PROGRESS REPORT

for

MUTAGENIC EFFECT OF RADIONUCLIDES INCORPORATED INTO DNA OF *Drosophila melanogaster*

AT-(40-1)-3728

ERDA Report No. ORO-3728-8

1974-75

Submitted to

Division of Biomedical and Environmental Research

Energy Research and Development Administration

Washington, D.C. 20545

by

William R. Lee, Professor
Department of Zoology and Physiology
Louisiana State University
Baton Rouge, Louisiana 70803
Phone (504) 388-1132

Progress Report for the pericd May, 1974 through May, 1975: To determine the mutagenic effect of ^{3}H incorporated into DNA of *Drosophila melanogaster* we are using the procedures we used for ^{32}P, ^{33}P, and ^{14}C. In this procedure ^{3}H is incorporated into DNA in the germ line by feeding male larvae in late second instar a pulse of the radionuclide. This stage of development of the male larvae coincides with the rapid expansion of nuclei in the testis. Following eclosion the males are mated for two days to females that are not labeled with the radionuclide. A sample of eggs is obtained during the mating and then the females are stored at 10°C for three weeks. Following storage the females are again placed on our standard Drosophila media at 25°C and a second sample of eggs is obtained. There is an average of 25 days between the laying of the first sample and the second sample of eggs because it takes a few days following storage at 10°C for the females to recuperate and begin egg laying. This storage of a few hundred labeled spermatozoa in unlabeled females is the equivalent of dilution experiments in single celled organisms where labeled cells are diluted in a non-labeled media to reduce the level of radiation from adjacent cells.

Genetic stocks are used as described in our previous publications (Lee, Sega and Benson, 1972 and Lee, Sega and Alford, 1967). In this mating scheme a cross is made that produces only male larvae for labeling with the radionuclide, and another cross is made that produces the parental females as virgins since no male progeny are produced. The F_1 generation can be scored for losses of the X or Y chromosome because of dominant markers, *Bar-Stone* and *yellow-plus*, on the Y-chromosome. All the F_1 and F_2 males are sterile permitting out-crossing of females to nontreated stocks for sex-linked recessive lethal tests in the F_2 and F_3. Genetic markers are incorporated to permit detection of nondisjunction in any generation, and lethals detected in the

F_2 or F_3 may be retested for an unlimited number of generations. In our test procedure all lethals detected in either the F_2 or the F_3 are retested in triplicate for two generations. The criterion for lethal is that no male with a treated chromosome will survive in any test culture. This stringent requirement for verification of lethals is essential in determining the mutational spectrum where the total mutation frequency is low; otherwise, detrimental mutations would be confused with F_1 females mosaic for a lethal in their gonad.

In our previous work with ^{32}P, ^{33}P, and ^{14}C there was no significant effect of the beta radiation with the dilutions used of labeled spermatozoa stored in unlabeled females. However, with tritium we expect a significant level of ionizing radiation from decay within the labeled sperm cell. Therefore, it is essential that we establish the mutational spectrum for ionizing radiation with our system. Accordingly an experiment was begun in the last reporting period and the preliminary results reported in our last progress report on an experiment with X-rays. This experiment was completed early in this reporting period and will now be reported in its entirety. In our transmutation experiments we compare the mutation rate of females newly inseminated with the same females stored for three weeks. Therefore the effect of storage on the mutation frequency needs to be considered. The change in the spontaneous rate during storage is minimal (Lee, Sega and Benson, 1972, See Table 1) and in most of our experiments would not be statistically significant.

Another factor to be considered in comparing stored with non-stored sperm where storage occurs in inseminated females is the increase in age of the female. A maternal effect on mutation frequency has been known since the work of Hildreth and Carson (1957). The effect of age on losses of sex chromatin has been shown by Wurgler and Maier (1972). While on sabbatical

during the fall of 1973 I had the opportunity of visiting in Dr. Wurgler's laboratory in Switzerland. Dr. Wurgler (personal communication) showed me extensive data showing an age effect on sex-linked recessive lethals at doses above 4000R. The length of storage was only six days but at room temperature whereas females in our transmutation experiments are stored for 21 days but at 10°C. The maternal effect varies for different stocks (Wurgler and Maier, 1972); therefore, I felt that it was necessary to do a control for our transmutation experiment using X-rays at a dose level (2000R) that would give a mutation frequency of about 5% to correspond to the frequency observed in our ^{32}P and ^{33}P experiments. We have observed frequencies due to beta radiation of less than 1% in our ^{14}C and ^{3}H experiments. A second dose of 4000R was selected to see if we could observe the effect of aging in our stock and storage conditions.

To test the effect of aging on the mutation frequency induced by ionizing radiation on our stocks under the storage conditions of our transmutation experiments the following experiment was conducted. One group of virgin females produced automatically by our mating scheme was stored as virgins for three weeks at 10°C. Following storage these aged virgins along with an equal size population of 3-5 day old (young) virgins from the same genetic cross were mated with males treated with X-rays. The treated males were the same stock as we used in our transmutation experiments and had been treated with 2000R and 4000R. The 2000R group were divided into two equal size populations. The first group was treated during the first half of the 4000R treatment, and the second group was treated during the second half of the 4000 treatment. After repeating the experiment twice, we found no indication of differences between the first and second repeats of the experiment nor between the first and second group of the 2000R treated males. Therefore, the results of the first

and second experiments along with the first and second group of treated males are pooled into Table 1. The treated males were divided at random into two equal groups and half were individually mated to aged females and half to young 3-5 day old females. A control was done by mating nonirradiated males to both aged and young females. The comparisons among the mutational response in our three different genetic tests (loss of X or Y chromatin in the F_1, sex-linked recessive lethals observed in the F_2, and sex-linked recessive lethals observed in the F_3) are shown in Table 1.

It can be seen (Table 1) that there are no significant differences for any of the genetic tests between the young and aged females mated to males treated either with 2000R or 4000R. Therefore, at the doses of ionizing radiation encountered in our transmutation experiments the increase in age of the females under our conditions of storage will not affect the results of storing radiolabeled spermatozoa in females. The control data showed a significant increase ($P = 0.01$) of loss of the X or Y paternal chromosome due to aging. The increase was entirely in the class of complete loss of both dominant markers; therefore, the chromosome loss could be due to either loss of the X or Y-chromosome from the male as a result of aging of the females' oocytes. Most of the increase was found in the first brood after removal from the $10^{\circ}C$ incubator, so it is apparently the aging of a late stage oocyte that caused the effect. Since the increase due to aging could be due to either loss of the X or Y-chromosome the data were analyzed comparing the mutant, yellow round eyed males, to both expected males and females of the appropriate class. There was no difference in using the females as the denominator instead of the males as shown in Table 1. This aging effect cannot be explained as the result of increased probability of nondisjunction because meiosis of the paternal genome occurred in the nonaged males. However,

this observed effect of aging could be due to the aged oocyte being less likely to successfully develop the proper mitotic apparatus during first cleavage or perhaps to the aged oocyte being less likely to replace protamines with histones in the paternal genome.

An additional finding of significance to our work is that there was only a minimal increase in the F_3 lethal rate (lethals detected in F_3 due to mosaic gonads in the F_1) induced with 2000R or 4000R of radiation. In fact, there is no dosage response of sex-linked recessive lethals observed in the F_3 between 2000 and 4000R. This lack of a dosage response of sex-linked recessive lethals observed in the F_3 may suggest a conversion of mosaics to completes at higher doses, and therefore it is of importance to us to know if at lower levels of radiation we would have perhaps observed a higher frequency of mosaics. Fortunately, it will not be necessary to do this larger scale experiment at lower dose levels because Matsudaira and Yamasaki (1975) have recently published results on frequencies of F_3 lethals with 500 to 2000R. Their results at the 2000R level are consistent with ours, and they show no dosage response over the range from 500-2000R. Therefore it appears that a small yet significant increase in sex-linked recessive lethals observed at the F_3 is induced by 500R (Matsudaira and Yamasaki, 1975) with no further significant increase at higher levels of radiation. We now have sufficient data to provide a background for determining the spectrum of mutations resulting from beta decay that may be encountered in our transmutation experiments.

In last year's progress report we reported the results of our test to develop a labeling procedure that would give adequate labeling to the sperm cells with minimal beta irradiation. This procedure is described in our 1974 progress report. In a series of preliminary experiments we included a small scale genetic test of labeling with (^{3}H methyl) thymidine and (5-^{3}H) deoxycytidine purchased from Amersham/Searle. Only data from the F_1 were

available at the time of our 1974 progress report. The final results of this small scale preliminary test are listed as experiment 1 and 2 in Table 2. These preliminary tests were never intended to be of sufficient size to give definitive results; however, they did show an increased mutation rate in the F_2 lethal test of (5-^{3}H) deoxycytidine over that observed for thymidine labeling. It is transmutation of ^{3}H at the 5 position in cytosine that should give the maximal genetic effect; therefore, I decided to do the first large scale genetic test on 5 labeled deoxycytidine. Accordingly deoxycytidine (5-^{3}H) was purchased from New England Nuclear and fed to the flies in accordance with our labeling procedure. A sufficient amount of material was labeled to test in excess of 4000 X chromosomes labeled with the 5-^{3}H deoxycytidine. According to information given me by the manufacturer the lot of 5 labeled deoxycytidine had been tested not only for radiochemical purity but also for labeling in the 5 position by bromination which should remove the ^{3}H in the 5 position. We always verified our radiolabeled material with our own test on a sample of the material that we used to label the flies. Therefore, the information from the verification test does not become available until after the flies are already labeled. This sequence was unfortunate, because after our testing we found the material supplied by the manufacturer was not labeled in the 5 position. Therefore, in the future we will schedule our verification test prior to the labeling of the flies.

For verification of the (5-^{3}H) deoxycytidine labeled material we do the following test. First, a sample is co-chromotographed with the deoxycytidine using a reverse Isotopic dilution procedure in which the labeled material is diluted with non-labeled material of known chemical purity. The specific activity is then determined before and after being chromatographed thereby giving us a quantitative measure as to the amount of the radiolabel that

co-chromatographed with deoxycytidine. This procedure has been shown to be more sensitive to small amounts of impurities involving the label than simply chromatographing and showing concurrence of the radiolabel with the chemical species by radioautographs. The ^{3}H-deoxycytidine was found to be in excess of 95% radiochemically pure. The second test we used is repeated lyophilization to detect loss of the label as measured by decrease in specific activity following lyophilization. This procedure would measure the loss of tritium from labile sites on the molecule. No significant loss of ^{3}H was found by repeated lyophilization. Therefore, we conclude that the material was radiochemically pure and labeled in a stable position. Hydrolysis of the deoxycytidine followed by chromatography in a 2-dimensional system that we have developed for separating not only the 4 bases but some modified bases used in our study of chemical mutagenesis was then performed. It was determined that essentially all of the label was on the cytosine moiety of the molecule. The information that the tritium was in a stable position and on the cytosine moiety of the molecule would limit the labeling to either the 5 or 6 position in the radiolabeled deoxycytidine. To determine if the tritium is in the 5 position we then did a bromination experiment in which first the specific activity of the labeled cytosine resulting from our hydrolysis experiment was determined. This cytosine had been purified by paper chromatography. To a neutral solution of the labeled ^{3}H-cytosine a stoichiometric amount of 1% bromine water solution was added. The brominated cytosine solution is chromatographed on paper (using butanol/water 86:14). The 5-bromo-cytosine spot is eluted from the paper with water, concentrated by lyophilization, and the specific activity determined by spectrophotometric and scintillation counting. It was found that 22% of the tritium remained in the 5-bromo-cytosine. Therefore, at least 22% of the tritium must have been in the 6 position since the substitution of bromine at the 5

position would have removed the tritium at the 5 position. We also think it likely in this procedure that we have lost some radioactivity at the 6 position; therefore, the 22% is probably a minimal estimate of labeling at the 6 position. In summary, the 5 labeled deoxycytidine purchased from New England Nuclear was not specifically labeled at the 5 position as stated by the manufacturer. Further inquiries with the manufacturer have revealed that this particular lot of deoxycytidine (5-^{3}H) had not been submitted for a test of specificity of labeling but instead was simply sold as specifically labeled based on its methods of manufacturing. Because the genetic tests were well underway by the time we realized that the tritium labeled deoxycytidine supplied to us by the manufacturer was not labeled as specified, we continued the genetic test since we were interested in the result from non-specifically labeled deoxycytidine as well. In the storage phase of this experiment (experiment 3; Table 2) an infection of mold occurred during storage and killed most of our stored females. Consequently, we did not get the large number of progeny following storage that had been planned for testing. Experiment 3 (Table 2) was very disappointing; nevertheless, it serves as a background for nonspecifically tritium labeled deoxycytidine.

Another experiment was initiated with deoxycytidine (5-^{3}H) purchased from Amersham/Searle. This material subjected to the same chemical test as the material from New England Nuclear was also found to be radiochemically pure with the label in the stable position on the cytosine moiety, but unlike the New England Nuclear product our bromination experiment with the Amersham/Searle labeled material yielded 5-bromo-cytosine without any detectable radioactivity. Therefore, all the ^{3}H was in the 5 position of this material within the limits of the sensitivity of our test, and we assumed that the Amersham/Searle product was specifically labeled at the 5 position. This experiment was conducted during the fall and winter months of this past year and the results are shown

as experiment 4; Table 2. Our procedure for storage worked very well with experiment 4, and we are able to present the results of a balanced design where we have nearly equal numbers of chromosomes tested in stored and unstored samples. However, we were limited by funds and could not add additional personnel necessary to do the large number of sex-linked recessive lethal tests desired. Therefore, while we were able to score over 5000 chromosomes in the nonstored F_1 we were limited to about 2000 chromosomes tested in the F_2 and F_3.

The amount of tritiated deoxycytidine incorporated into the DNA of the sperm cell was determined for each experiment. The procedure that we used for this determination has been described in our previous publications (Lee, Sega and Alford, 1967 and Lee, Sega and Benson, 1972). Briefly, this procedure consists of preparing for cytological examination the receptacle of inseminated females, counting the number of sperm cells within the receptacle (one of the sperm storage organs of the female), and counting microscopically the number of sperm cells within the receptacle. Receptacles containing a known number of sperm cells (2000-5000 cells) are then transferred to a glass millipore filter, washed with cold trichloroacetic acid and digested using the method of Mahin and Lofberg (1966). The digest from a known number of sperm cells is then counted in the liquid scintillation spectrophotometer and from the results of appropriate internal standards the disintegrations/minute/sperm cell (DPM/cell) are then determined. Variation among separate determinations is used as a measure of error, for this will include not only the statistical errors in both sperm cell counting and counting the radionuclides in the liquid scintillation spectrophotometer but also any errors in technique of transferring of material. The average DPM/cell is listed in Table 2 for each experiment followed by the error term of 1 standard deviation of the mean based on separate determinations of random samples from the inseminated females from the particular experiment.

The number of repeated determinations for each experiment is listed in parenthesis below the DPM/cell. Experiment 3; Table 2, has 16 repeated determinations on the level of incorporation because this experiment was done during the summer when we had extra help available. There is no statistically significant difference between the level of tritium incorporation among the four experiments. Therefore, it is of particular significance that experiment 4 has a significantly higher mutation rate in both the F_1 (loss of X or Y chromatin) and the F_2 than either of the experiments 2 or 3. Furthermore, the increase in the mutation rate in the F_2 during the 25 day storage period is on the border of significance using the tables of Kastenbaum and Bowman (*Mutation Research*, 1970) which are appropriate because we assume a Poisson distribution for mutation frequencies where the frequency of mutants is low in relation to the number of chromosomes tested. We find from their table that 51 mutants would have been required in the stored population to give a significant increase over the nonstored whereas actually 50 were observed. Therefore, it is likely that there is a real increase in the mutation rate of F_2 sex-linked recessive lethals during storage in experiment 4. These differences between experiment 4 and experiments 2 and 3 can best be explained on the basis of experiment 4 having the tritium incorporated at the 5 position of deoxycytidine as proven by its removal upon bromination at the 5 position whereas the generally labeled deoxycytidine of experiment 3 showed no effect over that observed by the preliminary experiment with tritium labeled thymidine (Experiment 2). The conclusion that the 5-labeled cytosine gave a significant increase in the mutation frequencies is tentative and requires further verification by repeating the experiment with 5-labeled deoxycytidine and by doing a comparably sized experiment with tritium labeled thymidine in which we are certain that none of the tritium will be in the 5 position of the

cytosine moiety. If our experiments this next year verify these findings, they will have the significance of verifying with eukaryotes the transmutation effect of tritium in the 5 position of deoxycytidine, and will permit us to make the calculations of the importance of this transmutation and the following deamination of cytosine that should result in a C→T transition. Preliminary calculations on the basis of the present data would suggest that there are approximately 54 disintegrations in euchromatin per X-chromosome using the correction factor suggested by H.J. Muller which was discussed in our previous publication (Lee, *et al.*, 1966). If the increase in mutation frequency during storage in experiment 4 is due to transmutation of tritium at the 5 position, then approximately 1 transmutation in 6600 in the X chromosome euchromatin will produce a recessive lethal mutation in spermatozoa. Apparently some immature stages are more sensitive to this transmutation effect, for the largest difference was between the F_2 test of nonstored females in experiments 2 or 3 in contrast with those of experiments 1 and 4 (Table 2). These comparisons are to be taken as tentative until we repeat with additional experiments involving 5-labeled deoxycytidine and the comparable controls of tritiated thymidine.

Table 1

Treatment	Loss of X or Y		F_2 lethals		F_3 lethals	
	Young ♀	Aged ♀	Young ♀	Aged ♀	Young ♀	Aged ♀
2,000 R	$\frac{52^{\alpha}}{2098}$	$\frac{48}{2118}$	$\frac{90^{\beta}}{1693}$	$\frac{83}{1509}$	$\frac{8}{1507}$	$\frac{13}{1119}$
	2.5%	2.3%	5.3%	5.5%	0.5%	1.2%
4,000	$\frac{31}{741}$	$\frac{47}{1003}$	$\frac{59}{451}$	$\frac{73}{706}$	$\frac{3}{370}$	$\frac{5}{557}$
	4.2%	4.7%	13.1%	10.3%	0.8%	0.9%
Control	$\frac{4}{2240}$	$\frac{24}{3664}$	$\frac{1}{881}$	$\frac{3}{1343}$	$\frac{3}{859}$	$\frac{4}{1285}$
	0.2%	0.6%	0.1%	0.2%	0.3%	0.3%
	P=.01$^{\lambda}$					

$^{\alpha}$Mutant males divided by males scored.

$^{\beta}$X-linked recessive lethal mutations divided by chromosomes tested.

$^{\lambda}$According to the tables of Kastenbaum and Bowman, 1970.

Table 2

Experiment	Tritium labeled precursor of DNA	DPM/Sperm cell (x 10^{-4})	Loss of X or Y		Lethals in F_2		Lethals in F_3	
			Nonstored	Stored	Nonstored	Stored	Nonstored	Stored
1	Deoxycytidine (5-^{3}H) Amersham/Searle (TRK 211) 21.2 Ci/mM Batch 15	150 ± 9 (4)	7^{α} 1137 0.6%	----	13^{β} 1083 1.2%	----	1 991 0.1%	----
2	Thymidine (methyl-^{3}H) Amersham/Searle (TRK 120) 19 Ci/mM Batch 90	111 ± 8 (5)	1 658 0.2%	----	2 541 0.4%	----	2 501 0.4%	----
3	Deoxycytidine (G-^{3}H)** New England Nuclear 31.8 Ci/mM Lot 824-052	121 ± 6 (18)	57 14281 0.4%	4 717 0.6%	24 4824 0.5%	5 543 0.9%	13 4067 0.3%	1 242 0.4%
4	Deoxycytidine (5-^{3}H) Amersham/Searle (TRK 211)	113 ± 7 (6)	58 5164 1.1%	31 2745 1.1%	28 1664 1.7%	50 2020 2.5%	12 1523 0.8%	10 1849 0.5%

$^{\alpha}$Mutant males divided by males scored.

$^{\beta}$X-linked recessive lethal mutations divided by chromosomes tested.

**This deoxycytodine was listed by the manufacturer as 5-^{3}H; however, our test showed that this was generally labeled.

REFERENCES

Hildreth, P.E. and G.L. Carson, 1957. Influence of the type of inseminated female on the lethal frequency in the X chromosome from the male. *P.N.A.S.* 43:173-183.

Kastenbaum, M.A. and K.O. Bowman, 1970. Tables for determining the statistical significance of mutation frequencies. *Mutation Res.* 9:527-549.

Lee, W.R., C.K. Oden, C.A. Bart, C.W. Debney and R.F. Martin, 1966. Stability of Drosophila chromosomes to radioactive decay of incorporated phosphorus-32. *Genetics* 53:807-822.

Lee, W.R., G.A. Sega and C.F. Alford, 1967. Mutations produced by transmutation of phosphorus-32 to sulfur-32 within Drosophila DNA. *P.N.A.S.* 58(4):1472-1479.

Lee, W.R., G.A. Sega and E.S. Benson, 1972. Transmutation of carbon-14 within DNA of *Drosophila melanogaster* spermatozoa. *Mut. Res.* 16:195-201.

Matsudaira, Y. and T. Yamasaki, 1975. On the relationship between the frequency of two types of lethal-mutation and X-ray doses in Drosophila. *Mutation Res.* 27:341-346.

Mahin, D.T. and R.T. Lofberg, 1966. A simplified method of sample preparation for determination of tritium, Carbon-14, or Sulfur-35 in blood or tissue by liquid scintillation counting. *Anal. Biochem.* 16:500-509.

Wurgler, F.E. and P. Maier, 1972. Genetic control of mutation induction in *Drosophila melanogaster*. I. Sex-chromosome loss in X-rayed mature sperm. *Mutation Res.* 15:41-53.