THE RELATIVE CONSTANCY OF THE X-RAY-INDUCED MUTATION FREQUENCY OF DROSOPHILA MELANOGASTER SPERM IN INSEMINATED FEMALES¹

S. ABRAHAMSON² AND J. D. TELFER³

Department of Zoology⁴, Indiana University, Bloomington, Indiana

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ALTHOUGH it has been known for many years that mutation rates were not constant within the male germ cells, being, for one thing, dependent on the age of the treated animal (OFFERMANN 1939), it remained for the crucial work of LÜNING (1952a and b) to dissect the daily changes in germ cell sensitivity in males, proving that chromosome breakability varied very definitely within short intervals with different stages of spermiogenesis and age at treatment. Among other things, it was shown that sperm X-rayed in females give a significantly higher mutation frequency than those irradiated in males within a few days before their discharge (LÜNING 1953, 1954; BONNIER and LÜNING 1953; ABRAHAMSON and TELFER 1954).

Evidence was also obtained, by MULLER, HERSKOWITZ, ABRAHAMSON and OSTER (1954), that the variation in breakability that exists among germ cells irradiated in the male can result in strong selective effects caused by dominant lethals. When the dose was increased there was an increasing selective elimination of offspring from the more mutable sperm. Thus the observed mutation frequency at a high dose but not at a low dose fell far below the frequency with which mutations had actually been induced, and the frequency-dosage relation was thereby falsified. In order that a more accurate determination of this relation might be made a project was then organized by MULLER to discover, if possible, conditions under which sperm of reasonably homogeneous and constant mutability might be obtained. It was thought that the most likely stage for this purpose would be that of spermatozoa in the inseminated female, since these were presumably in a stable, virtually mature condition, that could be prolonged for a considerable period.

In pursuit of evidence concerning this question it was decided that the present experiments should test the influence of the following conditions of aging on the observed mutation frequencies derived from sperm irradiated in females:

- (1) aging versus non-aging of the sperm in females before their irradiation;
- (2) aging versus non-aging of the sperm in females after their irradiation;
- (3) aging versus non-aging of the males before they discharged the sperm that were to be irradiated in the females.

In addition, further data were sought on the relation between the mutation frequen-

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² Eigenmann Fellow in Zoology.

³ Present Address: University of Louisville Medical School, Louisville, Ky.

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cies in sperm irradiated in the female and in those irradiated in the male within one or two days of their ejaculation.

MATERIAL AND METHODS

The stocks used in all the experiments to be described were of the following genotypes: "Multi-males", $sc^8.Y/y$ *In49 B*; *bw^D*, and "Multi-females", $X.Y$ *InEn y*; *st*. The $X.Y$ chromosome here used had been kindly supplied by DR. NOVITSKI. These stocks had been made up by MULLER for the purpose of simultaneous detection of sex-linked recessive lethals, sex chromosome losses, and translocations (for description of the stocks and genetic technique see MULLER 1954a).

Since the $sc^8.Y$ chromosome contains the normal allele of yellow, the male stocks are phenotypically grey bodied and brown, Bar eyed. The female stock has an X chromosome, the entire euchromatic region of which is inverted. The short arm of the Y chromosome is attached to the left end of the X , and the long arm of the Y is attached to the right end. Since this Y chromosome does not contain the sc^8 attachment the females appear yellow. Originally there was no free Y chromosomes present. However, crossing over between homologous X chromosomes can produce $X.Y$ chromosomes deficient for regions of the Y and also free partial Y chromosomes which may remain undetected in the stock.

The same X-ray machine was used throughout the course of all the experiments. Its output over a one-year period showed less than 10% variation, as determined by readings of a Victoreen dosimeter before and usually after the irradiation of the flies. A 1 mm Al filter was used and the flies, contained in perforated gelatin capsules, were irradiated by a G. E. Coolidge tube at 135 KVP, 20 ma, at a distance of 21.5 cm from the tungsten target. The dosage rate was about 220r/min.

In Experiment I, testing the effect of the aging of sperm in the females, a total of 1100 virgin "Multi-females", 24-48 hours old, forming series 61, were mated to non-virgin "Multi-males", 2-4 days old, for 24 hours, after which time the males were removed and discarded. To insure their non-virginity prior to use these males had been transferred 24 hours after hatching to fresh vials along with equal numbers of their sisters. The 1100 "Multi-females" after insemination were aged in bottles containing the standard sugar-agar medium (50 females per bottle). This sugar-agar medium was used (following the technique employed by MULLER in his experiments on the effects of aging of sperm on spontaneous mutation) to conserve sperm during aging by retarding egg laying. On this scheme the females were transferred to fresh sugar-agar medium every 5 days until the fifteenth day. At that time the females were etherized and placed into gelatin capsules for irradiation.

Two other series of inseminated females, 63 and 65, respectively, consisted of a total of 1850 virgin "Multi-females" (1-2 days old) that were mated to non-virgin "Multi-males" (3-4 days old) only 24 hours before irradiation. At the end of this 24 hours the males were removed and discarded and the females placed into gelatin capsules designating to which series they belonged. All the females of the three series were irradiated simultaneously, with 4000r of X-rays.

Immediately following the irradiation the females of series 61, containing sperm

aged 15 days prior to treatment, were placed, 50 females per vial, into freshly prepared vials containing standard culture medium and were transferred every 3 days for three successive "broods". About 750 of the females inseminated from 0 to 24 hours prior to irradiation, forming series 63, were also placed into culture vials, 50 females per vial, and transferred in the same manner as series 61. Although series 63 is relatively unaged it should be understood that some of the sperm tested in brood 3 were held for as long as 9 days subsequently to treatment. The remaining 1100 females, forming series 65, following the treatment were placed in bottles containing sugar-agar medium, 50 females per bottle, and aged for 15 days. During this 15-day period they were transferred to fresh sugar-agar medium every 5 days, and after this period they were transferred to culture vials, 50 per vial, for three 3-day broods, following a procedure identical with that employed for the other series.

The numbers of parents per bottle were here chosen so as to avoid overcrowding of offspring yet to provide enough of them to keep the food in good condition. The object of this was to minimize differences in selective effects on the developing F_1 that might otherwise, by more adversely affecting the mutants as compared with non-mutants in some series than in others, tend to make the frequencies found unrepresentative of those induced.

Another group of flies that was irradiated simultaneously with the above three was a group of 160 virgin "Multi-males", $2\frac{1}{2}$ – $3\frac{1}{2}$ days old. These males were immediately afterwards mated to virgin "Multi-females", 8 pairs of parents per vial, for 24 hours, forming series 67. Then the males were removed and mated to another group of virgin females for an additional 24 hours, forming series 68. The females of series 67 and series 68 were replaced in their vials, after removal of the males, for 2 days, and were thereafter transferred through two more broods, each of 3 days duration. The reason that smaller numbers of parents were used per culture in these cases was because of their higher productivity, caused by the facts that (1) the females' germ cells had not been irradiated and (2) the mutation rate is lower for sperm irradiated in the male than in the female.

The offspring of all the five series were examined for the presence of exceptional yellow males produced through complete loss of either the X or $sc^8.Y$ chromosome, long deletion of the X, or partial loss or mutation of the Y, involving the sc^8 attachment. These yellow males are easily distinguished from the "regular" class of gray males. The "regular" males were individually tested for translocations by backcrossing them to virgin "Multi-females". In the earlier work (ABRAHAMSON and TELFER 1954) it had been planned also to test the female offspring for recessive sex-linked lethals but after a considerable number of these tests had been carried out it was discovered that, through a rare coincidence, a preëxisting lethal had been present in the X chromosome of some of the parental males, so near to the left end as to be "covered" by the sc^8 portion of their Y. This circumstance invalidated the determinations of lethals.

Experiment II, performed at a different time, was designed to determine the difference, if any, produced by irradiating, in females, sperm derived from young males as compared with sperm derived from older males. To provide the sperm from

the young males, 1- to 2-day-old virgin "Multi-males" were mated to virgin "Multi-females" for two days. The inseminated females were thereupon irradiated with 4000r of X-rays, after which they were transferred through several 3-day broods, in bottles containing 25-30 females each. The same males as had been used for these inseminations were thereafter aged for 9 days with two consecutive groups of previously virgin females, for 4 and 5 days respectively, and then were mated to a new group of virgin "Multi-females" for 2 days. After that, the procedure followed for this older male series was the same as employed with the younger male series.

The offspring resulting from Experiment II were examined for exceptional males. In addition, it was found practicable in this experiment to carry out the test for sex-linked recessive lethals, since the preëxisting lethal was always covered by the $sc^8.Y$ chromosome. For this purpose, the F_1 heterozygous Bar-eyed females were individually mated in culture vials with two sibling males. The F_1 males were not tested for translocations, however, in Experiment II.

RESULTS

The summarized results of Experiments I and II are shown in tables 1 and 2, respectively. In table 1 the Y translocations are not included except when they also include II-III translocation because of the possibility some of the females failed to carry an X with all the Y-fertility genes, in which case some of the F_1 males carrying a chromosome translocation would have failed to breed.

TABLE 1

Summarized frequency of yellow males and of translocations (II-III including Y-II-III) obtained following treatment with 4000r X-rays

Series	$y \sigma^\sigma$ /total σ^σ	% $y\sigma^\sigma$	Translocations/ cultures tested	% translocations
61 (inseminated ♀ ♀ aged before irradi.)	8/286	2.8 ± 0.9	21(1)*/106	19.8 ± 3.8
63 (inseminated ♀ ♀ not aged)	21/586	3.6 ± 0.8	60(1)*/326	18.4 ± 2.1
65 (inseminated ♀ ♀ aged after irradi.)	17/678	2.5 ± 0.6	52/253	20.6 ± 2.5
67 (1st day sperm from irradi. σ^σ)	49/1842	2.7 ± 0.4	74(1)*/651 (2)**	11.4 ± 1.2
68 (2nd day sperm from irradi. σ^σ)	46/2631	1.8 ± 0.3	81(2)*/1240	6.5 ± 0.7

* Those cases representing probable translocations but could not be confirmed and are not included in the calculations.

** Represent doubtful but untestable cases of translocations and excluded in calculations.

TABLE 2

Summarized frequency of yellow males and sex-linked recessive lethals in paternal X from ♀ ♀ inseminated by young or old males prior to irradiation with 4000r X-rays

Series	$y \sigma^\sigma$ /total σ^σ	% $y \sigma^\sigma$	Lethal cultures/total cultures	% lethals
Young σ^σ	53/1393	3.8 ± 0.5	145/1073	13.5 ± 1.0
Old σ^σ	38/1192	3.2 ± 0.5	115/821	14.0 ± 1.2

DISCUSSION AND CONCLUSIONS

In Experiment I the values for translocations are more significant than those for yellow males because (1) the larger numbers of translocations obtained give them a higher statistical reliability; (2) translocations because of their comparatively small phenotypic effects, are relatively unaffected by differential viability that varies with culture conditions; and (3) translocations, on account of their frequency ordinarily varying as the $3/2$ power of the effective dose, are expected to show an especially high variation of frequency with dose and so to provide an especially sensitive index of mutagenesis.

While none of the three inseminated female series show significant differences from each other an idea of the limits of variability of the aged series can be obtained by taking into account the approximate error to which the ratios of the percentages found in any of the series considered two at a time would be subject (see MULLER and JACOBS-MULLER 1925). Thus considering the series (61) aged before irradiation and the non-aged series (63) it can be expected of samples of this magnitude in only 5% of the trials that frequencies as closely in agreement as the present one, and differing in the given direction, would be obtained, if the frequency of series 61 was really 57% larger than that of series 63, or if it was really 40% smaller. In other words, taking confidence limits of 95%, the frequency of series 61 would have a range from 57% larger to 40% smaller than that of series 63. Similarly, as regards the series (65) aged before irradiation and the non-aged series (63), the range for the frequency of series 65 is found to be from 50% larger to 25% smaller than the frequency of series 63.

All three series show translocation frequencies considerably higher than that of the first day sperm (series 67), and the difference is significant, being <0.05 in the comparison of series 61 with 67 and <0.01 both in the comparison of series 63 and 65 with 67. The comparison of the translocation frequencies of series 67 (1st day sperm) and 68 (2nd day sperm) shows the former to be significantly higher than the latter, P again being <0.01 . In fact, the translocation frequency is about doubled on passing from sperm treated 1–2 days before ejaculation to those treated 0–1 days before, just as it is doubled on passing from the latter to those treated after ejaculation.

The results presented indicate that the physiological conditions of aging the sperm when it is in the female, Experiment I, either before or after the irradiation, or aging males prior to insemination, Experiment II, have no appreciable effect on altering the mutation frequency and therefore demonstrate that the mutation rate in experiments performed at the same time with these different sperm is relatively constant. These physiological conditions do play an important role, however, as previous workers and the present results (series 67 and 68) have demonstrated, when the male is irradiated prior to insemination and successive ejaculates are tested.

It must be mentioned at this point, however, that a part of an investigation carried out in this laboratory by MULLER, HERSKOWITZ and OSTER (as yet unpublished) showed that translocation frequencies in the inseminated female were significantly different with the same dose of X-rays (1060r) when the experiments were performed at different times but under apparently as similar conditions as could be achieved. In one of these two experiments, utilizing approximately 1060r and 4240r, the trans-

locations appeared to follow the linear rather than $3/2$ power rule in their frequency-dosage relationship, whereas this was not the case in the other experiment, which utilized a lower range of doses (265r, 1060r and 2650r). Their results at approximately 4000r are nevertheless in excellent agreement with the results herein reported. Our own (earlier obtained) results at 600r (ABRAHAMSON and TELFER 1954), 1.18% II-III type translocations, when compared with the combined results, 19.4%, herein reported and those of MULLER, HERSKOWITZ and OSTER at about 4000r do follow the expected $3/2$ rule. The causes for the differences between the two results at 1060r and between the frequency-dosage relations in the experiments cited are as yet unknown. MULLER, HERSKOWITZ and OSTER are inclined to the interpretation that even the spermatozoa in the female may vary in mutability from one experiment to another, and even within the same female, the latter variation resulting in a selective effect that obscures the frequency-dosage relation.

The present data have again confirmed the phenomenon of apparently higher breakability of sperm chromosomes in the inseminated female than in sperm within a few days of their discharge from the male (LÜNING 1953; ABRAHAMSON and TELFER 1954). The reason for this phenomenon is still unknown. BONNIER and LÜNING (1953) have ruled out an influence of egg cytoplasm on the increased lethal rate in sperm chromosomes. LÜNING (1954a) has hypothesized that sperm stored in the female are more susceptible to breakage, possibly because of a different physiological environment.

If an appreciable proportion of the translocations found after irradiation of inseminated females had involved maternal chromosomes, one would expect to find a decreasing frequency of translocations when the less mature germ cells of the females were utilized in succeeding broods. This was, however, neither the case in our earlier work (ABRAHAMSON and TELFER 1954) nor in these experiments. Furthermore, in the present experiments nearly all the translocations were tested for an extra generation to determine their origin and all but one of these were found to be in paternal chromosomes. In still another experiment, utilizing the same stocks, virgin females were irradiated with 3600r X-rays. Over 1300 cultures derived from them were examined for translocations; the maternal translocation frequency in this experiment also was found to be extremely small; three cases were found, all involving the X.Y chromosome.

Still another leading hypothesis, that of differential restitution, postulated by BAKER and VON HALLE (1953), also can be considered as applicable to this discussion. This hypothesis states that the breakability of the chromosomes in the treated spermatozoa is the same in the different cases but that only restitutional and not other types of union can occur in 1st and 2nd day sperm, more of it occurring in the latter than in the former. (For a critique of this hypothesis see LÜNING 1954b). One new piece of evidence bearing on this question can be added here. Recent experiments in this laboratory by MULLER, HERSKOWITZ and OSTER (1955 and unpublished) have shown that when the inseminated females are irradiated at a higher CO_2 tension or in the presence of a different atmospheric environment, containing less oxygen, there are significantly fewer translocations than on irradiation in air. Since it has been demonstrated by MULLER, RAY-CHAUDHURI and MAKHIJANI (see MULLER 1939, 1940) that

no restitution occurs in sperm when it is stored in the female, it would appear that one would have to assume, to support the differential restitution hypothesis if applied to mature sperm stored in the female, either that restitution can occur under abnormal atmospheric conditions, or that the type of break produced under different conditions will influence the likelihood of its undergoing restitution rather than rearrangement at the time of fertilization. BAKER and EDINGTON (1952) have indeed postulated an effect of the latter type in the experiments of KAUFMANN and HOLLAENDER (1946), but it is regarded as unlikely by MULLER (1954, pp. 559-562). The differential breakage hypothesis of LÜNING is clearly the other main alternative. We believe that the unequivocal answer to this problem in *Drosophila* is still forthcoming.

SUMMARY

Experiments were performed to test the influence of aging on the mutation frequency of sperm irradiated in females under different conditions: 1. It was found that there was no significant difference in the II-III translocation frequencies obtained when sperm were not aged and irradiated, aged and irradiated, and irradiated then aged. 2. When sperm discharged from aged versus non-aged males were irradiated in females no significant difference was found in the sex-linked recessive lethal frequencies obtained.

Significantly higher mutation frequencies were again obtained, however, for sperm irradiated in females as compared to sperm irradiated in males. Moreover, sperm ejaculated on the second day after treatment had a translocation frequency about one half as great as those ejaculated on the first day after treatment, and the frequency of this latter group was almost one half that of sperm treated in females.

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