5.1[a]	69.1	46.6	33.1 ± 3.7	36.7	27.5
300 + 100	180	217	55.8 ± 5.1[b]	69.1	46.6	30.9 ± 3.8	36.7	27.5

[a]Significantly different from additivity.
[b]Significantly different from interaction.

lesions remain capable of interacting with each other to produce a chromosome aberration. In instances where the dose-response curve is best expressed as quadratic, the experiments are quite straightforward, because fractionation intervals that are shorter than the time required to repair the lesions produced by the first dose result in aberration yields that approximate that of the full dose. On the other hand, if the fractionation interval is greater than the time necessary for repair of the lesions produced by the first dose, the aberration yield approximates the additive of the two separate doses. The expected yields for interaction and additivity for the radiation regimens used in the current experiments are presented in Tables 25 and 26. It is seen that additivity is reached within 180 min regardless of the magnitude of the first dose. These data force us to conclude that, within the dose range studied, the time required for the oocytes to repair the lesions produced by the first dose is independent of the dose.

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1. J. G. Brewen *et al.*, *Mutat. Res.* **35**, 111–120 (1976).

OBSERVATIONS ON MOUSE CHROMOSOMES FOLLOWING INHALATION OF NITROGEN DIOXIDE

P. C. Gooch, H. E. Luippold, D. A. Creasia, and J. G. Brewen

The possible clastogenic effects of various components of atmospheric pollution have recently begun to be studied. The effects of ozone exposure

have been reported by several investigators. Observations will be reported here on the chromosomes of mouse leukocytes and spermatocytes following inhalation of nitrogen dioxide—a product of combustion and, therefore, an important constituent of urban air pollution.

C3H male mice were exposed to 0.1, 1, 5, and 10 ppm of NO_2 for 6 hr (0.6, 6.0, 30.0, and 60.0 ppm hr). The concentrations were produced by dilution of certified NO_2 with clean filtered air and confirmed by continuous monitoring with an NO_2 analyzer utilizing the chemiluminescent technique.

Blood samples were obtained by cardiac puncture of etherized animals immediately, 1 week, and 2 weeks after exposure. The blood from several animals was pooled at each sampling time and cultured for 48 hr. Colchicine was then added, and the cells were fixed at 52 hr. Following the blood sampling, the animals were held until 8 weeks after exposure, when spermatocyte preparations were made.

The results of the leukocyte chromosomal analysis (with 95% confidence limits indicated) are shown in Table 27. No increase was observed in either chromatid- or chromosome-type aberrations for any of the exposures tested. The analysis of the primary spermatocytes also showed no effect of the treatment (Table 28). Our results do not rule out the possibility that NO_2 could be clastogenic in other tissues or other systems. However, since the spermatocyte data were negative, it seems unlikely that inhaled NO_2 poses a significant genetic hazard in terms of chromosomal damage produced in the spermatogonial stem cells.

Table 28. Chromosomal analysis of mouse spermatocytes after NO_2 inhalation

Average Exposure (ppm hr)	No. of cells	Percent translocations
Control	600	0.0
0.6	300	0.0
6.0	300	0.0
30.0	300	0.0
60.0	250	0.0

THE USE OF CYTOSINE ARABINOSIDE FOR STUDYING THE RATE AND EXTENT OF DNA REPAIR IN MAMMALIAN CELLS FOLLOWING IRRADIATION OR CHEMICAL TREATMENT

E. A. Hiss and R. J. Preston

1-β-D-arabinofuranosylcytosine (ara C) has been shown by Regan and Dunn (unpublished results) to inhibit the repair of damage to the DNA induced by

Table 27. Chromosome aberration frequencies in mouse leukocytes after NO_2 inhalation

Average exposure (ppm hr)	Postexposure blood-withdrawal time (weeks)	No. of cells analyzed	Percent chromatid aberrations			Percent chromosome aberrations		
			Achromatic lesions	Deletions	Exchanges	Deletions	Rings and dicentrics	Exchanges
Control		400	1.75 ± 1.28	1.00 ± 0.98	0.00	1.50 ± 1.19	0.00	0.00
0.6	Immediately	300	1.67 ± 1.45	0.33 ± 0.65	0.00	0.67 ± 0.92	0.00	0.00
	1	125	0.80 ± 1.56	0.00	0.00	1.60 ± 2.20	0.00	0.00
	2	300	2.00 ± 1.58	1.33 ± 1.30	0.00	0.67 ± 0.92	0.00	0.00
6.0	Immediately	200	0.50 ± 0.98	1.50 ± 1.68	0.00	0.00	0.00	0.00
	1	300	0.67 ± 0.92	2.33 ± 1.71	0.00	0.33 ± 0.65	0.00	0.00
	2	150	3.33 ± 2.87	2.00 ± 2.24	0.00	0.00	0.00	0.00
30.0	Immediately	300	2.00 ± 1.58	2.00 ± 1.58	0.00	1.00 ± 1.13	0.00	0.00
	1	250	1.60 ± 1.56	1.20 ± 1.35	0.00	1.20 ± 1.35	0.00	0.00
	2	250	0.80 ± 1.10	0.80 ± 1.10	0.00	0.80 ± 1.10	0.00	0.00
60.0	Immediately	250	1.60 ± 1.56	1.60 ± 1.56	0.00	0.00	0.00	0.00
	1	300	2.33 ± 1.71	3.33 ± 2.03	0.00	0.33 ± 0.65	0.00	0.00
	2	250	0.40 ± 0.78	1.60 ± 1.56	0.00	0.00	0.00	0.00

ultraviolet radiation (UV). Since single-strand chain breaks were accumulated when human fibroblasts were irradiated with 200 ergs/mm^2 of UV and then incubated for 16 hr in the presence of hydroxyurea and ara C, it seems probable that ara C works by inhibiting either the polymerization or ligation steps. This observation has been used to study the initial velocity of UV excision repair, the inhibition of gamma-ray repair, and the inhibition of the repair of chemically induced DNA damage in human, Chinese hamster, or mouse cells.

The cell lines used were early passage normal human, transformed Chinese hamster (V-29), and normal mouse (10 T$^1/_2$) fibroblasts. ara C was used at a concentration of 10^{-4} *M*. The assay for DNA strand breaks was by alkaline sucrose gradient analysis. Following UV exposures of 25, 50, 100, 200 and 600 ergs/mm^2 the number of strand breaks present after incubation in ara C for 30 min was calculated from the molecular weight profiles. The results are depicted in Fig. 19 as a plot of the reciprocal of the breaks per genome after 30 min in ara C against the reciprocal of the dose. From this graph it is possible to estimate the maximum repair rate for each cell type (i.e., reciprocal of the *Y*-axis intercept) and the dose at which half the maximum velocity of repair is obtained (i.e., reciprocal of the *X*-axis intercept). Human and hamster cells repair UV damage very efficiently after doses of 25 to 600 ergs/mm^2, as expected. However, mouse cells repair damage poorly after doses less than 200 ergs/mm^2.

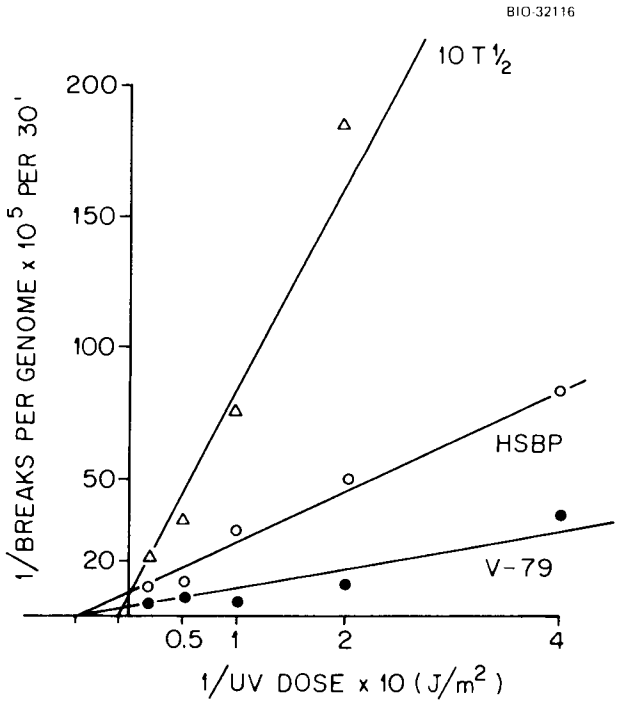

Fig. 19. Comparison of the extent of repair in mouse, hamster, and human cells as a function of UV dose.

ara C only slightly inhibits the repair of gamma-ray damage in Chinese hamster V-79 cells following an exposure of 10 kilorads. Complete repair is obtained even in the continued presence of ara C, although it takes longer than in the control (no ara C). This might be expected since the average size of the patch region after gamma rays is only a few bases, and the probability of ara C being incorporated is quite small. Furthermore, if more than one ara C needs to be incorporated to prevent repair synthesis or ligation by helix distortion, there will be a very low probability of inhibition after gamma rays.

Chinese hamster cells were also treated with several known mutagens: methyl-*N*-nitrosoguanidine, 4-nitroquinoline oxide, mitomycin C, and 8-hydroxyquinoline. When treated cells were allowed to repair the damage for 16 hr in the presence of ara C, the molecular weight was lower than for cells incubated in normal growth medium. Thus it appears that ara C also inhibits the excision repair of damage due to chemical mutagens.

This technique can, therefore, be used to study the amount of initial repair of damage to DNA by radiation and chemicals.

ALTERED CONCENTRATIONS OF PARENTAL-TYPE HEMOGLOBINS IN α-THALASSEMIC MICE

R. A. Popp, L. P. Stratton,* D. K. Hawley,† E. G. Tierney,‡ and G. P. Hirsch

Three independent mutations at the hemoglobin alpha-chain locus have been induced by X rays in (101 × SEC)F$_1$ mice.[1] One mouse (♂ 72) was sterile; the other two (♀ 27 and ♂ 352) were fertile, and they transmitted their hemoglobin anomaly to their offspring. Genetic and chemical studies proved that these hemoglobins lacked the SEC alpha-chain polypeptides.[2] Although each of the β-chain polypeptides is present, the starch gel electrophoresis patterns of hemoglobin from the α-thalassemic mice differ from those of normal F$_1$ mice.[1] Quantitative chemical analyses showed that the relative quantities of the β-chain polypeptides of SEC, 101-major, and 101-minor types in ♂ 72 and ♂ 352 were 0.60, 0.25, and 0.15, respectively, compared with 0.52, 0.36, and

0.11, respectively, in normal hemoglobin from (101 × SEC)F_1 mice. These alterations may result from the differential competitive affinities of the three kinds of β chains for the reduced quantity of α chains in erythrocytes of α-thalassemic mice. Isotope labeling has shown that synthesis of α chain was about 60% of that of β chain in α-thalassemic mice, but the relative rates of synthesis of the three kinds of β-chain polypeptides have not yet been determined.

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1. W. L. Russell, L. B. Russell, R. A. Popp, C. M. Vaughan, and K. B. Jacobson, submitted to *Proc. Natl. Acad. Sci. USA* (1976).

2. R. A. Popp, C. M. Vaughan, L. B. Russell, W. L. Russell, and K. B. Jacobson, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, pp. 90–91.

HEMATOLOGICAL STUDIES ON MICE POSSESSING RADIATION-INDUCED MUTATIONS AT THE HEMOGLOBIN LOCI

M. K. Enlow* and R. A. Popp

Hematological parameters of offspring from four of the five mice showing radiation-induced hemoglobin mutations[1] were studied to determine whether these mutations affect hematological indices other than hemoglobin synthesis. Many human hemoglobin variants cause hematological disturbances, and, in particular, a deficiency in polypeptide-chain synthesis is associated with an inherited anemia, thalassemia, which affects normal erythrocyte size, counts, and hemoglobin concentration.

The α-chain deficiency in progeny of mice ♀27 and ♂352 creates a hypercellular [12,350,000 vs 10,600,000 red blood cells (RBC) per cubic millimeter], microcytic (~ 37 vs 46 μ^3), hypochromic (~ 12 vs 15.5 pg of hemoglobin per cell) anemia, which is comparable to human α thalassemia. These hemoglobin variants, therefore, are mouse models of human α thalassemia.

The α-chain duplication in progeny of ♀86 creates a simple, slightly anemic condition, and progeny of ♀732 are hematologically normal. There is an imbalance in the amounts of α and β chains produced in ♀27, ♂352, and ♀86; however, the imbalance results in the former two from α-chain deficiency and in the latter from β-chain excess. It is significant that hematological disorders are associated with the former but not the latter, suggesting that α-chain deficiency *per se* rather than an imbalanced synthesis between the α and β chains is responsible for the clinical symptons of α thalassemia.

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ISOLEUCINE CONTENT IN HUMAN HEMOGLOBIN

R. A. Popp, E. G. Bailiff, G. P. Hirsch, C. C. Lushbaugh,* K. F. Hubner,* and L. G. Littlefield*

A sample of blood was obtained from a person (W.P.) who had received ~ 1490 R of chronic gamma irradiation for treatment of polycythemia vera. Chemical analysis showed an elevated isoleucine content in hemoglobin; the isoleucine substitution frequency was calculated to be 8.57 residues per 100,000 residues of other amino acids. The reticulocyte count in fresh blood was near 2%; thus an aliquot of blood was incubated for 2 hr in tissue culture medium containing [^{3}H]isoleucine to determine the extent of isoleucine incorporation into hemoglobin and to label reticulocytes that were synthesizing hemoglobin with appreciable quantities of isoleucine. A few heavily labeled reticulocytes were identified by autoradiography; these cells must be synthesizing hemoglobin containing coded isoleucine. Cells synthesizing such hemoglobin are likely to be those in which base substitutions have changed nonisoleucine into isoleucine codons.

A sample of blood was obtained from a person (R.H.) who had been accidentally exposed to ~30 R of neutrons in 1944. Chemical analysis of his hemoglobin showed an isoleucine substitution frequency of 18.71 residues per 100,000 residues of other amino acids. The patient has a myeloblastic leukemia; it is not known to what extent this might contribute to the elevated isoleucine content in the patient's hemoglobin.

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ISOLEUCINE BASE SUBSTITUTION IN RABBIT HEMOGLOBIN β CHAIN

G. P. Hirsch, M. W. Francis, and R. A. Popp

The highly purified hemoglobin β chain has been sequenced and the coded amino acids have been quantitated for the substitution of isoleucine. Strain III rabbits purchased from the Jackson Laboratory were selected for their hemoglobin which contains no genetically coded isoleucine in the β chain of the globin (although there are three coded isoleucines in the α chain). The α chain was used as a measure of synthesis rate during the incorporation of [^{3}H]isoleucine. After purification by molecular size (G-200 Sephadex) and ion-exchange purification on three CMC columns (utilizing the differences in the isoelectric point between carboxyhemoglobin, methemoglobin, and cyanmethemoglobin), separation of the isoleucine-containing α chain was done by chromatography (twice) on CMC. Tritiated isoleucine was recovered after sequencing as the phenylthiohydantin (PTH) derivative by high-pressure liquid chromatography (HPLC).

Based on the specific activity of isoleucine in the α chain and the yield of the coded amino acid in the β chain from the sequencer, the substitution frequency was calculated for the first eight amino acids of the rabbit β chain. The spontaneous rate of substitution of isoleucine for other amino acids ranges from 5×10^{-6} to 9.7×10^{-5} (Table 29). Table 29 also shows that the substitution frequencies appear to correlate with the predictive mutability of amino acids. The fidelity of the rabbit β chain is similar to that of human β and α chains, that is, 10^{-5}; therefore, it should be a sensitive method for testing somatic-cell base substitution induced by known mutagens and suspected environmental mutagens.

▼

AVERAGE SUBSTITUTION FREQUENCY OF CYSTEINE FOR OTHER AMINO ACIDS WITHIN A CYSTEINE-MINUS FRAGMENT FROM THE C57BL/6 α CHAIN OF HEMOGLOBIN

B. S. Bradshaw,* R. A. Popp, and G. P. Hirsch

Base substitution within the DNA of somatic cells can be measured by determining the frequency at which noncoded amino acids substitute for coded ones. We have used an ideal peptide to conduct such a study, the N-terminal fragment of the C57BL/6 α chain. A CNBr-cleared fragment from this protein has been isolated. Reticulocytes from anemic C57BL/6 mice were labeled *in vitro* with [^{35}S]Cys and [^{3}H]Ile. Most of the nonhemglobin protein was removed by sieving the supernatant from the cell lysate on Sephadex G-200. Globin was prepared by acid precipitation in cold acetone, and the chains were separated by using ion-exchange chromatography (Whatman CM23). After the cysteines in the α chain were aminoethylated, the α chain was cleaved at methionine (residue 32) with CNBr. The N-terminal fragment was isolated, first by sieving the CNBr preparation on Sephadex G-200, and second by ion-exchange chromatography on a Dowex 1-X2 column. The average substitution frequency of cysteine for other amino acids was determined to be 3.2×10^{-4} in one run and 4.3×10^{-4} in another run. The fragment with the average substitution frequency of 3.2×10^{-4} was cleaved further into smaller pep-

Table 29. Spontaneous substitution rates of isoleucine and predictive mutabilities for other amino acids

Position	Coded amino	Substitution frequency of isoleucine for coded amino acid	Mutable
1	Valine	1.9×10^{-5}	Yes
2	Histidine	9.7×10^{-5}	No
3	Leucine	7.3×10^{-5}	Yes
4	Serine	8.2×10^{-5}	Yes
5	Serine	2.0×10^{-5}	Yes
6	Glutamic acid	$<5.0 \times 10^{-6}$	No
7	Glutamic acid	6.3×10^{-6}	No
8	Lysine	1.7×10^{-4}	Yes

tides by trypsinization. An average substitution frequency varied for each of the tryptic peptides as follows: α T-1 was 1.4×10^{-4}, α T-2 was 6×10^{-4}, and α T-4 was 5×10^{-5}. Similar studies are being done to determine the average substitution frequency of proline and phenylalanine for other amino acids in this CNBr α-chain fragment.

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

ELEVATED ERRORS IN TRANSPLANTED MOUSE HEPATOMAS

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

The fidelity of protein synthesis is a fundamental property of cells. This fidelity determines the percentage of proteins and other cellular components which contain erroneous substitutions and, by implication, the accuracy of biochemical reactions in general. Previously discovered elevated errors in the liver tissue of old mice, which contained spontaneous hepatomas, suggested that some tumors may result from an escape from the protein degradation system which helps to maintain the fidelity of protein synthesis. An indirect estimate of errors in tumors was made by measuring the fidelity of protein synthesis in transplanted hepatomas. The incorporation of the amino acid analog, α-aminoisobutyric acid, was used as an index of the frequency by which an incorrect natural amino acid would be incorporated into protein. Both slow-growing and rapidly growing hepatomas incorporated the analogue 2.5 times as frequently (compared to alanine) as normal liver. Liver tissue in animals carrying these tumors incorporated analogue to the same extent as liver in non-tumor-bearing mice. These values are based on radioactivity recovered as α-aminoisobutyric acid after amino acid hydrolysis. Hepatoma tissue had a higher level of analogue available for incorporation than liver, suggesting that amino acid transport enzymes are elevated in the tumor tissue. This same condition has been observed in the tissues of mice in which error induction was produced by treatment with dimethyl sulfoxide.

KINETICS OF AMINO-ACID-ANALOGUE INCORPORATION IN AGING AND DIMETHYL SULFOXIDE-TREATED MICE

G. P. Hirsch and R. A. Popp

The amino acid analogue α-aminoisobutyric acid has been used to estimate the fidelity of protein synthesis *in vivo* because it is transported as a natural amino acid but incorporated very infrequently (80 times per million). Its rate of substitution is similar to values measured for the substitution of isoleucine for other amino acids in rabbit and human hemoglobin. This analogue was used in earlier studies on the fidelity of protein synthesis among 12- and 25-month-old C57BL/6 mice, where no significant loss of fidelity was detected when the assay was conducted at 24 hr after injection of the analogue. The kinetics of analogue incorporation are different than those of natural amino acids since the analogue remains in the circulation and tissues for several days. Thus, the measurement of errors through the incorporation of analogue into protein was extended to additional time points and a positive control was included. Dimethyl sulfoxide (DMSO) was administered to mice chronically via drinking water at 5% concentration to create an intracellular environment where protein conformations would be changed.

Liver and kidney tissues from 25-month-old mice differed in two statistically significant ways from the corresponding tissues in mice 5 months of age: (1) an increase in the accumulation of analogue in the tissue relative to that present in the plasma, and (2) a decrease in the amount of analogue incorporated. The decrease in analogue incorporated suggests that protein synthesis fidelity is better in the aged animals, except that young mice treated with 5% DMSO demonstrated the same changes when compared with the young controls. The percentage differences are shown in Table 30. The correspondence between the dynamics of old and DMSO-treated mice suggests that additional factors are influencing the level of analogue incorporation and may obscure the measurement of errors by analogue incorporation alone. Data obtained in this experiment using [^{14}C]alanine as a reference for normal protein synthesis and turnover suggest that amino acid reutilization is much more extensive in old and DMSO-treated mice, and this difference may explain why increased analogue incorporation was not observed.

Table 30. Effects of aging and dimethyl sulfoxide on uptake of α-aminoisobutyric acid (AIBA)
Relative change (%)

	Tissue/plasma ratio of AIBA		Wet weight (AIBA/mg)	
	Kidney	Liver	Kidney	Liver
$\frac{(\text{Old} - \text{young})}{\text{young}}$	49	29	−36	−14[a]
$\frac{(\text{DMSO} - \text{control})}{\text{control}}$	55	23	−34	−38

[a]Old not statistically significantly different from young.

AUTORADIOGRAPHIC ANALYSIS OF HYPOXANTHINE-GUANOSINE PHOSPHORIBOSYL TRANSFERASE ACTIVITY IN CHO CELLS AND THEIR MUTAGEN-INDUCED VARIANTS RESISTANT TO PURINE ANALOGUES

G. P. Hirsch, R. A. Popp, and A. W. Hsie

Catalytic products of the enzyme hypoxanthine-guanosine phosphoribosyl transferase (HGPRT) are incorporated into macromolecules in cells and, therefore, accumulate in a form which can be conveniently and quantitatively separated from the precursors. The ability to assay low levels of residual enzyme activity in clones of cells which grow in selective media containing appropriate purine analogue should allow a distinction to be made between point mutations, which often result in reduced catalytic activity, and deletions or frameshift mutations which yield variant clones with no residual activity. The frequency of clones of cells with residual enzyme activity was measured by incorporation of tritiated hypoxanthine in CHO-K_1-BH_4 cells treated with ethyl methanesulfonate (EMS) and then fixed in formaldehyde (3.7%) and washed extensively with cold water.

A comparison of grain counts in our autoradiographic analysis between cells of a previously characterized deletion mutant and normal cells cultured together indicated that residual activity of as little as 3% could be easily identified. When 8-azaguanine at a low level of 3.0 μg/ml was employed to select for resistant variants, 40% of the mutant clones induced by 50 or 200 μg/ml of EMS demonstrated residual activity. While at a high level of 7.5 μg/ml, none of 14 resistant clones (induced by 200 μg/mg of EMS) contained residual activity. Variant selection with another purine analogue, 6-thioguanine (1.7 μg/ml), did not allow the growth of resistant clones (induced by 50 μg/ml of EMS) with residual activity. Similar comparisons of other mutagens with these selective agents, including lower levels of 6-thioguanine, should provide additional support for the interpretation, based on the presence of residual enzyme activity, of the type of mutation induced by chemical and physical agents present in the environment.

USE OF COLONY-FORMING-UNIT ASSAY TO ASSESS CELLULAR DAMAGE (SOMATIC MUTATION) OF CELL-CYCLE SPECIFIC DRUGS

D. M. Popp and R. A. Popp

The colony-forming-unit (CFU) assay has been used to determine the effect of 6-thioguanine (6-TG) on a proliferating tissue, bone marrow.[1] A similar program has been initiated to study the effects of repeated exposure to busulphan (Myleran) on the hematopoietic stem cell. Busulphan has been reported to be less effective on rapidly dividing populations of cells, and, therefore, it has been suggested that it alone of the alkylating agents presently being investigated is effective on the resting stem cell. For this reason we chose busulphan as a possible inducer of somatic mutations in the hematopoietic stem cells of mice, since considerable evidence points to the conclusion that most of the hematopoietic stem cells are resting (nondividing) cells.[2] Initial studies to determine the toxic potential of busulphan and the regenerative potential of mouse bone marrow have been carried out. Male and female mice of strain B10.A received 20 mg/kg doses intraperitoneally (in DMSO and arachis oil) every 13 days. Two days and 13 days after each injection and 30 days after the fourth and final injection, bone marrow from both femurs and tibias was collected in saline. Nucleated cells are enumerated, and 100,000 to 1,000,000 bone marrow cells are injected into lethally irradiated isologous recipients. Ten days after injection the spleens are removed, weighed, and placed in Bouin's fixative. Stem cells with proliferative potential that settle in the spleen can be seen as round, white colonies on the surface of the spleen. In this way, the number of stem cells in the

Table 31. Cellularity and colony-forming unit (CFU) of bone marrow from B10.A mice sequentially treated with busulphan

Treatment (Tx)	Cellularity		CFU/mouse		CFU per million bone marrow cells	
	♀	♂	♀	♂	♀	♂
None	67×10^6 (100)[a]	96×10^6 (100)	13233 (100)	18240 (100)	198 (100)	190 (100)
1 Tx + 2 days	48×10^6 (72)	58×10^6 (60)	2560 (19)	4495 (25)	53 (27)	78 (41)
1 Tx + 13 days	54×10^6 (81)		3850 (29)		70 (35)	
2 Tx + 2 days	52×10^6 (77)	56×10^6 (58)	776 (6)	2240 (12)	15 (8)	40 (21)
2 Tx + 13 days	60×10^6 (90)		1050 (8)		18 (9)	
3 Tx + 2 days	39×10^6 (58)	50×10^6 (52)	0 (0)	5695 (31)	0 (0)	113 (59)
3 Tx + 13 days	64×10^6 (96)		635 (5)		10 (5)	
4 Tx + 2 days	34×10^6 (51)	48×10^6 (50)	1190 (9)	484 (3)	35 (18)	10 (5)
4 Tx + 30 days	76×10^6 (113)	98×10^6 (114)		12535 (69)		115 (61)

[a]Numbers in parentheses are percentages for a specific treatment in a particular category.

bone marrow after treatment with busulphan can be enumerated. Table 31 shows that the cellularity of the bone marrow never drops below 50% two days after treatment, even when the marrow has been exposed four successive times. The marrow regenerates to near normal values by 13 days and is within the normal range for cellularity after 30 days. The number of stem cells is more severely affected. Repeated injections cause a greater reduction in stem cells, so that after four injections the number of stem cells is severely reduced. After a recovery period of 30 days, the number of stem cells returns to only 60 to 70% that of normal.

These data show that the drug busulphan is more effective on the progenitor cell than it is on the more mature cell in cycle in the bone marrow. Genetic damage incurred by the resting cell may be more easily demonstrated, since greater numbers of progeny from an activated cell will be available for assay than from the more mature precursor cell which clones out and dies with the life span of the cell. The studies show that the mouse will survive four exposures to busulphan and will regenerate stem cells from a small reserve. Such animals will be used to look for somatic mutations in the hematopoietic stem cell.

1. D. M. Popp, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, p. 92.

2. T. M. Fliedner *et al. Adv. Radiat. Res., Phys. Chem.* **2**, 707–724 (1970).

EXPOSURE OF FETAL HEMATOPOIETIC STEM CELLS TO MUTAGENIC AGENTS: METHOTREXATE-INDUCED SOMATIC MUTATION

D. M. Popp and G. P. Hirsch

In an effort to induce somatic mutations (*e.g.*, $HGPRT^-$) in the hematopoietic stem cell, mice have been exposed to various mutagens during the last five

days of embryonic development. At this time there is a proliferative peak in CFU's in the liver and, therefore, this is the period most sensitive to mutagenic events in the hematopoietic stem cell. Two weeks after birth the hematopoietic stem cell becomes a resting cell,[1] proliferating little or not at all, and, therefore, is likely to be resistant to mutagenic events.

The drugs used to date have been methotrexate, 6-thioguanine, β-propriolactone, hycanthone, MMS, and busulphan. Offspring have been raised and are allowed to mature to be used in future experiments to assess accumulated somatic mutations. However, an interesting event has been noted using the folic acid antagonist methotrexate which will be reported here.

Pregnant females received a single injection of 10 μg/kg of methotrexate intraperitoneally at −1, −2, −3, −4, or −5 days of delivery. Very little embryo toxicity was noted 24 to 48 hr before birth, and litters were raised successfully. Embryo toxicity was noted from 3 to 5 days before birth; however, at about 2 weeks of age, when hair growth is evident, some offspring were observed to have varying degrees of white hairs, the degree of whiteness increasing with age. The expression of white hairs (gray babies) varies from animal to animal; some possess greater than 50% white coloration and others have only flecks of white here and there. In every case there is a unique distribution of white hair on the face resembling a mask. Table 32 indicates the frequency of this methotrexate-induced variant. It is evident that affected embryos are sensitive to this drug from 24 to 48 hr before birth, and that cells, other than hair follicles, that have been exposed to the drug may be likely targets for somatic mutations and profitable tissues for examination.

Table 32. Effect of 10 μg of methotrexate on embryos during the last 1 to 5 days of gestation

Days before delivery	No. of embryos born	No. of embryos weaned	No. of gray babies
1	7	3	0
	14 (two litters)	10	3
	7	6	1
	9	5	0
	16 (three litters)	15	6
	5	5	1
2	9	7	1
	5	3	0
	9	5	0
	6	5	0
3	7	5	0
	9	5	0
	12 (two litters)	11	0
4	7	4	0
	7	3	0
5	born dead		
6	born dead		

1. T. M. Fliedner *et al.*, *Adv. Radiat. Res., Phys. Chem.* **2**, 707–724 (1970).

BIOLOGICAL DESCRIPTION OF B10.F, A SHORT-LIVED CONGENIC MOUSE STRAIN

D. M. Popp

A strain of mouse in our colony has been shown to have a short life span with no apparent gross pathology predicating its early death. Studies have been undertaken to characterize this mouse strain and to determine if it is a mouse model of the human syndrome of *progeria*.

The mouse is strain C57BL/10.F-*H-2* (B10.F) and is congenic to C57BL/10, differing only at the *H-2* locus. These mice gray early (six months), suffer severe hair loss and emaciation, and die by 18 months. Skin ulcers and thin, wrinkled skin are common. A life-span study was done for 73 breeders; only three lived to be 25 months old, the mean age of death being $13^1/_2$ months. Death data taken from a random sample of cage cards from another B10 congenic line [B10.A(5R)] raised in our facility showed a mean age at death of 24 months.

Since congenic mice differ from B10 by a restricted genetic region, (B10 × B10.F)F_1, backcross, and F_2 mice were raised to determine if there is a genetic basis for the degenerative process. Of 40 F_1's raised to date, 22 have died and have a mean age at death of 20 months. Thirty-two B10 BC_1's, 54 B10.F BC_1's, and 73 F_2's are aging, and their age at death and H-2 types will be correlated.

Growth curves were determined for the B10, B10.A (another congenic strain), and B10.F strains from infancy to death. The growth curves do not differ significantly. The infant B10.F is somewhat larger, but between days 17 and 22 there is a stationary growth phase not observed for the B10 strain. The mature male weight for B10 is 32 g; for B10.F, 30 g; and for B10.A, 29 g. The mature female weight for B10 is 26 g; for B10.F, 28 g; and for B10.A, 25 g. The B10.F reaches its mature weight at about 24 weeks of age, whereas B10 and B10.A reach their mature weight at about 28 weeks. At 52 weeks, 59% of the

Table 33. Plaque-forming ability (to sheep red blood cells) of mouse strain B10.F at various ages

	4 weeks	5 weeks	2 months	3 months	4 months	6 months	$8^1/_2$ months	12 months
Thousands of plaque-forming cells per spleen	116	172	250	244	260	180	204	43

▼

B10.F mice, 100% of the B10 mice, and 97% of the B10.A mice being weighed are alive.

The immunological competency of the B10.F is being studied. Their ability to produce plaques to sheep red blood cells is normal through eight months of age but is very deficient by 12 months (Table 33). Additional quantitation of the immunocompetence of the B10.F strain is in progress.

ISOLATION OF H-2 ANTIGEN BY AUTOMATED AFFINITY CHROMATOGRAPHY

B. S. Bradshaw,* E. L. Candler, and R. A. Popp

We reported last year the preparation of an affinity column (AC26) using the immunoglobulin fraction of C3H anti-RFM H-2 serum.[1] After the affinity column was prepared, two characteristics of the column had to be determined: (1) whether AC26 was capable of binding RFM H-2 antigens and (2) the most effective incubation time for binding the H-2 active fraction. First, 14 ml of 3 *M* KCl extract taken from RFM lymphocytes and thymocytes, containing 15.4 mg of protein, was pumped onto AC26 at 1 ml/min. The sample was allowed to interact with the column for 16 hr at 4° C before the unabsorbed (U) and absorbed (A) fractions were collected, dialyzed against 0.01 *M* phosphate-buffered saline (pH 7.0), and concentrated. Second, two separate runs of 7 ml each of the 3 *M* KCl extract were processed in the cold (4° C) at a flow rate of 1 ml/min. The U and A fractions were pooled and treated as above. Third, another duplicate of the first run was made. All of the U and A fractions were tested by hemagglutination inhibition and found to contain active H-2 antigen. Polyacrylamide gel electrophoresis (PAGE) of the U and A fractions revealed that the A fractions of the runs that were incubated on the column 16 hr contained several bands, whereas the A fraction from the second run, when the column was pumped continuously, contained a single band.

When the sample (3 *M* KCl extract) was reduced to 5 ml and the column was pumped continually at 1 ml/min at either 4° C or at room temperature, the H-2 activity was found only in the A fraction. PAGE of the A fraction exhibited a single band. A Sephadex G-200 elution of this material revealed a profile showing a single peak, which was estimated to have a molecular weight of 48,000 D. The ^{51}Cr release inhibition assay confirmed the hemagglutination inhibition assay that the A fraction contained active H-2 antigen.

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

1. B. S. Bradshaw, R. A. Popp, and E. L. Candler, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, p. 95.

STRUCTURE OF BLASTOKININ

R. A. Popp, L. D. Wise,* and J. C. Daniel, Jr.*

The structure of blastokinin has as yet not been determined. Previous physical studies have suggested that rabbit blastokinin has a molecular weight of approximately 15,000 and that β-mercaptoethanol or dithioerythritol further reduces the molecular weight to approximately 7000, which suggests that blastokinin is composed of two polypeptide chains linked by at least one disulfide bond.

Five milligrams of blastokinin was subjected to automated amino acid sequencing. An amino acid sequence of Gly-Ile-Ser-Pro-Arg-Phe-Ala-His-Val-Ile-Glx-Asx was determined for the first 12 amino acid residues. Sequencing beyond the twelfth residue failed, possibly because of a carbohydrate side chain. Only one kind of amino acid was identified at each of the 12 positions, which suggested that the polypeptide chains being held together by disulfide bonds could be identical. However, the yield of amino acids at each position was 50% or less than that ordinarily expected if both polypeptides were being sequenced.

Ten milligrams of aminoethylated blastokinin was digested by trypsin, and the tryptic peptides were

separated by two-dimensional paper chromatography and high-voltage paper electrophoresis. After minimal reaction with ninhydrin to locate the tryptic peptides, each of 18 ninhydrin-positive peptides was eluted, hydrolyzed, and quantitatively analyzed for its amino acid content. The amino-terminal Gly-Ile-Ser-Pro-Arg peptide and the next Phe-Ala-His-Val-Ile-Glx-Asx-(Thr_2-Ser_3-Glx-Pro-Gly_2-Ala-Leu_4-Tyr)-Lys tryptic peptide were identified. However, the quantity of amino acids in these two peptides, and 10 others, was approximately half that found for six other peptides. The amino acid compositions of the peptides obtained in the higher yields are such that no combination could give an amino acid sequence like that reported for the first 12 residues. Approximately 60 amino acids are in peptides obtained in lower yield, whereas approximately 30 amino acids are in peptides obtained in the higher yield. In order to fit this with other physical data, we believe that blastokinin has a unique polypeptide chain of approximately 60 amino acids with Gly at its amino terminus. Blastokinin has two other polypeptide chains that are similar to one another; each contains approximately 30 amino acids and each has a blocked amino terminus. The 60-amino-acid-polypeptide chain is linked to the other two by one or more disulfide bonds, but the nature of the chemical bond(s) between the two 30-amino-acid-polypeptide chains has as yet not been determined. The proposed subunit structure would give rise to two components (each approximately 7000 mol. wt) in the presence of β-mercaptoethanol.

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RECOMBINATION OF ULTRAVIOLET-INDUCED PYRIMIDINE DIMERS IN HUMAN FIBROBLASTS

R. Waters* and J. D. Regan

The appearance of UV-induced pyrimidine dimers in DNA synthesized after UV irradiation has been monitored in human fibroblasts using a UV endonuclease. Pyrimidine dimers have been shown to be the only UV-induced substrate for this UV endonuclease;[1] the incision by this enzyme occurs virtually only in the dimer-containing strand.[2,3] The results indicate that a small percent of UV-induced pyrimidine dimers can be found in the DNA of human cells synthesized after UV irradiation. The mechanism responsible for this exchange operates in normal and in excision-deficient xeroderma pigmentosum (XP) cells and is thus independent of the incision step required for the excision repair of such dimers. If one assumes that recombined dimers are equally accessible to excision in the normal cells, the exchange of dimers has occurred at the same frequency in both normal and XP fibroblasts. This frequency (5%) is an order of magnitude less than that observed in excision-deficient *Escherichia coli* K-12.[4]

Currently, we are investigating the use of UV-induced pyrimidine dimers as DNA markers for monitoring recombination induced after chemical mutagen and carcinogen treatments.

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

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PARTIAL INHIBITION OF POSTREPLICATION REPAIR AND ENHANCED FREQUENCY OF CHEMICAL TRANSFORMATION IN RAT CELLS INFECTED WITH LEUKEMIA VIRUS

R. Waters,* N. Mishra,† N. Bouck,† G. DiMayorca,† and J. D. Regan

Postreplication repair of DNA and chemical transformation with the carcinogen 4-nitroquinoline oxide (4NQO) was studied in rat cell lines either uninfected or infected with Rauscher leukemia virus (RLV). The results indicate that equivalent amounts of carcinogen were bound to the DNA initially and removed during excision repair. However, differences occurred in that the infected lines alone are both sensitive to 4NQO-induced transformation and also exhibit a partial inhibition of the postreplication repair process after 4NQO or UV treatment. It remains to be determined whether this response represents a general effect of leukemia virus infection or is limited to RLV interaction with rat cells.

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THE INDUCTION AND REPAIR OF DNA-DNA CROSSLINKS INDUCED BY THE DUAL ACTION OF CHEMICALS AND LIGHT

A. A. Francis, R. Waters,* and J. D. Regan

Initial studies had indicated that the carcinogenic chrysene compounds may well bind photodynamically to DNA resulting in DNA–DNA crosslinks. This phenomenon was further investigated by the use of isopycnic cesium chloride centrifugation. The treatment of normal and excision-deficient XP fibroblasts with either 4-methyl chrysene, 5-methyl chrysene, or psoralen, followed by irradiation at 360 nm, induced crosslinking of DNA. These lesions were repaired in both cell lines. Since repair occurred in the XP cells in these experiments, the existing mechanism for the repair of DNA–DNA crosslinks in human cells apparently does not require the incision step involved in the repair of UV-induced pyrimidine dimers. Two cell lines from patients suffering with Fanconi's anemia exhibited a variation in the capacity of crosslink repair, one repairing these lesions in a normal manner and the other having a reduced capacity.

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

MUTAGENESIS OF YEAST BY HYDRAZINE: DEPENDENCE UPON POSTTREATMENT CELL DIVISION

J. F. Lemontt

Previous studies have shown that hydrazine is a weak chemical mutagen in *Haemophilus influenzae* with an unusual mode of action.[1] Induced mutation was detected at exposures (concentration times time) spanning nearly four orders of magnitude over which there was no effect on cell viability. Moreover, fixation of mutations was found to be entirely dependent upon DNA replication, which suggested that hydrazine mutagenesis in bacteria occurs by mispairing of altered bases during semiconservative DNA replication rather than by an error-prone repair system.[2]

The effects of hydrazine as a chemical mutagen in eukaryotic organisms have been investigated here with a sensitive forward-mutation system involving selection of canavanine-resistant mutants of the yeast *Saccharomyces cerevisiae*. Conditions of mutation expression were also studied. Little or no induced mutagenesis was detectable upon immediate assay following treatment in neutral buffer suspension. Posttreatment cell division in the complex growth medium was required, however, for expression of induced mutation. Frequencies of induced mutation rose to approximately tenfold higher than spontaneous levels for exposures between 0.1 and 12.0 min mol/liter. Cell survival remained at 100%. By contrast, single treatments with ethyl methanesulfonate, methyl methanesulfonate, MNNG, nitrous acid, hydroxylamine, and UV light were able to increase mutation frequency in this system upon immediate assay. Expression of hydrazine-induced mutation was detectable after treated cells had undergone less than one population doubling in the posttreatment growth medium. Such mutation expression could be blocked by the inhibitors hydroxyurea and cycloheximide, which block DNA synthesis (elongation) and protein synthesis (and initiation of DNA replication), respectively. The results indicate that DNA replication is required for fixation and expression of mutagenesis. It is suggested that in this eukaryote, hydrazine-induced DNA lesions, possibly *N*(4)aminocytosine, lead to mutation by causing base mispairing at DNA replication rather than by means of an error-prone repair mechanism.

1. R. F. Kimball and B. F. Hirsch, *Mutat. Res.* **30**, 9–20 (1975).
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LOSS OF HYDRAZINE-INDUCED MUTABILITY IN YEAST DURING POSTTREATMENT INHIBITION OF CELL DIVISION

J. F. Lemontt

The posttreatment cell-division-dependent mutagenesis induced by hydrazine in yeast may be blocked by agents that inhibit DNA replication or its initiation.[1] In order to determine whether or not the presumed premutational lesions in DNA retained their mutagenic potential after the growth inhibition period, mutagen-treated cells subsequently incubated for 1 day in growth medium containing cycloheximide were washed several times in distilled water and then seeded into fresh growth medium lacking the inhibitor. After a 1-day growth, such cells lost

nearly all (95 to 100%) hydrazine mutability, as compared with control cultures previously uninhibited and similarly grown up again. This suggests that premutational DNA lesions induced by hydrazine are removed or possibly modified and rendered nonmutagenic by some cellular process. After a 3-hr incubation in growth medium containing cycloheximide (1 μg/ml), induced mutability (assayed following 1-day uninhibited growth) decreased 50% as compared with controls incubated for 3 hr in growth medium lacking the inhibitor, followed by a further 1-day growth. This 3-hr control treatment was itself not long enough to permit significant expression of induced mutation, as determined by plating for viable titer and mutation frequency assay. If hydrazine premutational lesions are removable by a process similar to repair, certain yeast strains defective in error-free (excision) UV repair or error-prone (mutagenic) UV repair may also fail to remove these lesions. Experiments to test this hypothesis are currently in progress.

1. J. F. Lemontt, this report.

PROPERTIES OF MUTANTS RESISTANT TO ULTRAVIOLET-INDUCED MUTAGENESIS IN YEAST

J. F. Lemontt

Following isolation and genetic analysis of yeast mutants resistant to UV-induced forward mutation reported last year,[1] seven single genetic loci were chosen for further study. Pairwise meiotic segregation tests revealed seven nonallelic loci, designated *umr1*, *umr2*, ..., *umr7*. Five *umr* homozygotes (excluding *umr1* and *umr4*) failed to sporulate. One of these, *umr7*, blocked normal secretion of alpha mating hormone in the α haploid.

Haploids carrying each of the seven *umr* loci were outcrossed to strains carrying highly UV-revertible unlinked ochre-suppressible alleles (*his5-2*, *lys1-1*, and *ura4-1*) in an attempt to assess the effects of each locus on both UV forward mutation (*CAN1* → *can1*) and UV revertibility. A *UMR* wild-type segregant was similarly outcrossed in the same way to provide a control strain with comparable genetic background. Among the ten original *umr* isolates, one was later found to be only slightly UV-sensitive and not in fact mutation-resistant (mutants per survivor). It, too, was similarly outcrossed as a phenotypic wild-type control strain.

Strains carrying *umr4*, *umr5*, *umr6*, or *umr7* were found to exhibit normal UV revertibility of all three different test alleles, as compared with genotypic and phenotypic control strains. Yet these loci effectively reduced in varying degrees the ability of haploids to undergo UV forward mutation at a single locus conferring resistance to the arginine analogue canavanine. Little or no UV sensitivity resulted from the presence of these *umr* loci in cells. The *umr3* locus, however, appeared to reduce UV revertibility of all three auxotrophic markers; *umr2* reduced UV reversion of only *ura4-1*; *umr1* reduced UV reversion of *his5-2* and *ura4-1* but not of *lys1-1*. Very slight UV sensitivity was observed in these latter strains (dose-reduction factors at 10% survival less than 1.5).

Wild-type UV forward mutation with the *CAN1* system is routinely elicited by irradiation of cells immediately after plating on arginineless synthetic growth medium containing 40 μg/ml of canavanine sulfate. It seemed reasonable to suppose that *umr* mutants might only appear mutation-defective if they were to be more sensitive than the wild type to the toxic effects of canavanine. That is, they might die or become adversely affected by the analogue before mutation fixation and expression could be completed. Accordingly, the *umr* segregants studied above were tested for sensitivity to canavanine compared to wild type with respect to a number of different end points: growth inhibition (spot test), growth inhibition (colony-forming ability), UV mutability, and rate of uptake of ^{14}C-labeled amino acids into cells. Indeed, all *umr* mutants except *umr1* exhibited greater canavanine-induced growth inhibition than wild type, yet *umr1* transported arginine and tryptophan into cells at rates tenfold higher than wild type. The *umr6* strain, however, having only weak UV mutation resistance, was very sensitive to canavanine growth inhibition but transported amino acids at essentially wild-type rates. All the data suggest that increased canavanine toxicity does not necessarily lead to defective mutability at *CAN1* and that mutational deficiency cannot result solely from increased canavanine toxicity. Although exposure to canavanine was shown to block mutation fixation and/or expression, it is suggested that degree of growth inhibition is not strictly correlated with degree of mutation resistance. Moreover, the mechanism of canavanine toxicity is not at all well understood, but it is believed to somehow involve the incorporation of the analogue into protein, substituting for arginine. It is not known which cellular processes, when altered, kill the yeast cell. Other studies in bacteria and mammalian cells suggest that

DNA synthesis is especially sensitive to canavanine, compared to other amino acid analogues.

More data will be needed to determine whether or not certain *umr* genes control specific modes of UV mutagenesis in an error-prone repair pathway. It has been recently suggested that such specificity may reside with interaction between particular mutation (error-prone repair) enzymes and DNA damage induced only in genome regions of specific nucleotide sequence.[2] If the molecular mechanism of UV reversion depends upon such successful interaction, then a *umr* mutant might be enzyme-defective but still exhibit normal UV revertibility because the allele to be reverted may not include or be sufficiently close to the region of corresponding base sequence.

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THE EFFECT OF ULTRAVIOLET LIGHT UPON TRANSPORT OF L-ARGININE INTO YEAST CELLS

J. F. Lemontt and H. Taylor*

The uptake of L-arginine into yeast cells is known to occur by means of a specific permease enzyme, genetically controlled by the *CAN1* structural gene,[1] and by a more general amino acid permease enzyme, which is inhibited by ammonium ion under normal conditions.[2] Very little information is available concerning synthesis, integration at the site of action, or turnover of these molecules. It has been known for a long time that UV may be absorbed directly by protein, leading to inactivation of enzyme activity; but the exposures needed to produce these effects are typically hundreds or more times those needed to elicit DNA damage with resultant effects on transcription, DNA synthesis, mutation frequency, and whole cell reproductive survival. Preliminary experiments to investigate the effect of UV on arginine uptake were carried out with the hope of comparing the results quantitatively with the effects of UV on cell viability.

The initial velocity of the incorporation of [^{14}C]arginine into whole cells was measured under standard conditions in S288C (wild type). Cells were grown in minimal medium, harvested, and suspended in fresh minimal medium at $\sim 2 \times 10^7$ cells/ml, containing unlabeled arginine at 20 μg/ml. Part of the suspension was UV-irradiated prior to uptake assay, while the other part served as control.

UV was found to have an exposure-dependent inhibitory effect on the initial velocity of incorporation. The UV survival of the uptake (percent remaining activity), however, was found to be greater than the UV survival of cellular viability (dose-reduction factor $\sim$2). Post-UV exposure to photoreactivating black light (300 to 400 nm) was able to enhance cell survival but had no restorative effect upon inhibition of initial uptake rate. Non-UV-irradiated black-light-exposed control samples exhibited less than 100% rate of uptake. Thus it appears unlikely that UV damage to existing arginine permease or even UV damage to the *CAN1* structural gene can easily account for the effect of UV on arginine transport. The inhibitory effect could be due to an indirect action of UV, for example, upon the energy-generating metabolism needed for active transport.

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THE FAILURE OF ULTRAVIOLET AND IONIZING RADIATIONS AND CROSS-LINKING AGENTS TO INDUCE MUTATIONS IN *HAEMOPHILUS INFLUENZAE*; RELATIONS TO ERROR-PRONE REPAIR

R. F. Kimball

Earlier, preliminary data had suggested that the standard laboratory "wild-type" strain (Rd) of *H. influenzae* was not mutated by ultraviolet radiation. In this respect, it appeared to act like the *lex* (*exr*) mutant of *E. coli*, which lacks an inducible error-prone repair system required for mutation by UV and ionizing radiation and by several but not all chemical mutagens. It was desirable to find out how far *H. influenzae* Rd was like *E. coli lex*. For this purpose, attempts were made to induce mutation with UV light and X rays and with the two cross-linking agents mitomycin C and nitrogen mustard. All attempts to induce mutations in *H. influenzae* Rd with these agents failed. A mutant (*uvr-1*) of Rd that is unable to excise pyrimidine dimers probably was induced to mutate by UV but at a very low frequency near the limits of experimental detectability. Thus it

seems probable that pyrimidine dimers persisting to the time of DNA replication can lead to some mutation but by a very inefficient process. In all these respects, *H. influenzae* Rd and its derivatives closely resemble *E. coli lex*. Like the latter, Rd is mutated by nitrosoguanidine and EMS but not by radiations and by mitomycin C.

Beattie[1] has shown that mutations induced in *H. influenzae* Rd and its derivatives by *N*-nitrosocarbaryl (a nitroso compound somewhat similar to MNNG) arise in large part through errors in repair. The question therefore arises whether this error-prone repair system is different from the UV-inducible error-prone repair system in *E. coli* that is responsible for mutagenesis by UV or whether the system is really the same but simply not inducible in *H. influenzae* by UV although inducible by nitrosocarbaryl. Therefore, *H. influenzae* was pretreated with nitrosocarbaryl at an exposure sufficient to produce a small frequency of mutations and then treated with UV. No mutations attributable to the UV irradiation were detected. Consequently, the error-prone repair responsible for mutation induction in *H. influenzae* by nitrosocarbaryl is different from the error-prone repair system responsible for UV mutagenesis in *E. coli*. An error-prone repair system responsible for the induction of mutations by nitroso compounds in *E. coli* has not been reported nor, as far as can be told, looked for. It seems probable that it exists, however, since such compounds induce mutations in *E. coli lex*, which lacks the UV-inducible system.

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FAILURE OF BASE ANALOGUES TO INDUCE MUTATIONS IN *HAEMOPHILUS INFLUENZAE*

R. F. Kimball

The analogue of thymidine (dThd), 5-BrdUrd, has usually been assumed to induce mutations by causing errors in replication because this analogue is more likely than thymidine to be in a tautomeric form that could pair like cytidine. Thus it is assumed to act by mispairing during replication. Pietrzykowska,[1] however, obtained evidence that the frequency of mutations induced in lambda phage by this analogue was very strongly affected by the bacterial *recA* and *lex* and phage *red* loci. This suggests that a recombination-associated error-prone repair system may be involved rather than simple base mispairing. Witkin and Parisi,[2] on the contrary, found no effect of the *E. coli lex* locus on mutations induced in the bacteria by this analogue and concluded that there was no reason to believe that the *lex*-dependent error-prone repair was involved.

In the preceding report, evidence was given that *H. influenzae* Rd resembles *E. coli lex* in its response to mutagens. This, together with an unexpected failure to induce mutations in this species with BrdUrd several years ago,[3] led to a more careful evaluation of BrdUrd effects in this species. We have repeated the mutation experiments with concomitant measurements of the amount of BrdUrd incorporated and again found no mutation even when three-quarters of the DNA strands were nearly completely substituted with bromouracil (BrUra). There is very little bacterial death under these circumstances; consequently, differential death of bacteria with BrUra-substituted DNA cannot account for the lack of mutation. *H. influenzae* partially discriminates against BrdUrd when both BrdUrd and dThd are present, but when only BrdUrd is available it is incorporated to nearly the same extent as dThd.

Pietrzykowska[1] also found a considerable reduction in the frequency of induced mutation in lambda phage by another base analogue, 2-aminopurine, when the bacterial gene *recA* and the phage gene *red* were mutant. No mutations were induced in *H. influenzae* by exposures to this analogue that resulted in appreciable cell killing, but no tests for incorporation into the DNA have yet been performed.

In view of the evidence in the preceding report that *H. influenzae* wild type resembles *E. coli lex* in lacking an error-prone repair system for ultraviolet light (also absent in *E. coli recA*), our data are more compatible with Pietrzykowska's[1] than with Witkin and Parisi's[2] results. In any case, it appears that the replication system in *H. influenzae* recognizes BrUra only as a thymine and not a cytosine analogue, although it appears to partially discriminate against the incorporation of BrUra when both BrdUrd and dThd are present.

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2. E. M. Witkin and E. C. Parisi, *Mutat. Res.* **30**, 407–409 (1975).
3. R. F. Kimball and J. K. Setlow, *Mutat. Res.* **22**, 1–14 (1974).

THE INVOLVEMENT OF PREREPLICATION PROCESSES IN FIXATION OF MUTATION INDUCED BY *N*-METHYL-*N'*-NITRO-*N*-NITROSOGUANIDINE AND ETHYL METHANESULFONATE IN *HAEMOPHILUS INFLUENZAE*

R. F. Kimball and S. W. Perdue

Earlier evidence[1,2] showed that mutations induced by *N*-nitrosocarbaryl in *H. influenzae* were the result of fixation both during a prereplication process (presumably some form of repair) and at a replication-associated process, presumably a form of postreplication repair dependent on the *rec-1* locus. The latter process appeared to be considerably more efficient than the former, so that when DNA replication was delayed, most premutational lesions were repaired without mutation and only a small fraction were converted to mutation. Premutational lesions persisting to replication were several times more likely to cause mutation.

The same general types of experiments have been carried out with MNNG and EMS. Some of the mutations induced by both these agents were fixed prior to replication, just as was the case with *N*-nitrosocarbaryl. It also appears that with both agents there is a replication-associated fixation and that it is somewhat more efficient than the prereplication fixation. The evidence for this is fairly clear for EMS, somewhat less clear for MNNG. The role of the *rec-1* locus in replication-associated fixation has been somewhat difficult to test adequately because of the sensitivity of *rec-1* mutants to killing by these agents.

Thus all the alkylating agents that have been tried in *H. influenzae* produce an appreciable fraction of their mutagenic effect by fixation prior to replication. This is not consistent with the fairly frequently expressed view that they act only by causing mispairing at replication as a result of alkylation of bases. In this respect they differ from hydrazine, which seems to act in *H. influenzae* only by this latter mechanism.[3]

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EFFECTS OF X IRRADIATION ON A TEMPERATE BACTERIOPHAGE OF *HAEMOPHILUS INFLUENZAE*

M. E. Boling and M. L. Randolph

A number of alterations occurring in bacteriophages as a result of ionizing irradiation have been reported in the literature. All of these effects have been shown to be involved in the viability loss of certain phages. However, some alterations are of no consequence in certain phages. An example of this is single-strand breaks, which have been shown to be lethal for only one double-stranded phage. In general it has been found that nucleotide alterations are responsible for most of the inactivation of phages. Considerable work has been done in this laboratory using the *Haemophilus* phage system. Experiments were done to determine which, if any, of the various alterations resulting from X irradiation account for the inactivation of the phages. The results are as follows.

Inactivation of the bacteriophage HPlcl by X rays in a complex medium is exponential, and the D_0 was approximately 90 kR. Analysis of results of sucrose sedimentation of DNA from X-irradiated whole phage showed that the D_0 for intactness of single strands was about 105 kR and for intactness of double strands was about 280 kR. The D_0 for attachment of X-irradiated phage to the host was estimated to be about 1100 kR. X irradiation also causes lysis of phage, resulting in increased DNase sensitivity of the phage DNA. The lysis of the phage is an inactivating event and accounts for an unknown portion of the X-ray-induced survival loss.

Only the production of single-strand breaks approaches the rate of survival loss after X irradiation. However, single-strand breaks produced by UV irradiation in the presence of hydrogen peroxide equivalent to 215 kR of X rays showed no lethal effect on the phage.

In certain phages, double-strand breaks prevent the complete injection of phage DNA into the host cell. If this is true for our phage, it would be a rare occurrence, judging from the rate of double-strand break production.

None of the alterations measured in these studies account for the inactivation of the phage, either due to the rareness of the alteration or due to the

insignificance of the alteration. This is in keeping with the findings by others that it is base damage that accounts for most of the inactivation.

UV-sensitive mutants of the host cell exhibited no sensitivity to host cell reactivation of the X-irradiated phage, a fact suggesting that other repair systems are involved in X-ray repair.

UPTAKE OF RADIOACTIVE NITROSAMIDES INTO BACTERIA

R. K. Elespuru*

Methylating nitrosamides containing ^{3}H or ^{14}C in the methyl group were found to be taken up into *E. coli* and *H. influenzae* cells with very different efficiencies. Nitrosocarbaryl (NC), which contains a lipophilic naphthyl group, became concentrated inside the cell, while MNNG and methylnitrosourea bound to the cells with much less affinity. The concentration of MNNG and methylnitrosourea associated with *H. influenzae* cells was never as great as the extracellular concentration. An estimation of the effective concentration of mutagen in the cell could be made from the data obtained in these experiments. The differences in effective concentration accounted for some but not all of the differences between these three compounds in mutagenic effectiveness. Therefore there must be additional, as yet unidentified, causes for such differences.

The radioactivity from NC labeled in the methyl group was bound 2.5 times more effectively than the radioactivity from NC labeled in the naphthyl group. This suggests that the molecule was split, the naphthyl group was lost, and the methyl group was retained, presumably as a result of methylation of cellular materials.

E. coli whose membranes had been made more permeable by toluenization still bound the three nitrosamides in similar proportions to normal cells. The results suggest that permeability differences may not be important for uptake but are made somewhat difficult to interpret because just what toluene does to make the membrane permeable to certain molecules is not well known.

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FORMATION OF COMMON INTERMEDIATES FROM NITROSAMIDES

R. K. Elespuru*

One hypothesis concerning the mechanism of action of alkylating nitrosamides is that they break down to a common alkylating intermediate. Experimental evidence for this hypothesis was obtained by measuring the relative mutagenic efficiency of three classes of compounds: methylating nitrosamides, ethylating nitrosamides, and cyclic nitrosamines. The latter compounds are unlikely to break down to an intermediate. The different cyclic compounds gave different ratios of mutation frequencies at three genetic loci, as might be expected for compounds with different chemical structure that could react differentially with different regions of the DNA. Methylating and ethylating nitrosamides gave very different ratios from each other, but the ratios within these groups were very similar. These results are the ones expected if a common intermediate compound is responsible for mutagenesis by the methylating compounds and another one for mutagenesis by the ethylating compounds.

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FURTHER CHARACTERIZATION OF X-RAY-INDUCED DNA POLYMERASE I-DIRECTED REPAIR SYNTHESIS IN PERMEABILIZED *ESCHERICHIA COLI*

D. Billen and G. R. Hellermann*

We have previously shown that in an endonuclease I (endo I)-deficient *E. coli* strain (AB 3063) made permeable to nucleotides, X-ray exposure results in the introduction of primer-template sites for DNA polymerase I (pol I).[1] Exposure of permeabilized strain AB 3063 to 20,000 rads of X rays results in the introduction of 160 to 170 single-strand breaks per 10^{9}d in the cellular DNA, as compared with about 5 single-strand breaks per 10^{9}d observed in unirradiated cells.

The formation of breaks or nicks in X-irradiated DNA may be correlated with pol I repair activity,

since activation of DNA ligase by the addition of the cofactor for DNA ligase, nicotinamide adenine dinucleotide (NAD), to the assay mixture results both in ligation and in a cessation of pol I-directed repair synthesis. In X-irradiated cells the presence of NAD results in a marked reduction in the number of single-strand breaks (about 75% of single-strand breaks are rejoined in 20 min at 37°C) and a limited amount (short patch) of repair synthesis. Addition of nicotinamide mononucleotide (NMN), an end-product inhibitor of DNA ligase, increases the number of single-strand breaks as well as the extent of pol I-directed repair synthesis.

The nicks or breaks in DNA caused by X rays may be directly or enzymatically introduced. Direct breaks may require enzymatic activity for conversion to acceptable sites for pol I activity since this enzyme requires a 3′-OH terminus. Recent reports show that most breaks in DNA irradiated either *in vitro* or *in vivo* will not serve as primer sites for pol I, although they effectively bind the enzyme.[2,3] Hagen and coworkers[2] have used exonuclease III (exo III) to convert inactive sites to sites allowing DNA synthesis by pol I.

To determine the role of exo III in our system, a double mutant of *E. coli*, strain TN193 (*endA1 xth-1*), was constructed, as described elsewhere in this report. The *xth-1* mutant, BW9091, was originally isolated by Milcarek and Weiss[4] and shown to be deficient in exo III activity (5 to 10% of wild-type level). However, Yajko and Weiss[5] subsequently showed that all exo III-deficient cells were also endonuclease II (endo II)-deficient and concluded that both activities were performed by the same enzyme.

We reasoned that if exo III and endo II activities were involved in the production of primer sites for pol I, there should be a reduction in X-ray-induced repair synthesis in the *endA1 xth-1* mutant. This was not observed. We therefore tentatively conclude that exo III and endo II activities are not necessary for X-ray-induced, pol I-directed repair synthesis (assuming that the residual activity of this enzyme in the mutant is insufficient to allow normal response).

Other enzymes may carry out the necessary steps. Ljungquist *et al.*[6] have recently shown that there are at least two different endonucleases in *E. coli* K-12 (one of which is endo II) which specifically attack depurinated DNA.

We would like to suggest that it is the 3′→5′ exonuclease activity of pol I ("proofreading" activity) that is responsible for removal of the damaged nucleotide from the 3′ end at a spontaneous break in X-irradiated DNA. If DNA depurination or sugar damage has also occurred, then a specific endonuclease[7] would make a nick at the site, and the damaged nucleotide at the 3′ end would be removed by the 3′→5′ exonuclease of pol I. The 3′→5′ exonuclease of pol I is unable to attack 3′-PO_4 ends, however, and a break resulting in this type of terminus requires phosphatase activity before it can be converted to a pol I primer site. The resulting gap (one or several nucleotides) would be filled in by the polymerizing activity of pol I, and DNA ligation would complete the repair process. If, however, ligation is prevented (by a deficiency of DNA ligase or its cofactor, NAD) then the 5′→3′ exonuclease activity of pol I would initiate nick translation from the 5′ end of the break, finally leading to the exaggerated repair synthesis observed in our permeabilized cells under conditions of restricted DNA ligase activity.

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CONSTRUCTION OF A MUTANT OF *ESCHERICHIA COLI* K-12 DEFICIENT IN BOTH ENDONUCLEASE I AND ENDONUCLEASE II (EXONUCLEASE III) ACTIVITY

D. R. Stallions, G. R. Hellermann,*
and D. Billen

Toluene-treated *E. coli* that have normal levels of endonuclease I activity show high background levels of DNA repair directed by polymerase I when synthesis is measured in the standard assay mixture. This background can be greatly reduced by introduction of the *endA1* mutation, which results in a strain that is deficient in endonuclease I activity. In order to study DNA repair in various mutants in response to treatment with ionizing radiation or DNA-modifying chemicals, we, therefore, routinely introduce the *endA1* mutation in addition to those mutations under study.

TN193 F⁻ *thr-1 leu-6 proA2 lacY1 T6ʳ-31 xth-1 endA1 argE3 thi strʳ-31* is an *E. coli* K-12 strain deficient in both endonuclease I and endonuclease II/exonuclease III (*xth-1*) activity. It was constructed by transferring the *endA1* mutation from *E. coli* HS432 F⁺ *polB1 polC1026 thyA endA1 polA1 strʳ* into the AB1157 derivative, BW9091 F⁻ *thr-1 leu-6 proA2 lacY1 T6ʳ-31 xth-1 his-4 argE3 thi strʳ-31*. The transfer was accomplished by F-factor mating of HS432 with BW9091. The donor cells were selected against by their inability to grow at temperatures above 42°C, and *endA1* recombinants were selected for in medium lacking histidine (*endA1* and *his* have a high frequency of cotransfer). Suspect clones were screened for lack of endonuclease I activity by the methyl green test[1] and for lack of endonuclease II activity by sensitivity to methyl methanesulfonate.[2] More precise determinations of the level of endonuclease II activity in TN193 are planned.

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DNA POLYMERASE I-INDUCED REPAIR SYNTHESIS IN PERMEABILIZED BACTERIAL CELLS EXPOSED TO CHEMICAL MUTAGENS AND CARCINOGENS

D. Billen and G. R. Hellermann*

Mutagenic and carcinogenic chemicals seem to function by damaging DNA. Many (if not all) carcinogens bind to DNA and elicit repair synthesis (this subject has recently been reviewed by Stich *et al.*[1]).

Preliminary results of a study by a summer student (Ms. Lai Yet Lam) and a graduate student (Ms. Rosalie Elespuru Lijinsky) have shown that the methylating agents *N*-methyl-*N*-nitrosourea, *N*-nitroso-*N*-methylcarbamate, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and methylmethanesulfonate induce a DNA polymerase I (pol I)-directed repair synthesis in toluene-treated *E. coli*. This synthesis can be readily measured under minimal DNA ligase activity in the form of exaggerated repair synthesis (ERS).[2] These methyl donors induce DNA lesions leading to an enzymatic response which clearly resembles the response to X irradiation.[2]

These results are different from those observed with the *ethyl* derivatives of the same compounds (*N*-ethyl-*N*-nitrosourea, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine, and ethyl methanesulfonate). The ethyl donors do not induce pol I-dependent ERS at the concentrations tested. We plan to pursue this study by assessing the effects of alkyl donor concentration on the induction of ERS.

Since formation of primer sites for pol I must be reflected as nicks in the DNA, we plan to determine whether or not there is a relationship between the number of breaks induced and the extent of ERS. In preliminary experiments carried out by Ms. Lam, the methylating agents induced many more breaks than the ethylating agents. However, quantitation of the number of breaks needs to be done for toluene-treated cells exposed to these chemicals. We wish to determine whether or not the induction of similar numbers of breaks by the ethylating, as compared with the methylating, chemicals causes similar levels of pol I-directed repair synthesis.

The ERS seen after exposure to the methyl derivatives was not observed in a pol I-deficient mutant, *E. coli* D110. This is similar to X-ray-induced repair in pol I-deficient, toluene-treated bacteria.[2] We conclude that exposure of bacteria to these methylating agents leads to the induction of DNA pol I-primer sites, whereas ethylation of DNA is less likely to result in the formation of these sites. The use of toluenized *E. coli* in an assay system for measuring DNA repair synthesis provides a simple, efficient method of testing chemicals for their capacity to cause lesions in DNA and thereby of their potential as mutagenic or carcinogenic agents.

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TRANSFORMATION BY DNA POLYMERASE II IN *BACILLUS SUBTILIS*

C. T. Hadden* and M. E. Boling

The role of DNA polymerase II in genetic transformation of *B. subtilis* was investigated by comparing *polA* and PolI⁺ strains after exposure to UV light; to an inhibitor of DNA polymerase III, 6-(*p*-hydroxyphenylazo)-uracil (HPUra); or to UV-irradiated transforming DNA. Neither the level of

transformation nor inactivation of transforming DNA by UV was significantly different in the two strains, implying that DNA polymerase I is not needed for transformation or for repair of UV-irradiated transforming DNA. Addition of HPUra decreased the level of transformation by up to 60%. However, the inhibition of transformation by HPUra was quantitatively similar in both PolI$^+$ and *polA* cells of *B. subtilis* and in *H. influenzae* Rd, an organism in which DNA polymerase III is not sensitive to HPUra. Inhibition of transformation in *Pneumococcus* by HPUra is accounted for by inhibition of DNA uptake,[1] so it appears that DNA polymerase III is not important for transformation.

At low fluences of UV, DNA polymerase II-specific repair resynthesis of DNA can be detected in *polA* cells of *B. subtilis*. To determine whether transformation and excision repair might compete for a limited amount of DNA polymerase II in *polA* cells, competent cells irradiated with 10 J/m^2 of UV were transformed, and the formation of recombinant DNA was compared with the yield of transformant colonies. Although there was no selective killing of PolI$^+$ transformants, the relative survival of transformants of *polA* cells was about 25% of the expected level relative to recombinant formation. It is concluded that while repair of UV damage to cells requires DNA polymerase I for optimal efficiency, transformation and repair of UV-irradiated transforming DNA probably require only DNA polymerase II and that the limited amount of UV repair in *polA* cells by DNA polymerase II can be reduced by competition for DNA polymerase II by the transformation system.

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TRANSFORMATION ANALYSIS OF INACTIVATION AND REPAIR OF TRANSFORMING DNA IN *BACILLUS SUBTILIS*

C. T. Hadden*

Using the DNA transformation system of *B. subtilis*, irradiated DNA has been assayed in recipient strains which are *uvr-1*, *uvs-42*, *recA*, *recB*, or wild type for DNA repair. DNA was irradiated with UV, with X rays in phosphate buffer, or with X rays in Tris, which strongly protects DNA from hydroxyl radicals produced by ionizing radiation in water.[1] The observed sensitivities of donor DNA are summarized in Table 34. The relative sensitivity of the donor marker to UV is expressed as the fluence required for an increase in $(S_0/S)^{0.5}$ from 1 to 2.[2] It is clear that UV-irradiated DNA is repaired more in *uvr-1* cells than in *uvs-42* cells, although both are defective in repair of irradiated donor DNA, as would be expected from their excision defects. Surprisingly, the Rec$^-$ cells, which are also UV-sensitive, are only slightly more sensitive to UV irradiation of transforming DNA than wild-type cells. This means that fairly efficient repair must accompany the small amount of transformation that does occur in the Rec$^-$ cells. The same is true of X-ray-inactivated transforming DNA in *recA* cells, although Rec$^-$ cells are usually sensitive to ionizing radiation. Since hydroxyl radicals are important in the production of single-strand breaks in DNA,[3] it may be that these breaks, which are not repairable in the transformation system of *B. subtilis*, are almost entirely responsible for the inactivation of transforming DNA. Thus protection of DNA by Tris during X irradiation is a result of preventing single-

▼

Table 34. Inactivation of transforming DNA by irradiation

Recipient repair mutation	Nature of repair defect	UV inactivating dose (J/m^2)	X-ray D_{37} dose (kilorads)	
			Phosphate	Tris
Wild type	None	138	0.26	13.4
uvs-42	Incision	45	—	—
uvr-1	Excision or preexcision step	85	—	14.2
recA1	Probably modification at integration site	100	0.23	11.5
recB2	Replication of integrated DNA	100	—	—

strand breaks in the DNA rather than a result of inhibiting the formation of repairable lesions.

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ROLE OF DNA POLYMERASE III IN SURVIVAL OF UV-IRRADIATED POL I⁻ *BACILLUS SUBTILIS*

C. T. Hadden*

At fluences of UV light up to 10–15 J/m^2, DNA polymerase I seems not to be necessary for repair of UV damage in *B. subtilis*, since *polA* cells are no more sensitive to UV irradiation than PolI$^+$ cells in this range. However, at higher fluences, *polA* cells become considerably more sensitive (dose reduction factor 2 to 2.5 as compared with PolI$^+$ cells). This implies that DNA polymerases II and III in combination do only a limited amount of DNA repair. The use of temperature-sensitive mutations in DNA polymerases I and III and the chemical inhibitor 6-(*p*-hydroxyphenylazo)-uracil (HPUra) has shown that DNA polymerase III is essential for the repair seen at low fluences. If UV-irradiated *polA* cells are incubated in growth medium containing HPUra, which inhibits DNA polymerase III (or at a restrictive temperature for the PolIII^{ts} mutant) for 60 min before the cells are plated on nutrient agar, the shoulder disappears from the survival curve and the cells show a high sensitivity to UV. This does not occur in PolI$^+$ cells, nor in *polA* cells if DNA polymerase III is not inhibited.

Centrifugation in alkaline sucrose gradients shows that *polA* cells reseal the breaks appearing in DNA as a result of excision repair but that resealing is slower than in PolI$^+$ cells, as has been observed in *E. coli*.[1] In HPUra-inhibited *polA* cells, single-strand breaks accumulate rapidly after UV irradiation. Thus it appears that in *B. subtilis*, unlike *E. coli*,[2] in the absence of both DNA polymerases I and III, potentially repairable UV damage is rapidly converted to lethal damage reflected by the accumulation of breaks in the DNA.

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RESTRICTION OF FOREIGN DNA IN *recA BACILLUS SUBTILIS*

C. T. Hadden*

Dubnau *et al.*[1] have shown that in transduction experiments between *B. subtilis* subspecies W23 and 168, heterologous donor markers are strongly discriminated against. However, there is little discrimination in transformation of strains derived from *B. subtilis* 168 when the donor DNA is from *B. subtilis* W23. In *recA* cells of strain 168, transformation and heterologous transduction are reduced to 5–10% of the Rec$^+$ level, but homologous transduction is only moderately reduced.[2] This led Dubnau *et al.*[1] to propose that heterologous transduction and transformation by both homologous and heterologous DNA share a common pathway, while homologous transduction is carried out by a separate pathway. I have found that transformation of *recA* strains by W23 (heterologous) DNA results in a pattern of marker-specific discrimination similar to that seen in heterologous transduction of Rec$^+$ cells. However, once a marker of W23 origin is integrated into a 168 strain, it is no longer discriminated against when that DNA is used to transform other 168 cells. It is my conclusion that instead of different pathways of genetic integration, the cells exhibit different efficiencies of restriction of heterologous DNA. In Rec$^+$ cells, transforming DNA is protected from restriction because it is single-stranded before integration and modified by the recipient soon after integration. In transduction, double-stranded DNA is injected into the cells, and heterologous DNA is susceptible to restriction before it is integrated. In the *recA* cells, one result of the mutation may be that modification of integrated DNA is slow, so that upon replication of the unmodified sequences, the cell is killed by its own restriction system. A similar process may account for the UV sensitivity of this strain. DNA strand breaks resulting from excision of UV photoproducts are repaired at a normal rate in *recA* cells, but breaks reappear as the cells begin to grow. Presumably, repaired DNA is also slow to be modified, and as repaired (and unmodified) sequences are replicated the cell is killed by restriction.

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DNA REPLICATION IN UNIRRADIATED AND X-IRRADIATED CHINESE HAMSTER OVARY CELLS

D. Billen and A. C. Olson*

We have previously shown that Chinese hamster ovary (CHO) cells exposed to 1% Tween-80 in 0.25 *M* sucrose will incorporate exogenously supplied deoxyribonucleoside triphosphates into DNA in an ATP-dependent reaction.[1] We have further characterized this system and begun to use it in studies of the effects of X-ray and UV irradiation on DNA replication in these cells.

Density gradient analysis of the product synthesized with bromodeoxyuridine triphosphate in place of thymidine triphosphate (TTP) shows that the product is almost entirely the result of semiconservative synthesis. Autoradiographs of permeabilized cells that had incorporated [^{3}H]TTP showed that almost all of the counts were localized in the nucleus. CHO cells grown in suspension culture can also be permeabilized by the same treatment. Such cells are now being used for experiments, since handling of the cells prior to assay is minimal. Treatment of cells with Tween for 30 min results in less than 20% loss of viability. Permeabilized CHO cells will also incorporate labeled ribonucleoside triphosphates.

Since DNA synthesis in this system is completely dependent on the addition of the deoxyribonucleoside triphosphates and ATP, variations in specific activity of precursors due to endogenous pools is not a problem. We will also be able to study the role of the various cofactors of DNA and RNA metabolism that normally do not penetrate cells.

Convinced that this system can be used to quantitate DNA synthesis in CHO cells, we are presently investigating the effects of moderate doses of X rays on this system. If cells are exposed to 500–2000 rads after permeabilization, there is little or no effect on subsequent [^{3}H]TTP incorporation. Product analysis shows that, as with unirradiated cells, essentially all the DNA synthesis occurring after irradiation is semiconservative. Although we have not rebanded the parental DNA from these experiments, it is clear that any repair-type synthesis is minimal and difficult to assess because of the small quantity of precursor incorporation into parental DNA.

If, on the other hand, CHO cells are exposed to the same doses of X rays *before* permeabilization, incubated in growth medium at 37°C for various times, and then permeabilized and assayed, a 20 to 40% decrease in [^{3}H]TTP incorporation is observed. This decrease in activity is maximal when the cells are assayed between 30 and 120 min after irradiation. By 3 hr after irradiation, DNA synthesis has returned to control levels, indicating that this irradiation effect is transient.

These results may be interpreted in terms of the replicon model for DNA replication as follows: Once DNA synthesis is initiated in a replicon, elongation of the DNA strands is relatively insensitive both to X rays and to changes in the cellular state resulting from permeabilization. Initiation of DNA synthesis in previously inactive replicons, however, is delayed by X irradiation and inhibited in permeabilized cells during the assay. A decrease in [^{3}H]TTP incorporation reflects a decrease in the amount of synthesis required to complete those replicons active at the time of assay. If initiation is delayed by X irradiation, then cells held for 60 min will have fewer active replicons and show less [^{3}H]TTP incorporation. After 3 hr of postirradiation growth, initiation of DNA synthesis may have resumed as a result of repair of the lesions that caused the delay.

This interpretation of our data is substantially similar to one proposed earlier for other mammalian cells by Watanabe[2] and confirmed more recently by Painter and Young.[3] The former investigator used mouse leukemic cells and the latter investigators, CHO, HeLa, and mouse L cells. These authors have explained the two-component inactivation curve for precursor uptake into DNA in irradiated cells as being due to depression of initiation of replicons at low doses ($\leqslant$1000 rads) and inhibition of elongation at higher doses ($\geqslant$2000 rads).

We plan to extend these studies to include UV light.

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THE *rec-1* LOCUS IN *DROSOPHILA*

R. F. Grell

Our new *rec-1* locus lies close to or contiguous with the classical recombinationless gene *c(3)G* but fully complements with it. Three alleles, designated $rec\text{-}1^6$, $rec\text{-}1^{16}$, and $rec\text{-}1^{21}$, have been recovered. Each possesses distinctive properties.

Genetic studies, thus far limited primarily to the X chromosome, show that the heterozygotes of *rec-1*^{6} or *rec-1*^{16} with *rec-1*$^{+}$ give normal exchange values indicating recessiveness of these alleles, unlike *c(3)G*/+, which shows an increase in exchange. The third allele, *rec-1*^{26}, in combination with *rec-1*$^{+}$ shows slight dominance, in that crossing over is reduced to about 80% of normal. Here a polarity effect is observed, with the greatest reduction occurring distally, so that the region between the tip (1-0.0) and crossveinless (1-13.7) has 55% of normal exchange, whereas the most proximal region measured has 100%.

In the homozygote *rec-1*^{6}/*rec-1*^{6} and the heterozygote *rec-1*^{6}/*rec-1*^{16}, exchange is drastically reduced to 4% and 5.5% of normal, respectively. In these cases a reverse type of polarity is seen, with the greatest reduction proximally. By contrast, in the homozygote of *rec-1*^{26} at control temperature (25° C), exchange and polarity are similar to those found in the heterozygote *rec-1*^{26}/*rec-1*$^{+}$.

Among the three alleles, only *rec-1*^{26} is temperature-sensitive. A 6° elevation in temperature during day 6 of female development, corresponding to the first half of the temperature-sensitive period for enhancing exchange in our oocyte sample, fails to alter crossover frequency or polarity in the homozygote; a similar treatment on day 7, corresponding to the second half of the sensitive period, abolishes polarity by reducing exchange in the medial and proximal regions of the X to the same degree encountered distally without heat, so as to cause a uniform reduction to about 45% of normal.

When the temperature-sensitive allele is studied in combination with either temperature-insensitive one, interactions between the two permit conclusions as to the dominance or recessiveness of temperature sensitivity, crossover reduction ability, and polarity. In all combinations, temperature sensitivity is found to be dominant to insensitivity. The lesser reduction in crossing-over characteristic of *rec-1*^{26} is dominant to the major reductions found with *rec-1*^{6} and *rec-1*^{16} with the exception of the *rec-1*^{26}/*rec-1*^{16} heterozygote if heat-treated on either day 7 or days 6 and 7, when major reductions occur. With the 48-hr treatment, exchange is abolished in the proximal half of the X chromosome.

Polarity responses are complex, depending on the allelic combination and the temperature. In *rec-1*^{6}/*rec-1*^{26} at 25° C, codominance of their reverse polarities leads to a uniform reduction in the heterozygote; but with a heat treatment on day 7, the polarity of *rec-1*^{6} prevails. The *rec-1*^{16}/*rec-1*^{26} heterozygote shows the polarity of *rec-1*^{26} at 25° C; it shows codominance and no polarity with the treatment on day 7; while a treatment on days 6 and 7 evokes the polarity typical of *rec-1*^{16} and, as noted above, abolishes crossing-over in the proximal half of the chromosome.

The effects of the *rec-1* mutants on chromosome segregation and on fecundity have been studied. Heterozygotes of the mutants and wild type, where exchange is normal or nearly so, show negligible frequencies (less than 1%) of X nondisjunction; in the homozygote of *rec-1*^{26} heat-treated on day 7 when exchange is reduced to 45% of normal, X nondisjunction is 5%; in genotypes where exchange is greatly reduced to 4 or 5% of normal (*i.e.*, homozygous *rec-1*^{6}, the heterozygote *rec-1*^{6}/*rec-1*^{16}, and the heterozygote *rec-1*^{16}/*rec-1*^{26} heat-treated on days 6 and 7) X nondisjunction is conspicuously elevated to about 30% in each case. Similarly, fecundity is normal in the heterozygote of the mutants and wild type and greatly reduced when exchange is very low and nondisjunction very high, as in the three cases cited above. Again it becomes evident that there are strict correlations between exchange, disjunction, and fertility. Segregational irregularities and lethality both arise from a reduction in exchange, as was initially postulated by the distributive pairing model of meiosis.

Electron microscopic studies of *rec-1*^{6} and *rec-1*^{16} reveal synaptonemal complexes present in their normal nuclear location.

Electrophoretic studies of *rec-1*^{6} and *rec-1*^{26} by E. H. Grell fail to disclose any abnormality in nuclease activity.

The availability of a temperature-sensitive mutant which in allelic combination permits normal exchange at the permissive temperature but selectively eliminates exchange at the restrictive temperature provides a new tool for studying recombination as well as a new criterion for temporally localizing the process.

SERIAL RECONSTRUCTION OF OOCYTE NUCLEI

R. F. Grell and E. E. Generoso

Two independent labeling studies have shown that the S phase in the "first" oocyte formed in each ovariole occupies 24 to 30 hr, beginning close to oocyte formation at 132 hr of female development and terminating at about 162 hr, before the oocyte reaches stage 1. This 30-hr period, which is

predominantly if not entirely premeiotic interphase, also represents the temperature-sensitive period for enhancing intergenic crossing-over.[1] Further, we have been able to demonstrate the presence of synaptonemal complexes from 138 hr onward, that is, coincident with most of S; and recently unequivocal evidence of this coincidence in the form of electron microscopic autoradiographs showing both label and complexes within the same oocyte nuclei has been obtained.[2]

We have now begun a systematic study of the oocyte nucleus during this critical period when DNA replication, synaptonemal complex formation, and probably recombination are occurring. A prerequisite for such a study is a method of chronologically ordering the oocyte. Our "pupal system" provides a way to do this. The study is made at the electron microscopic level through serial reconstructions of successively older oocytes. To date we have obtained complete serial sections of four "first" oocytes from 144-hr females, three from 150-hr females, and a partial serial section of one from a 156-hr female.

As reported previously,[2] the complex is still incomplete and in the formation process at 144 and 150 hr, so that complete reconstruction is not possible at these times. No coalescence of all complexes at one point into a salivary-gland-type chromocenter is observed. Long fibrillar bands, which are wider than, but which often become continuous with, a well-defined synaptonemal complex, are a typical but anomalous feature of the early oocyte nucleus. They become less frequent as the oocyte ages.

Contrary to published reports that the synaptonemal complex for a particular species is constant in width at all stages, we observe a narrowing of the complex with age. Measurements of the width of complexes in the "first" oocyte from 144- and 150-hr females reveal a reduction in width in the 12-hr period by a factor of about 1/3.

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DISTRIBUTIVE PAIRING OF CHROMOSOMES 2 AND 4

B. Barton* and R. F. Grell

It has been proposed that there exists an "absolute specificity of nonhomologous pairing of the fourth chromosome with the X chromosome" at the distributive phase of meiosis and that this pairing is independent of size recognition.[1] As a consequence of this specificity, the 4 is postulated to be unavailable for nonhomologous pairing with either major autosome. Although data in the literature tend to contradict this notion, we have a particularly favorable situation for examining the question.

Flies of both sexes have been constructed which carry the two fourth chromosomes attached to a single centromere so as to form a small univalent compound 4 and in addition carry, as a duplication, a small deleted second chromosome. Both chromosomes are metacentric in shape and are of approximately equivalent length. In the female the two nonhomologues constitute the sole members of the distributive pool over 95% of the time; the two much larger X chromosomes are present the remainder of the time. In the male, which lacks both crossing-over and nonhomologous pairing, the compound 4 is present as a univalent, and the 2 duplication presumably either associates with one or both normal 2's or is also a univalent at meiotic prophase and/or metaphase.

Among 4119 progeny from females carrying the compound 4 and duplicated 2, 4114 carried one or the other chromosome and only 5 carried both or neither. In other words, the two nonhomologues of similar size segregated 99.9%, indicating that they paired distributively 99.8% and failed to pair only 0.24%. By contrast, among 2335 progeny from males, segregation of the two chromosomes occurred 50.1% and failed to occur 49.9%, demonstrating independent assortment. These results clearly invalidate the premise that nonhomologous pairing of chromosome 4 is restricted to the X and is independent of size recognition.

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GENETICS OF SOME NUCLEASES OF *DROSOPHILA MELANOGASTER*

E. H. Grell

Deoxyribonucleases are present as a number of molecular forms which may be separated by polyacrylamide gel electrophoresis and detected on gels by staining.[1] With native DNA as a substrate and incubation at pH 4.5, two bands of DNase activity are detected in young adult flies homozygous for the

DNase 1^{F} (Fast) and *DNase 1*^{I} (Intermediate) alleles, but only one component is observed in flies homozygous for the *DNase 1*^{S} (Slow) allele. Heterozygotes have the isozymes of the component alleles but no hybrid enzymes. The genetic locus of this enzyme is located on the right arm of the third chromosome at 61.8. The locus is in the deficiency Df(3R)P14 = Df(3R)90C2-D1;91A1-2. Homozygotes of an allele induced by ethyl methanesulfonate, *DNase1*^{lo}, have little or no activity of this enzyme. They have reduced viability and small size, and ovaries are underdeveloped in young females, although ovaries appear normal after a few days of adult life. Crossing-over is normal in *DNase1*^{lo} homozygotes.

On gels containing heat-denatured DNA incubated at pH 8.5, five DNase bands are usually observed in adults. The most anodal band, band A, is specified by a gene located on *3L*, based on analysis of a difference in this band in *D. melanogaster* and *D. simulans*. Bands B and C, the next most anodal bands, are both absent in a naturally occurring variant allele, *DNase 2*^{nl}(nl = negative 1). *DNase 2*^{nl} is located between *st* and *in* at 45.9 on *3L*. Abnormalities in viability, fertility, or development have not been observed in flies homozygous for *DNase 2*^{nl} even though they lack two major DNase bands.

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INDUCTION OF ENDOCYTOTIC UPTAKE OF YOLK PROTEIN *IN VITRO*

H. S. Wiley* and J. N. Dumont

The injection of chorionic gonadotropin into sexually mature *Xenopus laevis* females induces the synthesis and secretion of yolk protein (vitellogenin) by the liver and its subsequent endocytotic uptake by developing oocytes.[1] Once endocytotic uptake is stimulated in these oocytes, they can be removed (dissected) from their follicles and placed in culture, where uptake will continue for several days.[2] However, these earlier experiments indicated that nonendocytosing oocytes could not be induced *in vitro* to begin uptake after they had been dissected from their follicles. This evidence suggested either that the site of hormonal action resided in another target tissue or that some other hormone was responsible for the induction of endocytosis. To examine this question, pieces of *intact* ovary (ovarian fragments) from unstimulated females were cultured in the presence of gonadotropin. After 48 hr of culture, oocytes were removed from the follicles and tested for their ability to incorporate vitellogenin. The results indicate that a significant increase in the incorporation of vitellogenin is induced and that the magnitude of the induction is related to the concentration of gonadotropin in the culture medium, induction reaching a maximum at 100 IU/ml at which point ovulation of mature oocytes also occurs. Time-course studies of induction indicate that there is a lag of at least 12 hr between initial exposure to the hormone and the induction of uptake. After this time, uptake increases linearly for about 60 hr. Transmission electron microscopy reveals that uptake is indeed due to an increase in the amount of endocytosis. The results suggest that gonadotropin does not act directly on the oocyte but that its activity is mediated by another cell type. We suggest that the target cells may be the follicle cells which surround the oocyte. Scanning electron microscopy shows that if oocytes are dissected from their follicles and placed in culture, the follicle cells rapidly degenerate; this does not happen if pieces of intact ovary are cultured.

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PROGESTERONE INDUCES A BURST OF CALCIUM RELEASE FROM *XENOPUS LAEVIS* OOCYTES

T. G. Hollinger* and R. A. Wallace

Amphibian oocytes grow for months with their chromosomes arrested in first meiotic prophase. When fully grown, oocytes mature in response to steroid hormones by reinitiating meiosis. Limited information is available on the early events occurring during this period of hormone-induced maturation. In fact, the first observable sign of maturation has been reported only around the time the large oocyte nucleus, or germinal vesicle, ruptures. There are several well-known events which occur after nuclear dissolution. Since the basic biological steps initiated by steroid hormones remain undetermined and since

it is known that calcium ions are important for several biological responses that appear near the end of maturation, we decided to investigate the possible role of calcium during maturation of *X. laevis* oocytes.

We microinjected oocytes with 23 nl of ^{45}Ca-O-R2 solution (80,000 cpm) and measured the amounts released into the medium by individual oocytes. Control oocytes released gradually increasing percentages of ^{45}Ca for about 3 hr; then gradually decreasing percentages of ^{45}Ca appeared in the culture solution (Fig. 20). Oocytes exposed to progesterone released a burst of ^{45}Ca into the incubation solution (Fig. 20). After the hormone was removed, the amount of calcium released returned to control levels and stayed at control levels until the oocyte nucleus ruptured. Oocytes with ruptured germinal vesicles quickly stopped releasing ^{45}Ca until the incubation media accumulated almost no counts in a 1-hr time period. The ethanol carrier for the hormone itself caused at least some release of ^{45}Ca, but at a significantly lower rate. Although we know that progesterone free of the ethanol carrier will induce this efflux of the ion, we have yet to determine if ethanol itself, in perhaps higher concentrations, can mimic the effect of the steroid on calcium efflux. Ethanol will not, at any rate, induce oocyte maturation.

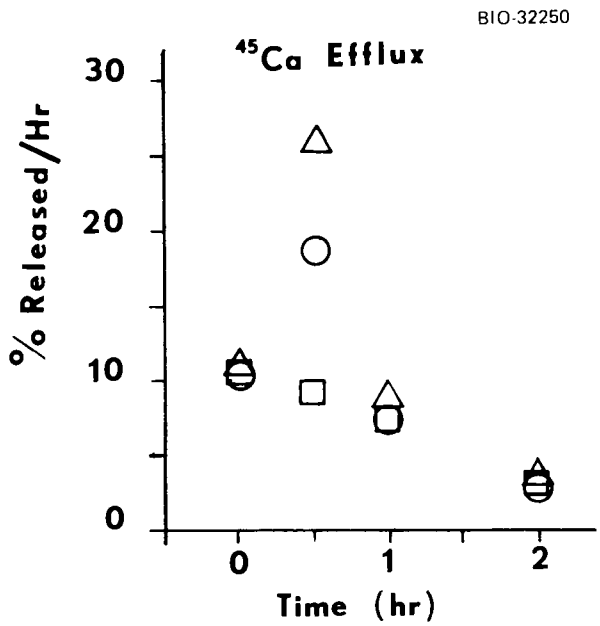

Fig. 20. Burst of ^{45}Ca efflux in response to progesterone. *Xenopus laevis* oocytes were microinjected with ^{45}Ca-O-R2 solution and allowed to equilibrate for 3 hr. At zero time we exposed them to 2 μg/ml of progesterone in 0.2% ethanol (triangles), to 0.2% ethanol (circles), or to fresh incubation medium only (squares). The amount of ^{45}Ca in the medium was measured and fresh O-R2 solution added at the times indicated. The percent of the total injected radioactivity released at 30 min differs among the points with a confidence level higher than 0.05%. At 1 hr the progesterone-treated group still differs from the control groups with a confidence level of 1%.

The burst of ^{45}Ca reported here represents the earliest observed event following exposure of oocytes to progesterone. If further examination confirms and extends these preliminary findings and if the burst of calcium efflux is physiologically significant, then several hypotheses can be tested that might provide new insights into a possibly new and unusual mechanism of hormone action.

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*Postdoctoral investigator supported by NIH Grant HD 03169 and by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

CALCIUM-INDUCED CLEAVAGE OF AMPHIBIAN OOCYTES AND EGGS

T. G. Hollinger* and R. A. Wallace

In preliminary experiments during which *Rana pipiens* oocytes (after germinal vesicle breakdown) and eggs were exposed to ionophore A-23187, we observed surface contractions and furrowing suggestive of cleavage. To directly evaluate the role of calcium in such responses, we microinjected Ca^{2+} into amphibian oocytes and eggs. The results illustrate that direct injection of Ca^{2+} induces contractions and cleavage-like constrictions in both oocytes and eggs.

Microinjection of calcium into maturing oocytes of both *R. pipiens* and *X. laevis* resulted in cleavage-like constrictions and formation of a structure resembling a two-cell embryo. Mg^{2+}, Na^{+}, or K^{+} did not induce any of these reactions. Greater than optimal amounts of Ca^{2+} induced a contraction reaction over the entire surface of the oocyte and did not cause any cleavage plane to form. Less than optimal amounts of the ion caused either a small "blastomere" to form near the injection site or no noticeable reaction. The location of the injected calcium determined the orientation of the resulting furrow; that is, the furrow forms perpendicular to the radius that passes through the injectate. Since the plane of cleavage is perpendicular to the injectate and since the volume of the injected blastomere is roughly proportional to the amount of calcium injected, we interpret these results as in-

dicating that calcium diffuses from the injectate towards the surface. At some critical concentrations the Ca^{2+} causes contraction. As Ca^{2+} continues to diffuse, it reaches some point where it is no longer effective. It is probably along this concentration line that the cleavage-like constriction appears.

Cleavage-like constrictions do not appear in Ca^{2+}-injected oocytes that have not been induced to mature, *i. e.*, stage VI oocytes with intact germinal vesicles, unless the investing vitelline envelope has been removed or loosened. Thus, one change that might occur during oocyte maturation is the loss of mechanical restriction to cleavage. When we removed the vitelline membrane from even stage IV occytes before injecting calcium, they also cleaved. This indicates that small growing oocytes already have the constrictive ability to cleave. Oocyte maturation may thus involve the organization of the control of cleavage, as the mechanical elements appear to be present even in growing oocytes.

*Postdoctoral investigator supported by NIH Grant HD 03169 and by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

LIPID DROPLET FORMATION IN CECROPIA OOCYTES

M. L. Pan* and R. A. Wallace

Although much is known about yolk protein deposition in insect oocytes, detailed biochemical analysis of lipid droplet formation in oocytes is lacking. Composition analysis of total lipid and incorporation of [^{3}H]palmitate into the lipid droplets of cecropia silk moth oocytes have recently been described,[1] and it was observed that (*a*) fat body converts [^{3}H]palmitate to diglycerides prior to its uptake by oocytes, (*b*) only oocytes actively sequestering vitellogenic blood proteins incorporate the diglyceride, and (*c*) incorporation of the label into lipid droplets occurred in the cortical cytoplasm and paralleled the formation of protein yolk spheres. The observations, when coupled with the recent evidence of one of us (M.L.P., unpublished) that fat body is the site of synthesis of the major vitellogenic blood proteins, indicate that lipid droplet formation is dependent upon the accessibility of vitellogenic blood proteins. One of the vitellogenic blood proteins of cecropia silk moth is a "carotenoid protein" which contains a high percentage of lipid. Results to date indicate that the carotenoid protein in blood and that in yolk, although antigenically identical, differ both in sedimentation rate in the preparative ultracentrifuge and in electrophoretic mobility on polyacrylamide and agarose gels. As determined by sodium dodecyl sulfate (SDS) gels, however, the carotenoid proteins in blood and yolk have subunits similar in size. These observations suggest that the carotenoid protein from blood releases part of its lipid during or after uptake into oocytes; that is, that the carotenoid protein may be involved in the formation of lipid droplets in the cecropia silk moth. In order to determine its role more definitively, the carotenoid protein must be isolated and purified. Preliminary experiments to determine the feasibility of this study have been initiated. To date, results of NaBr and sucrose gradient ultracentrifugation indicate that the carotenoid protein can be separated from other major blood proteins. Current experiments are being performed to free it from several minor small protein contaminants. When the carotenoid protein is purified, its chemical and physical characteristics will be determined and its physiological role in lipid droplet formation in the oocyte will be analyzed.

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STABILIZATION AND FRACTIONATION OF A MATURATION-PROMOTING FACTOR FROM *XENOPUS LAEVIS* EGGS

H. S. Wiley,* T. G. Hollinger,† and R. A. Wallace

Externally applied progesterone initiates the resumption of meiosis in amphibian oocytes by inducing the production of an intracellular maturation-promoting factor (MPF). Cytoplasm of mature oocytes will induce maturation when injected into immature oocytes, whereas cytoplasm from immature oocytes or injected progesterone will not. Studies to determine the nature and identity of MPF have been frustrated by the extreme lability of the substance, activity typically disappearing within 24 hr after extraction. However, we have recently been able to develop an easy extraction procedure that allows isolation of substantial and relatively stable MPF activity. Dejellied eggs obtained from *Xenopus* females injected with human chorionic gonadotropin were homogenized in two volumes of stabilization buffer (50 m*M* β-glycerophosphate,

pH 7) and centrifuged for 1 hr at 100,000 *g*. The supernatant had MPF activity which precipitated between 0.3 and 0.5 g/ml of ammonium sulfate. The precipitate was resuspended in buffer and further characterized by filtering through a series of ultrafiltration membranes. The active material passed through a 0.4-μm Unipore filter but was retained by a filter with 0.05 μm pore size. Essentially no MPF activity was detected at smaller or larger sized filters. When the fraction containing the active material was negatively stained and examined in an electron miscroscope, it was found to contain numerous vesicles ranging between 0.1 and 0.4 μm in diameter. This fractionation procedure provides preparations which are stable for at least a month at 4°C, thus allowing further work on the nature and characterization of MPF.

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† Postdoctoral investigator supported by NIH Grant HD 03169 and by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

THE ROLE OF CYCLIC NUCLEOTIDES DURING PROGESTERONE-INDUCED OOCYTE MATURATION

H. S. Wiley* and R. A. Wallace

Progesterone induces the resumption of meiosis in arrested oocytes and seems to work at a post-transcriptional level. Cyclic nucleotides have been implicated as the "second messenger" in a variety of other hormonal responses, so we investigated the possible involvement of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) in progesterone-induced oocyte maturation. Direct assays of the levels of the cyclic nucleotides revealed that the level of cGMP in oocytes did not vary over a period of 10 hr after progesterone exposure in *Xenopus* oocytes. Levels of cAMP either remained the same or decreased somewhat with time, depending upon the particular ovary used. Incubation of dissected oocytes with up to 1 m*M* cGMP neither caused nor inhibited normal maturation, whereas 1 m*M* cAMP completely inhibited progesterone-induced maturation. When we injected various amounts of cyclic nucleotides into oocytes, both strongly inhibited maturation. However, injected cyclic nucleotide phosphodiesterase did not affect maturation. Taken together, these results tend to rule out any positive role of cyclic nucleotides in the process of oocyte maturation.

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AN INVESTIGATION OF THE PLASMA MEMBRANE INVOLVED IN THE PINOCYTOSIS OF YOLK PROTEIN

S. Margon* and R. A. Wallace

The aim of this study was to determine whether there exist areas of specialization within the plasma membrane that can be associated with the specific binding and uptake of yolk protein during vitellogenesis in the spider crab (*Libinia emarginata*) oocyte. Oogenesis in the spider crab is highly synchronized, so that well-defined stages can be examined. Oocytes from days 4 to 7 contained many vesicles budding off the plasma membrane, and these had bristle coats and contained electron-dense material as visualized by transmission electron microscopy. The freeze-fractured surfaces of the oolema indicated numerous cleaved-off microvilli and pits and bumps of various sizes. It appeared to us that many of the pits were identical to the micropinocytotic vesicles visible in thin section. Intramembranous particles in the oolema were seen to be both large and small and to be randomly arranged throughout. The only exceptions to this random arrangement were closely packed arrays of large particles which appeared to represent points of contact between the microvilli of the oocyte and the follicle cells surrounding the oocyte. It is significant, however, that there were no special arrangements of particles seen in the vicinity of the pinocytotic pits. This result can be explained in three ways: (1) the proteins involved in recognition and binding are peripheral, (2) the proteins are present but disappear so rapidly into the forming pinocytotic pit that we have not seen them within the membrane, or (3) there are no protein molecules present, within the resolution of the freeze-fracture technique, which serve as membrane "receptor sites" for yolk proteins.

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YOLK DEGRADATION IN EXPLANTS OF *XENOPUS LAEVIS*

L. Opresko* and R. A. Wallace

It is common knowledge that yolk reserves are utilized as a source of nutrients for the developing embryos of some animal species. Within the past decade, interest in yolk has increased. Investigators have elucidated the chemical composition of a few kinds of yolk. There have also been several studies dealing with yolk formation and deposition in maturing oocytes; however, the methods and end products of yolk degradation remain almost totally unknown. This is surprising in view of the fact that yolk serves its primary function after degradation.

In order to facilitate the study of yolk digestion, a system was sought which would localize and accelerate yolk degradation in a limited number of developing cells. Embryonic explants from early gastrula presumptive ectoderm that contain moderate quantities of yolk and are made up of only 100 to 200 cells have proven to be ideal for this study. When these explants are cultured in nonnutritive medium, yolk breakdown commences within 24 to 48 hr. The culture medium used is standard O-R2 solution containing 0.1% lyophilized calf serum, gentamycin, and mycostatin. Maximum survival times exceeding 8 days have been obtained, but most of the explants succumb in 6 to 7 days.

Transmission electron microscope studies indicate that yolk platelet breakdown is probably accomplished through three independent processes. One method involves the orderly dissociation of the crystalline structure of the main body. This dissociation proceeds from the external surface inwards; the center of the platelet retains the original conformation, and the entire complex is surrounded by a few to many layers of membrane. In the second method, the outer area of the yolk body becomes very diffuse and irregular while surrounding a central compact mass that gradually decreases in size. This complex is contained within a single membrane and usually occurs in groups of two or three to many. The last method, which is also the most common, involves what appear to be autophagic vacuoles. These organelles are rather heterogeneous; they generally contain various cellular components in addition to yolk platelets. When vacuoles are present, their density varies from one to two per cell.

The explant system in conjunction with transmission electron microscopy and histochemical techniques will enable elucidation of the organelles and enzymes involved in yolk digestion. Cytochemical investigations are currently in progress.

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

THE EFFECTS OF PHENOL ON SOME BIOLOGICAL PARAMETERS OF THE CELLULAR MODEL SYSTEM *TETRAHYMENA PYRIFORMIS*

T. W. Schultz* and J. N. Dumont

Phenol is the major organic constituent of product or scrub waters from coal conversion processes[1,2] and, therefore, is a potential environmental and health hazard. The behavior, cytology, respiration, and growth rates of the cellular model system *T. pyriformis* were examined after exposure to varying concentrations of phenol.

Concentrations of phenol of 75 mg/liter (75 ppm) and higher cause alterations in cell motility, cell shape, and contractile vacuole activity within the first few seconds of exposure. Animals surviving the initial exposure recover normal motility; recovery time is inversely related to the concentration of toxicant. At 75 mg/liter of phenol, recovery to normal movement requires 8 hr. Oxygen uptake is abruptly reduced within 3 min of exposure to phenol in concentrations as low as 10 mg/liter (Fig. 21). At

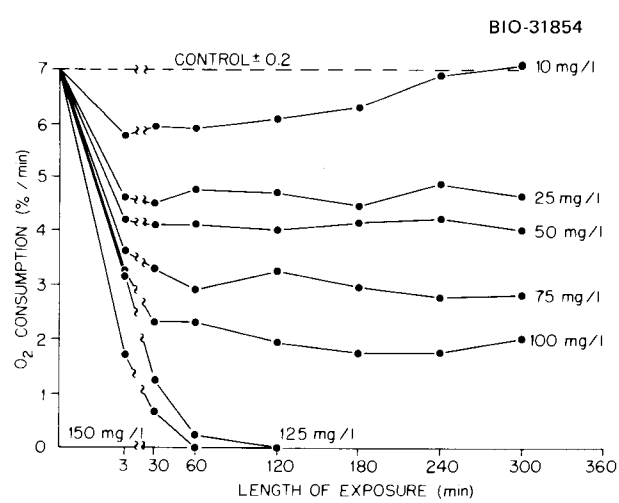

Fig. 21. Effect of phenol on the respiration rate of *Tetrahymena pyriformis* at 28° C.

concentrations of 150 and 125 mg/liter, oxygen consumption continues to drop sharply for 1 hr, at which point respiration ceases completely (Fig. 21). Oxygen consumption of *Tetrahymena* exposed to concentrations of 25 to 100 mg/liter drops initially, then plateaus after about 30 min (Fig. 21). This plateau level is maintained over the next 4½ hr. Examination of cultures 24 hr after exposure reveals that all animals in 100 mg/liter of toxicant are dead (nonmotile), while those in 75, 50 and 25 mg/liter show a graded increase in the numbers surviving. At 10 mg/liter, oxygen consumption returns to control levels in 300 min (Fig. 21). Concomitant with a decrease in oxygen consumption, there is an increase in the density of the mitochondrial matrix as revealed by electron microscopy. Recovery to normal rates of oxygen consumption is paralleled by a return to normal mitochondrial matrix density. Other ultrastructural alterations that are correlated with concentration of phenol are loss of structure in the mucocysts, rupture of the pellicle, and a reduction in glycogen content. Population densities[3] of *Tetrahymena* cultures exposed to phenol for 72 hr show an inverse relationship to concentration. At 5, 10, and 25 mg/liter, growth rate, as measured spectrometrically, shows a reduction to 89, 68, and 59% respectively (Fig. 22). The mean growth rate during exponential growth is not significantly affected ($P > 0.05$) by the concentrations of toxicant. However, the length of lag phase is directly related to the concentration. Phenol in a concentration of 50 mg/liter inhibited population growth during the 72 hr tested.

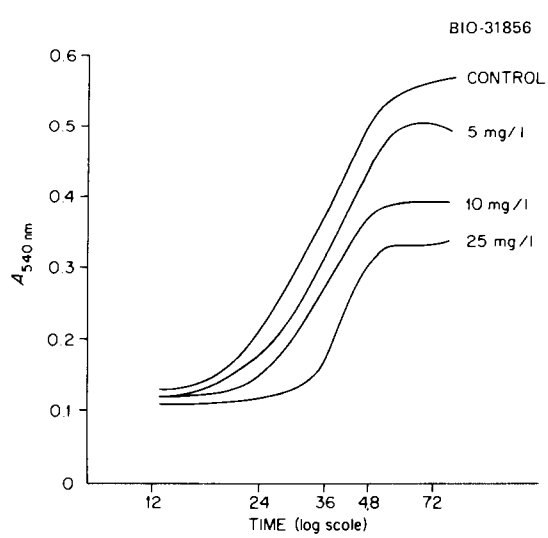

Fig. 22. Effect of phenol on the population growth of *Tetrahymena pyriformis* at 28° C.

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

1. C. E. Schmidt, A. G. Sharkey, Jr., and R. A. Friedel, Bureau of Mines TPR-86 (1974).

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CYTOTOXICITY OF SYNFUEL PRODUCT WATERS: SHALE OIL PROCESS WATER

T. W. Schultz,* J. N. Dumont, and L. M. Kyte

Aboveground processing (retorting) of Western oil shales is one of the more promising synfuel technologies. However, associated with this process is a product water effluent which is also a potential environmental and health hazard. The behavior and the respiration and growth rates of the cellular model system *T. pyriformis* were examined after exposure to varying concentrations of filtered shale oil product water. A concentration of 1% causes an increase in cell motility within the first minutes of exposure. After 60 min of exposure the cells approach normal motility. Motility then decreases with increased length of exposure. *Tetrahymena* exposed to 3% product water initially expresses a marked decrease in motility. After exposure for 60 min the cells, while varying in size, are spherical in shape and continue to exhibit reduced motility. Following 180 min of exposure to the toxicant, 50% of the cells are lysed, while those remaining are spherical and lack ciliary movement.

Oxygen consumption[1] parallels the behavioral pattern (Fig. 23). Cells exposed to 1% product water respire at a greater rate than controls for the first 60 min of exposure. Over the next 120 min the rates are roughly equal. However, following 180 min of exposure there is a slow reduction in the respiration rate of the treated cells, so that the total oxygen consumption of the treated and the control cells are equal after approximately 300 min. The oxygen consumption rate of cells exposed to 3% product water falls off sharply after 60 min exposure and ceases completely within 180 min. Five percent product water, while following the same pattern as 3%, is even more toxic.

Population densities[1] of *Tetrahymena* cultures exposed to shale oil product water at concentrations

from 0.001% to 0.01% for 72 hr show an inverse relationship to concentration (Fig. 24). Cultures exposed to 0.001% product have a slightly lengthened lag phase, then grow at the same rate as controls. At 36 hr these cultures plateau at about 60% of the final control absorbance value. The mean growth rate during exponential growth is significantly affected ($P < 0.05$) by concentrations of 0.005% and greater. Shale oil product water in concentrations greater than 0.02% inhibits population growth during the 72 hr tested. Cytological examinations are currently being conducted.

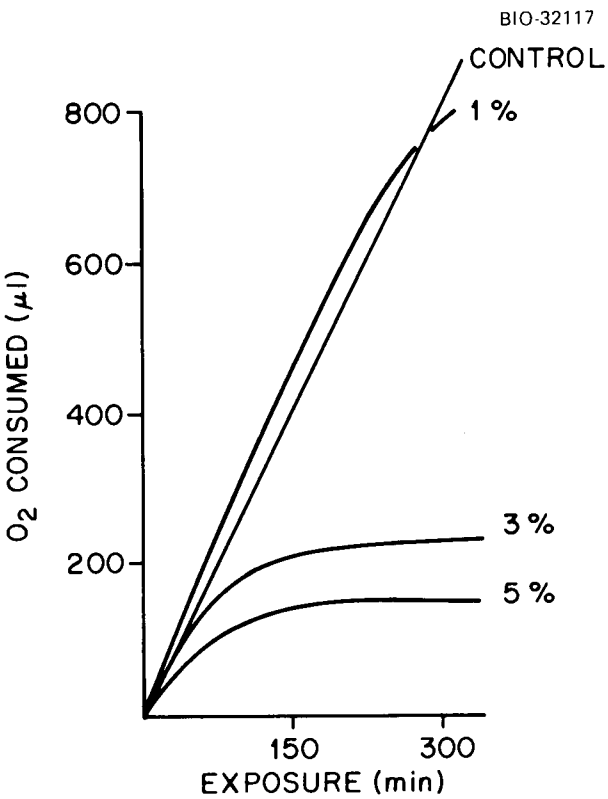

Fig. 23. Effect of shale oil product water on the oxygen consumption of *Tetrahymena pyriformis* at 28° C.

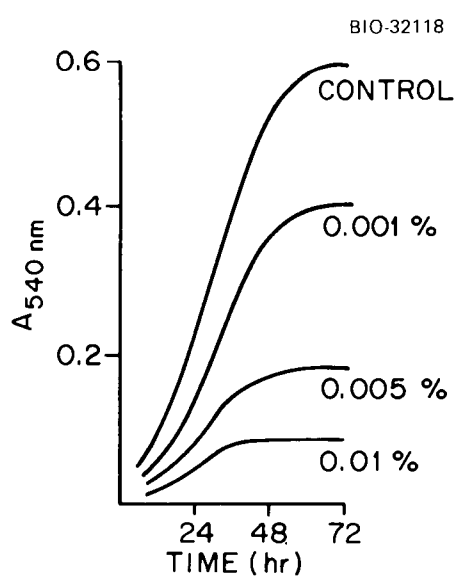

Fig. 24. Effect of shale oil product water on the population growth of *Tetrahymena pyriformis* at 28° C.

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

1. T. W. Schultz, J. N. Dumont, and L. M. Kyte, this report.

CYTOTOXICITY OF SYNFUEL PRODUCT WATERS: SYNTHANE PROCESS WATER

T. W. Schultz,* J. N. Dumont, and L. M. Kyte

Coal accounts for 75% of our nation's fossil-fuel resources, but to be of real industrial and commercial value it must be converted to oil or gas. Among the most promising of the gasification schemes is the Synthane process. A large volume of scrub or process water is associated with this process, and this is a potential environmental and health hazard.[1,2] In order to test the potential toxicity of this process water, the behavior, respiration, and growth rates of the cellular model system *T. pyriformis* have been examined after exposure to varying concentrations of Synthane product water. Cells exposed to 1% product water reveal little or no behavior change. *Tetrahymena* exposed to 3% toxicant exhibit little effect over the first 5 min of exposure. Following 60 min of exposure, motility is reduced and 25% of the cells are spherical in shape. By 150 min motility is further reduced and 50% of the cells are spherical. Over 75% of the cells are spherical after 180 min of exposure. Cells exposed to 5% product water immediately reduce their motility; 25% undergo varying shape changes. After 60 min, approximately 10% of the ciliates are lysed, while of the remaining cells, 95% are spherical and 90% immobile. By 150 min, more than 50% of the cells are lysed, and the remaining are immobile following 180 min exposure to the toxicants.

Oxygen consumption of 3- to 4-day-old stationary growth phase axenic cultures was monitored. Synthane product water causes a reduction in oxygen consumption that is nonlinear and dose-dependent (Fig. 25). Cellular respiration of *T. pyriformis* ex-

posed to 2% or less product water is not significantly different from controls. Cells exposed to 3% toxicant respire at a normal rate for the initial 90 min of exposure; respiration then decreases with increased exposure. Oxygen consumption of cells treated with 5% product water falls off more quickly so as to plateau after 180 min of exposure.

Population densities were measured spectrophotometrically. Growth was estimated as absorbance at 540 nm of axenic cultures of *Tetrahymena* grown at 28°C. Growth of cultures exposed to Synthane product water at concentrations from 0.004% to 0.01% for 72 hr shows an inverse relationship to concentration (Fig. 26). Cultures exposed to 0.004% toxicant grow at a somewhat slower rate than controls. At 48 hr these cultures plateau at about 66% of the final control value. The mean growth rate during exponential growth is significantly affected ($P < 0.05$) by concentration. Synthane product water in concentrations greater than 0.01% inhibit population growth during the 72 hr tested. Cytological examinations are currently being conducted.

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

1. C. E. Schmidt, A. G. Sharkey, Jr., and R. A. Friedel, Bureau of Mines TPR-86 (1974).

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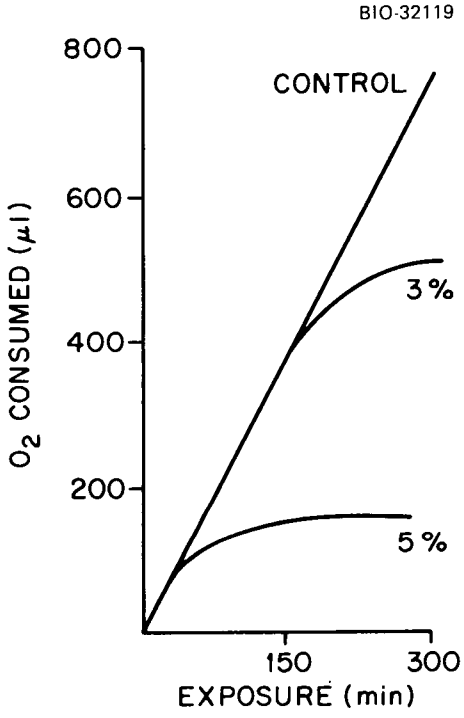

Fig. 25. Effect of Synthane product water on the oxygen consumption of *Tetrahymena pyriformis* at 28°C.

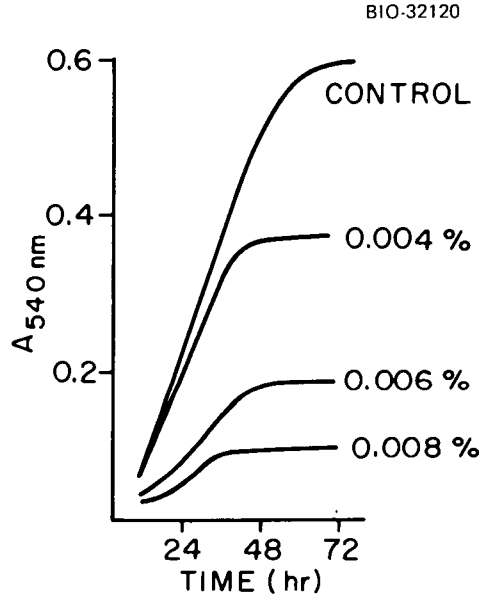

Fig. 26. Effect of Synthane product water on the population growth of *Tetrahymena pyriformis* at 28°C.

CALCIUM-INDUCED CORTICAL GRANULE BREAKDOWN IN *XENOPUS* OOCYTES

T. G. Hollinger* and J. N. Dumont

Amphibian oocyte maturation involves a series of hormone-initiated cellular changes terminating with the appearance of the capacity for the cortical granules to dehisce in response to appropriate stimuli (sperm, pricking). Recently it was demonstrated that this cortical granule reaction would be induced precociously by ionophone A-23187 if maturing oocytes were exposed to it shortly after their germinal vesicles had ruptured. Thus, cortical granules apparently have the capacity to release their contents sometime before maturation is completed. We investigated this possibility further by microinjecting various amounts of Na^+, K^+, Mg^{2+}, or Ca^{2+} directly into both large and small *X. laevis* oocytes. Only one ion, Ca^{2+}, caused a cortical response as determined by ultrastructural examination. Both full-grown (stage VI) and small vitellogenic (stage IV) oocytes exhibited this reaction when microinjected with 0.1 μg or more of Ca^{2+}. Thus, cortical granule breakdown is probably directly triggered by intracellular Ca^{2+}. Also, the capacity for these inclusions to dehisce as a response to Ca^{2+} is apparently present even in small (stage IV) oocytes. The response to Ca^{2+} may be an innate property of the granules, since the small oocytes contain cortical granules shortly after they first appear and migrate into the cortex but a long time before the oocyte can be activated (fertilized). Since cortical granule breakdown is normally not inducible until the end of maturation, we suggest that oocyte maturation at least partially involves changes in the compartments that regulate or adjust the level of free Ca^{2+}, either throughout the cell or in the cortex.

*Postdoctoral investigator supported by NIH Grant HD 03169 and by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.

CHANGES IN THE *XENOPUS* EGG SURFACE DURING AND SHORTLY AFTER THE CORTICAL GRANULE (FERTILIZATION) REACTION

J. N. Dumont and J. Drelich*

The oviposited egg of amphibians can be activated to develop by injury (pricking). This event simulates the entry of sperm into the egg. The immediate response to pricking (or sperm entry) is the dehiscence of cortical granules. These are membrane-bound organelles which lie beneath the cell surface and at fertilization fuse with the cell membrane and spew their contents into the perivitelline space to prevent the entrance of more than one sperm (polyspermy). The surface of the unfertilized egg is flat and studded with many short microvilli. Within 15 sec after pricking or activation, cortical granule dehiscence is apparent. The reaction is propagated from the prick site over the entire surface of the egg within 1.5 to 2.0 min. During this time, pits corresponding to dehiscing cortical granules are seen on the egg surface. Many of these pits contain material which was originally present in the cortical granule. About 2 min after the cortical reaction has begun, the surface of the egg becomes very densely populated with many long microvilli. Since the membranes of the cortical granules fuse with the cell membrane during dehiscence, resulting in a net addition of surface membrane, we postulate that the formation of additional microvilli is a mechanism to compensate for the large amounts of new membrane which become incorporated into the egg surface.

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SOME EFFECTS OF PHENOL ON *DAPHNIA PULEX* (CRUSTACEA: CLADOCERA)

T. W. Schultz* and J. N. Dumont

First-instar individuals of the crustacean zooplankter *D. pulex* were exposed to 15 mg/liter (ppm) of phenol, the major organic component of coal conversion process water,[1] at 20° C. Immediately upon exposure the daphnids cease swimming and feeding activity. Upon prolonged exposure (more than 12 hr), molting is inhibited, and death (cessation of heartbeat) is linear with respect to length of exposure over the period from 12 to 72 hr. Animals surviving initial exposure for up to 72 hr recover normal mobility within 5 min after resuspension in clean water. Cytological observations to date have not revealed detectable sublethal alterations. Individuals exposed to 2 mg/liter of phenol do not exhibit immediate behavior effects. However, while molting is not inhibited, no animal lives past the third juvenile instar (about 72 hr at 20° C). Adult *Daphnia* are not as readily affected as first-instar juveniles. The response observed is hypothesized to be due to the lipophilic nature of phenol and hence concentration within the nervous system.

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

1. T. W. Schultz and J. N. Dumont, this report.

II. MOLECULAR AND CELLULAR SCIENCES SECTION

Section Head — F. C. Hartman
Associate Section Head — S. K. Niyogi

Bio-Organic Chemistry and Enzymic Mechanisms

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F. H. Gaertner
F. D. Hamilton
F. C. Hartman
B. D. Mehrotra[a]
I. L. Norton
B. C. Pal
A. S. Shetty[a]
J. R. Totter

Biosynthesis of Nucleic Acids

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D. P. Allison
S. Dasgupta[b]
A. K. Datta[b]
K. Edwards III[b]
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J. F. McCarthy[b]
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D. M. Skinner
A. L. Stevens

Comparative Nucleic Acid Biochemistry of Abnormal Cells

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R. S. Foote[b]
J. X. Khym
C. G. Mead
L. R. Shugart
M. P. Stulberg
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Environmental-Cellular Interactions

J. S. Cook
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J. R. Azzi
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F. A. Burr[c]
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D. E. Foard
D. L. Hwang[b]
J. D. Regan
P. C. Will[b]

Genetic Damage: Repair and Physiological Consequences

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W. L. Carrier
J. E. Donnellan, Jr.[d]
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Macromolecular Structure and Function

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Molecular Genetics and Regulatory Processes

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Theoretical and Applied Cryobiology

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INTRODUCTION

ONE OF THE MAJOR organizational changes in the Division during the last year was the combination of the Biochemistry and the Biophysics and Cell Physiology Sections to form the Molecular and Cellular Sciences Section; 19 groups were reorganized to form 8. This regrouping serves to coordinate the disciplines of the various investigators involved, with the aim of acquiring a clearer understanding of biological phenomena in both normal and pathological states. Scientific excellence continues to be strongly emphasized.

Much of the Section's work is fundamental research which is utilized by all segments of the Division; in addition, many of the senior investigators are engaged in research that is either directly or indirectly related to the effects of environmental agents (chemical, viral, etc.) on biological systems. In general, such research is designed to provide insight into cellular and molecular processes pertinent to mechanisms of mutagenesis and carcinogenesis. Many of these results will be of long-range value because the studies are fundamental in nature.

It should be emphasized that investigations within this Section are coordinated with the work of the entire Division. Many of the activities of each Section are interwoven with those of the others in the form of collaborative endeavors. Examples of such joint projects are evident in the reports of all three Sections; such collaboration is one of the major strengths of the Division and, it is hoped, will continue to be encouraged.

THE RIBONUCLEOTIDE REDUCTASE SYSTEM IN *EUGLENA GRACILIS*

W. J. Allen and F. D. Hamilton

In 1974 our laboratory reported the isolation and properties of ribonucleotide reductase from *E. gracilis*.[1] The enzyme reduces the four common ribonucleoside triphosphates, requires dithiothreitol and adenosylcobalamin, and can be stimulated or inhibited by deoxyribonucleoside triphosphates. The purified protein is a tetramer, having four polypeptides with molecular weights of approximately 100,000. Subsequently, we have shown that *Euglena* contains two proteins that in combination will allow NADPH (reduced form of nicotinamide adenine dinucleotide phosphate) to be used as a reducing agent for ribonucleotide reduction.[2] It was also found that the previously purified reductase was not active in this NADPH-requiring system; instead, an enzyme obtained by a different and more gentle isolation procedure was active. This new enzyme is larger than the reductase described originally and contains at least three additional polypeptide chains as shown by sodium dodecyl sulfate (SDS) gel electrophoresis. The observation that only the larger enzyme functions in the NADPH-requiring system suggests that it is more closely related to the *in vivo* enzyme structure. Large-scale purification of the enzyme involves density gradient centrifugation and Biogel chromatography. Enzyme obtained from these procedures will be used to reinvestigate the kinetic properties of the ribonucleotide reductase reaction.

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2. S. Munavalli, D. Parker, and F. D. Hamilton, *Proc. Natl. Acad. Sci. USA* **72**, 4233–4237 (1975).

STUDIES OF THE CYTOSINE TRIPHOSPHATE REDUCTASE FROM *PSEUDOMONAS STUTZERI*

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

A cytosine triphosphate (CTP) reductase has been identified and partially purified from *P. stutzeri*. For activity, the reaction requires added dithiothreitol and adenosylcobalamin. Metal ions are not required, and 0.04 *M* EDTA gives a threefold stimulation of CTP reduction. The reaction is activated by deoxyribonucleotide 5′-triphosphates (dNTPs) and ATP; dATP, however, gives the maximal activation. The values of V_{max} for the dATP-activated and the unstimulated reaction are 1851 nmol/hr/mg and 161 nmol/hr/mg respectively. The corresponding values of K_m for CTP in the activated and unstimulated reactions are 2.3×10^{-5} and 1.3×10^{-4} *M*.

The CTP reductase in *P. stutzeri* is not very active if dATP is not included in the reaction mixture and in this respect appears to be similar to the reductase induced by T_4 infection of *Escherichia coli*.

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IDENTIFICATION OF A MEMBRANE FRACTION FROM *PSEUDOMONAS STUTZERI* CONTAINING RIBONUCLEOTIDE REDUCTASE AND DNA POLYMERASE ACTIVITIES

R. G. Holt,* R. K. Fujimura, and F. D. Hamilton

A CTP reductase has been isolated from *P. stutzeri* by ammonium sulfate fractionation and Biogel chromatography. The enzyme activity is only partially soluble in aqueous solutions and can be pelleted by centrifugation at 100,000 *g* for 2 hr. Reductase activity is not solubilized by treatment with urea or Triton X-100. Examination of the enzyme fraction has shown that it contains a 10- to 12-S RNA containing adenosine, guanosine, uridine, and a derivative of cytidine. The fraction also contains phosphatidylethanolamine and a small amount of free fatty acids. SDS gel electrophoresis of the fraction produced 15 to 20 protein bands. Other activities present in the fraction include a very active ATPase and a DNA polymerase. Attempts are being made to identify other enzyme activities in this fraction.

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

THE PROTEASE PROBLEM IN *NEUROSPORA*: VARIABLE STABILITY OF ENZYMES IN AROMATIC AMINO ACID METABOLISM

S. L. Lampkin IV,* K. W. Cole, A. Vitto, Jr.,† and F. H. Gaertner

A proteolytic activity isolated from *N. crassa* was shown to be responsible for the variable stability observed *in vitro* for enzymes involved in aromatic amino acid metabolism. For example, the activity of kynurenine formamidase was insensitive to the action of this protease preparation over a 24-hr period of incubation at 25° C, whereas chorismate synthase, anthranilate synthase, kynureninase, and the five activities of the *arom* multienzyme system were inactivated during this time. Anthranilate synthase and two of the *arom* system activates (dehydroquinate synthase and shikimate kinase) were inactivated by the protease preparation within 2 hr. Phenylmethanesulfonyl fluoride and a specific proteolytic inhibitor from *N. crassa* prevented inactivation of these enzymes. Spontaneous loss of activity at 25° C of purified samples of anthranilate synthase, dehydroquinate synthase, and shikimate kinase was also prevented by the inhibitors. A method for purifying the inhibitor from *N. crassa* was devised, and its use as a reagent in the analysis of proteolytic action has been demonstrated.

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ARTIFICIAL SUBUNIT STRUCTURE FORMED BY A PROTEOLYTIC ACTIVITY RELEASED DURING THE EXTRACTION AND ISOLATION OF THE *AROM* MULTIENZYME SYSTEM FROM *NEUROSPORA*: THE POSSIBILITY OF FIVE CATALYTIC ACTIVITIES ON A SINGLE POLYPEPTIDE CHAIN

F. H. Gaertner and K. W. Cole

The "aromatic complex," or "*arom* aggregate," of *N. crassa* catalyzes five consecutive reactions in the central pathway leading to the biosynthesis of the aromatic amino acids. Previously this multienzyme system was shown variously to have a molecular weight of 230,000 to 300,000 and to contain up to four subunits. Recently, a protease and a corresponding specific inhibitor have been isolated from *N. crassa*, and a new method for isolating the multienzyme system has been developed. With the aid of these findings, we have made the following observations: (*a*) Detergent (SDS) gel electrophoretograms of the "complex" isolated by two different methods are not comparable. In an earlier method, which involved more manipulations and time, the detergent gel banding patterns showed four polypeptides with

molecular weights totaling about 300,000. With the new purification procedure, there are only two major bands: the first with an apparent molecular weight of about 150,000 and the second with a molecular weight of 50,000. (*b*) When the freshly purified multienzyme system is incubated at 25° C, four new bands appear within 30 hr and a fifth is visible after 40 hr. (*c*) The formation of these new bands is prevented for up to 40 hr by the addition of phenylmethanesulfony fluoride or a purified preparation of the specific *N. crassa* protease inhibitor. (*d*) The multienzyme system appears to remain intact, as shown by standard polyacrylamide gel electrophoresis, even after it has suffered several specific proteolytic clips. These results demonstrate that the purified complex is contaminated with a small but influential quantity of the inhibitable *N. crassa* protease, and they show that this protease is capable of creating an artificial subunit structure in the multienzyme system. Before the true subunit structure can be defined, the multienzyme system must be isolated when protease is absent or inactive, and the detergent electrophoresis gel method must be shown to be valid for this system. However, if our results are substantiated, they suggest that the enzyme "complex" is actually a multifunctional enzyme with five enzymic activities contained on a single polypeptide chain with a molecular weight of approximately 150,000.

DETECTION OF AN ESSENTIAL SULFHYDRYL GROUP IN PHOSPHOGLYCERATE MUTASE WITH AN AFFINITY-LABELING REAGENT

I. L. Norton and F. C. Hartman

N-bromoacetylethanolamine phosphate was designed and used previously in this laboratory as an active-site-specific reagent for aldolase.[1,2] This reagent also rapidly and irreversibly inactivates 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase from rabbit muscle. From amino acid analyses on acid hydrolysates of the inactivated enzyme, we have identified a sulfhydryl group as the site of alkylation. Several experiments suggest that the group is at or near the active site: (*a*) The inactivation of the enzyme by the reagent exhibits a rate-saturation effect. (*b*) The concentration of reagent that gives one-half the maximal rate of inactivation is in the range of K_m for the substrate, and binding of reagent appears competitive with 2,3-bisphosphoglycerate. (*c*) Inactivation is associated with the covalent incorporation of a single mole of reagent per mole of catalytic subunit. (*d*) Reaction of the mutase with the reagent can be prevented by the presence of substrate in the reaction mixture and can be retarded by disruption of the three-dimensional structure of the enzyme with urea. Thus an accessible and three-dimensionally intact substrate binding site is essential to the rapid, selective alkylation. (*e*) The high degree of selectivity implied by the stoichiometry is confirmed by the finding of only one major labeled peptide in tryptic digests of the mutase alkylated with ^{14}C-labeled reagent. The amino acid composition of this peptide is Trp_1, Lys_1, $Cys(Cm)_1$, Asp_1, Ser_1, Glu_2, Gly_1, Ala_1, Leu_1, Phe_2.

1. F. C. Hartman, B. Suh, M. H. Welch, and R. Barker, *J. Biol. Chem.* **248**, 8233 (1973).
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COMPARATIVE STUDIES ON PHOSPHOGLYCERATE MUTASE WITH THE ACTIVE-SITE PROBE *N*-BROMOACETYLETHANOLAMINE PHOSPHATE

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

N-bromoacetylethanolamine phosphate has permitted the identification of a sulfhydryl group at or near the active site of phosphoglycerate mutase from rabbit muscle (see previous report). Comparative studies provide one criterion for ascertaining whether a given active-site residue is functional in catalysis, since residues essential to catalysis are generally species-invariant (with the assumption that the enzymes are derived from a common ancestral gene). We have thus investigated the reaction of the site-specific reagent with the mutase from chicken muscle and find the reaction to be entirely analogous to the corresponding reaction with the enzyme from rabbit muscle, *i. e.*, inactivation with a rate-saturation effect, binding of reagent competitive with binding of substrates, stoichiometric incorporation of reagent, alkylation of one sulfhydryl group as the only detectable modification. As in the case of the enzyme from rabbit muscle, the tryptic peptide that contains the essential sulfhydryl group has been purified by

ion-exchange chromatography. The sequences of the peptides

(rabbit) -Phe-Cys-Gly-Trp-Phe-Asp-Ala-Glu-Leu-Ser-Glu-Lys-

(chicken) -Phe-Cys-Gly-Trp-Tyr-Asp-Ala-Asp-Leu-Ser-Pro-Gly-Glx-Glx-Glu-Arg-

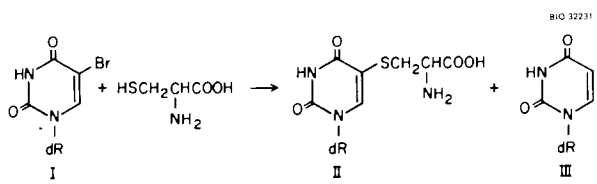
(as determined by automated Edman degradation and digestion with carboxypeptidases A and B) are highly homologous and therefore consistent with the postulate that the sulfhydryl group selectively alkylated by bromoacetylethanolamine phosphate is in the active-site region.

REACTION OF 5-CHLORODEOXYURIDINE WITH CYSTEINE

B. C. Pal

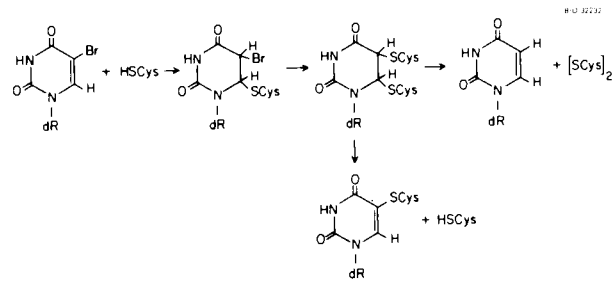
5-Chlorouracil[1] has been identified as one of the major chloroorganics in the effluent from sewage treatment plants and should be regarded as a pollutant of our aquatic environment. We have established that mice kept on a 5-chlorouracil diet do incorporate the base in liver DNA as 5-chlorodeoxyuridine.[2] These considerations led me to investigate the reaction of 5-chlorodeoxyuridine with cysteine.

It has been shown previously[3] that 5-bromodeoxyuridine (I) reacts with cysteine forming 5-cysteinyldeoxyuridine (II) and deoxyuridine (III):



To my surprise, 5-chlorodeoxyuridine reacted with cysteine to form quantitatively and exclusively compound II; not a trace of III was formed. The halogenated nucleosides were treated with 6 equivalents of cysteine in 1 *N* aqueous K_2CO_3 at 50°. The reaction was monitored by running an aliquot through a column of A-6 resin (BioRad, cation-exchanger, 20 × 0.63) maintained at 50° using 0.1 *M* ammonium borate, pH 7.4, as described previously.[4] This system separates 5-halodeoxyuridine, deoxyuridine, and cysteinyldeoxyuridine.

The proposed mechanism of reaction[3] of 5-bromodeoxyuridine with cysteine, which has been widely accepted,[5,6] is described below:



Apparently this mechanism does not operate in the case of 5-chlorodeoxyuridine. In order to rule out the possibility of participation of the deoxyribosyl moiety in this reaction, I prepared the methyl analogues of 5-chloro- and 5-bromodeoxyuridine by halogenation of 1-methyluracil with *N*-halosuccinimide. The methyl analogues reacted slowly but very similarly to the deoxyribosyl derivatives. When these reactions are carried out in $Me_2SO:H_2O$(1:1 V/V), the rate is slowed down remarkably, and in some instances the starting material is recovered intact. Based on these considerations, I propose that the reaction of 5-chlorodeoxyuridine with cysteine does not follow the same pathway as 5-bromodeoxyuridine and most likely 5-Cl is directly displaced by cysteine by an S_N2 mechanism. Sluggishness of the reaction in dimethyl sulfoxide medium can be explained by the premise that the aprotic solvent, dimethyl sulfoxide, activates the anions of 5-halodeoxyuridine (N-3 proton is ionized) and cysteine by removing the water of hydration of these ions. The two anions repel each other, which partially hinders the reaction. It is not known what role these reactions may play *in vivo* in mutagenesis by 5-chloro- and 5-bromodeoxyuridine.

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ELECTRON-SPIN-RESONANCE STUDIES OF PHOTOLYZED PEPTIDE SOLUTIONS

D. G. Doherty, R. Livingston,* M. A. Turner, and H. Zeldes*

Our earlier studies with simple dipeptides containing alanine, β-alanine, and glycine[1] established that OH radicals, arising from photolysis of a 1% hydrogen peroxide solution of the peptides, primarily abstracted hydrogen from the α carbon between the peptide nitrogen and the C terminal carboxylate group. Extension of this work to dipeptides with more complex side chains, such as L-prolylglycine and β-alanyl-L-proline, showed similar radical attack, although in the latter case the spectrum was affected by the presence of cis–trans isomers and dynamic phenomena. In the case of the valine dipeptides, hydrogen abstraction was almost entirely from the isopropyl side chain, giving rise to a mixture of radicals corresponding to the loss of one or the other kind of hydrogen in the isopropyl group. Glycyl-L-aspartate and β-alanyl-L-aspartate in aqueous solution at pH 3.5 gave a spectrum that was only partly resolved. However, a well-resolved spectrum was obtained when the lithium salt was examined. Two radicals were seen, one, as might be expected, formed by abstraction of a hydrogen from the methylene group in the side chain. There is considerable ambiguity about the nature of the second radical, and at present it seems that there has been a chemical modification of the side chain. A full discussion of these peptides, with the hyperfine couplings and g values, has been reported elsewhere.[2] When the methylene group of aspartic acid was removed, as in β-alanylaminomalonic acid and glycylaminomalonic acid, the usual single α carbon radical was formed. We are continuing our study with tri-, tetra-, and pentapeptides containing various side chain groups.

*Chemistry Division.

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CHEMILUMINESCENCE INDUCED BY CERTAIN MOLYBDENUM-CONTAINING OXIDASES

J. R. Totter, M. A. Turner, and D. G. Doherty

Several molybdenum-containing enzymes give off weak chemiluminescence during the time they are catalyzing oxidation of their substrates. The light seen may be greatly enhanced by adding the well-known chemiluminescent phthalazinediones or diaryl biacridines. It has been supposed that the emission in either case is related to the production of oxidizing radicals during the stepwise transfer of electrons to O_2. Comparisons of the light which may be produced by one unit of xanthine oxidase (as measured by uric acid production) in the presence of luminol (5-amino-2,3-phthalazine-1,4-dione) or of lucigenine (10,10′-dimethyl-9,9′-bicaridylium nitrate), each either in 0.04 M Na_3PO_4, pH 11.6, or in 0.1 M Na_2CO_3, have been made. Enzymes of different purities and those subjected to various treatments give rise to very different light intensities. It is difficult to ascribe these differing responses to any mechanism involving oxidizing radicals in solution and independent of the enzyme. It seems likely, therefore, that the light-emitting process, when fully understood, may provide a useful tool for studying changes in the conformation of the enzyme or in its constitution.

Studies of light production in relation to enzyme activity may also prove helpful in understanding the precise route of electron transfer in the molybdenum-containing enzyme group, which, in addition to xanthine oxidases and aldehyde oxidases, also includes nitrate reductases and part of the nitrogen-fixing system.

STUDIES ON THE MECHANISM OF LIGHT PRODUCTION BY PEROXYDISULFATE AND PHTHALAZINEDIONES[1]

J. R. Totter, M. A. Turner, and D. G. Doherty

It has been suggested that peroxydisulfate oxidizes luminol (5-amino-phthalazine-1,4-dione) only by a two-electron removal with the formation of bisulfate and sulfate anions. In a reinvestigation of this reaction, with determination of the stoichiometry of both luminol and peroxydisulfate disappearance, we have been unable to confirm this hypothesis. Experiments in 0.1 M K_2CO_3 solutions showed that with 0.06 M $K_2S_2O_8$ and 0.01 M luminol, the initial velocity of $K_2S_2O_8$ disappearance was 8.55×10^{-6} mol/liter per second, while luminol disappeared at a velocity of 3.9×10^{-6} mol/liter per second. With both compounds at 0.01 M and in the presence of 0.1 M H_2O_2, $K_2S_2O_8$ disappeared initially at a velocity of 2.4×10^{-6} mol/liter per second, while luminol was removed at an initial rate of 2.9×10^{-7} mol/liter per

second. Numerous tests were run with widely varying initial concentrations, and in no case was there found a one-to-one relationship.

1. To be presented at the Southeast Regional Meeting of the American Chemical Society, Gatlinburg, Tenn., October 27–29, 1976.

ASSAY OF α-AMINO-β-HYDROXY ACID ALDOLASE ACTIVITY WITH THE BACTERIAL LUMINESCENT SYSTEM

J. R. Totter, M. A. Turner, and D. G. Doherty

Serine hydroxymethyltransferase (2.1.2.1) appears to be identical with threonine aldolase and also rapidly splits allothreonine[1] to glycine and acetaldehyde. This apparent lack of specificity suggests that longer-chain α-amino-β-hydroxy acids might also cleave to glycine and long-chain aldehydes under the influence of the aldolase. The long-chain aldehydes, if formed, could be quantitated by use of bacterial luciferins which require this type of aldehyde for luminescence. α-Amino-β-hydroxypelargonic acid and α-amino-β-hydroxylauric acid were prepared and tested for activity as substrates of rabbit liver preparations containing serine aldolase. Both compounds were rapidly split to long-chain aldehydes which were active in bioluminescence. Experiments are in progress to adapt this method to the determination of atmospheric pollutants such as olefins and ozone.

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SYNTHESIS AND EVALUATION OF SERINE ANTIMETABOLITES

D. G. Doherty, M. A. Turner, J. R. Totter, and J. D. Regan

Previous reports have established the importance of the enzyme serine transhydroxymethylase in the metabolism of serine by human granulocytic leukemia cells.[1] The most active inhibitor found in the cell culture system was 1-(hydroxyamino)-cyclohexanecarboxylic acid. In an effort to utilize a simpler test system, we isolated the enzyme from both rabbit and rat liver and, in conjunction with an assay system using either alcohol dehydrogenase or a luminescent system, assayed the inhibitory activity of a series of variously substituted hydroxyamino carboxylic, phosphonic, and sulfonic acids. Compounds with carbon chains longer than three carbon atoms were inactive in the alcohol dehydrogenase system. Hydroxyvaline proved to be a good inhibitor of alcohol dehydrogenase. None of the compounds tested were more effective than the hydroxyamino-cyclohexanecarboxylic acid. Since collaborative studies are under way toward using this compound in dogs, we have devised a small-scale synthesis to prepare it with both ^{14}C and ^{3}H label for metabolic studies.

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THE PRIMARY SEQUENCE OF A CRUSTACEAN SATELLITE DNA

C. A. Chambers, M. G. Schell, and D. M. Skinner

Satellite DNA II of the hermit crab, *Pagurus pollicaris*, has been almost completely sequenced. The individual strands of the satellite contain different amounts of G + T residues, the heavy (H) strand containing 64% and the light (L) strand containing 41%. Such interstrand bias in base composition effects a 37 mg/cm^3 strand separation in alkaline cesium chloride gradients.[1]

Both of the single-strand DNAs were used as templates for RNA transcription using RNA polymerase and α-^{32}P-labeled nucleoside triphosphates. Each DNA was transcribed, first with all four ^{32}P-labeled nucleotide triphosphates, then with only one labeled triphosphate and three cold triphosphates to give a nearest-neighbor analysis for each triphosphate.

Degradation of the RNA transcripts with T1 and pancreatic RNase indicates that most of the L strand of the DNA consists of the repeating trimer CAG, whereas the H strand consists of the complementary trimer CUG. In the L strand, the sequence CUGCACUCAG follows every eight or nine trimer repetitions, whereas in the complementary H strand, the sequence CUGAGUGCAG follows every eight or nine CUGs. In retrospect, it is apparent that these

sequences were observed as DNA contaminants when the isopycnic twin[2] of this satellite, satellite I of the hermit crab, was previously sequenced.[3]

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INTERACTIONS OF THE SEPARATED STRANDS OF α-SATELLITE DNA OF THE GUINEA PIG WITH DOUBLE-STRAND DNAs

C. A. Chambers and D. M. Skinner

We have extended our studies on the interactions of separated H-(36% G) and L-(36% C) rich strands of the α satellite of the guinea pig. The H and L strands of the α satellite, recovered from centrifugation in alkaline cesium chloride gradients, have been found to form complexes with seven different double-strand (ds; companion) DNAs, including those of one bacteriophage, two prokaryotes, and four eukaryotes (two invertebrates and two mammals). It was shown that the complexes are not artifacts due to *in vitro* labeling of the satellite by ^{125}I or nick translation, by the methods of collection, the presence of divalent cations, or the fact that trace amounts of single-strand (ss) DNAs are used. More complex ss DNAs, such as that recovered from nicked RF M13, do not associate with ds DNAs.

We have taken advantage of the reduced complex formation with *Micrococcus luteus* DNA to determine the effect of salts on complex formation. Tritiated H strand was preincubated with *M. luteus* DNA in low-salt [0.10 *M* NaCl, 0.05 *M* Tris (pH 8)] or in high-salt (7.7 *M* CsCl) solution for one week at different temperatures; crab main-component DNA was then added, the cesium chloride was adjusted to 7.7 *M* if necessary, and the mixture was centrifuged. After preincubation in high salt the H strand remains complexed with the *M. luteus* DNA. Clearly, although complex formation is enhanced by high salt, it occurs in physiological salt concentrations as well.

Some of the companion ds DNAs were treated with S1 nuclease to obliterate single-strand regions. Such treatment resulted in an approximate twofold decrease in the molecular weight. The density at which the complexed H strand was observed with S1-treated DNA was 8 to 10 mg/cm^3 greater than the density of the complex with untreated ds DNAs. These data are most easily interpreted by the following model: in large-molecular-weight DNA, the complexes perturb the density only to a minor extent. With the smaller S1-treated DNAs, the interspersed noncomplexing sequences are no longer attached to the complexing DNAs and the density shift is more pronounced.

In experiments designed to determine whether complex formation was an association between ss regions in the companion ds DNAs, we have prepared phage DNAs that could be specifically characterized. Taken together, data on a linear ds DNA (T7) and a superhelical DNA with ss regions (PM2) show that neither ss regions nor nicks can account for complex formation.

The physical and chemical characterizations of satellites are quite advanced. By contrast, very little is known with certainty concerning their biological functions, although speculations have been made as to their role in "chromosome housekeeping." These data show the potential for molecular interactions of satellite DNAs.

THE REITERATION FREQUENCY AND ORGANIZATION OF SEQUENCES IN THE CRUSTACEAN GENOME

C. A. Holland,* N. T. Christie,* and D. M. Skinner

Total DNA of *Gecarcinus lateralis* has two satellites in neutral cesium chloride gradients ["crab poly(dA-dT)" and a GC-rich satellite].[1] Additional cryptic satellites (<1% total DNA) can be isolated from its purified main-component DNA in heavy-metal–Cs_2SO_4 gradients. Two or three cryptic satellites also present as minor components separate from the total DNA of *Geryon quinquedens* in Hg^{2+}–Cs_2SO_4 gradients; in Ag^+–Cs_2SO_4 gradients, a single cryptic satellite is isolable. Each satellite has a density very close to that of main-component DNA. In addition to satellite DNAs, sequences of lower frequencies of repetition occur in the genomes of all eukaryotes. These sequences, which comprise a different percentage of the genome of each organism, are present in varying reiteration frequencies in different animals and may function as control sites for gene expression. By studying the kinetics of reassociation of 300-nucleotide fragments, we have distinguished the repetitive from single-copy se-

quences in purified main-component DNA of the highly evolved land crab, *G. lateralis*, and of the total DNA of the primitive crab, *G. quinquedens*, which has no patent satellite DNAs.

Purified or total DNA was labeled *in vitro* by nick translation. Labeled 300-nucleotide fragments were combined with unlabeled 300-nucleotide fragments (present in 100× excess) and were denatured and incubated at 60° for different periods of time. The fraction of labeled DNA in duplex molecules was determined by hydroxyapatite chromatography.

The data show that both DNAs have three distinctive properties: (1) At least 50% of the DNA is present in sequences repeated more than 200 times. Since 25% of the genome of *G. lateralis* is isolable as satellites, at least 60% of the total DNA of the land crab is present in multiply reiterated sequences. The highly repetitive main-component DNA of *G. lateralis* reassociates with kinetics consistent with two second-order components: one comprising 18% of the main-component DNA and repeated 150,000 times, and a second comprising 17% of the DNA and repeated 4,800 times. (2) Although half the sequences in the DNAs of both crabs are highly repeated, there are no sequences that are repeated from 10 to 200 times. The lack of sequences of fairly low repetition frequency is unusual in eukaryotic genomes, most of which contain a range of sequences of frequencies of those repetitions. (3) The remaining 50% of the DNA of *Gecarcinus* and 35% of *Geryon* reassociate with a rate constant expected for nonrepetitive sequences of organisms with 1 pg of DNA per haploid genome.

These data show that the reiteration frequencies of the sequences in the genomes of a primitive and highly specialized crab are distinctive. Sixty percent of the sequences in the total genome are repeated more than two hundred times, whereas there are no sequences repeated a moderate (10–200 times) number of times.

Two patterns of interspersed repetitive and single-copy sequences have been found in the genomes of various eukaryotes. The genomes of several invertebrates as well as vertebrates are organized in a pattern of 300- to 400-nucleotide-long repetitive sequences interspersed with single-copy sequences of 1000 to 2000 nucleotides.[2] The genomes of *Drosophila melanogaster* and several other insects have a pattern of repetitive sequences about 5600 nucleotides long interspersed with long single-copy sequences of >10,000 nucleotides. To date, this pattern has been found only in the genomes of these three insect species. Since we found that the DNAs of both crabs lack sequences in the repetition frequency interspersed with single-copy sequences in the genome of the sea urchin, we have studied the sequence organization of the main-component DNA of *Gecarcinus*. It also seemed likely that the sequence organization of the crab DNA might resemble that of other arthropods.

To determine if repetitive and single-copy sequences are interspersed in the genome of the land crab, main-component DNA fragments of different lengths were self-reassociated to a C_0t where all repetitive sequences, but no single-copy sequences, have reassociated. Duplex molecules were collected on hydroxyapatite. As the fragment length increased to 1400 nucleotides, the percentage of DNA bound to hydroxyapatite increased to 81%. This is preliminary evidence that a short-period interspersion of repetitive and single-copy sequences exists in this DNA.

The hyperchromicities of the reassociated partially duplex molecules were monitored to determine the double-strand fraction of each. If repetitive sequences are interspersed with single-copy sequences in a pattern like that found in the sea urchin genome, then the fraction of the fragment in single-strand form should increase with increasing fragment lengths. As predicted, the hyperchromicities of the reassociated molecules decreased with increasing fragment length, indicating that a greater percentage of the longer molecules were single strands. These data indicate that although the main-component DNA of *G. lateralis* lacks sequences repeated from 10 to 200 times, it contains more highly repeated sequences which are interspersed with single-copy sequences in a short pattern of interspersion similar to that of the sea urchin rather than that of the arthropod, *D. melanogaster*.

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MOLTING AND REGENERATION IN *GECARCINUS LATERALIS*

J. F. McCarthy* and D. M. Skinner

Previous work in this laboratory has established that multiple-limb autotomy of intermolt crabs induces regeneration and precocious molting.[1,2]

Removal of partial regenerates before a critical period delays molting and inhibits the growth and rates of DNA synthesis of the remaining primary regenerates while secondary regenerates form. When these secondary regenerates grow to almost the size of the original primary regenerates, regeneration resumes and molting ensues.[3]

We are attempting to clarify the nature of the inhibitory mechanism by two approaches: (1) measurements of the serum titers of the arthropod molting hormone ecdysterone (β-ecdysone), and (2) parabiotic experiments to distinguish between hormonal and nervous mediation of this inhibitory effect.

Ecdysterone levels are measured by radioimmunoassay[4] using antiserum provided by Dr. J. D. O'Connor. In our hands, this method permits reproducible determinations of the ecdysone content of unpurified crab serum at concentrations as low as 100-pg β-ecdysone equivalents. Using this method, the ecdysone titer of individual animals may be monitored sequentially over the course of the molt cycle.

Concurrently with the sampling of hemolymph, we are monitoring the formation of gastroliths, calcareous concretions of calcium phosphate which form at specialized regions of the stomach epithelium during the premolt stage and serve as a reservoir of calcium during exoskeleton resorption. Gastroliths are visualized by X-ray photography *in vivo* (40 mR per exposure), and their growth is quantified. The X-ray procedure has had no discernible effect on the pattern of regeneration, on the duration of the molting period, or on the mortality rate.

With these new techniques, in addition to the histological examination of small samples of integumentary tissues from the branchial chamber, we are able to monitor hormonal titers, calcium metabolism, and exoskeletal degradation and synthesis over the course of regeneration and molting in individual animals and to determine the effect of the autotomy of partial regenerates on these processes.

Preliminary data indicate that following multiple limb autotomy and during basal regeneration [up to $R = 7$–10, where $R =$ (length of regenerate/width of carapace) $\times$ 100], ecdysone concentrations are slightly higher than intermolt levels. Ecdysone titers rise as the regenerates grow ($R \geqslant 10$). Autotomy of 1 or more regenerates at $R \simeq 15$ results in a rapid (within 1–4 hr) decline in ecdysone titers, which drop to basal levels after about four days. These data indicate that the inhibition of regeneration and premolt development following autotomy of partial regenerates is a consequence of a decreased titer of molting hormone.

Following eyestalk extirpation (which removes the molt inhibitory hormone localized in the eyestalk), ecdysone titers double and remain at that level for about 10 days, after which time the concentration increases precipitously. Limb regenerates grow linearly during this period. Immediately preceding ecdysis, ecdysone levels decline concomitant with the cessation of growth of the regenerates. Within a few days, the eyestalkless crabs undergo ecdysis but invariably die.

Although it appears that the inhibition of regeneration and molting preparations is correlated with a decreased titer of serum ecdysterone, the hormonal nature of the inhibitory effect can best be demonstrated by parabiosis, the union of the circulatory systems of two animals. We have further improved this method for *G. lateralis*.[5] The most successful regeneration results after parabiosis of intact crabs, followed in a few days by limb autotomy. Attempts to accelerate molting further by eyestalk extirpation, either before or after parabiosis, have resulted in extremely high mortality.

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

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TRANSCRIPTION PROPERTIES OF VEGETATIVE T4 DNA AND MODIFIED RNA POLYMERASE FROM T4-INFECTED *ESCHERICHIA COLI*

A. L. Stevens

A modified RNA polymerase from T4-infected *E. coli* can be isolated by DNA cellulose-column chromatography. The polymerase contains three of the four T4-specific polymerase binding proteins (15,000 and 12,000 mol wt, respectively, the gene 33 product; and 22,000 mol wt, the gene 55 product). The modified RNA polymerase lacks the sigma

subunit and the small polymerase binding protein of 10,000 mol wt. Vegetative T4 DNA has been isolated from the supernatant fraction of a Brij-lysis extract of T4-infected *E. coli*. The vegetative DNA has been used as a template for the modified RNA polymerase; the reaction has been compared with a similar reaction using mature T4 phage DNA as a template. The results show that (*a*) vegetative DNA is a better template than mature T4 DNA; (*b*) the reaction is not salt inhibited, as is the reaction with mature T4 DNA as a template; (*c*) little stimulation of the reaction is found upon addition of the sigma subunit, in contrast to a high stimulation by sigma with mature T4 DNA; and (*d*) the product of the reaction, as analyzed by competitive hybridization with *in vivo* RNA and asymmetry measurements, contains late T4 mRNA with a lower amount of symmetric RNA– when mature T4 DNA is used as a template, a high amount of symmetric RNA is found.

The reaction of vegetative T4 DNA and modified RNA polymerase has also been compared with that of vegetative DNA and host-core enzyme. The product of the reaction with the latter combination is also highly symmetric.

Further studies are in progress to determine the binding-protein requirement of the late T4 mRNA synthesis.

STUDIES OF AN INHIBITOR OF RNA POLYMERASE ISOLATED FROM T4 PHAGE-INFECTED *ESCHERICHIA COLI*

A. L. Stevens

Studies show that an inhibitor of RNA polymerase can be isolated from T4 phage–infected *E. coli* and that it also copurifies with the small polymerase binding protein of molecular weight 10,000.[1] Both host and T4-infected RNA polymerases are inhibited when T4 or T7 DNA is used as a template. The inhibition is promoted by salt. Little inhibition of activity is found with calf thymus DNA or poly (dA–dT) as a template. The inhibitor prevents the formation of rifampycin-resistant T4 DNA–enzyme complexes, suggesting an inhibitory role at the stage of enzyme–DNA binding or at the level of "open" enzyme–DNA complex formation. Inhibition of enzyme–DNA binding has been studied in two ways. With T4 DNA as a template, the competing-template method of Mueller[2] has been used. With T7 DNA, the direct-binding method of Hinkle and Chamberlin[3] has been used. Both methods show that the inhibitor prevents binding of the enzyme to DNA. The binding inhibition is promoted by salt, and once binding has occurred the inhibitor has no effect. No evidence can be obtained that the inhibitor binds to DNA.

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BASE-UNPAIRED REGIONS IN COVALENTLY CLOSED SUPERCOILED REPLICATIVE-FORM DNA OF COLIPHAGE M13

S. Dasgupta,* C. M. Snyder, S. Mitra, and D. P. Allison

The covalently closed double-strand replicative-form DNA (RF I) of coliphage M13 is supercoiled like many other covalently closed circular DNA molecules, but the susceptibility of M13 RF I to single-strand-specific S_1 endonuclease indicates the presence of a denatured region in these molecules. The size and location of the single-strand region(s) were investigated by electron microscopic and biochemical methods. M13 RF I molecules appear supercoiled in electron micrographs when spread over water; however, they open up with a localized denatured region in the form of a bubble (involving approximately 100 to 200 base pairs) in the presence of formamide-containing formaldehyde in the spreading solution. The location of the bubble is not unique, as was determined by the fixation of the bubble (one per molecule) in RF I with glyoxal followed by its digestion with Hind restriction endonuclease that cleaves the RF at a unique site. The fixed bubble occurs at several specific sites with varying frequency, with reference to the Hind cleavage site, but does occur predominantly at one site in the molecules. Furthermore, successive digestions with S_1 endonuclease and Hind restriction nuclease yielded several classes of fragments rather than two unique fragments (as in the case of a unique position of the bubble) or fragments of random size (as in the case of a randomly localized bubble). A comparison with the denaturation map of M13 RF I showed that the location of the denatured regions corresponds with the AT-rich regions in the DNA, as established by partial denaturation mapping of linear RF

generated by Hind nuclease digestion. However, while glyoxal reacts predominantly at one major AT-rich site in RF, S_1 endonuclease cleaves equally well any one of the five AT-rich sites in a molecule.

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

THE ROLE OF DNA POLYMERASE I-ASSOCIATED 5′-EXONUCLEASE IN M13 PHAGE REPLICATION

S. Dasgupta* and S. Mitra

5′-Exonuclease associated with DNA polymerase I has been implicated in the removal of the primer RNA at the 5′ end of nascent DNA fragments. Replication of the male-specific filamentous coliphage M13 was examined in the *E. coli* strain *pol*Aex I containing the temperature-sensitive 5′-exonuclease function. Examination of the different stages in intracellular phage DNA synthesis shows that (*a*) the synthesis of the parental replicative form (RF) from the infecting viral DNA is not inhibited at restrictive temperature; (*b*) under restrictive condition, the progeny RF DNA molecules contain a higher proportion of the open circular double-strand DNA with more than one nick (RF II); and (*c*) progeny single-strand DNA synthesis is moderately inhibited at the restrictive temperature.

The above data indicate that the 5′-exonuclease function is required in the sealing of RF II molecules into covalently closed circular RF (RF I), but this function is not completely indispensable and might be performed by alternate mechanisms, albeit less efficiently. Also, it is interesting to note that this requirement is observable mostly in the RF replication alone.

The pulse-labeled, newly synthesized RF II molecules purified from M13-infected, 5′-exonuclease-defective hosts under restrictive conditions can be converted into RF I with T4 DNA ligase and T5 DNA polymerase. These RF I molecules purified by equilibrium centrifugation in cesium chloride containing ethidium bromide are nicked in alkali, indicating the presence of RNA fragments in the RF II. On the other hand, when such newly synthesized RF II molecules are sealed by *E. coli* DNA polymerase I (which, unlike T5 DNA polymerase, contains 5′-exonuclease) and *E. coli* DNA ligase, the resulting RF I molecules are alkali resistant. It is concluded that newly synthesized M13 RF II molecules contain RNA pieces, presumably as primers at the 5′ ends of the nascent DNA chains, and these are subsequently removed during formation of RF I.

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

ROLE OF *ESCHERICHIA COLI* DNA LIGASE IN COLIPHAGE M13 DNA SYNTHESIS

S. Mitra

DNA ligase of host *E. coli* is essential for M13 phage DNA synthesis, as determined in the study of a temperature-sensitive ligase mutant of *E. coli*. Although the synthesis of intracellular-phage replicative-form (RF) DNA is comparable in the ligase mutant and in the wild-type revertant host, most of the newly synthesized RF DNA is present in the mutant in the nicked form (RF II) at the restrictive temperature. The synthesis of the progeny single-strand DNA is also strongly inhibited in the ligase mutant at the restrictive temperature. A large fraction of the intracellular progeny single-strand DNA that is synthesized is of unit length and linear. However, the small amount of progeny phage that is synthesized in the absence of ligase function contains only circular DNA, unlike in the case of $\phi X174$, where a large fraction of the progeny phage has been reported to contain linear DNA under a similar situation. This difference may be the consequence of the known difference in morphogenesis of the two phages. Therefore, host DNA ligase is necessary for sealing both RF II and for circularizing linear progeny single-strand DNA of the M13 phage. Inhibition of synthesis of progeny single-strand DNA might be due to the nicking of the template (complementary) strand in RF II DNA in the absence of ligase function.

SURVEY OF DNA ENDONUCLEASES IN CULTURED HUMAN FIBROBLAST CELLS

L. I. Hecker, S. Mitra, J. D. Regan, and E. Volkin

It was initially observed[1] that a strain of normal human fibroblasts (HSBP) had the ability to dis-

tinguish between homologous (human) and heterologous (bacteriophage T7) DNAs. Both types of DNA were taken up by the cells; T7 DNA was extensively broken down in only 3 hr, whereas human DNA remained in relatively large pieces for much longer periods of time and was observed in the nuclei of these cells.

It was reasoned that perhaps these HSBP cells had an enzyme which could distinguish between native and foreign DNAs. Enzymes of this type (restriction endonucleases) are very common in bacterial cells, but none have been found in eukaryotic cells. This investigation then would involve a search for a restriction endonuclease in HSBP cells.

During the first six months an extensive search was conducted to detect restriction endonucleases. Most early work was done using crude extracts; however, we later progressed to different chromatographic fractions. In these investigations, pH, buffers, types of ions, ionic strength, cofactors, and temperature were varied in a systematic manner. No restriction endonuclease could be detected. However, two major DNA endonucleases were detected: One had a pH optimum at 5.5 and required no Mg^{2+} for activity; the second had a pH optimum of 7.5 and required Mg^{2+} for activity; however, both calcium and magnesium ions were required for optimal activity. Of the two enzymes, that having an optimum pH of 5.5 was most active. These two activities could easily be separated using phosphocellulose column chromatography. Thus, for the enzyme with a pH of 5.5, an approximately 30- to 50-fold purification could be obtained in a single step. This enzyme was quite stable and would remain fully active for many days if stored at 0° to 4°C. This pH 5.5 endonuclease appeared similar to others reported in the literature that had been found to be associated with lysosomes and the spleen.

The pH 7.5 endonuclease was more interesting and appeared to be similar to pancreatic DNase, which is secreted by the pancreas into the intestine. Hence, it was of interest to study its function in cells that did not secrete it. The difference between this enzyme and the pancreatic enzyme, as well as those reported in the literature, was that its molecular weight, as judged from Sephadex G-75, appeared to be 60,000 to 70,000, as opposed to 30,000 to 35,000. Attempts were made to purify and characterize this enzyme. Although this enzyme was stable in concentrated form and crude extracts, it proved to be unstable upon attempts to further purify it using column chromatography. Partial fractionation was obtained by ammonium sulfate precipitation, but repeated attempts to fractionate on DEAE-cellulose, Sephadex G-100, hydroxyapatite, or CM-cellulose consistently resulted in the loss of all enzyme activity.

This type of enzyme has recently been found to occur in such diverse sources as beans and human serum, and enzymes from these sources are being actively studied by other investigators.

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PROMOTER AND OTHER "ACTIVE" REGIONS IN SINGLE-STRAND M13 DNA

S. K. Niyogi, B. H. Underwood, and S. Mitra

Studies from several laboratories suggest that there may be unique sites on single-strand phage M13 DNA that are involved in the synthesis, by *E. coli* RNA polymerase, of the RNA primer needed for the initiation of DNA replication. We are attempting to isolate and analyze such region(s) by a variety of methods: (*a*) Using the *E. coli* (host) binding protein (which is needed for the initiation of DNA replication on single-strand phage M13 DNA to its double-strand replicative form) to limit the binding of *E. coli* RNA polymerase to the specific region(s) or promoter(s) leads to predominantly unique-sized pieces about 40 to 50 nucleotides in length. (*b*) With poly(I) as a discriminator to remove "weakly bound" RNA polymerase molecules, the "strong" binding sites are composed of two size classes (about 45 and 90 nucleotides in length). (*c*) Isolation of naturally occurring double-strand hairpin regions in the single-strand M13 DNA by using a variety of specific endo- and exonucleases leads to specific size classes, depending on the enzymatic treatments.

The similarities and differences in size, base composition, secondary structure, sequence, and location in the M13 genome are now being investigated to obtain a clear and more complete picture of such regions and their regulatory roles in M13 phage development.

SALT-SENSITIVE TRANSCRIPTION OF SINGLE-STRAND M13 DNA

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

The initiation of replication of single-strand phage M13 DNA to its replicative form is dependent on an

obligatory transcriptional event using the host (*E. coli*) RNA polymerase. In studying this transcription *in vitro*, we find that it is strongly inhibited by moderate (0.2 *M*) concentrations of potassium chloride. The extent of inhibition is inversely proportional to the substrate concentration. The sigma factor, the initiation subunit of RNA polymerase, has little or no effect on the inhibition. Studies of the nature of the inhibition and the different steps involved lead to the following conclusions: (*a*) The rate of chain elongation is unaffected by salt. (*b*) Analysis of the RNA size distribution shows that potassium chloride does not lead to early chain termination. (*c*) Inhibition by potassium chloride is also observed in the presence of rifampin. (*d*) Incorporations of both [γ^{32}P]ATP and [γ^{32}P]GTP, as indicators of chain initiation, are inhibited proportionally with increasing potassium chloride concentrations. UTP and CTP do not initiate RNA chains in this system. (*e*) The rifampin-resistant preinitiation complex, when exposed to potassium chloride, decays to a certain level (depending on the potassium chloride concentration) and then remains constant; the effect is reversible.

It thus appears that potassium chloride inhibits the formation of the active preinitiation complex from an inactive complex. High salt concentration favors the equilibrium towards the formation of an inactive complex, whereas in the absence of salt the formation of an active preinitiation complex is facilitated.

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

ON THE RELATIONSHIP OF 3′→5′ EXONUCLEASE AND TURNOVER ACTIVITIES ASSOCIATED WITH T5-INDUCED DNA POLYMERASE

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

T5-induced DNA polymerase has an associated 3′→5′ exonuclease activity. This enzyme also catalyzes template-dependent conversion of dNTP to dNMP during synthesis, which presumably consists of incorporation into the primer template and subsequent removal by the enzyme (turnover). Since the 3′→5′ exonuclease activity has been implicated in the regulation of fidelity of DNA synthesis, and hence the spontaneous mutation frequency, we have asked an important but widely unrecognized question as to whether the 3′→5′ exonuclease and turnover activities are identical.

We have found that CTP is a potent inhibitor of 3′→5′ exonuclease action on nicked T7 DNA. However, CTP had no effect on polymerase and turnover activities. The nature of inhibition was linear noncompetitive and template specific. These and other kinetic studies indicate that the binding of CTP to the enzyme at the triphosphate-binding site is responsible for its inhibitory action on exonuclease.

We have considered two orthogonal models to explain the above-mentioned findings: (*a*) the common-active-site model [polymerase and exonuclease have a common or at least a partially overlapping (mutually exclusive) active site], and (*b*) the independent-active-site model (polymerase and exonuclease activity have independent active sites). On examining the predictions of these two models, it was found that model (*b*) is more likely to be correct.

The K_ms for exonuclease and polymerase activities were found to differ by a factor of 5, which is untenable with the common-active-site model. As predicted by template specificity, CTP had no effect on the degradation of single-strand DNA by 3′→5′ exonuclease. Thus, the formation of the enzyme:primer–template:CTP ternary complex seems to be necessary for the inhibition of exonuclease. These findings and the linear noncompetitive nature of inhibition by CTP can be explained by model (*b*). Thus, the polymerase and exonuclease active sites seem to be independent of each other.

The common active site for polymerase and turnover activities is expected and supported by the facts that (*a*) K_ms for polymerase and turnover activities are the same; and (*b*) even during the initial processive phase of synthesis, turnover activity is operative since there is no lag in the kinetics of dNMP production.

These findings, therefore, indicate that polymerase and turnover activities have a common and independent active site from the 3′→5′ exonuclease active site. The physical location of turnover activity at the polymerase active site suggests that this activity is involved in surveillance against misincorporation. The role of 3′→5′ exonuclease might be in trimming frayed ends at the 3′-OH ends and in generation of recombinational intermediates, etc.

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MECHANISM OF T5-INDUCED DNA POLYMERASE—INITIAL BURST KINETICS

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

Bacteriophage T5 induces synthesis of a new DNA polymerase upon infection of its host *E. coli*. We have found that during *in vitro* DNA synthesis catalyzed by this enzyme the initial rate is high and later, in the course of reaction, falls to a constant value and remains unchanged for prolonged times. On extrapolation of the line of constant rate of synthesis to zero time, we get an initial burst. The magnitude of the burst is proportional to the amount of enzyme used, and its apparent size is about 120 to 200 nucleotides per enzyme molecule, depending upon the primer template.

The following trivial explanations of the phenomenon have been taken into consideration and subsequently eliminated. The burst might be due to (*a*) a heat-labile component present in our polymerase preparation which might be either a contaminating-enzyme activity or a conformational isomer of T5 polymerase, (*b*) a higher rate of labeled precursor polymerization in the early phases of the reaction, (*c*) isotope discrimination, (*d*) an easily replicable region in the template (for example, a gap), and (*e*) substrate exhaustion.

Therefore, we believe that initial burst is an intrinsic property of the enzyme. Our experimental results suggest that after synthesizing a stretch of DNA, processively on the template, the enzyme goes into a "dead-end complex" and must dissociate from the primer template to resume synthesis. The slower rate of synthesis in the later phases can be explained if the dissociation step is rate limiting.

We know nothing about the nature of the process that leads to the formation of the "dead-end complex." This point is being investigated in detail.

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EFFECT OF SPERMIDINE ON T5-INDUCED POLYMERASE AND ITS ASSOCIATED 3′→5′ EXONUCLEASE AND TURNOVER ACTIVITIES

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

Spermidine has been found to stimulate/inhibit a number of DNA and RNA polymerases. However, the mechanism of its action on polymerases is poorly understood. We have, therefore, studied in detail the effect of spermidine on T5-induced DNA polymerase and other known enzymatic activities associated with it.

Spermidine at a concentration of 6.6 m*M* stimulates T5 DNA polymerase 4.5-fold with denatured calf thymus DNA as a primer template when saturating amounts of primer template, 4 dNTPs, and Mg^{2+} are used. A comparison of the kinetics of synthesis in the presence and absence of spermidine showed that there was no effect on the size of the initial burst; however, the rate of later synthesis is stimulated considerably. The size of the processively made product was similar in the presence and absence of spermidine.

Kinetic analysis showed that the K_m of the enzyme for DNA increased in the presence of spermidine, which indicates a lowering of apparent affinity for DNA. Apparent enthalpy of enzyme–DNA complex formation, obtained kinetically, showed no significant differences in the presence and absence of spermidine, which indicates that the lowering of affinity is due to an entropic contribution made by spermidine.

Thermodynamic studies showed that enhancement of the rate of later synthesis is not due to a lowering of Arrhenius energy of activation, which was found to be about 23 ± 2 kcal/mol in both cases. Thus, the rate of enhancement is due to a contribution to the preexponential factor in the Arrhenius equation. As was stated before, K_m was raised due to an entropic contribution made by spermidine. This indicates that the rate of enhancement might also be due to an entropic contribution which is buried in the preexponential factor in the Arrhenius equation.

We have stated before[1] that the dissociation of the enzyme–DNA dead-end complex is the rate-limiting step during the later phases of the reaction. Half-life measurement of this complex in the absence and presence of various amounts of spermidine showed that various-fold stimulations in the later rate and corresponding reductions in the half-life of the complex agree reasonably well. Thus, the rate of enhancement by spermidine seems to be due to a lowered half-life of the enzyme–DNA dead-end complex.

3′→5′ exonuclease was inhibited by spermidine. The nature of the inhibition was competitive, that is, an increase in K_m, as was observed for polymerase. The turnover activity was, however, not affected. This is yet another finding which indicates that the

turnover with 3′→5′ exonuclease activities might be different.

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TEMPERATURE-SENSITIVE DNA POLYMERASE INDUCED BY A BACTERIOPHAGE T5 MUTANT: RELATIONSHIP BETWEEN POLYMERASE AND EXONUCLEASE ACTIVITIES

R. K. Fujimura and B. C. Roop

DNA polymerase induced by bacteriophage T5*ts*53, a mutant with temperature-sensitive polymerase, was purified to about 95%, as judged by dodecyl sulfate gel electrophoresis. The 3′→5′ exonuclease associated with the polymerase had higher activity than that associated with the parent wild-type enzyme. The exonuclease was more stable to heat than polymerase, and it also degraded primer template, even in the presence of 4 dNTPs at higher temperature. However, our experimental results showed that the inhibition of DNA synthesis by higher temperature was primarily due to defects in polymerase function rather than to overactive exonuclease. The presence of primer-template DNA stabilized the polymerase to heat. Purified *ts*53 polymerase was also shown to discriminate against incorporation of BrdUMP, especially at higher temperature. This is in agreement with observations made *in vivo* with *ts*53-infected bacteria.[1]

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INITIATION OF DNA REPLICATION USING PURIFIED T5 DNA POLYMERASE

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

Purified T5 DNA polymerase without any additional proteins will initiate replication as long as there are primers with 3′-OH ends present and the primers are hybridized to longer DNA, which serves as a template. The polymerase is capable of displacing a preexisting strand from the template as the primer strand is elongated; thus, DNA with single-strand breaks will also serve as primer templates.[1] However, intact linear-duplex or circular-duplex DNA will not serve as primer template for purified T5 DNA polymerase, as is the case with all other known DNA polymerases. Replicating T5 DNA isolated from cells at the early stages of infection consists of "eyes," indicating that replication starts at the internal points of linear duplex without any apparent preexisting primers.[2] Thus, apparently, protein(s) other than DNA polymerase may be required for initiation.

Attempts were made to initiate replication from duplex DNA (linear or circular) and single-strand circular DNA *in vitro* with crude extracts from various mutants or with purified DNA polymerase and protein factors, with the following criteria as signs of the occurrence of initiation:

(*a*) Currently, most popular models suggest that RNA is required as a primer for initiation of DNA replication. If this were so, four rNTPs would be required. Thus far, all the crude extracts and purified protein factors from T5-infected cells that stimulate DNA synthesis did not require four rNTPs. One of the factors studied required ATP for synthesis with M13 DNA.

(*b*) If the replication is initiated by *in vivo* mechanisms, one should be able to observe "eye" formation in linear-duplex or circular-duplex DNA. None has been observed during T5 DNA replication *in vitro*; all the systems studied to date, using crude extracts or purified factors, appear to initiate synthesis by single-strand break formation followed by strand displacement. However, the number of these breaks per DNA molecule that causes strand displacement accompanied by DNA synthesis is very small. Thus, these could be caused by specific endonuclease(s) involved in initiation. A recently published paper suggests that there are at least four such endonucleases in T5-infected cells.[3]

On the basis of these approaches, we conclude that it is possible that an RNA primer is not required for initiation of T5 DNA replication, but some protein factors are definitely required.

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COMPLEMENTATION *IN VITRO* OF AMBER MUTANTS OF T5 DEFECTIVE IN DNA SYNTHESIS

R. K. Fujimura, B. C. Roop, and P. N. Bryan*

Crude extracts from $T5^+$-infected cells efficiently support DNA synthesis using T7 and T5 DNA (linear duplex), PM2 DNA (circular duplex), or M13 DNA (single-strand circle); however, extracts from non-infected *E. coli* pol A^- endo do not support synthesis. The only crude extracts from phage-infected cells that do not support DNA synthesis are those from cells infected with T5 DNA polymerase mutants (D7, D8, and D9) and D4 mutants (defect in DNA synthesis unknown). D7-, D8-, and D9-infected cell extracts will support DNA synthesis with all of the above-mentioned DNAs if the purified T5 DNA polymerase is added to the system. Noninfected cell extracts do not support the synthesis even if the purified T5 DNA polymerase is added. Thus, viral-induced proteins other than polymerase are required for synthesis with the above-mentioned DNAs. D4-infected cell extract appears to have an inhibitor of synthesis if the above-mentioned DNAs are used, but will support the synthesis with denatured DNA. Other DNA-synthesis mutants tested support synthesis in crude extracts. We have purified a factor that will stimulate DNA synthesis with PM2 DNA, T5 DNA, or M13 DNA just as efficiently as the $T5^+$ crude extracts, but its significance is still uncertain.

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STUDIES ON DNA REPLICATION WITH *cdc* MUTANTS OF *SACCHAROMYCES CEREVISIAE*

R. K. Fujimura

Some preliminary work was carried out with *cdc* mutants of yeast that are defective in initiation of DNA replication at high temperature. Nuclear fractions were isolated from *cdc* 4 cells grown at 25 and 37° C, and DNA synthesis *in vitro* was compared. The fraction from cells grown at 37° was inactive in DNA synthesis, whereas the fraction from cells grown at 25° was active. However, when nuclei were disrupted, both extracts from 25° and 37° were capable of synthesis at 37°. The following possible explanations can be considered.

(*a*) *cdc* cells at 37° have chromosomes in an inactive state of replication. This inactive state is due to the attachment of repressor proteins at initiation sites. *cdc* 4 cells have temperature-sensitive anti-repressor proteins. Disruption of the nuclei results in the following two possible phenomena: (1) the repressor proteins dissociated from initiation sites, making chromosomes active primer template; and (2) the fragmentation of chromosomes occurred and created 3'-OH ends, thus creating active primer templates.

(*b*) The nuclear membrane of 37°-grown cells is less permeable to precursor and DNA polymerase than 25°-grown cells.

To solve this problem, chromatin has to be extracted in an inactive state, and repressor and antirepressor proteins, if present, need to be isolated. Various techniques are being tested for protein, DNA, chromatin, and nuclei extraction and for protein fractionation.

NUCLEOSIDES IN MOUSE URINE

M. Uziel, L. H. Smith, and A. J. Bandy

The last stages of biosynthesis or maturation of RNA involve modification of the nucleoside residues. Most of these modified nucleosides are excreted intact or in slightly modified form, so they can be considered as characteristic markers of RNA catabolism and turnover. Thus, the appearance (disappearance) of a population of molecules may show a characteristic change in excreted nucleosides. To more completely evaluate the observed changes in RNA modification during thymic lymphoma generation, we have examined the excreted nucleosides in normal age control animals (20) and a group of animals which had a 50% incidence of tumors (10). The nucleoside affinity column was used to isolate the nucleosides.

The observed nucleosides in tumor-bearing animals can be divided into two groups, the known nucleosides found in normal urine and a group not present (not detectable) in normal urine. This latter group may prove to be most valuable as biomarkers for the precancerous state or measures of tumor mass. Significantly lower levels of excretion were observed in uridine (Urd), xanthosine (Xao), and adenosine; no change was observed in pseudouridine (ψrd), ac^4C, m^1G, and m^3C excretion; moderate increases were observed in t^6A, m^1A (m^1I), G, m^2_2G, and m^7G; and seven new peaks were observed.

Urine from a single animal with a known tumor that died a day later showed increases in three of the seven new peaks; a large decrease in ψ; and larger increases in Urd, ac^4C, G, A, C, m^3C, and m^1A. These excretion patterns do not fully correspond to the observed changes in tumor RNA. Thus, one cannot predict the urinary nucleoside-excretion levels from RNA composition differences. Since the corresponding modified bases have not yet been analyzed, the question of whether increased nucleoside (base) excretion depends only upon the increased cellular mass due to the tumor is not yet answered.

RNA METABOLISM AND THYMIC LYMPHOMA

M. Uziel, L. H. Smith, and A. J. Bandy

The X-irradiation-induced thymic lymphoma in C57BL/6 mice is characterized by a high incidence (50–70%) and a large tumor mass (5- to 20-fold increase). The biochemical properties observed by H. Kaplan include viral propagation and a transient increase in total RNA per cell in lymphomatous animals.

We have investigated the metabolism of the low-molecular-weight RNA (4–6 S RNA) with emphasis on the modified nucleoside composition. These derivatives are ubiquitous and characteristic in various types of eukaryotic RNA. We have observed a significant increase in amount of tumor 4–6 S RNA per gram of tissue, compared to normal, but there is not a corresponding increase in the amounts of modified nucleosides. Instead, there is no significant increase in any of the 24 nucleosides measured. Significant decreases were observed in the amounts of m^1A, m^3C, m^7G, m^5C, mC, Cm, Gm, m_2^2G, I, and $4(abu)^3U$. It is of interest to note that all these compounds, except I, require methionine for their synthesis.

This distribution of changes coupled with the increased levels of small RNA in tumor cells can result from several mechanisms: Either a new or expanded population of small RNA is being synthesized, which has a lower level of methylation, or the methylating system cannot maximally modify the RNA molecules as they are synthesized. In either case, the hypothesis proposed by others that tumor formation is accompanied by hypermethylation of RNA does not apply to the thymic lymphoma.

3-METHYLCYTIDINE: A NORMAL CONSTITUENT OF HUMAN AND MOUSE URINE

M. Uziel, L. H. Smith, and S. A. Taylor*

We have for the first time identified m^3C as a normal component of human and mouse urine. *Cis*-diols present in the pooled urine samples were selectively adsorbed to an affinity column containing immobilized boronate, and the recovered nucleosides were separated by high-resolution liquid chromatography and quantitated spectrophotometrically. Peaks containing 0.001 absorbance unit (*ca* 100 pmol) can be detected and quantitated. The concentration of nucleosides in urine is normally in the range of 0.2 to 550 nmol/ml, so 0.2 ml of mouse urine or 0.5 ml of human urine is sufficient for the analyses.

The m^3C isolated from 30 ml of human urine was characterized by cochromatography with known m^3C and by ultraviolet (UV) spectral analysis. The same procedures have led us to tentatively identify ac^4C as a normal constituent of human and mouse urine and to the tentative identification of m^7G as a normal nucleoside excretion product in mouse urine.

The identification of the remaining nucleoside peaks is continuing.

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RAPID AND SENSITIVE ANALYSIS OF URINARY NUCLEOSIDES*

M. Uziel, B. F. Salter,† and A. Solomon‡

A major problem in cancer therapy is the measurement of tumor mass. It has been observed that many cancer patients excrete increased quantities of the modified nucleosides and bases. Because of our success in analysis of mouse tumor urines using the combination of nucleoside affinity to boronate gels[1] and rapid, sensitive chromatographic analysis, we made the effort to assess whether these techniques would be applicable to human urine analysis.

We examined 15 normal and 23 cancer patients (11 types) and in the latter found that two-thirds showed increased levels of ψrd. This is about the same

proportion observed in other larger studies. In the normals we found that the excretion ratio of ψrd to creatinine was quite precise. The value was 200 ± 16 nmol ψrd/mg creatinine (male and female). This calculated to 68 mg pseudouridine excreted per day, which is in agreement with published estimates of 40 to 80 mg per day.

The properties of the boronate affinity gel were found to depend upon the commercially available precursors. After examining several preparations, it was found that the 200–400 mesh P-2 (Bio-Rad) provided the best support for nucleoside binding.

In collaboration with A. Solomon, analysis of tumor urines is continuing in order to assess the value of nucleoside analysis as a measure of tumor mass.

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*Research supported jointly by the Environmental Protection Agency and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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COMPARATIVE METABOLISM OF MODIFIED NUCLEOSIDES

M. Uziel, J. R. Cornelius,* and J. J. Keyes †

The observed increases in urinary excretion of nucleosides in tumor-bearing animals may be the result of one or more biochemical mechanisms that either increase synthesis or prevent further catabolism of each modified nucleoside. Two nucleosides that are characteristically found in transfer RNA (tRNA) and one nucleoside found in both tRNA and messenger RNA (mRNA) have been observed to show increased excretion (*ca* 100%) in the urine of a tumor-bearing mouse.

To test whether the increase was due to altered cellular excretion properties, the individual nucleosides were incubated at 37°C in phosphate-buffered saline containing freshly isolated or frozen, normal or tumor, thymocytes.

Significant hydrolysis occurs only when m^7G is the substrate and the free base 7-methylguanine is the only product. On a per cell basis, the tumor thymocytes degrade m^7G faster than the normal. There is no accumulation of radioactive m^7G within the cells. One can conclude that nucleoside stabilization is not the mechanism responsible for increased urinary excretion of m^7G.

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AFFINITY CHROMATOGRAPHY OF NUCLEOSIDES

M. Uziel, S. A. Taylor,* and A. J. Bandy

The boronate gel we had previously synthesized[1] was found to be extremely efficient in the binding of nucleosides. The binding is both pH- and salt-dependent. Maximal binding of adenosine under optimal conditions was found to be 0.26 *M* with a gel that was 0.5 *M* in boronate groups. This is almost tenfold greater than other boronate gel preparations.

There was difficulty in reproducing the binding efficiency with different batches of the commercial starting materials. This has been resolved by using as a starting support a 200–400 mesh particle of the poly(acrylylhydrazide) (Bio-Rad P2H). This preparation is about 0.66 *M* in boronate at pH 8.8 and retains all free diol-containing nucleosides, including ψrd.

This nucleoside affinity support has been used to isolate nucleosides from urine. This technique promises to be very productive in the quantitative analysis of nucleosides in urine and the application of these data to monitoring tumor mass in human cancer patients.

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

R. S. Foote* and M. P. Stulberg

Characterization of the *in vitro* protein-synthesis system from ascites tumor cells has been described previously.[1]

The first component to be tested with the system is tRNA from aged C57BL mice. Transfer RNAs from liver, heart, spleen, and kidney of young and aged mice have been isolated, purified, and stored at −80° C. These preparations are being tested for possible abnormalities in fidelity of specific protein synthesis as directed by a homogeneous mRNA obtained from encephalomyocarditis virus.

The main problem encountered lately is the difficulty of routinely preparing a system that is completely dependent on exogenously supplied tRNA. We have circumvented this problem by preparing separate fractions of the soluble and ribosomal fractions of the cells and subjecting the soluble portion to DEAE absorption to eliminate endogenous tRNA. We now can obtain at least a 20-fold stimulation of protein synthesis by addition of exogenous tRNA. In addition we have obtained the necessary amount of net synthesis needed to examine the specific protein product for fidelity by discontinuous slab-gel electrophoresis.

Our initial experiments have shown no difference in the rate or extent of protein synthesis displayed by tRNA from old and young mouse livers. Experiments on fidelity of synthesis, as well as an examination of tRNAs from other organs, are being conducted at this time.

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*Postdoctoral investigator supported by PHS fellowship AG 05054-01.

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THE TRANSLATIONAL ACTIVITY OF TUMOR RNAs

M. P. Stulberg, K. R. Isham, D. Roop,*
L. C. Waters, and B. C. Mullin†

This is a collaborative project between our laboratory and other investigators who are interested in the mechanism of tumorigenesis via transcription and translation of tumor RNAs. For the most part, they supply us with isolated and purified RNAs and we examine the control and regulation of translation of these messengers in our *in vitro* protein-synthesis system from ascites tumor cells.

After a comprehensive optimization of reaction conditions, a net translation of MOPC tumor mRNA was realized. This amount of translation was at least 8 times that reported in the literature; however, it was almost completely dependent on added stimulatory factor(s) present in the polysomal KCl wash from reticulocytes.[1] We are now identifying the specific products of translation to determine the authenticity of the messenger utilized.

The translation of AKR leukemia virus RNA and AMV viral RNA has not exceeded amounts noted in the literature. However, we have not as yet succeeded in optimizing reaction conditions. It is notable that in our hands all different mRNAs require different optimal ionic conditions, indicating that structure in solution may be important in the control of translation.

In all translations described, our ultimate goal is to obtain optimal synthesis of protein products and to ascertain the presence or absence of control factors effecting the propagation of the viral or tumor proteins.

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†Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

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TRANSLATIONAL CONTROL OF PROTEIN SYNTHESIS BY FACTORS PRESENT IN THE KCl WASH OF RETICULOCYTE POLYSOMES

M. P. Stulberg, R. S. Foote,* and K. R. Isham

We have tested the mRNA activity of numerous RNAs in our mRNA-depleted *in vitro* protein-synthesis system[1] to help establish intratissue compatibility of tested components.

Heterologous RNAs isolated from rabbit reticulocytes, myeloma tumor, AKR murine leukemia virus, and avian myoblastosis virus can be translated only in the presence of one or more factors present in the 0.5 *M* KCl wash of reticulocyte polysomes. In contrast, homologous RNAs from encephalomyocarditis virus and ascites tumor cells are markedly inhibited by the same factor(s). We are attempting to confirm this differential effect by isolating poly A mRNA from ascites cells as a more direct test of the inhibition of homologous mRNA translation.

Although this general phenomenon of differential translational control has been observed repeatedly, it is difficult, if not impossible, to interpret without

further fractionation and purification of the responsible factor(s) in the KCl wash preparation. This is presently being done, and isolated fractions will be compared with purified initiation factors known to be present in this preparation. However, preliminary experiments indicate that our inhibition effect is most likely independent of the initiation of translation and not similar to inhibitors characterized by other investigators.

Preliminary results of these investigations have been reported.[2]

*Postdoctoral investigator supported by PHS fellowship AG 05054-01.

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PURIFICATION OF tRNA(URACIL-5)-METHYLTRANSFERASE FROM *ESCHERICHIA COLI*

L. R. Shugart and B. H. Chastain

The enzyme tRNA(Uracil-5)-methyltransferase (EC 2.1.1.35) has proved refractory to isolation utilizing several different methods of purification;[1,2] however, the separation of the methylase from the enzyme *S*-adenosylhomocysteine nucleosidase can be accomplished by hydroxylapatite column chromatography without loss of either enzyme activity. Thus, the copurification of these activities through several steps appears to be fortuitous.[2] Nevertheless, subsequent to this step, the methylase activity can be stimulated by the addition of the hydroxylapatite column fraction containing the nucleosidase. Investigations are under way to determine whether or not this is a real observation or some artifact produced under the conditions in which the methylase activity is measured.

The methylase activity as isolated from the organism is complexed with nucleic acids, and it has proved extremely difficult to recover the enzyme protein free of these nucleic acids without a concomitant loss of enzyme activity.[3] These data suggest an enzyme stabilized by association with tRNA, and future purification of the methylase will be conducted with this property in mind.

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S-ADENOSYLHOMOCYSTEINE NUCLEOSIDASE FROM *ESCHERICHIA COLI*

L. R. Shugart

S-adenosylhomocysteine nucleosidase has been purified more than 200-fold. The enzyme at this stage of purification is stable and has essentially the properties reported by Duerre,[1] and, interestingly, *S*-adenosylhomocysteine can be released from it upon treatment with acid.

As noted previously,[2] this enzyme metabolizes an important intermediate produced during the transmethylation of cellular macromolecules. Currently, *in vitro* kinetic studies using 17 analogues of *S*-adenosylhomocysteine (10 modified in the adenine moiety and 7 modified in the homocysteine portion) are being conducted in an attempt to determine the interaction of the enzyme with its substrate.

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PROTEIN-NUCLEIC ACID INTERACTION

L. R. Shugart and B. H. Chastain

Transfer RNA can be an important model compound for studying the interaction of proteins with nucleic acids.[1,2] Recently we have focused our investigations on the involvement of minor nucleosides, in particular the methylated analogues, in the functional role of tRNA,[3,4] and currently we are extending these investigations to include several other undermodified species of tRNA isolated from the *rel*$^-$ mutant of *E. coli*.

Preliminary data indicate that methyl-deficient $tRNA^{fMet}$ can be aminoacylated and formylated to the same extent as the normal species. These studies will be extended to the estimation of the rates of reactions as reported for $tRNA^{Phe}$ from the same organism.[3,4]

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RNA SYNTHESIS AND PROCESSING AS A MEASURE OF PHENOTYPIC VARIABILITY IN CYTODIFFERENTIATION AND NEOPLASIA

J. W. Bynum,* J. D. Regan, and E. Volkin

To assess the interrelationship between cytodifferentiation and cell proliferation we utilized cell lines of different differentiated states and proliferative rates. The plasmacytomas, RPMI-8226 and MOPC-31C, are of the same differentiated state (phenotype) and they have the same generation time, but they originate from different species (genotype). The mouse neuroblastoma cell line exhibits phenotypic variability in culture; it can be grown in shaker culture (undifferentiated) or as a monolayer (differentiated). HeLa (HCAAT) is a clone selected for its ability to grow in suspension culture and has the fastest doubling time of all these cell lines. Our data suggest that RNA synthesis and processing are manifestations of the entire phenomenon of phenotypic variability, not of cell proliferation alone. Each cell type has its own unique set of RNA synthesizing and processing patterns that correlate with observed metabolic attributes related to the extent of cytodifferentiation and neoplasia. Mouse neuroblastoma cells of the same genotype but different differentiated states have different ribosomal RNA precursor processing patterns, while plasmacytoma cells of different genotypes but the same differentiated state have the same preribosomal RNA processing pattern. In addition, our observations indicate that chromatin-associated RNA is involved in cytodifferentiation and is closely related to phenotypic variability. When neuroblastoma cells are induced to differentiate, there is a two- to threefold increase in the labeling of chromatin-associated RNA. Both of the differentiated cell lines, human myeloma and mouse neuroblastoma, have slow-labeling, stable chromatin-associated RNA, while this same fraction from HeLa cells is labeled rapidly and is unstable.

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INHIBITION OF 18-S RIBOSOMAL RNA ACCUMULATION IN HUMAN MYELOMA CELLS BY EXOGENOUS ADENOSINE

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

The deleterious effect of exogenous adenosine (Ado) on mammalian cells in culture has been explained by (*a*) the inhibition of *de novo* pyrimidine biosynthesis or (*b*) nucleolar decomposition through the dissociation of nucleolar fibrillar and granular components. Human myeloma cells (RPMI-8226) that were pulsed for 1 hr with [^{3}H]uridine ([^{3}H]Urd) and chased for 6 hr in fresh medium containing 5 μg/ml of Ado were deficient in newly synthesized 18-S ribosomal RNA (rRNA). Cells chased in 5 μg/ml of exogenous Urd did not have this deficiency. Myeloma cells labeled with [^{3}H]Urd and [^{3}H]Ado simultaneously and chased for 6 hr with 5 μg/ml each of Ado and Urd were also deficient in labeled 18-S rRNA. Thus, the effect of Ado on pre-rRNA processing is not a labeling artifact nor is it alleviated by an equimolar concentration of Urd. There was no accumulation of pre-rRNA, yet there was a selective decrease in the accumulation of labeled 18-S rRNA. HeLa cells cultured under the same conditions exhibited a twofold increase in the labeling of 4-S RNA. Examination of the pyrimidine pools by high-pressure chromatography showed that these results could not be explained by pyrimidine starvation.

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DETECTION OF NUCLEOTIDE SUGARS IN THE ACID-SOLUBLE FRACTIONS FROM MYELOMA CELLS TREATED WITH [^{3}H]URIDINE

E. Volkin and J. X. Khym

Human myeloma cells that were pulse-labeled with [^{3}H]Urd for various periods gave rise to the expected [^{3}H]uracil ([^{3}H]Ura) nucleotides when the acid-soluble constituents of these cells were analyzed by our ion-exchange procedures. However, in addition to the expected [^{3}H]Ura nucleotides, several unidentified radioactive peaks were also observed to be present in large amount. Three of these latter peaks have now been tentatively identified as UDP-*N*-

acetylglucosamine (UDP-NAG), UDP-galactose (UDP-Gal), and UDP-glucose (UDP-Glc).

By modification of our ion-exchange procedures, these nucleotide sugars can also be detected and assayed directly by optical density in the presence of the overwhelming amounts of nucleotides that are present in the myeloma pool fractions. In the first part of our chromatograms a typical sequence, with analytical resolution, is CMP, UMP, AMP, UDP-NAG, UDP-Gal, UDP-Glc, CDP, and GMP. The resolution between some of the adjacent peaks is completely lost upon small changes of pH. The separation is best carried out, isocratically, with citrate buffer, pH 8.5.

In continuing studies, we hope to better define the role of nucleotides, sugar nucleotides, and related pool constituents in the synthesis and turnover of RNA and DNA molecules and membrane-coupled polysaccharides.

RAPID RESOLUTION OF 5'-MONO-, DI-, AND TRIPHOSPHATE RIBO- AND DEOXYRIBONUCLEOSIDE MIXTURES BY CONVENTIONAL ANION-EXCHANGE CHROMATOGRAPHY

J. X. Khym

Several different chromatographic methods are now available for the rapid separation of complex mixtures of either ribo- or deoxyribomononucleoside phosphates, but only limited success has been achieved in separating mixtures that contain all members of each class of nucleotide. However, by refinements of the conventional ion-exchange method[1] used in this laboratory to separate nucleotides, the resolution of mixtures that contain ribo- and deoxyribo-5'-nucleotides at the mono-, di-, and triphosphate levels, or any combination of these, has been accomplished. The separations are carried out on columns (0.62 × 20 cm) of Aminex A-28 with alkaline citrate solutions as the eluent. Initially, the eluent is held at a fixed dilute citrate concentration (25 m*M*, pH 8.2); this elution mode effects mainly the separation of ribo- and deoxyribonucleoside monophosphates. Nucleotides of each class at the di- and triphosphate levels are resolved with the use of a citrate gradient (25 to 500 m*M*, pH 8.6).

Under study now is the application of a compound gradient to replace the simple elution mode that is now being used. The use of a shallow gradient (both in pH and citrate concentration) at the beginning, increasing slightly in the middle, and finally increasing strongly at the end of the chromatogram should improve the resolution of those nucleotide pairs that are closely eluted.

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5'-NUCLEOTIDASE OF HeLa CELLS

E. T. Brake, P. C. Will,* and J. S. Cook

The isolation of plasma membranes from tissue-culture cells by virtually any method yields a preparation containing 5 to 15% of the total cell protein. Criteria of purity, especially with regard to loosely associated (peripheral) proteins, are not well established. For our work with logarithmically growing HeLa cells we use the plasma-membrane-bound 5'-nucleotidase as a marker; for use in quantitative experiments on induction and turnover of transport systems, we have more fully characterized this marker. The relevant properties are as follows.

(*a*) *Kinetic properties of the enzyme.*—Starting with purified membranes, we have developed an assay utilizing 5'-adenosine monophosphate (AMP) in 0.2% Triton X-100, determined the kinetic parameters with this substrate, and determined K_I's for the principal 5'-nucleotides. ATP and ADP are strongly inhibitory, 3'(2')-AMP is not. α,β-Methylene-adenosine-5'-diphosphate, a potent inhibitor, distinguishes 5'-nucleotidase from alkaline phosphatase; the latter enzyme has negligible activity under conditions of our assay. HeLa 5'-nucleotidase has a single optimum at pH 7.0 and a broad maximum activity between 35° and 50° C. It is stable to sonication and prolonged storage. It requires no added Mg^{2+} and is slowly inactivated by EDTA; the inactivation is not reversed by excess Mg^{2+}. The metal that is apparently a structural component of the enzyme has not been identified. Zn^{2+} is strongly inhibitory.

(*b*) *Cellular propertes of the enzyme.*—5'-Nucleotidase activity in broken cells is easily sedimentable. The cytosol is inhibitory, presumably because of endogenous nucleotides. In analytical sucrose gradients the activity codistributes with bound ouabain, indicating that more than 95% of the activity is to be found on the cell surface. The activity is independent of culture density until plateau phase. We conclude that under specified conditions the

activity of this enzyme is a reliable quantitative indicator for HeLa surface membranes.

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ENDOCYTIC MECHANISM FOR TURNOVER OF Na^+-K^+ ATPase IN HeLa CELLS

P. C. Will,* E. T. Brake, and J. S. Cook

The drug-release curve for the loss of [^{3}H]ouabain (a ligand for Na^+-K^+ ATPase on the cell surface) from pulse-labeled HeLa cells may be described as the sum of two decaying exponentials. The analysis of this curve includes a term for the simple dissociation of the drug from the cell surface and a term for turnover via internalization of the ouabain-binding site in the membrane; the latter process is faster and more significant for recovery of the cells from the effects of the drug.[1,2] The slowest and ultimately rate-limiting step is escape of drug from the cell interior.

With the use of inhibitors and cell-fractionation methods, we have quantitatively substantiated a number of predictions of this analysis. (*a*) The combination of NaN_3 (an inhibitor of ATP synthesis) and 2-deoxyglucose (an ATP sink in the hexokinase reaction) prevents translocation of the membrane binding site to the cell interior. Ouabain uptake is not a passive permeability phenomenon but an ATP-dependent process. (*b*) With internalization thus blocked, these inhibitors result in drug release by dissociation only, and this process is both first order and faster than drug release observed in actively metabolizing cells. (*c*) There are 8×10^5 to 10×10^5 binding sites on the surface of each cell. During steady-state turnover, 2×10^5 to 4×10^5 bound ouabains may be found inside the cell, but these binding sites are not exposed by gentle homogenization. Intracellular bound ouabain is easily sedimented (17,000 *g*, 12 min). These are the expected results if internalized binding sites are facing intravesicular spaces rather than being free in the cytosol. In preliminary experiments, intracellular binding sites have been unmasked by low concentrations of formaldehyde, which makes cellular membranes permeable to ouabain but does not inhibit ouabain binding. The results are consistent with an endocytic mechanism for the steady-state turnover of surface Na^+-K^+ ATPase.

*Postdoctoral investigator supported by subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville. Present address: Department of Anatomy, School of Medicine, Case Western University, Cleveland, Ohio 44106.

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ULTRAVIOLET-ENDONUCLEASE-SENSITIVE SITES IN HUMAN DNA FOLLOWING EXPOSURE TO AN ULTRAVIOLET-TYPE REPAIR-INDUCING CHEMICAL AGENT

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

Diarylcarbamate is a carbamate derivative which causes DNA damage in human cells. The subsequent excision-repair process exhibited bromodeoxyuridine (BrdUrd) photolysis with saturation kinetics for an average patch size of about 40 inserted nucleotide bases per repaired region in normal human skin fibroblasts. Defective repair of damage by this compound was observed in cells from a xeroderma pigmentosum (XP) complementation group A cell line which is known to be defective in dimer-excision repair following UV exposure.[1] Cells treated with 1′-acetoxysafrole show similar saturation kinetics but exhibit normal repair characteristics in the XP cells.

XP cells exposed to each compound were assayed for UV-endonuclease-sensitive sites.[2] Cells labeled with [^{3}H]thymidine were treated with 5×10^{-5} *M* concentrations of the given chemical for 90 min. Purified UV-endonuclease from a *Micrococcus luteus* preparation[3] was introduced into the cells following their exposure to an "osmotic shock" buffer solution and incubated for 5 min at 37°C. Cells were lysed on top of alkaline sucrose gradients, centrifuged, and analyzed for DNA weight-average molecular weights against cells not receiving UV-endonuclease (Fig. 27). Endonuclease-sensitive sites were observed in the cells exposed to diarylcarbamate but not in 1′-acetoxysafrole-treated cells. The assay appears to be very useful in distinguishing between DNA damage repaired by a UV-type mechanism as opposed to a gamma-ray or short repair system.

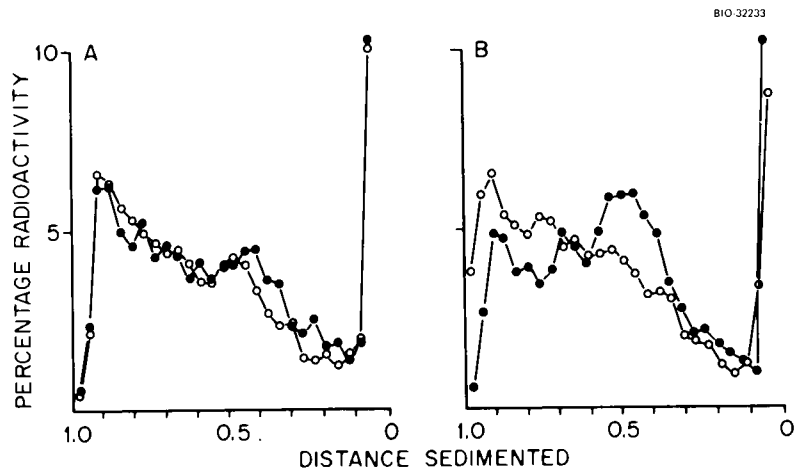

Fig. 27. Assay for UV-endonuclease-sensitive sites in the DNA of normal human cells following exposure to (A) 1'-acetoxysafrole and (B) diarylcarbamate [1,1-bis(phenyl)-2-propynyl-*N*-cyclohexylcarbamate] on alkaline sucrose gradients. (○), −UV-endonuclease; (●), +UV-endonuclease.

Studies for UV-endonuclease sensitivity are in progress using phenol-extracted DNA from exposed cells with different compounds known to induce both types of repair. These studies include quantitation of the number of sensitive sites.

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INHIBITION OF DNA REPAIR IN HUMAN CELLS BY CYTOSINE ARABINOSIDE: EFFECT ON NORMAL AND XERODERMA PIGMENTOSUM CELLS

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

Cytosine arabinoside (ara-C) inhibits UV-induced DNA repair in human cells by preventing closure of pyrimidine-dimer-excised regions when normal skin fibroblasts are exposed to 200 ergs of 254-nm radiation and allowed to repair for 20 hr in the presence of 5×10^{-5} *M* ara-C. Alkaline sucrose gradient profiles demonstrate a decrease in DNA molecular weight from that of cells receiving no ara-C (Table 35). Cells from complementation groups of XP that are defective in dimer excision show reduced numbers of DNA single-strand breaks per 10^8 daltons after UV and ara-C exposure as compared with normal cells (Table 35).

ara-C causes a 3 to 57% reduction in unscheduled DNA synthesis from 5×10^{-7} *M* to 10^{-5} *M* concentrations as well as significant incorporation of ara-C into the DNA. Repair inhibition observed on a dose-response curve (Fig. 28) suggests that a threshold level of ara-C incorporation is necessary to initiate inhibition of repair synthesis, followed by a gradual increase to total inhibition of repair as more ara-C is incorporated. In addition, incorporation of ara-C during repair replication supports the idea that the primary mechanism of action is competition with normal bases at the repair polymerase level, resulting in chain termination of repair synthesis.

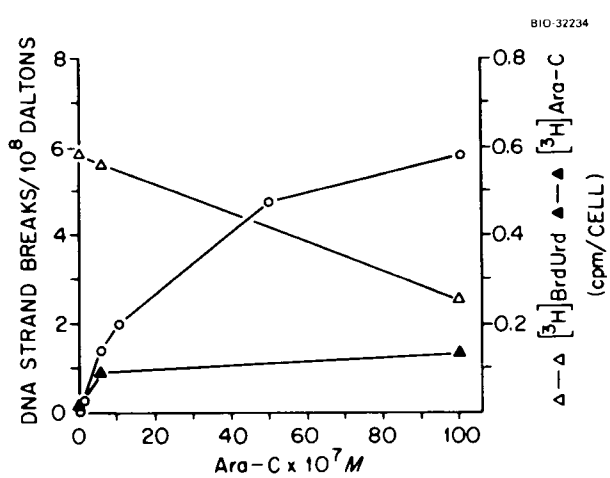

Fig. 28. Dose-response data for inhibition by ara-C of UV-induced DNA repair in normal human fibroblasts. UV dose = 200 ergs mm^{-2} of 254-nm radiation followed by a 20-hr repair period.

Table 35. Determination of DNA repair using inhibition by ara-C in normal and XP cells following 200 ergs/mm^2 of 254-nm radiation

Cell line	Description	Xeroderma complementation group	DNA single-strand breaks per 10^8 daltons[a]	Percent of normal repair[b]
HSBP	Normal skin fibroblasts	—	7.49	100.0
CRL 1223	XP	A	0.24	3.2
SGL	XP	A	0.32	4.3
CRL 1199 (PoCo)	XP	B	1.12	15.0
CRL 1158 (PeAr)	XP	C	0.54	7.2
CRL 1157 (CayWen)	XP	D	0.52	6.9
CRL 1259 (XP-2)	XP	E	4.60	61.4
CRL 1258 (PeHay)	XP	XP variant	11.92	159.1

[a]The number of DNA strand breaks was determined by taking the difference in reciprocal values of weight-average molecular weights of control and experimental DNA and multiplying times 2 $[2(1/M_w\ ^3H - 1/M_w\ ^{14}C)]$.

[b]An indirect measurement of dimer excision. Xeroderma groups A to E are defective in various degrees in their ability to excise pyrimidine dimers. The XP variant excises dimers normally and has normal repair replication.

DNA DAMAGE BY QUINOLINE DERIVATIVES AND THEIR INHIBITORY EFFECTS ON REPAIR OF ULTRAVIOLET RADIATION DAMAGE IN NORMAL HUMAN SKIN CELLS

W. C. Dunn, Jr., J. L. Epler, and J. D. Regan

Quinoline [benzo(*b*)pyridine] is a product of coal liquefaction as well as the base molecule of several antimalarial drugs and other therapeutic compounds. Using the BrdUrd photolysis assay for DNA repair, we examined several mutagenic quinoline derivatives for their ability to cause DNA damage as well as for DNA repair inhibition (Table 36).

Two of the compounds, quinoline-8-carboxylic acid and quinoline-8-sulfonic acid, induced unscheduled DNA synthesis at $10^{-4}M$ concentrations. Single-strand breaks per 10^8 daltons of 0.38 and

Table 36. DNA damage and UV-repair inhibition by quinoline derivatives in normal human skin cells

Compound	Dose[a] (M)	$\Delta(1/\text{mol wt}) \times 10^{8}$[b] Repair of compound damage[c]	UV repair[d]	Photodynamic effect
8-hydroxy-5-nitro-quinoline	10^{-4}	0.06	—	None
	10^{-5}	—	2.40	Mol wt reduction unequal in dThd- and BrdUrd-incubated cells[e]
8-nitroquinoline	10^{-4}	0.03	—	None
	10^{-4}	—	2.33	None
Quinoline-8-carboxylic acid	10^{-4}	0.19	—	None
	10^{-4}	—	4.10	None
Quinoline-6-carboxylic acid	10^{-4}	0.02	—	None
	10^{-4}	—	3.63	None
Quinoline-8-sulfonic acid	10^{-4}	0.13	—	None
	10^{-4}	—	4.98	Mol wt reduction equal in dThd- and BrdUrd-incubated cells
Quinoline-2-aldehyde	10^{-5}	0.03	—	None
	10^{-5}	—	4.01	None
Chloroquine	10^{-4}	0.03	—	None
	10^{-6}	—	4.87	None

[a]90 min for damage by compound, 20 hr for UV-repair inhibition by compound.

[b]$\Delta(1/\text{mol wt})$ is a measure of the magnitude of repair. A lower $\Delta(1/\text{mol wt})$ means less BrdUrd insertion, hence less repair.

[c]Greater $\Delta(1/\text{mol wt})$ indicates greater damage by compound.

[d]Following 200 ergs/mm^2 of 254-nm-radiation and 20 hr repair period in presence of compound.

[e]dThd, thymidine; BrdUrd, bromodeoxyuridine.

0.26 were observed for the respective compounds. None of the compounds tested inhibited the repair of UV-induced lesions. However, some photodynamic breakage on exposure to 313-nm light was observed with 8-hydroxy-5-nitroquinoline and quinoline-8-sulfonic acid, resulting in molecular weight reduction in the DNA of both UV-treated cells and untreated controls. Additional studies of mutagenic and carcinogenic compounds as DNA repair inducers or inhibitors, as well as their mechanisms of action, are forthcoming.

PHOTOSYNTHESIS

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

Joliot and Kok[1] have shown that oxygen production in photosynthesis involves four photochemical steps:

$$S_0 + h\nu \rightarrow S_1$$
$$S_1 + h\nu \rightarrow S_2$$
$$S_2 + h\nu \rightarrow S_3$$
$$S_3 + h\nu \rightarrow S_4$$
$$S_4 \rightarrow S_0 + O_2$$

The existence of the four steps is deduced from the waves in oxygen production per flash as a function of flash number when plants that have been relaxed in the dark for 5 min are illuminated with short bright flashes separated by 1 or 2 sec.

We have been trying to see if the four steps are different by measuring the volume changes at the time of the flash. We are using small pressure-sensitive piezoelectric cells, developed for underwater sound detection.

We find that the volume changes, for the first few microseconds, are the same for the first four flashes.

Since Joliot has shown that it takes 150 msec for the change of one S state to the next, we are now trying to measure the integrated volume change over the first half second after each flash.

*Consultant.

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STUDIES OF HIGH-METHIONINE-LOW-MOLECULAR-WEIGHT PROTEINS IN SOYBEANS

D. E. Foard, D. L. Hwang,* E. A. Beatty, W.-K. Yang, A. P. Pfuderer, and K. D. Lin†

Significant progress in isolating high-methionine proteins from soybean seeds occurred this year. This is an important step in our exploratory studies to determine the feasibility of genetically improving the methionine content of soybean protein. Five major peaks are separated on diethylaminoethyl- (DEAE-) cellulose from the peak with highest methionine obtained with Sephadex G-100. These five peaks have been purified and give single bands on polyacrylamide gels. Each acts as a single species in the analytical ultracentrifuge, and they give the following molecular weights: I, not determined; II, 7800; III, 7400; IV, 8300; V, 8600. The minimum molecular weight of I has been calculated from amino acid composition and from X-ray crystallographic observations[1] to be 6800. Other physical properties are as follows. Partial specific volumes: I, 0.703; II, 0.687; III, 0.689; IV, 0.685; V, 0.690. $E^{1\%}_{1\,cm,280}$: I, 3.66; II, 4.29; III, 5.29; IV, 4.99; V, 4.00. Each has an aspartic acid residue as its N terminal. The amino acid composition of these proteins is unusual compared with proteins in general in that half cystine values (determined as cysteic acid) range from about 13.5% to 17%; this phenomenon is, however, characteristic of legume trypsin inhibitors, which in fact all five prove to be. Each also has α-chymotrypsin inhibitory activities to variable extents. Inhibitor V is especially strong in this respect and appears from its inhibitory properties and amino acid composition to be identical with or very closely related to the well-known Bowman–Birk inhibitor (also known as inhibitor AA). The remaining four appear not to have been described previously. They are probably closely related to one another as inferred from amino acid composition and from "fingerprints" of tryptic digests. They are the richest proteins in methionine (determined as methionine sulfone) thus far described for soybean and contain the following percentages of the amino acid: I, 1.9; II, 5.0; III, 3.5; IV, 3.2; V, 1.5. These five proteins constitute about 6% of the total protein of the seed; trypsin inhibitors constitute an even greater percent-

age than this when the Kunitz inhibitor (molecular weight 21,500) is included in the calculation. (The Kunitz inhibitor is not a high-methionine protein.) Thus trypsin inhibitors probably play an important, although presently unknown, role in the seed. Basic studies of these proteins in the near future will be concentrated on the sequence of inhibitor II and immunological characteristics. Applied studies will continue to emphasize (*a*) genetic attempts to achieve soybeans having proteins with elevated methionine content and (*b*) development of an immunological assay to detect any such soybean among the large numbers of progeny involved.

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

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EVIDENCE THAT GUANOSINE 3′:5′-PHOSPHATE PREVENTS ADENOSINE 3′:5′-PHOSPHATE FROM ENTERING ULTRAVIOLET-IRRADIATED *ESCHERICHIA COLI* CELLS

P. A. Swenson and R. L. Schenley

When *E. coli* glucose-grown cultures are UV-irradiated with a fluence of 52 J/m^2 there is a transient cessation of respiration 60 min later. The cells that die are responsible for the transient low rate; the progressively faster rate thereafter is due to the fraction that survives and divides. Addition of adenosine 3′:5′-phosphate (cAMP) (5 m*M*) to irradiated cells causes additional cells to stop respiring, and cell survival decreases. Similar results are obtained with glycerol-grown cells given a lower fluence of UV (26 J/m^2). Our hypothesis is that UV derepresses an operon containing a gene for a protein that shuts off respiration and that the operon is subject to control by cAMP. However, guanosine 3′:5′-monophosphate (cGMP) (5m*M*) has no effect on maintenance of respiration and only a small effect on viability of UV-irradiated (52 J/m^2) cells grown on glycerol. Cyclic GMP does enter the irradiated cells and presumably binds to the cAMP receptor protein, as indicated by inhibition of the induced synthesis of β-galactosidase. We suggest that a different cAMP-binding protein is involved in the respiratory control operon and that cGMP does not bind to it. Although cGMP has no effect on respiration of UV-irradiated cells, it almost completely nullifies the effect of cAMP when the two are added together to irradiated cells. We suggest that cGMP competes with cAMP for a transport protein and thus prevents cAMP from entering the cell.

DELAYED ULTRAVIOLET-INDUCED CESSATION OF RESPIRATION BY INADEQUATE AERATION OF *ESCHERICHIA COLI* B/r

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

Inadequately aerated *E. coli* B/r cultures do not shut their respiration off 60 min after UV irradiation (52 J/m^2 at 254 nm) as they do when well supplied with oxygen. Since cessation of respiration is associated with cell death, this result suggests that oxygen toxicity by superoxide radicals generated by cell metabolism might be responsible for cell death. The specific activity of superoxide dismutase, which scavenges O_2^- radicals, increases twofold after 90 min of adequate aeration, but the specific activity of catalase remains constant. These results are the same for both a small- and large-scale method for preparing kilogram quantities of irradiated cells. Respiration and viability of irradiated cells are affected not at all by the presence of superoxide dismutase and only slightly by the presence of catalase. Metal ions such as Mn^{2+} and Fe^{2+}, inducers of superoxide dismutase, have no effect on respiration and viability. When irradiated cells are incubated under nitrogen for 90 min, the respiration, growth, and viability time-course responses are the same as for cells not exposed to anaerobiosis. We conclude that superoxide anions generated at the time of irradiation play no part in cessation of respiration and cell death and that inadequate aeration or anaerobiosis delays the UV-induced synthesis of proteins responsible for the irreversible cessation of respiration.

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THE ATP DEPENDENCE OF THE INCISION AND RESYNTHESIS STEPS OF EXCISION REPAIR

W. E. Masker

Excision repair of UV radiation damage in *E. coli* appears to involve alternate pathways and a number of enzymes with similar *in vitro* activities. In an effort to better understand the interplay of various en-

zymatic steps in repair pathways, we have made use of a quasi *in vitro* system which allows easy introduction of chemical factors required for the incision, resynthesis, and ligation steps of excision repair. The repair process in these toluene-treated cells has been well characterized and found, by a number of criteria, to closely resemble *in vivo* excision repair.[1] In the present study we succeeded in decoupling the steps of the excision-repair process and were able to examine repair resynthesis in toluene-treated cell preparations in which incision at the sites of pyrimidine dimers had already occurred. Our results confirmed that the incision step itself is ATP-dependent[2,3] and showed that in wild-type strains, repair synthesis, presumably carried out by DNA polymerase I, does not require additional ATP. However, in *pol*A strains (deficient in DNA polymerase I), repair resynthesis requires high concentrations of ATP even after incision takes place. This finding suggests that DNA polymerases II and III may carry out repair replication only as part of a complex that requires ATP for its formation.

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DNA REPAIR IN *ESCHERICHIA COLI* MUTANTS DEFICIENT IN EXONUCLEASES VI AND VII

W. E. Masker and J. W. Chase*

E. coli maintains at least three exonucleases capable of hydrolyzing single-strand DNA in the 5′-3′ direction. All of these—exonuclease VI (the 5′-3′ exonuclease associated with DNA polymerase I), exonuclease VII, and the 5′-3′ exonuclease associated with DNA polymerase III—are capable of the *in vitro* excision of thymine dimers introduced into DNA by UV irradiation.[1-3] Recently, mutants (*xse*A) deficient in exonuclease VII have been isolated.[4] We have combined the *xse*A mutation with mutants (*pol*A ex1, *pol*A ex2) temperature-sensitive for exonuclease VI[5] and have examined these strains for UV sensitivity, for postirradiation DNA degradation, and for ability to excise thymine dimers *in vivo*. The *xse*A mutants were found to have only slightly increased UV sensitivity. The exonuclease VI-deficient strains are somewhat UV-sensitive, but the double mutants *pol*A ex1 *xse*A and *pol*A ex2 *xse*A, which are deficient in both exonucleases VI and VII, are not significantly more UV-sensitive than the exonuclease VI mutants alone. The *pol*A ex1 and *pol*A ex2 mutants show serious postirradiation DNA degradation. A dose of 100 J/m^2 causes degradation of 60% of the DNA within 1 hr of incubation at 42°C. The double mutants, deficient in exonucleases VI and VIII, suffer similar DNA degradation.

A study of dimer excision in these strains showed that in spite of a 50-fold reduction in exonuclease VI activity, the *pol*A ex1 and *pol*A ex2 mutants are just as efficient as wild type at excising thymine dimers produced by up to 40 J/m^2 of UV irradiation. At higher doses, the exonuclease VI-deficient strains are less efficient than wild type at dimer excision, but the aforementioned DNA degradation makes it difficult to interpret dimer excision data for doses over 50 J/m^2. The *xse*A mutation, either alone or in combination with *pol*A ex1 or *pol*A ex2, has no measurable effect on dimer excision in the dose range 0 to 150 J/m^2.

The results obtained so far suggest that exonuclease VII does not play a major role in dimer excision *in vitro*. Residual exonuclease VI might be responsible for the efficient dimer excision measured in *pol*A ex1 strains exposed to up to 40 J/m^2 of UV; however, it is true that a major reduction in this enzyme causes no measurable effect on dimer excision. Thus, our results suggest that *E. coli* may maintain another nuclease responsible for dimer excision. Since the cell contains only a few molecules of DNA polymerase III,[6] the 5′-3′ exonuclease associated with this enzyme seems unsuited for dimer excision. However, a careful study of the involvement of DNA polymerase III in dimer excision must await isolation of mutants lacking the 5′-3′ exonuclease associated with this enzyme.

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ENZYMES THAT ACT ON ALTERED DNA

W. L. Carrier

It is a characteristic of most cells that they possess some capacity for repairing damage to DNA—the cellular structure most vulnerable to chemical and physical insults in the environment. One of the major repair systems acts before DNA replication by excising defects from the DNA of pro- and eukaryotic cells. Cells defective in this type of repair are more easily killed, mutated, and transformed by radiations and chemicals. The first step in excision repair is the action of an endonuclease that makes a single-strand break near the damage, and the quantitation of such breaks is a sensitive way to measure such endonuclease activity. Endonuclease activities from *Micrococcus luteus* act on UV-irradiated and on gamma-irradiated DNA as well as on DNA substituted with bromouracil (BrUra) and irradiated with UV. The latter activity acts on the uracil (Ura) residues that result from the photolytic debromination of BrUra and hence may easily be assayed by its ability to split extensively DNAs (such as that from PBS2) that naturally contain Ura. We are purifying these three activities and are investigating endonuclease activities to depurinated DNA and the abilities of DNA polymerase and polynucleotide ligase to rejoin the endonucleolytic scission.

PHOTOREVERSIBILITY OF ULTRAVIOLET-INDUCED PYRIMIDINE DIMERS IN HUMAN CELLS

W. L. Carrier and J. D. Regan

We have carried out experiments on the photoreversibility of UV-induced pyrimidine dimers in human cells. The experiments were begun after B. M. Sutherland (University of California, Irvine) reported that human cells are capable of the photoreversal of UV-induced pyrimidine dimers and possess photoreactivating enzyme activity. Sutherland also reported that XP cells (which do not excise dimers) have lowered levels of photoreactivating enzyme. We attempted to repeat these experiments but were not able to do so under our experimental conditions. In collaboration with B. M. Sutherland we have assayed the dimers in irradiated DNA to which she gave photoreactivating treatment; we have confirmed her result of fewer dimers remaining in the DNA of cells after this treatment. It now appears that there are growth-medium effects on the process of photorepair in human cells. We had grown the cells on a minimal medium (Eagle's), while Sutherland used an enriched medium (Dulbecco's). However, using a commercial preparation of Dulbecco's medium, we do not find photoreversal of dimers in human cells.

We are currently using modified growth media in an effort to determine the extent of this type of repair in human cells.

DAMAGE TO DNA FROM DECAY OF [^{3}H]- AND [^{14}C]THYMIDINE

R. J. Brake* and W. L. Carrier

Purified DNA from the bacteriophage PM2 was labeled with either [methyl-^{3}H]- or [2-^{14}C]thymidine and stored at 4°C in aqueous solutions (pH 7) containing a 0.1 *M* concentration of sodium phosphate, Tris-HCl, ethanol, or β-mercaptoethanol. The samples were monitored for 2 months for the accumulation of three types of damage from isotope decay: (*a*) strand breaks, (*b*) alkali-labile bonds, and (*c*) sites, presumably damaged bases, susceptible to the γ-endonuclease from *M. luteus*.[1]

In all solutions tested there were about equal numbers of enzyme sites and strand breaks and about one-fourth that many alkali-labile bonds. Compared with sodium phosphate, Tris and ethanol protected the DNA about three times as well against all types of damage and were indistinguishable in this effect. On the other hand, the rate of strand breakage in mercaptoethanol was increased at least twofold over that in phosphate, and the rates of formation of enzyme sites and alkali-labile bonds were even more enhanced.

Tris and ethanol are OH-radical scavengers, and thus the data might implicate this diffusible radical, rather than transmutation, in most DNA damage from decay of incorporated isotopes. Further studies are needed with mercaptoethanol to determine whether chemical damage from the compound or impurities, rather than radiation, account for the excessive damage to the DNA. In any case, Tris and ethanol are recommended as preferable to phosphate, and mercaptoethanol should not be used for storage of DNA.

Although the relative proportions of the three types of damage were not very different in ^{3}H- vs ^{14}C-labeled DNA, preliminary results on specific radioac-

tivity indicate that ^{14}C decay (tenfold more energetic than ^{3}H) causes four to eight times as much damage as ^{3}H decay when expressed per disintegration. Researchers who use labeled DNA should be aware that strand breaks account for less than half the damage caused by either ^{3}H or ^{14}C.

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EFFECTS OF RADICAL SCAVENGERS ON RADIATION DAMAGE TO DNA

R. J. Brake* and W. L. Carrier

Ionizing radiation causes damage to DNA that may be separated into at least three distinct classes: (*a*) strand breaks, (*b*) alkali-labile bonds, and (*c*) sites, presumably damaged bases, susceptible to the γ-endonuclease from *M. luteus*.[1] In dilute anoxic aqueous solution, radiation damage is almost entirely indirect, being mediated by the diffusible OH radical (OH·) and the aqueous electron (e_{aq}^-). We have tried to determine the nature of the damage produced by each radical by quantitating the three classes of damage to the supercoiled DNA from bacteriophage PM2 after irradiating in the presence of selective radical scavengers.

Potassium iodide and *t*-butanol, both scavengers of OH·, prevented at least 90% of the strand breaks and about 50% of the alkali-labile bonds but actually increased the yield of enzyme sites. Potassium nitrate, which quenches e_{aq}^-, has no effect on strand breakage but prevented about 50% of the alkali-labile bonds and 75% of the enzyme sites. Nitrous oxide, which converts e_{aq}^- to OH·, increased the yield of strand breaks but did not affect the alkali-labile bonds and decreased the yield of enzyme sites. Simultaneous addition of nitrous oxide and potassium iodide protected the DNA against all forms of damage, verifying that H· and direct effects play minor roles in our system.

These data are consistent with a model of radiation damage in which OH· causes mostly strand breaks but also a few alkali-labile bonds and enzyme sites, whereas e_{aq}^-, known to react exclusively with the DNA bases, causes mostly enzyme sites, a few alkali-labile bonds, and no strand breaks. The increase in enzyme sites upon scavenging OH· is unexplained, but we are investigating the possibility that biradical reactions, such as $OH\cdot + e_{aq}^- \rightarrow OH^-$, interfere with the course of enzyme site production.

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ANALYTICAL BIOCHEMISTRY OF DNA

R. O. Rahn, E. A. Hiss, and R. S. Stafford

We have continued to work on an assay for measuring the distribution of non-radioactive-labeled DNA present in an alkaline sucrose gradient. This problem arises for those special situations where incorporation of radioactive precursors into the nuclear DNA of cells is either not possible or undesirable. Two methods have been investigated: (*a*) postlabeling with ^{125}I and (*b*) fluorimetric analysis using ethidium bromide as a probe. A fundamental limitation we face is a maximum of 2×10^5 cells per 35 ml of gradient (*i. e.*, 1 μg of DNA) because of sedimentation artifacts at higher DNA concentrations. [There exists the possibility of avoiding this artifactual situation by pretreatment of the cells with phenol to remove some of the protein.]

Analysis requires precipitation of the DNA from the gradient fractions, regardless of the method of assay. A considerable effort has been made to quantitatively recover the DNA from the gradient without resorting to high concentrations of "carrier," which would interfere with the assay. A variety of carriers, precipitants, and sedimentation techniques have been employed. Currently, we are able to recover more than 90% of the DNA using as little as 2 μg polyriboadenylic acid (poly A) per milliliter of gradient fraction. Precipitation is with cold 5% perchloric acid followed by sedimentation at 3000 rpm for 60 min. The use of swinging-bucket rotors facilitates recovery of the DNA in semimicro volumes and allows large numbers of samples (over 100) to be spun simultaneously. Poly A interferes minimally with the analysis since ^{125}I reacts specifically with cytosine and very little with poly A, and the ethidium bromide binding to poly A is much weaker than to DNA. Furthermore, if, alternatively, the diaminobenzoic acid fluorescence assay is used (which measures deoxyribose), the ribose of poly A is nonreactive.

At the present time, labeling of DNA with ^{125}I results in 200 cpm/ng with a background of 500 to 2000 per sample, depending upon the amount of poly

A employed as carrier. Hence, 50 ng of DNA should give a signal over background ratio of about 10. With ethidium bromide, the assay is less sensitive; 50 ng of DNA has not more than a 2:1 signal-to-background ratio. However, it is felt that the reproducibility may be greater with the fluorescence assay, and hence, over all, the accuracy for the two methods may not be very different.

We have initiated several preliminary projects to facilitate automatic sampling of the fluorescence samples. In collaboration with Gary Salzman at Los Alamos, we have employed a high-speed flow system to measure the fluorescence of propidium iodide-stained DNA solutions pumped from sucrose gradients. This system employs a laser to stimulate the emission; hence the sample volume being excited can be kept very small. We have constructed a mixing chamber which allows for continuous neutralization of the DNA as it is pumped from alkaline sucrose gradients. Another effort is being made, in collaboration with John Mrochek of the Chemical Technology Division, to use the "centrifugal fast analyzer" operating in the fluorescence mode to measure "simultaneously" the intensity of up to 17 samples.

PHOTOCHEMISTRY OF ADENINE IN DNA AND MODEL SYSTEMS

R. O. Rahn, R. S. Stafford, W. E. Masker, R. K. Fujimura, F. J. Bollum,* and D. L. Williams †

We have previously demonstrated that the adenine photoproduct formed in poly dA and first reported by Pörschke is converted by acid hydrolysis into a product easily characterized on paper chromatograms. In order to determine whether this product is formed in DNA, synthetic DNA containing [^{14}C]adenosine was prepared using either T5-polymerase or T7-polymerase. At fluences sufficient to convert 20 to 25% of the adenine in poly dA into photoproduct, less than 2% of the adenine in the DNA appeared as photoproduct. Denaturation of the DNA prior to UV did not significantly alter these results. It is concluded that the rate of formation of the adenine photoproduct in UV-irradiated DNA is probably too small to be biologically significant, although the difference in behavior between single-strand DNA and poly dA is not understood. We have also obtained evidence that the so-called Pörschke photoproduct may, in fact, be a secondary photoproduct derived photolytically from a precursor. This evidence is based on the observation that the Pörschke adenine photoproduct located at R_f 0.29 on our chromatograms forms with an apparent lag, while a minor product located at R_f 0.21 has a faster initial rate of formation and then levels off. The radioactive dinucleotide p(d[^{14}C]A)$_2$ has been prepared in order to determine whether similar evidence for a precursor can be obtained using DEAE-cellulose chromatography, the method originally employed by Pörschke for isolating the photoproduct. The advantage of DEAE is that no hydrolysis is necessary in order to study the photoproducts derived from the dinucleotide. These experiments are in progress and should allow us to find the proper conditions for large-batch purification of the photoproducts.

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KINETIC FORMALDEHYDE ANALYSIS OF DNA ULTRAVIOLET-IRRADIATED IN THE PRESENCE OF SILVER IONS

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

DNA from *E. coli* was irradiated at 254 nm in the presence of silver in order to preferentially enhance the rate of formation of pyrimidine-dimer damage over nondimer damage. The irradiated DNA was treated with formaldehyde in order to measure the unwinding velocity of the defects associated with the pyrimidine dimers. This velocity was found to be 0.18 base pair per minute per pyrimidine dimer, which is nearly eight times less than that found for a double-strand break (1.37 base pairs per minute) obtained by use of sheared DNA whose size was determined by electron microscopy. The rate of reaction of the DNA with formaldehyde varied linearly with the pyrimidine dimer concentration and showed no inflection due to clustering. Treatment of irradiated DNA with UV-endonuclease enhanced the formaldehyde reaction by about sevenfold, consistent with the conversion of a dimer into the faster-reacting defect associated with a single-strand break. These results indicate that the distribution of dimers in DNA is random and not clustered and that previous interpretations of clustering by Lazurkin and coworkers were based on the false assumption that dimer and chain-break defects unwind with similar velocities when treated with formaldehyde.

ULTRASTRUCTURE OF CHROMATIN

A. L. Olins* and M. Hsie-Hsu†

We have visualized and helped to establish the existence of ν bodies (nucleosomes), the small histone–DNA particles which are the first order of DNA folding in chromatin. During the last year we have turned our attention to the high-resolution analysis of the ν-body substructure in chromatin spilling out of nuclei and in isolated monomer ν bodies (ν_1). We have analyzed and classified many micrographs and have proposed a model for the internal structure of the particles.[1] We are actively looking for methods which will improve our high-resolution images of ν bodies. We have made very good progress in this direction by improving our carbon film making, by altering our staining procedure, and by setting up dark-field illumination in the electron microscope.

Recently our attention has been turned toward new problems: the higher-order organization of ν bodies and high-resolution electron microscopy of transcription.

Nuclease-digested chromatin can be fractionated on sucrose gradients. The three slowest peaks correspond to monomers (ν_1), dimers (ν_2), and trimers (ν_3) respectively. The ultrastructure of these ν bodies is the same as that observed in ν bodies on long chromatin fibrils spilling out of freshly isolated nuclei. We have also studied a faster sedimenting fraction, which is a mixture of multimers ν_6 through ν_{10}. These structures are as sensitive to salt concentrations as whole chromatin and can be extended and compacted by altering the salt. We are looking for conditions which will give characteristic and reproducible compact structures which correspond to the 200- to 250-Å chromatin fibers. These small fragments avoid the tangling and overlapping of fibers which we encounter in some of the parallel studies with freshly isolated nuclei.

Our studies of the ultrastructure of the ν body at the binding site of RNA polymerase have led us to many interesting systems. In the process we have established the existence of ν bodies in chromatin from the following sources: chicken reticulocytes, mouse fetal liver, polyoma virus (gift of M. Hobish, The Johns Hopkins University), and "active" and "inactive" chromatin fractions isolated by E. Berkowitz of Harvard University. We have also come to appreciate the ultrastructural sensitivity of active chromatin to freezing and other preparative techniques. To preserve the ultrastructure of transcription complexes we must have fresh chromatin and avoid electron-beam damage. We are, therefore, continuing these studies on lampbrush chromosomes and growing SV40 minichromosomes in collaboration with George C. Lavelle.

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BIOPHYSICAL CHARACTERIZATION OF CHROMATIN ν BODIES

D. E. Olins, R. D. Carlson,* and E. B. Wright

The structure of eukaryotic chromosomes represents a hierarchy of macromolecular organization beginning at the level of DNA and ending at the chromatid. The first level of DNA folding is the spheroid chromatin ν body (or nucleosome), which results in a six- to sevenfold compacting of the DNA in association with a "core" of histone molecules.

In collaboration with J. P. Breillatt we have employed the K-VIII zonal ultracentrifuge to fractionate micrococcal nuclease digests of isolated chicken erythrocyte nuclei, and we have achieved the preparation of gram quantities of monomer ν bodies (ν_1).

A single digest fractionated in three separate zonal centrifuge runs yielded ν bodies that have been characterized hydrodynamically in several solvent systems. Intact chromatin and ν_1 show salt-dependent aggregation, and ν_1 was, therefore, characterized in a solvent (10 m*M* KCl, 0.2 m*M* EDTA, pH 7.0) where the particles are monodisperse. By analytical ultracentrifugation, we have obtained the data presented in Table 37.

In addition, the ν_1 preparations have been characterized in terms of DNA fragment length per ν body, histone molar ratios, solubility, electrophoretic mobility in polyacrylamide gels, and electron microscopy.[1]

It is interesting to note that the calculated diameters of equivalent anhydrous and hydrated

Table 37. Analytical ultracentrifugation of ν_1 in 10 m*M* KCl buffer

$S^{\circ}_{25,B}$	12.15×10^{-13} sec
$D^{\circ}_{25,B}$	3.91×10^{-7} cm^2 sec^{-1}
$\bar{v}$ (ml/g)	0.669
Mean molecular weight from S° and D°	232,000
Mean molecular weight from equilibrium sedimentation	236,000
Diameter of equivalent anhydrous sphere	79.4 Å
Diameter of equivalent hydrated sphere	112.4 Å

monomer particles are almost identical to the largest low-angle X-ray reflections observed for dry and for wet monomer ν bodies and for chromatin or nuclei. This comparison supports the suggestion made previously that the chromatin particles pack in a lattice, and that the drying of chromatin shrinks the particles while still keeping them close packed.

Current studies are being directed at the spectroscopic characterization of ν bodies and the exploration of alternative conformational states.

*NIGMS postdoctoral investigator (Fellowship GM 55427).

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X-RAY CRYSTALLOGRAPHIC STUDIES ON RICIN (TOXIN) V-3

J. R. Einstein and C. H. Wei

X-ray intensity data for native crystals of ricin V-3 in mother liquor at room temperature have been measured with a modified Oak Ridge computer-controlled diffractometer to a resolution of 3.56 Å (roughly 10,000 reflections). To improve the signal-to-background ratio of the measurements, the crystal-to-detector distance was increased from 210 to 560 mm. The measurements were by omega step scans over an omega range of 0.6°. Three reference reflections were measured periodically during the data collection, and their average intensity decreased by 25%. Absorption corrections were obtained by the Furnas method from measurements of several reflections made at 10° (psi) intervals of rotation about their reciprocal-lattice vectors.

A number of heavy-atom-containing compounds have been screened in attempts to find isomorphous crystalline heavy-atom derivatives of ricin V-3. The crystals, grown at low ionic strength, have first been transferred into protein-free solutions containing 12% methylpentanediol and 5 m*M* phosphate buffer, pH 6.5, so that the heavy-atom compounds could be diffused into the crystals. The crystals can be maintained in this medium for many weeks at 2° to 6°C, but they dissolve within hours at room temperature. X-ray photographs were obtained with precession cameras fitted with cold-air specimen-cooling equipment. Promising mercury and platinum derivatives were obtained. An air cooling system has been installed on the diffractometer so that counter data may be measured for these derivatives.

A METHOD OF REDUCING ERRORS IN ELECTRON-DENSITY FUNCTIONS OBTAINED BY THE MULTIPLE ISOMORPHOUS REPLACEMENT METHOD FOR MACROMOLECULAR CRYSTALS

J. R. Einstein

In the isomorphous replacement method, the phases of reflections in the diffraction pattern are estimated by a procedure which involves combining data from several crystalline heavy-atom derivatives. The mathematical procedures in common usage, due primarily to Blow and Crick, are based on simplifying assumptions not in accord with probability theory. As has been recognized, the data are, therefore, weighted somewhat incorrectly. This is one of the causes of errors in electron-density functions, which are often uninterpretable in part or give rise to erroneous structural parameters.

A different mathematical procedure has been devised for combining isomorphous replacement and anomalous scattering data. The procedure, which is theoretically correct yet practicable in terms of computing time, has been tested by the computation of many examples (in press). Phase estimates by this procedure can differ significantly from those obtained by the Blow–Crick procedure. Tests of the computer program with full sets of protein data are planned or in progress, in collaboration with groups at MRC-Cambridge, Columbia University, University of Texas, and Wayne State University.

COMPARATIVE STUDIES OF RICINUS TOXINS OBTAINED FROM SMALL- AND LARGE-TYPE CASTOR SEEDS

C. Koh, L. R. Pollack,* and C. H. Wei

We have previously isolated and purified two toxic ricins, ricin III_L and ricin V-3, from small-type castor seeds, and their biological and biochemical properties have been studied.[1] Another ricinus toxin, designated ricin D, has been prepared by a group of Japanese scientists from large-type seeds and has several reported properties which appear to correspond to those of ricin III_L but not ricin V-3. Although the crystallization of ricin D has repeatedly been mentioned by the same Japanese researchers, we have not been able fully to reproduce their results. Ricin V-3 is still the only ricinus toxin for which crystals suitable for X-ray work have been reported in the literature.[2]

Our finding that small-type castor seeds contain at least two major toxins raises the possibility that ricin D is similar (if not identical) to our ricin III_L. This prompted a detailed comparison of ricin D and ricin III_L.

Defatted large-type castor beans were treated according to the Funatsu procedures[3] as well as our own procedures. We obtained ricin D and also, in relatively small amounts, a second toxin which appears to correspond to ricin V-3. Our purified ricin D preparation is indistinguishable from ricin III_L with respect to isoelectric point, polyacrylamide disc gel electrophoresis pattern, SDS electrophoresis pattern, affinity chromatographic behavior on Sepharose 4B, and crystallization. Both ricins D and III_L are more acidic (p*I* 7.4) than ricin V-3 (p*I* 8.6), as is also shown by their greater electrophoretic mobilities at pH 9.5. A coelectrophoresis of ricins D and III_L resulted in only a single band. Preliminary results of amino acid analyses indicated that ricin V-3 preparations obtained from small- and large-type seeds have amino acid compositions closely similar to each other, whereas our ricin D preparation has slight differences from ricin V-3. (A complete comparison of our ricin D preparation with ricin III_L and Funatsu's ricin D is in progress.) In order further to compare ricins V-3 from small- and large-type seeds, we are in the process of crystallizing the latter.

Our results so far strongly suggest that large-type ricinus seeds contain two distinct toxins (ricin D and "V-3"), which are closely similar if not identical to the two toxins in the small-type variety (ricins III_L and V-3).

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X-RAY CRYSTALLOGRAPHIC STUDY ON A SOYBEAN TRYPSIN INHIBITOR[1]

D. L. Hwang,* E. A. Beatty, D. E. Foard, and C. H. Wei

During the course of attempts to isolate soybean proteins with high amounts of sulfur-containing amino acids, we developed a simple method of extraction and purification which resulted in the isolation of five different, but closely related, inhibitors of trypsin and α-chymotrypsin.[2] Among the five inhibitors, we have succeeded in obtaining crystals of one species (hereafter designated inhibitor 1)—the first case known to us of the crystallization of a soybean trypsin inhibitor to give crystal size and quality suitable for X-ray diffraction studies.

Disc gel electrophoresis and SDS-polyacrylamide gel electrophoresis of the purified inhibitor I preparation showed a single band in each case. The $\epsilon 1\%/1$ cm value at 280 nm was determined as 3.66. The N-terminal residue was found to be aspartic acid by thin-layer chromatography of the dansylation reaction product. Determination of the antitryptic activity showed that 1 μg of inhibitor 1 was capable of inhibiting 0.72 μg of active trypsin to 50% of its activity level. The amino acid compositions were different from those previously reported for soybean inhibitors. There are 64 amino acid residues in a molecule of molecular weight 6800—the smallest known among this family of proteins.

The purified inhibitor I was crystallized by the vapor diffusion technique. Clear droplets (30 to 50 μl) of the protein solution in 0.02 *M* sodium acetate buffer (pH 4.5) containing 0.1 *M* NaCl were kept in a glass reservoir which contained a 100-ml solution of 10% saturated ammonium sulfate. The crystals usually start to appear after a few hours at room temperature and grow to at least 0.5 mm in length within 2 days.

X-ray diffraction patterns were recorded at room temperature with nickel-filtered copper $K\alpha$ radiation from an Elliott rotating-anode generator operated at 40 kV and 40 mA. The diffraction patterns showed that the crystals belong to the monoclinic system. The only observed systematic absences were $0k0$ for k odd, indicating the probable space group to be $P2_1$.

Twelve strong reflections in the 2θ range of 22° to 29° were centered with an Oak Ridge computer-controlled diffractometer using copper $K\alpha$ (λ = 1.5418 Å) radiation, and cell parameters were refined by the least-squares method. The cell parameters thus obtained are a = 25.919(7) Å, b = 43.23(1) Å, c = 19.905(5) Å, and β = 103.63(2)°. The unit cell volume is 21,673 Å^3.

Intensity data were collected from a thin tabular crystal (elongated along the b axis) measuring 0.03 × 0.10 × 0.49 mm. The mosaic spread of the crystal was estimated to be only 0.25° about each of the three reciprocal axial directions. The intensities of 1273 independent reflections with $2\theta \leqslant 33°$ (equivalent to a minimum crystal spacing of 2.71 Å) were measured with the θ-2θ scan technique. The crystal was unusually stable to X radiation. Measurable intensities extended beyond 3-Å spacings. A high-resolution study thus appears to be feasible.

The density of the crystals, determined by flotation, was found to be 1.23 g/cm^3. The molecular weight of the asymmetric unit, protein plus solvent, was calculated to be 8028. The amount of protein in the crystal is therefore $85n$% by weight, where n is the number of molecules per asymmetric unit. The ratio of crystal volume to protein weight is $1.59/n$ Å^3 per dalton. For $n \geqslant 2$, the ratios are far below the commonly observed range of 1.68 to 3.53,[3] while for n = 1, the ratio of 1.59 lies on the lower limit for the observed range; therefore, n = 1 appears the likely choice. Since the calculated partial specific volume of inhibitor 1 is 0.705 cm^3/g, the solvent content in the crystal would then be 26% by volume.

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

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X-RAY INTENSITY DATA COLLECTIONS FOR SOME COMPOUNDS OF BIOLOGICAL IMPORTANCE

C. H. Wei

As part of our work devoted to the structural elucidation of small molecules of biological importance, we reported last year the structural analysis of a highly active schistosomicidal agent, hycanthone methanesulfonate.[1] We have now completed collection of three-dimensional X-ray data for two hycanthone analogues which possess much less mutagenic activity without the loss of anti-schistosomal potency.

8-Chloro-2-[2-(diethylamino)ethyl]-2H-[1]-benzothiopyrano-[4,3,2-*cd*]indazole-5-methanol monomethanesulfonate (hereafter designated IA-4)[2] crystallizes with probably two molecules in a triclinic unit cell of symmetry either $P1$ or $P\bar{1}$ and parameters a = 8.7282(8) Å, b = 11.444(2) Å, c = 12.338(2) Å, α = 111.71(2)°, β = 94.28(2)°, and γ = 99.68(2)°. The crystal density is calculated to be 1.21 g/cm^3 if two formula species are assumed in a unit cell. Approximately 3700 intensities were measured by the θ-2θ scan technique up to a 2θ maximum value of 125°. It is anticipated that the structure can be solved by the heavy-atom technique.

Des-chloro IA-4 base[2] (another hycanthone analogue) crystals belong to the trigonal system, space group probably $R\bar{3}$, with rhombohedral cell parameters a = 21.337(1) Å and α = 118.5582(3)° and hexagonal cell parameters $a = b$ = 36.685(2) Å and c = 7.7432(5) Å. A complete data set with $2\theta \leqslant 125°$ was collected by the θ-2θ scan technique, and about 3300 (including duplicates) intensities were obtained. The calculated crystal density is 1.17 g/cm^3 assuming that there are 18 formula species in a hexagonal unit cell.

In addition to the above-mentioned hycanthone analogues, a set of intensity data has also been collected for a derivative obtained by treating the hydrogen form of P^1,P^4-diguanosine 5′-tetraphosphate (di-GDP) with 48% HBr.[3] This unknown compound crystallizes in a monoclinic space group of symmetry $P2_1/c$ with refined parameters a = 4.591(1) Å, b = 9.886(2) Å, c = 18.985(3) Å, and

$\beta = 99.62(2)°$. The intensity data were collected by the ω-scan technique up to $2\theta \leqslant 134°$, whereas for a 2θ range of 134 to 136°, the θ-2θ scan technique was employed. Thus a total of about 1500 independent reflections were measured. Characterization of this unknown compound awaits further analysis of the intensity data.

Note added in proof:

X-ray structure determinations for three compounds have now been computed.

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CALCULATIONS OF ENERGY DEPOSITION BY NEUTRONS

M. L. Randolph

Three aspects of calculations of energy deposition by neutrons in tissue and tissue-like materials have received attention this year.

There has been continuing collaboration with R. S. Caswell and J. J. Coyne at the National Bureau of Standards in the development of full tables of neutron kerma factors (kerma per unit neutron fluence) for an ICRU report on *Neutron Dosimetry for Biology and Medicine* expected to appear in 1976, Below incident neutron energies of 20 MeV, problems which cause most uncertainty include (*a*) how to apportion the cross section for a given reaction to its various product energy states when only the entire cross section is known; (*b*) how, at low (keV) incident energies, to calculate the energy of final nuclei after (n,γ) reactions which yield a cascade of gamma rays; and (*c*) how to treat special problems which arise at incident energies below 20 keV (see last paragraph of this report). At incident neutron energies of 20 to 30 MeV, the lack of a detailed systematic compilation of cross-section data requires extensive approximations, which we do by using the nuclear optical model, extrapolations, and normalizations from data at about 20 MeV.

Calculations have been made of the contribution to kerma of beta particles from neutron-induced short-lived ($t_{1/2} < 20$ days) radioisotopes. For incident energy of 14.5 MeV, this contributes an additional 21% to the "heavy-particle" kerma for sodium, 12% for aluminum, and 10.5% for silicon. For thermal neutrons, much larger effects are found: the average beta-particle decay energy contributes 2430 times the contribution to kerma from "heavy particles" for ^{7}Li, 3300 times for fluorine, 1300 to 4900 times for sodium, 570 times for magnesium, 2060 times for aluminum, 1130 times for phosphorus, 67 times for chlorine, 1440 times for gold, 7.7 times for potassium, and 0.35 for iron.

The ratio of kerma in water to the fluence of neutrons with energies 0.0253 eV to 300 keV has been calculated, taking into account energy transfers to product particles of all 11 energetically possible nuclear reactions, isotopic abundances, chemical binding effects, etc. For thermal neutrons, the reactions $H(n,\gamma)D$ and $^{17}O(n,\alpha)^{14}C$ contribute about 85% and 15% of the kerma. At this energy the relatively enormous energy transfers by these reactions mask chemical binding effects in n,p scattering. The conventional kerma-to-fluence ratio has a minimum at about 3 keV, above which neutron energy the $H(n,n)H$ reaction dominates. Looking at the interaction products, we note that nuclear recoils may be uncharged, charged but unable to ionize (*e.g.*, a 2-keV proton), or charged and able to ionize. Corresponding to the inclusion of all these recoils, only charged recoils, or only recoils which can ionize, calculations were made of "total," "classical," and "ionizing" kerma. Our values for total and classical kerma at low energies are somewhat greater than those of others[1,2] because we include the ^{17}O reaction but are otherwise similar. Ionizing kerma is about four orders of magnitude smaller than classical kerma at 1 keV and less than one-third of classical kerma at less than 10 keV. Ionization at these energies is produced by alphas and ^{14}C ions. The extension of these calculations to include other elements and compositions is planned. The *a priori* expectation is that when nitrogen is present, the differences between these kinds of kerma will be less pronounced because the appreciable cross section for the exothermic $^{14}N(n,p)^{14}C$ reaction tends to make this the dominant factor in kerma calculations at incident energies less than 5 eV.

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MODELING OF DNA KINETICS

M. L. Randolph, W. H. Olson,*
and V. R. R. Uppuluri †

Previous comparisons of experimental data with theoretical models of the kinetic processes of DNA

degradation in *Haemophilus influenzae* cells after X-ray irradiation led us to other modeling studies which, in part, test or expand upon earlier assumptions.[1,2] One assumption was that for X-ray irradiation, single-strand breaks were equally likely at all possible sites. This still seems to be a good approximation for the special case of X-ray irradiation of long DNA chains, but in the general case, which should include, for example, mechanical shearing, the approximation is unsatisfactory. Therefore, a general solution to the problem of break distributions and hence posttreatment distributions of strand lengths was derived which, at least formally, allows for unequal break probabilities, which might be related simply to relative distance from the end of a strand.[3] Another parameter we are investigating is the effect of multiple replicating forks in log-phase cells on the pre- and posttreatment distributions of strand lengths. In principle, although not necessarily in practice, comparison of detailed distributions determined from sucrose gradient centrifugation measurements with these various theoretical distributions may enable us to choose the most likely of postulated cellular kinetic mechanisms.

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ANALYSIS OF THE SUM OF EXPONENTIALS AND FLUORESCENCE-LIFETIME MEASUREMENTS

J. W. Longworth

During the last year, several of the critical features of measuring fluorescence lifetime have been clarified by work in many laboratories. Theoretical studies have shown that the maximum-likelihood solution is identical to the least-squares solution and is a global optimum at convergence. Good initial estimates for the number of exponentials needed for fitting with initial parameter values are provided by an eigenfunction expansion developed by Provencher at the Max-Planck-Institut für Biophysikalische Chemie, Göttingen, Federal Republic of Germany. Sipp and coworkers of the LURE (Engineering) group, using the storage ring (ACO) in Orsay, France, as a source of synchrotron light pulses, have convincingly shown that there is a time shift with photon energy for photomultipliers. Several workers have realized the importance of the time shift and systematic shifts in lamp profile and time-to-amplitude converter response in any analysis of multiexponential decay. The latter two systematic features can be minimized by the contemporaneous collection of fluorescence and lamp data. An estimate for the time shift can be obtained by using ordinary nonlinear least-squares analysis of the decay of a single exponentially decaying standard fluorescence. The shift value can then be incorporated into subsequent data analyses of unknown sample decay profiles.

Initial estimates, further refined by a second-order corrected Newton–Raphson minimization, can then be optimized by a general nonlinear least-squares procedure that considers all the observation vector components to be subject to error. Consideration of all second partials of parameters and observables permits accurate statistical analysis of nonlinear functions. Hence, the major goal of fluorescence lifetime measurements can be achieved—the number of exponentials involved—and the associated parameter values with consonance limits can be estimated, after technical artifacts and systematic errors of measurement have been eliminated. The task remaining is to develop the suite of programs to accomplish the analysis from the separate components I have developed and to implement contemporaneous data collection.

LUMINESCENCE OF BENCE-JONES PROTEINS AND LIGHT CHAINS OF IMMUNOGLOBULINS

J. W. Longworth, A. Solomon,* and M. G. Badea†

Luminescence from triplet states of tryptophyl residues in several Bence-Jones proteins decayed with a nonexponential form. Increasing the temperature from 100° to 200° K caused a progressive prolongation in the decay length. Tryptophyl residues occur vicinal to a disulfide link in both variant and constant domains. A mechanism suggested from model-system studies is an initial formation of a cation of tryptophan and an electron adduct of disulfide. Subsequently, a recombination occurs between the radical pair by a quantum mechanical tunneling, creating a tryptophan triplet. Solvation increases with temperature, increasing the barrier to tunneling and slowing the decay.

Fluorescence and phosphorescence have been measured at several temperatures between 100° and

200° K, and the quenching was modeled with a simple Arrhenius kinetic scheme. A general nonlinear least-squares minimization was used to estimate parameter values, and confidence limits were calculated to second order.

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GROWTH- AND HEART-RATE-INHIBITING PHEROMONES

A. P. Pfuderer

A toxic digylceride with pheromone activity has been purified from *Carassius auratus*. At maximum effect, the diglyceride depressed the fish's heart rate over fourfold. A similar diglyceride analogue which also affects the heart rate in *C. auratus* has been found in tung oil.

Another toxic diglyceride fraction from tung oil has been observed to inhibit growth in *Xenopus laevis* tadpoles and may be related to a natural growth-inhibiting diglyceride found in *Xenopus*. This second inhibitor was also active as the methyl ester. Evidence indicates that this methyl ester or a similar toxic compound is produced when α-eleostearic acid methyl ester is passed through the gas chromatograph.

This second inhibitor has been purified by high-pressure reverse-phase liquid chromatography and preparative gas chromatography. Initial mass spectra indicate that the second inhibitor has a chain length of 18 carbon atoms and three double bonds, and initial spectral data indicate that two of the bonds are conjugated.

The mode of action of the heart-rate-depressing pheromone has been shown to involve the central nervous system. Work has also been started on the mode of action of toxic phenols from coal conversion effluents, which also affect the central nervous system.

STUDIES ON THE FUNCTION OF ASCORBATE SULFATE

F. J. Finamore and R. P. Feldman

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

One aspect of our work involves a comparison of the efficacy of ascorbate sulfate and ascorbic acid in preventing atherosclerosis in rabbits. Rabbits on a high-cholesterol diet were divided into three groups: one group received subcutaneous injections of physiological saline three times a day, 5 days a week, for 10 weeks; another group received subcutaneous injections of L-ascorbic acid (0.37 mmol) according to the same timetable; and the third group was administered an equivalent amount of L-ascorbate 2-sulfate as outlined above. Each week the serum levels of total and free cholesterol and triglycerides were measured. At the end of 10 weeks the animals were killed, and the cholesterol content of the livers, spleens, and adrenal glands was measured. Gross and histopathological examinations of the organs were also performed. The aortas were examined for plaque deposition, the deposits were excised and pooled according to groups, and the total mass and cholesterol contents of the pooled plaques were determined.

Administration of ascorbic acid or ascorbate 2-sulfate did not prevent hypercholesterolemia or elevated levels of serum triglycerides. No significant differences among the groups were found either in tissue cholesterol levels or in the extent or type of lesions found. Although plaque deposition appeared to be similar in the aortas of these animals, a marked difference was found in total mass and cholesterol content of the plaques: the plaques of the saline-treated group had a total mass and cholesterol content approximately 2.5 times that found in the group injected with ascorbic acid and about 1.5 times that found in the animals treated with ascorbate 2-sulfate. These results indicate that ascorbic acid, in particular, minimizes the total quantity of plaque deposition, even though it is ineffective in preventing hypercholesterolemia, elevated serum triglycerides, and accumulation of cholesterol by several tissues.

PRIMARY SEQUENCE OF *EUGLENA* CHLOROPLASTIC $tRNA^{Phe}$

L. I. Hecker, S. H. Chang,* M. Silberklang,† C. K. Brum,* U. L. RajBhandary,† and W. E. Barnett

The first primary sequence for a tRNA from a eukaryotic organelle has been determined. The

nucleotide sequence of the $tRNA^{Phe}$ of *Euglena* chloroplasts is pG-C-U-G-G-G-A-U-A-G-C-U-C-A-G-D-U-G-Gm-U(U*)-A-G-A-G-C-G-G-A-G-G-A-C-U-G-A-A-A*-A-ψ-C-C-U-U-G-U-m^7G-Py*-C-A-C-C-A-G-T-ψ-C-A-A-A-U-C-U-G-G-U-U-C-C-U-A-G-C-A-C-C-A. The sequence of this tRNA was analyzed by labeling fragments produced by T_1-RNase and pancreatic RNase with ^{32}P at the 5′ end, using polynucleotide kinase. Labeled oligonucleotides were separated by two-dimensional electrophoresis and sequenced by partial digestions with either snake venom phosphodiesterase or nuclease P_1.

The nucleotide sequences of all (10) $tRNA^{Phe}$'s show considerable sequence homology (~50%), and all have a chain length of 76 residues. Interestingly, cytoplasmic $tRNA^{Phe}$'s from mammalian (calf, rabbit, and human) tissues are all identical in primary sequence, as are pea and wheat germ $tRNA^{Phe}$.

Briefly stated, the two simplest models which account for tRNA structural genes within the eukaryotic organelle genome are (*a*) that they originated from *within* (*i.e.*, they evolved from tRNA cistrons in the nuclear genome) and (*b*) that they originated from *without*, perhaps from a prokaryotic endosymbiont. Our intent is to determine the degree of sequence homology between the organelle tRNAs and their counterparts from the nuclear genome and prokaryotic (in this case the blue-green algae *Agmenellum*) sources. It is anticipated that these comparisons will provide insight into the evolutionary origin of the organelle genome.

At present we may say that, based on overall sequence and modified base content, chloroplastic $tRNA^{Phe}$ is more similar to prokaryotic $tRNA^{Phe}$'s than cytoplastic eukaryotic Phe's (from sources other than *Euglena*). $tRNA^{Phe}$'s from the cytoplasm of *Euglena* and *Agmenellum* have been purified and are in the process of being sequenced.

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HYBRIDIZATION STUDIES WITH CHLOROPLASTIC tRNAs AND DNA

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Further hybridization studies with unfractionated and purified species of [^{125}I]tRNA from *Euglena* chloroplasts indicate (*a*) that the chloroplastic genome of *Euglena* contains *one* structural gene for each of 26 organelle tRNAs, (*b*) that hybridization between chloroplastic tRNA and nuclear DNA is undetectable, (*c*) that photoinduction of chloroplastic tRNAs is at the level of transcription rather than maturation (modification) of preexisting precursor molecules, and (*d*) that the tRNA cistrons are not closely linked to the rRNA cistrons, as is the case in certain bacterial systems.

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

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

K. B. Jacobson and J. B. Murphy

The properties of the two forms of tyrosine:tRNA ligase from *Drosophila* are being compared. The two forms are designated D (discriminating) and ND (nondiscriminating); the ND form charges both the A and B isoacceptors of $tRNA^{Tyr}$ obtained from the suppressor mutant *su(s)*[2], whereas the D form distinguishes between the two isoacceptors and charges only the A isoacceptor. On storage at −20° or −196° C, the D form of the enzyme is always converted to the ND form in a few weeks or months.[1]

The quantity of tyrosine:tRNA ligase was determined at various developmental stages of *Drosophila*. The specific activity of the enzyme varied less than threefold during the larval, pupal, and adult stages. The relatively constant amount of enzyme indicates that there is no time in development that requires modulation of the tyrosine:tRNA ligase. In this series of studies, all of the stages yielded the ND form of the enzyme.

The conversion of the D to the ND form of tyrosine:tRNA ligase occurs during storage in the frozen state. To understand these enzyme forms better, their molecular weights were estimated by a molecular-sieving procedure. Both the D and the ND form were obtained at a position appropriate for 100,000 daltons. Therefore one is not a subunit of the other.

Since proteolytic cleavage of small peptides from certain amino acid:tRNA ligases has been observed, we examined our enzyme preparations for protease. It is clear that several proteases are present in the *Drosophila* extracts, and one or two chromatographic species of protease are constant contaminants of the ligase preparations. During the

examination of various developmental stages of *Drosophila*, the protease activity was measured; the third-instar larva had the highest levels of proteases, and 6-day-old adults had the lowest. Yet the tyrosine: tRNA ligase was obtained in the ND form from both developmental stages, which indicates that the conversion of the D to the ND form requires only minimal amounts of protease if, indeed, proteolytic cleavage is actually involved in the conversion.

The conversion of the D to the ND form of the tyrosine:tRNA ligase does not occur under some conditions. When the D form is chromatographed on agarose or carboxymethyl cellulose, the form retains its isoacceptor specificity. Prior to learning this, we had presumed that purification procedures would allow the conversion; since this is not so, the purification of the ligase will be attempted. Having a pure enzyme would allow greater insight into the basis for the isoacceptor specificity.

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ISOACCEPTORS OF TYROSYL tRNA OF *DROSOPHILA*: COMPARISON OF WILD TYPE AND SUPPRESSOR MUTANTS AT THE *su(s)* LOCUS

K. B. Jacobson, E. H. Grell, and J. B. Murphy

Tyrosyl tRNA has two major isoacceptors in wild-type *Drosophila* ($tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$) and in the $su(s)^2$ mutant ($tRNA_A^{Tyr}$ and $tRNA_B^{Tyr}$). In 1971[1] we reported only the presence of $tRNA_A^{Tyr}$ in $su(s)^2$ and in $su(s)^{c1}$, but subsequently we learned that we had been using the D form of tyrosine:tRNA ligase for those experiments. Using the ND form of the ligase, the presence of $tRNA_B^{Tyr}$ in $su(s)^2$ could also be demonstrated. A number of alleles at the *su(s)* locus are known, and they were examined for the presence of $tRNA_B^{Tyr}$.

A number of mutants, only some of which contain the *su(s)* mutation, were examined for the tyrosyl tRNA major isoacceptors by charging with the D form of tyrosine:tRNA ligase and chromatography on RPC-5. In the following mutants that contain no *su(s)* allele, both $tRNA_1^{Tyr}$ and $tRNA_2^{Tyr}$ were found: *speck, purple, purple cinnabar, vermilion-brown, brown*. For $su(s)^2$ alone or in the combinations $su(s)^2$; *pr*, $su(s)^2v$;*bw*, or $su(s)^2$;*sp*, only $tRNA_A^{Tyr}$ was obtained. In the naturally occurring mutants $su(s)^s$ and $su(s)^1$, only $tRNA_A^{Tyr}$ was found, but in $su(s)^{ab}$, both $tRNA_A^{Tyr}$ and $tRNA_2^{Tyr}$ resulted. This indicates that $su(s)^{ab}$ may have a different mechanism of suppression than the others.

Two new mutations at the *su(s)* locus, $su(s)^{c1}$ and $su(s)^{x1}$, were isolated during the earlier studies. At that time they both contained $tRNA_A^{Tyr}$ alone. During the current experiments, $su(s)^{x1}$ again gave only $tRNA_A^{Tyr}$, but $su(s)^{c1}$ gave both $tRNA_A^{Tyr}$ and $tRNA_2^{Tyr}$. In the 6 years since $su(s)^{c1}$ was last tested, a number of alterations in the X chromosome and Y chromosome were deliberately introduced by crossing-over. We are now removing these traits by further crossing-over to determine if the presence of one of them has been responsible for this modification of $tRNA_B^{Tyr}$ so that it now reacts with the D form of tyrosine:tRNA ligase. The appearance of the second $tRNA^{Tyr}$ isoacceptor in $su(s)^{c1}$ must be understood, since we have presumed that the tRNA alteration, as compared with wild type, is involved in the suppressor mechanism. The presence of the second isoacceptor may indicate (*a*) that the $tRNA_B^{Tyr}$ is not involved in suppression, (*b*) that this tRNA has been further modified so that it retains the structural trait of $tRNA_B^{Tyr}$ but has an additional alteration that now allows it to react with the D form of the ligase and to cochromatograph with $tRNA_1^{Tyr}$, or (*c*) that the $tRNA^{Tyr}$ alteration is not controlled at the *su(s)* locus at all but at some other locus. It is important to resolve these possibilities.

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TYROSYL tRNA IN *DROSOPHILA*: MULTIPLE FORMS IN WILD TYPE AND IN THE $su(s)^2$ SUPPRESSOR

N. K. Howes* and K. B. Jacobson

During investigations of the role of tyrosyl-tRNA in gene suppression in *D. melanogaster*, it was found that tyrosyl tRNA from this source could be separated into two peaks by chromatography on reverse-phase columns[1] (called peaks 1 and 2 in wild type and peaks A and B in the $su(s)^2$ suppressor). Peak 1 differs from peak 2 in having a Q-base substitution for guanine.[2] While peak 2 can be distinguished from peak B by the ability of the *D. melanogaster* ligase enzyme to charge it with tyrosine,[3] we have been unable to distinguish peak 2 and peak B by physical methods, including RPC-5

chromatography over the range of pH 3 to pH 6, a range of magnesium concentrations, temperature (35°–50° C), and the addition of 6 *M* urea.[4] Chromatography on the anion-exchange resin Aminex A28 or on agarose with a decreasing gradient of ammonium sulfate was also unable to distinguish $tRNA_2^{Tyr}$ from $tRNA_B^{Tyr}$.

To investigate the basis for the discrimination of peaks 2 and B by tyrosine:tRNA ligase, their possible sequential differences were examined. Peaks 2 and B were charged with ^{3}H- or ^{14}C-labeled tyrosine and subjected to partial T_1 RNase digestion, and the oligonucleotides were separated by RPC-5 chromatography using a 0.1 *M* to 0.7 *M* NaCl gradient. Seven oligonucleotides labeled at the 3′ end with tyrosine were resolved, each having a constant ^{3}H:^{14}C ratio after cochromatographing on RPC-5 columns.

Similar partial digests of peaks 1 and 2 and peaks A and B showed that peaks 1 and A had six oligonucleotides in common with the first six obtained from digests of peaks 2 and B. The seventh oligonucleotide, present only in digests of peaks 2 and B, appears to be generated by enzymic cleavage at the guanosine in the anticodon region. This guanosine is believed to be replaced by the Q base in both peaks 1 and A, which results in no cleavage by T_1 RNase.

Thus it appears that peaks 1 and 2 from wild-type *Drosophila* and peaks A and B from the *su(s)*[2] suppressor mutant have good sequential homology in the region between the 3′ end and the anticodon. Experiments are now being undertaken to determine the sequence of these tRNAs in the 5′ region by chemically labeling the tRNAs with [^{32}P]- or [^{33}P]phosphate, followed by partial and complete T_1 RNase digestion.

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

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LOCALIZATION OF THE *PURPLE* MUTANT OF *DROSOPHILA MELANOGASTER* IN THE PTERIDINE BIOSYNTHETIC PATHWAY

J. J. Yim* and K. B. Jacobson

The *purple* mutant of *Drosophila* is known to be defective in the biosynthesis of several pteridines, specifically sepiapterin and drosopterins. Suppression of *purple* by *suppressor of sable* or *suppressor of purple* restores the level of these pteridines, producing a wild-type phenotype.[1] As a prelude to studying the mechanism of suppression of *purple*, we have been investigating the enzymatic lesion involved in *purple*.

Since the guanosine 5′-triphosphate (GTP) cyclohydrolase I[2] and sepiapterin synthase[3] catalyze early reactions in the pteridine pathway, we compared their levels of activity in various *purple* genotypes. We have shown that there is no significant difference in GTP cyclohydrolase activity in *purple*, *suppressor-purple*, and wild-type flies. The sepiapterin synthase level, however, is reduced in *purple* mutants as compared with that observed in wild-type and *suppressor-purple* flies. This was determined as follows: *Drosophila* extracts (either crude extracts or 40 to 60% ammonium sulfate fractions) were incubated with [U-^{14}C]neopterin triphosphate which had been prepared enzymatically from [U-^{14}C]GTP. After incubation at 42° C for various times, alkaline phosphatase and sepiapterin were added, and the incubation was continued for an additional 20 min. Each mixture was heated at 100° C for 5 min and subjected to centrifugation. The resulting supernatant material was then applied to a cellulose thin-layer plate. After two-dimensional chromatography, the radioactive materal that migrated with the sepiapterin standard was analyzed for radioactivity. The amount of sepiapterin synthase activity present in *purple* mutants is about one-third of that in the wild type and *suppressor-purple*. This difference indicates that the enzymatic lesion of *purple* involes sepiapterin synthase activity and that *suppressor of sable* somehow restores this activity. Research in progress is designed to determine the mechanism of this suppression.

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

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

W. R. Farkas* and K. B. Jacobson

We reported that *Drosophila* tRNA reacts with the guanylating enzyme of rabbit reticulocytes and that

four chromatographic species of tRNA were found to contain the [^{3}H]guanine (Gua). We also reported that *Drosophila* contains an active guanylating enzyme.[1]

These studies are continuing. We examined various developmental stages of *Drosophila* and found that the specific activity of the guanylating enzyme fluctuates very little. This stability indicates that the enzyme is not used for restricted developmental stages only but is probably essential at all stages.

The four chromatographic species of *Drosophila* tRNA that accept Gua are being identified. It is significant that the hypermodified Gua called Q base is contained in only four species of tRNA. In the identification of the Gua-accepting tRNAs, we are considering all tRNA but would be especially interested in the involvement of the Q base in this exchange reaction.

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DEVELOPMENT OF A MOUSE MODEL TO STUDY CELLULAR KINETICS OF TUMORIGENESIS

J. E. Tobler and K. B. Jacobson

This research is designed to provide a model system in the mouse to study the cellular kinetics of tumorigenesis. The project takes advantage of mammalian X-chromosome inactivation and requires a gene that is X-linked, mutant viable, and histochemically discernible. Three X-linked genes—alpha-galactosidase (*α-gal*), glucose-6-phosphate dehydrogenase (*g-6-pd*), and sparse fur (*sp-f*)—should fit these requirements and are currently being used to develop the model. Glucose-6-phosphate dehydrogenase has been shown to be present in erythrocyte, kidney, muscle, and heart tissue,[1] and we have developed a screen (described below) to obtain a mutant for this enzyme.

We have examined the α-galactosidase system and have found it to be particularly well suited for such a model system for the following reasons: (*a*) the enzyme is present in spleen, heart, brain, liver, testes, ovary, small intestine, lung, and blood serum, (*b*) developmental profiles of enzyme activity show that the tissues have characteristic profiles of activity which plateau after 5 to 6 weeks of age, and (*c*) blood serum contains α-galactosidase and can easily be extracted and examined for enzyme activity. One problem encountered during the examination is the fact that there are at least two different electrophoretically distinct forms of the enzyme. Since neither the model nor the mutant search would tolerate a multiple gene system, attempts were made to isolate and compare the properties of the respective isozymes. DEAE-cellulose (DE 23) and agarose failed to separate the two forms. DE 52 chromatography separated the two forms, which were subsequently shown by agarose chromatography to have molecular weights of 30,000 and 120,000 respectively.

The two electrophoretic forms were unresolved on DE 52 chromatography. Preparative gel electrophoresis was used to separate the two electrophoretic forms. Neuraminidase treatment of the separate forms caused them to migrate together on both starch and acrylamide gels, although this treatment had no effect on the specific activity of the enzyme. Since heat stability curves, inhibitor sensitivities, K_m characteristics, and pH optimum curves failed to distinguish between the two forms, we believe that the electrophoretic forms represent the same structural enzyme with differing amounts of neuraminic acid residues attached.

A mutant screen is currently in progress to examine male mice which have a treated X chromosome from their mutagenized mother. The procedure allows determination of anemia, glucose 6-phosphate dehydrogenase activity in lysed red blood cells, and α-galactosidase in blood serum. To date, some 400 mice have been examined, but no mutants have been found.

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SURVIVAL OF FETAL RAT PANCREASES FROZEN TO −78° AND −196°C

P. Mazur, J. A. Kemp,* and R. H. Miller

It has recently been shown that diabetes mellitus induced in rats with streptozotocin can be reversed by the implantation of one to three pancreases from syngeneic 16- to 18-day fetal rats. The ability to establish banks of frozen tissue-typed fetal pancreases might facilitate similar procedures in allogeneic animals, including man. However, long-term low-temperature storage requires temperatures below

−70° C, and confirmed instances of organs surviving cooling to such temperatures are rare—most are irreversibly damaged below −20° C. Nevertheless, recent reports of the successful freezing of isolated islets of Langerhans combined with recent achievements in the freezing of early mammalian embryos encouraged us to attempt to freeze the intact fetal pancreas. Survival was based on the ability of the pancreases to incorporate [^{14}C]amino acids into protein during a 4-hr incubation in a growth medium. The optimal freezing procedure yielded pancreatic tissue after 48 hr to several months at −196° C that synthesizes 80% as much protein in a 4-hr incubation period as does tissue from unfrozen controls, and it yields tissue which becomes fully vascularized after transplantation into adult recipients. High survival required high concentrations of dimethyl sulfoxide (DMSO) (2*M*) and very slow cooling (0.3° C/min). The optimum procedure also included slow warming (4 or 15° C/min) and unusually slow dilution procedures. We believe (but have not yet demonstrated) that these latter two factors contributed importantly to survival. Our survival assay must measure protein synthesis by exocrine cells chiefly, since only 1% to 3% of the pancreas represents islet of Langerhans cells.

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NUCLEATION TEMPERATURES OF INTRACELLULAR ICE FORMATION IN MOUSE OVA

S. P. Leibo

Last year it was reported[1] that low-temperature microscopy had shown that intracellular ice formation in mouse ova cooled in DMSO solutions increased proportionately with increasing cooling rates above 1.3° C/min. This observation confirmed a previously reported interpretation[2] that the death of ova cooled faster than about 1.2° C/min was due to intracellular ice formation. Furthermore, the microscopic study showed that 100% of the ova cooled faster than 5° C/min froze intracellularly and that the nucleation temperature of intracellular ice formation was about −40° to −45° C.

The phenomenon of intracellular ice formation in mouse ova is now being studied further by measuring survival as a function of the temperature to which the ova are cooled at 15° C/min, a rate sufficient to ensure intracellular freezing. The ova were suspended in phosphate-buffered saline (PBS) alone or in PBS solutions of glycerol and DMSO at 0° or 20° C for varying times prior to being cooled to various subzero temperatures. The results suggest that the nucleation temperature of intracellular ice formation depends on the nature of the suspending medium, but not on its concentration or the extent to which a protective solute has permeated the ova. For example, temperatures at which survival decreased precipitously were −7° C for ova in PBS, −25° C for ova in 1 *M* glycerol, and about −40° C for ova suspended in 1 *M* and 2 *M* DMSO at 0° and 20° C prior to cooling. Preliminary results further suggest that the nucleation temperature may depend on the cooling rate, at least at rates 100 times the rate yielding maximum survival.

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PRELIMINARY ESTIMATES OF THE PERMEABILITY OF EARLY MOUSE EMBRYOS TO GLYCEROL

P. Mazur, N. Rigopoulos, and S. P. Leibo

The permeability of early embryos to solutes is of physiological significance, and it is central to understanding their response to subzero temperatures. The present study is concerned with determining the permeability coefficient for glycerol and its temperature dependence. Coefficients were calculated from measurements of cell volume vs time in 1*M* glycerol at 0°, 15°, or 22° C for one-, two-, four-, and eight-cell embryos. Embryos were transferred to 1 *M* glycerol in saline and photographed during the ensuing shrinkage and swelling. Volumes were estimated from planimetric measurements of the cross-sectional area, assuming the embryos to be spherical. We have also developed procedures for estimating volumes by the use of an analogue-to-digital converter and subsequent computer processing.

The increase in cell volume with time is described by two coupled equations.[1] One expresses continuous osmotic equilibrium between the intra- and extracellular solution. The other relates the rate of glycerol entry to the difference in the concentration of intra- and extracellular glycerol. Permeability coefficients (*P*) were calculated by determining the numerical value of *P* required in the latter equation to

make the calculated volume equal to the observed volume at a selected time. Calculated and observed plots of volume over the whole time course of permeation agreed satisfactorily. Development from the one-cell to the eight-cell stage produced a tenfold increase in P, from about 3×10^{-5} to about 3×10^{-4} cm/min at 22° C. The value of P for two-cell embryos at 3° C was about tenfold lower than the value at 22° C. The permeability of the eight-cell embryo to glycerol at 22° C is comparable with that of the human red cell.

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RESPONSE OF FERTILIZED MOUSE OVA TO FREEZING AND THAWING AS A FUNCTION OF THE PERMEATION BY GYLCEROL

S. C. Jackowski* and S. P. Leibo

Glycerol permeation and its role in protection against freezing damage have been studied using fertilized mouse ova. Measurements of the rate of glycerol entry have been made, and, using these measurements, the freezing sensitivity has been examined. Using equations derived by Mazur, Leibo, and Miller,[1] permeability coefficients (P_{glyc}) have been estimated from photographic observations of cell volume vs time in 1 M glycerol at 0°, 10°, and 22°C. In addition, coefficients have been estimated for ova isolated at various times after fertilization. Preliminary calculations show that P_{glyc} for ova isolated 7 hr after fertilization increases from 5×10^{-6} to 1×10^{-5} and to 2.5×10^{-5} cm/min at 0°, 15°, and 22° C respectively. Furthermore, P_{glyc} at 0° C increases from 7×10^{-7} to 7×10^{-6} cm/min for ova isolated 1 hr and 17 hr after fertilization respectively.

Freezing sensitivity has been examined by isolating ova at various times after fertilization and suspending them in 1 M glycerol at 0° or 22° C for varying times prior to cooling at a rate of 1° C/min to −196° C and then warming at 2° to 4° C/min. Survival is the percentage of treated ova that develop normally in culture. In all cases, survival after freezing increases as a function of time in glycerol prior to freezing. Furthermore, survival also increases as a function of the temperature of incubation in glycerol prior to freezing. That is, the variables that increase glycerol permeation also appear to increase survival after freezing. However, the survival of ova isolated 14 hr after fertilization was 70% vs only 20% for ova isolated 7 hr after fertilization, despite the fact that the former are only slightly more permeable to glycerol than the latter. Therefore, we tentatively conclude that while glycerol permeation may indeed enhance survival, it is not the sole factor that determines a cell's freezing sensitivity.

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1. P. Mazur, S. P. Leibo, and R. H. Miller, *J. Membr. Biol.* **15**, 107 (1974).

THE USE OF PERMEABILITY COEFFICIENTS IN PREDICTING THE OSMOTIC RESPONSE OF HUMAN RED CELLS DURING THE REMOVAL OF INTRACELLULAR GLYCEROL

P. Mazur and R. H. Miller

Although some cells survive freezing in the absence of intracellular additive, others may require its permeation for optimum survival. Moreover, even when an additive has not permeated prior to freezing, the development of high solute concentrations and the possible alterations of membranes during freezing may drive additive into the cell. Once additive is in a cell it can cause serious osmotic problems during thawing and during its subsequent removal. Knowledge of the permeability coefficients of the solute permits one, in theory, to calculate the changes in volume that a cell will be subjected to when attempts are made to remove protective additives by dilution. Theoretically, one should be able to rapidly determine by computer modeling those dilution procedures which will minimize osmotic damage. To test whether this expectation is met in practice, we made use of recent determinations of the permeability coefficients of human red cells in glycerol.[1] The coefficients were used to calculate the cell volume changes when cells which had been fully equilibrated with 2 M glycerol were subjected to two sorts of dilution procedures. One procedure involved stepwise dilution with various volumes of isotonic PBS at various time intervals. The other procedure involved transferring the cells to a nonpenetrating solute (1.0 M sucrose) for various times, followed by abrupt 20× dilution with PBS. In the red cell, hemolysis occurs when the volume exceeds rather precisely determinable values, and from these determinations we were able to calculate the amount of hemolysis that would be expected at different steps in a given dilution procedure. Red cells were then subjected to these dilution procedures, and the amount of hemolysis produced by each step of the dilution was determined experimentally. The agree-

ment between the predicted amount of hemolysis and that actually observed is good.

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CORRELATION BETWEEN THE SURVIVAL OF SLOWLY FROZEN HUMAN RED CELLS AND THE COMPOSITION OF THE UNFROZEN PORTION OF THE EXTRACELLULAR MEDIUM AT VARIOUS SUBZERO TEMPERATURES. I. SURVIVAL VERSUS TEMPERATURE

H. Souzu* and P. Mazur

One widely accepted explanation of slow-freezing injury is that damage results when the concentration of electrolyte reaches a critical level in partly frozen solutions during freezing. We have conducted experiments on human red cells to further test this hypothesis. Cells were suspended in saline or PBS containing 0 to 3 *M* glycerol, held for 30 min at 20° C to permit solute permeation, and frozen at 0.5° or 1.7° C/min to various temperatures between 0° C and −100° C. Upon reaching the desired minimum temperature, the samples were warmed at rates ranging from 1° to 550° C/min and the percent hemolysis was determined. The results for a cooling rate of 1.7° C/min indicate the following: (*a*) Between 0.5 *M* and 1.8 *M* glycerol, the temperature yielding 50% hemolysis (LT_{50}) drops slowly from −20° to −35° C. (*b*) The LT_{50}'s over this range of concentrations are relatively independent of the warming rate. (*c*) With glycerol concentrations of 1.95 *M* and 2.0 *M*, the LT_{50} drops abruptly from −35° C to below −100° C and becomes dependent on the warming rate. The LT_{50} is lower with slow warming than with rapid. With still higher concentrations (2.5 *M* and 3.0 *M*), there is no LT_{50}, that is, more than 50% of the cells survive freezing to −100° C, and survival again becomes independent of the warming rate. Results for cooling at 0.5° C/min were similar, except that the LT_{50}'s were some 10° to 20° C higher.

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CORRELATION BETWEEN THE SURVIVAL OF SLOWLY FROZEN HUMAN RED CELLS AND THE COMPOSITION OF THE UNFROZEN PORTION OF THE EXTRACELLULAR MEDIUM AT VARIOUS SUBZERO TEMPERATURES. II. SURVIVAL VERSUS COMPOSITION OF EXTRACELLULAR MEDIUM

P. Mazur, W. F. Rall,* and H. Souzu†

One theory of freezing damage suggests that slowly cooled cells are killed by being exposed to increasing concentrations of electrolytes as the suspending medium freezes. A corollary to this view is that protective additives such as glycerol protect cells by acting colligatively to reduce the electrolyte concentration at any subzero temperature. Recently published phase-diagram data for the ternary system glycerol–sodium chloride–water[1] allow further analysis of the data on human red cell survival at subzero temperature presented in the companion study.[2] Appropriate liquidus phase-diagram information for those solutions used in our red cell freezing experiments was obtained by interpolation of Shepard *et al.*'s liquidus data. The results of phase-diagram analysis of red cell survival indicate the following: (*a*) The correlation between the LT_{50} and the electrolyte concentration attained at that temperature in various concentrations of glycerol is poor. With increasing concentrations of glycerol, the cells were killed at progressively lower concentrations of NaCl. For example, LT_{50} for cells frozen in the absence of glycerol corresponds to an NaCl concentration of 12 wt %, whereas for cells frozen in 1.85 *M* glycerol in buffered saline, the LT_{50} corresponds to 3.9 wt % NaCl. When the NaCl concentration is expressed on a molal basis, the correlation is improved. (*b*) There is some correlation between LT_{50} and the fraction of cell water remaining unfrozen at that temperature; 50% hemolysis occurs when about 90% of the water has been converted to ice.

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1. M. L. Shepard, G. S. Goldston, and F. H. Cocks, *Cryobiology* **13**, 9–23 (1976).

2. H. Souzu and P. Mazur, this report.

III. CANCER AND TOXICOLOGY SECTION

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INTRODUCTION

CANCER AND TOXICOLOGY SECTION research centers around studies of the health effects of chemical and physical agents in the environment. Such agents include known and suspected chemical carcinogens and low- and high-LET (linear energy transfer) ionizing radiations; many are products or potential products of energy-producing systems. Their effects are being studied at the molecular, cellular, and organismic levels. For example, the metabolism of certain carcinogens is being studied *in vitro* in tissue culture systems and *in vivo* in rodents, with the objective of determining the nature of the transforming event or the mechanisms by which carcinogens induce tumors. With this knowledge, it may be possible to find ways to prevent carcinogenic action, make early diagnoses, provide effective treatment, or substitute safer compounds.

Another important aspect of the studies in this Section is the investigation of the interaction of chemical and physical agents at varying dose levels in producing toxicity or neoplastic growth, since certain chemicals (sometimes innocuous of themselves) potentiate or promote the carcinogenic action of other compounds, while other chemicals may act as inhibitors. Because the quality and extent of the deleterious effects of various agents depend in part on the genetic and physiologic constitution of the cells or organism affected, some studies are directed toward defining the ranges of response that correlate with constitution. Such studies include, for example, the examination of (*a*) sex-dependent differences in toxic and tumorigenic responses to radiation or chemicals and (*b*) the effects of immunologic malfunction. This is critically important to man, because genetic and physiologic constitution vary widely in the human population. Many of the deleterious changes effected by the biological, chemical, and physical agents under study are evidenced as neoplastic growth, indicating alterations of the regulatory processes that normally control cell and tissue growth. Some effort in this Section is therefore directed toward the investigation of regulatory mechanisms at various levels of organization.

Research in the Cancer and Toxicology Section is jointly sponsored by the National Cancer Institute, the Environmental Protection Agency, and the U.S. Energy Research and Development Administration.

EFFECTS OF BUTYLATED HYDROXYTOLUENE, DIETHYLNITROSAMINE, AND X RAYS DURING TUMORIGENESIS IN MICE

N. K. Clapp,* R. L. Tyndall,* J. P. Daugherty,†
K. A. Davidson, and C. F. Gottlieb

As evidence continues to incriminate the environment as a major source of carcinogens for humans, more information is needed to determine whether environmental agents act additively, synergistically, or antagonistically and how such interactions may be rapidly detected. Therefore, by applying a variety of experimental approaches, we are evaluating possible relationships of induced pathologic conditions to physiologic changes observed during treatment and during tumor development.

The antioxidant butylated hydroxytoluene (BHT), which is widely used as a food preservative, protects against acute lethality by the potent chemical carcinogen diethylnitrosamine (DEN) and by ionizing radiation. We are now determining the mechanism of BHT's effect on DEN carcinogenesis, whether BHT modifies the late effects of ionizing radiation, and whether the two known carcinogens, DEN and radiation, interact. Male and female BALB/c mice are being used. Doses of the agents are (*a*) 0.75% BHT in the feed throughout life, (*b*) DEN in the drinking water for 7 weeks for an average cumulative dose of about 300 mg/kg, and (*c*) a leukemogenic dose (250 rads) of X rays. Suitable control groups are also included. The mice will be killed at either 12 or 18 months of age for pathological examination. In addition to obtaining pathology data (Clapp), we are making serial observations in individual mice on plasma changes (Tyndall), on the metabolic fate of the chemicals (Daugherty), on hormonal dependence and the source of esterases (Davidson), and on immune status (Gottlieb).

Some of our results are reported in companion papers. We have observed that (*a*) hormonal status affects DEN carcinogenesis, (*b*) BHT does modify DEN tumor induction, (*c*) plasma esterase changes are better indicators of chemical treatments than standard clinical chemistry tests (tests for levels of SGOT, AP, LDH, GGTP), (*d*) studies of metabolic patterns are productive in increasing an understanding of the significant molecular interactions involved in the transforming of a normal cell into a cancer cell, (*e*) there were no dramatic alterations in immune competence either during treatment or during the tumorigenic period and no significant differences in immunocompetence between tumor-bearing and non-tumor-bearing animals, and (*f*) interactions between DEN enzyme activities and testosterone levels in the test animals are evident.

Each of these research areas is being actively pursued with the objective of gaining a better understanding of the mechanisms of action of carcinogens, as well as establishing possible biosensors which may reflect the status of an animal during the critical early periods of the carcinogenic process.

*Consultant.

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

A SEX-DEPENDENT PROTECTION OF BUTYLATED HYDROXYTOLUENE AGAINST DIETHYLNITROSAMINE CARCINOGENESIS IN BALB/c MICE

N. K. Clapp,* R. L. Tyndall,* W. C. Klima, and L. C. Satterfield

Previous studies which indicated that BHT protected against acute lethality by DEN led us to investigate the effects of BHT upon DEN carcinogenesis. BALB/c mice of both sexes (8 weeks old) received either (*a*) 0.75% BHT in the feed throughout life, (*b*) DEN in the drinking water for 7 weeks (mean cumulative dose of about 330 mg per kilogram of body weight), (*c*) DEN plus BHT, or (*d*) no treatment. Mice were killed at 12 and 18 months of age, because a preliminary experiment had showed that the first deaths occurred by 12 months and that most of the remaining mice were sick or moribund by 18 months. In addition, plasma enzyme changes and alterations in immune competency are being evaluated in these mice.

Tumor incidences by treatment groups are shown in Table 38. DEN-treated mice developed tumors of the forestomach (squamous) and lung (adenomas) in both sexes. BHT protection against DEN effects was observed at 18 months, as evidenced by the increased survival of females (43% vs 68%—$p < 0.01$) and an associated decreased incidence of squamous cell carcinomas (100% vs 68%—$p < 0.005$). No differences in survival or stomach tumor incidences were observed in males treated with DEN with or without BHT, nor were the incidences of lung tumors affected in either sex.

The mechanism whereby BHT modifies the DEN effect on the forestomach is not clear at this time, although the hormonal status of the animal is important in DEN carcinogenesis, as seen by the higher incidences of tumors in females given DEN and the BHT modification of the DEN process observed only in females. The 18-month pathological data correlated with observations of alterations in plasma esterases observed 15 months earlier in that amelioration of DEN-induced esterase changes occurred in females but not in males treated concomitantly with BHT. The selective beneficial effect of BHT on an induced malignant tumor (forestomach) and the failure to alter incidences of the benign lung tumor may relate to hormonal status and variations in tissue metabolism. Studies are now under way to relate these two variables to DEN carcinogenesis.

*Consultant.

A COMPARISON OF THE EFFECTS OF BUTYLATED HYDROXYTOLUENE TREATMENT UPON SURVIVAL AND CARCINOGENESIS AFTER X RAYS OR DIETHYLNITROSAMINE

N. K. Clapp,* L. C. Satterfield, and W. C. Klima

In an effort to further evaluate the interactions of BHT, DEN, and ionizing radiation, BALB/c mice (both sexes) received BHT (0.75% in the feed) beginning at 8 weeks of age, either for a 4-week treatment or for life span. DEN was given at 12 weeks of age either intraperitoneally (150–200 mg per kilogram of body weight) or orally in the drinking water for 7 weeks (cumulative dose of about 300 mg per kilogram of body weight). X rays (250–650 R) were given in a single dose at 12 weeks of age. Thirty-

Table 38. Incidences of tumors in BALB/c mice after BHT and DEN (330 mg per kilogram of body weight) alone or in combination

Mice killed at 18 months of age

Treatment	No. mice at risk	No. mice surviving 18 months	Incidence of tumors (%)	
			Stomach (squamous cell carcinomas)	Lung (adenomas)
Females				
Controls	50	50	0	20
BHT	50	47	0	15
DEN	49	21	100	100
BHT + DEN	50	34	68	97
Males				
Controls	100	89	0	34
BHT	100	98	0	35
DEN	100	53	75	81
BHT + DEN	100	45	80	80

day survival, life shortening, and tumor induction are being evaluated.

BHT pretreatment protected against 30-day lethality in both sexes from both X rays (LD_{50}s increased—females, 628 R to 643 R; males, 581 R to 622 R) and ip DEN (LD_{50}s increased—~175 mg/kg to ~225 females and ~250 males), while oral-DEN groups with or without BHT had no 30-day lethality. In 30-day survivors of X rays, gross necropsy data indicated that neither mean survival times nor incidences of thymic lymphoma or myeloid leukemia were affected by BHT pretreatment in either sex. Some mice receiving BHT pretreatment plus ip DEN and some controls are still alive, and data on those groups are not available at this time. When BHT was fed for life, oral-DEN-treated female mice had increased survival and significant decreases in malignant forestomach tumors; no differences were seen in males.

Since DEN needs to be "activated" by intracellular metabolism while ionizing radiation does not, the mode of action for BHT protection against acute lethality may be different for the two agents. BHT may protect by free radical scavenger activity (radiation) or by altering enzymic induction (DEN). While the mechanism of BHT protection against DEN carcinogenesis in females is not clear at this time, the lack of BHT effect upon X-ray-induced leukemogenesis and tumorigenesis in both sexes further supports the interpretation that different mechanisms may be involved in BHT's protection against acute lethality by the two carcinogens.

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IMMUNE COMPETENCE OF MICE TREATED WITH DIETHYLNITROSAMINE AND/OR BUTYLATED HYDROXYTOLUENE; LACK OF CORRELATION WITH CARCINOGEN TREATMENT OR TUMOR STATUS

C. F. Gottlieb, G. M. Peterman, and N. K. Clapp*

Immunosuppression as an explanation for carcinogenesis has received considerable attention in recent years, and the circumstantial evidence supporting it is extensive. Cancer tends to be a disease of old age, both in man and animal, and correlates well with the decline in immunity with age. An increased frequency of *de novo* tumors is observed in individuals with compromised immunity, either those with naturally occurring immunological deficiency diseases or those deliberately depressed to permit acceptance of organ homografts. Immunosuppression has also been suggested as a partial explanation for the carcinogenicity of ionizing radiation.

To gain more definitive evidence on this "immunological surveillance" hypothesis, we have evaluated the immune status of barrier-derived BALB/c mice of both sexes treated with DEN and BHT, either singly or in combination. BHT, a food additive suspected of modifying the action of DEN, was administered at a concentration of 0.75% in the food for life, starting at 8 weeks of age. A 7-week DEN treatment was given orally in the drinking water, beginning 3 weeks after the initiation of BHT, with cumulative doses of about 330 mg/kg. Untreated mice of similar age and sex served as controls. Immunoresponsiveness was measured on an

individual-mouse basis by determining the DNA synthetic response of spleen cells to the T-cell mitogen phytohemagglutinin (PHA) and the B-cell mitogen *Escherichia coli* lipopolysaccharide and the production of antibody-forming cells (plaque-forming cells) *in vitro* against sheep erythrocytes. Routine histological examination of tissues collected at gross necropsy was performed on all mice. Mice were killed at three times: 18 weeks of age (termination of the DEN treatment) and 12 and 18 months of age. The results were analyzed by treatment group and time after treatment as well as by tumor type and organ site. Although age-associated decline in the immune indices was noted (most pronounced in the plaque-forming-cell, less so in the PHA, and only slightly in the lipopolysaccharide responses), it was concluded that carcinogen-treated and tumor-bearing mice were equally immunocompetent with age-matched control mice. Most significant of these findings was the failure to observe a difference in the immune responsiveness of tumor-bearing mice as compared with normal (non-tumor-bearing) control mice.

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SUBCELLULAR DISTRIBUTION OF RADIOACTIVITY IN TUMOR-SUSCEPTIBLE AND NON-TUMOR-SUSCEPTIBLE TISSUES OF MALE RFM AND BALB/c MICE FOLLOWING A SINGLE CARCINOGENIC DOSE OF RADIOACTIVELY LABELED DIETHYLNITROSAMINE

J. P. Daugherty* and N. K. Clapp †

The incidence of primary tumors following dimethylnitrosamine (DMN) and DEN in RFM and BALB/c strains of mice has been described by Clapp *et al.*[1] Differences were noted between DMN and DEN tumor induction within strains and between strains for each carcinogen. The differences between tumor types within strains, as well as between strains for each nitrosamine, may reflect differences in the affinity, transport, and/or metabolism of DMN and DEN in the different strains, in different tissues of the same strain, or by different cells within the same tissue. This system provides an excellent model for a study of the activity of cells to bind, degrade, eliminate, and repair altered constituents in relationship to the neoplastic process.

Radioactively labeled DEN (^{14}C) was administered (150 mg per kilogram of body weight) by gastric intubation to groups of three 8-week-old male RFM or BALB/c mice, and the animals were killed at timed intervals following administration of the carcinogen (15 min to 120 hr). Tumor-susceptible tissues (esophagus, stomach, liver, and lung) and a non-tumor-susceptible tissue (heart) were removed and processed as described by Daugherty and Clapp.[2]

Radioactivity was detected in the tumor-susceptible tissues following the administration of DEN; the non-tumor-susceptible tissue did not accumulate appreciable levels of radioactivity. The stomach incorporates the greatest amount of radioactivity at the early time intervals ($^1/_2$ hr), and the liver reaches a maximum at 4 hr. The tissues are currently being fractionated into subcellular fractions, and the radioactivity associated with the various classes of macromolecules within each subcellular fraction is being determined.

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

† Consultant.

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ALTERATIONS IN PLASMA ESTERASES OF MICE TREATED WITH PHYSICAL AND CHEMICAL AGENTS

J. P. Daugherty,* R. L. Ullrich, N. K. Clapp,† R. L. Tyndall,† M. C. Jernigan, L. M. Adams, L. C. Satterfield, and W. C. Klima

The various molecular forms of plasma and tissue esterases have been shown to be sensitive to changes in the physiological state of the organism—either naturally (pregnancy) or artificially induced (treatment with carcinogens, toxins, and antioxidants).[1,2] Such alterations in esterases may be reflective of the pathological processes induced by these chemical agents.[2] Therefore, we have undertaken analysis of mouse plasma esterases using a continuous-gradient (4% to 30%) polyacrylamide gel electrophoresis system developed in our laboratory.

Male RFM mice were irradiated with neutrons (0 to 655 rads) at 12 weeks of age and killed at 12 months of age, and the tumor incidence is described in an accompanying report.[3] Male and

female BALB/c mice received (*a*) 250 rads of X rays at 11 weeks, (*b*) X rays plus 0.75% BHT for life, and (*c*) X rays plus DEN for 7 weeks. The tumor incidence of these mice has been reported previously.[4]

Several alterations in esterase patterns were observed. The majority of the alterations were consistent at each dose of neutrons and generally increased as the dose of radiation increased. The esterase band migrating in front of the albumin esterase region disappeared at the dose of neutrons where maximum lung tumor induction was observed and reappeared at higher doses of neutrons. There was also a progressive decrease in the slowly migrating esterase bands as the dose of neutrons increased. The X-ray-treated animals are currently being analyzed for esterase alterations in order to compare esterase alterations due to chemical carcinogens as well as physical carcinogens.

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

† Consultant.

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SEPARATION OF METABOLITES OF DIMETHYLNITROSAMINE IN THE ACID-SOLUBLE COMPONENTS OF MOUSE LIVER CYTOSOL BY COLUMN CHROMATOGRAPHY

J. P. Daugherty,* M. H. Zehfus,† and N. K. Clapp ‡

Studies on chemical carcinogenesis at the molecular level require information on the kinetics of the metabolites formed from the parent compound. Radioactively labeled DMN was administered by intragastric intubation (10 mg per kilogram of body weight) to male RFM mice. The animals were then killed and the livers removed at timed intervals (15 min to 16 hr). The liver cytosol fraction was isolated as described previously,[1] and the kinetics of the metabolites in the acid-soluble components were followed by fractionating this subcellular fraction by Sephadex LH-20 column chromatography. Fractions of 1 ml were collected and scanned from 220 to 320 nm, and the radioactivity of each fraction was determined. The elution profile was resolved into 29 regions, with radioactivity incorporation varying between regions. In some regions there was a definite increase in radioactivity incorporation through the first 4 to 8 hr, and this was followed by a decrease through 16 hr. Other regions, although changing in concentration, did not incorporate radioactivity. The results indicate that radioactivity from [^{14}C]DMN is found in numerous compounds, even after 15 min, and that a more detailed understanding of the early events of tumorigenesis is contingent upon a full understanding of DMN metabolism, not just focusing on one or a few possible metabolites. The elution patterns obtained from the liver cytosol are now being compared with the elution patterns of known metabolites of DMN, including formaldehyde, formic acid, methylamine, *N*-methylhydrazine, *N*-methylhydroxylamine, methanol, and *N,N*-dimethylhydrazine.

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

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1. J. P. Daugherty and N. K. Clapp, *Life Sci.*, in press.

THE KINETICS OF APPEARANCE OF COVALENTLY BOUND METHYL LABEL (^{14}C) IN LIPIDS AND PROTEIN OF MOUSE LIVER AND LUNG FOLLOWING A SINGLE INJECTION OF RADIOACTIVE DIMETHYLNITROSAMINE

J. P. Daugherty* and N. K. Clapp †

Although DMN has been investigated extensively since its discovery as a hepatoxin, little is known of the subcellular distribution of the parent compound and/or its metabolites in the various classes of macromolecules. A single carcinogenic dose (10 mg per kilogram of body weight) of radioactively labeled DMN was administered to male RFM mice. Tissues (lung and liver) were removed at timed intervals (15 min to 16 hr) and fractionated into subcellular fractions (nuclear, mitochondria-lysosomal, ribosomal, membrane-microsomal, and cytosol) according to the procedure of Daugherty and Clapp.[1] The radioactivity associated with macromolecular

species (DNA, lipid, protein, RNA) of each subcellular fraction was determined. The association of radioactivity with DNA and RNA has been reported.[2]

In the majority of subcellular fractions of both tissues, the lipid moieties incorporated more radioactivity than did the protein molecules, but both incorporated more than did the DNA and less than did the RNA. The relative amounts were 20, 11, 2, and 1 for RNA, lipid, protein, and DNA at 8 hr in the liver cytosol. The greatest amount of radioactivity was associated with the liver cytosol fraction, where the lipid incorporated 34% and the protein incorporated 6% of the radioactivity in that fraction at 8 hr. In general, this same proportion of radioactivity was observed at other time intervals. The lung cytosol peaked at 4 hr, and the lipids and protein bound 23% and 3% of the radioactivity respectively. Similar kinetics were observed with the nuclear and ribosomal fractions. The radioactivity in the mitochondria–lysosomal fraction of the liver peaked at 1 hr and remained constant through 16 hr. The membrane–microsomal fraction of the liver reached a maximum at 2 hr, remained at this level through 8 hr, and then decreased. In the lung lipids, the radioactivity of the mitochondria–lysosomal fraction peaked at 2 hr and remained constant through 16 hr. Radioactivity in the membrane–microsomal subcellular fraction continued to increase throughout the last time interval.

Current studies are under way to follow the subcellular distribution of radioactivity from a single injection of [^{14}C]DEN. It is hoped that such a comparative study will provide information on the organ and cellular specificity of the nitrosamines.

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

† Consultant.

1. J. P. Daugherty and N. K. Clapp, *Life Sci.*, in press.
2. J. P. Daugherty and N. K. Clapp, *Biol. Div. Annu. Prog. Rep., June 30, 1975*, ORNL-5072, p. 159.

PLASMA TESTOSTERONE LEVELS IN ATHYMIC "NUDE" MALE MICE

K. A. Davidson, J. M. Holland, J. W. Hall, and L. C. Gipson

Athymic nude mice are not only immunologically incompetent but their reproductive capacity is also greatly reduced. The relationship between the immune and endocrine systems has not been clearly defined, but the evidence suggests that neonatal thymectomy leads to ovarian dysgenesis and that hypophysectomy leads to immunodeficiency. To establish whether hormone levels are altered in athymic mice to a degree that may help explain reproductive deficiency, we conducted the following experiments.

The mice used in this study were reared in germ-free isolators so that variations due to infectious diseases would be minimized. Nude male mice and normal heterozygous littermates were bled at 5, 14, 25, and 45 days of age. Plasma was separated, and testosterone levels were measured by radioimmunoassay. As shown in Table 39, plasma testosterone levels in nude males were increased relative to normal littermates. Between 14 and 45 days, nude mice had significantly higher testosterone levels than normal littermates. The 45-day-old nude had circulating levels of testosterone which were five times those of normal littermates.

These results suggest that the pituitary feedback mechanism regulating testosterone levels may be altered in *nu/nu* mice. Experiments are in progress to determine the circulating levels of estradiol in female nudes and normal littermates. Eventually we hope to establish whether the hormonal abnormalities are mediated through the thymic defect or whether they are pleiotropic manifestations of the primary mutation. These observations also suggest caution in drawing conclusions from experiments based on nude mice, especially if hormone levels are significantly deranged.

Table 39. Plasma testosterone levels in athymic "nude" mice and normal heterozygous littermates

Age (days)	Testosterone (pg per milliliter of plasma)		Probability[a]
	Nude	Normal	
5	445 ± 59[b] (13)[c]	496 ± 47 (16)	$p = 0.50$
14	547 ± 50 (16)	411 ± 39 (22)	$p < 0.05$
25	932 ± 90 (19)	455 ± 37 (24)	$p < 0.001$
45	4782 ± 334 (18)	833 ± 87 (20)	$p < 0.001$

[a]Probability of difference between nudes and normals, Student's t test.

[b]Mean ± standard error.

[c]Number of determinations.

EFFECT OF DIETHYLNITROSAMINE ON TESTOSTERONE-INDUCED ENZYMES IN INTACT AND CASTRATED MALE BALB/c MICE

K. A. Davidson, R. L. Tyndall,* N. K. Clapp,* L. C. Gipson, and J. W. Hall

Previous investigations using polyacrylamide gel electrophoresis showed that the carcinogen DEN induces alterations in plasma esterases in both male and female BALB/c mice.[1] It was also shown that some of these esterases are testosterone-dependent; that is, they are quantitatively altered by castration and can be restored to normal by testosterone injections.[2] These results suggested that DEN may stimulate testosterone production in the mouse. Another enzyme, kidney β-glucuronidase, is known to be induced by testosterone. Male BALB/c mice were castrated at 6 weeks of age; beginning at 10 weeks of age, these mice were given DEN in their drinking water for 7 weeks. The mice were then bled to collect plasma samples for esterase analysis, and kidneys were removed for β-glucuronidase analysis. Age-matched nontreated intact and castrated mice and intact treated mice were used as controls.

Esterase profiles confirmed that, relative to nontreated intact mice, castration resulted in diminution of esterase bands 6, 8, 9, 10, 11, 12, and 14 and enhancement of band 7 (Table 40). DEN treatment of castrated mice gave results identical to those with intact DEN-treated mice. Eliminating the major source of testosterone in male mice by castration does not affect the induction of plasma esterases by DEN. Thus it appears that DEN acts directly in inducing these esterases rather than indirectly via testosterone production.

The results with β-glucuronidase were quite different (Table 40). The level of this enzyme was very low in nontreated castrated mice and was not significantly affected by DEN treatment. However, β-glucuronidase activity was reduced in intact DEN-treated mice to about 50% of the level in intact untreated controls.

Analysis of the effect of DEN on the activity of two different enzymes, both of which are induced by testosterone, reveals that DEN has opposite effects on the activity of these enzymes. The activity of most plasma nonspecific esterases is enhanced in intact and castrated mice, whereas the activity of kidney β-glucuronidase is depressed. One possible explanation for these results is that DEN acts directly to induce plasma nonspecific esterases and at the same time depresses testosterone production, as revealed by the decrease in kidney β-glucuronidase activity. An alternate explanation is that DEN has no effect on testosterone production but stimulates testosterone excretion, which results in depression of plasma testosterone levels and kidney β-glucuronidase activity. Consequently, experiments are planned to determine the rate of excretion of testosterone in DEN-treated and control mice using tritium-labeled testosterone and to determine testosterone levels in the plasma of male and female BALB/c mice following DEN treatment.

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1. R. L. Tyndall, S. Colyer, and N. K. Clapp, *Int. J. Cancer* **16**, 184 (1975).
2. R. C. Allen and D. J. Moore, *Endocrinology* **178**, 655 (1966).

LATE SOMATIC EFFECTS OF IONIZING RADIATION IN MICE AS A FUNCTION OF DOSE AND DOSE RATE

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A portion of the larger study outlined by Storer *et al.*[1] is now under intensive histopathological study. This portion utilized 10-week-old female BALB/c and RFM mice at two dose rates, 8 rads/day and 40 rads/min, of ^{137}Cs gamma rays in dose ranges of 0 to 400 rads (Table 41). The larger preceding study

Table 40. Effect of castration and DEN treatment on plasma esterase and kidney β-glucuronidase activity in male BALB/c mice

Treatment	Band 6[a]	Band 7	Bands 8–10	Band 11	Band 12	Band 14	β-glucuronidase activity[b]
Intact, no DEN	N	N	N	N	N	N	26.3 (25.5–28.0)[c]
Intact plus DEN	I	D	I	I	I	I	13.8 (12.4–17.3)
Castrated, no DEN	D	I	D	D	D	D	3.8 (3.3–4.1)
Castrated plus DEN	I	D	I	I	I	I	2.5 (1.6–3.3)

[a]Band of esterase activity. N, normal activity; I, increased activity; D, decreased activity.
[b]Micrograms of phenol per milligram of kidney.
[c]Range of values.

Table 41. Radiation experiments conducted with female mice
All mice were necropsied, and approximately one-third were examined histologically

Total radiation dose (rads)	Total number of mice in subgroup	
	RFM	BALB/c
Control		
0	757	873
Low dose rate (0.007 rad/min)		
50	1475	1297
100	1530	1343
200	1538	1383
400	863	1007
High dose rate (40 rads/min)		
50	775	870
200	773	881

used only one strain of mouse (RFM) and a "high" dose rate (40 rads/min). Early analyses of the mortality rates and disease incidence of those mice suggested the desirability of studying the effects of low dose rate (0.007 rad/min) and including a second mouse strain. Controls and duplicates of previously studied irradiation doses were included to check on the inherent variability in such a biological system in a prolonged study. All mice are dead, and histology coding, storage, and retrieval of the pathologic findings are well advanced. Analysis of the findings and intergroup comparison will be done as the subgroup workups are completed. Studies of the control groups of the two mouse strains have been completed and show marked differences in the spontaneous diseases in the two strains. It is anticipated that the irradiated groups will differ from the controls in induction of certain diseases, in decrease in incidence of other diseases, and in changes in average life span, which may be both dose- and dose-rate-dependent.

1. J. B. Storer *et al.*, *Biol. Div. Annu. Prog. Rep., June 30, 1974*, ORNL-4993, pp. 149 150.

PRENATAL SURVIVAL OF MOUSE EMBRYOS IRRADIATED WITH FAST NEUTRONS OR X RAYS DURING A RADIOSENSITIVE STAGE OF CENTRAL NERVOUS SYSTEM DEVELOPMENT

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A study is in progress on the effects of irradiation with neutrons and X rays at stages of embryonic development that are particularly susceptible to radiation-induced malformations of the brain. One such critical time is gestation day 8.5; in most of the experiments to date, the embryos were irradiated at this time.

Seven-week-old mice (CD-1 strain, Charles River Mouse Farms) were allowed mating opportunity for 12 to 13 hr beginning at 7 PM. The mating groups consisted of one male and three females; the dark period in the animal room was from 8 PM to 8 AM. In the morning the males were removed, and the females were examined; those with a copulation plug were grouped for later exposure to X rays or fast neutrons or for sham irradiation. At various times after irradiation the embryos were examined. The neutron source was the Health Physics Research Reactor at ORNL, and the source of X rays was a 250-kV constant-potential machine.

Preliminary results indicate a relative biological effectiveness for neutrons of approximately 4.5 for survival to gestation day 16 after irradiation at 8.5 days. The following radiation doses were given: 30, 40, and 50 rads of neutrons and 100, 160, 200, and 300 rads of X rays.

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RESISTANCE OF GERMFREE ATHYMIC MICE TO HYDROCARBON SKIN CARCINOGENESIS

J. M. Holland, L. C. Gipson, and M. S. Whitaker

We have observed that germfree athymic mice are resistant to initiation-promotion skin carcinogenesis under conditions that induce multiple benign and malignant squamous-cell tumors in age- and sex-matched normal littermate controls.

Thirty-six healthy female athymic nude mice and 21 female normal littermates between 6 and 10 months of age were treated with 200 nmol of dimethylbenz[*a*]anthracene (DMBA) under germfree conditions. Carcinogen was applied in a 50-μl volume of spectrophotometric-grade acetone to the intrascapular skin. One week later the same mice were treated with 10 μg of 12-O-tetradecanolylphorbol-13-acetate (TPA) in a 50-μl volume of acetone. TPA promotion continued twice weekly until the tumor response saturated.

After 16 weeks of promotion, the results were as follows: 19 of 21 normal littermate mice had developed one or more typical squamous-cell tumors in the area of treatment, while no tumors were observed in any of the 36 athymic nudes. The average latency, or time after initiation to the development of macroscopic skin tumors, in normal littermate mice was 68 $\pm$ 5 days, with an average of 2.4 tumors per mouse after 16 weeks.

In addition to the total absence of tumors in athymic mice, there also appeared to be a lack of response to the promoter. Normal littermate mice respond to twice weekly TPA with a typical mild acute inflammatory response that subsides and is succeeded by a more chronic hyperplastic response characterized by epidermal thickening, hyperkeratosis, and parakeratosis, as well as accelerated hair regrowth. Nude mice exhibited none of these changes, suggesting that resistance to skin-tumor induction may be the result of failure to respond to promoter, although it is not possible on the basis of information currently available to exclude other factors, such as a genetically determined difference in carcinogen metabolism, a lack of available or sensitive target cells, or homeostatic imbalance due to thymic aplasia that indirectly inhibits tumor development. Whichever, if any, of these alternatives prove to be correct, this observation affords an opportunity to explore some of the many genetically controlled host variables controlling sensitivity to a carcinogen.

OPTIMUM CONDITIONS FOR THYMUS RECONSTITUTION OF GENETICALLY ATHYMIC MICE

J. M. Holland, E. H. Perkins, M. S. Whitaker, and L. C. Gipson

For many reasons it is frequently necessary to prepare thymus-reconstituted athymic nude mice. This may be accomplished in various ways and with varying results. It has been argued that more stable recovery of T-cell competence is obtained if young adult thymus is used as donor tissue prior to the onset of involution that begins at sexual maturity. Alternatively, others argue that fewer complications arise when newborn thymus is used. From the perspective of the graft recipient, age is also a potentially important variable; the older the nude mouse when grafted, the more likely that it will be in the early phases of wasting disease. If this is true, then thymus grafts would be less likely to survive. Conversely, the older animal may represent a more stable hormonal background upon which to establish a graft. In order to obtain information that would permit a choice of procedure and to establish objective criteria for acquisition of T-cell competence, we conducted the following experiments. Equal numbers of eight- to ten-generation-backcrossed C3H nude mice were divided into four groups. Group A mice were grafted with 1- to 3-day-old C3H thymus when 3 to 5 days old (newborn into newborn). Group B were grafted with 4- to 6-week-old thymus when 3 to 5 days old (young adult into newborn). Group C were grafted with 1- to 3-day-old thymus when they were 4 to 6 weeks old (newborn into young adult), and group D were grafted with 4- to 6-week-old thymus when they were 4 to 6 weeks old (young adult into young adult). In all instances, the sexes of the thymus donor and the recipient were matched, and the graft, consisting of both thymus lobes, was implanted subcutaneously between the shoulders.

To determine which graft technique resulted in the greatest probability of stable T-cell recovery, thymic function was measured in grafted mice by several techniques. Thirty days after implanting thymus, skin allografts were made using BALB/c mice as donor. After skin grafts, mice were observed for 60 days in order to score acceptance or rejection. At the end of this time, mice were killed, and the capacity of a spleen cell suspension to respond to a panel of mitogens was determined. In addition, spleen cells from reconstituted athymic, athymic, and normal littermate mice were assayed in a modified graft-vs-

host (GVH) assay for their T-cell-dependent proliferative capacity in the spleen of lethally irradiated noncompatible recipients. Table 42 summarizes the results of control studies on athymic and littermate spleen cell cultures stimulated with various mitogens *in vitro*. The data were calculated from two separate experiments using three-spleen pools for both athymic and littermate control.

The response of nude spleen cells to the T-cell mitogens PHA and concanavalin A (con A) was approximately 7% of control. The response to the predominantly B-cell mitogen, bacterial lipopolysaccharide, was also slightly reduced (about 85% of control). This suggests that some T-helper cell activity may be necessary for a normal response, even to homopolymeric antigens. The capacity of spleen cells from athymic donors to proliferate in response to an allogenic stimulus was also depressed, but extreme variation was observed. We explain this variation on the basis that incorporation of thymidine measures total cell proliferation and does not discriminate between immunologic (T-cell-dependent) and nonimmunologic cell proliferation (hematopoietic recovery). The utility of this end point must, therefore, depend on the development of techniques for the selective elimination of nonimmunologic cell proliferation or the estimation of the relative fraction of thymidine incorporated by hematopoietic vs immunoreactive cell types.

Using the criteria of mitogen responsiveness, GVH-inducing capacity, and visible rejection of full-thickness skin allografts, nude mice were compared after thymus reconstitution. For each of the donor-recipient combinations, skin-graft acceptances and rejections were noted. Included in the rejection category were grafts judged to be undergoing slow or delayed rejection characteristic of weak (non-H-2) responses. Based on the fraction of nude mice that were sufficiently thymus-reconstituted to reject a skin allograft, the combination of adult thymus in adult nudes was clearly inferior. The other donor-recipient combinations were relatively effective, with the combination of newborn thymus into adult nude the most efficacious.

The question of the significance of allograft rejection as a criterion of T-cell status was addressed by measuring the response of spleen cells to mitogens *in vitro* and their capacity to respond to alloantigens in the GVH reaction. Spleens were pooled on the basis of whether the animal had accepted or rejected the skin grafts within each donor-recipient group. The response was expressed as a percent of age-matched normal littermate values. In animals rejecting their skin grafts, there was measurable recovery of T-cell competence. It is of interest to determine what percent of control PHA or con A response is necessary in order to initiate and sustain allograft rejection as well as a GVH response. A secondary question not answered by these experiments is: How stable is the recovery of T-cell competence?

In conclusion, we believe we have demonstrated good correlation between allograft rejection and T-cell mitogen response and a similarly positive correlation between allograft rejection and GVH response. Although the GVH response does not appear to be as sensitive as the mitogen response, mitogen response can be used to evaluate thymus reconstitution much more readily than either skin graft or GVH reactions. Using the micro peripheral blood leukocyte method, it should be possible to monitor individual reconstituted nude mice over time. None of the graft-recipient combinations completely restored normal T-cell competence using these criteria. However, functional immune competence was achieved with greater apparent efficacy with one of the four possible combinations.

Table 42. Mitogen and GVH responsiveness of normal littermate and athymic nude mouse spleen cells

Phenotype	Mitogen response[a]				GVH response[b]
	LPS	con A	PHA	Control	
Normal	34.2 ± 0.8	46.9 ± 0.6	44.0 ± 4.4	2.6 ± 0.2	59.5 ± 6.5
Athymic	29.1 ± 3.0	3.6 ± 0.1	3.0 ± 0.1	2.9 ± 0.06	21.5 ± 23.9

[a]Thousands of counts per minute per 5×10^5 spleen cells.

[b]Thousands of counts per minute per spleen in lethally irradiated BALB/c mice injected iv with C3H nude or littermate spleen cells.

PLASMA ESTERASE ALTERATIONS IN HEPATOMA-BEARING MICE

R. L. Tyndall* and J. M. Holland

Our recent studies have shown specific plasma esterase alterations in mice treated with the carcinogen DEN or the antioxidant BHT or both chemicals in combination. These alterations occurred within days after exposure and closely correlated with the eventual tumor formation. In addition, mice fed a variety of other carcinogens reproducibly showed the same plasma esterase alterations. Most of the chemicals tested were both potent inducers of hepatocellular tumors and hepatotoxins. Studies were undertaken to determine if the esterase alterations induced by exposure to hepatic carcinogens, months before any histologic changes occur, mirror those seen in the plasma of mice bearing spontaneous or X-ray-induced hepatomas, where the presence of a carcinogen or toxin was not a contributing factor. Eight-week-old C3H mice were either X-rayed with 250 R or were not treated. Twelve months later, plasma was collected, and all mice were necropsied and examined grossly and histologically. Plasma proteins were separated electrophoretically, and the esterase profiles were determined using α-naphthyl acetate as a substrate. Approximately 100 mice were examined. All mice with X-ray-induced or spontaneous hepatomas had a marked increase in two specific plasma esterase bands. While these two esterase bands are also increased in carcinogen-treated mice, other esterase band changes in mice exposed to carcinogens were not apparent in hepatoma-bearing mice. Analysis of plasma from mice bearing transplantable hepatomas showed two patterns of esterase alterations. One pattern was identical to that seen in mice bearing primary hepatomas, whereas another pattern showed diminution of three specific esterase bands.

These results suggest that plasma esterase analysis may be diagnostic of primary hepatomas and, in addition, can differentiate between some but not all transplantable hepatomas. These data also support the concept that early plasma esterase alterations in mice fed carcinogens capable of inducing hepatocellular tumors may serve as a useful prescreen for such chemicals. Much work is required to establish the basis for the observed changes.

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ISOLATION OF *ACANTHAMOEBA* FROM CULTURED TUMOR CELLS

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On culturing human choriocarcinoma cells in an enriched medium, a subpopulation of small mobile cells emerged. By selective passage, these cells were freed of the choriocarcinoma cells. Morphologic and serologic analysis identified these cells as an *Acanthamoeba*.

The amoeba was pathogenic for a variety of cultured malignant and normal mammalian cells. Unlike known species of pathogenic *Acanthamoeba*, however, this strain did not produce the typically fatal meningoencephalitis when inoculated intranasally into mice.

Histochemical analysis showed the cells to be rich in alkaline phosphatase and nonspecific esterases. While morphologically similar and serologically cross-reactive with known species of *Acanthamoeba*, the "Oak Ridge strain" was not identifiable with any known species. Likewise, electrophoretically separated esterase profiles of cell extracts differed markedly from those of species which showed some serologic relationship. The culturing of four other established tumor cell lines in a manner analogous to that which yielded the Oak Ridge strain also resulted in the emergence of acanthamoebae. Two of these isolates were shown to be *Acanthamoeba culbertsoni*; this is only the second time that this species has been found since its original isolation from cultured monkey kidney cells over a decade ago. The remaining two amoeba isolates have yet to be identified.

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REPRODUCIBILITY OF PLASMA ESTERASE ALTERATIONS IN MICE FED CHEMICAL CARCINOGENS

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We recently reported the alteration of plasma esterase profiles in mice fed a variety of chemical carcinogens; we also reported the lack of change in

mice fed related noncarcinogenic chemicals. In order to test whether the microflora environment of test mice would influence the reproducibility of the esterase change, we tested a variety of carcinogens, noncarcinogens, and the hepatotoxin carbon tetrachloride (CCl_4) in barrier-derived and maintained, barrier-derived and conventionalized, and commercially available BALB/c mice.

Mice treated with the potent carcinogens DEN, dinitrosopiperazine, dipropylnitrosamine, dimethylhydrazine, urethane, and dimethyldinitrosopiperazine had similarly altered plasma esterase profiles after 7 days' exposure to the chemicals. Some esterase alterations persisted after cessation of treatment. Exposure to high concentrations of the weakly carcinogenic or noncarcinogenic compounds nitrosohydroxyproline, nitrosomethoxymethylamine, 1-nitroso-4-methylpiperazine, nitroso-2,6-dimethylpiperidine, and ethyl methanesulfonate caused no obvious plasma esterase alterations. The ingestion of carbon tetrachloride resulted in a plasma esterase alteration different from that seen in carcinogen-treated mice. Analysis of serum from test mice for levels of serum oxalacetic transaminase (SGOT), alkaline phosphatase (AP), lactate dehydrogenase-lactate substrate (LDH), and D-γ-glutamyl transpeptidase did not differentiate between mice treated with selected carcinogens and those treated with noncarcinogens and/or carbon tetrachloride.

The esterase alteration in carcinogen-treated mice was not affected by the source of the test mice. Conversely, the microflora environment of the mice noticeably affected the plasma levels of LDH and SGOT, thus adding to the unpredictability of these enzymes in differentiating between the effects of carcinogens, noncarcinogens, and carbon tetrachloride. The effects of other carcinogens, initiators, promoters, and toxins on plasma esterase profiles are now being studied to determine the possible value of such early changes as a rapid *in vivo* prescreen for environmental carcinogens or toxins.

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THE INFLUENCE OF DOSE AND DOSE RATE ON THE INCIDENCE OF NEOPLASTIC DISEASE IN RFM MICE AFTER NEUTRON IRRADIATION

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Over the last few years we have been conducting experiments with female RFM and BALB/c mice comparing the late biological effects of various doses of neutrons at a low dose rate, using ^{252}Cf, with the same doses delivered at a high dose rate from a reactor. These studies are sufficiently complete to allow analysis of the tumor incidence in the RFM portion of the experiment. In addition, tumor incidence data from a previous study using high-dose-rate gamma-ray exposures (JBS, unpublished data) have allowed us to compare the relative tumorigenicity of neutron and gamma radiation in a number of tumor systems. In general, a comparison of acute neutron- and gamma-irradiation data indicated that tumors were induced more effectively with neutrons than with gamma rays, particularly at low doses.

For thymic lymphoma induction by neutrons, linear dose relationships were obtained at both dose rates, whereas a dose-squared relationship was obtained for gamma rays. At low doses, little dose-rate dependence was seen with neutrons; however, at the highest dose, the low dose rate was more effective.

An increased incidence of lung adenomas was seen after neutron irradiation with doses as low as 20 rads, whereas the incidence after gamma-ray irradiation decreased with increased dose except at approximately 300 rads. In spite of the apparent sensitivity, the neutron dose-response curve for lung tumors was not linear, since doses of 5 and 10 rads failed to cause an increase in tumor incidence above that of the control group.

Examination of the influence of dose rate on tumor induction suggests that neutron irradiation at low dose rates may have induced thymic lymphomas and possibly lung adenomas more efficiently than at high dose rates under some circumstances. However, low-dose-rate neutron irradiation was less effective than high-dose-rate irradiation in inducing ovarian and pituitary tumors.

These data indicate that the influence of dose rate on the tumorigenicity of high-LET (linear energy

transfer) irradiation may be more complex than previously thought. It appears that the presence, magnitude, and direction of any dose-rate effect may depend upon a number of factors (including age-dependent changes in sensitivity, the total dose, and the tissue type), all of which would determine the relative contributions made by the initiating, promoting, and modifying mechanisms involved in the particular carcinogenic process. Further experiments are being undertaken to examine these dose-rate relationships in more detail and to establish the basis for the effects observed.

LUNG-TUMOR INDUCTION IN FEMALE RFM MICE AFTER LOCALIZED IRRADIATION

R. L. Ullrich and M. C. Jernigan

Most investigators have reported that the incidence of murine lung tumors after exposure to whole-body neutron irradiation is lower than in the control animals. However, these studies have been complicated by the fact that many animals may have died as a result of mortality from other radiation-induced disease or injury before any lung tumors could be detected. Recent data from our laboratory using age-adjustment procedures to correct for differences in mortality among groups receiving whole-body neutron exposures have indicated an increase in the incidence of lung adenomas in female RFM mice with doses as low as 20 rads.

As a result of these data, we have undertaken a more detailed examination of some of the factors that may influence the incidence of these lung adenomas using an assay system previously described by Yuhas and Walker.[1] In this system, animals are given localized thoracic radiation exposure. Nine months after treatment the animals are killed, their lungs cleared, and the tumors scored as percent incidence and as tumors per mouse. This localized irradiation procedure allows the use of doses sufficient to influence lung-tumor incidence without the early death from other causes that can complicate whole-body studies.

Data obtained thus far indicate results similar to those obtained using the age-adjustment procedure; namely, the incidence of lung tumors and tumors per mouse increased with increasing dose over the range from 25 to 100 rads and began to decline over the range of 200 to 300 rads. Although both approaches yield similar results, the lung-clearing assay has the advantage of a relatively short time between treatment and analysis with negligible mortality and also allows a more quantitative analysis of the carcinogenic process. Additional experiments using this system to examine some of the possible biological and radiobiological factors that may influence or modify the carcinogenic process after exposure to high-LET and low-LET radiation are currently under way.

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1. J. M. Yuhas and A. E. Walker, *Radiat. Res.* **54**, 261–273 (1973).

THE EFFECTS OF COMBINED IRRADIATION THERAPY AND IMMUNOTHERAPY WITH *CORYNEBACTERIUM PARVUM* ON TUMOR GROWTH AND METASTASIS

R. L. Ullrich and L. M. Adams

Since the immune system is only able to exert its antitumor actions on a limited number of tumor cells, immunologic approaches to tumor therapy may be most effective in combination with conventional cytoreductive therapies, such as radiation therapy. This type of combined therapy could allow the effective control of distant metastasis while at the same time achieving better local tumor control.

We have been studying the effectiveness of combined radiation therapy and immunotherapy with *C. parvum* using the line 1 alveolar cell carcinoma. The purpose of these studies is to identify the radiobiological and immunological factors of importance in this type of cancer therapy. The line 1 tumor is a transplantable murine tumor, which, unlike most others, kills the host by means of metastatic spread. In fact, attempts to cure the tumor with radiation often fail, in spite of local control, because of metastatic spread. This system allows us to study not only the interaction of radiation therapy and *C. parvum* on local control but also on metastasis.

Although treatment with *C. parvum* by itself has little influence on the growth and metastasis of this tumor, we have been able to obtain significantly higher levels of local control than with radiation alone and have also achieved significant control over the metastatic spread using combined radiotherapy and immunotherapy. In fact, even in those animals in which we do not obtain local control, we have increased the survival time from 45 to 90 days using combined therapy. This implies that even in animals whose tumors were not locally controlled, we have been able to control metastatic spread to some

degree. We are currently examining in more detail the influence of the route of *C. parvum* treatment and of the treatment timing on the overall effectiveness of the combined therapy. Studies have also begun which are designed to define the components of the immune system which are involved in the control of metastasis.

MECHANISMS OF NITROSAMINE CARCINOGENESIS*

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In continuing studies of the possible relation of *N*-nitroso compounds formed *in vivo* to human cancer, the feeding of several secondary and tertiary amines used as drugs and agricultural chemicals together with nitrite to rats has yielded some significant positive results. In such experiments, aminopyrine, oxytetracycline, and methapyrilene have given rise to liver tumors; chlordiasepoxide to tumors of the nervous system; and dimethyldodecylamine to bladder tumors. Lucanthone without nitrite has induced liver tumors. No other compounds have induced tumors alone or together with nitrite.

The *N*-nitroso derivatives of several *N*-methyl carbamate insecticides (including carbaryl and methomyl) have induced tumors of the forestomach. Mouse-skin-painting tests of these compounds and a variety of other nitrosamides have been negative, except for methylnitrosonitroguanidine (MNNG), ethylnitrosonitroguanidine, and methylnitrosourea.

Examination of the relation between chemical structure and carcinogenic activity has involved the testing of a series of substituted cyclic nitrosamines and a series of aliphatic nitrosamines given orally to rats at equimolar doses. The results point to cleavage of a hydrogen atom from the molecule, which is alpha to the nitroso function as the rate-limiting step in carcinogenesis. Replacement of the alpha hydrogens by deuterium has reduced the carcinogenic activity of dimethylnitrosamine (DMN), nitrosomorpholine, and nitrosoazetidine. Methyl substitution at these positions reduces carcinogenic activity, sometimes to zero, whereas in the cyclic series, methyl substitution at other positions often enhances carcinogenic activity. Carboxyl and cyano groups eliminate carcinogenic activity, while halogens enhance it greatly. Hydroxyl substitution greatly reduces carcinogenic activity in the aliphatic series but enhances it in the cyclic series. Nitrosomethyldodecylamine is a bladder carcinogen in rats. Nitroso-2,6-dimethylmorpholine induces esophageal tumors in rats, but pancreatic-duct tumors in hamsters.

Examination of the mechanism of nitrosation of tertiary amines has continued, but it is not yet elucidated. Ready disproportionation of 1-nitrosopiperazine and, to a lesser extent, 1-methyl-4-nitrosopiperazine in dilute acid has been observed, and this is the first example of such a reaction with an aliphatic nitrosamine.

A sensitive detector has been developed for nitrosamides and has been applied to the analysis of nitrosated pesticides.

A comparison of the metabolism of three ^{14}C-labeled carcinogenic cyclic nitrosamines with different organ specificities is in progress. Although at high acutely toxic doses the proportion of carbon excreted as carbon dioxide is quite small, at lower (carcinogenic) doses the proportion converted to carbon dioxide is quite high (30 to 75%). This indicates that the cyclic nitrosamines are metabolized as readily as the open-chain compounds and that the limiting factor is the amount of enzyme present in the liver. Of the administered dose of radioactivity, most is found in the cytoplasm of both target and nontarget organs. There is very little activity in the nucleus and almost none in the DNA of the nucleus. Most of the radioactivity is protein-bound, an especially large amount occurring in the liver.

A compendium of the mass spectra of *N*-nitroso compounds has been compiled as an aid to their identification.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

† Consultant.

STUDIES ON THE MECHANISM OF ACTION OF PHORBOL ESTER TUMOR PROMOTERS*

T. J. Slaga, A. Viaje,† S. H. Yuspa,‡ U. Lichti,‡ and H. Hennings‡

Dose-response relationships on the abilities of several phorbol ester tumor promoters to promote skin tumors after DMBA initiation and to bring about edema, inflammation, and epidermal hyperplasia were determined in female Charles River CD-1 mice. The promoting ability of the potent synthetic promoter phorbol 12,13-dioctanoate ($P.diC_8$) was determined over a dose range of 0.1 to 10

μg per application. Administration of P.diC$_8$ two times weekly at dosages of 4, 6, 8, and 10 μg gave little variation in tumor response. A dose-dependent tumor response occurred at dosages of from 1 to 4 μg of P.diC$_8$. Only one papilloma was observed when P.diC$_8$ was given twice weekly at a dose of 0.1 or 0.5 μg. A similar dose-response relationship was observed for the ability of P.diC$_8$ to stimulate epidermal hyperplasia. When other phorbol esters were investigated, the results indicated an excellent correlation between their promoting ability and their ability to induce epidermal hyperplasia. However, the correlation fails when other compounds outside the phorbol ester series (acetic acid, cantharidin, and ethyl phenylpropiolate) are compared.

We also studied the effects of phorbol ester tumor promoters on ornithine decarboxylase (ODC) activity and DNA synthesis in mouse epidermal cell cultures. Phorbol ester tumor promoters stimulated ODC activity and DNA synthesis in mouse epidermal cell cultures. The maximum stimulation of ODC occurs 6 to 12 hr after a 1-hr pulse dose of promoter, whereas DNA synthesis reaches a maximum at 72 to 96 hr after exposure. The tumor-promoting potency *in vivo* of a series of phorbol esters correlates well with their ability to stimulate ODC activity and DNA synthesis *in vitro*. Non-promoters are inactive in this *in vitro* system. These results suggest that mouse epidermal cell cultures are a valid model for studying the mechanisms of tumor promotion and may be a convenient assay system for assessing promoter potency.

*Research supported in part by NIH Grant CA-17605.

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MECHANISM OF ACTION OF STEROIDAL ANTI-INFLAMMATORY AGENTS THAT INHIBIT SKIN CARCINOGENESIS*

T. J. Slaga, J. A. Schwarz,†
S. Thompson,† and A. Viaje‡

The effects of steroidal anti-inflammatory agents on both the initiation and promotion phases of skin tumorigenesis were investigated. We first studied the relationship between the inhibition of mouse skin tumor promotion and the inhibition of epidermal DNA synthesis by the steroidal anti-inflammatory agent fluocinolone acetonide (FA). Simultaneous doses of either 10, 1, or 0.1 μg of FA and a phorbol ester tumor promoter resulted in almost complete inhibition of promotion, whereas 0.01 and 0.001 μg of FA resulted in inhibition rates of 75% and 15% respectively. With the exception of 0.001 μg of FA, which had a borderline effect, the above doses of FA inhibited epidermal DNA synthesis by at least 60% for 24 hr in adult mice. In contrast, when applied topically to newborn mice, FA did not inhibit epidermal DNA synthesis but stimulated it by about twofold at 48 hr after treatment. Newborn mice are also insensitive to the stimulatory effect of phorbol esters. In cultures of newborn epidermal cells, treatment with FA caused an early stimulation of DNA synthesis followed by a 50% inhibition 2 to 3 days after a pulse treatment. In adult mice, there appears to be a relationship between the inhibition of tumor promotion and epidermal DNA synthesis.

Topical applications of 75 μg of dexamethasone or 10 μg of FA were found to be effective inhibitors of mouse-skin initiation and complete carcinogenesis. Aryl hydrocarbon hydroxylase (AHH) in epidermis was only slightly stimulated by 75 μg of dexamethasone, with a peak of 180% of controls at 2 hr. Dexamethasone did not inhibit benz[*a*]anthracene-induced AHH activity *in vitro* when added to the assay, even at concentrations greater than equimolar amounts of the substrate, benzo[*a*]pyrene (BP). Other steroids tested were effective in inhibiting AHH *in vitro* in the following order: estradiol > estrone > estriol > testosterone > progesterone. Topical application of either 75 μg of dexamethasone or 10 μg of FA together with 3-methylcholanthrene inhibited the induction of AHH by 3-methylcholanthrene by at least 50% for 18 hr. Dexamethasone and FA may inhibit tumor promotion by inhibiting DNA synthesis, thus counteracting the induction of hyperplasia induced by tumor promoters, whereas their inhibition of tumor initiation may be related to their inhibition of the metabolism of initiators to ultimate carcinogenic form(s).

*Research supported in part by NIH Grant CA-17605.

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‡ Research Assistant supported by Grant 7R01 CA20076 from the NIH.

THE ROLE OF EPIDERMAL ARYL HYDROCARBON HYDROXYLASE IN THE COVALENT BINDING OF POLYCYCLIC HYDROCARBONS TO DNA AND ITS RELATIONSHIP TO TUMOR INITIATION*

S. G. Buty,† S. Thompson,† and T. J. Slaga

Mouse epidermis contains an NADPH-dependent aryl hydrocarbon hydroxylase which is inducible by polycyclic aromatic hydrocarbons. In general, unsubstituted polycyclic aromatic hydrocarbons caused a greater induction of epidermal aryl hydrocarbon hydroxylase than substituted ones (1,2,3,4-dibenzanthracene > 1,2,5,6-dibenzanthracene > 3-methylcholanthrene >> DMBA), which did not correlate with their ability to initiate tumors in mouse skin. The covalent binding of radioactive 3-methylcholanthrene to DNA by epidermal aryl hydrocarbon hydroxylase *in vitro* (as a function of time after induction with unlabeled 3-methylcholanthrene *in vivo*) indicated that the specific activity of binding reached a peak by 12 hr. A correlation appears to exist between the tumor-initiating ability of several hydrocarbons and their ability to covalently bind to DNA *in vitro* using mouse epidermal homogenates and NADPH as an electrophile generating system (DMBA > 3-methylcholanthrene > BP > 1,2,5,6-dibenzanthracene > 1,2,3,4-dibenzanthracene). Also, the specific activity of covalent binding of BP to different nucleic acids was as follows: polyguanylate > polyadenylate > DNA.

*Research supported by NIH Grant CA-17605.

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EFFECTS OF BENZOFLAVONES AND TRICHLOROPROPENE OXIDE ON POLYNUCLEAR AROMATIC HYDROCARBON METABOLISM AND INITIATION OF SKIN TUMORS*

T. J. Slaga, D. L. Berry, M. R. Juchau,† and S. Thompson‡

7,8-Benzoflavone (7,8-BF) was found to be a potent inhibitor of the initiation of skin tumors by 3-methylcholanthrene (MC) and DMBA. However, 7,8-BF enhances the initiating ability of 1,2,5,6-dibenzanthracene (DBA) and BP in mouse skin. 5,6-Benzoflavone (5,6-BF) inhibited tumor initiation by MC and DMBA but to a lesser degree than 7,8-BF. Dose-response studies of the capacity of 7,8-BF to inhibit tumor initiation by DMBA revealed that 7,8-BF was an effective inhibitor at concentrations equivalent to that of DMBA, and a maximum inhibition was observed at 20 times that of DMBA. Epidermal aryl hydrocarbon hydroxylase was increased by 5,6-BF and inhibited by 7,8-BF when given either topically or ip. Both flavones when added directly to the assay tubes greatly inhibited the *in vitro* epidermal aryl hydrocarbon hydroxylase activity from control mice and mice pretreated with benz[*a*]anthracene. When added *in vitro*, 7,8-BF and 5,6-BF inhibited the epidermal NADPH-dependent covalent binding of radioactive MC, DMBA, DBA, or BP by 50% or more. Trichloropropene oxide (TCPO) only slightly increased the *in vitro* covalent binding of the above polycyclic hydrocarbons to DNA. TCPO was effective in increasing the skin-tumor-initiating ability of MC, BP, and BP-4,5-epoxide but not the initiating ability of DMBA and DBA. MC-11,12-epoxide and BP-4,5-epoxide were very weak initiators when compared with the parent hydrocarbons. A correlation appears to exist between the tumor-initiating ability of several polycyclic hydrocarbons and their ability to be converted *in vitro* by epidermal microsomes to an electrophilic intermediate(s); however, the action mechanisms of the benzoflavones and TCPO are still in question.

* Research supported by NIH Grant CA-17605.

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THE EFFECTS OF ANTIOXIDANTS AND ANTI-INFLAMMATORY AGENTS ON MOUSE-SKIN-TUMOR INITIATION AND ARYL HYDROCARBON HYDROXYLASE*

T. J. Slaga, W. Bracken,† and A. Viaje†

The antioxidants BHT, butylated hydroxyanisole (BHA), and vitamins C and E are effective inhibitors of DMBA tumor initiation in a two-stage system of tumorigenesis. These antioxidants did not significantly induce epidermal aryl hydrocarbon (BP) hydroxylase (AHH), nor did they have any effect when added directly to the *in vitro* AHH assay. However, BHT and BHA, when applied topically to mice, inhibited the *in vitro* epidermally mediated

covalent binding of radioactive BP and DMBA to DNA. When BHT and BHA were added *in vitro*, they did not inhibit the epidermally mediated covalent binding of the hydrocarbons to DNA. The inhibition of polycyclic hydrocarbon tumorigenesis by antioxidants may be related to their ability to prevent the *in vivo* activation of hydrocarbons to carcinogenic epoxides and/or other electrophilic intermediates.

The effects of various anti-inflammatory agents on mouse skin tumorigenesis and AHH were also investigated. Oxyphenbutazone, a nonsteroidal anti-inflammatory agent, inhibited tumor initiation by 3-methylcholanthrene but was less effective than the steroidal anti-inflammatory agent dexamethasone. Oxyphenbutazone was not found to induce AHH activity in mouse epidermis, whereas Indomethacin and Seclazone had a slight inducing effect. When these agents were added directly to the *in vitro* AHH assay, they did not inhibit AHH activity. However, additional experiments have shown a decreased epidermally mediated covalent binding of 3-methylcholanthrene to DNA *in vitro* when the epidermal homogenates were isolated from mice pretreated with either dexamethasone or oxyphenbutazone and 3-methylcholanthrene at 3 or 12 hr before killing.

*Research supported by NIH Grant CA-17605.

† Research Assistant, supported by Grant 7R01 CA20076 from the NIH.

INHIBITION OF THE TUMOR-INITIATING ABILITY OF THE POTENT CARCINOGEN 7,12-DIMETHYLBENZ[*a*]ANTHRACENE BY THE WEAK TUMOR INITIATOR 1,2,3,4-DIBENZANTHRACENE*

T. J. Slaga and R. K. Boutwell†

Aryl hydrocarbon hydroxylase (AHH) in mouse epidermis was inducible by topical application of several tumor-initiating polycyclic hydrocarbons. The weak tumor initiator 1,2,3,4-dibenzanthacene (1,2,3,4-DBA), at a dose level of 200 nmol, increased AHH activity more than tenfold over the acetone controls at 12 hr after treatment. Administration of the same quantity of the potent initiator DMBA induced AHH activity by approximately fourfold at 12 hr after treatment. Simultaneous treatment with 200 or 100 nmol of DMBA and with 1,2,3,4-DBA resulted in AHH activity that was 546% and 732%, respectively, of the controls 12 hr following treatment. The amount of AHH activity was less than that observed when 1,2,3,4-DBA was administered alone. Doses of 20 nmol or more of 1,2,3,4-DBA, when given at about the same time as DMBA, effectively inhibited the DMBA initiation of skin tumors in a two-stage system of tumorigenesis. The results suggest that the weak initiator 1,2,3,4-DBA may program the epidermal AHH system to metabolize the strong carcinogen DMBA to noncarcinogenic intermediate(s).

*Research supported in part by NIH Grants CA-17605, CA-07175, and CA-05002.

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METABOLISM OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE IN MOUSE-SKIN HOMOGENATES ANALYZED WITH HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

J. DiGiovanni,† T. J. Slaga, D. L. Berry, and M. R. Juchau†

The metabolism of DMBA was analyzed with high-pressure liquid chromotography using epidermal homogenates from mice pretreated with 3-methylcholanthrene. Metabolism was undetectable in the absence of pretreatment. Specific activities in epidermal homogenates from pretreated mice were found to be approximately 100 to 1000 times lower than those observed in comparable incubations containing hepatic microsomes from rats pretreated with 3-methylcholanthrene. The major metabolite found was 7-hydroxymethyl-12-methylbenz[*a*]anthracene. Each of the known hydroxymethyl metabolites was found in detectable quantities. The *K*-region diol was not detectably present in incubations with mouse-skin homogenates or rat-liver microsomes from animals pretreated with 3-methylcholanthrene. 7,8-Benzoflavone was a potent inhibitor of both DMBA tumor initiation and metabolic transformation *in vitro*. 5,6-Benzoflavone also inhibited both tumor initiation and metabolism *in vitro*, but to a lesser degree than 7,8-benzoflavone. BHT inhibited DMBA tumor initiation by approximately 50%, while having little effect on or enhancing metabolite formation. 17-β-Estradiol, testosterone, and 1,1,1-trichloro-2,3-propene oxide (TCPO) had no effect on initiation of tumors with DMBA. However, 17-β-estradiol drastically in-

hibited metabolite formation *in vitro*. TCPO either had little effect on or enhanced the formation of all DMBA metabolites, with the exception of one (as yet unidentified) polar metabolite whose formation was inhibited. This metabolite was tentatively identified as a diol.

*Research supported in part by DHEW Grant CA-17605 and Training Grant GM-00109.

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BIOTRANSFORMATION OF BENZO[*a*]PYRENE IN MOUSE EPIDERMIS*

D. L. Berry, T. J. Slaga, S. G. Buty,† and M. R. Juchau‡

Mouse epidermal homogenates metabolized BP, as evidenced by high-pressure liquid chromatography. The NADPH-dependent metabolism was linear with time up to 60 min. The formation of dihydrodiols relative to phenols was less than in liver. Epoxide hydrase activity using BP-4,5-oxide as a substrate was less in mouse epidermis than in mouse liver. Covalent binding of BP to DNA *in vitro* by an NADPH-dependent epidermal homogenate was linear for 90 min, after which binding plateaued. A second addition of NADPH resulted in a linear increase in DNA binding for approximately 90 additional minutes. The specific activity of BP binding to DNA by epidermal homogenate *in vitro* reached a peak at 12 hr after induction with unlabeled BP. Either a single treatment or pretreatment with trichloropropene oxide at 6 hr prior to, zero time, and 6 hr after initiation caused a slight increase in BP-4,5-oxide initiating ability. Pretreatment with croton oil 18 hr prior to initiation with BP-4,5-oxide followed by TPA (12-O-tetradecanoylphorbol-13-acetate) promotion slightly enhanced initiation.

*Research supported by DHEW Grant CA-17605 and Training Grant GM-00109.

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SKIN-TUMOR-INITIATING ABILITY OF THE 4,5-EPOXIDE, 7,8-EPOXIDE, 7,8-DIOL-9,10-EPOXIDE, AND 7,8-DIOL OF BENZO[*a*]PYRENE*

T. J. Slaga, A. Viaje,† D. L. Berry, and W. Bracken†

The skin-tumor-initiating abilities of both *K*-region and non-*K*-region epoxides of BP were determined in mice using a two-stage system of tumorigenesis. By testing potential carcinogens as tumor initiators in this system, one eliminates the promoting phase from being a limiting factor. BP-4,5-epoxide and BP-7,8-dihydrodiol-9,10-epoxide (anti) were found to be weak tumor initiators, whereas BP-7,8-epoxide had about one-third as much activity as the parent hydrocarbon, BP. However, the 7,8-dihydrodiol of BP was found to be approximately as potent as BP, suggesting that it may be a proximate carcinogen. This finding was true in terms of the latency period, the number of papillomas per mouse, and the percent of mice with tumors.

*Research supported in part by NIH Grant CA-17605.

† Research Assistant supported by Grant 7R01 CA20076 from the NIH.

TRANSPLACENTAL INDUCTION OF CARCINOGEN HYDROXYLATING SYSTEMS WITH 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN*

D. L. Berry, P. K. Zachariah,† M. J. Namkung,† and M. R. Juchau†

The effects of a highly toxic herbicide contaminant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), upon rates of hydroxylation of *N*-2-fluorenyl acetamide (FAA) and of BP were studied in fetal livers, placentas, maternal livers, and adrenal glands of pregnant Sprague-Dawley rats. Aryl hydrocarbon hydroxylase activities were increased 14- and 100-fold in maternal and fetal livers, respectively, but only minimal increases were observed in placentas and adrenal glands. Similar results were obtained with respect to rates of 7-, 5-, *N*-, 3-, and 1-hydroxylations of FAA. Histologic examinations revealed extensive cellular damage in placentas, as well as pathologic changes in fetal and maternal livers. Electron microscopy indicated enlarged mitochondria and

extensive glycogen deposition in maternal and fetal livers, along with increases in amounts of rough endoplasmic reticulum in fetal livers. Analyses of BP metabolism with high-pressure liquid chromatography also revealed that the formation of diols (relative to phenols) was markedly increased in fetal and maternal livers following treatments with TCDD. The results suggested that the relative lack of response of fetal hepatic mixed-function oxidase systems to commonly employed inducing agents (polycyclic aromatic hydrocarbons) was not due to a lack of appropriate structural and/or regulatory genes required for the expression of enzyme induction during fetal life.

*Supported by Research Grants HD-04839, DHEW; CRBS-250, National Foundation (March of Dimes); and Training Grants GM-01160 and GM-00109, DHEW.

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TRANSPLACENTAL STIMULATION OF BENZO[*a*]PYRENE METABOLISM IN EXTRAHEPATIC TISSUES BY 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN*

D. L. Berry, P. K. Zachariah,† T. J. Slaga, and M. R. Juchau†

Treatment of pregnant rats with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) resulted in a dose-dependent induction of a mixed-function oxidase system in fetal and maternal hepatic and extrahepatic tissues. At doses of 6 μg/kg, aryl hydrocarbon hydroxylase (AHH) activity was increased 24-, 22-, and 4-fold in fetal lung, kidney, and skin, respectively, while maternal lung, kidney, and adrenal AHH activity was increased 4-, 2-, and 2-fold respectively. High-pressure liquid chromatographic analysis of BP metabolism after TCDD induction indicated that fetal lung, kidney, and skin produced significant quantities of benzo[*a*]pyrene-7,8-dihydrodiol (BP-7,8-diol), benzo[*a*]pyrene-4,5-dihydrodiol (BP-4,5-diol), and 9- and 3-phenols of BP. The fetal liver produced benzo[*a*]pyrene-9,10-dihydrodiol (BP-9,10-diol), BP-4,5-diol, BP-7,8-diol, and 9- and 3-phenols of BP. Maternal lung also produced BP-9,10-diol, whereas maternal adrenal gland yielded primarily the 9-phenol of BP. Epoxide hydratase activity was increased two- to threefold in maternal lung, fetal lung, and skin following TCDD pretreatment but was not affected significantly in liver, kidney, or placenta. Treatment of pregnant rats with TCDD increased the covalent binding of BP to DNA in preparations containing maternal liver, lung, and placenta as well as fetal liver, lung, and skin. Pretreatment with TCDD resulted in increased epoxide hydratase and AHH activities in extrahepatic tissues; but only AHH was increased in hepatic tissues, indicating that the inducing capabilities of both phenobarbital and 3-methylcholanthrene differ from but share some similarities with both phenobarbital and 3-methylcholanthrene. Thus, TCDD appears to provide an exceptionally potent and broad-spectrum transplacental induction of carcinogen-transforming enzymes in extrahepatic tissues.

*Supported by Research Grants HD-04839 and CD-17605, DHEW; CRBS-250, National Foundation (March of Dimes); and Training Grants GM-01160 and GM-00109, DHEW.

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ANALYSIS OF THE BIOTRANSFORMATION OF BENZO[*a*]PYRENE IN HUMAN FETAL AND PLACENTAL TISSUES WITH HIGH-PRESSURE LIQUID CHROMATOGRAPHY*

D. L. Berry, P. K. Zachariah,† T. J. Slaga, and M. R. Juchau†

The biotransformation of BP was studied *in vitro* in the presence of microsomal fractions of placental and fetal tissues from humans and monkeys (*Macaca nemestrina*). Metabolites formed in the incubation flasks were extracted and separated by means of high-pressure liquid chromatography utilizing a microparticulate column. In general, the formation of diols and quinones in fetal and placental homogenates was undetectable following 15-min incubations. The formation of phenolic metabolites, however, was easily measurable in fetal liver and lung and in the placenta but not in fetal spleen, kidney, pancreas, or adrenal gland. The latter observation contrasted with high specific activities measured in the fetal adrenal gland with the fluorometric assay for aryl hydrocarbon hydroxylase activity. In placentas from cigarette smokers, relatively large quantities of an unidentified metabolite(s) appeared in metabolic profiles. This metabolite(s) did not cochromatograph with any of the standard metabolites, and the retention time was between those of the 9,10- and 4,5-diols. The same placental tissues catalyzed the covalent binding of BP to DNA and were far more active in this regard than any of the other fetal tissues investigated. The data

indicated a correlation between metabolic profiles and capacity for catalyzing covalent binding to DNA for fetal and placental tissues.

*Supported in part by Research Grants HD-04339 and CA-17605, DHEW; CRBS-250, National Foundation (March of Dimes); and Training Grants GM-01160 and GM-00109, DHEW.

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OXIDATIVE BIOTRANSFORMATION OF 2-ACETYLAMINOFLUORENE IN FETAL AND PLACENTAL TISSUES OF HUMANS AND MONKEYS. CORRELATIONS WITH ARYL HYDROCARBON HYDROXYLASE ACTIVITIES*

M. R. Juchau,† M. J. Namkung,† D. L. Berry, and P. K. Zachariah†

The mixed-function oxidation of ^{14}C-labeled 2-acetylaminofluorene (AAF) was investigated in placental and fetal tissues of humans and monkeys *(M. nemestrina) in vitro*. The major metabolite formed in most tissues was 7-hydroxy-AAF. Rates of the hydroxylation reactions varied widely among the tissues investigated and were generally one to two orders of magnitude lower than those measured in rat hepatic tissues. High correlations among rates of 7-, 5-, and 3-hydroxylations and between 1- and *N*-hydroxylations of AAF were observed. The latter two reactions were less responsive to inhibition by carbon monoxide. Rates of 3-hydroxylation of BP also were highly correlated with rates of 7-, 5-, and 3-hydroxylations of AAF but were not correlated with rates of 1- and *N*-hydroxylations in human placental microsomes. A lack of statistically significant correlations was observed among rates of many of these hydroxylation reactions studied in primate fetal tissues in most instances, but statistically significant correlations among rates of 3-hydroxylation of BP and 1- and *N*-hydroxylations of AAF were observed in those preparations. The results suggested two separate mechanisms for the genetic control of rates of placental aromatic ring- and *N*-hydroxylation reactions as opposed to apparent multiple genetic controls for rates of these hydroxylation reactions in primate fetal tissues.

*Supported by Research Grant HD-04839, DHEW; Training Grant GM-01160, DHEW; and CRBS-250, National Foundation (March of Dimes).

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EFFECT OF THE *Fv-1* GENE ON MURINE LEUKEMIA VIRUS PROVIRAL DNA SYNTHESIS*

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We are currently investigating the mechanism by which the *Fv-1* gene restricts murine leukemia virus replication in mouse cell cultures. We and investigators from several other laboratories have evidence which eliminates the adsorption and penetration events as possible steps sensitive to the restriction by the *Fv-1* gene.[1,2] Recent evidence by Jolicoeur and Baltimore[3] indicates that the *Fv-1*-sensitive step is prior to viral RNA transcription. Our efforts have centered on detecting the synthesis of proviral DNA in permissive and restrictive cells by hybridization to [^{3}H]cDNA complementary to the AKR virus.

We have synthesized ^{3}H-labeled complementary DNA (single strand) by the endogenous reverse transcriptase reaction of detergent-disrupted virions. The cDNA was purified by chloroform–isoamyl alcohol extraction and ethanol precipitation. The precipitate was alkali-digested, neutralized, and chromatographed on Sephadex G-50. Only the material which eluted in the column flow-through was retained as the purified cDNA.

The AKR viral 70 S RNA was isolated by SDS-phenol extraction and velocity sedimentation in sucrose. The unlabeled 70 S RNA was incubated in excess with [^{3}H]cDNA for 24 hr at 66°C in 0.33 *M* NaCl, 3.0 m*M* EDTA, and 0.1% sodium dodecyl sulfate (SDS). The hybridization was assayed by resistance to S1 nuclease *(Aspergillus oryzae)*. As indicated in Fig. 29, the maximum hybridization was 87% with a background resistance to S1 of approximately 3%. The hybridization kinetics of this cDNA with AKR virus 70 S RNA (assayed by S1 nuclease) is shown in Fig. 30.

Our present approach is to fractionate the cell DNA into low-molecular-weight (Hirt soluble) and high-molecular-weight (Hirt precipitable) components. The Hirt soluble fraction has been shown to contain unintegrated proviral DNA from permissive NIH/3T3 cells and restrictive BALB/3T3 cells following AKR virus infection. Preliminary results suggest that proviral DNA is synthesized in both permissive and restrictive cell types. We are presently quantitating the copies of proviral DNA in Hirt supernates by hybridization kinetics. Papers presented at the recent Cold Spring Harbor meeting on RNA tumor viruses (May 26–30, 1976) by

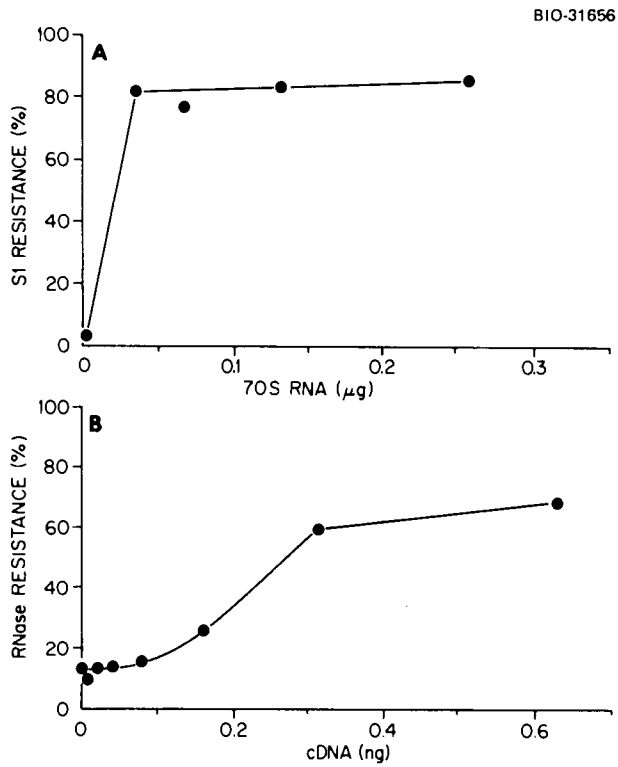

Fig. 29. Hybridization of AKR [^{3}H]cDNA to unlabeled AKR 70 S RNA in excess, assayed by S1 nuclease.

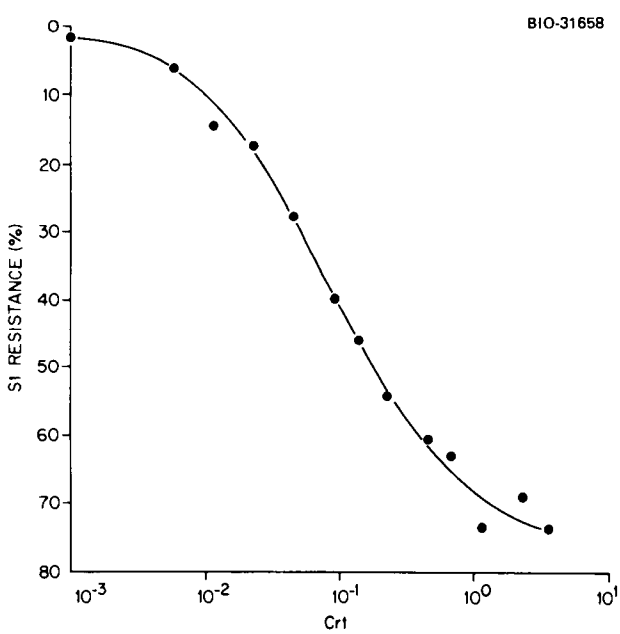

Fig. 30. Hybridization kinetics of AKR [^{3}H]cDNA to AKR 70 S RNA at 62°C in 0.3 *M* NaCl, 3 m*M* EDTA, 0.1% SDS, assayed by S1 nuclease.

Jolicoeur and Baltimore and also R. Soeiro *et al.* demonstrate that proviral DNA may be synthesized in the restricted cell but it fails to be integrated.

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*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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THE EFFECT OF HYDROCORTISONE ON THE *Fv-1* GENE RESTRICTION OF MURINE LEUKEMIA VIRUSES*

S. C. Jones,† R. W. Tennant, W.-K. Yang, and A. Brown†

The $Fv\text{-}1^n$ and $Fv\text{-}1^b$ alleles of mice reciprocally restrict N- and B-tropic viruses in cell culture, resulting in a multiple-hit phenomenon in the nonpermissive system. However, others[1] have reported that the multiple-hit kinetics can be altered by the addition of dexamethasone.

We have used several sensitive assay methods for murine leukemia viruses to test the effects of a micromolar concentration of hydrocortisone on viral replication. Results from both the fluorescent antibody-staining and the reverse XC (infectious center) assay indicate that the percentage of initially infected cells does not change when cells are pretreated with hydrocortisone. In contrast, the progeny virus per infected cell is increased in the presence of the hormone; as much as a fivefold higher titer of murine leukemia virus is seen in the direct XC assay when hydrocortisone is added after infection. Experiments are under way to determine if this increase is due in part to a heightened sensitivity of the indicator XC cell. However, conversion of multiple-hit kinetics in the nonpermissive cells to one-hit kinetics, as seen in a permissive system, does not occur. The effect of hydrocortisone, therefore,

appears to be on some event(s) in virus replication rather than on cell sensitivity.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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FLOW MICROFLUOROMETRIC ANALYSIS OF CELLS INFECTED WITH MURINE LEUKEMIA VIRUSES*

R. E. Hand, Jr., R. W. Tennant, J. M. Quarles,† W.-K. Yang, G. C. Lavelle, and K. Nall

Flow microfluorometric (FMF) assay with a Cytofluorograf model 4081 in combination with immunofluorescence provides a method of quantitatively determining cells infected with murine leukemia viruses. The technique offers the advantages of the visual immunofluorescence method in detecting individual cells containing viral protein and is capable of assaying up to 5000 cells/sec, with specificity equivalent to direct visual immunofluorescence, thereby providing a statistically valid sampling in a fraction of the time required by visual counting.

A fixation procedure using 50% cold acetone prevents cellular autolysis and clumping but does not interfere with subsequent staining or induce gross changes in morphology. Briefly, cells either infected with murine leukemia virus or uninfected are removed from culture dishes with 0.4% EDTA, fixed in acetone, washed in buffered saline, incubated with fluorescein-isothiocyanate-labeled antibody, and washed and examined by FMF. A system of electronic discriminators permits either total cell counts or counts of subpopulations (fluorescent cells as a percentage of total cells) to be displayed both as a cathode-ray scattergram and as a digital display.

The Moloney leukemia virus is a laboratory-adapted strain, highly infectious for many mouse cell cultures, and was therefore most suitable for application to FMF. Two other less well adapted types of mouse leukemia (Gross and BALB/c) viruses were also used, and the titration patterns of these viruses in both the FMF and visual immunofluorescent assays were compared. While the slopes of the visual and FMF titrations are the same, there is a consistent displacement of the visual curve 20 to 30% higher in the Gross and BALB/c than the FMF curve. In cells infected with Moloney leukemia virus, the same data were obtained by the visual assay and the FMF assay at high multiplicity of infection. This displacement problem is apparently due to the threshold setting, which eliminates weakly fluorescent cells from being counted as positive.

By using the discriminator as a threshold and by incrementally advancing along the horizontal storage oscilloscope display, the percentage of positive cells can be recorded manually for each 1000 cells counted. Using this technique the staining characteristics of each virus and cell used in the FMF assay were defined. When the percent of positive cells is plotted against the threshold setting, a discrimination pattern is generated and a net positive curve can be obtained (Fig. 31). A histogram can be developed by plotting the percentages against the threshold ranges. As shown in histograms B, D, and F, the Gross virus in SME cells (F) and the BALB/c virus in BALB/c cells (D) show a percentage of the positive cells occurring in the trailing edge of the negative cell population. This observation explains why 20 to 30% of the infected cells are lost when a threshold is empirically set to exclude the negative control cells. However, this problem does not occur with the Moloney leukemia virus in NIH3T3 cells (B). It should be possible for the Cytofluorograf system to surpass the visual method in sensitivity and specificity. Addition of a multichannel analyzer and computer analysis of the data allows the manual discrimination of each virus-cell system to be accomplished automatically.

*Research sponsored by the Virus Cancer Program of the NCI.

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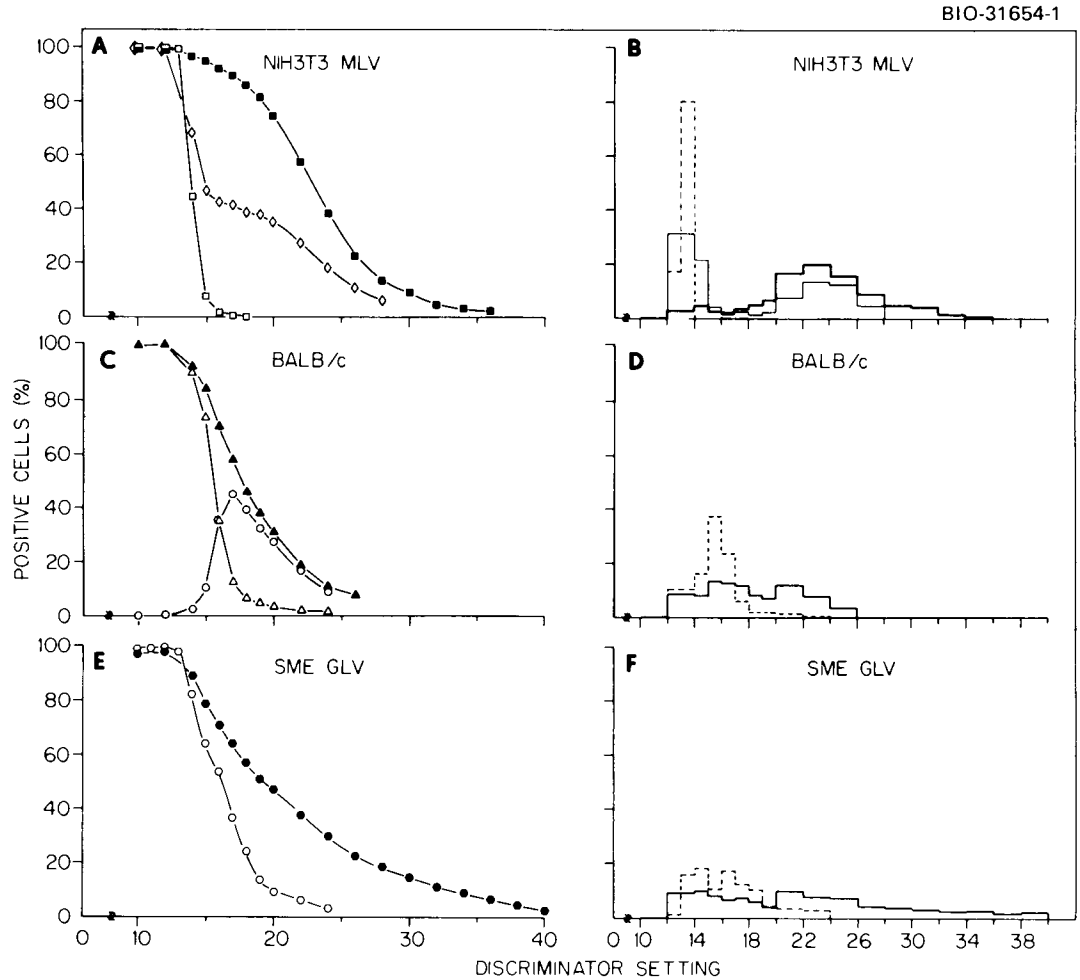

Fig. 31. Analysis of Cytofluorograf sensitivity. NIH3T3 cells infected with Moloney leukemia virus were examined by FMF at photomultiplier tube gain of 60, and BALB/c embryo cells infected with WN1802B, SME cells infected with Gross leukemia virus, and corresponding uninfected controls were examined by FMF at photomultiplier tube gain of 67. An electronic discriminator (threshold) was passed through the cell population from completely open to closed. Data were plotted as percent positive cells (A, C, E, G) or percent positive cells within each threshold range (B, D, F, H) vs threshold setting. A: (■), NIH3T3 infected with Moloney leukemia virus; (□), NIH3T3 controls; (◇), equal mixture of infected and uninfected cells. B: (▬), NIH3T3 infected with Moloney leukemia virus; (----), NIH3T3 controls; (—), equal mixture of infected and uninfected cells. C: (▲), BALB/c cells infected with WN1802B; (△), BALB/c cell controls; (○), net positive cells. D: (—), BALB/c cells infected with WN1802B; (----), BALB/c cell controls. E: (●), SME cells infected with Gross leukemia virus; (○), SME cell controls. F: (—), SME cells infected with Gross leukemia virus; (----), SME cell controls.

TRANSPLACENTAL HOST-MEDIATED (*IN VIVO-IN VITRO*) MODEL FOR CHEMICAL CARCINOGENESIS*

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The transplacental carcinogenesis system developed by DiPaolo[1] in hamsters has been successfully applied in our laboratory to identification of carcinogenic chemicals.[2] We have conducted an evaluation of the technique using selected pesticides and some nitrosated derivatives obtained from Dr. William Lijinsky (ORNL). In this system, pregnant hamsters were injected with the test compounds on day 10 of gestation, and mass embryo cultures were prepared on day 13. The cells were subcultured every 4 to 6 days and cloned and scored for morphological transformation and growth in 0.3% agar at subcultures 3, 7, and 10.

Cultures from solvent-treated and untreated animals yielded no transformants or growth in agar. Several nitroso compounds and chemicals of known carcinogenic potential, including DEN, urethan,

benzo[*a*]pyrene (BP), and nitrosocarbaryl, induced morphological transformants which formed colonies in agar. The advantage of using the agar test to aid in determining transformants was particularly evident in experimental groups where morphological criteria were not unequivocal but where agar assay yielded growth indicative of transformation.

Of the series of pesticides tested, only landrin showed ability to induce transformation, while of the nitrosated derivatives, nitrosocarbaryl, nitrosobaygon, nitrosomethomyl, and nitrosolandrin induced transformants, varying from 0.1% to 1.8% of the colonies examined. The results of growth in soft agar showed all of the nonnitrosated pesticides to be negative, while all of the cultures from animals treated with the nitrosated derivatives produced colonies.

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1. J. A. DiPaolo, *Arch. Pathol.* **95**, 380 (1973).
2. *Proc. Am. Assoc. Cancer Res.* **17**, 72 (1976).

STUDIES ON THE MECHANISM OF TERATOGENESIS IN A RODENT MODEL SYSTEM

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Some members of the parvovirus group, such as Kilham rat virus (KRV), induce teratogenic effects in rats and hamsters and provide an important model system for investigating the mechanisms of viral teratogenesis. KRV was shown to have an affinity for certain developing fetal tissues, which suggested a requirement for actively dividing cells for its multiplication. This theory was confirmed by the work of Dr. Tennant and co-workers in this laboratory, who demonstrated that the virus is reproduced by, and consequently destructive for, only those cells which undergo cellular DNA synthesis after infection by the virus. Our approach to the study of parvoviruses as teratogens, using rat virus as a pertinent laboratory model, is directed at aspects of the replication of the viral DNA within infected cells *in vitro*. These studies concern specific events in virus multiplication which are dependent upon cellular DNA synthesis, a unique structural feature of the viral DNA, and a unique DNA polymerase which is associated with purified virus.

Earlier work from this laboratory by Dr. Tennant and co-workers demonstrated that cellular DNA synthesis was required for multiplication of KRV in rat cells *in vitro*. One step in the multiplication of the virus which could be dependent upon cell DNA synthesis is replication of the double-strand DNA intermediate (replicative form). The experiments were conducted by infecting synchronized cells with KRV and pulse-labeling with [^{3}H]thymidine at times after infection. The low-molecular-weight DNA fraction, which contains viral DNA, is extracted from the cells, purified, and hybridized to purified viral DNA which is bound to nitrocellulose filters. This procedure detects DNA complementary to virion DNA (which consists of single monopolar strands). The progression of cells through the cell cycle was followed by autoradiography. The results showed that virus-specific DNA was synthesized during middle to late S phase of the cell cycle. Cells deprived of serum or irradiated before infection synthesized greatly reduced amounts of virus-specific DNA. This finding constitutes the first direct demonstration of a specific viral function which is cell-cycle-dependent, and it pinpoints the period in the cell cycle when the cellular requirement is available. The work won Ms. Anna Li, a University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences graduate student, the George Cameron Award from the Kentucky-Tennessee branch of the American Society for Microbiology, Lexington, Kentucky, October 1976.

Single-strand viral DNA is never recovered in the extracted fractions of cells, even at times when progeny single strands are believed to be made. The reason is thought to be that progeny single strands are encapsidated as quickly as they are made, are not released by the extraction procedure (method of Hirt), and are thus lost. Analysis of progeny DNA by molecular hybridization to complementary strand DNA, by all estimates, would be very time-consuming and costly because complementary strand DNA is only found intracellularly. What was needed, therefore, was a method which would release viral DNA under nondenaturing conditions, but without shearing cellular DNA. We have found that guanidine hydrochloride fulfills these requirements. After pulse-labeling infected cells, the cells are layered intact on top of a sucrose gradient which contains 4 *M* guanidine hydrochloride, a small amount of proteinase K is added, and the sample is incubated for several hours at room temperature during which lysis of cells and encapsidated virus takes place. After subsequent sedimentation, two

distinct species of low-molecular-weight DNA are found: one cosediments with viral DNA marker and represents newly synthesized progeny single strands; the other cosediments with a marker of the size expected for the double-strand replicative form. Under appropriate pulse-chase conditions, the movement of label from the position of replicative form to that of progeny single strands is striking. In addition, there is a complete separation between viral molecules and high-molecular-weight cell DNA. This one-step isolation of the intracellular forms of KRV DNA is more sensitive and quantitative than any other method in popular use among animal virologists and may prove useful in analyses of more complex viruses, such as the herpesviruses, and of cellular DNA.

In the course of studies on the intracellular forms of KRV DNA, we found that DNA from purified virions had anomalous behavior when chromatographed on hydroxyapatite by the popular batch-elution method. When chromatographed by means of a phosphate gradient, KRV DNA eluted at a phosphate concentration intermediate between completely single-strand and completely double-strand DNA markers. This suggested that the viral chromosome, which appears entirely single-strand by electron microscopy, actually contains one or more small double-strand regions. Susceptibility of hydroxyapatite-purified DNA to degradation by the single-strand-specific endonuclease S1 and by the 3′-specific single-strand exonuclease I (from *E. coli*) has shown the following: the 3′ end is not double-strand; a duplex region appears to be present at a position 40% from the 3′ end; after combined treatments with the enzymes, a resistant duplex "core" can be isolated which comprises about 5% of the molecule and has a melting temperature of 87°. That is, the duplex region is adequately well base-paired and large enough to be quite stable. The number of these regions (if more than one), the base composition, and the structure of the 5′ end of the chromosome have not yet been determined. The biological function of these intramolecular self-complementary regions is not clear, but roles in transcription of encapsidation are possible. This finding earned an invited paper at the ICN-UCLA Winter Conference on Animal Virology, Squaw Valley, California, March 1976. The research is a collaborative effort between the Viral Carcinogenesis Group and the Molecular and Cellular Sciences Section.

Because KRV DNA replication follows a complex pattern of single-to-double- and double-to-single-strand DNA production, a susceptible cell must be able to furnish DNA polymerases of more than one template specificity. In view of the very small size of the virus (purified virions contain only three polypeptides), the report several years ago from another laboratory that purified virus contained a DNA polymerase is surprising. Several laboratories looked for, but did not find, the polymerase in other parvoviruses. It appears we may now be the first to confirm that finding. The enzyme has been purified from KRV which was isopycnically banded twice. It is a single polypeptide seen by polyacrylamide gel electrophoresis in the presence of SDS with a molecular weight of 75,000 ± 3000 daltons. It was purified by sonic disruption of purified virions followed by DEAE-cellulose (diethylaminoethyl cellulose) and DNA-cellulose chromatography. The polymerase has the same molecular weight as one of the capsid proteins, and it may be that this protein is the polymerase. However, it is not yet possible to distinguish between several possibilities: (*a*) that the enzyme is a preexisting cellular enzyme which is incorporated into the virion, (*b*) that the enzyme is a cellular enzyme which is not normally present but is induced upon infection with KRV, and (*c*) that the enzyme is coded for by KRV DNA. Regardless of which possibility proves to be correct, the answers will contribute to our understanding of the relationship between a teratogenic virus and its target cells.

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STUDIES ON THE MECHANISM OF INDUCTION OF ENDOGENOUS LEUKEMIA VIRUS*

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The genetic sequences of leukemia viruses are carried at specific loci in the genome of all mouse strains, and virus replication can be induced by exposure of cultured cells to certain chemicals or radiation.

In previous studies we reported that the induction of virus in AKR mouse cells by iododeoxyuridine (IdUrd) requires two cell-division events. DNA

synthesis is required for the incorporation of IdUrd, and a second round of synthesis is required for expression of the stable provirus intermediate which is formed from the incorporation of IdUrd. In another study we showed that virus could be induced from AKR cells by low-dose-rate gamma irradiation, but these studies demonstrated that the stable provirus intermediate state induced by IdUrd was not formed following gamma irradiation.

As a working hypothesis we assume that the formation of the stable provirus intermediate in IdUrd-treated cells results from the irreversible incorporation of the compound into cellular DNA. The lack of a stable provirus in gamma-irradiated cells is probably related to the efficient repair of induced lesions in the DNA of irradiated cells by unscheduled DNA synthesis.

To test this hypothesis, we have examined the effect of a repair inhibitor on virus induction in AKR cells. AKR cells were deprived of serum for 18 hr and then irradiated in the presence of 3 μg/ml of quinacrine or without quinacrine. The cells were irradiated for either 20 or 40 hr at a dose rate of 17.5 rads/hr. Following irradiation, the cells were refed with medium containing serum and $10^{-6}M$ hydrocortisone. Three days later the cells were subcultured in medium containing 10^{-6} M hydrocortisone and 2 μg/ml polybrene (to enhance virus attachment). At 4 days after irradiation, the AKR cells were overlaid with NIH Swiss secondary cells (which amplified two- to tenfold) for 4 days, followed by an overlay of rat XC cells which develop syncytial plaques. The cells which were serum-deprived and irradiated in the absence of quinacrine showed minimal virus induction, whereas the serum-deprived cells irradiated in the presence of quinacrine resulted in a five- to tenfold increase in the frequency of virus induction. Thus far, these experiments have suggested that the inhibition of repair synthesis may be important, and experiments are continuing to determine if quinacrine can induce a stable provirus intermediate.

We have also tested several methods of synchronization and finally achieved good results using a combination of serum and isoleucine deprivation for 72 hr, although 8 to 10% of the cells continued to incorporate [^{3}H]thymidine even after this period of starvation. After refeeding with complete medium, up to 70 or 80% of the cells were found to initiate DNA synthesis within 10 hr. The synchronized AKR cells were exposed to X rays at dose rates up to 1000 rads/min and tested for induction. The cultures were held for 24 hr, refed with complete medium containing $10^{-6}M$ hydrocortisone, allowed to grow to confluence, and then overlaid with rat XC cells for the development of syncytial plaques. The results indicate that induction occurred when the cells were irradiated approximately 16 hr after release from starvation, when the peak of DNA synthesis occurs. While the induction was less efficient than either IdUrd or low-dose gamma radiation (the syncytial plaques were both less frequent and much smaller, indicating that fewer cells were induced and that less virus per induced cell was synthesized), these results confirm the requirement for cell division by acute X-irradiation induction. The results can also be interpreted as indicating that in the synchronized cultures, DNA synthesis required for virus expression occurs before the inducing lesions are removed by cellular repair processes.

Nitroso compounds and polycyclic hydrocarbons are known to have carcinogenic activity in a wide variety of animal species, and several have been shown to have mutagenic and transforming activity in cell-culture systems. Certain nitroso compounds, particularly nitrosamines, may be important environmental carcinogens since they can be readily formed from nitrites and secondary amines under conditions similar to those existent in the mammalian stomach. It is believed that these compounds derive their activity, at least in part, by their ability to bind to mammalian DNA, causing repairable lesions in the DNA, and may, therefore, be able to induce expression of endogenous RNA tumor viruses. The cell system chosen to test this hypothesis is the AKR mouse cell line, which is readily inducible when the cells are allowed to replicate in the presence of halogenated pyrimidines. We believe that a situation similar to that of gamma-irradiation and X-irradiation treatment may be occurring with chemical treatments of cells. If repair of the chemically induced lesion occurs prior to the onset of DNA replication, no virus is induced. We have, therefore, initiated the testing of several compounds using an assay which should allow detection of virus induction even if the chemically induced lesions are repaired.

Cultures of AKR cells which are 95 to 98% confluent are treated for 2 hr with 2×10^{-3} M hydroxyurea to stop replicative DNA synthesis. Repair functions, however, do occur. Cells are then treated with the chemical to be tested in the presence of hydroxyurea and IdUrd using two 12-hr treatments. Any lesions which are repaired will be repaired with IdUrd instead of thymidine. Cells are then incubated for 24 hr in the presence of excess thymidine to wash out the unincorporated IdUrd and

hydroxyurea. The cells are then subcultured and allowed to grow to confluence, at which time they are tested for virus expression by the XC-plaque assay, by fluorescent antibody binding, and by assay for reverse transcriptase activity. Simultaneously, the chemicals are being tested for transforming ability by subculturing the treated cells several times and then looking for the ability of the cells to grow in soft agar. If a lesion is induced by a given chemical and then repaired in the presence of IdUrd, it is possible that the lesion can be "fixed" into a state that would allow virus expression upon subsequent DNA replication and cell division. We have shown that treatment of 95- to 98%-confluent cultures of AKR with IdUrd does induce virus expression but that if the treatment is carried out in the presence of hydroxyurea, thus allowing no incorporation of IdUrd into DNA, induction is blocked. Treatment of cells with nitrosoethylurea in the presence of IdUrd and hydroxyurea does not allow virus induction. Experiments are currently in progress to test the ability of 3-methylcholanthrene, DMBA, BP, 7,8-dihydroxybenzo[*a*]pyrene, ethane methylsulfonate, and the nitrosated pesticide nitrosocarbaryl to induce virus expression in this system. If one or more of these chemicals should induce virus expression, further experiments are planned to investigate the nature of the lesion causing induction and the mechanism involved.

*Research sponsored by the Virus Cancer Program of the National Cancer Institute.

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THE FATE OF EARLY CARCINOGEN-INDUCED LESIONS IN TRACHEAL EPITHELIUM*

R. A. Griesemer, P. Nettesheim, A. C. Marchok, D. C. Topping,† and D. H. Martin

Hyperplastic-metaplastic lesions were produced in transplanted rat tracheas by exposure to 310 μg or 75 μg DMBA delivered over a 2-week exposure period. Tissue recovery was studied for up to 32 weeks. After cessation of carcinogen exposure, most of the hyperplastic-metaplastic epithelium was rapidly replaced by mucociliary epithelium. In the DMBA-exposed tracheas, a few metaplastic foci remained at 4 and 8 weeks. In all three exposure groups, portions of the mucosa were occupied by atrophic–pleomorphic epithelium that persisted throughout the observation period. Of the tracheas exposed to 75 μg of DMBA, 50% showed some characteristics of preneoplasia at 32 weeks. Palpation data on survival groups has shown no tumors at 8 months in animals exposed to 75 μg of DMBA and two tumors in 12 transplants exposed to 310 μg of DMBA.

Similar early lesions were produced in tracheas by exposure to 145 μg of DMBA or 550 μg of BP delivered over a 4-week period. In the DMBA-exposed tracheas the hyper-metaplastic epithelium was replaced by mucociliary or atrophic epithelium after cessation of exposure. Of these tracheas, 50% contained metaplastic and verrucous lesions after 16 weeks of recovery, and 83% contained preneoplastic lesions after 32 weeks, including some dysplastic and pleomorphic lesions containing cellular atypia. Palpation data on survival groups at 8 months have shown one tumor in 24 transplants exposed to 145 μg of DMBA and 8 tumors in 12 transplants exposed to 550 μg of DMBA.

*Research sponsored by the National Cancer Institute.

†Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

CHARACTERIZATION OF THE TRACHEAL TRANSPLANT DURING STAGES OF ESTABLISHMENT AND COMPARISON OF THE MODEL WITH NORMAL TRACHEAS*

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We have previously reported the feasibility of using entire tracheas, transplanted subcutaneously into isogenic recipients, as a target tissue for a quantitative exposure of epithelium to chemical carcinogens. This study was designed to characterize temporal changes occurring during establishment of the transplants, to compare long-established transplanted tissue with that of the host, and to determine the validity of the model for carcinogenesis studies on respiratory epithelium.

Rats with transplanted tracheas were injected with tritiated thymidine and killed at 2, 3, 4, 5, 6, 7, 14, 21, and 28 days and 6 months following transplantation. Host and transplanted tissues were cut into pieces and blocked sequentially to allow for anatomical localization of histological sections.

Newly transplanted grafts demonstrated degeneration and sloughing of part of the epithelial lining. The total number of cells dropped to 58% of normal at 3 days following transplantation. A remarkable amelioration of the epithelial morphology occurred at 4 days, when the blood supply was reestablished, and the cell population returned to normal size after 7 days. Sloughed cilia were present in the lumen during the first 5 days. Short cilia were seen to reappear at 6 days following transplantation, and by 14 days, 70% of all columnar cells possessed a heavily ciliated margin. The labeling index of all cells increased to 4.61% at 3 days and was greater than 10% at days 4 and 5. By 7 days the labeling index had fallen to 1.32%, and it was comparable with that of normal undiseased tissue (less than 0.3%) at 14 days.

Labeling was observed in all cell types during days 3 to 7 but was primarily confined to basal cells at day 14 and later. In 6-day transplants, a burst of secretory activity occurred which was characterized by large numbers of periodic-acid-Schiff-positive cells of varied appearance. These cells appeared primarily at the margin of the cartilaginous rings.

Vascularity of the transplants was visualized by intracardial injections of India ink followed by fixation, staining, and clearing of the tissue. Vascularization of the transplant was observed at 3 days and was widespread at 4 days, the preformed pathways being reutilized. Established transplants contained a more diffuse network of vessels than did normal tracheas, which contained single vessels between tracheal rings with arborizations extending to the adjacent cartilage. Electron microscopic morphology of the tracheal transplants at various stages of establishment is now being investigated.

This study indicates that transplanted tracheas establish and possess the epithelial morphology and proliferative characteristics of host tracheas within 2 weeks after surgical transplantation.

*Research sponsored by the National Cancer Institute.
†Postdoctoral investigator, Carcinogenesis Training Grant CAO5296 from NCI.

STUDIES ON DEVELOPMENT OF METHODS FOR PROTRACTED DELIVERY OF CARCINOGENS TO RESPIRATORY EPITHELIUM*

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Previous reports from our laboratory have demonstrated the feasibility of using carcinogen-impregnated beeswax pellets for localized quantitative delivery of polycyclic hydrocarbons to respiratory epithelium. Release from such pellets into tracheal transplants has been shown to be dependent on carcinogen concentration and to be approximately first order. We undertook the present studies to provide for a lower and more sustained rate of delivery of carcinogen to respiratory epithelium.

We have developed an *in vitro* method for investigating vehicles and release rates. The rate of release of BP from beeswax pellets into fetal calf serum *in vitro* was found to approximate the first-order characteristics of release previously observed *in vivo*. All of the carcinogen was released within 2 weeks. Binding of BP to carbon particles before introduction into the beeswax matrix was found to markedly slow the release of the carcinogen into fetal calf serum. BP and carbon in a ratio of 1:4 in beeswax released only 60% of the carcinogen in 2 weeks, while a mixture of 1:9 released less than 30% in 2 weeks. These rates of release *in vitro* approximately parallel *in vivo* release data in hamster tracheal transplants.

A vehicle composed of beeswax mixed with cholesterol has been effective in reducing the total delivered dose but not in eliminating the high initial delivery of BP.

Coating pellets with polymers has been found to be an acceptable method of reducing carcinogen release to very low levels. Polyethylene tubing and cellulose acetate coatings have been shown to be barriers capable of delivering less than 0.1% of the encased carcinogen per day. Release of carcinogen from pellets coated with cellulose acetate, while extremely low, appears to provide for low-level zero-order release. Release rates of from 0.02% to 0.9% per day have been achieved, depending on the thickness of the polymer applied to the pellets.

*Research sponsored by the National Cancer Institute.
†Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

LONG-TERM CULTURE OF EPITHELIAL CELLS FROM NORMAL RAT TRACHEAS*

A. C. Marchok, J. C. Rhoton, R. F. Epler, and P. Nettesheim

We have developed a system for maintaining tracheal epithelial cells in primary culture for many weeks. Explants are made from adult rat tracheas and planted in tissue culture dishes containing an enriched Waymouth's MB 752/1 medium plus 10% fetal bovine serum. The medium enrichment consists

of added amino acids, fatty acids, sodium pyruvate, and putracine. Insulin (10 μg/ml) and hydrocortisone (0.1 or 1.0 μg/ml) are also required. Using these culture conditions, the sheets of epithelial cells that rapidly grow out from explants are heterogeneous and include ciliated, secretory, and many dividing cells. After removal of the explants the epithelial cell cultures continued to expand in size for many weeks. Small foci of piled-up squamous cells containing keratohyalin granules appear in some of the older cultures. Addition of 0.1 to 1 μg/ml of either retinol or retinyl acetate causes sloughing of the keratinized cells and induces the formation of refractile granules and vacuoles in many of the cells throughout the culture. These cultures are being utilized for further study of the factors which regulate proliferation and differentiation of the specialized cell types present in the normal respiratory as well as the metaplastic epithelium after exposure to toxic agents.

*Research sponsored by the National Cancer Institute.

TWO ASSAY SYSTEMS FOR DETERMINING THE EFFECTS OF VITAMIN A AND CARCINOGEN ON RAT TRACHEAL SECRETIONS*

J. N. Clark,† A. C. Marchok, and P. Nettesheim

It is known that vitamin A is necessary for the maintenance of a normal mucociliary epithelium. In the absence of vitamin A, the tracheal epithelium undergoes a metaplasia to a keratinizing squamous epithelium. In the presence of toxic agents, such as carcinogens, the rat tracheal mucosa usually undergoes a period of secretory hyperplasia followed by squamous metaplasia. We are interested in studying the underlying mechanisms involved in the maintenance, hypersecretory, and hyposecretory changes in the mucociliary epithelium. As the first step, we wish to identify the changes in the quality and quantity of the specific mucins secreted during the transition to hypersecretion and hyposecretion. Up to now, such studies have been hampered by the difficulty of collecting enough of the secretory products. Using the tracheal transplant system, we can elicit the mucosal changes and also sample large amounts of secretions which collect in the transplant. The high-molecular-weight mucin fractions are separated from the secretions first by gel filtration on Sepharose 6B columns followed by DEAE Sephadex and finally by polyacrylamide gel electrophoresis. The fractions will be further characterized by sugar and amino acid analysis of the mucin glycoproteins.

Kinetic studies of the synthesis and secretion of the mucins under different experimental conditions (no vitamin A, high vitamin A, exposure to toxic agents) are being carried out in the tracheal organ culture system described in previous reports. This system permits the use of radioactively labeled amino acids and carbohydrates to follow the synthesis and secretion of the glycoproteins. The tissue glycoproteins and the secretory products in the medium are submitted to the fractionation and purification described previously. The identity of the labeled products will be substantiated by comigration with purified mucus on the polyacrylamide gels.

*Research sponsored by the National Cancer Institute.
† Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

BIOLOGICAL ROLE OF ETHER LIPIDS IN GROWTH AND NEOPLASIA*

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The content of ether lipids is characteristically elevated in tumors as compared with normal tissues. The biological role of these lipids in the development of neoplasia is, however, unknown. A comparison of normal and tumorigenic epithelial cells *in vitro* has, therefore, been initiated to examine the relationship of ether lipid metabolism to neoplastic potential.

BP-induced transplantable tumors of rats have been used as a model system to establish the presence of ether lipids, particularly alkyldiacylglycerols, in tumors of the respiratory tract. Alkyldiacylglycerols were found at a level of approximately 2% of the total lipid content in four squamous cell carcinomas, as compared with undetectable levels in normal rat lungs. The levels did not appear to vary with the sex of the host animals or with the degree of necrosis found in the tumors.

Cloned cell lines from one of the transplantable tumors (BP-A) are being used as a model in which to study turnover of the ether lipids. The two cell lines being used show characteristic differences in doubling time and morphology. One cell line, having epithelioid morphology and growth pattern, formed anaplastic carcinomas upon injection of 1×10^4 cells into syngenic hosts. By electron microscopy, the cells were found to contain desmosomes and tono-

filaments and to have extensive microvilli. A second cell line appeared less highly differentiated *in vitro* but induced keratinizing squamous cell carcinomas in syngenic hosts upon injection of 1×10^6 cells. A higher alkyldiacylglycerol content was characteristic of the more tumorigenic line (16% and 7% respectively). Populations of normal epithelial cells are also being grown *in vitro*. Although the limited growth potential of normal cells has so far precluded mass determinations of the ether lipids, preliminary experiments have shown that turnover studies with the normal cells are possible. In particular, the turnover rates of the alkyl and alk-1-enyl lipid species are being compared in the cell lines and normal epithelial cells. The results of these studies are expected to show whether the presence or metabolic stability of any of the ether lipid species is correlated with the neoplastic potential of epithelial cells.

In addition, the alkyl phosphoglycerides have recently been implicated in the adhesion and growth of cells *in vitro*. One of the transplantable tumors (BP-3) is being used to test the effect of *in vitro* conditions on the ether lipid content. It has been possible to establish a cell line by directly plating pooled cells from this tumor. Although no alkyldiacylglycerol is present in the serum in which the cells are grown, the cells have elevated levels of this lipid (approximately 5%) at early passage times. As previously shown for cultured L fibroblasts,[1] the lipid composition of the BP-3 cells is different from that of the serum. Changes in the lipid content are presently being studied in relation to possible changes in the plating efficiency and growth rate of these cells during adaptation to *in vitro* conditions.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

†Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

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THE EFFECT OF THE TUMOR PROMOTOR TPA AND THE CHEMICAL CARCINOGEN MNNG IN A TRACHEAL ORGAN-CULTURE-CELL-CULTURE SYSTEM*

V. E. Steele,† A. C. Marchok, and P. Nettesheim

In order to determine whether induction and promotion to neoplasia can be carried out *in vitro* in respiratory tract epithelium, we are studying the effect of the potent skin-tumor-promoting agent 12-O-tetradecanoylphorbol-13-acetate (TPA) using rat tracheal organ cultures. Tracheal explants treated with 10^{-8} to 10^{-5} *M* TPA in dimethyl sulfoxide (0.5% final concentration) showed a dose-dependent decrease in DNA synthesis in the whole explant after 12 hr. After recovery to control rates by 3 days, the DNA synthetic rate increased to more than twice control levels by day 6. These results were supported by a detailed autoradiographic study of [^{3}H]thymidine incorporation into specific tissue layers. The increase in radioactive labeling occurred mainly in the epithelial cells. Addition of insulin and hydrocortisone to the cultures enhanced the effect; increasing the serum level abated the effect. Limiting the exposure time to 24 hr increased the [^{3}H]thymidine uptake by 5 times above control levels after 6 days. Surprisingly, a 1-hr exposure increased incorporation threefold after only 24 hr.

Treatment of tracheal explants in organ culture with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) alone resulted in increased rates of outgrowth of epithelial cells from tracheal explants, improved maintenance in cell culture, higher cell densities, and changes in cell morphology. Some of the primary epithelial cell cultures derived from MNNG-exposed organ cultures could be subcultured (cells from unexposed cultures cannot be subcultured).

At present we are initiating a series of experiments which will include exposure of the organ cultures to MNNG followed by TPA exposure in order to determine whether we can initiate and promote respiratory tract epithelium *in vitro*.

*Research sponsored by the National Cancer Institute.

†Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

A METHOD FOR THE ISOLATION AND *IN VITRO* GROWTH OF SUSPENSIONS OF TRACHEAL EPITHELIAL CELL SUSPENSIONS*

M. Terzaghi, M. L. Williams, and P. Nettesheim

The ultimate aim of this project is to quantitate changes occurring during oncogenesis in carcinogen-exposed tracheal epithelium. Several means of removing cells to obtain cell suspensions have been tried. Although pronase (1%) yielded 1×10^6 to 3×10^6 cells per trachea (larynx to bifurcation) with a

viability (dye exclusion) of 90%, the actual plating efficiency *in vitro* was unacceptably low. Trypsin [EDTA (0.2%) with chicken serum (5%)] yielded about 6×10^5 to 8×10^5 cells with a lower viability (50 to 60%) but a relatively high plating efficiency *in vitro*. When such cells were plated *in vitro*, it was found that approximately 48 hr elapsed before these cells begin to synthesize DNA (as indicated by continuous labeling studies with [^{3}H]thymidine). Once cell division is initiated, the doubling time is 25 to 30 hr. By 100 to 150 hr most cells seeded have taken up labeled thymidine. This is interesting in the light of the fact that the majority of the cells originally have specialized functions (i.e., they are ciliated or secretory cells) and most of them do not synthesize DNA and do not divide *in vitro*.

By use of this technique of trypsinizing and culturing tracheal epithelial cells *in vitro*, we hope to be able to quantitatively study carcinogen-specific effects prior to the development of frank tumors.

*Research sponsored by the National Cancer Institute.

DIFFERENTIAL GROWTH AND MAINTENANCE IN CELL CULTURE OF TRACHEAL EPITHELIUM EXPOSED *IN VIVO* TO CARCINOGEN*

A. C. Marchok, J. C. Rhoton, R. F. Epler, R. A. Griesemer, and P. Nettesheim

To study the evolution of neoplasia in the respiratory tract, we are using the tracheal transplant system to uniformly expose the mucosa *in vivo* to carcinogen. After exposure to carcinogen at predetermined dose rates for various periods of time, attempts are made to detect preneoplastic and neoplastic changes by identifying alterations in the *in vitro* growth behavior of epithelial cells derived from the exposed tissue. For these studies, explants made from the transplants are maintained under conditions which stimulate outgrowth of epithelial cells. The cultures are then monitored for the number of explants that produce outgrowths, the rate of epithelial cell outgrowth, and changes in cell morphology. This is correlated with the number of outgrowths that can be maintained as primary cell cultures under media conditions suboptimal for unexposed epithelial cells.

In a current experiment, rat tracheal transplants were exposed *in vivo* to 75 μg (group II) and 310 μg (group III) of DMBA, delivered in 2 weeks. Explants from these and normal tracheas (group I) were cultured in Waymouth's 752/1 medium plus 10% fetal bovine serum (W-FBS) or this medium plus 0.1 μg of insulin and 0.1 μg of hydrocortisone per milliliter (Wihc-FBS). The rates of epithelial outgrowth from group I were similar in both media. These outgrowths could *not* be maintained beyond 7 days without explants. The rate of outgrowth from group II explants was faster, and differentiation into a mulitlayered squamous epithelium occurred. Group II, initiated in W-FBS, could only be replanted a few times, while some explants in Wihc-FBS were replanted 18 times. Group III could only be cultured in Wihc-FBS. The rate of outgrowth in this group was initially slower, but differentiation to multilayered squamous epithelium was marked. Outgrowths from groups II and III were maintained in cell culture for many weeks. Some of these have been successfully subcultured into distinct cell lines. These are being tested for growth in soft agar and tumorigenicity *in vivo*.

*Research sponsored by the National Cancer Institute.

METHODS FOR DEFINING ALTERED BIOLOGIC BEHAVIOR OF TRACHEAL EPITHELIAL CELL POPULATIONS EXPOSED *IN VIVO* TO 7,12-DIMETHYLBENZ[*a*]ANTHRACENE*

M. Terzaghi, M. L. Williams, A. C. Marchok, and P. Nettesheim

We have been sytematically screening normal, preneoplastic, and tumor-derived tracheal cell populations for *in vivo* and *in vitro* markers which can ultimately be used to study oncogenesis in tracheal epithelium. The characteristics studied are as follows: (*a*) the effect of decreasing serum concentration on *in vitro* growth parameters such as plating efficiency, population doubling time, and plateau density, (*b*) the concentration of concanavalin A necessary to agglutinate the test cell population, (*c*) the capacity to grow in agar, and (*d*) tumorigenicity and/or *in vitro* morphology in the nude mouse and normal or immunosuppressed syngenic rat.

We have not found differences in growth parameters at different serum concentrations *in vitro* a reliable way to assess the relative oncogenicity of different tracheal cell populations. Concanavalin A agglutination was positive (75% agglutination with 50 to 100 μg/ml at 37° C) in all neoplastic and

preneoplastic lines tested and negative in normal nonexposed cells. Plasminogen activator production was positive in two out of five preneoplastic lines tested and negative in normal and tumor-derived cell populations. Growth in agar seems to be a reliable "late" marker of transformation and well correlated with tumorigenicity in the hosts tested.

*Research sponsored by the National Cancer Institute.

ULTRASTRUCTURE OF RESPIRATORY EPITHELIAL CELLS *IN VITRO**

C. A. Heckman, A. C. Marchok, P. A. Maxwell, and P. Nettesheim

Cultured respiratory epithelial cells are being used in our laboratory for *in vitro* transformation studies. In order to develop criteria for distinguishing normal and transformed epithelial cells *in vitro*, the fine structure of the cells is being studied by scanning and transmission electron microscopy. Outgrowths of rat tracheal explants are being used to obtain monolayers of epithelial cells.

Explants maintained in an enriched medium[1] were studied at various times after initiation of outgrowths. Although the ciliated and mucous cell populations on the explants retained many of their characteristic ultrastructural features, an increased density of microvilli on the cell surfaces was apparent. Near the cut edges of the explants, a marked flattening of the cells occurred. Cell spreading was even more pronounced on the monolayers. Substantial diminution of surface features accompanied cell spreading, yet nearly all epithelial cells retained discrete, low microvilli. In contrast, fibroblasts found in the monolayers had smooth cell surfaces with few microvilli.

Epithelial cells of the monolayers also show fine structural features peculiar to the *in vivo* epithelial cells of the trachea. Ciliated cells, although relatively few, are present in the monolayers. Tonofilaments and desmosomes are observed in nearly all of the cells (Fig. 32). In addition, lateral interdigitations, proteinaceous inclusions resembling mucous droplets, and specialized intercellular junctions have been observed. Some smaller cells in the monolayers contain numerous desmosomes and tonofilament bundles. While these cells resemble basal cells of the intact trachea, they have not yet been observed to form basal laminae or hemidesmosomes at the surface of the petri dish at times up to 20 days *in vitro*.

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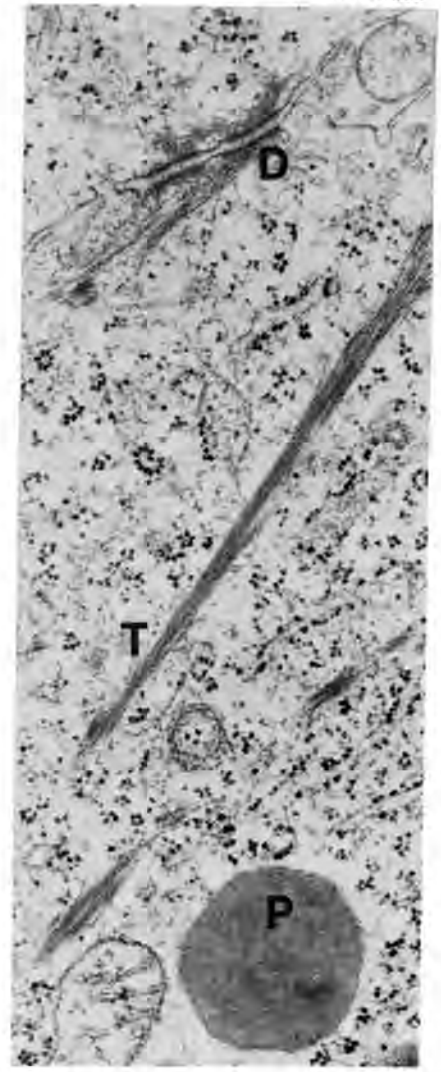

Fig. 32. Desmosomes (*D*) and tonofilament bundles (*T*) are characteristic of epithelial cells in 7-day monolayer outgrowths. Proteinaceous inclusions similar to mucous droplets (*P*) are also observed. 43,700×.

At longer times of maintenance of the monolayer cultures, the cells have extensive areas of desmosomal connections and many intracellular lipid inclusions (Fig. 33). Since lipid droplets are not characteristic of epithelial cells in the normal trachea, their presence may indicate a significant alteration in cellular metabolism associated with *in vitro* maintenance.

The origin and proliferative capacity of cells in the monolayer are currently being studied by light-microscopic autoradiography following [^{3}H]thymidine incorporation. The major sites of cell renewal will be established by this method. It is anticipated that further studies will concern changes in the

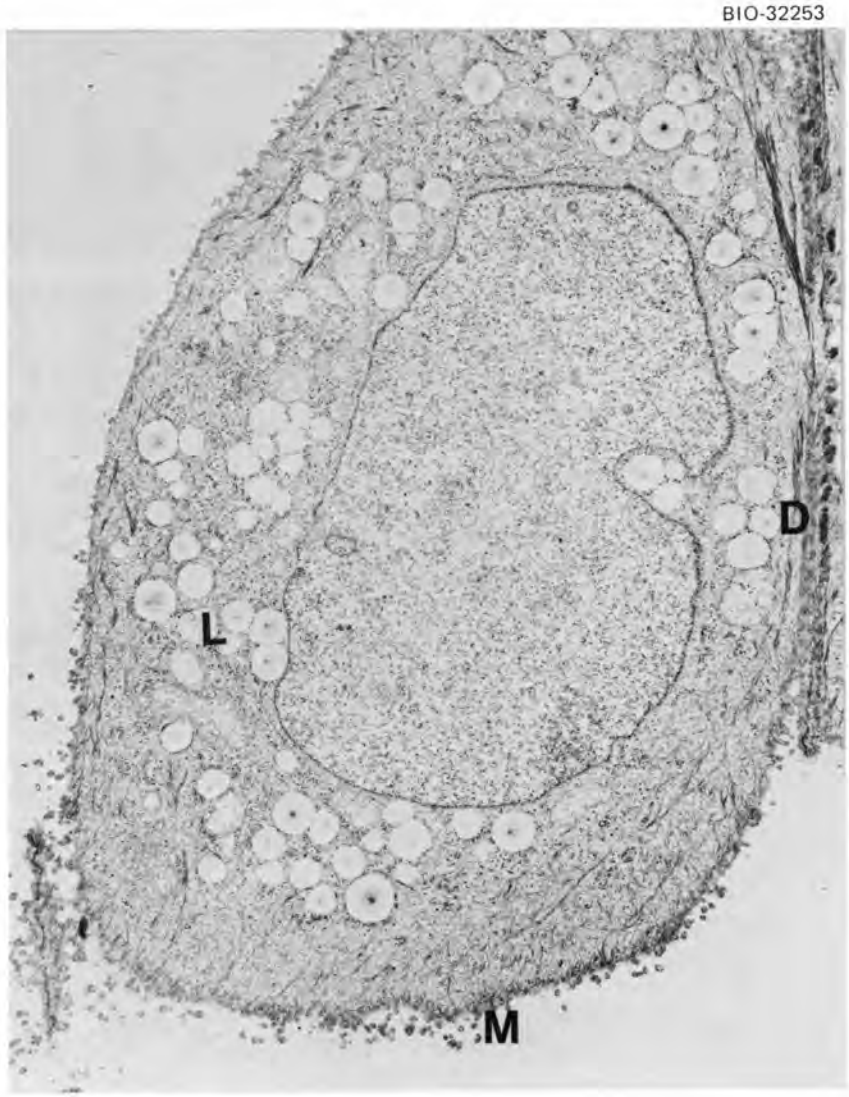

Fig. 33. A typical cell from a monolayer maintained for 20 days shows extensive regions of desmosomal contact (*D*) and numbers of lipid inclusions (*L*). Short microvilli (*M*) are apparent at the cell surface. 8000×.

proliferative capacity and fine structure of preneoplastic cell populations with reference to the epithelial outgrowth model.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

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IMMUNOLOGICAL STUDIES WITH A TRANSPLANTABLE SQUAMOUS CELL CARCINOMA IN MICE. I. DETERMINATION OF IMMUNOGENICITY *

R. J. Jamasbi† and P. Nettesheim

Tumorigenicity studies in isogenic recipients (DBA/2 mice) showed that intramuscular (im) inoculation of 10^4 MSC-10 tumor cells regularly

induced tumors in 50% of the recipients, whereas 10^3 cells are nontumorigenic. It was also shown that with im inoculation of 5×10^4 tumor cells, metastases occur in 10% of the animals at 3 weeks and in 90% at 4 weeks after inoculation.

Attempts were made to induce tumor-specific immunity in syngenic hosts by tumor transplantation. Mice were "immunized" either by repeated intraperitoneal (ip) injection of heavily irradiated tumor cells or by im inoculation of tumor cells and subsequent surgical removal of tumor grafts. Within one to two weeks following the "immunization" procedure, the mice were tested for the development of resistance to MSC-10 inoculations using graded doses of live tumor cells (from 10^4 to 2×10^5 cells). No tumor immunity could be demonstrated using different routes of immunization, different time intervals between immunization and challenge, and different challenge doses. Lymphoid cells from tumor-bearing and tumor-excised mice showed no increased antitumor activity in the *in vivo* neutralization test (Winn test); however, they did exhibit significantly enhanced cytotoxicity in the *in vitro* assay. The sera from such mice did not contain measurable amounts of antitumor antibody detectable with the indirect fluorescence technique. However, anti-MSC-10 antibodies were induced in rats by repeated immunization with tumor cells mixed with Freund's adjuvant.

We conclude that the lung squamous-cell carcinoma MSC-10 is a "weakly" antigenic tumor but that its antigenicity is not strong enough to induce tumor immunity in host animals which would provide effective protection of such hosts.

*Research sponsored by the National Cancer Institute.

† Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

IMMUNOLOGICAL STUDIES WITH A TRANSPLANTABLE PULMONARY SQUAMOUS CELL CARCINOMA IN MICE. II. NONIMMUNOLOGICAL ENHANCEMENT OF TUMOR DEVELOPMENT IN WHOLE-BODY X-IRRADIATED HOST ANIMALS*

R. J. Jamasbi† and P. Nettesheim

Experiments were conducted to investigate whether the impairment of host immunological competence, particularly thymus-dependent immune reactions (*e.g.*, by adult thymectomy and whole-body X irradiation) can significantly alter the development and progression of transplantable "weakly" antigenic tumors. Severe long-lasting impairment of humoral and cellular immunocompetence was induced by thymectomy followed by X irradiation with 600 R in DBA/2 mice (as evidenced by suppression of the sheep-red-blood-cell hemagglutinin response and of the skin graft rejection). Such animals showed an increased tumor incidence compared with controls when tumor cells were inoculated 48 hr after whole-body X irradiation. This effect of X irradiation was not reversed by restoration of immunological competence through infusion of normal lymphoid cells.

Six weeks after whole-body X irradiation (with or without thymectomy) the animals showed a "normal" level of resistance toward tumor transplantation regardless of their immunological "status." Thus thymectomized, X-irradiated mice were still severely depressed immunologically, but tumor incidence and tumor growth rate following tumor cell inoculation were the same in this group as in normal controls or in immunologically reconstituted mice.

The experiments indicate that whole-body X irradiation of tumor cell recipients, prior to tumor cell inoculation, creates a condition which favors tumor cell survival or growth. This "permissive state" exists only shortly after X irradiation and seems not to be correlated with the host's level of immunocompetence.

*Research sponsored by the National Cancer Institute.

† Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

HOST-SPECIFIC IMMUNE MECHANISMS TO RESPIRATORY CARCINOMAS IN RATS*

R. J. Jamasbi† and P. Netteshiem

The appearance of tumor-associated antigens seems to be a common occurrence in human and animal tumors. It is of considerable interest to determine when during the process of carcinogenesis these antigens appear. To study this problem during the evolution of respiratory tract cancers, it was necessary to develop background information on the antigenicity of such tumors and on the type of immune reactions that can be mounted against them.

For this purpose, studies were conducted with seven rat respiratory-tract carcinomas to investigate their immunogenicity and to study host immune responses against them. Of the seven carcinomas used, one (a lung carcinoma) was developed in a male

rat by intratracheal injection of 3-methylcholanthrene. Five were induced in tracheal transplants by BP, two of them in female and three in male rats, and the seventh tumor was induced in a tracheal transplant with DMBA in a male rat. A sarcoma was induced by subcutaneous implantation of BP. The tumors were passaged *in vivo* five to seven times before they were utilized in the immunological studies.

Microscopic examination showed that the histological characteristics of the carcinomas were stable, typical of well-differentiated squamous cell carcinomas. One of the tumors, however, was composed of two elements, a keratinizing squamous cell carcinoma and an undifferentiated carcinoma. This tumor was also found to metastasize early. The TD_{50} values (tumor-cell dose inducing tumors in 50% of inoculated recipients) were determined for each tumor and were found to range from 1×10^4 to 5×10^5 live tumor cells, depending on the tumor line. All the tumors showed some degree of antigenicity as determined by induction of transplantation immunity. An inverse correlation was found between metastasizing capacity and antigenicity.

The existence of cellular immunity in tumor-bearing animals was demonstrated with the *in vivo* neutralization test of Winn. The development of humoral immunity is presently under investigation.

Of considerable interest was the existence of antigenic cross reactivity between all seven respiratory tract carcinomas. This was demonstrated by inducing cross protection (transplantation immunity) with each one of the tumors against every one of the other six tumor lines. This seems to be rather unusual, since cross reactivity between chemically induced tumors, if and when it exists, is usually not exhibited in the form of transplantation immunity. This cross protection was found to be specific for the respiratory tract carcinomas, since it could not be induced against the sarcoma. It was further shown that the protection was not due to tissue-specific antigens. Whether embryonic antigens are involved is presently being investigated.

*Research sponsored by the NCI.

†Postdoctoral investigator, Carcinogenesis Training Grant No. CA05296 from the NCI.

INHALATION COCARCINOGENESIS STUDY*

D. A. Creasia and P. Nettesheim

The previous inhalation cocarcinogenesis study investigated the interaction of topically applied and inhaled irritant gases in respiratory tract tumor induction. Preliminary evaluation of the data from this study suggests a positive cocarcinogenic effect of some of the inhalants tested. Interpretation of the data is complicated, however, by the fact that some degree of injury to the upper respiratory tract occurs when the carcinogen is introduced by intratracheal instillation of the carcinogen. Thus efforts are made to develop experimental models for cocarcinogenesis studies in the respiratory tract that circumvent this complication. A study was initiated in hamsters with the same respiratory irritants as used previously and a systemically applied carcinogen, DEN. The experimental protocol is listed in Table 43. No data are as yet available.

* Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

Table 43. Experimental protocol for inhalation cocarcinogenesis study

Group		Treatment
1	Irritant:	none
	Carcinogen:	0.5 mg DEN weekly for 10 weeks
2	Irritant:	10 ppm NO_2 weekly for life
	Carcinogen:	0.5 mg DEN weekly 48 hr after NO_2 for 10 weeks
3	Irritant:	10 ppm NO_2 weekly for life commencing 2 weeks after DEN treatment
	Carcinogen:	0.5 mg DEN weekly for 10 weeks
4	Irritant:	30 ppm formaldehyde weekly for life
	Carcinogen:	0.5 DEN weekly 48 hr after formadelhyde for 10 weeks
5	Irritant:	30 ppm formaldehyde weekly for life commencing 2 weeks after DEN treatment
	Carcinogen:	0.5 mg DEN weekly for 10 weeks

DEVELOPMENT OF TOLERANCE IN HAMSTERS TO LETHAL DOSES OF AN OXIDANT AIR POLLUTANT*

D. A. Creasia and P. Nettesheim

Nitrogen dioxide is a common oxidant air pollutant found in both urban and industrial atmospheres. Historically, experimental evidence has existed showing that in laboratory animals a single preexposure to a low level of NO_2 will protect the animals from a subsequent exposure to a lethal dose of NO_2. This protection may, depending on the preexposure dose, last for more than a year. This protection phenomenon is common to all oxidant air pollutants and classically has been termed tolerance development. In the past, using incorporation of [^{3}H]thymidine as a label for dividing cells, we have shown a different type of tolerance development. Briefly, we have shown that in the lungs of hamsters exposed to 10 ppm NO_2 for 5 hr there is a wave of cell proliferation which reaches a maximum at 2 or 3 days after exposure and gradually subsides to control levels by day 5 after NO_2. Following repeated exposures animals no longer respond to a subsequent NO_2 challenge with a proliferative response. Such animals have developed a state of "adaptation."

It was of interest to determine whether such animals had also become "tolerant" in the classical sense of the word (resistant against lethal exposures). Rats are exposed daily to 10 ppm of NO_2 for 3 weeks (control animals remained unexposed). After 3 weeks, preexposed and control rats were subjected to 65 ppm NO_2 for 5 hr, which was known from previous studies to be an LC_{50} (concentration lethal to 50% of the rats). Seven out of 16 preexposed rats and 6 out of 12 control rats died, indicating that the frequent, closely spaced NO_2 preexposures had *not* rendered these animals tolerant. This suggests either that the adaptation phenomenon is unrelated to tolerance or that it is insufficient to confer adequate protection against an LC_{50} of NO_2. Studies are now in progress to determine whether NO_2-tolerant rats show "adaptation" (defined as lack of cell proliferation in response to NO_2 exposure; see above).

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

TOBACCO SMOKE BIOASSAY PROJECT*

W. E. Dalbey, R. A. Griesemer, and P. Nettesheim

During the past year we have continued development of bioassays for the impact of chronic tobacco-smoke exposure on respiratory tract tissues.

Rats were chosen for the inhalation exposure because of the desirability of rodents in general for large chronic experiments and the susceptibility of rats in particular to polycyclic aromatic hydrocarbons. The rat had not been extensively used for the tobacco-smoke exposure due to its apparent susceptibility to respiratory tract infections and acute tobacco-smoke toxicity. We found that these problems can be overcome through the use of specific-pathogen-free rats and an exposure regime which allows time for the gradual introduction of rats to the daily exposure schedule and for detoxification between cigarettes.

A chronic inhalation exposure experiment is under way to determine (*a*) the feasibility of lifetime exposure of rats to cigarette smoke, (*b*) the overall sequential biological impact of smoke exposure on rats, and (*c*) means of maximizing the dose of inhaled smoke. Eight rats are being exposed to seven cigarettes a day until death. An additional 80 rats are split into those receiving seven or ten cigarettes a day; individuals from both groups are to be killed after 12, 18, or 24 months of exposure. Appropriate controls are provided.

Smoke-exposed rats have exhibited low but stable body weights. Mortality data have been very favorable. After 80 weeks of exposure, 50% of the rats exposed to ten cigarettes a day and only 20% of those receiving seven cigarettes a day had died. Mortality in the lower dose group may not be significantly different from controls, despite an estimated retention of 0.3 mg of particulates from each cigarette. These mortality data are far better than for other published rat and hamster studies.

Study of 5 of the 11 smoke-exposed and control groups killed up to this time has been completed, including one group of age controls. There appears to be an accentuation of age-related changes in the electrocardiograms of smoke-exposed rats. Smoke exposure may also be related to an increase in total static respiratory compliance. For example, static compliance was 0.157 ± 0.019 ml/cm HOH in age controls and 0.256 ± 0.005 ml/cm in rats receiving

seven cigarettes a day for 12 months. Also being measured are respiratory minute volume, frequency dependence of dynamic compliance, total pulmonary resistance, and pressure, flow, and volume during a forced deflation maneuver. These data are being analyzed. No significant changes have been observed in red blood cell count, hematocrit, blood hemoglobin, white blood cell count, or a series of biochemical measurements performed on blood and urine.

Microscopic examination of tissues from the rats that have been killed has revealed a number of abnormalities resulting from smoke exposure. Hyperplasia and squamous metaplasia are observed in the tracheal epithelium. Focal chronic alveolitis is found throughout the lungs. These areas are packed with smoke particles and alveolar macrophages. In and around the lesions the alveoli are disrupted and severely dilated. Alveolar pores are widened. A marked increase in the fibrous and fibrillar elements of the alveolar walls is evident, and thickening of pulmonary vessels was observed. Preliminary examination of tissues with a scanning electron microscope reveals a similar picture: disrupted alveolar architecture with large open spaces, increased alveolar fenestration, and threadlike strands of alveolar tissue. The relation of these changes to human emphysema will require further study.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

DECLINE IN PHYTOHEMAGGLUTININ RESPONSIVENESS OF MOUSE SPLEEN CELLS WITH ADVANCING AGE ACCOUNTED FOR BY REDUCED FREQUENCY OF PHYTOHEMAGGLUTININ-RESPONSIVE CELLS

C. F. Gottlieb and G. M. Peterman

Age-associated decline in immune competence has been extensively documented. We have reexamined the age changes in phytohemagglutinin responsiveness of spleen cells from BALB/cNIH mice[1] with a microsystem for mitogenic studies which we have developed.[2] In the preliminary experiments reported here, we have used the microsystem to perform limiting dilution analysis of PHA-stimulated spleen cell cultures from mice of different ages.

Limiting dilution analysis is a quantal assay based on the Poisson distribution, with the critical assumption that there is a single limiting event necessary for response. The probability of nonresponding cultures, as given by the Poisson distribution, is $P(0) = \exp(-\Phi x)$, where $P(0)$ is the probability of no response, Φ is the frequency of responding cells, and x is the number of cells cultured. The frequency of responding cells in a cell population (Φ) can be determined by plotting the logarithm of the fraction of nonresponding cultures [estimate of $P(0)$] for each of several different numbers of cells per culture (x). The slope of the resulting curve is $-\Phi$.[3]

Spleen cells from young (3 months) or old (20 months) BALB/cNIH female mice were cultured at concentrations ranging from 5×10^3 to 2×10^5 cells per culture. At each cell concentration, 24 replicate cultures were stimulated with PHA, while another 12 replicates served to establish the unstimulated level of incorporation of tritiated thymidine ([^{3}H]dThd). Responder cultures were defined as those cultures giving a response equal to or greater than the mean plus two standard deviations of the control cultures.

The frequency of PHA-responsive cells in the spleens of young mice was determined to be $(7.5 \pm 4.6) \times 10^{-5}$, while the value for the old was $(2.2 \pm 0.8) \times 10^{-5}$. This 3.4-fold difference in frequency accounts for a major portion of the five- to tenfold difference in [^{3}H]dThd incorporation evidenced by cultures of equal numbers of spleen cells from young or old mice. We therefore conclude that most, if not all, of the age-associated decline in PHA responsiveness of spleen cells in this mouse strain is due to the presence of fewer numbers of responsive cells.

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AGE-RELATED DECLINE IN T-CELL HELPER FUNCTION

R. L. Krogsrud* and E. H. Perkins

The cooperation among cell types involved in the humoral immune response may be a site of deficiencies responsible for the overall decrease in antibody-forming capacity of aged mice. The evidence that macrophage function is unaffected, B-cell proliferation in response to mitogens is only slightly affected, and T-cell proliferation is decreased is not sufficient to explain the decline in immune function of aging animals. Therefore, we designed experiments to test

the possibility that T-cell helper function declines with age. Hence, T-cell populations from different age groups of $BC3F_1$ mice primed with carrier (bovine gamma globulin) were mixed in various numbers with a fixed number of B-cell populations from young mice primed with hapten (dinitrophenol) and injected into immunologically inert lethally irradiated mice along with antigen (dinitrophenylated bovine gamma globulin). The immune response was assayed 7 days later by a modification of the Jerne plaque technique specific for the dinitrophenyl determinant.

We found that the response was decreased fourfold using the T cells from 34-month-old mice (the median life span for the strain) as compared with young adults (at maximal immune capacity). More important, though, analysis of cell dose vs response curves indicates that two separate functions of the T helper cells are adversely affected by increased age. First, the increased number of older mouse T cells needed to activate the B-cell population to reach a plateau level of plaque-forming cells showed that they were less efficient in stimulating the antigen-specific B cells. Second, we observed that the plaque-forming-cell response continued to rise beyond the plateau level with the addition of more T cells from young mice, but when we added more T cells from old mice the rise in slope was markedly decreased or absent. This implies that the T cells of old animals are deficient in regulating the proliferation of the B-cell clones.

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DECREASED LYMPHOPROLIFERATIVE ACTIVITY FOLLOWING SYNGENIC TUMOR INOCULATION IN AGED MICE

E. H. Perkins and L. H. Cacheiro

Interaction between cell-mediated immunity and progressive tumor growth is a central issue in tumor immunology. If it were possible to produce increased numbers of specifically sensitized lymphocytes (sensitized T cells), a more vigorous and sustained attack on tumor cells should be elicited. Thus the significance of cellular proliferation, particularly of T lymphocytes, is readily apparent.

Our past studies demonstrate that T-cell, but not B-cell, proliferation is impaired in old animals in response to T- and B-cell plant mitogens. Therefore, groups of young adult (3-month) and old (24-month) BALB/c mice were inoculated in the right hind footpad with 10^6 syngenic weakly immunogenic line 1 (alveolar cell carcinoma) tumor cells. To measure and express the relative rates of cellular proliferation in draining and contralateral lymph nodes at 2-day intervals thereafter, five mice from each group received an intravenous (iv) injection of 20 μCi of [^{3}H]dThd. Thirty minutes later the nodes were removed, and either they were dissolved individually in 0.5 ml of Protosol for counting in a Beckman-230 liquid scintillation spectrometer, or they were fixed and radioautographs were prepared for differential staining of specific cellular components (T and B cells and macrophages). A logarithmic increase in the amount of [^{3}H]dThd incorporated into the draining lymph nodes was seen for the 10-day observation period, whereas the activity for the contralateral nodes for both young and old animals was constant and comparable. However, cellular proliferation ([^{3}H]dThd incorporation) in the draining nodes of old animals was minimal, being approximately two-, three-, and fourfold less on days 6, 8, and 10, respectively, than that observed in young animals.

The mechanisms of action and the limiting conditions for eliciting effective cell-mediated systemic tumor immunity have not been fully explored. The comparative model described here is being utilized for kinetic studies to ascertain the significance of cellular proliferation and the interaction and contribution of the various cellular components of lymphoid tissue during the lymphoproliferative response to tumor growth.

THE EFFECT OF MOUSE STRAIN, THYMIC FUNCTION, AND AGE ON THE ABILITY OF SPLEEN CELLS TO RECOVER THEIR IMMUNOHEMATOPOIETIC FUNCTIONS FOLLOWING SUBLETHAL IRRADIATION

W. J. Peterson

The bone marrow, thymus, and lymphohematopoietic microenvironments are determining factors in recovery of immune competence following sublethal irradiation. Because of age-related degenerative changes in all of these parameters, it was anticipated that immune competence of irradiated old mice would show an altered pattern of recovery. Therefore, three age groups (3 to 7, 15, and 23 to 34 months) of C57BL/6J mice were treated with either 250 R, 500 R, or 600 R. At various intervals thereafter

their spleen cells were assessed for recovery of humoral and cell-mediated immunologic activity and for thymus-derived (T), bone-marrow-derived (B), and stem cell compartments. Two age groups (3 to 7 and 23 months) of C3H/Anf Cum mice were also treated with the two highest doses of irradiation, and their spleen cells were tested only for recovery of T and B cell compartments. The results showed that recovery of immune competence following 250 R was independent of age. In contrast, an age-related delay was noted in recovery from 500 R. The only case of complete recovery using C3H/Anf Cum mice was recovery of the ability of spleen cells from young mice to respond to B-cell-specific lipopolysaccharide. The ability of spleen cells from young mice to respond to T-cell-specific PHA and of spleen cells from old mice to respond to both mitogens failed to show complete recovery. The ability of spleen cells from young C57BL/6J mice to form colony-forming units in the spleen of lethally irradiated recipients and to respond to lipopolysaccharide returned progressively to control level by the 2d week. However, the ability of these cells to produce antibody against sheep RBCs, induce a graft-vs-host reaction, and respond to PHA did not recover completely until the 12th week. The same colony-forming-unit recovery pattern was observed using spleen cells from irradiated old mice. On the other hand, the ability of these cells to produce antibody against sheep RBCs, induce a graft-vs-host reaction, and respond to lipopolysaccharide was depressed for 8 weeks before returning to normal by the 12th week. Furthermore, their ability to respond to PHA remained below normal until the 11th month. It is concluded that delayed recovery of immune competence in irradiated old mice is most likely due to two factors: (*a*) defective ability of stem cells to produce immunocompetent precursor cells and (*b*) decreasing efficiency of the thymus to process precursor cells into immunocompetent T cells.

REVERSAL OF DIFFERENTIAL TUMOR GROWTH AND METASTASIS IN NUDE MICE BY THYMUS GRAFTS

C. B. Skov,* J. M. Holland, and E. H. Perkins

In spite of the fact that congenitally athymic "nude" mice accept grafted tissues from xenogenic as well as allogenic and syngenic donors, they have proved no more prone to develop spontaneous or chemically induced tumors. Furthermore, nude mice do not readily accept all tumor grafts, and highly metastatic tumors either do not metastasize or do so much less readily in nude recipients. The reduced efficiency for metastasis of transplanted tumors in nude mice provides a provocative and promising means for investigating immunological and other biological mechanisms of tumor development.

Our earlier experiments with artificial metastasis of syngenic tumors in nude mice established that significantly fewer metastases developed in nude mice. Several approaches were explored in an effort to demonstrate a differential potential in nude and littermate mice for supporting growth of new tissue. No differences were found in the rate of liver regeneration after partial hepatectomy, in wound healing, in formation of clones by stem cells after iv injection of bone marrow into lethally irradiated recipients, or in the expression by serum or spleen cells of natural cytolytic activity toward tumor cells.

The growth and natural metastasis of a syngenic mammary adenocarcinoma has now been investigated. After being transplanted intramuscularly in the thigh of nude and littermate mice, this tumor consistently metastasizes to the lungs of both groups within 4 weeks after becoming palpable. Nude mice, however, develop 2 to 4 times fewer metastases per lung.

To determine whether the thymus would influence the growth and/or metastasis of this tumor, nude mice were grafted with syngenic thymus from young adult donors several weeks before being challenged with the tumor. Tumor size was monitored weekly by measuring the front-to-back and inside-to-outside thigh diameters with vernier calipers. Approximately 5 weeks after tumor challenge, the lungs were excised for microscopic enumeration of tumor foci. Spleens, lymph nodes, and thymus grafts were also taken from these animals in order to histologically and functionally compare the immune status of thymus-grafted nude mice with nude and littermate controls. The immunocompetence of spleen cells from these donors was assessed by measuring the proliferative response (incorporation of tritiated thymidine) of the cells in a graft-vs-host assay. Spleen cells from thymus-grafted nude mice were from 60 to 90% as responsive as cells from normal littermates in the graft-vs-host response when tested 7 to 8 weeks after thymuses were successfully grafted. Although tumors grew progressively in all mice, tumor size was greatest in littermate, intermediate in thymus-grafted nude, and smallest in nude mice. The numbers of metastatic foci in the lungs of thymus-grafted nude and littermate mice were not different but were significantly greater than in nude mice.

Other experiments were conducted to test whether the pattern of growth and metastasis would be altered by immunization. Thus, nude, thymus-grafted nude, and littermate mice were immunized subcutaneously in the left hind footpad with irradiated tumor cells, *Corynebacterium parvum*, or both. The mice were challenged with tumor in the right thigh 10 days later, and growth and metastasis were monitored as before. Rates of growth were unaltered in all groups; however, the incidence of metastasis in littermate and thymus-grafted nude mice was reduced significantly in those animals immunized with both agents together. No protection resulted from treatment with either agent alone.

These studies show that the thymic influence is a major factor in regulating tumor growth in this system. Our current efforts are directed toward understanding whether the reduced metastasis in nude mice is either a positive or negative process mediated through an immunological or hormonal mechanism.

*Supported by Postdoctoral Fellowship 1 F22CA 00875-01, Division of Cancer Research, NIH.

PRETHYMIC LYMPHOCYTES IN THE PERITONEUM

P. N. Bryan* and J. W. Goodman

Peritoneal cells from C57BL/6 (B6) mice have the ability to augment the growth of B6 bone marrow grafts in lethally irradiated B6D2F_1 recipients. Previous experiments to determine whether thymus-derived lymphocytes in the peritoneum are responsible for augmentation have been ambiguous. Peritoneal cells reacted with anti-θ antibody still had the ability to augment; however, peritoneal cells from thymectomized, irradiated, and reconstituted mice have reduced augmenting ability (J. W. Goodman, unpublished data).

We did an experiment to see whether pre-T lymphocytes, which might be causing augmentation, are present in the peritoneum. Cells taken from peritoneal cavities of B6 and B6D2F_1 mice were cultured 72 hr with either concanavalin A or thymosin, a thymic extract prepared by us. θ-Bearing cells were then stained with fluorescent antibody and counted under a phase-contrast microscope. In peritoneal cells from B6 mice the percentage of lymphocytes bearing θ antigen increased from 30% in control cultures to 42% in thymosin-induced cultures and to 62% in cultures induced with concanavalin A. In cultured cells from B6D2F_1 mice the percentage of θ-bearing lymphocytes increased from 32% in controls to 48% in thymosin-induced cultures and 56% in cultures induced with concanavalin A. Since the normal, unstimulated peritoneum contains about 20% lymphocytes, one can estimate that 3% to 5% of the cells can be induced to become θ-bearing lymphocytes.

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EFFECT OF THYMOCYTES ON RED CELL PRODUCTION

J. W. Goodman

Among the kinds of data adduced to substantiate the observation that thymocytes increase growth rate of proliferating blood-forming cells were studies of the kinetics of red cell production. Using 24-hr ^{59}Fe incorporation, we studied uptake for several days during the period of exponential marrow growth. From regression lines fitted to the logarithmically transformed data, doubling times could be estimated. In two experiments using rat marrow and thymocytes transplanted to different F_1 hybrid recipient mice we found the slope for thymocyte plus marrow compared with that for marrow only gave a ratio of 1.6 in both cases. The slopes that were compared in the two experiments were significantly different from each other and represented doubling times of around 23 hr in the case of marrow only and 14 hr for marrow plus thymocytes.

It has been suggested to us on the basis of recently published data[1] that conclusions based on 24-hr ^{59}Fe uptake studies were inaccurate or even erroneous because 24-hr values represent variable amounts of the 72-hr uptake. In other words, the 24-hr uptake values might not have been representative of the whole proliferative portion of the erythron. We undertook the following experiment to dispel any doubt that the 72-hr uptake values would have led us to the same conclusions as those based on 24-hr data. The irradiated mouse recipients were F_1 hybrid type (C57BL/6 × BALB/c F_1) and different from the two used in earlier experiments. The rats also were genetically different from those in the earlier two experiments. The number of chimeras sampled was in the range of 10 to 12 in this experiment, approximately one-third of the number represented at each point in the earlier experiments. Data were transformed

and plotted in a similar fashion. Slopes were estimated, and the ratio of slopes at 24 hr (thymus plus marrow: marrow only) was 1.3, the difference being significant ($P = 0.05$). When the same groups were sampled 72 hr after ^{59}Fe injection, the two groups gave slopes that yielded a ratio of 1.8, the difference being again significant ($P < 0.05$). Thus, the 72-hr value provides even more substantiation for our inference about the influence of thymocytes on the rate of cell division, because the ratio of slopes is greater at 72 hr than at 24 hr, clearly indicating a more rapid expansion of thymus- plus marrow-derived erythroid colonies.

1. R. Schofield, *Exp. Hematol.* **3**, 22 (1975).

AUGMENTATION OF HEMOPOIETIC COLONIES BY IRRADIATED THYMIC AND LYMPH-NODE LYMPHOCYTES

J. W. Goodman and N. L. Basford

Thymic and lymph-node lymphocytes are capable of augmenting growth of transplanted bone marrow in certain poor-growth combinations. By some criteria, such as number of microscopic splenic colonies or 24-hr ^{59}Fe incorporation into circulating erythrocytes, lymphocytes exposed to 900 R have been found to be as effective as unirradiated cells. However, by the criterion of macroscopic colony number, 900-R lymphocytes are not regularly so capable of enhancing hemopoiesis. In seeking to characterize the augmenting process, we decided to examine the differentiation patterns of chimeras' regenerating hemopoietic tissues. In experiments already published we found that lymphocytes shifted differentiation from erythropoiesis toward granulopoiesis. When lymphocyte donors had been irradiated, the pattern of differentiation was unchanged from that seen when marrow alone had been administered. That result, plus the subsequent finding that specifically tolerant lymphocytes failed to promote granulopoiesis, led us to conclude that a lymphokine is elaborated during graft-vs-host activity which acts as a specific stimulator for granulocyte production. Having recently verified in quantitative terms that mice injected with thymic lymphocytes have larger colonies that those that receive only marrow cells, we were curious to know the relative sizes of colonies in recipients of 900-R lymphocytes.

Irradiated hybrid mice (900-R B6D2F$_1$'s) were injected with thymic or lymph-node lymphocytes from either parental (P) or allogenic (A.SW) donors, some of which had been exposed immediately before the experiment to 900 R. Eight or ten days later, spleens were removed from the chimeras, and sections were prepared for histologic examination and for counting, classification, and measurement of colonies. Cross-sectional areas were calculated and compared. Preliminary results from two experiments indicate that irradiation of thymic lymphocytes reverses the shift to granulopoiesis but does not affect the augmented size of granulocytic colonies that develop. Irradiation of lymph-node lymphocytes, on the other hand, does not decrease the augmented total number of colonies developing. If substantiated by additional data, these findings would suggest that augmentation of granulopoietic colony size can be effected by nondividing thymic but not lymph-node lymphocytes and that the process is independent of the extent of granulopoiesis relative to other hemopoietic activity in the spleen.

HEMOPOIETIC BURST SIZE STUDIES

J. W. Goodman and S. G. Shinpock

Evidence was sought to corroborate physical measurement differences found in hemopoietic colony sizes when thymocytes were administered together with bone marrow transplants. Uptake of [^{3}H]dThd was used to assess colonies of all kinds synthesizing DNA in the spleens of chimeras 8 to 10 days after transplantation.

We used the method of [^{3}H]dThd incorporation into colonies described by Zipori and Trainin.[1] Ten microcuries of [^{3}H]dThd was injected intraperitoneally into each mouse 3 hr before the animal was killed and its spleen was removed. Individual spleens were placed in saline which contained a small amount of saturated picric acid a few minutes before enumeration of colonies under a dissecting microscope. The spleen was then washed in saline, and a suspension was prepared in ammonium chloride to remove red cells and thus to minimize color quenching. After two washings the pellet was resuspended in sodium lauryl sulfate, transferred to a 1-in.-square piece of Whatman chromatography paper, and allowed to dry in air. DNA was extracted by removing extraneous material with two washings of 10% trichloroacetic acid followed by two of 5% trichloroacetic acid, one of cold absolute alcohol, and one of ethyl ether. When completely dry, each square was placed in a vial of scintillation fluid, and the sample was counted in a liquid scintillation counter.

In three experiments, all of which were sampled on day 10, the average counts per minute per colony for the group receiving bone marrow only was 54, as compared with a count of 134 for the colonies from spleens of mice that had received thymocytes in addition to marrow. This difference was significant at the 95% confidence level. The assumption was made in these experiments that the only cells synthesizing DNA were those repopulating hemopoietic colonies. When large numbers of thymocytes (2×10^7 or more) have been administered, this is not strictly true. However, at 10 days after transplantation the cells derived from thymocytes that are dividing in the white pulp areas are no longer numerous and probably do not contribute significantly to the [^{3}H]dThd counts per colony. To control this possible source of error, a fourth experiment was carried out at 8 days after transplantation, this time at a 2-hr sampling interval after ip injection of the label. Surface colonies were similarly assessed in weak picric acid, but Protosol was used to dissolve whole spleens individually. DNA was not extracted. The average number of disintegrations per minute of radiation-control spleens (that contained no nodules) was subtracted from the average value for marrow-only spleens. The difference was then divided by the average number of colonies for that group. Likewise the average number of disintegrations per minute of thymus-only spleens (containing no nodules) was subtracted from thymus-plus-marrow spleens to arrive at the number of disintegrations per minute per colony in that group. Values obtained were: bone marrow only, 889 counts per minute; bone marrow plus thymocytes, 3431. There seems to be little doubt that the colonies in the latter group are more actively synthesizing DNA.

1. D. Zipori and N. Trainin, *Exp. Hematol.* 3, 1 (1975).

AMPLIFICATION OF COLONY-FORMING CELLS BY THYMOCYTES

S. G. Shinpock and J. W. Goodman

Mouse bone marrow contains hemopoietic colony-forming cells which give rise to splenic colonies when transplanted into heavily irradiated recipients. These colony-forming cells not only are capable of giving rise to fully differentiated blood cells but also have the capacity of self-renewal. Thymocytes have been shown in earlier experiments in our laboratory to be involved in the control of proliferation and differentiation of hemopoietic cells in transplantation situations where suboptimal growth occurs, for example, particular semiallogenic, allogenic, and xenogenic combinations.[1] The object of this study was to determine whether thymocytes are able to increase the self-renewal capability of bone marrow colony-forming cells.

Cells which are exposed during DNA synthesis to dThd labeled with ^{3}H of high specific activity undergo a loss in colony-forming ability due to the lethal effect of the decay of incorporated tritium. Consequently, the extent of loss of colony-forming ability of a rapidly expanding population of hemopoietic cells can be used as a measure of the fraction of these cells synthesizing DNA (in S phase.).

In our experiments, bone marrow (4×10^5 cells) from C57BL/6 (B6) mice with or without large numbers of thymocytes was injected into lethally irradiated B6D2F$_1$ hybrids. At various times after transplantation, the spleens of the resultant chimeras were removed, pooled, and suspended in RPMI 1640 containing 10% fetal calf serum and [^{3}H]dThd (500 μCi/ml). After incubation for 20 min at 37° C, the cells were diluted, and an equivalent of one or a fraction of one spleen was injected into each lethally irradiated B6 recipient, that is, a host syngenic with the original marrow graft. Eight days later, spleens from the secondary recipients were removed, fixed in Tellyesniczky's solution, and evaluated macroscopially for the presence of hemopoietic nodules in order to assess the number of colony-forming units in the retransplanted spleen that had survived exposure to [^{3}H]dThd.

Using the thymidine suicide technique, we have found that when thymocytes have been transplanted with marrow, a significantly larger fraction of colony-forming cells are in S phase at times early after transplantation. The fraction of cells in DNA synthesis during the brief exposure to [^{3}H]dThd is undetectable before day 12 in the group that received bone marrow only. On day 12 the fractional survival of this group was 0.39 in one experiment, indicating that 61% of the colony-forming cells were in S phase. Data from mice that had been given thymocytes with bone marrow indicate an enhanced self-renewal ability of the original bone marrow graft. On day 5 the fraction of cells surviving was as low as 0.24 in one case, indicating that 76% of the colony-forming cells were in S phase at the time of exposure to [^{3}H]dThd. By day 12 the fractional survival per spleen was 0.74, showing a decline in proliferative rate toward the normal steady-state level for colony-forming units in adult mouse bone marrow and spleen.

It thus appears from these preliminary results that thymocytes may be able to amplify division or self-renewal of colony-forming cells. We intend to explore this finding in detail and to examine other augmentative cells, such as lymph node lymphocytes and peritoneal cells, for their ability to enhance self-renewal of bone marrow colony-forming units.

1. J. W. Goodman and S. G. Shinpock, *Transplantation* 13, 203 211 (1972).

STIMULATION OF MEGAKARYOCYTOPOIESIS BY ACUTE THROMBOCYTOPENIA IN RATS

T. T. Odell

Rats were made acutely thrombocytopenic by injection of antiplatelet serum. Marrow sections and squash preparations were made at intervals during 120 hr. Determinations were made of mitotic index, stage of maturation, ploidy level, and cell size of megakaryocytes; number and size of platelets were measured. Increased endomitosis among megakaryocytes was followed by an increase in the proportion of immature megakaryocytes, a greater average ploidy level of recognized megakaryocytes, and larger megakaryocytes. Maximum changes in these several parameters occurred between 32 and 72 hr after induction of thrombocytopenia. By 120 hr all megakaryocyte parameters were near normal. For about 3 days, beginning at about 36 hr, platelet numbers increased rapidly. Average platelet size rose and returned to normal within about 60 hr. Changes in ploidy and size of megakaryocytes were measured in the immature and mature maturation stages. The results suggest that the initial stimulus in response to acute thrombocytopenia acts primarily on diploid precursors, programming them to mature into a population of megakaryocytes with an average ploidy approximately one level greater than in normal rats and a proportionate increase in cell size. The larger megakaryocytes presumably produce more platelets, accounting for a major part of the increased rate of platelet production. Since the changes in megakaryocytes begin to reverse before circulating platelet numbers have reached the normal level, reversal of the stimulus appears to be initiated by some change other than platelet mass.

CHANGES IN THE MITOTIC INDEX OF MEGAKARYOCYTES OF MICE AFTER ACUTE THROMBOCYTOPENIA

T. T. Odell and D. A. Boran

Studies of the responses of the megakaryocyte system of rats to acute thrombocytopenia have revealed a variety of alterations, including changes in megakaryocyte ploidy and size, in the proportions of megakaryocytes in various maturation stages, in the 30-min [^{3}H]dThd labeling index, and in the endomitotic index. Similar information is needed for mice because of their extensive use in studies of hematopoiesis and particularly because of their potential use in investigations of the effects of presumptive thrombopoietic agents on megakaryocytopoiesis. Changes in ploidy and in endomitotic index were the most striking alterations observed in rats. Since we can measure the mitotic index more easily than ploidy, experiments were undertaken to determine whether the endomitotic index of megakaryocytes of mice responds to thrombocytopenia in a manner similar to that seen in rats and to establish the time parameters of the response in mice.

Acute thrombocytopenia was induced by injection of antiplatelet serum in a dose sufficient to reduce the peripheral platelet count to about 2% of normal. New platelets had begun to enter the circulation at a rapid rate by 48 hr and reached a peak of about 150% of normal on day 5. Mice were killed at intervals during 12 to 144 hr after injection of antiserum, and marrow sections were taken for determination of the mitotic index of megakaryocytes. The mitotic index rose to a value about twice that of the controls at 32 hr after antiplatelet-serum injection and then declined. At 96 hr the mitotic index was significantly less than that of controls by the *t* test, and it returned to normal by 6 days.

The results demonstrate that the endomitotic index of megakaryocytes of mice increases as part of the response to a need for platelets. This is followed by a transient oscillation below normal. Additional experiments will be undertaken to determine whether this end point can be used to measure effects of presumptive thrombopoietic agents on megakaryocytopoiesis.

THE MOLECULAR BASIS FOR CHRONIC HEART FAILURE

N. W. Revis

The role of toxic air contaminants (both organic and inorganic) and of obstructive pulmonary diseases in the development and the progression of heart failure has been of major interest to this group. Our studies have been directed toward delineating the molecular events in the failing heart. We have observed that in the experimentally induced failing heart of rats or hamsters the concentrations of both noradrenaline and 3′:5′-cyclic adenosine monophosphate (cAMP) are significantly decreased. Based on these observations, we have extended our studies by measuring the responsiveness of the failing heart to glucagon and noradrenaline. Results of these studies showed that responsiveness to glucagon was significantly decreased, while responsiveness to noradrenaline was normal. The responsiveness was determined by measuring the accumulation of cAMP.

Determination of the binding constant for these two hormones to the plasma membrane of the failing heart showed that glucagon binding was significantly depressed, while noradrenaline binding was increased by twofold. The above finding suggests that the plasma membrane of the failing heart has been altered. Accordingly we have been attempting to measure the concentrations of two phospholipids, phosphotidylinositol and phosphotidylserine, in the purified plasma membrane of the normal and the failing heart. The responsiveness of the normal myocardium to glucagon has been shown to require phosphotidylserine, while noradrenaline responsiveness requires phosphotidylinositol. In preliminary studies the concentration of phosphotidylserine was found to be significantly decreased, while phosphotidylinositol was significantly increased. The significance of these results remains to be elucidated. However, these findings may provide a possible model upon which the failing heart can be based.

In addition to the studies mentioned above, this group has been involved in studying the *in vitro* metabolic effect(s) of two occupational and urban inorganic air pollutants—cadmium and zinc—on lung, heart, kidney, and the aorta of rats and hamsters. These divalent cations were chosen because (*a*) both of these elements have been implicated in predisposing the cardiovascular system to disease in rodents and (*b*) cadmium is associated with both pulmonary emphysema and renal tubular necrosis in both humans and rodents. Although there is information available on these two trace elements, very little is known about the molecular mechanism responsible for pathological changes.

It has been predicted that the concentrations of both cadmium and zinc will be increased in the air and water by the large-scale effort involved in mining coal and in converting it into liquid and gaseous fuels. If the prediction is correct, it establishes the urgency of determining, at the molecular level, the effects of these two trace elements on biological systems.

In the lung, heart, kidney, and aorta of rats and hamsters, the activity of monoamine oxidase and catechol-*O*-methyltransferase and the binding of noradrenaline to plasma membrane were measured in the presence and absence of either cadmium or zinc. Results from these studies show that in the presence of either trace element, the activity of these two enzymes and the binding of noradrenaline to the plama membrane are decreased. Dithiothreitol prevented the decrease in enzyme activity in the presence of the two elements but did not prevent the observed decrease in the binding of noradrenaline. These results suggest that the two elements inhibited the activity of these two enzymes by binding to the sulfhydryl groups in the enzyme. The fact that dithiothreitol did not prevent the decrease in noradrenaline binding in the presence of these two elements suggests that the charged receptor does not contain sulfhydryl groups. Additional studies are planned to determine whether these findings are of physiological significance.

TRANSLATION OF MESSENGER RNA SPECIFIC FOR TYROSINE AMINOTRANSFERASE IN OOCYTES OF *XENOPUS LAEVIS*

J. M. Nickol,* K.-L. Lee, T. G. Hollinger,† and F. T. Kenney

The level of the hepatic enzyme tyrosine aminotransferase (TAT) is increased by administration of the glucocorticoid hydrocortisone. This induction is caused by an increase in the rate of synthesis of the enzyme, but the exact biochemical mechanism responsible for this hormonal stimulation is unknown. Indirect evidence (kinetics of induction and the effect of inhibitors on induction) suggests that the hormone acts transcriptionally to increase the amount of messenger RNA (mRNA) encoding TAT. To directly test this possibility, we have used the *in vivo* heterologous translation system

of *Xenopus laevis* oocytes to quantitate the relative amounts of TAT-specific mRNA present on polysomes isolated from control and hydrocortisone-induced rat liver.

Polysomal RNA containing polyriboadenylic acid [poly(A)] isolated by oligo(dT)-cellulose chromatography was microinjected into manually dissected oocytes. These injected oocytes were then incubated in a salt solution containing [^{3}H]leucine to allow for the translation of the newly introduced messages into labeled proteins. The amount of TAT synthesized by the oocyte, in response to this exogenous RNA, was quantitated by immunoprecipitation of the newly synthesized proteins using a specific antiaminotransferase antibody. SDS–polyacrylamide gel electrophoresis of these immunoprecipitates shows a single large peak of radioactivity migrating to the position of authentic TAT subunit. Various controls strengthen the conclusion that this protein, which is synthesized by the oocyte in response to liver poly(A) RNA and which has immunochemical and electrophoretic properties identical to TAT, is actually the enzyme whose message we are attempting to quantitate.

Titrations of polysomal poly(A) RNA from control and induced rats are linear for a certain RNA range and then reach a plateau at increasing RNA concentrations. Injection of equivalent amounts of the two RNA preparations shows a fivefold increase in the amount of TAT synthesized by oocytes injected with RNA from hydrocortisone-treated rats. Since the total yield of poly(A) RNA was the same for both preparations and their messenger activity is identical, as shown by their ability to direct the synthesis of all proteins in a wheat germ translation system, we conclude that hydrocortisone increases the amount of functional TAT-specific mRNA present on the polysomes.

Current research interests include: (*a*) investigation of changes in the amount of mRNA encoding for TAT throughout the hydrocortisone induction period to correlate changes in rate of synthesis during TAT induction with changes in TAT-specific mRNA; (*b*) investigation of the amount of TAT-encoding mRNA present during induction by other hormones, insulin, and adenosine 3′:5′-phosphate (cAMP), which also cause an increase in the rate of TAT synthesis. Although the mechanism of action of these hormones is unknown, they are believed to act at some translational step in protein synthesis.

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† Postdoctoral investigator supported by Grant HD03169 from the National Institutes of Health.

STUDIES ON THE MECHANISM OF ACTION OF HYDROCORTISONE, INSULIN, AND ADENOSINE 3′:5′-PHOSPHATE IN THE INDUCTION OF HEPATIC TYROSINE AMINOTRANSFERASE

N. Pomato,* J. M. Nickol,† K.-L. Lee, and F. T. Kenney

The synthesis of tyrosine aminotransferase is increased by hydrocortisone, insulin, and cAMP in rat liver and cultured hepatoma cells. Evidence from this laboratory has indicated that hydrocortisone probably acts at the level of transcription, whereas insulin and cAMP act posttranscriptionally. We have attempted to further distinguish between the mechanisms of action of these inducers in a rat liver *in vitro* protein-completion system.

The protein-completion system (S-20) was isolated from a postmitochondrial rat liver supernatant which was passed through a Sephadex G-25 column. We have demonstrated that the S-20 is capable of completing the synthesis of nascent polypeptide chains *in vitro*, which had already been initiated *in vivo*. In addition, no reinitiation takes place *in vitro*. By allowing the completion of nascent polypeptide chains to occur in the presence of radioactive amino acids and by specific immune precipitation of the tyrosine aminotransferase synthesized, we are able to quantitate the amount of the enzyme being completed.

Using this system, we have been able to compare the time course of induction of tyrosine aminotransferase by hydrocortisone, insulin, and cAMP. The induction of the enzyme by insulin and hydrocortisone, as measured in this system, closely parallels the rate of synthesis of the aminotransferase measured *in vivo*. There is very little change in the amount of enzyme completed *in vitro* when cAMP is used as the inducer.

Since the S-20 system employed here represents strictly a measure of active polysomes engaged in protein synthesis, our results indicate that the effect of hydrocortisone and insulin is to increase the number of polysomes, whereas the primary mechanism of action of cAMP in stimulating aminotransferase synthesis does not involve an increase in this parameter. If the indirect evidence obtained from kinetic results and inhibitor studies, which indicate that hydrocortisone increases the amount of tyrosine aminotransferase mRNA whereas cAMP and insulin do not, is supported by the quantitation of the mRNA in a cell-free protein synthesizing system, then it can be concluded that the probable mechanism of action of insulin on aminotransferase synthesis is through an increase in the rate of initiation whereas cAMP probably acts by increasing the rate of translation and/or termination of the enzyme.

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GLUCOCORTICOID CONTROL OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE

F. T. Kenney, L. E. Roberson, and F. J. Bollum*

The thymus-specific enzyme, terminal deoxynucleotidyl transferase, disappears very rapidly ($t_{1/2} \cong$ 6 hr) when sensitive mouse lymphomas of thymic origin are treated with glucocorticoids.[1] This is one of the earliest changes observed in the well-known destruction of thymocytes by glucocorticoids and may be a significant component of it. Our interpretation of the kinetics of this response suggests that: (*a*) the transferase normally undergoes a rapid rate of turnover, and (*b*) the steroid stops synthesis of the enzyme quickly and completely. To analyze this hypothesis using isotopic–immunochemical techniques, we have obtained samples of the mouse P-1798 lymphoma, a steroid-sensitive tumor made available to us by F. Rosen of Roswell Park Memorial Institute for Cancer Research. We have determined that tumor extracts contain high levels of the transferase, and we will shortly initiate turnover studies. If our hypothesis is correct, a significant negative counterpart to the induction response to glucocorticoids will be available for further study. Also, this system may prove to be of particular importance in view of the essential role of T cells in cell-mediated immunity and the immunosuppression resulting from their sensitivity to glucocorticoids.

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1. F. J. Bollum, in *Research in Molecular Biology*, Vol. 6 (ed. by R. Z. Kahn). Verlag GmBH, Wiesbaden, 1975.

POTENTIATION OF HEPATIC MESSENGER RNA TRANSLATION BY RIBOSOMAL RNA: A POSSIBLE MECHANISM OF REGULATION OF MESSENGER RNA TRANSLATION BY INSULIN

S. Sakonju* and K.-L. Lee

Insulin stimulates total protein synthesis in liver, muscle, and other target tissues. Indirect evidence indicates that this hormonal effect does not involve increased cellular mRNA synthesis. Recently, it has been suggested that complex formation between 18 S ribosomal RNA (rRNA) (3′ end) and mRNA (5′ end) appears to function in mRNA translation. We have initiated experiments to determine if such a complex formation is facilitated by insulin. The following results are consistent with the premise: (*a*) sucrose gradient analysis of hepatic polysomal poly(A)-containing RNA isolated by oligo(dT)-cellulose column reveals that considerable amounts of functional poly(A)-RNA (assayed in a wheat germ cell-free system) are in the complex form with 18 S rRNA; (*b*) heat treatment (80° C for 3 min) dissociates the complex. Analysis of message activity of the complex in the wheat germ system before and after heat treatment indicates that heat treatment decreases the message activity without changing the average size of protein synthesized; (*c*) 18 S rRNA potentiates the translation of ribosomal RNA-free, poly(A)-containing RNA in the wheat germ system; (*d*) the amounts of the mRNA–rRNA complex are reduced in the livers of diabetic rats and can be restored by insulin treatment.

We are investigating the effect of 18 S rRNA on the translation of a specific mRNA, that which codes for hepatic tyrosine aminotransferase, and will determine whether the induction of this protein by insulin can be correlated with the effect of the hormone on mRNA–rRNA complex formation.

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EFFECT OF VITAMIN B_6 DEFICIENCY ON THE SYNTHESIS AND DEGRADATION OF HEPATIC AMINOTRANSFERASES

K.-L. Lee, P. L. Darke, and F. T. Kenney

We have previously shown[1] that the level of a rapidly degraded vitamin B_6-dependent enzyme [TAT ($t_{1/2}$ = 1.5 hr)] is reduced in rats fed B_6-deficient diets. Direct analysis of transferase synthesis and degradation revealed that the changes in the TAT level in B_6-deficient rats are primarily caused by a decrease in the rate of enzyme synthesis; there is no significant change in the rate of enzyme degradation.

We have extended these studies to another B_6-dependent enzyme, alanine aminotransferase [AAT ($t_{1/2}$ = 3.5 days)], to determine if this slow-turnover enzyme is subject to regulation by dietary vitamin B_6. During B_6 deficiency, the total hepatic AAT activity was decreased to 60% of the control level. Immunochemical titration revealed that the enzyme from B_6-deficient rats did not differ appreciably from the enzyme of control rats by this criterion. However, 40% more antibody was needed to neutralize equivalent amounts of the B_6-deficient enzyme than was required for the control enzyme. These results suggest that 40% of the AAT molecules in B_6-deficient rat liver are recognized by the antibody but have no enzyme activity. Supplementation of assay mixtures with a coenzyme (pyridoxal phosphate) did not alter the total enzyme activity. However, supplementation of the B_6-deficient diet with pyridoxine rapidly changed the inactive enzyme to an active enzyme. Label-and-chase analyses of AAT degradation in B_6-deficient rats showed it to be normal ($t_{1/2}$ = 3.5 days). Thus, turnover rates of active and inactive (coenzyme-free) enzyme molecules are the same. Pulse measurements of AAT synthesis indicated no change in this rate if the results were expressed as radioactivity incorporated into AAT per antigenic unit.

These results clearly demonstrate that the cofactor, pyridoxal phosphate, protects neither TAT nor AAT against degradation *in vivo*. Thus, the hypothesis that differences in coenzyme binding may explain heterogeneity of rates of protein degradation is not supported by experimental evidence.

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MOLECULAR WEIGHT OF POLY(A)(+)RNA

M. C. MacLeod*

Previous studies[1] have demonstrated that the apparent molecular weight of poly(A)(+)RNA can vary by a factor of 2 depending on the method of determination; values obtained by sucrose-gradient velocity sedimentation are lower than those obtained by polyacrylamide gel electrophoresis. This is thought to be caused by a conformational nonhomology between the poly(A)(+)RNA and the rRNA and transfer RNA (tRNA) used as molecular-weight standards. The nonhomology is probably caused, at least in part, by the secondary structure (base stacking) of the poly(A) moiety in aqueous solution. Gratzer and co-workers[2] have developed techniques for electrophoresis in 99% formamide under conditions where all RNAs studied [including poly(A) but not including natural poly(A)(+)RNA] are essentially devoid of secondary structure and, therefore, are conformationally homologous.

I have analyzed the number average molecular weight ($\bar{M}_n$) of samples of poly(A)(+)RNA obtained by Mg^{2+} precipitation of rat liver polysomes followed by oligo(dT)-cellulose chromatography. The $\bar{M}_n$ determined by electrophoresis in either 99% formamide or in aqueous buffer is about twice the $\bar{M}_n$ determined by sedimentation in aqueous sucrose gradients. Thus, the present best estimate of the $\bar{M}_n$ of liver poly(A)(+)RNA is about 7×10^5 daltons. This represents, on the average, about 800 more bases than are necessary to code for the average soluble liver polypeptide.

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TOWARD A MOLECULAR UNDERSTANDING OF VIRUS INDUCTION*

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Incorporation of 5-iododeoxyuridine (IdUrd) into the genome of virus-negative AKR mouse embryo cells in culture results in the expression of endogenous, C-type RNA tumor virus.[1] The

mechanism of IdUrd induction is unknown but must involve changes in the composition and/or structure of the chromatin which contains the proviral genes. Thus, the most direct approach to the solution of this problem would be to isolate proviral chromatin from uninduced and induced cells. Recent advances in the preparation and fractionation of intact mammalian chromatin,[2] coupled with the availability of nucleic acid probes for viral-specific sequences, make such an approach feasible. The basic steps we envision are: (*a*) construction of a viral complementary DNA (cDNA) probe (reverse transcriptase) containing poly(dG) tails (terminal transferase); (*b*) hybridization of the probe to the viral-specific, chromatin-associated RNA in partially purified, template-active chromatin from induced cells; (*c*) hybridization of the proviral chromatin-cDNA complexes to poly(dC)-Sepharose. Preliminary work, to date, has focused on the adaptation of previously developed chromatin preparation procedures[2] to the AKR cell line. Although slight modifications of labeling and cell fractionation procedures have proven necessary, no undue problems have been encountered thus far.

Since, in a typical experiment, IdUrd treatment induces virus expression in a maximum of 25% of the cells,[1] it would be advantageous to be able to purify those cells which are induced. Since induced cells express viral antigens on their surfaces, affinity methods using antiserum against viral proteins should be useful. A viral-specific immunoadsorbent has been prepared, and preliminary experiments with labeled virus preparations have given positive results. Further tests with pure populations of virus-positive and virus-negative cells are now under way.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and by the U.S. Energy Research and Development Administration.

†Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI.

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

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TWO FORMS OF REVERSE TRANSCRIPTASE FROM LEUKEMIC SPLEENS*

F. T. Kenney and L. E. Roberson

To prepare an antiserum as a reagent in analyses of virus regulation, we undertook a large-scale preparation of reverse transcriptase from the spleens of RLV-infected mice[1] using methods of affinity chromatography we have developed.[2]

A dialyzed extract from 245 g leukemic spleen and containing 2×10^7 units of enzyme was chromatographed on poly(G)-Sepharose, yielding two discrete peaks of activity. The first of these contained appreciable quantities of the cellular DNA polymerase γ, which were removed by chromatography on poly(Um)-Sepharose to yield a form of reverse transcriptase (form I) like that purified from murine viruses by several groups. This enzyme sedimented in sucrose gradients at molecular weight 60,000 to 70,000 and contained a single major polypeptide (SDS-polyacrylamide gel electrophoresis) of this molecular weight. The second peak, eluting at high salt concentrations from poly(G) (form II), sedimented in sucrose gradients containing detergent (0.1% NP 40) at molecular weight 140,000 to 150,000 and contained major polypeptides in addition to those found in form I of molecular weight 60,000 to 70,000. Both were exceedingly labile and required high ionic strength for full activity; this requirement was found to reflect inhibitors in the enzyme preparations, probably oligomers of guanylic acid arising from nuclease digestion of poly(G) during chromatography.

A second large-scale preparation (475 g) yielded only form I when chromatographed on poly(G)-Sepharose. We are currently attempting to define the variable in preparation which determines distribution of the two enzyme forms.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and by the U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

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HORMONAL CONTROL OF LEUKEMIA VIRUS EXPRESSION IN HEPATOMA CELLS*

S. Sakonju,† K.-L. Lee, and F. T. Kenney

Glucocorticoids promote expression of activated oncornavirus genomes.[1] In a previous report from our laboratory, we showed that this effect appears to be mechanistically comparable with other systems in which these steroids control the synthesis of specific proteins.[2] However, neither our experiments nor

those reported by other laboratories were able to distinguish whether the steroids directly promote expression of the viral genome or do so indirectly, for example by stimulating synthesis of an unrelated gene product that controls viral transcription. To make this determination and also to determine whether integration of oncornavirus genomes alters normal cellular regulatory mechanisms, we have initiated experiments involving hormonal control of viral genomes integrated in the rat H-35 hepatoma, a cell line in which control of the synthesis of specific proteins has been extensively analyzed.

Preliminary analyses by fluorescent antibody staining and by reverse transcriptase assay of pelleted culture media indicated that H-35 cells synthesize little or no endogenous virus, and attempts to activate one (IdUrd, 2-deoxyglucose) were unsuccessful. The cells could be infected with murine Moloney leukemia virus (MLV) but only after multiple prolonged exposure and only to a maximum of 40 to 50% of the total cells (by fluorescent antibody staining). Effects of hydrocortisone on these cells have been somewhat variable for reasons that are not understood. Present results indicate: (*a*) the steroid strongly stimulates virus expression as measured by reverse transcriptase of pelleted culture media; (*b*) intracellular reverse transcriptase is increased at a rate comparable to the inducible TAT, suggesting direct control by the hormone of transcription of the viral genome; (*c*) the viral infection appears to cause a significant change in the response of TAT to hydrocortisone in a direction indicating stabilization of the usually labile enzyme and/or its equally labile mRNA. These preliminary results will be analyzed further in MLV-infected H-35 cells and in cells infected with the homologous rat leukemia virus.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

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REGULATORY PROCESSES IN DAMAGED CELLS*

K.-L. Lee, P. L. Darke, and F. T. Kenney

Cellular damage, caused by polycyclic hydrocarbons and other hazardous agents generated in fossil-fuel conversion technologies, may include alterations in the regulatory mechanisms that determine the cellular content of enzymes and other proteins. To investigate this possibility we have used, as a model, the inducible tyrosine aminotransferase in H-35 hepatoma cells. Simple kinetic measurements of the level of this enzyme in various experimental conditions permit an analysis of the control of transcription (by glucocorticoids) and the control of translation (by insulin and cyclic nucleotides), as well as turnover of the enzyme and its specific mRNA. Lesions identified by kinetic analysis can be characterized further by more rigorous methods as required.

Carcinogenic hydrocarbons (BP and DMBA) were tested under a variety of conditions. A significant stimulation of transcriptional induction, but not of translational induction, suggests that the hydrocarbons may alter cellular levels of cAMP; this possibility is being examined. A beginning analysis of trace metals known to be present in aqueous wastes in coal conversion has not yet revealed any significant effects on the regulatory parameters examined.

*Research supported jointly by the Environmental Protection Agency and the U.S. Energy Research and Development Administration.

UPTAKE AND METABOLISM OF HEXOSES BY HUMAN CELLS *IN VITRO**

D. W. Salter† and J. S. Cook

One of the well-known concomitants to malignant transformation of cultured cells from nonhuman species is their enhanced capacity for uptake of small metabolites; glucose is an outstanding example. This apparent induction of transport may also be observed when cells are stimulated from quiescence to growth by any of a number of means, and again when cells are challenged by deprivation of the transported substrate. In quantitative comparative studies by others with rodent and avian cells, stimuli to enhanced transport show considerable overlap in the response, which suggests that the mechanisms for the transport enhancement are similar in all cases. Few equivalent studies have been made with human cells. We have now characterized in detail the hexose-transport system of the human diploid fibroblast strain HSWP and have been investigating its response to glucose deprivation; we chose this stimulus since it leads to the most rapid (about 17 hr) and most reproducible response (two- to threefold

increases), apparently involving all cells in the population. Our principal findings are as follows.

Characteristics of transport.—Uptake is stereospecific; there is no detectable uptake by diffusion of L-glucose or mannitol. D-glucose and 2-deoxy-D-glucose are taken up with K_m's of 5 to 7 mM; 3-O-methylglucose, glucosamine, 5-thio-D-glucose, and galactose compete for the same transport system with higher K_m; fructose is not transported. There are no titratable groups in the transport system between pH 5.5 and 8.5 in human cells. The V_{max} for growing cells is the same as that for serum-deprived and confluent cells, although large differences may be observed in glucose-dependent macromolecular syntheses. Glucose deprivation leads to a tripling of transport V_{max} with no detectable change in the K_m for all sugars tested. The results suggest an increase in the number of transport sites.

Transport inhibitors.—Phloretin inhibits transport with a K_I of 10 to 15 mM and binds with a K_D of the same value. The binding is inhibited by diethylstilbesterol and quercetin with an effectiveness equal to their own abilities to inhibit sugar transport. At high pH, the phloretin binding is inhibited more than transport inhibitory capacity. In rate studies, transport inhibition and disinhibition occur more rapidly than equilibrium binding, which eventually exceeds 10^9 sites per cell. Binding is not inhibited by glucose, nor are the number of sites altered with changes in transport capacity. We conclude that nonionized phloretin (and quercetin) binds to and perturbs the lipid bilayers of the cell, altering the environment and activities of the transport proteins. Much of the binding is intracellular, but only the binding to the surface bilayer inhibits transport.

Changes in glucose metabolism with changes in transport.—Enhancement of transport following glucose deprivation is cycloheximide- and puromycin-sensitive but does not require RNA synthesis. Preliminary experiments suggest that the enhancement may be caused by inhibition of the degradative side of turnover—a conservative mechanism in stressed cells. After a relatively long label (5 min) with tracer glucose, we find in deprived cells a tenfold increase over controls in total intracellular radioactivity, little or no radiolactate (in control cells at 5 min, 70% of the label returns to the medium as a steady-state excretion of lactate), and a *ca* 60-fold increase in glucose-6-phosphate and fructose-6-phosphate. The free glucose level is also elevated. It appears that phosphofructokinase activity is depressed in these cells, and/or they have initiated gluconeogenesis to main synthesis of essential metabolites in the pentose cycle. Recovery of metabolism after the restoration of glucose to the medium is independent of macromolecular synthesis.

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*Research supported jointly by the National Cancer Institute and by the U.S. Energy Research and Development Administration.

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THE SERINE REQUIREMENT OF BONE MARROW CELLS OF CERTAIN EXPERIMENTAL MAMMALS*

F. M. Faulcon, W. H. Lee, J. B. Jones,† and J. D. Regan

We have previously shown that human bone marrow cells and human bone-marrow-derived leukemias require a "nonessential" amino acid, serine.[1] We have also demonstrated the molecular basis for this requirement[2] and have designed and synthesized a serine antimetabolite[3] which could conceivably be employed therapeutically in such leukemias.

Before such a drug can be used in human leukemia patients, it must be shown to be effective against experimental tumors. Most of these tumors are of murine origin. Murine bone marrow cells and murine bone-marrow-derived leukemias do not require serine. Thus, a serine antimetabolite would be expected to have only a marginal effect against murine bone-marrow-derived leukemias. Murine leukemias therefore do not present an adequate animal experimental trial for serine antimetabolites.

We have examined a number of experimental mammals for the serine requirement of their bone marrow cells. Typical results of a number of experiments are shown in Table 44. Dog bone marrow cells show a serine requirement that is quantitatively undistinguishable from the human requirement. Results with cat cells are equivocal and do not exhibit an impressive requirement. Rabbit and guinea pig cells appear to have a serine requirement but rat cells do not. Thus, bone-marrow-derived leukemias from the rat would not be reasonable experimental tumors

Table 44. Effect of serine deprivation on macromolecular synthesis in bone marrow cells of dog, cat, rabbit, guinea pig, and rat

Animal	Medium[a]	DNA synthesis[b]		RNA synthesis[b]		Protein synthesis[b]	
		4 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Dog	1	100	100	100	100	100	100
	8	36	23	39	32	85	90
	9	28	17	30	26	87	81
Cat	1	100	100	100	100	100	100
	8	70	68	59	66	72	64
	9	84	85	62	77	75	65
Rabbit	1	100	100	100	100	100	100
	8	78	57	52	27	38	35
	9	57	47	77	35	49	44
Guinea pig	1	100	100	100	100	100	100
	8	85	89	78	98	90	96
	9	66	82	88	78	85	101
Rat	1	100	100	100	100	100	100
	8	89	101	72	81	75	78
	9	81	105	66	84	71	76

[a]Medium No. 1 contains all the "nonessential" amino acids; medium No. 8 is the same as medium No. 1 but lacks serine; medium No. 9 lacks all seven "nonessential" amino acids.

[b]Percent of controls (medium No. 1).

for testing serine antimetabolites, while dog, rabbit, or guinea pig leukemias would.

*Research supported jointly by the National Cancer Institute and by the U.S. Energy Research and Development Administration.

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EXTENT OF DNA REPAIR FOLLOWING TREATMENT OF HUMAN CELLS WITH SAFROLE AND RELATED COMPOUNDS*

A. A. Francis, W. C. Dunn, Jr., N. R. Drinkwater,† J. A. Miller,† and J. D. Regan

Safrole (4-allyl-1,2-methylenedioxybenzene), which occurs naturally in sassafras and was a flavoring agent in certain soft drinks until 1960, is now known to be a hepatocarcinogen in rats and mice. Present evidence indicates that safrole requires metabolic activation to act as a carcinogen. 1′-Hydroxysafrole is a major metabolite of safrole and is more hepatocarcinogenic for both rats and mice than safrole.[1,2] This proximate carcinogen is not mutagenic or electrophilic. In contrast, the synthetic ester 1′-acetoxysafrole is mutagenic and electrophilic and is a stronger carcinogen than 1′-hydroxysafrole at local sites of administration in the rat.[2] Evidence has been obtained that the electrophilic ester 1′-sulfonoxysafrole can be formed from 1′-hydroxysafrole by rat and mouse liver preparations.[3] Similarly, the naturally occurring compound estragole (4-allyl-1-methoxybenzene), in basil and tarragon, is hepatocarcinogenic in the mouse, and its proximate carcinogenic 1′-hydroxy metabolite in this species becomes electrophilic upon synthetic esterification to form 1′-acetoxyestragole.[4]

In the present study, safrole and estragole, their 1′-hydroxy and 1′-acetoxy derivatives, and two related synthetic allylic esters have been tested for their ability to cause repairable DNA damage to human cells in culture as measured by the bromodeoxyuridine (BrdUrd) photolysis assay. Results are shown in Table 45. The repair seen in endonuclease-deficient xeroderma pigmentosum cells indicates that all these compounds elicit the type of DNA repair seen compounds elicit the type of DNA repair seen following ionizing radiation damage. The repair seen with safrole may indicate its metabolic conversion to 1′-hydroxysafrole or another more active derivative.

Table 45. DNA repair measured by photolysis of BrdUrd-substituted strands after chemical insult and incubation in BrdUrd-containing medium

Compound	Final concentration on cells (M)	No. excess strand breaks after 10^6 ergs mm^{-2} 313-nm radiation
Safrole	10^{-4}	0.28
1′-Hydroxysafrole	10^{-4}	0.28
1′-Acetoxysafrole	10^{-4}	0.46
Estragole	10^{-3}	0.14
1′-Hydroxyestragole	10^{-3}	0.16
1′-Acetoxyestragole	10^{-3}	0.36
1′-Acetoxyallylbenzene	10^{-4}	0.20
1′-Acetoxy-1-allyl-4-methoxynaphthalene	10^{-4}	0.24

▼

The most active compound in the series, 1′-acetoxysafrole, was the most cytotoxic derivative, as measured by cell death.

*Research supported jointly by the National Cancer Institute and U.S. Energy Research and Development Administration under contract with Union Carbide Corporation.

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REPAIR OF DNA DAMAGED BY MUTAGENIC METABOLITES OF BENZO[*a*]PYRENE IN HUMAN CELLS*

J. D. Regan, A. A. Francis, W. C. Dunn, Jr., and D. M. Jerina†

The repair of human DNA damaged by metabolically formed derivatives of benzo[*a*]pyrene has been examined with emphasis on the functional groups necessary to elicit the maximum response. We have found that only 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and its isomer, 7α,8β-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene will elicit damage to DNA which is recognizable by the UV excision repair system in human cells. Other metabolically synthesized benzo[*a*]pyrene epoxides without the hydroxyl groups do not elicit damage repairable by this enzyme system. Dihydroxynaphthalene epoxide derivatives with the same functional groupings have no measurable activity in our assay. These results indicate that both the benzo[*a*]pyrene ring structure and the dihydroxy epoxide groups are necessary to elicit DNA damage repairable by ultraviolet (UV) excision repair enzymes. These results are in agreement with mutagenic properties of these compounds and support the conclusion that 7α,8β-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene may be the ultimate metabolically activated form of benzo[*a*]pyrene.

*Research jointly supported by the National Cancer Institute and the U.S. Energy Research and Development Administration.

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ISOLATION AND CHARACTERIZATION OF MOUSE SERUM ALBUMIN MESSENGER RNA

P. C. Brown* and J. Papaconstantinou

We previously reported that monospecific antibodies directed against mouse serum albumin can bind specifically to mouse liver polysomes, presumably by immunologically recognizing nascent albumin polypeptides.[1] We have carried this work further and have immunoprecipitated albumin-synthesizing polysomes and have used this material as a source of albumin mRNA.

Polysomes of BALB/c mouse livers were incubated at 4°C in the presence of monospecific rabbit anti-mouse-serum-albumin antibody, carrier serum albumin, heparin (as an RNase inhibitor), 0.4% Triton X-100, and sodium deoxycholate. After 75 min, the immunoprecipitate was collected by low-speed centrifugation. The pellet was gently

resuspended in buffer with heparin and 0.2% Triton X-100 and respun. RNA was extracted from the final pellet by the SDS–phenol–chloroform method. Poly(A)-containing RNA was subsequently selected by oligo(dT)-cellulose chromatography.[2]

Indirect precipitation of albumin polysomes was not successful. In these experiments, polysomes were first incubated with rabbit antialbumin and then incubated with goat antibodies to rabbit immunoglobulin. Apparently, in the short incubation period required, the second antibody was incapable of forming a large enough precipitate to be pelleted by low speed centrifugation.

The translational capacity of the RNA from immunoprecipitated polysomes was determined in a cell-free protein-synthesizing system derived from wheat germ.[3] Total poly(A)-containing RNA from pelleted mouse liver polysomes was used initially to characterize this system. After incubation of the wheat germ preparation with RNA and ^{14}C-labeled leucine, the sample was centrifuged to pellet ribosome-bound radioactivity. The incorporation of labeled amino acid into total protein was determined by trichloroacetic acid precipitation of an aliquot of the resulting supernatant. From another aliquot of the supernatant, the incorporation of radioactive label into albumin was determined by radioimmunoassay using antibody to highly purified serum albumin and carrier nonradioactive albumin. When required, either precipitate was dissolved and electrophoresed through 10.5% polyacrylamide gels containing 0.1% SDS. Of particular interest in the initial characterization studies was the finding that albumin synthesis, as determined by radioimmunoassay and gel analysis of the precipitated product, was maximal at a potassium concentration (100 m*M*) which was less than optimal for the incorporation of radioactive label into trichloroacetic-acid-precipitable material. We conclude that the lower potassium concentration (<100 m*M*) favors the initiation of translation but is not optimal for successful completion of translation. Albumin synthesis was also significantly improved by the inclusion of spermine (30 μg/ml).

When the wheat germ system is programmed with total liver poly(A) RNA, the percent of total trichloroacetic-acid-precipitable radioactivity which is immunoprecipitable as albumin is 4–5%. Since albumin *in vivo* comprises 8–10% of the total liver protein synthesis,[4,5] we conclude that our *in vitro* system and subsequent radioimmunoassay is about 50% efficient in translating and detecting albumin. When the *in vitro* system is programmed with RNA from specifically immunoprecipitated polysomes, albumin synthesis increases to approximately 50% of the total incorporation. Thus, immunoprecipitation of albumin polysomes results in a 10- to 12-fold enrichment in albumin mRNA sequences and yields a preparation which, in terms of its translational capacity, probably exceeds 90% purity. We believe that the products synthesized under the direction of specific albumin mRNA in the wheat germ system consist of many prematurely terminated polypeptides which are incapable of being precipitated by antibodies to complete albumin. Indeed, gel analysis of the TCA-precipitable products synthesized under the direction of albumin mRNA shows a major peak of radioactivity (about 50%) coelectrophoresing with the albumin marker, while the remaining counts are heterogeneously distributed among lower-molecular-weight polypeptides.

Analysis of the sedimentation profile of purified albumin mRNA through 15 to 30% sucrose gradients containing 0.1% SDS reveals a rather broad band of material sedimenting at 18 S and a smaller, sharper peak at 28 S. Subsequent efforts to eliminate these obvious ribosomal RNA contaminants have not been successful. Thus, at this time, we cannot demonstrate physical homogeneity of the albumin mRNA preparation. The translational capacity of fractions taken from these sucrose gradients revealed sharp peaks of both total protein synthesis and albumin synthesis in the 16- to 17-S fractions. Since these two activities cosedimented symmetrically, we believe that the mRNA content of our albumin mRNA preparation is essentially homogeneous.

We are currently investigating the sedimentation properties of albumin mRNA under fully denaturing conditions in an attempt to accurately determine its size and to possibly separate albumin mRNA from ribosomal RNA contaminants. Once these are completed, we plan to synthesize a DNA probe complementary to albumin mRNA using viral reverse transcriptase. With this probe, we will be able to detect transcriptional modulation of albumin genes throughout the cell cycle in synchronized mouse hepatoma cells.

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REGULATION OF DIMETHYL SULFOXIDE-INDUCED HEMOGLOBIN SYNTHESIS IN FRIEND LEUKEMIA CELLS. INHIBITION OF HEMOGLOBIN SYNTHESIS BY HYDROCORTISONE

P. R. McClintock,* J. P. Rebek,†
and J. Papaconstantinou

It has been shown in other laboratories that the dimethyl sulfoxide-mediated induction of hemoglobin synthesis in Friend leukemia cells is accompanied by an increase in the production of Friend virus particles. On the basis of this observation, the question arises of whether the induction of hemoglobin synthesis and the stimulation of virus production are closely linked. Since hydrocortisone is one of the glucocorticoids known to stimulate oncornavirus synthesis in cells, experiments were done to determine whether this steroid hormone will stimulate the synthesis of hemoglobin. The results of these experiments, which were reported last year, showed in fact that 10^{-6} *M* hydrocortisone is a potent inhibitor of hemoglobin synthesis. In addition, there is little effect on the growth and viability of the cells in concentrations of hydrocortisone ranging from 10^{-5} to 10^{-8} *M*. The specificity of the inhibition is indicated by the fact that a related steroid, tetrahydrocortisone, has no effect on hemoglobin synthesis.

In these experiments, hemoglobin levels were measured by spectrophotometric analysis of a total cell lysate at 510 nm. In our recent studies, we confirmed these observations by showing the inhibition of appearance of benzidine-positive (B^+) cells in the presence of hydrocortisone. In addition, we have shown (by B^+ cell analysis) that dexamethasone inhibits hemoglobin synthesis without showing any effect on the replication and growth of these cells. Since growth and replication are not affected, the site of inhibition seems to be at the level of transcription. Studies designed to localize the site of inhibition and the mechanism of action of hydrocortisone and dexamethasone on these cells are being carried out.

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PURIFICATION OF α-FETOPROTEIN FROM MOUSE AMNIOTIC FLUID AND HEPATOMA CELLS

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and J. Papaconstantinou

Fetal development in a variety of mammalian species, including man and rodents, is accompanied by the synthesis of a fetal specific α-globulin, α-fetoprotein (αFP). This α-globulin is a major component of fetal serum, and its site of synthesis has been localized in yolk-sac cells and fetal liver parenchymal cells. The synthesis of αFP peaks during embryonic development and drops rapidly during the final stages of prenatal development. αFP is virtually absent in the serum of healthy adult animals.

The synthesis of αFP is reinitiated in adult animals during liver regeneration, in animals with a primary hepatocellular carcinoma, in embryonic teratocarcinoma, and in viral hepatitis.

In our previous studies we have shown that Hepa, a mouse hepatoma cell line, synthesizes and secretes a variety of serum proteins. These include albumin, αFP, and transferrin.

The specific temporal repression of αFP synthesis in fetal liver development and the induction of αFP synthesis in adult liver by a variety of chemical carcinogens together offer a system in which the mechanism of regulation of gene expression can be studied. Furthermore, the availability of hepatoma cells in tissue culture which synthesize αFP provides materials for *in vitro* as well as *in vivo* studies. One of our immediate aims in this program is to isolate αFP-mRNA by immunoprecipitation of αFP polysomes. In this communication, we report the isolation of pure αFP and rabbit anti-mouse-αFP antibody by affinity chromatography, to be used for the preparation of αFP-mRNA and in other studies involving the regulation of synthesis of this protein.

Amniotic fluid and serum from hepatoma-bearing animals are the major sources of αFP, and both of these have been used in the fractionation procedure described below.

Amniotic fluid.—Amniotic fluid was collected from pregnant females on the 14th day of gestation. The embryos were stripped from the amniotic sac into 30-ml beakers where the amniotic fluid was

collected. The embryos were washed in saline, and the wash was concentrated by filtration. The amniotic fluid and concentrated embryo wash were combined, and the αFP was precipitated by ammonium sulfate in the 0.4 to 0.7 saturation fraction. The precipitate was spun down and dissolved in 0.005 *M* sodium phosphate buffer, pH 7.0. This solution was placed on a DEAE-cellulose column, from which the αFP and albumin co-elute with 0.1 *M* sodium phosphate buffer, pH 5.7, plus 0.1 *M* NaCl. A similar fractionation was carried out with serum from hepatoma-bearing mice and with the secretion product produced by the hepatoma cells in tissue culture. The fraction containing αFP and albumin was resolved by affinity chromatography.

Affinity chromatography.—Monospecific antibody to mouse albumin was coupled to cyanogen bromide–Sepharose 4B. The αFP-albumin fraction from the DEAE-cellulose column was passed through this column. Analysis of the breakthrough fraction from this column showed a highly purified αFP with slight albumin contaminant. These studies are being repeated to determine the amount of antialbumin-Sepharose needed to absorb a given amount of albumin.

In the past we have been able to prepare a mixed antiserum in rabbits having high titers for both albumin and αFP. This antiserum has now been resolved by passage through a Sepharose–albumin column. The highly purified anti-αFP antibody produced by this column has been linked to Sepharose. This material is presently being used to isolate αFP from the DEAE fractions as well as from amniotic fluid and serum.

With the development of procedures for the preparation of pure αFP and antibody to αFP, we are now tooling up to proceed to the next stage of our program, namely, the isolation of αFP-mRNA by immunoprecipitation of polysomes from mouse hepatoma cells.

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KINETICS OF TRANSFERRIN SYNTHESIS BY CULTURED MOUSE HEPATOMA CELLS

J. Papaconstantinou and R. E. Hill*

Mouse hepatoma cells derived from the solid tumor BW7756 have been cloned and maintained in culture for several years. In our previous studies we have shown that these cells synthesize and secrete albumin and α-fetoprotein. The level of albumin synthesis is comparable with that reported for *in vivo* synthesis, namely, 8 to 10% of the total protein. Thus, the biochemical phenotype of these hepatoma cells is stabilized and maintained in long-term cultures.

In our electrophoretic analyses of the proteins secreted by Hepa cells, it was noted that a significant band(s) had electrophoretic mobilities comparable with that reported for transferrin. Studies were carried out to identify this protein and to further describe the biochemical phenotype of these mouse hepatoma cells.

Through the use of immunodiffusion, it was shown that the proteins secreted by Hepa cells react strongly with transferrin antibody. Radioactive secretion product was reacted with transferrin antibody. The precipitate was analyzed by SDS–acrylamide-gel electrophoresis. A single radioactive peak having a molecular weight of 76,000 was identified. This value is comparable with the molecular weight of transferrin. Electrophoretic analysis of the supernatant from the antibody precipitation showed radioactive peaks having the mobility of albumin and αFP. The third peak, transferrin, was completely absent in this gel but was detected in the control electrophoretogram. From these data we conclude that transferrin is synthesized and secreted by mouse hepatoma cells.

In another series of experiments, attempts were made to determine the rate of synthesis and secretion of transferrin in log- and stationary-phase cells. Using the immunoprecipitation technique, the following observations were made. (*a*) There is a lag period of approximately 1 hr before radioactive transferrin can be detected in the medium. We interpret this lag period to be due to the time required for transferrin to move through the cellular secretory apparatus. (*b*) The rate of secretion of transferrin in stationary-phase cells is greater than the rate of secretion in log-phase cells. (*c*) The rate of secretion is similar to the rate of synthesis of transferrin in both stationary phase and log phase. (*d*) The intracellular transferrin pool is significantly higher in stationary phase than in log phase. This has some bearing on the observed increase in the rate of secretion of transferrin in stationary-phase cells. (*e*) In stationary-phase cells, transferrin makes up approximately 2% of the total protein synthesized, whereas in log-phase cells it comprises 1% of the total protein synthesized. Since it is possible that the [^{3}H]leucine pool varies in stationary- and log-phase cells, we cannot attribute

the increase in specific activity of transferrin to an increase in the rate of synthesis; another explanation could be based on an increase in the specific activity of the [^{3}H]leucine pool.

We conclude that Hepa cells have maintained the ability to synthesize two serum proteins, albumin and transferrin, at the levels observed for normal adult liver synthesis. In addition, these cells synthesize αFP (5% of the total protein synthesis), which is normally observed in fetal liver and adult hepatoma cells.

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CELLULAR RNA SPECIFIC FOR TRANSFER OF *Fv-1* LOCUS RESTRICTION OF MURINE LEUKEMIA VIRUSES*

W.-K. Yang, R. W. Tennant, J. O. Kiggans, B. Schluter,† J. A. Otten, F. E. Myer, and A. Brown†

We have previously demonstrated that mouse embryo culture cells which belong to a given *Fv-1* genotype and are susceptible to an ecotropic MLV can be rendered partially resistant to the infection of this virus by treatment with cell-free extracts made from the cells of a different *Fv-1* genotype which restricts the infection[1]. Tests of the cell extracts with various hydrolytic enzymes showed that the inhibitory activity was sensitive to inactivation by ribonuclease, but not by deoxyribonucleases or by proteases. Specific activity was subsequently detected in RNAs isolated by phenol deproteination from both $Fv\text{-}1^{n/n}$ and $Fv\text{-}1^{b/b}$ mouse-embryo culture cells. With the use of RNA preparations, particular precautions become necessary in the treatment procedure to prevent nonspecific activities, such as inhibition of virus absorption and stimulation of cell growth. Differential phenol extraction at pH 7.6 and pH 9.0 indicated that most of the activity was present in the RNA fraction extracted at pH 9.0. Sucrose-gradient sedimentation analyses showed that the specific RNA migrated at 20 to 22 S for both alleles of the *Fv-1* gene. Demonstration of the specific activity, especially in the purified RNA preparations, has been found to depend on appropriate experimental conditions which favor RNA uptake in undegraded form. For purification purposes, we utilized RNA preparations from the livers of young adult mice, which were shown to contain *Fv-1* gene-specific inhibitory activity and could be obtained in large quantity. Affinity chromatography on poly(U) Sepharose as well as oligo(dT) cellulose achieved purification of the activity in the poly(A) RNA fractions. Preliminary hybridization experiments indicated that the specific RNA had no protective effect against S-1 nuclease on MLV-specific copy DNA after hybridization and thus may not have a structure sequence homologous to MLV RNA; also, RNA preparations from MLV-infected mouse-embryo cells did not show enhanced specific activity. The results, therefore, indicate that cellular-specific, rather than viral-specific, RNA is responsible for the transfer of resistance against ecotropic MLV.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

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MECHANISM OF *Fv-1* LOCUS RESTRICTION OF MURINE LEUKEMIA VIRUS INFECTION: PRELIMINARY RESULTS OF TRANSFECTION STUDIES*

I.-C. Hsu,† W.-K. Yang, R. W. Tennant, and A. Brown‡

Infection by ecotropic MLVs is restricted in mouse-embryo fibroblasts which carry the restrictive allele of the *Fv-1* gene. The precise biochemical mechanism of the *Fv-1* locus restriction is unknown at present. Using specific cell extracts, we have previously obtained results showing that MLV infection is inhibited by the *Fv-1* gene product at an early state of virus infection. In the present study, we have employed the transfection technique to investigate the mechanism of *Fv-1* locus restriction. Since DNA preparations rather than virion particles are used in this technique, an inhibitory effect of the *Fv-1* locus would imply restriction at proviral DNA integration or at later steps in the virus infection cycle, whereas a negative result would imply restriction at or before the proviral DNA formation step.

Using Marmur's procedures for DNA preparation and Graham and van der Eb's procedures for DNA inoculation, we could obtain transfection of N-tropic MLV DNA in permissive NIH 3T3 cells in 4 to 10 weeks. The progeny viruses from the transfected cells showed the same N-tropism as the parent virus. Transfection of ecotropic MLV DNA was extremely inefficient; hence, detection of transfection must depend on amplification of the initial infection by

horizontal transmission during serial cell subculturing. Since horizontal transmission of MLV is minimal in the *Fv-1* restrictive cells, detection of transfection in these cells becomes difficult. This may explain our initial results showing that no transfection was obtained in secondary BALB/c mouse-embryo cells inoculated with DNA prepared from N-tropic virus-infected cells or in NIH 3T3 cells inoculated with DNA prepared from B-tropic virus-infected cells. To circumvent this difficulty, we have undertaken three experimental approaches. First, efficiency of transfection is improved by using hydrocortisone, insulin, and polybrene. Second, an infectious center assay is employed by immediate co-cultivation with permissive cells following DNA inoculation. Third, infectious DNA preparations are isolated from $Fv\text{-}1^n$ (or $Fv\text{-}1^b$) cells infected with B-tropic (or N-tropic) MLV. Our preliminary results indicate that NIH 3T3 cells can be transfected to produce B-tropic MLV with an efficiency similar to that of N-tropic MLV, suggesting that restriction of MLV infection by the *Fv-1* loci or their gene products is at steps prior to proviral DNA integration into host DNA. The results are consistent with our previous finding that *Fv-1* loci act at the early stage of MLV infection.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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TRANSFECTION IN MOUSE FIBROBLASTS WITH DNA ISOLATED FROM CELLS INFECTED WITH MURINE LEUKEMIA VIRUSES*

I.-C. Hsu† and W.-K. Yang

Infection of cells with RNA tumor viruses involves reverse transcription of the viral RNA genome into DNA and subsequent insertion of proviral DNA into the cellular genome. In 1972, Hill and Hillova reported that infection can also be achieved by treating noninfected cells with DNA isolated from virus-infected cells. In addition to its general biological significance, the transfection phenomenon with RNA tumor viruses has two important specific aspects: (*a*) that uptake and utilization of a foreign DNA molecule by eukaryotic cells can be measured by sensitive methods of virus assay and (*b*) that the phenomenon may be intimately related to the oncogenic process. The experimental results of our study on transfection in the MLV system can be summarized as follows:

(*a*) Treatment of cells with infectious DNA preparation is more efficient by employing Graham and van der Eb's calcium chloride procedure than by pretreatment of recipient cells with 25 μg/ml of DEAE-dextran.

(*b*) Infectious DNA can be prepared from chronically infected cells by Marmur's procedures. Shearing of the cellular DNA to appropriate size can enhance the efficiency of DNA transfection.

(*c*) The N- and B-tropic MLV systems showed extremely low efficiency of transfection in comparison with the NB-tropic (Maloney) MLV system. We have not been able to demonstrate transfection using DNA preparations isolated from the Hirt supernatant of cells infected with N-tropic MLV for 9 hours.

(*d*) DNA preparations from chronically infected cells showed consistently increased transfection activity if the cells were pretreated with hydrocortisone and insulin.

(*e*) Treatment of recipient cells with bromodeoxyuridine followed by light illumination, a condition causing breakage of cellular DNA, did not induce an increase in the efficiency of transfection.

(*f*) The "competence" of the recipient cells varied greatly among the various mouse-cell lines tested.

(*g*) Following DNA treatment, serial passages of the recipient cells were generally needed, and progeny viruses usually do not appear until 4 to 12 weeks later. We have developed a procedure in which the recipient cells are treated with polybrene, hydrocortisone, and insulin in the culture medium; with this procedure, positive detection of transfection can be achieved in 2 weeks.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

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UPTAKE OF RNA BY SECONDARY MOUSE-EMBRYO CULTURE CELLS*

M. H. Morales,† J. O. Kiggans, D.-M. Yang, and W.-K. Yang

Since transfer of the *Fv-1* locus restriction is achieved by treatment of recipient mouse-embryo

culture cells with RNA preparations, it is obvious that uptake and utilization of the specific RNA by the recipient cells are critical for the induction of resistance. However, very little is known about these phenomena in the mouse-embryo secondary culture cells. We therefore made such studies, using highly [^{3}H]uridine-labeled RNA prepared from both BALB/c (B) and NIH Swiss (N) mouse-embryo secondary cultures.

Experiments were performed using total RNA preparations initially and sucrose-gradient-separated fractions afterward, with emphasis on two aspects: (*a*) the effects of various facilitators and (*b*) the optimal cell-culture conditions for the uptake of the labeled RNA. To be consistent with our bioassay procedure for the *Fv-1* locus restriction products, 1.5×10^5 cells were plated in 35-mm^2 dishes and used 18 to 24 hr later for the uptake studies. RNA uptake by mouse cells was found to be very limited (about 0.1%) under ordinary conditions but was greatly enhanced (up to 10%) if the cells were pretreated with polycations such as DEAE-dextran, polybrene, and polyornithine. The calcium chloride method of Graham and van der Eb showed an increased adsorption on the cell surface but no apparent enhanced uptake. Polyornithine appeared to be the best facilitator of RNA uptake but was found to be toxic (at about 10 μ/ml in a 1-hr period) to the mouse cells, which are more susceptible than cultured cells from other mammalian species. Polybrene and DEAE-dextran showed no obvious toxic effects on the mouse cultures at 25 μg/ml for 1 hr. Sucrose-gradient sedimentation analyses revealed rapid degradation of RNA upon uptake by the cells. To minimize the rapid degradation, various experimental procedures were tested; these included use of the natural RNase inhibitor (isolated and semipurified from mouse liver), removal of traces of fetal calf serum in the culture medium, and utilization of recipient cells at definite stages of *in vitro* cultivation. The natural RNase inhibitor, a 4 S acidic protein preparation, gave no enhanced uptake but an apparent protection of RNA. Complete removal of serum from the culture medium, especially with a 4-hr starvation prior to RNA inoculation, was found to be particularly useful. The time interval between the refeeding of mouse primary cultures and their use in preparing secondary cultures also appeared to have a considerable effect. Employing a procedure with a combination of various apparently optimal conditions, we obtained high uptake (up to 25% of input RNA at 10 μg/ml) and reasonably minimized degradation of RNA. Preliminary results indicate that this procedure may increase the sensitivity of our bioassay of *Fv-1* locus-specific RNA products.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

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FEASIBILITY OF PURIFICATION OF AKR MURINE-LEUKEMIA-VIRUS SURFACE GLYCOPROTEINS BY LECTIN-AFFINITY CHROMATOGRAPHY*

D. W. Fountain† and W.-K. Yang

The feasibility of using plant lectins, selected for their capacity to bind to AKR MLV, as specific affinity agents for the purification of viral surface glycoproteins has been studied. Iodinated (^{125}I) proteins of the virus were prepared so that either (*a*) by using intact virions, iodination was confined mainly to surface membrane components or (*b*) by using solubilized viral extracts, all major viral polypeptides were iodinated, each in proportion to its relative abundance in the virion. Concanavalin A-Sepharose and phytohemagglutinin-Sepharose conjugates bound iodinated surface proteins to various degrees. The recovery of bound material was effected either by elution with lectin-specific glycosides or by sequential elution with low-pH–high-salt buffers and a denaturing agent. SDS-gel electrophoretic analysis showed that the major bound species has a molecular weight of about 70,000. An apparent fractionation of the 70,000-molecular-weight proteins by the elution protocols was also observed. Lectin-affinity chromatography of the solubilized AKR MLV extract, where gp 69/71 is a minor iodinated component, showed the feasibility of this method for the purification of gp 69/71. Apparently, in addition to gp 69/71, specific fractions of the viral proteins p 30 and p 15 were bound and recovered from the gel.

*Research supported by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

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

SOYBEAN LECTINS *IN VIVO**

D. W. Fountain,† D. E. Foard, W. D. Replogle,‡ and W.-K. Yang

While the physiological significance of carbohydrate-complexing lectins present in leguminous seeds is largely unknown, it may be related to the ability of the plants to fix atmospheric nitrogen within root nodules formed following the infection of root tissues by *Rhizobium* spp. bacteria. Circumstantial evidence suggests a specific role of lectins in the initial recognition and binding of the bacteria to the host root-hair surface. Lectins have also been thought to function in such diverse aspects of the plant's physiology as inhibition of hyphal growth and spore germination of phytopathogenic fungi, as mediators of plant-cell extension growth by plant growth regulators, and as membrane recognition sites for the regulation of the dynamics of intracellular compartmentation. Some or all of these possible functions require the presence of lectins at sites which may be distant from their original location within seed tissues. To date, however, nothing is known regarding the relationship of lectins stored in the dry seeds to lectin activities detected in these various studies of mature plants or tissues. We have investigated the early stages of germination of soybean seeds to gain some information on this question.

Soybean seeds were surface-sterilized and incubated in sterile glass-distilled water for various periods up to 72 hr. At the end of each period, the remaining water from around the beans was collected and lyophilized, and the beans were extracted for total nonglobulin protein. Lectin activity was assayed by a standard hemagglutination assay.

Lectins were present within seed tissues at all times tested. The hemagglutination titer (lectin activity per microgram of protein) rose over the first 20 hr of germination and declined to 40 hr. The kinetics of the initial increase in activity was similar to that of water uptake by the seeds (complete by 16 hr), suggesting an early hydration-dependent mobilization of lectin reserves. Following an initial lag period of 8 hr corresponding to rapid initial water uptake, considerable quantities of protein were secreted into the water surrounding the seeds. Lectin activity in this material was detectable as early as 4 hr from the start of water uptake (when seed hydration was only 50% complete) and was present at all subsequent times. The hemagglutination titer increased rapidly from 4 hr to a maximum at 8 hr and declined thereafter, suggesting dilution of the lectin at later times by nonlectin protein.

These results suggest that soybean lectins may be mobilized and preferentially exported from the seeds early in the water-uptake phase of soybean germination. While the physiological significance of this is as yet unknown, it is a most provocative observation.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

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MEMBRANE GLYCOPROTEINS OF CULTURED CELLS OF A HUMAN BREAST CANCER: ANALYSIS BY LECTIN-GEL CHROMATOGRAPHY*

W.-K. Yang, L. P. Trigg,† D. W. Fountain,‡ and D.-M. Yang

ALAB is an established cell line derived originally from breast cancer tissue of a human patient (Naval Research Laboratory, University of California). These epithelioid cells are useful target cells for studying lymphocyte-mediated immunity against human breast cancers [1,2] and have been used in the transfer-factor research project of G. D. Novelli's group in the Biology Division. Considering the possible antigenic relationship between human breast cancers and mammary tumor viruses, we are particularly interested in studying cell-surface glycoproteins. In this report, we briefly describe the results of such a study on ALAB cells, using lectin-gel affinity chromatography.

Among various methods tested for labeling the surface of ALAB cells, the lactoperoxidase method of radioiodine labeling has consistently given the best results in specific radioactivity conjugated to surface proteins. The plasma membrane of ALAB cells can be isolated in high purity by hypotonic homogenization of the cells and subsequent isopycnic sucrose density gradient centrifugation. Proteins labeled by the lactoperoxidase method were found to be associated with the plasma membrane and gave multiple-band profiles on SDS-gel electrophoresis. No internal glycoprotein appeared to be labeled by the lactoperoxidase method. Total cell proteins could also be labeled using the iodine monochloride method. By acid precipitation and ethanol-ether

extraction, the incorporated radioactivity was found to be associated with both lipid components (about 70%) and protein components (about 30%) of the surface membrane. When a solubilized extract of surface-labeled ALAB cells was chromatographed on a Con A–Sepharose column, approximately 10% of the protein radioactivity was bound, of which subsequent elution recovered about 4% by 0.5 *M* α-methyl-D-glucoside at 0°C, about 3% by 0.5 *M* α-methyl-D-glucoside at 45°C, and about 3% by 4 *M* urea/HCl at pH 2.2. SDS-polyacrylamide gel electrophoresis of the eluted fractions demonstrated that selected populations of surface proteins do bind to Con A–Sepharose and may be differentially eluted. Similar studies with a *Phaseolus vulgaris* phytohemagglutin gel suggest that this lectin gel may have more selectivity for the surface glycoproteins of ALAB cells. Antigenicity studies of the lectin-gel-eluted fractions from ALAB cells are in progress.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

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DNA- AND RNA-DEPENDENT DNA POLYMERASES: PROGRESSIVE CHANGES IN RABBIT ENDOMETRIUM DURING THE PREIMPLANTATION STAGE OF PREGNANCY

W.-K. Yang, R. L. Tyndall,* and J. C. Daniel, Jr.†

The present study of DNA polymerase activities of rabbit endometrium during the preimplantation stage of pregnancy was undertaken mainly for two purposes. First, since during this time the uterus undergoes rapid cellular proliferation, it is of interest to examine how enzymic processes of DNA replication are activated and also which DNA polymerase activity is involved. Second, preliminary observations of "virus-like" particles in the endometrium of pregnant rabbits have caused us to search for phenomena in the uterus that may be associated with the particles and that could help define their possible viral relationship.

DNA polymerase activities were studied in rabbit endometria at estrus and through the first 7 days of pregnancy. Extracts of total endometrial homogenates by high salt gave two peaks of polymerase activity by sucrose-gradient centrifugation: the 6 to 8 S polymerase showed a considerable increase in specific activity which reached a maximum at 5 to 7 days after coitus, whereas the 3 to 4 S polymerase showed only a slight increase after 2 days of pregnancy. A 5 to 6 S RNA-dependent DNA polymerase activity, extracted by the combined use of hypertonic salt solution and nonionic detergent from particulate subcellular fractions, appeared in the endometrium after coitus, reached a maximum at day 3 or 4, and declined thereafter. This RNA-dependent DNA polymerase activity, isolated by phosphocellulose chromatography at distinct regions of salt gradients, showed a preference for template primers such as $(rA)_n.(dT)_6$ and $(rC)_n.(dG)_6$ but did not utilize 70 S RNA of RNA tumor viruses. Attempts to demonstrate oncornavirus expression by other means have been unsuccessful with the endometrium of 3-day-pregnant rabbits. The progressive changes of the 5 to 6 RNA-dependent DNA polymerase specific activity appeared to correlate best with the DNA synthesis activity of the rabbit endometrium at the preimplantation stage of pregnancy.

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PRIMING FUNCTION OF CELLULAR PROLINE TRANSFER RNA IN REVERSE TRANSCRIPTION OF MURINE LEUKEMIA VIRUS GENOME RNA*

W.-K. Yang

A functional role of unique species of cellular tRNAs as primer molecules in reverse transcription of RNA tumor virus genomes may be implicated by an *in vitro* study including three experimentation steps (*a*) differential thermal dissociation of 60 to 70 S RNA to detect the associated tRNA species which has the highest melting temperature, (*b*) hybridization of cellular tRNAs and 35 S RNA of RNA tumor virus to demonstrate specific association, and (*c*) reverse transcription of the *in vitro* reconstituted 35 S RNA–tRNA primer complex by the viral polymerase preparation. Such a study has been done with avian RNA tumor viruses, and tryptophan tRNA was

shown to constitute the primer molecules of these viruses. Tryptophan tRNA has two characteristic molecular properties specifically required for the primer function—partial complementary sequence with 35 S RNA of avian RNA tumor viruses and recognition by the reverse transcriptase of these viruses. In MLV systems, a step *a* experiment performed by L. C. Waters has demonstrated proline tRNA to be the associated tRNA species having the highest melting temperature with 35 S RNA of MLV. In the present report I will summarize the results of experiments which conclusively demonstrate the primer function of proline tRNA in MLVs.

Previous studies by many investigators have shown that purified 70,000-molecular-weight reverse transcriptase of MLVs has very poor activity with its endogenous 60 to 70 S RNA template primer. This finding raised the possibility that such reverse transcriptase preparations may not be the holoenzyme and that some specific subunit (or native structure) may have been lost in the process of purification, which involves treatment of the enzyme with detergent and high salt. Recently Alan M. Wu of the Bionetics Research Laboratory and Robert C. Gallo of the National Cancer Institute have noted the separation of two polymerase activities in their studies of MLV polymerase. Subsequent characterization showed that one polymerase activity has a molecular weight higher than 140,000 and appeared to utilize natural templates better than the other polymerase activity, which is of 50,000 to 70,000 molecular weight. Their polymerase preparations have been employed for the present study.

(*a*) When mixed with labeled tRNAs of mouse cells, the high-molecular-weight polymerase showed binding of specific species of tRNA, which comigrated with prolyl-tRNA in the reversed-phase chromatographic system (RPC-5). This specific binding was further established by using proline $tRNA_3$ which was purified from crude mouse tRNA by RPC-5 chromatography. Control tRNA showed essentially no binding to the high-molecular-weight viral polymerase. The low-molecular-weight viral polymerase was unable to bind proline $tRNA_3$ or other tRNA molecules.

(*b*) A kinetic study of reverse transcription showed that the high-molecular-weight polymerase utilized MLV 60 to 70 S RNA much better than the low-molecular-weight polymerase. Heat denaturation of the 60 to 70 S RNA markedly decreased catalysis by the high-molecular-weight polymerase.

(*c*) A study of reverse transcription catalyzed by the high-molecular-weight polymerase clearly demonstrated that MLV 35 S RNA, a poor template by itself, showed much enhanced template activity when hybridized with proline $tRNA_3$.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration.

CELLULAR DNA REPAIR SYNTHESIS AND RNA TUMOR VIRUS INFECTION*

W.-K. Yang, D.-M. Yang, W. H. Lee, and J. D. Regan

Cells infected with or transformed by RNA tumor viruses generally contain the viral genome as double-strand DNA covalently integrated into the genome of the infected host. Viral genome integration apparently constitutes a key step in the establishment of RNA tumor virus infection, although the biochemical mechanism of this step is still unknown. In the present study we examined the relationship of cellular DNA repair synthesis and RNA tumor virus infection, with special attention to two questions: *(a)* During early stages of infection, is there a stimulation of the DNA repair mechanism in the host cell? (*b*) How does an impaired DNA repair mechanism of the host cell affect the infection of RNA tumor viruses?

To answer the first question the following experiment was performed. Normal human-fibroblast cultures were infected with RD-114 cat endogenous virus or with simian sarcoma virus at a high multiplicity of infection. The cultures were then incubated for the next 12 hr with medium containing bromodeoxyuridine (BrdUd) in the absence of cellular DNA synthesis. After photoillumination to cause DNA breakage at the sites of BrdUrd incorporation, the size of the cellular DNA was analyzed by alkaline sucrose gradient centrifugation. The results showed no change in size of DNA in either of the infected cell cultures. Thus, by employing the BrdUrd method, we were unable to detect stimulation of the DNA repair mechanism in normal human fibroblasts infected with Rd-114 cat virus or with simian sarcoma viruses.

To answer the second question we studied infection of Rd-114 virus and simian sarcoma viruses in cultured fibroblasts derived from normal human individuals (control cells) and xeroderma pigmentosum patients (XP cells). The cells were infected with various titers of viruses, and the infection was then assessed by measurements of the reverse transcriptase

activity in the medium and by observation of transformed-cell focus formation. Preliminary results of the experiments can be summarized as follows: (*a*) The three lines of control cells examined were all susceptible to infection with RD-114 and with simian sarcoma viruses. Virus production and cell transformation after infection were strongly correlated with the rate of cell proliferation; the most rapidly growing cell culture showed the most productive infection with the two viruses. (*b*) Infection of both normal and XP cells with Rd-114 virus appeared to be correlated with the rate of cell proliferation. Infection by simian sarcoma viruses in the XP cells was usually much depressed, as measured by reverse transcriptase activity and by transformed-cell focus formation. Comparison of various XP gene complementation groups suggested a positive relationship between the depressed simian sarcoma virus infection and impaired DNA repair process. However, a few exceptions were noted. Detailed analyses in this regard are in progress.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration.

PROGRESS IN PURIFICATION OF TRANSFER FACTOR AND DEVELOPMENT OF A SUITABLY SPECIFIC ASSAY FOR ITS ACTIVITY

G. D. Griffin,* H. G. Sellin, S. I. Simms, D.-M. Yang, and G. D. Novelli

The success of attempts to isolate and purify the active molecules in crude transfer-factor preparations depends to a large degree upon the specificity and appropriateness of the assay used to measure transfer-factor activity. Of the various assays proposed to measure transfer-factor activity, we feel that an *in vitro* cytotoxic assay is measuring most nearly the end point which we wish to determine, namely, the destruction of tumor cells by transfer-factor-activated lymphocytes. Assay procedures which utilize as end points blastogenesis or skin test reactivity may measure aspects of cellular immunity which may or may not relate to cell-mediated immune reactions against tumor cells.

We are currently employing a micro modification of the cytotoxicity assay developed by Byers and Levin[1] in which ^{51}Cr-labeled tumor cells are distributed into individual wells (5×10^3 cells per well) of the micro titer plate and transfer-factor-activated lymphocytes are seeded upon the target cells and allowed to attack for 3 hr. The release of ^{51}Cr into the supernatant medium after this time provides a measure of the lysis of the tumor cells by the lymphocytes. Some advantages of this assay system are the following: relatively small amounts of blood are required as a source of lymphocytes, since very few tumor cells are used per well (an obvious corollary is that the ratio of lymphocytes to tumor cells can be increased to quite high ratios without using excessive quantities of blood); the use of small quantities per individual assay allows many more transfer-factor fractions to be assayed at a given time; the reproducibility of the assay, both among replicates using the same transfer factor and from one assay to the next, is very good, in general; and finally, specificity of the cytotoxic reaction is very high, in that transfer-factor preparations specific for a certain tumor activate lymphocytes only against that tumor and not against other tumors.

Using this assay system, we have continued to pursue the goal of isolating the active component in transfer factor. Ion-exchange chromatography on Aminex A-28 resulted in a distribution of activity throughout a volatile salt gradient, and when dilutions were made of the various fractions, inhibition of the assayable activity was detected in all fractions, particularly those fractions eluting with 0.7 to 1.0 *M* ammonium acetate. If chromatography on Sephadex G-25 was done on crude transfer factor, again the activity was distributed throughout the gradient, but now the main inhibitory material was in the first several fractions. This suggests that inhibitory molecules are behaving differently depending upon the chromatography system, and current efforts are being made to eliminate the bulk of inhibitors by appropriate combinations of systems. If we are able to remove these inhibitors, it will enable us to more readily approach the problem of isolating an active moiety of transfer factor, although, from the results to date, we suspect that a number of different molecules (all of which may be various-sized digestion products of a larger molecule) may be found to have transfer-factor activity, but all of these molecules may be closely related in terms of composition. The accompanying abstract[2] will discuss work directed toward isolation and characterization of a possible parent molecule for transfer factor, namely, immune RNA.

*Postdoctoral investigator, U.S. Public Health Service Fellowship 1F22CA01755-01.

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2. H. G. Sellin, G. D. Griffin, and G. D. Novelli, this report.

USE OF IMMUNE GUINEA PIGS FOR STUDY OF CELLULAR IMMUNE COMPETENCE

H. G. Sellin, G. D. Griffin,* and G. D. Novelli

In the past, successful transfer of cellular immune competence with subcellular materials has been restricted to *in vivo* human systems. Use of human systems severely limits the directions of possible research aimed toward increased understanding and possible clinical application of immunotherapy for deficiencies in *cellular* immunity. Development of animal systems will greatly facilitate study in these areas. If transfer of cellular immune competence can be accomplished successfully in animals, attention should be turned to isolation and identification of the active moiety, be it transfer factor, immune RNA, or another as yet unidentified molecule, and to its mode of action.

In an attempt to develop an animal system, guinea pigs have been immunized by injection of approximately 10^8 cells per animal. The cells, designated ALAB and grown in tissue culture, originated from a human mammary carcinoma. Injections were in the footpads, peritoneum, and/or nape of the neck. Primary immunization was followed by secondary booster immunization with an equal cell number approximately 1 month later. The animals' state of immunity was assayed *in vitro* by measuring the cytotoxicity of each immunized animal's peripheral lymphocytes toward ALAB target cells labeled with ^{51}Cr. One month following the immunization the lymphocytes were cytotoxic toward the target cells, and the lymphocytes retained reactivity for a minimum of several months. The reactivity of the lymphocytes was directed toward human antigens, since the lymphocytes were equally active toward ALAB, a human mammary carcinoma line, and toward Te85, a human osteogenic sarcoma line. The lymphocytes from ALAB-immunized guinea pigs did not show, in this instance, any tumor specificity. Lymphocytes from naive, unimmunized animals did not attack either line of target cells.

Having guinea pigs with active immunity for ALAB, work is now being directed toward isolation and identification of the active moiety. Lymphoid tissues, specifically spleen and regional lymph nodes including inguinal, axillary, mesenteric, and popliteal nodes, are used as the starting materials. Initial attempts at isolation of an immune RNA using standard hot or cold phenol techniques have been complicated by low RNA yields and high contamination with DNA. Further effort will be required for successful RNA extraction and development of separation techniques useful for a wide spectrum of RNAs. Development and refinement in the *in vitro* assay for the active moiety are also needed. Some parameters in the assay requiring definition are the environment necessary for *in vitro* activation of naive lymphocytes to attacker status, the length of activation, the length of attack on target cells, etc.

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*Postdoctoral investigator, U.S. Public Health Service Fellowship 1F22CA01755-01.

THE STUDY OF TRANSFER FACTOR USING MOUSE AND HUMAN CELL LINES

M. E. Whitson,* N. K. Clapp,† W. H. Lee, J. D. Regan, and G. D. Novelli

The relationship between transfer factor and "immune RNA" is currently under investigation using both mouse and human systems. Squamous cell carcinomas of the forestomach of BALB/c mice induced by diethylnitrosamine (DEN) were established in tissue culture. Cells are in the sixth to eighth passage and have been injected into both nude and BALB/c mice to observe the development of tumors histologically similar to the original specimens. Guinea pigs immunized with DEN tumor cells will be used as the source of either immune RNA or transfer factor. The transfer-factor or immune RNA preparations will be assayed for their ability to stimulate nonimmune mouse lymphocytes to cytotoxicity against the DEN tumor cell line. Future plans include treatment of BALB/c mice with a number of chemical carcinogens. At intervals after treatment the mouse-lymphocytes would be removed and screened for their ability to be stimulated by transfer factor or immune RNA.

Two human tumors are being examined as possible model systems for the study of transfer factor. A squamous cell carcinoma of the lung recently has been grown in tissue culture (five passages). Cells from the lung tumor line will be used as target cells in an *in vitro* assay to screen for cytotoxic lymphocytes from family contacts of the lung tumor patients. Transfer-factor preparations from cytotoxic lymphocytes will be available for further fractionation and test purposes.

Multiple myeloma cell lines will be used in studies similar to those described above for the human lung tumor cells. Transfer factor prepared from lymphocytes cytotoxic for multiple myeloma cells

can be compared with the lung tumor transfer factor for characterization studies.

*Postdoctoral investigator, Carcinogenesis Training Grant CA05296 from NCI and subcontract 3322 from the Biology Division, ORNL, to the University of Tennessee, Knoxville.
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TRANSFER RNAs IN MOUSE THYMIC TUMORS AND IN NORMAL MOUSE THYMUS

G. D. Griffin,* L. H. Smith, M. Uziel, and G. D. Novelli

A number of reports have indicated changes in certain isoaccepting species of tRNAs in material extracted from tumor cells as compared with tRNA from noncancerous tissue. Very little is known about when these altered tRNAs develop during the course of tumorigenesis or the sequence modifications (if indeed there are any) which cause these tRNAs to be detected as abnormally behaving species in chromatographic systems.

We have chosen the mouse thymic tumor system as a particularly appropriate one to study, because its development takes place in an ordered fashion and it should be possible to look for the appearance of aberrant tRNA species at various times during the process of tumor growth. A particular difficulty using this system has been the problem of nuclease degradation of the tRNAs during the extraction process. We have found it helpful to use diethyl pyrocarbonate, a potent enzyme-inactivating agent, in order to prepare tRNA from these cells in reasonable yield. It is possible that this agent is also modifying individual bases in tRNA, and appropriate nucleoside analyses will be done to check this point. (The low temperature used for diethyl pyrocarbonate treatment and the low concentration used, 3% vol/vol, should keep nucleoside modification at a low level.) The tRNA preparations made by using diethyl pyrocarbonate and a cold phenolic extraction technique seem to be quite good in terms of amino acid acceptance, using a mouse-liver synthetase enzyme mixture. We are currently preparing to compare tumor and normal thymus isoaccepting species of tRNA using techniques of RPC-5 reversed-phase chromatography. If any aberrant species are found, it is our goal to try to determine what changes (if any) in primary structure produce this chromatographic behavior difference.

*Postdoctoral investigator, U.S. Public Health Service Fellowship 1F22CA01755-01.

A SYSTEM POTENTIALLY USEFUL FOR DETECTING SUBSTANCES WITH IMMUNE-TRANSFERRING ACTIVITY

L. H. Smith, L. E. MacAskill,* and G. D. Novelli

Detection, standardization, and identification of subcellular materials with immune-transferring activity have met with considerable difficulty. Recent advances in tissue culture techniques, however, provide possible approaches to at least the detection of these materials. Using an *in vitro* culture system, Dennert and Hatlen[1] have shown that when naive mouse splenocytes are incubated for several days with one of several kinds of stimulator cells, the splenocytes become cytotoxic to the stimulator target cells. Cytotoxicity is measured as a release of radioactivity from ^{51}Cr-labeled target cells. We are in the process of evaluating this *in vitro* system as a means of detecting and assaying substances with potential immune-transferring activity, for example, transfer factor and immune RNA.

To date we have reproduced the *in vitro* conditions for maintaining in short-term (5 days) culture splenocytes from C57BL mice, and we have found that about 30 to 40% of these cells are viable (trypan blue exclusion) on day 5. Currently, we are studying the growth characteristics of EL4 leukemia cells, which, coupled with trinitrophenyl, will be used as stimulator and target cells.

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

A. N. Best and G. D. Novelli

Last year we reported the initiation of a collaborative research effort with the Respiratory System Group to investigate possible fluctuations in nucleoside levels in rat urine during the progress of squamous carcinomas in tracheal grafts.[1,2] With some types of malignant tumors, patients have shown elevated urinary levels of certain nucleosides, namely, pseudouridine, 1-methylinosine, and N^2-dimethylguanosine.[3]

Our immediate concern has been to develop satisfactory analytical procedures. Preliminary

chromatography of rat urine (0.1 to 1.0 ml) on P-2 gel can achieve a partial separation of the major nucleoside fraction from some metabolic breakdown products. An analysis of a small portion of this fraction by cation-exchange chromatography showed that about 290 nmol of pseudouridine was excreted per milliliter of urine (24-hr collection, intake limited to water) from a normal rat. A small amount of 1-methylinosine was detected.

Since boronate gel columns offer a possibility of good nucleoside separation, it was decided to halt the analytical experiments until we could obtain some of this material from a large batch being prepared by Mayo Uziel. We are ready to test the boronate gel method and analyze for nucleosides by Uziel's high-resolution technique.[4]

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STUDIES ON THE TRANSFER RNA OF METHANE-PRODUCING BACTERIA

A. N. Best

Purified bulk tRNA from *Methanococcus vannielii*, a fast-growing methanogenic coccus using formate for its carbon source, has been shown to be deficient in ribothymidine and N^7-methylguanosine. The presence of 1-methlyadenosine, N^2-methylguanosine, and N^2-dimethylguanosine reflects a different pattern of methylation from *E. coli* tRNA.[1]

With a mixed synthetase preparation from *E. coli*, the *M. vannielii* tRNA can be aminoacylated with at least 13 amino acids. Aminoacylation with methionine using synthetase preparations from both bacteria showed different elution patterns with reversed-phase chromatography. Under the conditions employed, methionyl tRNA of *E. coli* has two characteristic peaks, the first at 0.55 *M* NaCl for $tRNA_f^{met}$ and the second at 0.61 *M* for $tRNA^{met}$. Heterologous aminoacylation with *E. coli* synthetase of *M. vanielii* tRNA showed one peak, which coincided with the *E. coli* $tRNA^{met}$ position. With homologous aminoacylation, the *M. vannielii* tRNA had one peak at the *E. coli* $tRNA^{met}$ position and a second peak at a higher salt concentration. It is probable that the first peak is the initiator $tRNA^{met}$, since heterologous aminoacylation will usually occur with the initiator $tRNA^{met}$.[2]

The elution patterns on reversed-phase chromatography with other amino acids have not been tested.

M. vannielii methionyl-tRNA can be formylated by a partially purified *E. coli* synthetase preparation containing transformylase. This activity has not been observed in *M. vannielii* preparations. Folate derivatives have been implicated in the incompletely elucidated pathway of methane production. Possibly due to other metabolic demands, formylation of initiator $tRNA^{met}$ does not occur during cell growth.

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IDENTIFICATION OF POTENTIAL PRIMER TRANSFER RNA MOLECULES IN RNA TUMOR VIRUSES*

L. C. Waters, B. C. Mullin,† L. G. Hardin, E. G. Bailiff, and R. A. Popp

We have developed a method to qualitatively and semiquantitatively identify small amounts of tRNA. We have previously reported on our use of the method to implicate tryptophan tRNA and proline tRNA as primers, or initiators, of DNA synthesis in the avian myelobastosis virus (AMV) and the AKR murine leukemia virus (AKR-MLV) respectively. Our preliminary data using the Rauscher murine leukemia virus supported our suggestion that primer tRNAs were specific for the host species from which the virus originated. To date we have analyzed a total of nine different viruses as to the identity of a tRNA most likely to serve a primer function, and three classes have emerged. In two avian viruses, AMV and Rous sarcoma virus, tryptophan tRNA is clearly indicated as primer. We have evidence that proline tRNA might serve as primer in four murine leukemia viruses, two feline leukemia viruses, and also a simian sarcoma virus. In RD-114 virus, which is endogenous to cats but does not replicate in cat cells, our data suggest that glycine tRNA might be the DNA synthesis primer in this system. On the basis of the results with murine and feline viruses it seems clear that primers in viruses from different species can be the same. The primer class might more accurately

reflect the phylogenetic origin of the virus. It has been shown by nucleic acid sequence homology that feline leukemia virus is more closely related to MLVs than to the cat xenotropic virus RD-114. Additional data from other xenotropic viruses would be useful.

Having identified the interacting tRNAs in the viral genomes, we are continuing to probe the mechanism of this interaction. In addition, we are initiating a long-range program to study the involvement of RNA in DNA replication in other systems.

*Research supported jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

† Postdoctoral investigator supported by Carcinogenesis Training Grant CA05296 from NCI.

INTERACTION OF SPECIFIC TRANSFER RNAs WITH AVIAN MYELOBLASTOSIS VIRUS 35 S RNA*

B. C. Mullin,† L. G. Hardin, and L. C. Waters

We have determined that 30% of the primer $tRNA^{Trp}$, when hybridized to the 35 S RNA genome of AMV, is resistant to S_1 and pancreatic nuclease digestion. The resistant fragment of the tRNA has since been shown by others to consist of the first 16 to 21 nucleotides at the 3′ end of the tRNA. We have prepared hybrids between tRNAs other than $tRNA^{Trp}$ and the viral 35 S RNA and have found these hybrids to be poor template primers for DNA synthesis. In particular, $tRNA^{Lys}$ was shown to hybridize in a 1:1 molar ratio with the 35 S RNA, yet the resulting hybrid was not an active template primer. In an effort to determine the nature and function of the interactions between nonpriming tRNAs and the 35 S RNA, we are developing nucleic acid sequencing methods including RPC-5 reversed-phase column chromatography and two-dimensional cellulose thin-layer chromatography.

*Research sponsored jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

† Postdoctoral investigator supported by Carcinogenesis Training Grant CA05296 from NCI.

PROLINE TRANSFER RNA INTERACTIONS WITH AKR MURINE LEUKEMIA VIRUS 35 S RNA AND REVERSE TRANSCRIPTASE*

B. C. Mullin,† L. G. Hardin, and L. C. Waters

We had previously shown that the last tRNA fraction to be dissociated from AKR-MLV 35 S RNA by heat contained predominantly proline tRNA. To study this interaction we attempted to show specific hybridization of proline tRNA, from a mixture of tRNAs, with the viral 35 S RNA. Some 4 S RNAs other than proline tRNA hybridized under our conditions. However, heat dissociation experiments showed that, of the hybridized 4 S RNA, a fraction chromatographically similar to proline tRNA was most tenaciously bound.

In the AMV system, tryptophan tRNA has been shown to interact with the viral DNA polymerase. This is considered as strong supporting evidence that tryptophan tRNA indeed has a primer function *in vivo*. We have used a variety of techniques in an attempt to show an interaction between proline tRNA and the reverse transcriptase purified from mouse spleens infected with Rauscher leukemia virus, mouse fibroblasts infected with AKR-MLV, and AKR-MLV. No such interaction was shown. Others have evidence to indicate that there is a high-molecular-weight form of the murine tumor virus reverse transcriptase which does bind proline tRNA and which transcribes viral RNA more efficiently than does the enzyme form generally obtained.

*Research sponsored jointly by the Virus Cancer Program of the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

† Postdoctoral investigator supported by Carcinogenesis Training Grant CA05296 from NCI.

NEW SYSTEMS FOR AUTOMATED AFFINITY CHROMATOGRAPHY: THE MODEL C AND MINI-C CYCLUMS*

E. L. Candler, D. D. Willis, and M. A. Brown

The Human Cancer Diagnostics and Therapeutics Laboratory has been actively engaged in the development and application of immunosorbent technology for cancer-related studies. Part of our effort has been

Fig. 34. The Model C Cyclum for large-scale automated affinity column separations.

devoted to the development of two instruments to facilitate the use of immunoaffinity chromatography. One system, termed the Model C Cyclum, has been designed for fairly large-scale separations involving the use of immunosorbents ranging from 5 ml to 2 liters in bed volume. This instrument, shown in Fig. 34, is fully automated and can be easily programmed to perform affinity chromatography operations, such as sample application, elution of unadsorbed and adsorbed proteins, dialysis, fraction collection, and column equilibration, in a precise and repeatable manner. The ability of the instrument to perform repetitive separations allows one to make the most efficient use of an immunosorbent and to process large quantities of sample. Once programmed, the instrument can perform as many as 1000 repetitive affinity column separations unattended, thus providing a considerable saving in technician time and expediting the research objective.

The other system, designated the Mini-C Cyclum, has been constructed to do small-scale affinity column separations on immunosorbents prepared with precious reagents available only in small quantities. It is best suited for affinity columns ranging from 1 to 10 ml in bed volume. Like the larger system, the Mini-C Cyclum (Fig. 35) is fully automated and can perform any number of preset repetitive separations. The components of the Mini-C Cyclum have been designed and arranged to minimize the separation time and dilution of the eluted fraction.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

H-2 ACTIVE FRAGMENTS ISOLATED BY AUTOMATED AFFINITY CHROMATOGRAPHY*

E. L. Candler, B. S. Bradshaw,† R. A. Popp, and M. A. Brown

A number of recent studies have indicated that neoantigens of tumors induced by chemical carcinogens and oncogenic viruses may be modified histocompatibility antigens. Proof of this relationship will ultimately require extensive immunochemical studies of the antigens in pure form. We have initiated a collaborative study to determine the feasibility of isolating mouse histocompatibility antigens by affinity chromatography.

We have previously reported the preparation of an affinity column containing antibody to H-2 antigenic

Fig. 35. The Mini-C Cyclum for small-scale automated affinity column separations.

specificities 7 and 9.[1] Studies have been conducted to determine the most efficient method of using this affinity column to isolate H-2 activity from 3 *M* KCl extracts of RFM mouse cells. Batch-type operations in which 15 ml of extract was incubated in the column overnight did not result in complete adsorption of the antigens. Complete adsorption could be obtained in continuous-flow operations by processing 5 ml of the extract at a time. Since the time required to complete this separation and to equilibrate the column for another run was only 3 hr, as much as 30 ml of extract could be processed in an 18-hr period.

The affinity column was mounted in a Model C Cyclum which was preset to process 100 ml of the 3 *M* KCl extract in 20 consecutive 3-hr cycles. Each cycle was programmed so that 5 ml of extract was placed on the immunosorbent and was washed through the column with phosphate-buffered saline until the optical density (monitored by spectrophotometer at 280 nm) of the unadsorbed material returned to zero. Then 10 ml of 3 *M* ammonium thiocyanate was passed through the column to elute the adsorbed material. Most of the eluting fluid was removed from the adsorbed fraction prior to collection by routing the effluent stream from the column through hollow-fiber dialyzers containing phosphate-buffered saline. The unadsorbed and adsorbed fractions from the 20 runs were pooled, dialyzed against phosphate-buffered saline, and concentrated with Amicon PM 10 membranes.

When the fractions were assayed for H-2 activity by the ^{51}Cr release inhibition test, all activity was found in the adsorbed fraction. Acrylamide gel electrophoresis of the adsorbed fraction revealed the presence of a single band, which was later determined to have a molecular weight of approximately 48,000. From 100 ml of the crude extract, 2.56 mg of apparently pure H-2 active protein was obtained. In view of the fact that we have not been able to detect

any significant loss of activity in the affinity column after 47 runs, we believe that the affinity column technique will provide sufficient quantities of material for characterization and immunochemical study.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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

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ISOLATION OF FETAL ANTIGENS IN HUMAN LEUKEMIA*

E. L. Candler, C. H. Granatek,† and M. A. Brown

Immunologic studies have indicated that some neoantigens associated with human leukemia cells are cross-reactive with surface components of midgestation mouse fetal liver cells.[1,2] We report here preliminary efforts to isolate these interspecies fetal antigens by affinity chromatography.

Antiserum to BALB/c 15-day fetal liver cells was prepared in rabbits and then adsorbed extensively with both normal human blood cells and adult mouse liver. DEAE-cellulose-purified immunoglobulin from the antiserum was coupled to Sepharose 4B by cyanogen bromide. The immunosorbent was packed in a small column and placed on a Model C Cyclum for automated affinity chromatography. Three molar potassium chloride extracts of mouse or human fetal liver cells and four different morphological types of human leukemic cells (ALL, CLL, AML, and CML) were processed through the column in small volumes (3 to 4 ml). Elution of the adsorbed material was accomplished with 3 *M* ammonium thiocyanate. The adsorbed fractions from each extract were dialyzed against phosphate-buffered saline, concentrated with Amicon PM 10 membranes, and examined for fetal antigen activity using rabbit antiserum to 15-day mouse fetal liver cells.

The results of three different assays (complement fixation, inhibition of antibody binding to surface antigens, and radioimmunoassays) have indicated that all isolated fractions contain cross-reactive fetal antigen. Polyacrylamide gel electrophoresis of the adsorbed fractions has revealed the presence of three to four distinct bands with comparable electrophoretic mobility. Efforts to identify the immunological relationship of the electrophoresis bands in the six adsorbed fractions have indicated that the lymphocytic and myelogenous leukemias may have different interspecies embryonic antigens. Preliminary studies have indicated that some of these fetal antigens may be viral in nature.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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AUTOMATIC REPETITIVE CHROMATOGRAPH (ARK)*

J. W. Eveleigh, D. E. Levy, and D. D. Willis

A versatile instrument has been constructed that automatically performs preparative chromatographic separations in an efficient and reliable manner (Fig. 36). Incorporating a unique programmable fluid valve, for which patent protection is currently being sought, and several other innovative devices, the system has successfully been applied to many separation methods in common use in the biochemical laboratory. Included in these preprogrammed preparative applications are ion exchange, gel filtration, adsorption techniques, and affinity and immunosorbent chromatography separations. Additionally, programs involving the simultaneous use of two or more columns combining these techniques have been used with a large saving of time and effort. This extremely versatile instrument has incorporated in its design safety devices that allow reliable continuous operation throughout long periods completely unattended. This efficient mode of operation has resulted in enormously increased yields compared with the current manually directed methods.

The function of the instrument to repetitively perform a chromatographic separation until the sample is completely processed becomes extremely attractive when valuable immunological reagents in limited supply are being utilized. An application of this nature was recently successfully performed for the University of Tennessee Medical Research Center

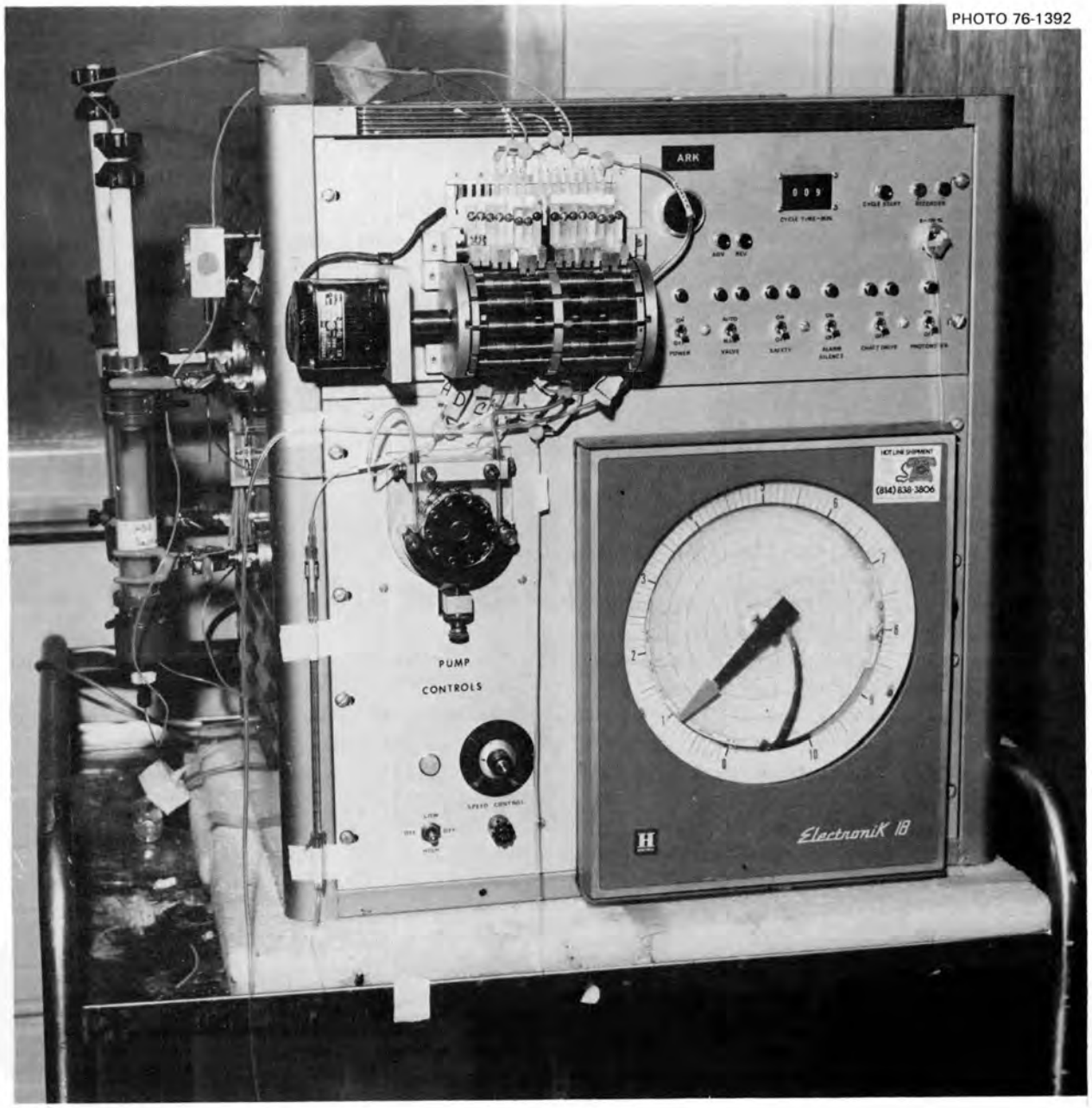

Fig. 36. The ARK.

using the instrument. A 10-ml column of erythropoietin was repetitively cycled 96 times unattended over a period of 5 days to provide specific antibodies from whole antisera. The system is being used routinely in the program to provide purified proteins and specific antibodies required in other projects.

A report is being prepared for open publication once patent protection has been obtained.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

IMMUNOSORBENT TECHNOLOGY*

J. W. Eveleigh, D. E. Levy, R. A. Ryba,† M. K. Sarcar,† and S. R. Sanders†

Research has continued in developing new immunosorbents for specific applications and in determining the basic characteristics of these materials. Immobilized reagents have been prepared using polycarbonate, cellulose derivatives, and porous and solid glass, as well as commercially available material such as Sepharose and Sephadex. These widely differing supports have allowed basic studies of the kinetic and immunochemical reaction mechanisms involved in preparative and analytical applications. Determination of reaction rate constants, affinity constants, and the effect of environmental factors, such as temperature and pH, has provided the data necessary to effect an increase in operational efficiency. Using these findings, the protocols of recycling systems have been modified, resulting in the routine production of daily yields of gram quantities of monospecific antibodies.

Investigation of the factors involved in applying immunosorbents to *ex vivo* depletion of specific antibodies and antigens is being continued. We are currently using model systems; a sealless rotor system is, however, currently being constructed to extend these studies into actual animal depletions with the ultimate objective of human therapeutic applications.

An Industrial Cooperation Bulletin (No. 232) has been prepared and issued on the technology and associated instrumentation of this project. The response from commercial users, particularly pharmaceutical companies, has been good, and the methodologies developed in the program are being increasingly applied by industry.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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AUTOMATED IMMUNOASSAY*

J. W. Eveleigh and S. K. Thieme†

Feasibility studies of the use of defined pore polymeric films as a means of performing rapid automated radioimmunoassays have been completed. Basic studies of the system have shown that antibodies and antigens can be reproducibly immobilized within the pores of these membranes and that immunological reactions can effectively be performed on the discs. The kinetics of these reactions are, however, still under investigation to determine the speed that this reaction can be utilized. Our objective is to allow the quantitative assay of antigens in a rapid manner and thus avoid the lengthy and space-consuming incubations that are necessary in the current methodologies. To facilitate these investigations, collaboration with the Analytical Chemistry Division is anticipated in the near future when extension of the proposed method into the specific analysis of hormones, drugs, and carcinogens will be undertaken.

Development of the associated instrumentation is concurrently being appraised in the light of these results, and it is hoped to have a working prototype once patent status has been completed.

*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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IMMUNOLOGICAL REAGENTS FOR CHEMICAL CARCINOGENS*

J. W. Eveleigh, J. T. Meier,† R. E. Cape,‡ G. Tennyson,§ and S. R. Sanders ‖

Preliminary studies of this project, for which specific funding has been requested, have been successfully accomplished. The use of carcinogens as haptens in invoking an immune response has been previously demonstrated only for a limited number of easily derivatized polyaromatic hydrocarbons. One of the objectives of this current project is to produce specific antibodies against defined chemical entities, including specific carcinogen metabolites. This in practice entails the covalent linking of these compounds to carrier proteins and the subsequent use of associated immunosorbents for purification of the specific antibodies.

A chemical scheme has now been perfected to allow a wide selection of these compounds to be linked to proteins and to inert supports. The bonding involves the phenolic hydroxyl group, which is present in the most well-characterized derivatives of the polyaromatic hydrocarbons, and will allow

assays of many significant metabolites. This chemistry has been primarily tested on BP derivatives but will be extended to other carcinogens in the very near future.

An immunoassay to detect specific antibodies to these carcinogens has been developed, and immunizations of rabbits are under way. It is hoped to combine these studies with those involving development of a rapid radioimmunoassay, thus providing a practical application to current requirements in the Carcinogenesis Program.

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*Research supported jointly by the National Cancer Institute and the U.S. Energy Research and Development Administration under contract with the Union Carbide Corporation.

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SEMINARS

One hundred eight seminars were given at the Biology Division by visiting scientists from research organizations in the United States and abroad during the period from July 1, 1975, through June 30, 1976. Speakers came from 26 states and 11 foreign countries. In addition there were numerous informal seminars at which Division members spoke about their own research.

Listed below are the seminars presented by the visiting scientists.

Speaker	Affiliation	Subject
Mohamed Bahie Abou-Donia	Analytical Pharmacology Laboratory, Duke University Medical Center	Organo-phosphorus pesticide-induced delayed neurotoxicity
David E. Amacher	Somatic Cell Genetics Branch, National Institute of Environmental Health Sciences	Removal of acetylaminofluorene adducts and pyrimidine dimers from the DNA of cultured mammalian cells
Lawrence Anderson	Naval Biomedical Research Laboratory	Murine epithelial cells *in vitro*
Rodney Baker	Oak Ridge Associated Universities	Interactions of methylenedioxyphenyl (1,3-benzoenedioxole) compounds with microsomal cytochrome P-450
Russell J. Barrnett	Department of Anatomy, Yale University School of Medicine	Biogenesis of plasma membrane in secretory cells of salt glands with relevance to phospholipid synthesis and Na^+,K^+-ATPase
William T. Beck	University of Southern California School of Pharmacy	Aspects of polyamine regulation in mammalian cells
Ellen M. Berkowitz	Department of Biochemistry and Molecular Biology, Harvard University	Characterization of an active fraction of chicken reticulocyte chromatin
Frederick J. Bollum	Department of Biochemistry, University of Kentucky	Terminal deoxynucleotidyl transferase biology
R. K. Boutwell	McArdle Laboratory for Cancer Research, University of Wisconsin	Studies on the mechanism of action of tumor promoters
Daniel Branton	The Biological Laboratories, Harvard University	Freeze-fracture studies of macromolecular distribution control in membranes
Ricardo Brentani	Laboratory of Experimental Oncology, University of São Paulo, Brazil	Collagen messenger RNA
John I. Bruce	Lowell Institute of Technology	Control and treatment of schistosomiasis in southeast Asia
G. Bunick	University of Pennsylvania	Status report on the X-ray structure of yeast inorganic pyrophosphatase
Frederick Burns	Institute of Environmental Medicine, New York University Medical Center	Biological and physical factors affecting radiation carcinogenesis in rat skin
Norman F. Carver	Jalbert Physics Institute	Recent developments in the quest for controlled thermal fusion
Paul W. Chun	The J. Hillis Miller Health Center, University of Florida	Electrokinetic properties of the sickle cell
A. D. Conger	Department of Radiobiology, Temple University School of Medicine	Why is the nucleus so damn big? Relation of nuclear volume, DNA, chromosome size per cell, and nuclear metabolism to each other for 107 species
Donald Crothers	Department of Chemistry, Yale University	Studies on the structure of RNA
Lloyd A. Culp	Department of Microbiology, Case Western Reserve University	Studies on the mechanism of substrate adhesion of normal and virus-transformed cells

Norman Davidson	Division of Chemistry and Chemical Engineering, California Institute of Technology	Gene mapping by electron microscopy
Ronald W. Davis	Department of Biochemistry, Stanford University School of Medicine	Gene amplification and cloning
Dale DeHamer	University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences (dissertation seminar)	A biochemical study of lethal mutations at the *c* locus in the mouse
D. G. de Rooij	Laboratory of Histology and Cell Biology, Rijksuniversiteit of Utrecht, The Netherlands	Spermatogonial stem cell response to radiation and myleran
Giampiero diMayorca	Department of Microbiology, University of Illinois School of Medicine	The molecular mechanism of carcinogenesis: A mutagenic event
Anthony Dipple	Frederick Cancer Research Center	Carcinogen-nucleic acid interaction in chemical carcinogenesis
Allen B. Edmundson	Division of Biological and Medical Research, Argonne National Laboratory	3D structure and divergent evolution of immunoglobulin domains
Udo Ehling	Institut für Biologie, GSF, Neuherberg, Munich, Germany	Estimation of the risk from chemical mutagenesis in mice
Rosalie K. Elespuru	University of Tennessee-Oak Ridge Graduate School of Biomedical Sciences (dissertation seminar)	Mutagenesis by nitrosocarbaryl and related compounds in *Escherichia coli* and *Haemophilus influenzae*
Henry Eyring	Department of Chemistry, University of Utah	A physical chemist's view of biology
Roger Fenna	Institute of Molecular Biology, University of Oregon	Chlorophyll arrangement in a bacterial chlorophyll protein
Susan Fischer	University of Texas System Cancer Center, Houston	Aryl hydrocarbon hydroxylase activity in cultured human lymphocytes
Irwin Fridovich	Department of Biochemistry, Duke University	Oxygen: Bane or boon
Earl Frieden	Department of Chemistry, Florida State University	The role of macromolecules in inducing metamorphosis
Carl Gehrs	Environmental Sciences Division, Oak Ridge National Laboratory	Methods for determining the effects of chemicals on aquatic populations
Eduard Gfeller	Veterans Hospital, Birmingham, Alabama	Inheritance of schizophrenia
Alfred Gillhorn	Center for Biomedical Education, City College, City University of New York	Medical education for the last quarter of the Twentieth Century
James E. Glasgow	Department of Molecular Biophysics and Biochemistry, Yale University	Interaction of platelets with human cell cultures
D. G. Gosslee	Computer Sciences Division, Oak Ridge National Laboratory	The use of biometry in solving biological problems
Dale E. Graham	Department of Biological Sciences, Purdue University	Gene regulation, genome structure, and evolution
Jennifer Dean Hall	Department of Biochemical Sciences, Moffett Laboratories, Princeton University	Murine testicular teratoma cells transformed by SV40 tumor virus
G. Wesley Hatfield	Department of Medical Microbiology, College of Medicine, University of California, Irvine	Autoregulation of isoleucine-valine biosynthesis

Ralf Hutter	Technische Hochschule, Microbiology Institute, Zurich, Switzerland	Genetics and physiology of melanin production in *Streptomyces*
Masan Imada	Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder	Membrane proteins in neuroblastoma cells
Elwood V. Jensen	The Ben May Laboratory for Cancer Research, University of Chicago	Estrogen receptors and hormone dependency in mammalian tissues
Ronald W. Johns	Department of Microbiology, U.S. Army Medical Laboratory, Fort Sam Houston, Texas	Serodiagnosis of fungal infections
Nathan O. Kaplan	Chemistry Department, University of California, San Diego	Immobilized drugs and enzymes
Walter Kauzmann	Chemistry Department, Princeton University	The structure of water: Biological implications
Stephen Kennel	Department of Immunopathology, Scripps Clinic and Research Foundation	Viral proteins as cell surface antigens
David Krahn	McArdle Laboratory for Cancer Research University of Wisconsin	Liver homogenate mediated mutagenesis in Chinese hamster V79 cells by chemical carcinogens
Richard L. Krogsrud	University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences (dissertation seminar)	Age-related decline in two parameters of the humoral immune response: B cell proliferation and T cell helper function
Robert Jerry Langenbach	Eppley Institute for Research in Cancer, University of Nebraska Medical Center	Glycol lipid and morphological alterations after *in vitro* chemical transformation
Frank W. Larimer	Department of Biological Sciences, Florida State University	Neutral amino acid transport in *Neurospora crassa*
Martin G. Lewis	Cancer Research Unit, McGill University, Montreal, Canada	Antibodies and anti-antibodies in malignancy
Y. S. Lu	The University of Michigan Medical School	Immunologic studies of herpes-virus-induced chicken lymphoma-Marak's
P. K. Maitra	Tata Institute of Fundamental Research, Molecular Biology Unit, Bombay, India	Glycolytic mutants in yeast
Thomas Malone	Extramural Research and Training, National Institutes of Health	Some recent trends in the support of research training programs at NIH
William McBride	Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, Scotland	The mechanisms underlying the antitumor action of *Corynebacterium parvum*
Patrick R. McClintock	University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences (dissertation seminar)	The regulation of dimethyl sulfoxide-induced hemoglobin synthesis in Friend leukemia cell clone 745
Alton Meister	Department of Biochemistry, Cornell University Medical College	Metabolism of glutathione by the gamma-glutamyl cycle
Ann D. Mitchell	Biochemical Cytogenetics Program, Biomedical Research Department, Stanford Research Institute	Development and validation of a new DNA repair synthesis assay for the prescreening of chemical carcinogens
Sohan Modak	Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland	Cytoplasmic information DNA in eukaryotic cells: Does it exist?

Paul F. Mullaney	Biophysics and Instrumentation Group, Health Research Division, Los Alamos Scientific Laboratory	Flow systems cell sorting and analysis techniques
Alfred H. Narten	Chemistry Division, Oak Ridge National Laboratory	Conjectures on the structure of amorphous solid and liquid water
Joseph S. Nasyrov	Institute of Plant Physiology and Biophysics, Academy of Sciences of the Tadjk SSR	Genetic aspects of photosynthesis
Carroll Nix	Department of Biology, George Mason University	Unstable redundancy in 5 S RNA of *Drosophila*
J. K. Poggenburg	Operations Division, Oak Ridge National Laboratory	How to put a label on your work: Seeing beyond the microscope
Efraim Racker	Section of Biochemistry, Molecular and Cell Biology, Cornell University	Reconstitution and mechanisms of oxidative phosphorylation and of ion pumps
K. T. Rao	Department of Biological Sciences, Florida State University	Acetate transport in *Neurospora crassa*
Brian R. Reid	Department of Biochemistry, University of California, Riverside	NMR studies in the solution structure of tRNA The crystal structure of Tyr-tRNA synthetase in the protein RNA recognition problem
Jean Paul Revel	Division of Biology, California Institute of Technology	The gap in intercellular communication
Richard J. Reynolds	University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences (dissertation seminar)	Excision repair in ultraviolet-irradiated *Saccharomyces cerevisiae*
Marvin Rich	Division of Biological Sciences, Michigan Cancer Foundation	Breast cancer viruses
Marian Rivas	Department of Medical Genetics, University of Oregon Medical School	Human linkage studies and prevention of genetic disease
Alfred L. Robertson	Amersham/Searle, Arlington Heights, Illinois	Liquid scintillation counting
Charles O. Rock	University of Tennessee–Oak Ridge Graduate School of Biomedical Studies (dissertation seminar)	Harderian glands, ether lipids, and tumors
James P. Rolls	Upjohn Company, Kalamazoo, Michigan	The use of ^{13}C NMR spectroscopy and ^{13}C-enriched precursors in biology
Irwin Rose	Institute for Cancer Research, Fox Chase, Philadelphia	The mechanism of biotin enzymes
Richard L. Russell	Department of Biology, California Institute of Technology	Genetic and anatomical analyses of sensory behaviors in the nematode *Caenorhabditis elegans*
William J. Rutter	Department of Biochemistry and Biophysics, University of California, San Francisco	Control of growth and transcription of specific genes during cytodifferentiation
Claus H. Schröder	Deutsches Krebsforschungszentrum, Institut für Virusforschung, Heidelberg, Germany	Generation of HSV angelotti virions containing highly defective viral DNA
J. H. Schröder	Institute for Radiation and Environmental Research, Neuherberg, Munich, Germany	Teleosts as a tool of mutation research
Nadrian Seeman	Department of Chemistry, Massachusetts Institute of Technology	The specific recognition of nucleic acid sequences: Infidelity in the grooves
L. Dennis Smith	Department of Biological Sciences, Purdue University	Synthesis and conservation of RNA during oogenesis

Thomas Smith	Graduate School of Biomedical Sciences, University of Texas Health Science Center, Dallas	The mechanism of multiple allosteric interactions
F. H. Sobels	Laboratory of Radiation Genetics and Chemical Mutagenesis, Leiden, The Netherlands	Studies on radiation and chemical mutagenesis in *Drosophila*
Charles D. Stiles	Department of Biology, University of California, San Diego	Growth control: heterologous tissue culture cells in the congenitally athymic nude mouse
Bernard Strauss	Department of Microbiology, University of Chicago School of Medicine	DNA repair in mammalian cells
Juan A. Subirana	Departamento de Quimica Macromolecular, Escuela Te'cnia Superior de Ingenieros Industriales, Barcelona, Spain	Studies of chromatin structure by electron microscopy and X-ray diffraction
Lewis Sullivan	Morehouse College	Effect of alcohol on hemopoiesis
William N. C. Sun	Frederick Cancer Research Center	Genetics of Chinese hamster cells
S. S. Tevethia	Department of Pathology, Tufts University School of Medicine	Immunobiology of SV40 transplantation-rejection antigen
W. E. Thiessen	Chemistry Division, Oak Ridge National Laboratory	Tertiary structural differences between microbial serine proteases and pancreatic serine enzymes
Paul Todd	Department of Biophysics, Pennsylvania State University	Survival-related repair processes in cultured mammalian cells exposed to UV light
Margaret M. Tolbert	Department of Chemistry, Tuskegee Institute	Studies on the stereochemistry and biological properties of 5-methyltetrahydrohomofolate
Ruby Valencia	Mutagenesis Laboratory, WARF Institute Inc., Madison, Wisconsin	Use of *Drosophila* in mutagenicity testing
Raymond C. Valentine	Department of Chemistry, University of California, San Diego	Genetic engineering of microorganisms as related to nitrogen fixation
Julian VanLancker	Department of Pathology, University of California, Los Angeles Health Science Center	The enzymology of DNA repair in liver carcinogenesis
Dimitri Viza	University of Paris, France	Leukemia antigens
Ekkehart Vogel	Department of Radiation Biology and Chemical Mutagenesis, State University of Leiden, The Netherlands	Metabolic activation of chemicals to mutagens in *Drosophila*
George Wald	Biological Laboratories, Harvard University	Some new pathways in vision research
Stephen G. Waley	University of Oxford, England	Structural studies on triose phosphate isomerase
Charles A. Walker	School of Pharmacy, The Florida Agricultural and Mechanical University	Circadian rhythms of biogenic amines in the CNS and drug activity
Wolfgang Wechsler	Max Planck Institute, Cologne, Federal Republic of Germany	Growth and differentiation of cell clones isolated from CNS tumors
Allen Wilcox	Pharmacia Fine Chemicals, Piscataway, New Jersey	Polyacrylamide gel electrophoresis

T. G. Wilson	University of Tennessee–Oak Ridge Graduate School of Biomedical Sciences (dissertation seminar)	Studies on the purple mutant in *Drosophila melanogaster*
John E. Womack	Max Planck Institute for Molecular Genetics, Berlin, Federal Republic of Germany	Origin-RNA and the *E. coli* origin plasmid
Milton Yatvin	Radiology Research Laboratory, University of Wisconsin Medical School	Characteristics of a DNA-membrane complex in irradiated *E. coli*

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