STABILITY OF DROSOPHILA CHROMOSOMES TO
RADIOACTIVE DECAY OF INCORPORATED PHOSPHORUS-32

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PHOSPHORUS-32 incorporated into DNA in Drosophila melanogaster germ cells offers a method of analyzing the genetic effects—if any—of a known number of transmutations and associated energies of recoil in the DNA, provided the genetic effect of the “long range” and therefore nonspecific beta radiation can be eliminated or accurately determined. In multicellular organisms the separation of the mutagenic effect of beta radiation from transmutation and associated energy of recoil is difficult because: (1) beta radiation from P\textsuperscript{32} in surrounding somatic tissue is intense; and (2) in order to incorporate P\textsuperscript{32} into DNA, all stages of meiosis—with their great variation in radiosensitivity—are subjected to beta radiation. Because of variation in sensitivity of different stages of meiosis, Bate-Man (1955) concluded, “The difficulties in the way of a valid comparison of the qualitative effects of P\textsuperscript{32} and X rays are almost insuperable.” However, OfteDal (1959) calculated that one in 100 transmutations of P\textsuperscript{32} to S\textsuperscript{32} resulted in a sex-linked recessive lethal.

In order to avoid difficulties that previous authors have experienced in working with higher organisms, we based our research upon mutations induced in mature sperm stored in unlabeled Drosophila melanogaster females. Mature sperm containing DNA labeled with P\textsuperscript{32} was obtained from 2-day-old males that had been fed P\textsuperscript{32} as larvae. Females recently mated to P\textsuperscript{32} labeled males were divided into two groups. One group was allowed to produce progeny immediately. The other was first stored on sugar agar media at 18°C for three weeks to inhibit oviposition and then permitted to oviposit freely on cornmeal media. The mutation rate of the first group’s progeny was subtracted from that of the second to measure excess genetic damage, if any, caused by P\textsuperscript{32} decay in sperm during the storage period.

The purpose of the experiment reported here is to distinguish between mutations induced by beta radiation and those induced by the combined actions of transmutation and energy of recoil in mature sperm and to measure the mutagenic effectiveness, if any, of transmutation and recoil of P\textsuperscript{32}. Tests were conducted for both chromosome loss and sex-linked recessive lethals.

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**MATERIALS AND METHODS**

**Stocks and mating scheme:** The larvae fed P$^{32}$ were of the genotype B$^8$.Y/In(1)dl-49 v ptg oc Fl In(1)BM$. Upon eclosion, males were mated to automatic virgins (described by Lee 1963, 1965a) of the genotype In(1)dl-49 v Fl g In(1)BM$^1$ Maxy (Maxy chromosome described by Muller and Schalet 1957). This cross normally produced only one type of male which had a narrow Bar-shaped, orange eye (genotype: B$^8$.Y/In(1)dl-49 v Fl g In(1)BM$^1$) and only one type of female which was phenotypically vermilion-eyed with the mild Bar of Muller. Loss of either the X or Y chromosome or the tip of the long arm of the Y (containing B$^8$) will produce an exceptional male easily detected by the nearly normal shaped (BM$^1$), orange eye. Small interstitial deletions in the treated X would often “uncover” one of the 14 recessive visible mutants scattered along the Maxy chromosome for which the F$_1$ females were heterozygous. These mutations could be detected either as complete or mosaic mutations.

F$_1$ males were scored for loss of the marker B$^8$, and F$_1$ females were scored for visible mutations and placed in bottles to mate with males (genotype: sc$^8$.Y.B$^8$/Y$^{os}$ sc$^8$.sn$^{5.1}$ w). F$_1$ females were tested for a sex-linked recessive lethal by brooding them one per vial and later scoring each female’s progeny for absence of F$_2$ vermilion-eyed males. Lethal cultures were restested by selecting the phenotypically normal (except for BM$^1$) F$_2$ females and crossing back to (sc$^8$.Y.B$^8$/Y$^{os}$ sc$^8$.sn$^{5.1}$ w) males. In a few cases in which the F$_1$ female mated only to her brother, it was impossible to retest the culture. The culture was taken to be a lethal if there were 12 or more (vermilion garnet) females. The number 12 was determined by studying the sex ratio in control cultures and accepting the .001 probability level as significant. “Borderline” cases were too few to affect the results.

**Treatment:** In a preliminary experiment adult males were injected with P$^{32}$, and, in a parallel test, a similar group was injected with tritiated thymidine. Radioautographs prepared of sperm transferred to females from the males injected with tritiated thymidine failed to show any period of uniformly labeled sperm, even though the males had been placed with five virgin females each day for 14 days. Therefore, a method of feeding larvae that would minimize the dose of radiation from the media and yet uniformly label the sperm cells ejaculated during the first three days of adult life was developed (Lee 1965b). This method consisted of floating 42 to 48-hour old, late second instar larvae out of media in a concentrated sucrose solution and placing them on moistened filter paper. Molting to the third instar served as the criterion for a uniform stage of development. As the larvae molted to the third instar, they were picked out with a brush and transferred for 24 hours to P$^{32}$ labeled yeast, which had been grown in 63 mm (inside diameter) Stender dishes containing cornmeal media and 50 μC P$^{32}$. After feeding, the larvae were removed from the radioactive media and washed free of P$^{32}$ surface contamination. This feeding period of only 24 hours should cover the time when most of the cells in the posterior part of the testis change from spermatogonia to first spermatocytes. While there are a few first spermatocytes in a 48-hour-old larva, the bulk of the testis is composed of first spermatocytes in a 72-hour-old larva (Kerksis 1933); therefore, most of the sperm cells in the 1 to 3 day ejaculates should have undergone the last DNA synthesis during this period. That synthesis of DNA occurs during the first half of the third instar was confirmed by feeding tritiated thymidine during this period and preparing radioautographs of sperm taken from females that had mated to treated males from the same breeding scheme as in the P$^{32}$ experiment.

Following treatment with P$^{32}$ the larvae were placed on cornmeal and kept at 27°C to complete their development in a minimum length of time. Within 24 hours following emergence, males were separated from females and stored 24 hr on cornmeal media. The males were then randomly divided into two groups: (1) One fifth were placed one per vial on cornmeal media and given five virgin females. After 24 hr both males and females were discarded. The eggs laid during the 24-hr period were fertilized an average of eight days after the paternal larvae had fed on P$^{32}$. (2) Four fifths of the males were placed one per vial in vials containing sugar agar and five virgin females that had been on sugar agar since emergence. Under these conditions copulation occurs but egg laying does not. After 24 hr males were removed and females stored on fresh sugar agar media at 18°C for three weeks. Females were then transferred to fresh cornmeal agar
(to induce egg laying) every three days until three successive broods had been produced. Most of the progeny were produced in the first two broods; consequently, for the second group, 32 days was taken as the mean length of time for labeling until fertilization of the egg. Because of the nature of the $^{32}P$ decay curve, a variation of several days this long after labeling is inconsequential. Therefore, we have two random samples from the same population, but one produced its brood an average of 24 days following the first. The difference between mutation rates induced in these two populations is the result of $^{32}P$ disintegrations in mature spermatozoa while stored in nonlabeled females. The F$_1$ males of each population were scored for loss of either the X or Y chromosome, whereas the F$_1$ females were scored for visible mutations and gynandromorphs and tested for sex-linked recessive lethals according to the previously described mating scheme.

Dosimetry: Two methods were used. In the first, seminal receptacles and spermathecae were dissected from females inseminated with $^{32}P$ labeled sperm, and radioautographs were made using techniques described by Kaplan et al. (1964) and Taylor (1956).

The second method utilized a Tri-Carb scintillation counting system to determine the number of $^{32}P$ atoms incorporated per cell. To maximize the amount of semen in each female, treated males were allowed to mate with only one female. The seminal receptacle from the inseminated female was dissected out, fixed in 5% formalin for 10 minutes, stained using the method suggested by Oster and Balaban (1963), and squashed with a siliconized cover slip. The stained squash was examined microscopically for spermatozoa, which, if present, were counted twice. Samples were prepared for counting radioactivity by washing material from the slide onto a millipore filter with 5% trichloroacetic acid at 4°C. Both slide and coverslip were then scanned under the microscope to detect any remaining fragments of tissue. To prepare for use in a scintillation counter, the millipore filter was placed tissue side up in a polyethylene vial, bleached by moistening with 0.5% solution of sodium hypochlorite to prevent the color of the stain from interfering with scintillation counting, dried, and submerged in a 10 ml solution of fluor (40 mg 2,5-diphenyloxazole and 1 mg 1,4-bis-2-(5-phenyloxazolyl)-benzene dissolved in toluene. Each sample, representing sperm transferred from a single male to a female, was then counted for 500 minutes in a Tri-Carb scintillation counting system (Packard 314E). Counting was done only over a relatively narrow range of the peak energy of $^{32}P$ so as to maximize the ratio of $^{32}P$ counts squared to background.

The efficiency of the counting system was determined by preparing standards in the same manner as the samples, except the millipore filter was moistened with known amounts of $^{32}P$ from a Nuclear-Chicago $^{32}P$ standard solution. The number of disintegrations per cell during the period of storage was calculated from the number of counts per cell in 500 minutes, efficiency of the counting, and the known decay constants for $^{32}P$.

RESULTS

Genetic tests: The results of experiments in feeding $^{32}P$ to male larvae as measured by loss of the $B^Y$ (or X) chromosome and sex-linked recessive lethals are shown in Table 1. Test 1 (Table 1) is a test on the progeny produced immediately following mating of males from group 1, that, as larvae, had been fed $^{32}P$ an average of eight days prior to mating; test 2 is a test on progeny from males of group 2, that, initially were from the same populations as group 1 males, but their sperm were stored an average of 24 days in females before fertilization occurred. The object of the experiment is to determine the difference between test 1 and test 2, this difference being a measure of the mutagenic effect of radioactive decay in $^{32}P$ labeled mature spermatozoa stored in females.

Before a comparison between tests 1 and 2 can be made, a number of factors that might possibly bias the results should be checked. Within any one of these experiments except experiment I, the paternal larvae were fed $^{32}P$ simultaneously.
TABLE 1

Results of genetic tests

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Loss of Y (or X) (percent)</th>
<th>X chromosome lethals (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Test 1</td>
<td>Test 2</td>
</tr>
<tr>
<td>Control</td>
<td>0.074 (22738)†</td>
<td>0.153 (9155)</td>
</tr>
<tr>
<td>I</td>
<td>0.8 (1146)</td>
<td>0.6 (521)</td>
</tr>
<tr>
<td>II</td>
<td>1.4 (4236)</td>
<td>1.2 (1342)</td>
</tr>
<tr>
<td>III</td>
<td>1.1 (2185)</td>
<td>1.4 (2011)</td>
</tr>
<tr>
<td>IV</td>
<td>1.4 (5214)</td>
<td>1.2 (5657)</td>
</tr>
<tr>
<td>Weighted mean*</td>
<td>1.3 ± .2 (12781)†</td>
<td>1.2 ± .2 (9531)</td>
</tr>
</tbody>
</table>

Test 1 is from eggs fertilized within 24 hours after mating. Test 2 is from eggs fertilized an average of 24 days after mating.

* 95% confidence interval.
† Number of tests in parentheses.

Experiment I is a sum of several small experiments in which the males were fed P^32 over a period of one month, but in all experiments the males from each bottle were divided proportionally between the first and second groups. Although the proportion of P^32 labeled males for each group remained constant throughout the experiment, the proportion of progeny in the two groups varied widely, indicating the possibility of nonadditivity among the experiments. Also, the possibility of an unequal distribution of dose between the two tests within each experiment must be checked, for, in any experiment where the mutagen is fed, there may be considerable dosage variation among individuals. To determine the variation in dose among groups, radioactivity of each male in experiments II-IV was determined with an end-window GM tube on the day following mating. As activity decreases rapidly in labeled males (BATEMAN 1955), this measurement is relative only among males measured on the same day.

All measurements were made in the day following mating; therefore, the measurements should suffice to check on any possible bias among groups. Results of this test (Table 2) showed no significant difference among groups, although

TABLE 2

Radioactivity of males used in genetic test

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Counts/Minute/Male Test 1</th>
<th>Test 2</th>
<th>Counts/Minute/Male weighted with progeny tested Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>92</td>
<td>94</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>III</td>
<td>73</td>
<td>80</td>
<td>69</td>
<td>68</td>
</tr>
<tr>
<td>IV</td>
<td>89</td>
<td>81</td>
<td>86</td>
<td>80</td>
</tr>
<tr>
<td>Weighted average</td>
<td>. .</td>
<td>. .</td>
<td>79.6</td>
<td>75.5</td>
</tr>
</tbody>
</table>
Group 2 males in experiment IV were 10% lower than Group 1 in radioactivity. Experiment IV provided 60% of the total chromosomes tested in test 2, whereas only 40% were from test 1; therefore, the overall effect of this aberrant sample was that males in Group 2 received 5% less P³²—weight for number of progeny produced per male—than those in Group 1. The effect of this on the genetic test will be considered later.

Another possible bias in comparing the first test with the second is that males containing the most P³² might produce fewer sperm than those with a small amount of P³². Furthermore, females inseminated with a small amount of semen may contribute to the first test but may be less likely than well inseminated females to produce progeny in the second test that followed three weeks of storage. These possibilities, by reducing the mutation rate in the second test more than in the first test, would create a serious bias. To test for this possible bias, a separate record was kept of the progeny of each P³² labeled male and the radioactivity count for each male was weighted by multiplying it by the number of progeny produced (Table 2). The effect of weighting the radioactivity of the male with the number of progeny he produced was not significant and in each case reduced the difference between the two groups. Therefore, variation in amount of incorporated P³² did not affect the number of progeny in a way that could bias the results.

Another factor to be considered in comparing the first and second groups is the spontaneous mutation rate in sperm during storage (Müller 1946). A consensus based on several large experiments by H. J. Müller and associates (personal communication) has shown an accumulation of .06% spontaneous sex-linked recessive lethals per week in sperm stored in inseminated females. Therefore, an increase in the spontaneous rate of .18% would be expected during the 3-week storage period used in this experiment. From Müller’s results, the increase in spontaneous mutation rate expected during storage is less than the sampling error in this experiment and of about the same magnitude, but in an opposite direction from the effect due to a 5% decrease in radioactivity of Group 2 males. Therefore, no correction has been made in these data for the above effects.

The lack of any unusual bias in any one experiment is further shown by the homogeneity of data; a homogeneity chi-square test among the four experiments (Table 3) showed no significant heterogeneity. It is, therefore, possible to sum the results of the four experiments and to compare the average of the first and second tests for all four experiments.

<table>
<thead>
<tr>
<th>TABLE 3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Chi-square test of homogeneity among experiments</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Loss of $B^{s}Y$ (or X)</th>
<th>Sex-linked lethals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test 1</strong></td>
<td><strong>Test 2</strong></td>
</tr>
<tr>
<td>$X^2$*</td>
<td>3.2</td>
</tr>
<tr>
<td>P</td>
<td>.4</td>
</tr>
</tbody>
</table>

* Three degrees of freedom.
A check on the sensitivity of loss of the $B^*Y$ (or X) chromosome in this stock to X rays was made with the following results: 1,000r, gave 28/2589 or 1.1% ± 0.3% corrected for the spontaneous rate.

There was no indication of change in either direction during 24 days storage (Table 1) for either loss of the $B^*Y$ (or X) chromosome or sex-linked recessive lethals. An increase of 0.3% in the mutation rate of either class should have been detected. Mutation rates indicated by loss of the $B^*Y$ (or X) and sex-linked lethals induced by $P^{32}$ at the time of mating (test 1 corrected for spontaneous rate) were 1.22% and 1.53% respectively. During the 24-day storage period, there was no increase of mutation rate in either test even though there occurred 1.44 times as many disintegrations as during the period from $P^{32}$ ingestion to mating. If all stages of germ cell development are equally sensitive to the mutagenic action of transmutation and recoil and if 14% of the mutation rate induced in the first test had been due to transmutation and recoil, there should have been a significant increase of 0.3% during the 24 days when the sperm was stored in females. Since there was no increase indicated, at least 86% of the mutations induced in the male by the $P^{32}$ must have been due to beta radiation.

Only one male mosaic for the Bar-S eye was found in test 2 and none were found in test 1. The results of scoring $F_1$ females for visibles at the "Maxy" loci are as follows: In test 1 there were eight complete visible mutations (complete for the phenotype of the mutant and transmitting the mutant to all progeny that receive the treated chromosome) and three incomplete-mosaic mutants among 12,483 $F_1$ females. In test 2, there were two complete mutations and one mosaic among 8,931 $F_1$ females. These numbers are too low to make a meaningful comparison of frequencies, but the significance of the scarcity of these mutations will be discussed later. No gynandromorphs have been observed.

Dosimetry: The initial efforts with dosimetry in these experiments utilized radioautographs. To remove $P^{32}$ or $H^3$ compounds of low molecular weight, slides to be radioautographed were immersed in 5% trichloroacetic acid for 5 min at 4°C (Davidson 1950). Radioautographs of seminal receptacles and spermathecae from females inseminated by males labeled as described in Methods, and seminal vesicles of labeled males dissected upon eclosion showed that all males labeled with $P^{32}$ or $H^3$ by the feeding method (previously described) produced semen containing the radionuclide incorporated into molecules that are insoluble in cold trichloroacetic acid. Labeling of sperm with $P^{32}$ appeared uniform as judged visually by comparing darkening of the film with the number of sperm cells. Labeling with $H^3$ thymidine showed conclusively that at least one period of DNA synthesis occurred during feeding of the first half of the third instar. No effort was made to place the radioautographic results on a quantitative basis because we felt the scintillation counter was better for quantitative results.

An effort was made to determine the number of disintegrations per cell during the period when sperm were stored in females and to calculate the minimum limit for the number of $P^{32}$ decay events required to induce either chromosome breakage or sex-linked recessive lethals by transmutation and associated energy of recoil. A Tri-Carb scintillation detector (described in Methods) was used to determine the number of $P^{32}$ disintegrations per sperm cell per 100 minutes (corrected for the efficiency of the Tri-Carb—46%—and for radioactive decay). This determination was made individually on the sperm from 18 males that had a whole-body radioactivity—as measured by the end-window GM tube—that was similar to males used for the genetic test. Individual tests were made on males in order to determine if all males were labeled.

The small number of sperm cells (about 200) transferred to a female by a male during its
The second day following emergence contains only a small amount of P$^{32}$. Extreme precautions must be taken in counting samples because the counts from P$^{32}$ are only a fraction of the background.

The Tri-Carb was programmed to count each sample for 100 minutes and then to count the background and a Cl$^{36}$ standard. This counting program was repeated five times giving a total of 500 minutes count on each sample with a background and Cl$^{36}$ count between each 100-minute count on the sample. There was no significant change noticed in the efficiency of the Tri-Carb as measured by the Cl$^{36}$ standard or in the background during the counting of these samples. The total counts during 500 minutes for each male exceeded the background count by at least two standard deviations. Therefore, each male incorporated a significant amount of P$^{32}$ into its sperm cells.

The uniformity of this labeling was indicated by visual inspection of radioautographs and the homogeneity test of the genetic results. The actual counts on the sperm of each male varied, as expected, by a factor of five because the small amount of P$^{32}$ in each sample gave a Poisson distribution to the number of counts observed on each sample. A square-root transformation was made to correct for the Poisson distribution and the mean and SD were calculated. The mean was significant at the 0.5 probability level. The mean transformed back to standard units and corrected for the counting efficiency of the Tri-Carb was 2.50 disintegrations per cell per 100 minutes at the time of incorporation of P$^{32}$ into the cell. Since we have evidence from the homogeneity of genetic data and radioautographs that labeling was more uniform than indicated by the count of each male's sperm sample, only one standard deviation was subtracted from the mean to give the minimum estimate of 0.804 disintegrations per cell per 100 minutes at the time of labeling.

The number of P$^{32}$ atoms incorporated per cell was calculated with the formula $N = -\Delta N/\Delta t \lambda$, where $N$ equals the number of P$^{32}$ atoms per sperm cell, $\Delta N$ equals disintegrations per 100 min per cell corrected for efficiency of the Tri-Carb, $\Delta t$ equals time counted in minutes, and $\lambda$ equals the decay constant. This equation for this experiment reduces to $N = \Delta N(297.14285)$. An average of 743 P$^{32}$ atoms was incorporated into each cell with a minimum estimate of 239 P$^{32}$ atoms per cell.

The number of P$^{32}$ atoms that underwent decay during the period of storage (average of 24 days) was computed by calculating the number of P$^{32}$ atoms remaining after 8 days (the beginning of storage) and after 32 days (the end of storage). The difference between the number of P$^{32}$ atoms remaining at 8 days and those remaining at 32 days was taken as the number of radioactive disintegrations per sperm cell during the period of storage in the unlabeled female. The average number of disintegrations per sperm cell during storage was 347 and the minimum estimate was 111 disintegrations per cell.

Since Mirsky and Pollister (1942) found that over 90% (by dry weight) of fish spermatozoa is extractable as DNA and protamine, it is expected that the preponderance of phosphorus remaining in sperm cells after washing with cold trichloroacetic acid is in DNA. The difference between the phosphorus remaining after washing with cold trichloroacetic acid and that remaining after washing with hot trichloroacetic acid (90°C) is taken as the phosphorus incorporated into nucleic acid (Pollister and Ris 1947; Davidson 1950) and is a good estimate of the amount of phosphorus in DNA, for the amount of RNA in sperm cells is low (Mirsky 1947). Extraction of Drosophila sperm transferred from a single male with hot trichloroacetic acid did not leave enough P$^{32}$ in the sperm to give a significant count; however, since the count following extraction with cold trichloroacetic acid is low, though significant, there could have been some P$^{32}$ left
in the sperm following extraction with the hot trichloroacetic acid. Extraction of larger amounts of \( P^{32} \) labeled honey bee semen showed that 75% of the phosphorus remaining after extraction with cold trichloroacetic acid was removed with hot trichloroacetic acid. Therefore, it is assumed that 75% of the \( P^{32} \) in Drosophila sperm washed with cold trichloroacetic acid is incorporated into DNA. The statistical limits set by the low counts observed from sperm of an individual male are wide; therefore, an error of even a factor of two in the estimate of the proportion of \( P^{32} \) incorporated into DNA is not serious. H. J. Muller (personal communication) suggested that two fifteenths of the \( P^{32} \) in DNA should be in the X-euchromatin because evidence from mutation frequencies indicates that ratios of euchromatin of the X, second, and third chromosomes are 2:5:5; whereas, the euchromatin constitutes four fifths of all the chromatin of condensed chromosomes. Therefore, the average number of \( P^{32} \) disintegrations per X-chromosome euchromatin was 35 and the minimum estimate was 11.

The dose of beta radiation received by the stored sperm from \( P^{32} \) was calculated on the basis of three models: (1) the cylindrical head of the sperm cell, (2) the cylindrical seminal receptacle, and (3) the spherical coiled seminal receptacle. Two models are considered necessary for the seminal receptacle, for this tube-like structure is normally coiled on the surface of the vagina.

The proportion of energy from beta radiation that would escape objects the shape of these three models was calculated from the formulae of Richards and Rubin (1950). These formulae are based on the assumption of a uniform loss of energy along the path of the beta particle. This assumption is not correct; however, these formulae should overestimate the biological effect of the retained fraction of radiation since the end of the beta track would have a higher ion density and therefore a higher relative biological effectiveness. The fraction of energy escaping \( F \) from a sphere of volume \( V \) is \( F = 1 - 0.435 (V/R)^{1/6} \), where \( R \) equals the mean range of the beta particle in water (for \( P^{32} \), \( R = 3.25 \) mm). The fraction of energy escaping from an infinite circular cylinder \( F_i \) of radius \( A \) is \( F_i = 1 - 4/3 (A/R) + 1/2 (A/R)^2 + 1/12 (A/R)^4 \), and for a finite cylinder of length \( L \) the fraction of energy, \( F \), escaping from both ends and sides is \( F = F_i + (1 - F_i) (1 - L/2R) \). The fraction of energy escaping from each of the three models is given in Table 4.

From the number of disintegrations per cell corrected for efficiency of the Tri-Carb and for decay to the beginning of the storage period (8 days after labeling), there were \( 7.6 \times 10^{-6} \mu \)c of \( P^{32} \) per sperm cell. For the average of 198 sperm cells per female, there was an average of \( 1.5 \times 10^{-6} \mu \)c of \( P^{32} \) transferred to the female by the labeled male. The concentration of \( P^{32} \) for each of the three models based on the assumption that the sperm head or seminal receptacle has a density equal to water is shown in Table 4. The dose in rads that each of the three models would have received from beta radiation of internal origin (Table 4) was calculated by multiplying the fraction of energy retained \( (1 - F) \) by the dose that would have been received if the volume had been infinite.

The dose \( D \), on the assumption of an infinite volume, was calculated on the basis of the formula of Marinelli (1954):

\[
D_{\beta (\text{rad})} = 73 \bar{E} \cdot T^{1/2} \cdot C,
\]

where \( \bar{E} \) equals the mean energy for the beta particle, \( T^{1/2} \) equals the half-life, and \( C \) equals the concentration in \( \mu \)c/g. Marinelli's formula is based on complete decay. Only 69% of the \( P^{32} \) present at the beginning of storage would decay.
P³² decay in Drosophila chromosomes

**TABLE 4**

*Calculated dose of beta radiation from incorporated P³²*

<table>
<thead>
<tr>
<th>Model</th>
<th>Fraction of energy retained†</th>
<th>p³² concentration (µc/gram)</th>
<th>Dose retained in model‡ (Rads)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cylindrical sperm head</td>
<td>10⁻⁷</td>
<td>7 × 10³</td>
<td>0.3</td>
</tr>
<tr>
<td>* r = 0.2µ, l = 9µ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cylindrical seminal receptacle</td>
<td>2 × 10⁻³</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>* r = 14µ, l = 2mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spherical seminal receptacle</td>
<td>48 × 10⁻³</td>
<td>0.4</td>
<td>10</td>
</tr>
<tr>
<td>r = 0.1mm</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Measurements from DEMEREC (1950) pp. 44 and 526.
† Calculated from the formulae of RICHARDS and RUBIN (1950).
‡ Dose calculated on the assumption of an infinite volume from the formula of MARINELLI (1954) then multiplied by the fraction of energy retained to determine the dose retained.

during the 24-day storage of sperm cells in females; therefore, 0.69 of the dose (D) calculated from MARINELLI's formula was multiplied by the fraction of energy retained (calculated from the formulae of RICHARDS and RUBIN 1950) to obtain the dose received during the 24-day storage (Table 4).

The dosages calculated by these formulae are probably an overestimate because of the higher relative biological effectiveness of the end of the beta track; nevertheless, the geometrical form considered that produces the highest intensity of beta radiation gives a negligible dose (Table 4). Negative results from the genetic test are further evidence of the negligible dose of beta radiation during storage of the sperm.

There was no indication of any mutagenic effect of transmutation and recoil when the mutagenic effect of beta radiation was eliminated experimentally; however, the statistical limit of the maximum mutagenic effectiveness of transmutation and recoil was calculated. A minimum estimate of 11 P³² disintegrations per X euchromatin along with a 0.5% increase in the mutation rate that would have been detected if present gives the maximum mutagenic efficiency of one P³² disintegration in 2,000 that will produce a "complete" recessive lethal. This estimate differs by a factor of 200 from the results obtained with virus (STENT and FUERST 1955). A more reasonable estimate of the Drosophila data would combine 35 disintegrations per X euchromatin with a 0.3% limit on the mutation rate to give one P³² disintegration in 11,000 that will produce a "complete" recessive lethal.

**DISCUSSION**

Radionuclides offer an advantage in that the chemical position of the nuclide undergoing radioactive decay, the number of nuclides undergoing decay, and the initial effect of transmutation and associated energy of recoil of the nuclide can be determined by physical measurements. The genetic consequence, or, of equal interest, the lack of a genetic consequence from a given number of radioactive decays can then be determined by genetic analysis.

The initial effect of transmutation and associated energy of recoil of the P³²
nuclide is known by the physics of its radioactive decay. When radioactive decay of $^{32}$P occurs, a beta particle is ejected from the nucleus with a maximum of 1.71 Mev and has a maximum range in water of 7.9 mm. The energy of any one beta electron is determined by the angle of the beta particle to the neutrino. The resultant average energy of the beta particle is 0.70 Mev. In spite of the large range in energy of beta radiation from $^{32}$P, in nearly all cases the beta particle will distribute most of its energy well beyond the molecule in which the decay occurred. Therefore, the mutagenic effect of beta radiation is from $^{32}$P atoms not likely located in the molecule in question. The probability of a molecule being “hit” by one of these beta particles depends upon the concentration and geometrical distribution of $^{32}$P in the system. This will be considered later in relation to the Drosophila experiments.

When the beta particle is emitted under the law of conservation of momentum, energy of recoil is applied to the $^{32}$S nucleus. If reasonable values are assigned to the energy of recoil, to energy of the P-O bonds, and to the mass of material affected, KAMEN (1950), POWERS (1956), and STRAUSS (1958) calculate that almost every disintegration should lead to the rupture of an ester bond. Also, transmutation of the $^{32}$P to $^{32}$S would likely rupture an ester bond owing to probable instability of the sulfate diester, even if both S-O bonds survived the effects of nuclear recoil (STENT and FUERST 1955). Therefore, it seems probable that every $^{32}$P disintegration causes a break in one of the DNA strands at the site of $^{32}$P decay.

Extensive experimental work on the effect of radioactive decay of $^{32}$P incorporated into DNA of viruses and bacteria has been reviewed by STENT and FUERST (1960). When $^{32}$P labeled viruses were stored in sufficient dilution so that control lysates containing an equal amount of nonincorporated $^{32}$P were stable, HERSHEY et al. (1950) showed that the inactivation of virus particles was due to the “short range” transmutation and recoil and that the number of surviving viruses fell linearly with the number of $^{32}$P atoms that had decayed up to the time of assay. THOMAS (1959) has shown by means of radioautographs that decay of $^{32}$P incorporated into viral DNA breaks the molecule with a frequency that is in rough quantitative agreement with the frequency of viral inactivation. In six different strains of double-stranded DNA bacterial viruses stored at 4°C, one $^{32}$P decay in ten will inactivate the virus particle (STENT and FUERST 1955). The efficiency at which $^{32}$P decay in one strand could rupture both strands, thus breaking the molecule and inactivating the virus, was decreased 45% by reducing the storage temperature to −196°C, and was increased by raising the storage temperature above 4°C. The synthesis of viral specific protein following infection of a host bacterium progressively stabilizes the viral DNA to inactivation by decay of incorporated $^{32}$P atoms. This progressive stabilization to decay of $^{32}$P atoms is prevented by treatment with chloramphenicol, a substance which inhibits protein synthesis.

The critical test of the action of $^{32}$P decay came when TESSMAN, TESSMAN and STENT (1957) and TESSMAN (1959) found that the viruses S13 and $\phi$X174 are inactivated with the efficiency of one inactivation per $^{32}$P decay, even at a tem-
temperature of \(-196^\circ\text{C}\). Sinsheimer (1959) has found that \(\phi X174\) has a single-stranded DNA molecule instead of the usual double-stranded DNA. Therefore, each \(^{32}\text{P}\) decay ruptures a single strand of the DNA polynucleotide; but, in the case of double-stranded DNA, both are ruptured with a frequency that is less than one. The number of \(^{32}\text{P}\) decay events is therefore a minimum estimate of the number of single strands of DNA broken.

Phosphorus-32 incorporated into \(\text{Paramecium aurelia}\) produces death following autogamy at a higher frequency than an equal amount of beta radiation from \(^{89}\text{Sr}\), \(^{90}\text{Sr}\), or \(^{90}\text{Y}\) (Powers 1956). This work is particularly significant since \(\text{Paramecium}\) has true chromosomes, yet as a microorganism can be studied in a manner similar to virus and bacteria. Transmutation and the associated recoil effect are mutagenic in eukaryotic organisms, although we do not know if the mutations are “complete” or mosaics because of the number of generations between incorporation and autogamy.

Early work with \(^{32}\text{P}\) mutagenesis in Drosophila (reviewed by Oftedal 1959) had a variety of objectives in addition to separating the mutagenic effect of beta radiation from the possible mutagenic effect of transmutation and energy of recoil. However, an effort was made to determine the mutations that should have been produced by beta radiation and to subtract these from the total number of mutations produced. The difference between the observed mutation rate and that expected from beta radiation was ascribed to transmutation. Extensive experiments along this line of investigation were conducted by Oftedal (1959) who used Yttrium-91 \((1.52\text{ Mev beta radiation})\) in a parallel test with \(^{32}\text{P}\) to determine the mutation rate due to beta radiation. The difficulty in this method is the necessity of determining the mutation rate when the mutagen is applied during all stages of spermatogenesis. Because of great variation in germ cell sensitivity during spermatogenesis and variation in the rate of producing sperm, this approach cannot be considered critical in separation of the mutagenic effect of beta radiation from transmutation and associated energy of recoil.

Walen (1962) found no significant difference between the distribution on the X chromosome of lethals induced by ingested \(^{32}\text{P}\) and X rays. Bateman and Sinclair (1950), and Bateman (1955) approached this problem differently by comparing the ratio of dominant lethals, sex-linked recessive lethals, deleted X’s, and “probable” visibles for the two mutagens, ingested \(^{32}\text{P}\) and X rays. Hindered by the same difficulty, variation in sensitivity during different stages of germ cell development, Bateman arrived at the pessimistic conclusion quoted in the introduction of this paper.

Mature sperm was used in experiments reported here to avoid difficulties of variation in radiosensitivity during germ cell development and to eliminate the effect from beta radiation, as Hershey et al. (1950) did with microorganisms. Indeed, sperm cells are microorganisms in which loss of beta radiation is comparable to that in \(\text{Escherichia coli}\) because of the small diameter of the cylindrical sperm head—0.28 to 0.5 \(\mu\) (Demerec 1950). It is significant that when the effects of beta radiation were removed experimentally, there was no increase in loss of the \(B^8\text{Y}\) (or X) or in sex-linked recessive lethals that could be attributed to trans-
mutation and recoil even though there were at least 11 breaks in the X-chromosome euchromatin during storage of the mature sperm in unlabeled females. Apparently all the mutations observed in the first test and in the experiments of OTFEDAL (1959) were due to beta radiation.

Loss of $B^s$ on the Y chromosome could be the results of a number of different types of chromosome aberrations; however, all these aberrations would require at least one chromosome breakage. Therefore, loss of this marker is taken as a relative measure of chromosome breakage.

The physics of radioactive decay and results of experiments with viruses show that the initial effect of transmutation and recoil of $^{32}P$ incorporated into DNA is the breakage of a single strand of the DNA double helix with a probability of 1 in 10 to 1 in 20 (depending upon temperature) of breaking both strands in viruses. It is assumed that the initial event of breaking at least one strand of DNA for each radioactive decay occurs also in the Drosophila chromosome, but that the genetic consequence is different because of the structure of complex nucleoproteins observed in electronmicrographs of chromosomes from eukaryotic organisms (Ris and Chandler 1963).

In the Drosophila experiment if breaks in the phosphorus diester bond produced by transmutation and recoil remain unhealed and if protein associated with DNA is not capable of maintaining the structural continuity of the eukaryote chromosome, then the breaking of both strands of the DNA double helix (as occurs at a frequency of one in ten at 4°C in double-stranded DNA viruses) in either the X or Y chromosome would often result in chromosomal loss producing an X0 male that would be easily detected by absence of the narrow Bar eye. If only one strand is broken and remains unhealed (as occurs for each transmutation and recoil in single-stranded viruses) and if there is no structural support from associated proteins in the eukaryote chromosome, then each transmutation and recoil in the Y chromosome should produce a male with mosaic tissue. The consequence of producing a male embryo mosaic for a Y chromosome marked with $B^s$ depends on the distribution of the nuclei to the eye imaginal discs. From an extensive study of 213 mosaics in which two thirds were caused by loss of one X chromosome at first cleavage division, Patterson and Stone (1938) found that 15% of the individuals had both eye-imaginal discs composed entirely of mutant nuclei, 39% had mosaicism in the eye imaginal discs, and 46% had eye imaginal discs composed entirely of nonmutant tissue. Therefore, we would expect at least 15% of the mosaic embryos to show complete loss of $B^s$, and 39% to show some tissue of both types. Since there was no increase in loss of $B^s$ that could be attributed to transmutation and recoil and only one mosaic for $B^s$ was found among 22,312 males (combining test 1 and 2), breakage of the phosphorus diester bond in either one or both strands of the DNA molecule in mature sperm does not often lead to either chromosome or chromatid aberrations.

Loss in a small portion of the paternal X would in many cases "uncover" one or more of the 14 recessive mutations on the maternal X (Maxy chromosome) and be detected either as a mosaic or complete by the appearance of one or more of the recessive phenotypes in the F₁ female. Since there were only 10 complete
and 4 mosaic visible mutations observed in 21,414 F1 females (combining test 1 and 2), this type of chromosome damage is not a frequent result of breaking the phosphorus diester bond. Mosaics resulting in loss of a large part of paternal X would produce a gynandromorph; however, gynandromorphs may not have been detected because the recessive lethal in the "Maxy" chromosome might act as a cell lethal. It is noteworthy that the same type of chromosome aberration in the paternal X that produces a gynandromorph would in the Y chromosome often produce a male complete or mosaic for loss of B8. As shown in the previous paragraph, this is not often the result of breaking the phosphorus diester bond. Apparently transmutation and recoil of P32 incorporated into the eukaryote chromosome does not destroy the chromosome's continuity.

If breakage of the phosphorus diester bond on the X chromosome should result in loss of one or more nucleotides or in repair with a substitution of the wrong nucleotide, a mutation that would fit the operational definition in Drosophila genetics of a "point" mutation would occur. Estimates for the fraction of "point" mutations in Drosophila that are lethal range from one fifth to one half and are based on the ratio of lethals to detrimentals or the percent of lethals at visible loci (Timoféeff-Ressovsky 1935; Kerkis 1938; Muller 1954; Käfer 1952; Altenburg and Browning 1961).

If a lethal produced is complete so that all cells in the F1 embryo carry the lethal, it will be detected as a sex-linked recessive lethal; however, if a mosaic F1 embryo is produced because of breakage of only one strand (as occurs nine tenths of the time in double-stranded DNA viruses stored at 4°C), the results will depend upon the distribution of mutant nuclei to gonad in the mosaic embryo. Carlson and Southin (1963) found that mutant nuclei in Drosophila mosaics were non-randomly organized, and that one third of the flies mosaic for a visible mutation, dumpy, transmitted the mutation as a gonadal complete; therefore, we would expect one third of the mosaic lethals to be detected as sex-linked recessive lethals if the proportion of mutant tissue in the embryo is similar (25% to 50%) to that observed by Carlson and Southin. Therefore, from 1/5 to 1/2 of the complete mutations and from 1/15 to 1/6 of the mosaic mutations—single strand breaks—induced should have been detected as sex-linked recessive lethals in the F2 generation; but, in fact, neither lethal mutations nor chromosome breakage were detected that could be attributed to transmutation and recoil of P32 incorporated into DNA in mature spermatozoa.

The refractory nature of the eukaryote chromosome to genetic damage caused by transmutation and recoil of P32 as judged by these tests can be explained in three ways: (1) The broken strand could be held in place by its complement or by both its complement and protein until an enzyme repairs the broken diester bond—probably following fertilization of the egg. This repair hypothesis does not seem unreasonable because phosphorylation of the nucleoside occurs readily in Drosophila, as shown by the incorporation of H3 thymidine into DNA; and repair of DNA strands following excision of thymine dimers in bacteria has been demonstrated (Setlow and Carrier 1964; Boyce and Howard-Flanders 1964; Pettijohn and Hanawalt 1964). (2) If there are many DNA molecules for
each locus (as suggested by Ris 1961), mosaic embryos would have such a small proportion of mutant nuclei that it would be very improbable for only mutant nuclei to be included in the germ line to give a gonadal complete. Further evaluation of this hypothesis must await completion of an experiment in which the F2 are tested. (3) A considerable amount of DNA in eukaryote chromosomes may not be carrying genetic information or may be redundant; however, this hypothesis presents some problems from an evolutionary point of view.

SUMMARY

Transmutation of P32 to S32 and the accompanying energy released by steric changes in the molecule and recoil of the S32 nucleus have been separated from the mutagenic effect of accompanying beta radiation by storage of P32-labeled spermatozoa in unlabeled Drosophila females. Because of the high energy of the P32 beta particle, only a negligible dose is received by sperm from beta radiation during storage. Mature sperm containing DNA labeled with P32 was obtained from 2-day-old males that had been fed P32 as larvae. Females recently mated to P32 labeled males were divided into two groups. One group was allowed to produce progeny immediately. The other was first stored on sugar agar media at 18°C for 3 weeks to inhibit oviposition, and then permitted to oviposit freely on cornmeal media. The mutation rates of progeny of the two groups were compared to measure additional genetic damage, if any, caused by P32 decay in sperm during the storage period. Incorporation of P32 was determined by counting spermatozoa microscopically, washing with cold trichloroacetic acid, and determining the radioactivity with a liquid scintillation detector. The average number of P32 disintegrations during storage per X-chromosome euchromatin was 35 and the minimum estimate was 11. There was no significant change during storage in the rate either of chromosome breakage (as measured by loss of the marker B5 on the marked B5/Y chromosome) or of sex-linked recessive lethals that could be attributed to transmutation and recoil. By combining the minimum increase in mutation rate that could have been detected statistically with the minimum estimate of P32 disintegrations, it was concluded that less than one P32 disintegration in 2,000 in the X chromosome will produce a "complete" recessive lethal.

LITERATURE CITED

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P^32 Decay in Drosophila Chromosomes


