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AUG 31 1965

MASTER

REPORT ON THE TERMINAL PERIOD,

FROM DATE OF 1963 REPORT TILL JUNE 30, 1964

ON U.S. ATOMIC ENERGY GRANT CONTRACT NO. A.T.(11-1)-195 (MULLER)

Date of this statement August , 1965

1. Title of Research Project: The influence of radiation in altering the incidence of mutations in Drosophila.

2. Institution: Indiana University Foundation, Research Division, Bloomington, Indiana

3. Leader of Project: H. J. Muller, Distinguished Service Professor, Department of Zoology, Indiana University, Emeritus since July 1, 1964

4. Work accomplished on grant in terminal period:

Because this report will be accompanied or shortly followed by a summary report on the work done during the entire period of 13 years, from 1951 through 1964, of this grant, the work of this terminal period will be described in less detail in relation to its amount, complexity, and significance, than that of the preceeding years had been. The unfortunate lateness of both of these last reports, for which apology is herewith tendered, has been caused by a combination of several circumstances, set forth below.

For one thing, the project leader, who reached retirement at Indiana University on June 30, 1964, before the data obtained since the last report could be duly collated, moved on that date to a new position in California. Prior to that date much time had to be expended in preparing for the moving and in connection with all the extra activities that are connected with the closing of an academic year. On top of that, as usually happens with new positions, much immediate work had to be undertaken after arrival in California, in order to settle down and to adjust to the new and very different appointment.

The project leader was then far removed from his Research Executive, and from his other former co-workers and assistants on this project, and this greatly hindered his collation and interpretation of the voluminous and complicated data. Meanwhile, in the new position, it was urgent for him to undertake new projects. Finally, for the past five months, he has been laid up with a protracted and incapacitating illness, and although he is definitely recovering the process is very slow and has not until very recently allowed him to return his attention to the data or the reports.

As for the preceding two years so also in the terminal period, the major effort was devoted to the attempt to obtain more definitive evidence as to whether ionizing radiation applied to Drosophila oögonia is more effective in producing gene mutations, as judged by the frequency of recessive sex-linked lethals, when administered as a concentrated, intensive ("acute") treatment than when administered in the same total dosage as a protracted, mild ("chronic") treatment.

RELEASED FOR ANNOUNCEMENT
IN NUCLEAR SCIENCE ABSTRACTS

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In the attack on this problem, the set-up devised and instituted chiefly by Dr. William R. Lee, Research Executive on the 1962-'63 grant, and already described in our previous report, was first utilized. It may be recalled that its theory was to attain precisely equal total doses of acute and chronic treatments bearing a known ratio of dose-rates to one another by having as the source of acute and chronic radiation, respectively, a given concentrated solution of a radioactive substance ($Cs^{137}Cl$) and a known dilution of the former (1 in 336), the lengths of exposure being exactly reciprocal to the concentrations, and all other conditions of exposure that determine radiation filtering, scattering and distance being alike. As a further check on relative (and absolute) dose, mature spermatozoa were simultaneously irradiated within the same females as those carrying the oögonia to be tested for sex-linked lethals, the offspring of the former (sperm) being differently marked genetically, and obtained and tested earlier, than the offspring (later derived from other matings) of the latter (oögonia).

This test of sperm turned out to have been essential, since it was unfortunately found that the first such pair (chronic and acute) of irradiations gave rise to substantially higher mutation frequencies, in both male and female germ cells, than the (several months) later ones, as if a considerable part (similar but rather more of the low concentration) had somehow escaped! More likely is the gradual evaporation of some of the water in the solutions (somewhat more from that of less concentration because of the more retaining influence of the solute in the other) with resultant shortening of the vertical columns of the radioactive source and a lesser average exposure of the flies, which are negatively geotropic. (For future experiments involving the given dilution principle, which is in itself unexceptionable, we would therefore urge the use of mixtures of dry powders rather than solutions.) Thus the total doses and their ratios had to be estimated by the results from the spermatozoa, and these total doses, being little more than half the intended ones, induced lethals at frequencies having larger relative errors than desirable.

These errors, in the gonial results, were further increased by the fact that, as the unirradiated controls showed, there was an unusually high (nearly three times the usual) "spontaneous" mutation frequency in both oögonia and spermatozoa of the stocks used, to be subtracted from the frequencies found in the irradiated series. The errors were also enlarged by reason of there having been so many and sometimes such large "clusters" of oögonial lethals (each resulting from the multiplication of one mutant oögonium). Although this source of error could have been reduced by ignoring all clusters larger than two, and (as we have calculated) more "significant" results thus obtained, that procedure would have been invalid, for there were doubtless more daughters bred per treated female after one type of treatment than after the other, and in the case of one treatment fewer irradiated oögonia may have survived, and these would therefore have given rise to a larger proportion of the offspring than in the case of the other treatment, and so to more of the large clusters.

Excluding the results from the aberrant first irradiation, whose sperm tests were too few for satisfactorily establishing relative doses, and combining those (which were substantially alike) from the other two irradiations, it turned out that, when all clusters were included in the reckoning and were allowed their due errors, the more highly concentrated solution, which delivered to the flies a total of about 2000r, had resulted in a frequency of sex-linked lethals induced in the oögonia about 1.45 times as high, per r, as did the dilute solution. The latter had delivered about 2350r spread out over a period ($10\frac{1}{2}$ days) that was 336 times as long as the 45 minutes given the former. The actual dose-rates therefore had been some 2667r per hour for the concentrated versus 9.35r per hour for the dilute treatment, a dose-rate ratio of about 286 instead of 336, provided that

mature spermatozoa are really quite unaffected by dose-rate differences. However, the errors of the "sperm-corrected" oögonial frequencies obtained at the two widely differing dose-rates turn out to be so large that the difference between them was somewhat less than the standard error of that difference.

Therefore, these findings could by no means be considered significant in proving a dose-rate effect -- at least, not in itself -- despite the sophistication, in some respects, of the methods used. At the same time, this difference does lie in the same direction as has been shown recently by the trend of our other experiments. Moreover, the actual frequencies per r obtained at the two given dose-rates agree fairly well with those obtained in our other experiments at those dose-rates, as will be pointed out below. We will postpone a more general over-view of our results, however, until our over-all report.

In our preceding report it was mentioned on manuscript p. 9 that we had just begun, by employing still another physical technique, the study of whether differences in the nearly 100-fold range of "acute" dose-rates lying from some 3000r per hour (and thus like the "acute" rate with the $Cs^{137}Cl$ irradiation) and 240,000r per hour resulted in different mutation frequencies. This method, by utilizing the powerful 300-KVP X-ray machine at Purdue University that Dr. A. B. Burdick had invited us to use, enabled us, by stepping down the amperage in a controlled way and leaving the distance of the flies from the target and all the other physical circumstances unaltered, to give two treatments, of which one was known to be twenty times as low as the other in intensity but, as a result of its duration having been made twenty times as long, was known to constitute the same total dose. Then, by increasing the distance , another pair of doses, spanning a still lower but known range of rates, could be given similarly, the higher-rate one matching as far as possible the previous lower-rate one, the results from the tests of spermatozoa indicating the degree of this matching. As before, spermatozoa and oögonia were irradiated within the very same flies. Here again, then, the techniques were such as -- barring unforeseen errors -- to allow the dose-rate ratios to be precisely predetermined, and later checked upon by the biological as well as physical dosimetry.

These experiments were carried out on a considerable scale, mainly by Dr. Rinehart and his associates and assistants. In but a few cases the unirradiated controls fortunately gave the low spontaneous frequencies usually found, in both male and female germ cells. The results of the irradiations were clear, and consistent among themselves, as well as with those of our other experiments at such dose-rates. The most concentrated ("acute") X-ray treatments, those at 240,000r per hour, proved to have a mutagenicity for oögonia virtually identical with that of 2.1% for 4000r found for the previous very acute treatments, which had employed cobalt-60 gamma irradiation at nearly twice this rate, in the "hot room". X-irradiations at 15,000r and 12,000r per hour were again virtually identical in their results with one another and with those of the more highly acute irradiations.

Only the series irradiated at 3000r gave somewhat different results, namely, about 1.8% for 4000r, just as had been found for the $Cs^{137}Cl$ irradiations at about that dose-rate. It is true that in the 3000r per hour X-ray irradiation at Purdue the difference between the effect found and those of the more acute treatments

given there was only just "significant". Balancing this reservation, however, was the fact that an earlier cobalt-60 irradiation at some 6000r per hour had given results which, though overlapping them both in its standard error, were nicely intermediate between those of the 3000r per hour treatments and all the highly acute irradiations.

It is also to be noted that these X-ray treatments carried out at Purdue gave a good demonstration of the equal mutagenicity to spermatozoa of the dose-rates over the entire range there used. For the results from this biological dosimetry matched those from the physical dosimetry, as well as the calculations based on the changes in current, time, and distance. Thus, whatever doubt there might be about whether 3000r per hour is less effective than are more acute dose-rates, there can be no doubt of the substantially equal effectiveness of dose-rates from 12,000r per hour to the highest tested. And there can be no doubt that the frequencies here found by these methods are significantly higher, at least 1.5 times as high, as those found in our earlier experiments, although by other methods (perhaps not giving results so well comparable), at "chronic" dose-rates. Our evidence concerning the range of rates over which this seeming transition in effectiveness probably occurs will be reviewed in our over-all report.

Another series of experiments of the year here being reported on dealt with influences, if any, of dose-rate differences on the mortality induced as an after-effect in the individuals that were themselves exposed to radiation. As in our earlier experiments on such mortality in Drosophila, types having chromosome constitutions differing in such ways as to cause chromosome breakage and loss to make cell death more or less likely were compared. Unfortunately, the irradiations that had been relied upon for giving the differing dose-rates -- those with the $Cs^{137}Cl$-solution techniques -- proved to give, at the chronic levels, doses that were too low to be sufficiently damaging by themselves. Thus they, and the parallel acute treatments, were in the later work both supplemented by a more acute X-ray treatment.

A description of the details of these necessarily intricate experiments would be too lengthy for this report. Neither have the calculations based on the complex results obtained yet been sufficiently worked out. It is nevertheless safe to say at this stage that, as expected in view of our earlier results, these results do give evidence of chromosome loss as the pathway by which the cell damage is produced. Secondly, this damage also is to some extent dose-rate dependent, being lower at chronic rates. This difference may well express the fact that a significant proportion of the losses were, at the more concentrated irradiations, caused by misunions of different chromosome fragments that had resulted from two or more breaks, arising within a limited period of space and time along independent ionization tracks.

Pursuing radiation mutagenesis in another direction, the project was followed up under the project leader's direction and the A.E.C.'s support, of investigating the concept -- for which evidence had been reported by others -- that there is a "recovery" from some of the preliminary mutagenic effects (on genes as well as on chromosome breaks) in the mature spermatozoa of young males when sperm are held over for a day between their irradiation and ejaculation. This study was carried out successfully by William E. Trout, III (now Ph.D.) as a part of that for his doctoral degree (see our bibliographic reference). Mutation frequencies were determined among ring X-chromosomes (thus excluding most structural changes) of young males from ejaculations into successive females, performed every half-day subsequent to irradiation, both in cases in which (1) they ejaculated each half-day, (2) they skipped ejaculation on one or more half-days although given the opportunity, (3) they were not given the opportunity during the first two half-days.

The results indicated strongly (1) that the sperm that are fully mature at irradiation are more radiosensitive and are (when the male is given the opportunity) ejaculated first, (2) that when the earlier ejaculations are prevented the later one(s) include a mixture of any sperm that were fully mature, along with those that were less mature at the time of irradiation, (3) that when a fertile male fails to utilize the opportunity to ejaculate during the first one or two half-days it is usually because he did not yet contain enough sperm that were fully mature (and therefore especially radiosensitive) at the time of irradiation, and, finally (4), that no evidence remains for the idea that there is a "recovery" from gene-mutagenesis, or even for structural changes, in sperm when they are held over within Drosophila males. Unbeknown to us, the same conclusions were being drawn by Dr. George Lefevre on the basis of work, in part very similar, that was being carried out independently and simultaneously at Harvard University. Meanwhile too, partly with the grant's aid in our laboratory, H. Traut (German Ph.D.) reached like conclusions.

Another doctoral candidate working with the A.E.C. grant's support under the project leader's direction, Dale E. Wagoner (also Ph.D. now), was concerned with the exceptionally high radiosensitivity of the heterochromatin in regard to the induction of structural changes. By the use of stocks especially constructed for the purpose by the project leader, X-chromosomes were obtained that had almost no heterochromatin as seen at mitosis (and therefore, also, as present in the spermatozoon) and almost double the usual amount, the latter X-chromosome being almost double the former in its total size as seen at mitosis. As expected on the basis of the project leader's much earlier (but usually ignored) work on the subject, the frequency of recovered translocations (after the irradiation of the mature spermatozoa contained in inseminated females) proved to be not very different in these two chromosomes of radically different sizes and certainly nothing like in the almost 2:1 ratio of their size. This is true despite the fact that in normal X-chromosomes about one-third of the breaks that result in structural changes occur in their heterochromatin, which constitutes about one-third of their length as seen at mitosis, and that, similarly, about as many breaks that result in structural changes occur in the Y-chromosome, which is nearly all heterochromatin and about the same total size as the X at mitosis, as in the X itself.

The interpretation of this result is clarified by the consideration that the portions of the heterochromatin resulting in its (relative to its active gene content or its size at interphase, as represented by the salivaries) large size as

seen at mitosis are constituted of special, closely neighboring genes, that give rise at mitosis and in sperm to chromatic enlargements ("blocks"), having little or no influence on the chromosomes' radiosensitivity, breakability, or tendency to give mis-unions of fragments. Thus the exceptionally high proclivity of heterochromatin to result on irradiation of spermatozoa (or "spontaneously") in breaks that mis-join is not an expression of a genuine proportional relation between the size of a chromosome region, as seen at mitosis, and its tendency to undergo breakage and mis-union, as so many have thought, but an expression of a much higher like proclivity of a given length of its actual "gene-string" to undergo these changes as compared with the same length of a euchromatic region. For the seeming proportionality in the case of mitotic chromosomes can be destroyed by removing or adding blocks.

In the same work, Wagoner unexpectedly obtained evidence for the concept (earlier proposed by us on theoretical grounds) that the exceptionally high radiosensitivity of heterochromatin to the induction of structural change represents, in considerable measure at least, if not entirely, a much greater likelihood of its fragments than of euchromatin fragments, after having arisen by breakage, to engage in mis-union rather than in restitution. This evidence lay in his finding that the translocations undergone between the X-chromosome and the major autosomes, although similar in their total frequency in the mitotically short and long X-chromosome, consisted in the case of one of these X's of about twice as many mis-unions involving large chromosome II as equally large chromosome III, whereas in the case of the other X the ratio was just the reverse, the normal X undergoing both types of translocations with about equal frequency.

These radical differences can hardly be explained as due to differences of breakage frequency of the heterochromatic regions of the contrasted X's, in view of the total frequencies of the structural changes undergone by the heterochromatin being about alike and the autosome structure being alike. Thus, as Wagoner has pointed out, they must be reflections of drastic differences in the likelihood of the heterochromatic parts of the contrasted X's undergoing different types of mis-union. This effect would be a result of the types of association taken up by the parts (before and/or after fragmentation) in the cell, after union of pieces had been made possible. That being the case, the same general phenomenon, which should also extend to the likelihood of parts being placed so as to mis-join rather than restitute, might well account also for the much higher tendency of heterochromatin than a portion of euchromatin having the same "gene-string" length to undergo structural changes. That is, it becomes gratuitous to postulate that the heterochromatin is actually more "breakable".

Another major series of experiments, rather fundamental in the significance of their results, dealt with the question -- investigated in regard to radiation-induced gene-mutations by Muller, Carlson and Schalet (1961) -- of whether some spontaneous gene-mutations, like most of those induced by irradiation of spermatozoa, result from alterations of the pre-existing gene (in both its strands, in a complementary way), or whether as so often assumed they represent "errors in replication," as do many chemically induced ones. The stage chosen for this study was for technical reasons that of spermatozoa which are being held in inseminated females. For it is easy in Drosophila to double the frequency of mutations derived from the father by causing the female, through underfeeding, to retain her sperm

for some three weeks before laying many eggs. The offspring developing from the eggs then laid, having double the usual number of sex-linked lethals that had arisen in their paternal X chromosomes, must have had some 50% of these result from "spontaneous" mutations that were somehow initiated during their presence in the long-retained spermatozoa. Thus a comparison, among descendants of immediately used versus long-retained sperm, of the frequency of "whole-body" lethals -- apparent on testing the daughters (F_1) -- and "fractional" (mosaically occurring) ones -- often not evident until tests are made of the grand-daughters (F_2) derived from non-lethal-seeming F_1 females -- will indicate how often (if at all) the "spontaneous" gene-mutations initiated in the stage of mature spermatozoa involve both strands of the already existing gene rather than only one strand, or a daughter strand arising by replication of one present in the spermatozoon.

The work, ably managed by Dr. Rinehart, had to be on a large scale because of the relatively low frequency of spontaneously arising sex-linked lethals and because two generations of testing (by special methods) were required. However, the results showed clearly that, although the expected rise in mutation frequency with sperm retention occurred, there was no increased frequency of those of a fractional (mosaic) kind. It could therefore be calculated that, as in the case of mutations induced in mature spermatozoa by irradiation, those arising from causes that initiate them "spontaneously" during that stage involve for the most part the whole pre-existing gene, in both the complementary strands of the Watson-Crick structure, changing each in a manner complementary to the change in the other.

It is therefore proposed that in these mutations also our earlier suggestion applies, namely, that a base happened to be removed from each of two appositely lying nucleotides, to be replaced by a different base than before, and that whatever base became incorporated in the one, the complementary one became incorporated on the other side. This complementarity in the incorporation process could simply be the result of the already completed nucleotide exerting a guiding influence on what base should be inserted opposite to it, as happens after mutagenesis by ethyl-ethane-sulfonate in experiments reported by Freese and Bauch. Or it could be caused, mainly at least, by the fact that the two bases in question were more available locally, by reason of their having been removed (and perhaps still connected by hydrogen bonds), as in the author's working hypothesis of "rotational substitution". The latter more specific details, however, are not essential to our more general interpretation of removal of bases from both complementary nucleotides of the already existing gene, followed by the incorporation of bases different from those previously present but still complementary to each other. This consideration of course applies equally to the radiation-induced gene-mutations in question.

As in previous years, various special and often very elaborate stocks were devised and constructed by the project leader, at the cost of considerable time, during the last year of the grant, to serve as genetic machinery for use in the mutation work. Moreover, diverse new mutants of special interest were found and studied by him.

The study of data obtained during the last and even during earlier years of the grant, and the preparation of articles dealing with them, is expected to proceed for a long time yet.

Articles reporting work done in part or wholly by aid of this grant, that have been issued since the sending in of the previous report (May 29, 1963)

Muller, H. J., Oster, I. I., and Zimmering, S. 1963. Are chronic and acute gamma irradiation equally mutagenic in Drosophila? In Repair from Genetic Radiation Damage (ed. F. Sobels), p. 275-304; discussion on p. 305-311. Oxford, London, New York and Paris: Pergamon Press.*

Muller, H. J. and Oster, I. I. 1963. Some mutational techniques in Drosophila. In Methodology in Basic Genetics (ed. Walter J. Burdette), p. 249-274; discussion on p. 274-278. San Francisco: Holden-Day, Inc.*

Muller, H. J. 1964. The relation of recombination to mutational advance. Mutation Research 1: 2-9.

Muller, H. J. 1964. Synthesis [of Symposium on Radiation Genetics]. In Genetics Today (Proc. 11th Int. Cong. of Genetics), p. 265-274.*

Muller, H. J. 1964 (Nov.). Genetic effects of chemicals. In "Interbureau By-Lines" (of the U.S. Dept. of Health, Education and Welfare's Food and Drug Administration), p. 133-145.

Traut, H. 1964. Evidence for differential radiosensitivity rather than recovery in sperm samples from X-irradiated Drosophila melanogaster males. Genetics 50: 165-171.

Trout, W. E. 1964. Differential radiosensitivity as an explanation for so-called recovery in Drosophila sperm. Genetics 50: 173-179.

Muller, H. J. 1965 (Jan.). New mutants. Dros. Info. Serv. No. 40: 35-36.

Muller, H. J. 1965 (Jan.). An improved stock, "vix," for scoring visible mutations that arise at specific loci in the X of the female. Dros. Info. Serv. No. 40: 94-95.

*Articles that were listed in the previous report (May 29, 1963) as having been submitted but not yet published.