MUTATION RATES AT SPECIFIC AUTOSOMAL LOCI IN THE MATURE AND IMMATURE GERM CELLS OF DROSOPHILA MELANOGASTER [1]

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STUDIES utilizing mutation rates at specific loci as a measure of radiation damage to biological material allow a systematic test of limited sections of genetic material. The damage is measured in terms of gene mutations, since these could be retained in the gene pool of a population and possibly be expressed over a number of generations.

A differential sensitivity to X-radiation for the different stages of spermatogenesis of Drosophila has been suggested by a drop in the number of sex-linked lethals obtained from mature males 14 to 16 days after treatment (HARRIS 1929; HANSEN and HEYS 1929). MOORE (1934) found fewer sex-linked visible mutations from adults which had been treated as larvae than from treated adults. The present study was designed to determine mutation rates at 8 autosomal loci in sperm and spermatogonial cells of *Drosophila melanogaster*. The early larval stages were treated to limit the test to spermatogonial cells and the mature sperm test was limited by using the males for only 4 days after treatment. These procedures, including the use of specific loci, were employed to avoid some of the complications encountered in previous studies of this type.

The specific loci test can be used to provide a reliable index for comparisons of genetic damage produced by X-radiation with that produced by other types of radiation. It is also the best of the few general methods for comparative studies of radiation damage in different biological forms.

MATERIALS AND METHODS

General procedure

A standard stock of *Drosophila melanogaster,* designated Oregon-R, Oak Ridge, was established from the Oregon-R stock by inbreeding pairs for 4 generations, then combining several of the most fertile strains from each of the original pairs. The mutant test stock was the third chromosome *res* complex. The linkage positions (BRIDGES and BREHME 1944) are as follows: *ru* (roughoid), 0.0; *h* (hairy), 26.5; *th* (thread), 43.2; *st* (scarlet), 44.0; p^p

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(peach), 48.0; *cu* (curled), 50.0; *sr* (stripe), 62.0; e^s (sooty), 70.7. Mutations of spontaneous origin were chosen in order to reduce the probability that deletions or chromosome aberrations were associated with the mutants. A cytological check of the Oregon-R and *res* marker stocks by C. L. WARD showed that there were no inversions or detectable deletions.

The general procedure for establishing mutation rates consisted in mating X-irradiated males to homozygous *ru h th st* p^p *cu sr* e^s females. Any mutation at any one of the 8 loci as well as dominant mimics and modifiers, phenocopies, and partials can be detected in the F_1 individuals.

The procedure for testing the F_1 variants for classification into one of several categories is as follows:

P_1 Oregon-R ♂ treated × *ru h th st* p^p *cu sr* e^s (*res*) ♀

1. Test of F_1 ♂ variants:

F_1 ♂ variants (*h′* induced) × *res* ♀♀

F_2 classes:

Presence of *h′* or *h* in all offspring excludes dominant mimics and modifiers on the X, second and fourth chromosomes, phenocopies and partials excluded.

Crossover test: (pairs tested)

F_2 (*h′/res*) ♀ × *res* ♂

F_3 classes:

Presence of *h′* or *h* in all offspring repeats previous test and excludes dominant mimics and modifiers on the third chromosome except any which may be so close to the *h* locus that no crossovers are detected. Crossover values may not be normal because of the presence of induced inversions or translocations. Normal individuals indicate a dominant mimic or modifier on the third chromosome.

2. Test of F_1 ♀ variants:

F_1 ♀ variant (*h′* induced) × F_1 ♂♂ (treated chromosome/*res*)

F_2 classes:

Normal individuals received treated chromosome from F_1 ♂.

Crossover test—detectable by presence of *h′* or *h* alone or with any one or combination of *res* mutants:

This test excludes all nonmutant variants as regular crossover test. Presence of any one or combination of *res* mutants without *h′* or *h* indicates a nonmutant type.

Additional crossover test:

Strains of the original mutant were obtained by crossing individual F_2 ♂ (*h′* or *h/res*) to *res* ♀♀. Individual ♀ *h′* or *h/res* (from F_2 ♂ strains) × *res* ♂.

Classes obtained:

Presence of *h′* of *h* in all offspring excludes all nonmutant variants as in other crossover test.

The crossing-over data from the F_1 female variant gave immediate proof as to whether or not the variants were true mutations but the crossing over introduced difficulties in establishing stocks of the induced mutations, since the marker mutant may have crossed into the irradiated chromosome. Individual F_2 males which showed the mutation (either induced or from the *res* complex) were crossed to *res* females, a minimum of 10 strains from individual F_2 males being established whenever possible. The greater the number of mutant strains established, the smaller the chance that all of them would contain the allele from the *res* complex. The center mutants of the *res* complex, *th, st, p^p, cu* and *sr* would require a double crossover to be crossed into the treated chromosome, but only a single exchange would be necessary to recover *ru* or e^s alone. Since the region between *sr* and e^s is 8.2 units long about 8 individuals in a sample of 100 will show a single or multiple exchange involving this region. The *ru* mutant from *res* would be more likely to be crossed into the treated chromosome, since the *ru-h* region is 26.5 units in length. The induced allele could be distinguished phenotypically or cytologically from the tester allele in some cases, thus insuring its recovery. In only 1 or 2 cases the induced mutant resembled the test mutant so closely that the two could not be distinguished phenotypically or cytologically. A viability test eliminated a large number of doubtful cases. A lethal, semilethal, or sterile condition of the homozygous mutant showed that the induced mutant was recovered. Up to 9 strains of certain mutants were tested and all strains of a given mutant gave the same viability result. It should be noted that the possibility of crossing out the *res* mutants does not affect the determination of the mutation rate since the crossover tests prove the original variant to be a true mutation. Crossing over affects the possible validity of the viability test only.

Spermatogonial test

Mature sperm as well as three types of immature germ cells, spermatids, spermatocytes, and spermatogonia are present in the adult males of Drosophila. In irradiated adult males, there is no sure way to tell which type of immature cells is being tested. To ensure that spermatogonia were tested, larvae which contained only spermatogonia were irradiated. According to KERKIS (1933) and GLOOR (1943), only spermatogonia are present in the larval gonads 24 hours after the larvae hatch from the egg at a temperature of 25°C, the first spermatocytes appearing about 48 hours after the larvae hatch from the egg.

Aged Oregon-R, Oak Ridge flies (usually 4 to 7 days old) which had produced larvae in culture bottles were placed in empty pint milk bottles. These bottles were then inverted in Petri plates lined with damp filter paper which was used as a source of moisture. A piece of damp filter paper the size of the mouth of the bottle and coated with a mixture of yeast-banana agar, Y-2 yeast, and a drop of acetic acid added to stimulate egg laying, was used for collecting eggs. The flies were allowed to lay for a 2-hour period, after which

the filter papers with the eggs were removed to small culture vials containing banana-agar food. To provide moisture necessary for normal hatching a small amount of saline was placed in the vial with one edge of the filter paper touching the water. After 21 hours at 24°C, the filter paper was removed and any larvae which had hatched in this period were washed off with saline and discarded. The remaining eggs were then allowed to hatch over a 2-hour period and these larvae were transferred onto a large piece of filter paper which contained a mushy solution of banana-agar food and yeast. In order to ensure that all larvae would be of uniform age, great care was taken to allow no unhatched eggs to remain. The larvae were aged for 20 hours in a glass Petri plate and transferred to a lucite Petri plate for X-irradiation. These 20- to 22-hour larvae were irradiated with 900 roentgens of 250 kvp X-rays delivered by a Coolidge self-rectifying tube with inherent filtration of 3 mm of aluminum and a half value of 0.4 mm of copper. Using a current of 15 ma, the intensity obtained at 84 cm distance was approximately 85/r/ minute.

A dose of 900 r was decided upon after a preliminary test of the viability and fertility of adults which had been treated as larvae. Doses of 800 and 900 r gave 60 to 75 percent recovery of adults from treated larvae. Only a few larvae died in the pupal stage and the fertility of the adult males was approximately 80 percent.

Increasing the dose to 1000 r caused a tremendous increase in the death rate of pupae and a drop to about 40 percent in the fertility of the males. Raising the dose to 1200 r caused no further decrease in the number of adults that hatched but these adults were very weak and the fraction of fertile males dropped to about 20 percent.

After X-irradiation with 900 r the larvae were placed in a bottle containing yeast-banana-agar food. The adult males, which hatched from these larvae were aged 6 to 7 days and were mated individually to several *res* females in all experiments but one. In this case each male was mated successively, to a single different female for a 24-hour period until four such matings were made.

Information on the effect of irradiation on spermatogonia was obtained from 7 experiments in which from 126 to 400 P_1 males were tested. In the first larval test, in which there was no way to determine the number of mutations to expect from the sample, an average of 290 F_1 offspring from each male were checked. After recovery of several cluster mutations the sample size was reduced to 113 to 140. Offspring from each male were recorded separately. Males from which one or more mutations were recovered were retained and larger samples obtained for determination of cluster sizes as expressed in the ratio of mutant to total offspring. Several mutant stocks were established from each cluster, using F_1 male mutants when possible. These mutants were tested as described under *General procedure*. The mathematical treatment of spermatogonial mutation rates will be discussed under Mutation rates.

Sperm test and control

Adult males, 6 to 7 days old, were X-irradiated with 3000 r and mated, in pairs, to *res* females. After 24 to 36 hours the males and females were separated and each male mated to another *res* female. Each treated male, remated every 24 hours, was mated in sequence to a total of four *res* females over a period of 96 hours and then discarded. This technique was employed to detect any spontaneous mutations which might be carried heterozygous by the male and to limit testing to the later stages of spermatogenesis—presumably, only mature sperm. The F_1 offspring of each male were recorded separately. Variants which resembled any of the 8 mutants were tested as described under General procedure.

Such a paired-mating technique is not sufficient to detect spontaneous clusters in the treated males, as evidenced by the recovery of 2 different mutations at the same locus from the progeny of a single treated male and of recovery of mutations at 2 and 3 different loci from the progeny of a single male. The separate control test must therefore serve as a comparison test. Even though spontaneous clusters cannot be detected by this type of test, it does serve to limit the sperm sample obtained from the male. Larvae for the control, mature sperm and spermatogonial tests were collected in the same way to prevent any possible physiological difference in the 3 tests. Adult males for the control test were mated in the same way as those in the mature sperm test.

Viability test

X-ray-induced mutations recovered from spermatogonia and mature sperm were tested for viability in the homozygous condition. Only 1 strain of the mutant was tested if the mutant originated from a male; 1 to 9 strains were tested if the mutant was obtained from a female. In most cases the spermatogonial clusters produced a few mutant males so that stocks of these could be established from males.

An ebony mutant was used to test all induced mutants except that peach was used for those at the ebony locus. The mutant strains which had been carried through the male heterozygous with *res* were crossed to ebony-11 or peach. Ten to 25 paired matings of the F_1 phenotypically normal individuals were used. Genetically, the F_1's were heterozygous for the test mutant, ebony or peach, and the mutant being tested. A mutant ratio of 1 test mutant: 2 normal: 1 induced mutant would be expected from such a cross if the induced mutant survived in a homozygous condition and was as viable as the test mutant. A ratio of 1 test mutant: 2 normal with the third class absent showed that the induced mutant was homozygous lethal. A large reduction in size of the induced mutant class indicated lowered viability; this condition was considered semilethal. Ratios of 1 test mutant: 3 normals indicated that the mutant was not phenotypically detectable in a homozygous condition, even though the mutant phenotype was expressed when heterozygous with the mutant contained in the *res* complex. This method has an advantage over a balancer

method since crossing over would decrease the chance of lethals, present at another locus, being homozygous in all offspring.

A different method was necessary to test the *sr* mutants, since some ebony allele must be present in a heterozygous or homozygous condition for the mutant to be detected phenotypically. For testing the *sr* mutants the *sr/res* males were mated to $Dl^{13}/In\ (3R)C,\ Sb\ e\ l(3)\ e$ females. The phenotypic *Sb*, non-ebony F_1 individuals were mated in pairs. The appearance of normal non-*Sb* F_2 individuals showed that the homozygous *sr* mutant lived since the balancer chromosome contained a lethal and cannot be recovered in a homozygous condition. The heterozygotes of the mutant and balancer showed *Sb*. The normal F_2's (homozygous *sr*) were crossed to *res* to test whether the mutant was present homozygous or crossing over had occurred to give normal individuals.

RESULTS

The F_1 variants recovered from the mature sperm test are included in table 1. The variant was placed in the " Mutations " category if it could be proved by the crossover test to involve the locus which it phenotypically resembled. This type of variant was the only one used for calculating mutation rates. A total of 71 mutations was recovered from a sample of 49,512 F_1 offspring. Of 324 males tested, 262 produced no mutation, 54 produced 1, 7 produced 2, and 1 produced 3 different mutations. The 3 mutations in parentheses were lost before a stock strain could be established but the number of crossovers checked was considered sufficient to prove them mutations.

TABLE 1

Variants recovered from mature sperm X-irradiated with 3000 r (Sample size: Control, 45,504; mature sperm, 49,512).

Variants	Loci tested								Total
	ru	*h*	*th*	*st*	p^p	*cu*	*sr*	e^s	
(Control)	0	0	0	0	0	0	0	0	0
Genetic mutations	3 (2)	6	4	9 (1)	13	8	12	13	68 (3)
Phenocopies and mimics	89	1	2	67	3	10	8	17	197
Sterile variants	35	5	0	10	9	3	7	7	76
Dead before completion of test	65	5	3	9	4	10	14	4	114
Partials	19	1	0	0	2	3	7	0	32
Total	213	18	9	96	31	34	48	41	490

Many variants were semisterile and produced only a small number of offspring. Other variants, tested for 2 or 3 weeks and remated several times to the *res* stock, proved to be sterile. Both of these classes were placed in the " Sterile Variants " category. Variants which died in 2 weeks of testing were recorded in the " Dead " column. The " Phenocopies and Mimics " category includes those variants which were not inherited but were due to physiological conditions and those which involved some other locus or loci in the genome.

The " Partials " included variants with unilateral and partial expressions which were not inherited.

According to this classification of variants the total number of mutant and sterile variants happens to be about equal but the same relation does not exist for each of the 8 loci. The roughoid phenotype accounts for about one-half the total variation in each of the nonmutant classifications but is next to the smallest class of mutations. A rough eye is obviously an exceptionally frequent phenocopy resulting from irradiating Oregon-R, Oak Ridge stock. This was not true for other phenocopies of mutants except scarlet. This complete lack of correlation between the numbers of mutant and nonmutant variants for the different loci requires genetic testing of all variants. Also, it is impossible to assign with assurance the sterility effects to the loci which are seemingly involved or to decide how many of the sterile variants might be true mutations.

TABLE 2

Variants recovered from mature sperm X-irradiated with 3975 r (Patterson, 1934, unpublished). (Sample size, 50,159).

Variants	Loci tested								Total
	ru	*h*	*th*	*st*	*cu*	*sr*	e^s	*ca*	
Genetic mutations	16	1	3	8	3	4	13	22	70
Phenocopies and mimics	7	0	0	1	6	1	1	7	23
Sterile variants	17	1	10	5	4	8	8	8	61
Dead before completion of test	1	0	1	1	1	0	1	0	5
Partials	3	1	2	1	2	2	1	4	16
Total	44	3	16	16	16	15	24	41	175

The absence of any noticeable drop in the number of mutations recovered —a drop would be an indication of the exhaustion of sperm which were mature at the time of treatment—indicates that only mature sperm (or equally sensitive late stages) were recovered within the 4-day period after irradiation of the adult male. The total number of 71 mutants were distributed over the 4 days of testing to give values of 0.149 percent for the first day, 0.111 percent for the second, 0.148 percent for the third, and 0.172 percent for the fourth day.

Table 2 includes unpublished data which were obtained by J. T. PATTERSON of the University of Texas in 1934. PROFESSOR PATTERSON has consented to allow these data to be included since 7 of the 8 loci used in his experiments were used in the present study. This permits a comparison of the mutation rates in mature sperm at these 7 loci in the Oregon-R, Oak Ridge and in a Texas strain of *D. melanogaster*. The *ru, h, th, st, cu, sr* and e^s loci were used in both experiments. The mutant, *ca* (claret) is located to the right of the e^s locus, at 100.7 on the third chromosome. A dose of 3975 r of X-radiation was used in the PATTERSON experiment and a sample of 50,159 individuals was checked.

Tables 1 and 2 show that the phenocopy and mimic variants were only about one-third as frequent as the mutations in PATTERSON's experiment using the Texas strain as compared with experiments using the Oregon-R strain. The sterile and mutation categories were fairly similar in the 2 experiments. The range and frequency of the nonmutant types of variants differed in the 2 normal stocks tested; however, this difference is unimportant in comparing mutation rates.

In table 3 the 95 percent confidence intervals for the frequency of mutation at the various loci are compared. These values were obtained from MOLINA's tables (Poisson's Exponential Bionomial Limit—MOLINA 1942) and STEVENS' tables (FISHER and YATES 1949). Data for the Oregon-R stock indicate possible but not certain differences in mutation rates among some of the loci. An overlap is found between the lower limits of the value for the loci with the highest observed frequency (e^s and p^p) and the values for other loci. The

TABLE 3

Comparative frequencies of mutations at specific loci.

Locus	Frequency			95% confidence limits		
	Observed		Adjusted			
	Oregon-R (3000 r)	Texas (3975 r)	Texas (3000 r)	Oregon-R (3000 r)	Texas (3975 r)	Texas (3000 r)
ru	5	16	12.08	1.62–11.67	9.14 –25.99	6.90–19.62
h	6	1	0.75	2.20–13.06	0.025– 5.57	0.02– 4.20
th	4	3	2.26	1.09–10.24	0.62 – 8.77	0.47– 6.62
st	10	8	6.04	4.80–18.44	3.45 –15.81	2.60–11.93
p^p	13	....	....	6.92–22.27	....	....
cu	8	3	2.26	3.45–15.81	0.62 – 8.77	0.47– 6.62
sr	12	4	3.02	6.20–20.97	1.09 –10.24	0.82– 7.73
e^s	13	13	9.81	6.92–22.27	6.92 –22.27	5.22–16.81
ca	....	22	16.60	....	13.79 –33.35	10.41–25.17

data for the Texas strain show greater differences among the mutation frequencies of some of the 8 loci. The 8 mutants can be divided into the following general classes, on the basis of frequency: *ca* and *ru* loci have high frequencies; e^s and *st* loci have somewhat lower frequencies; mutations at the *h, th, cu,* and *sr* loci occurred still less often. The 4 loci with the lowest frequencies have about the same range, and the confidence intervals do not overlap that of *ca.* The other loci are intermediate in that the confidence intervals overlap either the high *ca* range or lower values of *h, th, cu* and *sr.*

A comparison of mutant frequencies between the Oregon-R and Texas strains (mutation rate for Texas strain has been adjusted for 3000 r) shows that the confidence intervals for similar loci overlap in all 7 cases. The greatest differences exist between the mutation rates of p^p and e^s in the Oregon-R strain and among those of *h, th, cu* and *sr* in the Texas strain. The 95 percent confidence interval for the total number of mutations at the 7 similar loci in the 2 experiments overlap. In this comparison the p^p locus in the present experiment and *ca* locus in PATTERSON's experiment were omitted. After omission

of 13 p^p mutants the 95 percent confidence interval for the remaining 58 is 44 to 75. In PATTERSON's experiment, 48 mutations were found, excluding the 22 at the *ca* locus. Adjusted to the radiation dose used in the present investigation, a rate of 36.2 mutations with 27 and 48 as the 95 percent confidence limits was obtained.

Data for spermatogonia presented in table 4 include 10 separate mutations recovered from 1797 males which were irradiated as larvae. In 3 cases a single mutant individual was recovered from the total sperm sample; in the remaining 7 cases 2 or more mutants were detected. The actual number of mutants recovered from the total sperm sample (total number of offspring recovered from the male) in each case is given in the third column. From the ratio of mutant to normal in the total sperm sample, given in the fifth column of the

TABLE 4

Spermatogonial mutations.

Locus	Number of independent occurrences of mutation	Number of mutant individuals in each sample	Total number of individuals in each sample	Ratio of mutant in total sperm sample
b	2	5	598	1/120
		17	770	1/45
cu	2	1	225	1/225
		4	297	1/74
sr	3	2	161	1/80
		1	342	1/342
		2	181	1/90
e^s	3	31	469	1/15
		1	110	1/110
		18	388	1/21

Total number of males tested = 1797.
Total number of sperm tested = 273,574.

table, the number of spermatogonia present in the adult male can be estimated. Each spermatogonium contains 2 sets of chromosomes; therefore the number of spermatogonia is obtained by dividing the denominators of these ratios by 2. The 1/120 ratio obtained in the first hairy cluster indicates that 60 spermatogonia were present in the males; the other hairy cluster gives a value of 23 or 24 spermatogonia. The wide variation in the ratios of different clusters shows that the number of spermatogonia also vary. Judged by this type of estimation the number of spermatogonia vary from a low of 7 or 8 to more than 100 per male.

Viability test

Table 5 contains the data for the viability test of the X-ray-induced mutations. Twenty of the 58 tested mutations induced in mature sperm lived in a homozygous condition, 35 were lethal, and 3 were semilethal. The ratio of viable to lethals would therefore be 1 : 1.8. About one-half of the mutations

TABLE 5

Viability of homozygotes of mutations obtained from X-irradiated sperm and spermatogonia.

	Loci tested								Total
	ru	*h*	*th*	*st*	p^p	*cu*	*sr*	e^s	
Sperm									
Viable	1	1	0	1	5	5	0	7	20
Lethal	3	2(1)	4	7	3	1	7(1)	5(1)	32(3)
Semilethal	0	1	0	0	1	1	0	0	3
Total	4	4(1)	4	8	9	7	7(1)	12(1)	55(3)
Spermatogonia									
Viable	....	0	....	....	....	2	0	2	4
Lethal	....	1	....	....	....	0	2	0	3
Semilethal	....	1	....	....	....	0	0	0	1
Total	....	2	....	....	....	2	2	2	8

from spermatogonial tests were viable in a homozygous condition and one-half lethal or semilethal. The finding of the 3 conditions, lethal, semilethal, and viable among spermatogonial mutants is important although the numbers are not large enough to determine their true proportions.

Mutation rates in mature sperm

The mutation rates (table 6) are expressed as the rate of mutation per roentgen of X-radiation of each locus. The rate for mature sperm was obtained by dividing the number of mutations produced at one locus by the number of individuals checked and the total dose in roentgens. The average rate for all 8 loci tested was obtained by dividing the total number of mutations recovered by the dose, number of individuals checked, and the number of loci used. An average mutation rate for these third chromosome genes of 5.72×10^{-8}/r/locus is obtained if the 3 cases are excluded in which stocks were not established. The rate is 5.98×10^{-8}/r/locus when they are included.

The mutation rates at specific loci varied from 2.01×10^{-8}/r to 8.75×10^{-8}/r. The variation between the different loci may be significantly dif-

TABLE 6

Mutation rates at specific loci and average rate for the third chromosome of Drosophila melanogaster.

Sample size and dose	Rate at specific loci/r $\times 10^{-8}$									Average rate/r $\times 10^{-8}$
	ru	*h*	*th*	*st*	p^p	*cu*	*sr*	e^s	*ca*	
Oregon-R strain										
49,521	2.01	4.04	2.7	6.06	8.75	5.4	8.08	8.75	....	5.72
	(3.4)			(6.7)						(5.98)
3,000 r										
Texas strain*										
50,159	8.02	0.5	1.5	4.01	....	1.5	2.0	6.5	11.03	4.4
3,975 r										

* Patterson, unpublished.

ferent from chance variation but no exact statistical test is available. There was no correlation between the mutation rate and the cytological position of the gene on the chromosome.

A similar treatment of PATTERSON's data gives an average rate of 4.39×10^{-8}/r/locus (table 6). A statistical comparison of the two experiments has been considered previously.

Mutation rates in spermatogonia

Several characteristics of Drosophila spermatogenesis must be discussed before the mathematical treatment of mutation rates for spermatogonial cells can be considered. In the spermatogenesis of *D. melanogaster* the primary germ cells migrate from the polar cap in the developing egg to the testes of the early larvae. SONNENBLICK (1941) states that a number of the primordia are lost in this migration. RABINOWITZ (1941) gives an average number of 40 primordia which begin this migration. The number of cells which are incorporated into the gonads fall into 2 groups, for some gonads contain 5 to 7 cells, others contain 9 to 13 germ cells. Between the formation of the testes and hatching of the egg 1 or 2 mitoses occur. The larvae at the time of hatching from the egg may have gonads with 8, 10 or 12 primordia while others have 36 to 38 primordia per gonad (SONNENBLICK 1941). The histological data thus show that the number of primordia incorporated into these early larval gonads varies, or that a different number of divisions has occurred. KERKIS (1933) and GLOOR (1943) report that only spermatogonia are present 24 hours after the larvae hatch from the egg. KERKIS's observation of the presence of many division figures at this stage indicates a rapid multiplication of the spermatogonia. Granted that mitotic divisions occur as indicated by KERKIS's data, it is still not known whether each spermatogonial division occurs at the same time or whether there is an asynchronous type of division. Each spermatogonium could divide the same number of times before producing spermatocytes, or some spermatogonia could undergo more divisions than others. It should be noted that the larvae were X-irradiated during this early stage of spermatogenesis, that is, 20 to 22 hours after hatching from the egg.

The first spermatocytes are produced from the spermatogonia about 48 hours after the larvae hatch from the egg. Primary spermatocytes are produced in groups of 16 from 4 synchronous divisions of each spermatogonial element; from these groups, 64 haploid sperm are produced (PONTECORVO 1944; TIHEN 1946). Since there is no crossing over in the male, half the sperm (32), contain similar haploid strands from one chromosome and half contain similar strands from the other, except when a spontaneous mutation had occurred. Therefore, when a mutation is produced in the spermatogonial stage, a number of sperm with the same mutation may be recovered in the mature sperm sample. The spermatogonia continue to form spermatocytes which in turn form mature sperm. Any mutation present in the spermatogonia would also continue to appear in the sperm as long as the spermatogonial stem cell is active.

Genetic data on the cluster mutations offer the only method of studying the type of sampling obtained from the spermatogonia and also the relative activity of the mutant and nonmutant spermatogonia. Only 2 of the cluster mutations were suitable for such study. The affected male with an *ebony* cluster was mated to several *res* females and remated several times to new females. Offspring were collected over a period of a month. The following mutant and normal offspring were collected at 4 successive remating intervals.

Number of mutants	*Total sample*	*Ratio*
7	146	1:21
5	142	1:28
14	126	1:9
5	55	1:11
31	469	1:15

In the second cluster, involving the mutant hairy, the affected male was remated to a separate female every 24 to 36 hours; the ratios are based on the total number of mutant sperm recovered among offspring from each female. The time can be expressed in terms of successive (daily) fertilizations by the male.

Female No.	*Mutants*	*Total sample*	*Ratio*
1	2	108	1:54
2	6	239	1:40
3	2	202	1:101
4	3	88	1:29
5	4	133	1:33
	17	770	1:45

Homogeneity within the 2 clusters was measured by computing the χ^2's. For the first cluster, $\chi^2 = 7.66$ which for three degrees of freedom corresponds to a probability of .056. In the second cluster, $\chi^2 = 2.53$ which for 4 degrees of freedom corresponds to a probability of .63. Since the smallest expected frequencies in the first and second clusters are 3.63 and 1.94, respectively, the χ^2 approximations may be inaccurate. As a rough guide, however, homogeneity is indicated in the second cluster; the value for the first cluster is in the questionable range.

The genetic data from these two clusters show a rather random distribution of mutant sperm among the normal sperm. No large clumps of mutant sperm were recovered in any one part of the sample. Sperm produced from a number of different spermatogonia must be mixed in the vagina of the female when insemination occurs or large clumps of mutant sperm (32 are produced from one division of the mutant spermatogonia) would have been detected. LÜNING (1952b) has also reported a mixing of sperm from different inseminations. This mixing of sperm (from different stem cells) in the vagina obscures testing to determine the synchronization of the stem cell divisions. However, synchronous and asynchronous types of divisions can be inferred from the consistency or variation in the ratios of mutant and normal sperm over the sampling period. Asynchronous divisions of a few nonmutant spermatogonia,

which are in a relatively larger number than the mutant spermatogonia, would produce slight variations in the ratio of mutant to normal sperm and would be more difficult to detect than an additional division of the 1 mutant spermatogonium. The stem cell divisions may not be perfectly synchronized but, whatever the mechanism or mechanisms may be, the ratios vary no more than might be expected from chance. The absence of any progressive decrease in the number of mutant sperm as compared to normal sperm throughout the sampling period shows that mutant spermatogonium is not becoming inactive before nonmutant spermatogonia. These two clusters showed no selective action against the mutant spermatogonium in this respect.

Estimation of the mutation rate for spermatogonia is complicated by the following fact: The sample of F_1 flies collected from each male, which had been treated when a larva, was usually between 115 and 150. However, if a mutant individual was detected in this sample, the male parent was retained and remated. Most of the samples of F_1 progeny that contain mutants (column 4, table 4) are, therefore, considerably larger than those which contain no mutants. The most satisfactory method for estimating the mutation rate seems to be to calculate the mean of the rates found in the progeny of each of the 1797 males. Since most of the males had no mutant offspring, this mean is simply

$$\frac{1}{1797}\left(\frac{5}{598}+\frac{17}{770}+\ldots+\frac{18}{388}\right)=1.09\times10^{-4},$$

for all 8 loci and for 900 r, or 1.52×10^{-8}/r/locus. The expression within brackets is obtained from columns 3 and 4, table 4. This estimate is, however, biased. If the samples in which mutants were found had not been augmented, then cases in which a mutation occurred in the spermatogonia but was not present in the sperm sampled, would have been compensated for by a corresponding excess proportion of mutants in the samples that contained mutants. With augmented samples this compensation will not be complete and the above method of calculating the rate will be expected to underestimate it. However, in the present data, this bias is not likely to be large, and probably does not invalidate the conclusions drawn from the estimated rate.

To facilitate the estimation of confidence limits, the value for the rate may be regarded as approximately what would have been obtained if the calculation could have been made from 10 observed occurrences of mutation in the (actually unknown) number of chromosomes tested. The 95 percent confidence limits of 10 are 4.80 and 18.39 (FISHER and YATES 1949). Expressing these in terms of the rate of 1.52×10^{-8}, they become $(0.728 \text{ and } 2.79)\times10^{-8}$. Since the upper limit falls well below the lower limit of the 95 percent confidence interval for the mutation rate in mature sperm, it appears that, even when allowance is made for the bias in the estimate of the spermatogonia rate and for error in the estimation of its confidence interval, the rates in sperm and spermatogonia are significantly different.

DISCUSSION

The previous studies on mutation rates at specific loci in Drosophila have used mature sperm and have often utilized loci with high mutation rates. The highest rate reported for any locus of *D. melanogaster* is that of the white locus by TIMOFÉEFF-RESSOVSKY (1933). A rate of 15 to 20×10^{-8}/r was obtained for this locus when the allele from one strain, designated as American strain, was tested. A mutation rate of 11×10^{-8}/r was obtained for the same locus when another strain was tested. BONNIER and LÜNING (1949) obtained a rate of approximately 8×10^{-8}/r for white if only those mutants which had been tested by progeny tests were included. The rate approached 12×10^{-8}/r when the untested variants were included. The same approximate values were obtained for the forked locus in the same experiment. TIMOFÉEFF-RESSOVSKY and DELBRÜCK (1936) obtained a rate about one-half this value for forked but VALENCIA and MULLER (1949) obtained a value around 2.5×10^{-8}/r for the same locus. These studies show that different alleles of the same gene may have different mutation rates and may produce different patterns of mutant alleles (TIMOFÉEFF-RESSOVSKY 1933). The high mutation rates obtained for these genes seem to represent the upper range of Drosophila mutation rates. Three loci, pink, curled, and ebony, of the 8 tested in the present study gave rates of around 8×10^{-8}/r but the remaining 5 gave lower rates. The roughoid and claret loci of PATTERSON's study gave high rates but the hairy locus gave a rate of 0.5×10^{-8}/r. Rates lower than that of the hairy locus were obtained for the sex-linked mutants, *ras, sc* and *ac* by VALENCIA and MULLER (1949). An average rate for a number of loci, some of which have high rates and others which have low rates, will give a better general estimate for mutation rate in Drosophila. The average rates of 5.98×10^{-8} and 4.39×10^{-8}/r found in the present experiment and by PATTERSON therefore seem more representative than rates at a specific locus.

A differential sensitivity of mature and immature germ cells of Drosophila to X-ray treatment has been indicated by the lower number of mutations recovered from immature than from mature cells. A drop in the number of sex-linked lethals recovered from irradiated adults has been found by several workers (HARRIS 1929; HANSEN and HEYS 1929; DEMEREC and KAUFMANN 1941) to occur 14 to 16 days after treatment in *D. melanogaster*. This drop was explained by HARRIS (1929) as either germinal selection against the lethal or differential sensitivity of the two stages to X-radiation while HANSEN and HEYS believed the difference to be due solely to the latter explanation. KOSSIKOV (1937) found that the same type of drop in the number of sex-linked lethals occurred 6 days after treatment when adult males of *D. simulans* were irradiated. He attributes a differential sensitivity to the mature and immature cells and bases the possibility of selection against hemizygous lethals in the immature cells on the comparison of data from irradiated males and females. Since the reduction division does not occur in eggs until after fertilization, his data from females are not entirely comparable to those for the male.

The work of SHAPIRO and SEREBROVSKAIA (1934) and SHAPIRO (1936) on induced lethals of the X and second chromosome in mature and immature germ cells offers data which might indicate that both factors may be involved to produce the differences found between the X chromosome and autosomes. Adult males of *D. melanogaster* were irradiated and mated to appropriate marker females. The data from the first 6 days of the test were designated for the mature sperm test. New females were crossed to the treated males after 6 days and were left with the males for 14 days. The males were remated to virgin females on the twentieth day and data were collected from the twentieth- to twenty-sixth-day mating periods for immature cells. The comparative frequencies of recessive lethals in the X and second chromosomes were found to be 1 : 2.66 for the mature sperm and 1 : 5.60 for the immature cells. SHAPIRO explains the difference in these ratios by germinal selections against lethals hemizygous in the male, and estimates that about 50 percent of the lethals which arise in the immature cells do not reach the spermatozoa stage.

The use of adults with a designated time interval for mature and immature cells leaves some doubt as to the actual stage being treated. The classification of mutants as lethals in this type of test is hard to evaluate since several situations can produce a lethal action. A difference in the number of sex-linked visible mutations recovered by MOORE (1934) for treated adults and larvae gives a better indication of a difference in the 2 stages. By mating to attached-X females, the sex-linked lethals were eliminated. A greater number of viable sex-linked visible mutations were recovered from the mature sperm than from sperm of males which had been treated as larvae.

A comparison of the mutation rates at specific autosomal loci in the mature sperm and spermatogonia shows a lower rate for the latter. For these loci, the confidence interval of 0.728 to 2.79×10^{-8}/r/locus for the spermatogonial rate falls below the values of the confidence interval of 4.64 to 7.34×10^{-8}/r/locus obtained for mature sperm. The data seem to indicate a significant difference in the rates of visible mutations for the 2 stages. This difference in the rates cannot be explained by any kind of germinal selection against mutations in the hemizygous condition since autosomal loci were used. The selection against the hemizygous X in the male may occur but in general the available data from Drosophila do not support germinal selection.

Histological examination of irradiated immature stages of *Pediculus corporis* and *D. melanogaster* by PONTECORVO (1944) indicates that no germinal selection occurs. In Pediculus the meiotic divisions occur early in spermatogenesis so that the germ cells are haploid earlier than in Drosophila. In Pediculus with a dose of 4000 r PONTECORVO reports the following:

" It should be noted that all usual chromosome rearrangements are produced and can be seen (testing of spermatozoa for dominant lethals confirms it) : but the cells carrying these rearrangements, many of which are ' lethals,' go through six mitoses and spermateleosis undisturbed. Apart from the direct observation of no cells individually killed, counting the spermatozoa in each bundle (easily done in cross sections) confirms that no germinal selection takes place in all these stages. The only exception is that occasionally a whole

cyst is killed; but this is almost certainly a secondary effect of some injury to nongerminal tissue."

For Drosophila, PONTECORVO continues:

"What histological observation is possible in *Drosophila* confirms that the same situation, *mutatis mutandis*, holds here too. Irradiated testes show necrosis of a small number of apical cells (homologous with the premeiotic stages in the louse) and no necrosis of cells in the spermatogonial divisions, meiosis and spermateleosis, again with the exception of occasional cysts degenerating as a whole."

The data from gynandromorph studies by PATTERSON and STONE (1938) do not show any serious selection during embryological development against aneuploid cells as compared to cells with the normal component of chromosomes. The number of gynandromorphs in each of the detectable classes produced from treated males and carrying an extra fragment of a broken chromosome in the male tissue was as follows: Six were three-fourth male tissue, 25 were one-half male, 13 were one-fourth male, and 5 contained less than one-fourth male tissue. The one-half and three-fourth gynandromorphs could not have been recovered in these relative frequencies if drastic selection had occurred against the aneuploid cells.

A test of tolerance to heterozygous deletions of portions of the third chromosome of *D. melanogaster* was reported by PATTERSON, BROWN and STONE (1940). Individuals hypoploid for different regions of the third chromosome were obtained by segregation from 2 heterozygous translocations which were broken at different points on the chromosome. Some of the hypoploid males survived and were fertile with heterozygous deletions as long as the longest deletions recovered from irradiated sperm in the present experiment. Competition with normal gametes did not eliminate these gametes.

Since dominant Minutes are often produced by small deletions, the recovery of such Minutes from treated spermatogonia in the present experiment indicates the survival of this type of deletion. About one-half the total number of Minutes were recovered from the spermatogonia as groups of two or more from the same tested male; the remaining Minutes were recovered as single individuals from different males. The Minutes from both sperm and spermatogonia were phenotypically similar and were usually weak and semisterile. In a few cases from both tests, they produced a few offspring, some of which showed the dominant Minute effect. In the mature sperm data 96 Minutes were recovered in a sample of 49,512 at 3000 r; in spermatogonia, 106 Minutes were recovered in a sample of 273,574 F_1's at 900 r. In the control tests, 14 Minutes were recovered in a sample of 45,504, with 3 of the 14 recovered from 1 male. Therefore proportion of Minutes to normal would be 1 : 3250 for the controls; 1 : 2580 for spermatogonia at 900 r and 1 : 516 for mature sperm at 3000 r. These two types of data support the assumption that deletions will survive in the immature cells of Drosophila.

Translocations when heterozygous in the males give about 50 percent aneuploid sperm from nondisjunction (BROWN 1940). The aneuploid sperm

produce zygotic mortality as shown by egg counts. The chance of recovering a translocation present heterozygous in spermatogonial cells would be reduced one-half by this zygotic mortality. However, this reduction is not a determinate factor in the present experiment, since a small number of this type of aberration would be expected at 900 r. This possible source of reduction in variants will not account for the difference in the sperm and spermatogonial mutation rates.

In testing the variants from irradiated sperm WARD found that about half the mutations tested had no detectable chromosome aberrations, the other half being composed of deletions, inversions, and translocations (WARD and ALEXANDER 1952, and unpublished). This 1 : 1 ratio at 3000 r would not be expected at 900 r judging from the translocation study on *Drosophila virilis* by BAKER (1949). The frequency-dose relation showed that the percentage of sperm with interchanges increased more rapidly than the first power of the dose for 1000, 2000, and 3000 r of X radiation. On this basis, approximately 0.2 as many two-break rearrangements would be expected with a dose of 900 as with 3000 r. Mutants with no chromosome rearrangements (point mutations) would be expected to increase linearly with dose and 0.3 as many point mutations would be expected at 900 r (OLIVER 1932). The ratio of point mutations to chromosome aberrations should approach a 3 : 2 ratio for 900 r as compared to a 1 : 1 ratio for 3000 r. If the number of position effect mutants calculated on this basis from the mature sperm data is added to the number of spermatogonial mutants, the confidence intervals for the two stages would still not overlap. An average mutation rate can be calculated for mature sperm on point mutations without including the position-effect mutants. With about half the mutants from mature sperm at 3000 r being associated with visible chromosomal abnormalities, the rate of point mutations is about 3.0×10^{-8}/r/ locus. Although this is twice the rate of point mutations recovered from spermatogonia, the 95 percent confidence ranges overlap to some degree.

Mutation rates for the mature sperm and the premeiotic spermatogonia show a different amount of genetic damage from X-radiation. The data from different Drosophila studies do not support the assumption that drastic germinal selection occurs. This difference in radiation damage may be due, in part, to the difference in probability of inducing variants caused by chromosomal aberrations. With the present sample size for spermatogonial cells, the absence of such aberrations among the recovered spermatogonial mutants can be considered indicative but not conclusive evidence that they do not occur. LÜNING (1952a) found a difference in rate of breaks measured as dominant lethals and hyperploid males in the various stages of the postmeiotic cells of spermateleosis. This difference was not found in the test for " point mutations " and gynandromorphs. There was a definite drop in the number of hyperploid males, mutations, and gynandromorphs when cells which were treated in meiotic or premeiotic stages were tested. These tests indicate a reduction in the genetic effects of radiation in premeiotic cells but an increase in sensitivity in the spermatid stage even above that of sperm.

A comparable study of mutation rates at specific loci in another species of

Drosophila has been reported by GIRVIN (1949). Seven loci on the X chromosome of *D. virilis* were used in his experiment. When only the proven mutations were counted and the rates for the 7 loci were calculated in the same way as in the present experiment, the following values were obtained: yellow, 4.69×10^{-8}/r; crossveinless, 2.24×10^{-8}; vermilion, 4.64×10^{-8}; singed, 11.34×10^{-8}; dusky, 3.52×10^{-8}; white, 12.67×10^{-8}; apricot, 13.81×10^{-8}/r: average rate for 7 loci, 7.56×10^{-8}/r. A determination of the confidence limits for this average would be difficult since a different number of individuals were checked for some of the loci. This rate would undoubtedly overlap the range of 4.64 to 7.34×10^{-8}/r/locus obtained for the third chromosome of *D. melanogaster* in the present experiment. The average rate of 4.39×10^{-8}/r/locus obtained by PATTERSON for the third chromosome of *D. melanogaster* is also quite similar to the *D. virilis* rate. This comparison of average rates shows no obvious difference in the rates for the 2 species.

In general, the mutation rates at specific loci induced in Drosophila sperm have been found to be lower than the spermatogonial mutation rates reported by RUSSELL (1951) for autosomal loci of the mouse. The mean observed rate for the spermatogonia of the mouse of $(25 \pm 3.7) \times 10^{-8}$/r/locus for 7 autosomal loci is significantly higher than the mean spermatogonial rate of 1.5×10^{-8}/r/locus obtained for 8 autosomal loci of Drosophila. The mean mutation rate for spermatogonia in the mouse is also significantly higher than the mean mate of 5.98×10^{-8}/r/locus obtained for the mature sperm of Drosophila.

The recent demonstration that several more loci are members of pseudoallelic series makes it necessary to consider the effect of pseudoalleles on specific loci studies. Pseudoalleles have been reported for the Star locus by LEWIS (1945), for lozenge by GREEN and GREEN (1949), for vermilion by GREEN (1952, and unpublished), and for white by LEWIS (1952). Very similar cases have been reported for scute and achaete by DUBININ (1933) and by SEREBROVSAÎA (1938). Since a mutation in any one of the component parts of a pseudoallelic series will produce a mutant allele, the relative radiation hazard is still expressed by the mutation rate. However, the number of component parts which are contained in a pseudoallelic series could be reflected in the mutation rates. A locus with several component parts could give a higher mutation rate than a single component locus.

SUMMARY

1. Mutation rates were obtained for 8 specific loci on the third chromosome of *Drosophila melanogaster*. With a dose of 3000 r of X-radiation, the rates for mature sperm varied from 2.7×10^{-8}/r for the thread locus to 8.75×10^{-8}/r for the peach and ebony loci. The 8 loci gave an average rate of 5.98×10^{-8}/r/locus. Of the 58 X-ray-induced mutations, 20 were viable, 35 were lethal, and 3 were semilethal when homozygous.

2. Spermatogonial cells in larvae 20 to 22 hours old were irradiated with 900 r of X-radiation. Single mutant individuals and clusters of the same mutation were recovered from adult males which has been treated as larvae. A variation in the size of the clusters indicated that the number of sperma-

togonia under test varied from 7 to more than 100. The spermatogonial mutation rate is estimated to be approximately 1.52×10^{-8}/r/locus. Four spermatogonial mutants were viable, 3 lethal, and 1 semilethal in the homozygous condition.

3. A lower average mutation rate obtained for spermatogonia than for sperm can be better explained by a differential genetic sensitivity of the 2 stages than by germinal selection.

4. The average mutation rate for the 8 loci of the third chromosome for *D. melanogaster,* from treated sperm, is similar to the average sperm rate obtained by GIRVIN (1949) for the X-chromosome of *D. virilis.* The average spermatogonial rate of 1.5×10^{-8}/r/locus for *D. melanogaster* is significantly lower than the spermatogonial rate of $(25 \pm 3.7) \times 10^{-8}$/r/locus obtained by RUSSELL (1951) for the mouse.

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