A NONLINEAR RELATION BETWEEN X-RAY DOSE AND RECOVERED LETHAL MUTATIONS IN DROSOPHILA[1]

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SINCE the demonstration by OLIVER (1930) of the linear relation between the frequency of recessive lethal mutations induced by application of ionizing radiation to the sperm of Drosophila and the dosage of radiation used, numerous confirmatory experiments have been reported. It has usually been held that a strictly linear relation exists throughout the range of doses which can be studied, even at the highest ones. If this is true, however, important problems of interpretation regarding mutational mechanisms are raised, because of the connection which is known to exist between some lethals and some structural chromosome changes, in view of the fact that the frequency of the chromosome changes increases more rapidly than the first power of the dose (see TIMOFÉEFF-RESSOVSKY 1939; LEA and CATCHESIDE 1945; HERSKOWITZ 1946, 1951; FANO 1947; MULLER 1950, 1954). It has therefore seemed desirable to obtain further data concerning the lethal frequency-dosage relation, over a dosage range within which there would be a considerable alteration in the proportion of lethals connected with structurally changed chromosomes.

In most previous work on dosage relations little attention has been paid to the exact age of the males used or the exact period, after irradiation, when the sperm were discharged, so long as the period of low lethal frequency beginning about 12 days after irradiation was avoided. However, a series of recent papers by LÜNING (1952a, b, c, d) have shown the importance of these factors. He concludes that chromosome abnormalities, and the lethals connected with them, are at a maximum in offspring derived from sperm released between 7 and 11 days (passed at a temperature of 25°C) after irradiation, especially if the males were not more than a few days old when they were exposed. In other words, in young males, those sperm which are not due to be discharged for some 7 to 11 days are at a stage which is especially susceptible to the induction of chromosome changes. It was therefore decided to use this stage in our own experiments, in order to emphasize effects dependent on chromosome changes.

METHODS

Adult males of a wild-type (" Oregon R ") stock were irradiated when they were less than 24 hours old from the time of eclosion. Approximately 4350

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males were given 4000 r, 1100 males 1000 r, and 350 males no irradiation (these serving as controls). As usual, a 1 mm thick Al filter was used and the flies were exposed in gelatin capsules, supported on a thin board (about 3 mm thick), with no heavy material in the neighborhood. The peak kilovoltage was 135. A Victoreen dosimeter was used to check the output, just before (and sometimes also after) the flies were treated. In order to have the higher dose as nearly as possible four times the average of the lower dose, the flies for the two doses were irradiated simultaneously, according to the following scheme. The capsules containing the flies for the higher dose were kept in the X-ray field during the entire interval necessary for them to receive the 4000 r, but this interval was divided into quarters, with intermissions of about one minute between them. During each of these quarters a different capsule with flies for the lower dose was in the field, one low-dose capsule being substituted for another during each intermission. This procedure was gone through on each of three successive days, since on any one day only one-third of the total number of flies used was available for irradiation.

Within two hours after exposure the irradiated males were placed in uncrowded culture vials, with equal or slightly larger numbers of wild-type virgins (Oregon R). They were left with these for 7 to 7½ days, at 25°C. In the middle of this period both males and females were transferred to freshly made-up vials, in order to maintain the flies in good condition. The control males were handled similarly. The purpose of these first matings was not to obtain offspring for testing but only to promote elimination of sperm that were ready for discharge, during the week following irradiation. At the end of this period, the males were transferred to another set of vials, into which females of " Basc " stock (see below) were placed, while the wild-type females and the cultures in which they had been kept were discarded. The newly made cultures with the Basc females were the first set (" brood 1 ") from which offspring were to be obtained for the lethal tests.

In about half of the brood 1 cultures the Basc virgins used were ½ to 2½ days old at the time they were placed with the males, while in the rest they were 7 to 8 days old, separate records being kept of the results from the two age-groups of mothers, since there had been a suspicion that maternal age might influence the results. As it was desired not to have great differences in the rigor of selection, as affected by crowding, among the developing offspring of the three dosage groups to be compared, the numbers of parental flies per vial were graduated approximately according to their productivity, as dependent on the amount of radiation which they had received. Thus, the control vials each received 3 males and 3 females, the low-dose vials 3 males and 4 females, and the high-dose vials 24 males and 30 ± 6 females. All vials used were provided with the standard yeast-enriched food, and all parental cultures were kept at 25°C.

After two days in the brood 1 vials the parental males were discarded and the parental Basc females were transferred to a second set of vials, constituting brood 2. After 4 days in brood 2 the females were transferred to the

last brood, numbered 3, and left there 4 to 6 days. Because of the fact that the males were discarded at the time of removal of the parents from brood 1, when they had lived 9 days and 9 to 10 hours since being irradiated, the offspring of all three broods alike were derived from sperm discharged 7 to 9½ days after irradiation.

The Basc stock (which has been referred to by some geneticists as " Muller 5," but for which we prefer the present designation, derived from its contained mutations) is constructed to facilitate the finding of sex-linked recessive lethals. It contains the dominant marker Bar (*B*) and the recessive apricot (*apr*), as well as (to avoid the occurrence of crossover offspring) a long inversion-combination (In sc^{81} L-In sc^{8} R) associated with changes in the locus of scute (*sc*), and also a moderate-sized inversion (InS) included within the long one. The regular (not nondisjunctional) F_1 females from the cross of Basc mothers with wild-type fathers are readily distinguished from those produced by nondisjunction, contamination, or " non-virginity " (prior mating of the mothers with males of their own stock). These desired F_1 females need not be obtained as virgins, since for the detection of lethals in their paternal X-chromosome the most suitable mating is with Basc males like their brothers. These females were therefore bred, one per vial, with Basc males (those from stock cultures being preferred to their brothers, however, because of the lower fertility of males with irradiated chromosomes). The presence of a lethal in the paternal X was evidenced in the next generation (F_2) by the absence of wild-type males. Doubtful cases were carried further by the breeding of those F_2 females which had a phenotype like that of the F_1 females.

RESULTS

On account of the large numbers which were desired in this experiment, the task of making up and scoring the F_1-F_2 and later cultures was divided among three groups of workers, respectively led by (1) HERSKOWITZ and ABRAHAMSON, (2) MULLER and (3) OSTER. Since, however, no differences in results other than those to be expected from random sampling were obtained the numbers will not be presented separately for these groups. A similar situation exists regarding the results from the three different broods, from flies treated on the three successive days, and from the P_1 females of the two age groups. That is, the results in all cases failed to show consistent differences from one another. For this reason only the combined results are presented in the accompanying table.

Among the controls 18 F_1 were found to contain lethals, but 16 of these, all derived from the same parental culture, obviously represented a single mutation that had occurred at a very early embryonic stage of a parental male. Earlier work by the senior author (MULLER 1946, and unpublished) has shown the frequency of such cases to be so low as to make it very unlikely for any other cases of the kind to have arisen in the present experiment. If, for this reason, we do not count this case, the observed control frequency of $0.05 \pm 0.04\%$, shown in the table, agrees well with that of 0.06% found in

the earlier work for second-week sperm of previously mated males. If on the other hand the group of sibs is included, the frequency becomes 0.45 ± 0.4%.

The frequency obtained with the low dose is, as expected on the basis of LÜNING's findings (or even more than on that expectation), considerably and significantly higher than the frequency of about 3% (including spontaneous lethals) ordinarily obtained by application of 1000 r to spermatozoa, when sperm discharged during the first week after irradiation are used. That the high rate in the present case was a result of the stage at which these sperm were irradiated is further shown by information supplied to us by DR. J. I. VALENCIA, who obtained in our laboratory, shortly after the present experiment was completed, a frequency of recessive sex-linked lethals of very nearly 3%, from sperm of young Oregon-R males discharged within some five days after irradiation with 1000 r. Thus our present rate is approximately double that for earlier-released, more fully mature sperm.

On the other hand, the induced frequency of 9.45% ± 0.5% obtained with the high dose is significantly lower than that of 11% (including spontaneous lethals) expected on the principle of a linear frequency-dosage relation under

TABLE 1

Frequency of sex-linked lethals derived from sperm released 7 to 9½ days after irradiation of 0–1-day-old males.

Dose	0 (controls)	1000 r	4000 r
Lethal X chromosomes	2(+16 sibs)	759	402
Total tested X chromosomes	3893	11,295	4,246
% lethals with std. error	0.052 ± 0.035 (ignoring group of sibs)	6.73 ± .25	9.50 ± .50
% induced lethals & error	0	6.68 ± .25	9.45 ± .50

ordinary circumstances, when the frequency at 1000 r is only 3%. (In citing this figure as 11% rather than 12% account is taken of "overlapping," i.e., of the scoring of only one lethal when more than one arise in the same chromosome.) But, in relation to the frequency of 6.7% actually obtained at 1000 r, that of 9.5% obtained at 4000 r departs far more from the expectation for linearity. Using the values for induced mutation frequency given in the table, which are corrected for a control frequency taken as 0.05% ± 0.04%, the ratio of the induced frequency for the high dose to that for the low dose is found to be 1.41 (± 0.09) : 1. This would make 1.65 : 1 the upper limit for the ratio of frequencies at a confidence level of 100 : 1, in contrast to the ratio of 4 : 1 for the doses used. Even when the control frequency is taken at the improbably high value of 0.45% ± 0.4%, the ratio of frequencies turns out to be 1.44 (± 0.15) : 1, with an upper limit of 1.83 : 1 at the 100 : 1 confidence level or 1.94 : 1 at the 1000 : 1 level. Thus we may conclude that when the dose was quadrupled the frequency of lethals was not even doubled; that is, the frequency at the higher dose was less than half what it would have been on the linearity principle.

CONCLUSIONS

These experiments show that, under some circumstances, factors are at work which cause a considerable reduction, at high doses, in the frequency of recovered lethals, below that to be expected on a linear frequency-dosage relation. It is significant that in the present experiment, in which this effect was so marked, many or most of the sperm were at a stage which had two or more times the susceptibility of the fully mature sperm of young males to the lethal-producing and (judging by LÜNING's work) the chromosome-breaking action of ionizing radiation. If now the very probable assumption is made that not all the sperm which became discharged during the interval in question (from 7 to 9½ days after irradiation) were at the time of irradiation at just the same stage, and possessed of just the same susceptibility, the theoretical basis is provided for explaining, as a consequence of selection, the unexpectedly low value found for the high dose.

The reasoning on which this conclusion is based is as follows. If the sperm in question (either those of different males or of the same male) had been heterogeneous in their susceptibilities, then the more susceptible ones, those in which there was a higher induced recessive lethal frequency, would also have a higher chromosome breakage frequency. Since some of the broken chromosomes (those which were aneucentric or aneutelic) would tend to give dominant lethal effects in the zygotes, or, if the break had occurred at a spermatogonial stage, lethal effects in the germ cells themselves, it would follow that a smaller proportion of the more susceptible than of the less susceptible germ cells would produce viable offspring. That is, zygotic and/or germinal selection would work more against the production of viable offspring from the more susceptible germ cells, which contain more lethals than from the less susceptible ones, which have fewer lethals. Thus this influence reduces the frequency of lethals which are recovered (i.e., found) below that at which they are produced. Moreover, at the lower dose used by us, where there are relatively few chromosome breaks, this selective influence would be much weaker than at the higher dose. Hence the frequency of *recovered* recessive lethals would be depressed much more, relatively to the frequency of recessive lethals that had actually been induced, at the higher dose than at the lower dose. This would cause a departure from linearity in the observed direction, i.e., a flattening of the curve, even if the frequency of actually induced lethals had had a linear relation to dose.

Although it is not unlikely that the males differed somewhat from one another in their susceptibility, at the time of irradiation, of those sperm which were to be released 7 to 9½ days later, perhaps in correspondence with the speed at which the sperm of different males completed their development, or were moved along in the ducts, it is probable that, in addition, those sperm of any given male which were destined to be released in the period in question differed from one another in their susceptibility, perhaps falling into several distinctly different groups corresponding with their stages of development.

More extensive testing of offspring of individual males could give evidence on this point.

Another uncertain matter, alluded to above, is whether the less susceptible sperm of the present experiment were mainly those which had been more nearly mature at the time of irradiation, and therefore similar to most of those discharged soon after irradiation, or were mainly germ cells in spermatogonial stages (although these are believed by some authors not to become discharged until 12 days or more after irradiation). It has been well known since the work of HARRIS (1929) that the irradiation of spermatogonia gives a far lower lethal frequency than that of mature spermatozoa, and as was shown later it also results in a far lower frequency of dominant lethals and other chromosome aberrations among the resulting spermatozoa. Although the drop in these frequencies seems to begin only with the twelfth day after irradiation, and then to be very sharp, whereas in our experiment the sperm were discharged during the earlier (7-to-9½-day) part of the 7-to-11-day period of maximum frequencies, it is not unlikely, especially in view of work by FRIESEN (1936) on the timing of induced spermatogonial crossing over, that some irradiated spermatogonia were included among the germ cells which furnished the offspring studied by us. This would help to explain why the frequencies obtained by us with 4000 r were even lower than those which would have been expected from fully mature spermatozoa treated with this dose. In fact, there is no reason to suppose the number of germ cell types dealt with in this experiment to have been limited to two or even three; they probably formed a cline of considerable range, with more than one peak.

The question now arises, could not some selection of the kind in question take place even when the sperm under consideration are those released at an earlier period, a shorter time after irradiation? That this is probably the case when the males used are not very young at the time of irradiation (and therefore, perhaps, not so abundantly supplied with fully mature sperm), is indicated by LÜNING's findings that the sperm of such males, even those discharged within the first five days after irradiation, give frequencies of chromosome changes intermediate between those produced by the earlier and by the later (7-to-11-day) released sperm of males irradiated when newly hatched (see also the related finding by OFFERMANN 1938). LÜNING is inclined to interpret his result as meaning that in the older males (or in a group of older males) the sperm discharged in any given period are more heterogeneous than in young males, in regard to the stage at which they had been at any given period before their discharge. If this is true, then, as our own results indicate, there would be a selective effect of this heterogeneity when these males are used shortly after irradiation, similar to but not as strong as that occurring when sperm discharged 7 to 9½ days after the irradiation of young males are used. Hence results from older males, even when used soon after irradiation, would give spurious values for mutation frequency, values which tended to flatten the observed curve below the actual one.

If however this is true of old males it may even be true, although to a

lesser extent, for young males, and even when these are bred within a few days after their exposure to radiation. This consideration makes all previous experiments on the dosage-frequency relations of genetic effects more or less suspect, especially when high doses are concerned. This stricture is reenforced when it is recalled that in much of the work no great attention was paid to the age of the males used for irradiation, or to the length of time after irradiation at which the sperm were discharged which produced the offspring tested for lethals.

It may be objected that the very fact that the frequency-dosage relation observed in earlier work has appeared to be linear argues for the validity of the observed lethal frequencies, as representative of the frequencies actually induced. Despite the appeal of this argument, it is in some degree circular, inasmuch as it is the question of linearity (especially at high doses) which is at issue. The selective effect found so markedly in our results from sperm taken 7 to 9½ days after irradiation would have had to exist to only a very slight extent, in the data reported in earlier work, in order to be enough to cover up that rather small rise in actual lethal frequency above linearity at high doses, which would be expected if most recessive lethals associated with chromosome changes were expressions of position effects. Hence it is entirely conceivable, in the light of our present results, that the frequency with which lethals are produced increases more rapidly than the dose at high doses. A definite decision of this question awaits the carrying through of further dosage experiments, under conditions in which there are good grounds for inferring that the sperm are practically homogeneous in regard to their susceptibility to the induction of mutations. In the meantime, attention may be called to the rise above linearity for high doses which has been reported by STAPLETON, HOLLAENDER and MARTIN (1952) for visible mutations induced by X-rays applied to spores of Aspergillus (see also the results on Neurospora by SANSOME, DEMEREC and HOLLAENDER 1945, as analyzed by MULLER 1954).

The flattening of the frequency-dosage curve for mutations obtained in the present experiment with X-rays is very reminiscent of the flattening of this curve that has been found in material of diverse kinds, including Drosophila pole cells, when ultraviolet light is applied (for recent discussion of the ultraviolet curve see MULLER, ALTENBURG, MEYER, EDMONDSON and ALTENBURG 1954). In the case of ultraviolet also this flattening has in some cases been shown to be due, at least in part, to a selective effect, but this has usually been based on heterogeneity in the degree of exposure rather than in susceptibility. For germ cells of adult male Drosophila, however, in which the higher dose of ultraviolet actually resulted in a lower recovered frequency of recessive lethals, as observed by SELL-BELEITES and CATSCH (1942), differences in susceptibility were inferred to form the basis of the effect. It seems likely that even in work with ionizing radiation such differences would occasionally have been encountered in sufficient strength to give rise to significant deviations from the linear " expectation." They may well have been responsible for some of the irregular results reported in experiments dealing with the

frequency-dosage relations for mutations induced by X-rays in plant material (see e.g., STUBBE 1933; GUSTAFSSON 1947), although these may in larger measure represent differences in susceptibility itself (and not in selection) among different groups of treated individuals.

At the same time, it should be emphasized that at lower doses the selective effects here in question would usually be too weak to disturb the observed frequencies materially. Hence the linear relation established for these lower doses must be accepted as real, and as representing the workings of the primary mechanism of production of " point mutations " by radiation, whatever complications may be found at the higher doses.

SUMMARY

When 1000 r of X-rays were applied to adult males of *Drosophila melanogaster* which had hatched not more than 24 hours previously, and offspring derived from sperm ejaculated by these males 7 to 9½ days after irradiation were tested, a frequency of 6.5% ± 0.3% of induced recessive lethals was found in the exposed X chromosomes. This frequency, being more than double that ordinarily obtained, confirms LÜNING's finding that germ cells at the given period (i.e., that of 7 to 9½ days prior to ejaculation, in newly hatched males) are especially susceptible to mutagenesis by ionizing radiation. However, a dose of 4000 r, under otherwise identical circumstances, resulted in only 9.3% ± 0.6% of induced recessive lethals. This marked flattening of the lethal frequency-dosage curve at high doses is interpreted as an effect of selection, operating more strongly at higher doses to kill off preferentially, by chromosome breakage, the descendants of the more susceptible germ cells, in which recessive lethals had been induced at a higher frequency. It is inferred that the germ cells of the period in question are heterogeneous in their susceptibilities, and that there is a strong positive correlation between susceptibility to the chromosome-breaking and that to the recessive-lethal-inducing effect of X-rays.

It is pointed out that heterogeneity of a similar kind probably exists, to a lesser extent, in the germ cells of a period shortly before ejaculation, when older males are used, and that it may even be present to some extent in the germ cells of that period in young males. In view of these considerations, and the fact that in most earlier work the importance of exactly controlling paternal age and germ-cell stage was not realized, the significance of earlier data purporting to show the continuing linearity of the lethal frequency-dosage relation at high doses becomes uncertain, and conclusions based on a supposed linearity in this dosage region should be held in abeyance until more definitive data can be obtained, on material of maximal homogeneity.

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