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and
RENEWAL PROPOSAL FOR THE PERIOD SEPT. 15, 1960 TO SEPT. 14, 1961,
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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, Professor of Zoology
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4. Work accomplished on grant:

Nearly all the objectives set in the technical program proposed by us last year, for work during 1959-'60, have already been attained, to a first order of approximation, in the studies ~~already~~ conducted since the time of that proposal. We intend, however, to review our progress on these matters here in a different order from that therein given, an order which, in the light of the results themselves, relates them in a more meaningful manner.

We regard as our most important conclusion of this year regarding genetic effects expressed in later generations the definite establishment for immature germ cells of *Drosophila* of a lower mutagenic effectiveness of gamma radiation delivered at a relatively low dose-rate than of that delivered at a relatively high one. The gamma irradiations and dosage measurements were carried out at the Brookhaven National Laboratory with the kind cooperation of Dr. H. J. Curtis, Dr. Dale M. Steffensen, Dr. Leonard La Chance and their assistants.

Adult inseminated *Drosophila* females of nearly the same age were exposed to 4000r of gamma radiation from a Cobalt-60 source, one group being irradiated evenly over 2 weeks at 12r per hour and the other group being given the same dose concentrated into approximately half a minute. The type, amount, and distribution

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of shielding and of accessory scatter-producing material were alike in the two groups. Non-irradiated control females were bred similarly, and in one series of experiments another group of females was exposed, for comparison, to 4000r of X-rays, delivered in about half an hour. The genetic set-up, which had been especially devised and constructed for the purpose (Muller D.I.S. 33: 149) allowed the testing with especial facility and reliability of the daughters ("P₁") of the experimental females ("P₁") for lethals that had arisen in the latter's X-chromosomes. Daughters derived from different "broods," i.e. from eggs laid at varying lengths of time after irradiation, were tested, in the earlier series, in order that ^a period might be chosen, for the more definitive comparisons, in which a plateau of mutant-gene frequency had been attained. The induced mutation frequency was determined by subtracting the control value from that obtained in the treated series.

As expected on the basis of our previous work, chromosomes derived from germ cells exposed to acute irradiation in a late oocyte stage showed frequencies of induced lethals similar to those derived from irradiated spermatozoa, and some five times as high as those exposed in the relatively early oögonial stages of 15 or more days prior to oviposition. Important for our work was the finding that the frequencies were not sensibly different among offspring from the brood of eggs laid 15 to 20 days after irradiation than among those from the brood of eggs laid 25 to 30 days after irradiation. For this establishment of a plateau of effect for so long a period made it legitimate to compare the frequencies of the acutely and chronically treated groups even though the latter represented a much less exactly delimited interval between exposure and oviposition than the former.

It was found that the acute exposures of oögonia to gamma or X-rays delivered 15 or more days prior to oviposition gave, as expected, very similar results, namely, about 1.8% of recessive lethals induced in the X-chromosome by 4000r, despite the fact that the intensity of the gamma radiation here was some 50-fold that of the

X-rays. On the other hand, the chronic gamma exposure to the same total dose produced, in oögonia at stages 15 or more days prior to oviposition, only about a third as many, namely 0.6%, of these lethals. The statistical error was enough to leave it uncertain whether the reduction here was as great as that of about 75% occurring in immature mouse germ cells, as found by the Russells. On the other hand, the error was not enough to cast doubt on the fact of reduction, and it was clear that the values in the chronic groups were distinctly less than 50% of those in the acute groups.

It is to be noted that our chronic treatments were more than an order of magnitude more intense than those of the Russells'. Moreover, this difference in intensity is in effect even more marked in view of the lower temperature of our biological material than theirs (25°C as compared with about 37°C), since this is, in chemical terms, equivalent to a shortening of the exposure time. It is possible, therefore, that (unlike what seems to be true in the work with mice), intensities have not yet been reached with *Drosophila* that are so low as to have the lowest mutagenic effectiveness: in other words, we may not yet have plumbed the "rock bottom" of the effectiveness. It would, however, be very difficult, for technical reasons--chiefly because of the relatively high spontaneous mutation rate, the low induced rate, and the short life span of *Drosophila*--to obtain significant data from this organism for rates much less intense than those already tested.

At any rate, the essential agreement between the *Drosophila* results and those in mice make it probable that a protective mechanism against the mutagenic action of ionizing radiation, one capable of "disarming" some two thirds to three quarters of the potential "hits" when these are widely scattered in time and place, is a phenomenon of extensive occurrence among the ordinary interphase cells of organisms in general. It is very likely therefore that it is at work in the immature germ cells of man also. It should be recalled, however, that results long ago obtained by the

author and his collaborators in *Drosophila*, as well as by Timoféef-Ressovsky, Stern and others, and more recently by the Russells in mice, have shown clearly that this mechanism does not operate in mature spermatozoa.

We have also carried out tests of the mutagenicity of chronic gamma treatments of the same kind, when given to oögonia of the same stage, in *Drosophila* being kept in a state of prolonged inanition by deprivation of yeast and of all dietary constituents except sugar. The intention here was to test whether the enzymes or other cellular materials needed for the protective action were present in smaller amount or less effective under these circumstances. The mutation frequency found was however not significantly higher than when better nourished, actively reproducing females were used. The disposal of this possibility will allow the simplification of our techniques of treatment in further tests. We will be further reinforced in this regard if an experiment now being conducted, to determine whether the chromosomes of oögonia of virgin females have a susceptibility to chronic irradiation similar to those of inseminated, actively reproducing females, shows that this supposition is in fact correct. Meanwhile, our tests of acute irradiation have already shown that with the intense treatment the mutagenicity is not appreciably different for the oögonia of the relatively inactive ovaries of ^{starved flies} virgins and for those of actively reproducing females.

Since it is evident from the linear relation, at high dose-rates, between recessive lethal mutation rate and total dose, that at these dose-rates the protective mechanism has become saturated and thereby rendered negligible, the possibility is raised that when radiation of high linear energy transfer is used, such as that derived from fast neutron treatment, there will be a similar saturation, on a local scale, that will allow even very low dose rate chronic treatment with such radiation to have as much effectiveness as acute treatments, or at least an effectiveness more nearly like that of acute treatments than is the case with gamma- or

X-rays. We therefore undertook a comparison of the mutagenicity of acute and chronic fast-neutron treatments, similar to that carried out with gamma radiation. Our preliminary results did show a difference in the mutation rates which, although in the same direction as with gamma rays, appeared (not yet significantly) to be of lesser magnitude. Unfortunately, however, it has been found, on physical retesting of the set-up for the chronic neutron irradiation given at Brookhaven, that the chronic treatment included some 40% of gamma contamination whereas the acute treatment included only about 5%. This contamination, in the total dose of 1500 rads, would be enough to explain our findings on the basis of the gamma radiation alone. The question is soon to be reinvestigated, with a set-up in which we are assured that the gamma contamination will be not substantially more in the chronic than in the acute series.

Our acute neutron treatments, in which the gamma contamination had been definitely low, have meanwhile given us some other results of interest. For one thing, they have confirmed earlier findings by the senior investigator and the Valencias that 1500 rads of fast neutrons derived from fission of uranium-235 have approximately the same mutagenic effectiveness in producing recessive sex-linked lethals, when delivered as an acute treatment to spermatozoa in the male, as 4000r of "acute" gamma or X-rays similarly delivered. Secondly, it is shown, for the first time, that this type and dose of neutron irradiation (1) of oöcytes, and (2) of oögonia, has, likewise, about the same mutagenicity, in producing these mutations, as 4000r of acutely delivered gamma or X-rays. Thus the RBE of the fast neutrons is consistently about 2.7 for these mutations, at these very different cell-stages and degrees of chromosome condensation, that differ from one another in their mutational susceptibilities by factors of as much as 5 or 6.

Some of these comparisons were carried out with a genetic set-up that allowed the detection, by inspection of the F₁, of small deficiencies (two-break deletions

that arise with a frequency linearly related to dose). These were of several different types, including both those having at least one break in a heterochromatic region (losses of the locus of non-yellow from scute-8 Y-chromosomes and scute-8 Y-chromosomes and of Bar from Y-chromosomes provided with it), and those (Notch-wings) having both their breaks in euchromatin. In the case of all types it was found that their frequency after neutron irradiation was roughly twice that after gamma or X-ray irradiation with the doses (1500 rads and 4000r, respectively) that were alike in their recessive lethal-inducing effectiveness. This was clearly true when spermatozoa were irradiated. The data for other stages were much more meager, in part because of the very low frequency of induction of such deficiencies in germs, yet the results from them showed significant differences in the same direction. Thus the RBE of neutrons for the production of two-break deletions whose breaks are so near together as to be referable to the ionization-track of the same fast particle is of the order of ⁴5. We believe that this higher RBE for these events than for "point mutations" can best be interpreted on the ground of the higher probability afforded for two ^{hits to be} nearby hits when the linear energy transfer is greater. This interpretation implies the premise that each break takes place at a site very close to that at which the ionization that occasioned it arose, i.e., that the length of the pathway from ionization to break is very short (of the order of one tenth micron or less, as our calculations indicate).

The same conclusion (of a very short mutagenic pathway for an ionization) is indicated for point mutations, by Carlson's case, described in our report of last year, of a point mutation, induced by the X-raying of a given spermatozoon, in one sub-locus of one chromosome strand and in a different but adjacent sub-locus of the same gene of the "sister" (presumably the complementary) strand. Further work by Carlson under this grant has confirmed the prevailing point-mutational

nature of mutations induced at this locus by X-rays, through the finding that in most cases but one sub-locus has been changed. Moreover, as Carlson and the present author are pointing out in an as yet unfinished interpretive paper on this and related work, the change so induced can be inferred from the distribution of mutant tissue to consist in an alteration of an already formed strand, rather than in a "mistake" in the replicative construction of a daughter (complementary) strand. Not infrequently the alteration must involve an exchange of complementary bases between the two original strands, thus changing both in a corresponding fashion and resulting in a "whole-mutant" offspring from an irradiated spermatozoon. Here the breaking of each of the two conjoined bases from its "backbone" strand was presumably accomplished by the localized action of two nearby ionizations formed in the track of the same fast particle, a process that would be occasioned with higher frequency, for a given total dose, (i.e. at a higher RBE) for radiation of higher linear energy transfer, as we have found to be true for recessive lethals.

Proceeding with our study of the overall effects on fitness of radiation-induced mutations present in heterozygous condition, we have now amassed, principally through the efforts of Mrs. Elisabeth Ehrlich, a much larger body of data than that mentioned in our report of last year. It will take a considerable time to complete the mathematical processing of these data. Such processing is essential since as we expected the over-all effects in the heterozygote are so small as usually to be lost to view in the variance caused by random sampling, differences in cultural conditions and other uncontrollable factors. A similar large-scale study, carried out under our National Institute of Health grant this year by Dr. Raphael Falk, is nearly ready for publication under that grant. It may be stated here that none of this work gives any support to the ideas advocated by Dobzhansky, Bruce Wallace, and some other geneticists that there is a tendency for heterozygosis per se of mutant

genes in general (including those induced by radiation), to have a favorable effect on fitness. A determination of the average magnitude of their unfavorable effect will, however, require work on such an enormous scale, or with material so specially constructed genetically for the automatic accumulation of the results of natural selection, that we have decided to conduct all our future work along these lines under our Public Health Service grant, thus leaving our AEC grant more scope for our other projected lines of research.

Not least among our present lines of research under our AEC grant is the study of the genetic basis of the somatic damage done by radiation to the individuals that were themselves exposed to it. Our predoctoral student Ostertag, working under the senior investigator's direction, has during the current year obtained what we believe to be important further information concerning this matter. The evidence that a deficiency, if already present in one member of a pair of homologous chromosomes, greatly increases the individual's vulnerability to radiation-induced mortality, has now been extended, in principle, to include not only the X-chromosome (an effect reported on by us last year), but also the two major autosomes (the "second" and "third" chromosomes). This shows, among other things, that the individual cells, unlike the early zygote as a whole, do not require a balanced complement of the major autosomes but can survive and function even if provided with only one member of a given pair, even though possessing two members of the other pair and of the X-chromosomes, for if this were not true the loss of one of them would not cause greater damage ^{when} the homologous chromosome contained a deficiency.

This evidence and some of that presented last year showed that it is the absence of the chromosome (consequent upon its breakage) that causes the somatic cell deaths leading to the induced mortality, rather than the formation of chromosome bridges that kill the daughter cells by entangling their nuclei. For deaths of

the latter type would be independent of the presence of deficiencies. Nevertheless Ostertag's results show that a certain type of chromosome--an X-chromosome with arms of the Y attached to it that had been found by Novitski to give rise to lethal chromosome bridges at meiotic divisions when by crossing over within a heterozygous inversion it acquired two centromeres--does tend to cause somatic cell death by bridge formation. This conclusion is based upon its presence, even as one member of a pair, occasioning a considerable rise in the induced somatic mortality. Evidently the broken chromosome here, forming a dicentric isochromosome, has a centromere arrangement strong enough to stretch the dicentric chromosome into an effective bridge, rather than leaving it caught in the middle of the division figure where it would be lost from both daughter nuclei. Individuals with such chromosomes, then, would have a heightened somatic vulnerability to radiation.

The question is thereby raised: how common are such chromosomes and do they predominate in some types of organisms. And do ordinary chromosomes ever behave in this way under certain conditions or in certain stages of development? That they may do so in *Drosophila* during the incipient stages of zygote development is already indicated both by the early work of Lamy and Muller and by recent tests by Ostertag. For in these studies males of ^{very} non-early stages were not more readily killed by radiation than females, a result indicating that in these stages the breakage of the Y is about as damaging as that of the X, even though the mere absence of the Y is known to be well tolerated in most cases.

Evidence from a different angle that chromosome breakage followed by non-restitution of the pieces lies at the basis of the induced somatic mortality was obtained by testing the effect of immersing the larvae in an atmosphere of nitrogen for several hours immediately after they were irradiated. Abrahamson in our laboratory had shown that such post-treatments given to oöcytes increased the frequency of the half-translocations studied by him and, as stated in our last report, Falk

had found this to be true also of full translocations induced in spermatids. The most probable interpretation was that since joining of broken ends was hindered in the absence of oxygen (see Wolff's studies in *Fredericantia*) the complementary broken pieces had more time to drift apart when there was a nitrogen post-treatment and therefore managed less often to reconstitute the original chromosome even though oxygen was later restored. Whatever the interpretation may be, however, the fact remains that if unrestituted chromosome breakage lies at the basis of the induced somatic damage it should, as in these genetically studied cases, be exaggerated by nitrogen post-treatment. This was in fact found to be decidedly the case in the tests conducted by Ostertag, even when due allowance was made for the mortality induced by the nitrogen treatment alone.

Even though much of the chromosome damage, especially at lower dose-rates and doses, probably results from individual breaks that are one-hit effects and therefore occur at frequencies linearly related to dose, it is not to be expected that the mortality would show a similar relation. For as the number of cells killed is increased the chance of death should at first be little affected and gradually rise more and more rapidly, until it finally levels off as it approaches 100%, thus following a sigmoid curve. This is the curve actually found by Ostertag, on comparison of the mortality occasioned by different doses given at the same stage (that of the late third-instar larva).

Because of the dependence of chromosome loss on cell division and the virtual absence of somatic cell divisions in the adult of *Drosophila*, it is to be expected that irradiations given after maturity has been attained would have far less effect on mortality than those given to immature stages, as we have found to be the case. In conformity with the cell-loss interpretation of the damage, however, the adult survivors of damaging irradiations given to larvae should be impaired in their functions and therefore exhibit a higher mortality throughout their subsequent life. Ostertag's studies of mortality have now been extended through a

considerable period of the adult life. They show that, although the greatest induced mortality following the irradiation of late larvae occurs, as expected, ~~following~~ ^{during} the critical pupal and immediately post-pupal periods, when nicely interadjusted transformations of form and function are required for survival, nevertheless the visibility of the adult of later stages is also reduced very distinctly, to a degree strongly correlated with the amount of the earlier noted effect. Frequently too the adults are visibly decrepit, lame and weak. This increased mortality persists throughout life. Unlike the ~~normal~~ ^{usual} curve of the mortality rate of untreated organisms, which in flies as elsewhere follows approximately the Gompertz law of having its logarithm rise linearly with age, the radiation-induced mortality-rate behaves as though it consisted of an approximately constant ^{amount} ~~increment~~ superimposed upon the Gompertz curve. It must be remembered that this represents the permanent effect of only one irradiation.

Articles, reporting work done wholly or in part by aid of this grant, that have or will be issued or delivered between date of last report and expiration date (Sept. 14, 1960) of this year's grant

I. Those listed in last report as in press, but issued since that time

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- Oster, I. I. 1959. The spectrum of sensitivity of Drosophila germ cell stages to X-irradiation. Radiation Biology, pp. 253-267.
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II. Those not listed in last report, but issued or delivered
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- Muller, H. J. 1960. Evolution and genetics. Atti d. Accad. Naz. d. Lincei,
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- Oster, I. I., S. Zimmering and H. J. Muller. 1959. Evidence of the lower
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of the sc⁸.Y.B^S. Dros. Info. Serv., No. 33: 175-176.

Zimmering, S., I. I. Oster and H. J. Muller. 1960. The high effectiveness of fast neutrons in inducing minute deletions. Science, 131: 1322.