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THE LACK OF PROPORTIONALITY BETWEEN MUTATION RATE AND ULTRAVIOLET DOSE IN *DROSOPHILA* *

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1. INTRODUCTION

ALTHOUGH mutagenesis by X and gamma radiation is effected by individual " hits ", these hits may comprise clusters of ionisations, and each ionisation may itself undergo degradation to clusters of excitations and of other effects involving lesser energy, and of varied kinds. As ultraviolet light, on the contrary, usually results in individual excitations, independently produced, but largely concentrated within given types of materials, it would seem in some respects better suited for the analysis of mutagenic phenomena. Any studies utilising it for this purpose should, however, proceed on the basis of information gathered in preliminary investigations, in which data have been obtained regarding the manner in which the production of mutations, and the recovery by the investigator of the mutations produced, is influenced by change in dose, and by change in the conditions of applying the dose. In the series of studies here to be reported some of these questions have been investigated in our material.

2. MATERIALS AND METHODS OF TREATMENT

Although much work has been reported on the relation between the mutation rate and dosage of ultraviolet, most of this has been done on microorganisms, and it was therefore thought desirable to investigate the problem further with *Drosophila* because of the unusual suitability of this material for the genetic analysis of the results.

However, the adult *Drosophila* is not very transparent to ultraviolet light. Only a very small fraction of ultraviolet that strikes the surface of an adult male can penetrate as deeply as the gonad. In order for appreciable amounts of ultraviolet to reach the germ cells of the adult fly, it is therefore necessary to use excessive doses, and these are highly damaging to the individual. Moreover, when the adult fly is treated, it is advisable to compress the abdomen between two plates, both to hold it in place during treatment and also to bring the gonads (testes, since oogonial and oocyte nuclei are still harder to reach) closer to the surface. As the degree and type of compression unavoidably vary somewhat from fly to fly, as well as the transparency of its surface layers to the ultraviolet, there is considerable uncontrollable variation in the dose that gets to the germ cells. Hence it is impracticable, for quantitative experiments on ultraviolet dosage, to treat the adult males, as is customary with X-rays. Recourse was therefore had to ultraviolet treatment of embryos, according to the technique first applied by Geigy (1931) and developed

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further, with special reference to mutation frequency studies, by E. Altenburg (1933-36).

At a certain stage in the development of the fertilised egg—the " polar cap " stage—the cells of the early germ track of *Drosophila* are located just below the vitelline membrane, at the amicropolar end of the egg, where they form a cap of cells (the polar cap, fig. 1). When the chorion (or shell) of the egg is removed, these polar cap cells are separated from the outside surface by only the thin, transparent vitelline membrane and thus are almost directly exposed to any ultraviolet light that strikes the overlying surface. Moreover, the polar cap cells are now readily visible under the microscope, so that it is possible to examine the eggs just before or after treatment and see whether or not they are in the polar cap stage. In the present experiments, only eggs from which the shells were removed (dechorionated eggs) were treated. Moreover, the eggs were examined under the microscope just before (Rice group) or just after (Indiana group) treatment, and any not in the polar cap stage were rejected. In addition, in the post-treatment examinations, those eggs, often constituting a majority of the total, were rejected which already showed visible signs of injury, such as vacuolisation and interruption of embryonic development, since these were found by experience to be in the great majority of cases moribund.

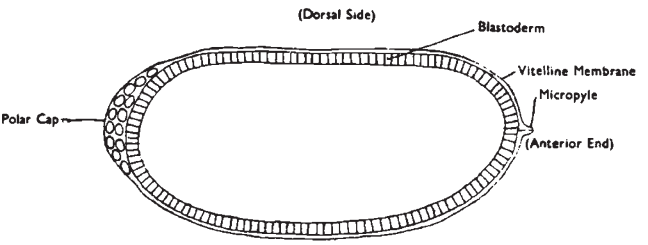

FIG. 1. A *Drosophila* egg at the polar cap stage of development, with chorion removed.

The polar cap stage sets in about two hours and fifteen minutes after fertilisation, at 25° C., and it lasts for about seventy minutes (see, for instance, the excellent summary by Sonnenblick, 1950). Most of the polar cap cells then migrate into a pocket, the proctodeal invagination, located under the dorsal surface of the egg just a little distance in front of their original position. Once in the pocket they are well below the surface of the egg and no longer directly accessible to ultraviolet light. Treatments must therefore be completed before the polar cap stage ends.

At 2½ to 3 hours after the beginning of development, the budding of cells into the polar cap from the underlying syncytium has ceased ; the blastoderm has been formed and its nuclei have taken on a characteristic elongated shape, and the polar cap cells are no longer found in division. There are then some 55 (37 to 73, according to Rabinowitz, 1941) pole cells present. Rabinowitz states that some of these migrate into the yolk, during the polar cap stage, and there disintegrate. When the pocket is formed, only some 30 or 40 pass into it, and finally only 10 to 26 of these, about half as many in the male (10 to 14) as in the female (18 to 26), become incorporated (without any intervening mitosis) in the gonads (Sonnenblick, 1941). As Poulson (1947) has shown, some of the pole cells which enter the pocket but do not get into the gonads take part in the formation of the midgut. At 16 hours after fertilisation, some 6 hours after the establishment of the gonads, cell division of the germ cells is resumed (Sonnenblick, 1941).

Any mutant gene originating in a chromosome of a polar cap cell is multiplied, in the course of mitotic cell division, to the same extent that the cell multiplies. Such mutants therefore are often found in " clusters ". Thus, for example, if the sperm cells of the mature male were derived from five polar cap cells, in one of which a lethal was induced, and if all these polar cap cells had multiplied equally,

then about $\frac{1}{8}$ of the sperm cells with the kind of chromosome in question would contain the lethal. Thus if 10 such chromosomes had been tested for lethals, $\frac{1}{8}$ of these 10, on the average, or a cluster of 2, would contain the lethal. These two lethals would be at the same locus, and a test for their allelism would give positive results. By contrast, two lethals of independent origin, derived from two different cells of a polar cap, would most likely be at different loci, and hence an allelism test would prove negative.

Recent results on mutant cluster size obtained at Indiana University, in tests of some 37 second chromosomes of a given genome per polar cap, after an ultraviolet dose low enough to give only about 5 per cent. of second chromosome lethals, have indicated that about 13 pole cells proliferate to form spermatozoa, since on the average a given lethal was found in $\frac{1}{13}$ (7·6 per cent.) of all the tested chromosomes derived from the same genome of the same treated polar cap. This figure agrees well with the previously mentioned one of 10 to 14 based on cell counts of the primordial testis. It can be shown mathematically that even when the different cells of each polar cap proliferate to different extents, so as to give rise to clusters of different sizes, the average cluster size, obtained by dividing the number of lethals found by the number of clusters they were in and then dividing this quotient into the average number of chromosomes tested per genome of a polar cap, is still the reciprocal of the total number of germinally functioning cells per polar cap.

TABLE 1

Increase in Size of "Runs" with Age of Father

Treatment			Imaginal age of P_1 ♂ in weeks	No. of second chrom. tested	No. of lethal second chrom.	Per cent. of lethal chrom.	Average size of runs (in per cent.)
Time (min.)	Distance	Relative dose					
3	50 cm.	14	0-1	180	35	19·5	29·3
			1-3	213	27	12·7	41·0
6	150 cm.	3·6	0-1	173	35	20·2	23·5
			1-3	184	28	15·2	46·9

This only holds, however, in situations in which it can be assumed that the pole cells containing the lethals found have not had their multiplication materially depressed below that of those cells from which no lethals were obtained, for if they had been more retarded the relative size of the clusters derived from them would be reduced and the calculated number of pole cells would thereby be rendered larger than the actual number. A test of whether this was the case in our experiments which indicated some 13 pole cells would be obtained by determining whether or not, with still further decrease of the dose, the cluster size became larger again, as it would if a part of the small size had been caused by selective physiological damage.

Proceeding in the other direction, that of increase of dose, our experiments show that at a dose high enough to produce as many as 10-20 per cent. of second chromosome lethals in the sperm of the first week after hatching, the clusters are considerably larger, forming one-fifth to one-fourth of the total, as would be the case if only 4-5 or fewer pole cells had proliferated to give rise to gametes. In general, our results on the size of clusters show that the higher the dose, the more primary germ cells have been inactivated, as might be expected from the killing action of the ultraviolet.

The number of pole cells which act as progenitors to sperm released after the first week becomes more limited as time passes. This was shown by the results of Harris (1929), working under Muller's direction, on the large clusters of lethals (averaging about 25 per cent. of the total) in sperm released some three weeks after X-raying of the adults, and by the similar results of Friesen (1936) on the clusters of X-ray induced crossovers in such sperm. These results lead to the conclusion that there are usually only about two cells per testis (four per individual) which, like apical cells, continue indefinitely to give rise, by their proliferation, to the end stages, the spermatozoa. When, however, large enough doses of ultraviolet have been given to reduce to four or five the number of pole cells which give rise to the spermatozoa of the first week, a still smaller number of these pole cells, namely only 2 to 3 per individual, is represented among the spermatozoa of the second and third weeks. Table 1 gives results obtained at Indiana University, which illustrate this relation. The word " run " is here used in place of " cluster ".

The same males were used as fathers in weeks 1 to 3 as in week 0 to 1, and the lethals obtained from the later sperm proved, as expected, to be of identical origin (allelic to) those from the earlier sperm (except in the case of a single later lethal). However, not all the lethals of the earlier sperm reappeared in the later sperm although those that did were present in larger " runs ". Approximately 10 second chromosomes were tested per genome (20 per male) in the first week, and somewhat more in the combined second and third weeks. The first week's offspring here reported upon also form a part of the material given in table 4. Those males were purposely chosen for the tests of the later weeks which, according to preliminary fertility indications, would give a higher than average per cent. of lethals in the first week. Thus there was in the later weeks some regression of the rate, caused by a portion of the apparently greater early effects having arisen from the selection of those random fluctuations in the time distribution of the effects which happened to be in the direction of a higher early rate. The observed drop in rate with age must therefore be, in part at least, spurious. The fact that selection was used also makes the material unreliable for a comparison between mutation rates produced by different doses. However, this influence does not explain the increase in the size of runs with age since it would work in the opposite direction, inasmuch as selection in the first week for higher rate would include selection for larger runs. This makes the observed increase in size of runs with age of father the more significant, and shows that the later sperm are on the average derived from fewer pole cells than are the earlier sperm.

It follows from the increase in cluster size with age that when sperm of the first week are used a larger proportion of treated pole cells is sampled than when late sperm are used. Since larger samples give rise to smaller statistical errors, we therefore made it a practice to use sperm of the first week so far as practicable, although when a young male did not yield enough offspring these were sometimes supplemented by those derived from the same male in its second week.

Ultraviolet light is particularly damaging to a *Drosophila* egg (embryo) at the polar cap stage of development. If the entire egg is treated, the dose of ultraviolet that can be tolerated is not sufficient to induce a mutation rate much above the spontaneous rate. In the present experiments, therefore, the egg as a whole was shielded from the ultraviolet and only the polar cap exposed. In the earlier experiments this was done by placing the eggs along the sharply cut edge of a moist blotter or along the edge of a piece of tinfoil, with little but the polar caps projecting beyond the edge, into the region illuminated by the ultraviolet (fig. 2*a*).

However, by this method, as at first used, the rays of the ultraviolet are at right angles (90°) to the long axis of the egg, striking the polar cap from one side (at about 15° to its own plane) so that the polar cap cells on the further side are very much in the shadow of those first struck. This uneven treatment must cause the mutation rate at the higher doses to appear lower than it actually is, since the ultraviolet must selectively kill the cells which receive the highest treatment and

have the highest rate. A second method for shielding the eggs was therefore used in some of the later experiments at Rice Institute. This consisted in putting the polar cap ends of the eggs into small holes in a piece of aluminium foil, so that only the polar cap projected through the hole and was exposed to the ultraviolet light (fig. 2*b*). By this method the ultraviolet is parallel to the long axis of the egg and strikes the plane of the polar cap, which is slightly tilted, almost from above, *i.e.* at about 75°. It therefore strikes the cells much more uniformly than it does in the case of the first method, by which the polar cap is treated from the side. In the experiments here reported which were carried out at Indiana University, although the first method of shielding was used, the light was caused to fall at an angle of 45° to the egg axis, or at about 60° to the plane of the polar cap (fig. 2*c*).

The source of the ultraviolet light in the present experiments was a 15-watt G.E. germicidal lamp, most (over 90 per cent.) of the ultraviolet radiation of which is of 2537 Å wave length. This is close to the region of the ultraviolet spectrum which is most highly absorbed by nucleic acid and which is most efficient in the production of mutations. However, the lamp itself is far from being a point source of radiation, since the luminous portion of the 15-watt tube is about 18 inches

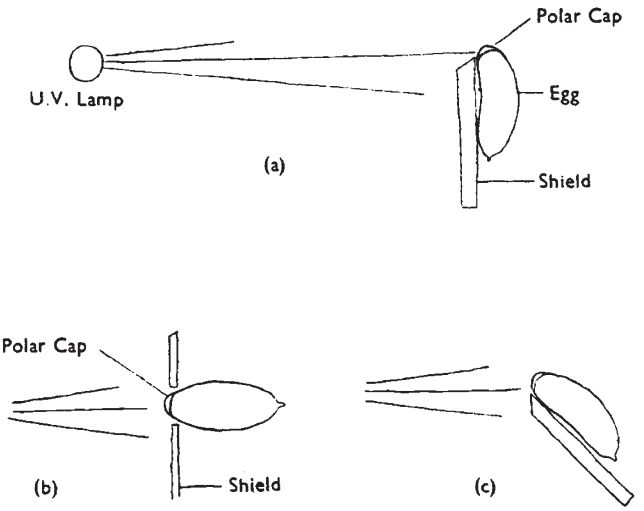

FIG. 2.—Methods of treating the polar cap. (*a*) From the side (rays that strike polar cap are at right angles to long axis of egg), (*b*) from above (rays parallel to long axis of egg), (*c*) rays at 45° to long axis of egg (and therefore at about 60° to plane of polar cap). In (*a*) and (*c*) the egg is resting on its dorsal surface on the shield. In (*b*) the dorsal surface happens to be parallel to the plane of the page, and the egg is supported only in the hole of the shield.

long and ¾ inch wide. Therefore the intensity of the light received by the eggs is not exactly proportional to the inverse square of the distance of the eggs from the lamp, as it would be for a point source. However, the proper correction was made for this fact in calculating the relative intensities of the light received by the eggs at the different distances from the lamp, in the work reported by the Rice Institute group, while in that of the Indiana University group the intensities were measured by a photometer.*

For the purposes of the present experiment it seemed best in a part of the work to vary the dosage of the ultraviolet light by varying the intensity rather than the duration of the treatment. For if the effect of a hit is short-lived, then this effect

* The authors are indebted to Dr J. E. Evans, of the Physics Department of The Rice Institute at the time this work was done (and now at Los Alamos), for help in making the calculations referred to.

might not last from one interval or subdivision of the treatment to the next, so that the addition of a second equal interval would add mainly those mutations which were produced entirely within this interval. That is, if two or more hits were necessary for a mutation, many of those produced in the first interval would be unable to cooperate with those produced in the second interval, so as to cause a mutation. On the other hand, if the dosage is increased by increasing the intensity of the ultraviolet light (and the time kept constant), then as the dose is raised the chances of a coincidence of two or more hits within a given interval are increased logarithmically. Much of the ultraviolet work involving rate-dosage relationships which was done before that herein reported is open to criticism because only duration, and not intensity, of the radiation was used in variation of the dosage. Experiments in which the duration is varied are also useful, however, when comparison of their results can be made with those from the experiments in which the intensity is varied.

3. PRELIMINARY EXPERIMENTS AND GENETIC TECHNIQUES

An extensive series of preliminary experiments was run to determine the maximum dosage of ultraviolet tolerated by eggs in the polar cap stage. But the results of these experiments were rather indefinite, because the dose tolerated varied from one lot of eggs to another. Moreover, the eggs are subject to damage in being shelled and otherwise handled, and in particular they are unduly subject to drying out after being shelled. The damage thus done may vary from one day to another, depending on personal factors, and this influences the percent of eggs that survive a given treatment. In the later experiments, increased proficiency in the handling of the eggs decreased the damage due to the handling. The doses finally selected for treatment in the earlier experiments proper were those that as a rule did not kill or sterilise over about a third of the eggs in most of the preliminary experiments. But in the later experiments higher doses were employed. In some of these the percent of eggs that survived handling and treatment and that developed into fertile males was rather small (less than 2 per cent.). As the shelling and accurate lining-up of the eggs is an arduous process and has to be done in the limited interval of about one hour between the collection of the eggs and expiration of the polar cap stage, the number of eggs successfully treated and bred in any one experiment was rather limited.

In the earlier experiments lethals in the *X* chromosomes were looked for, by means of a recent genetic technique and stocks developed by Muller, but it turned out that the induced rates were so low that no conclusions could be drawn from these experiments. The low recovered rates may have been due in part to the relatively low dosages of ultraviolet employed.

It was then decided to look for lethals in the second chromosome since this has about twice as much euchromatin as the *X* and therefore would be expected to have about twice as high an induced mutation rate. With X-rays in fact, the rate has been reported to be two and a half times that of the *X*. Moreover, heavier doses of ultraviolet

light were employed to further increase the rate. The lethals were detected by means of Muller's *sifter* technique, which has been described more fully elsewhere (Muller, 1951).

In accordance with that form of the *sifter* technique which was used in most of the work done with it at the Rice Institute, males of second chromosome composition *cn bw/cn bw sp* are treated in the polar cap stage of development (*cn* = cinnabar, *bw* = brown, *sp* = speck). These males are mated to females which contain Curly cinnabar^2 and speck^2(*Cy, cn*^{2} *sp*^{2}) in one second chromosome and morula blistered (*mr bs*) in the other, thus *cn bw sp/cn bw* ♂ × *Cy cn*^{2} *sp*^{2}*/mr bs* ♀. This is termed the P_1 cross. The Curly cinnabar offspring (F_1) are of two classes (1) *cn bw sp/ Cy cn*^{2} *sp*^{2} (speck) and (2) *cn bw/Cy cn*^{2} *sp*^{2} (non-speck). About 10 F_1 males of each class, or some other convenient number derived from each P_1 male, are selected as P_2 and mated to females of *sifter* stock. The *sifter* stock is a translocation heterozygote for two *œnothera*-like complexes involving rearrangements of the second and third chromosomes. One of these complexes (the *Cy complex*) contains, among other things, Curly with its left and right inversions and the Pale insertion (P^i). The other contains the Pale deletion (P^-). The stock is viable because P^i in the one complex compensates for P^- in the other. In outcrosses, however, the only viable offspring are those that receive the *Cy* complex. Those that receive the P^- die because they do not receive the compensating P^i. To summarise :

P_1 *cn bw sp/cn bw* ♂ × *Cy cn*^{2} *sp*^{2}*/mr bs* ♀ (♂ irradiated in polar cap stage)

P_2 10 brother F_1 *cn bw sp/Cy cn*^{2} *sp*^{2} ♂♂ and 10 brother F_1 *cn bw/Cy cn*^{2} *sp*^{2} ♂♂ from each P_1 ♂, individually bred × *sifter* ♀♀ (having " *sifter Cy* " chromosome)

P_3 *cn bw sp/sifter Cy* ♂♂ and ♀♀ and *cn bw/sifter Cy* ♂♂ and ♀♀ (10 mass cultures of each kind, one from each of the 10 brother F_1)

F_3 Look for homozygotes (*cn bw sp/cn bw sp* and *cn bw/cn bw*).

The *cn bw* combination of the above homozygotes, when uncomplicated, produces white eyes, in conspicuous contrast to the rather bright red eyes of the *Cy* heterozygotes, so that a nonlethal culture (one containing homozygotes) can be identified at a glance by observing a few white-eyed flies through the glass container. However, in a considerable fraction (about two-thirds) of the homozygotes the expression of *bw* is suppressed by the normal allele of *bw* present in a chromosome section (the Pale insertion, P^i) which was derived from the P_2 *sifter* parent ; hence the eyes of these flies appear cinnabar (bright red) instead of white. However, all the homozygotes, whether white or cinnabar, are recognisable by their straight (non-*Cy*) wings. A nonlethal culture might therefore be identified by means of a few straight cinnabars, in case it happened to contain no

whites, although ordinarily the nonlethal cultures are most readily identified by means of the whites.

It might sometimes happen by accident that a P_1 female (a Curly) was non-virgin. The F_1, F_2 and F_3 derived from her would in that case fail to have a chromosome of treated origin. They would, however, appear Curly in the F_3. *A* P_3-F_3 culture might therefore appear lethal, simply because it did not contain the *cn bw* (*sp*) chromosome. In order to have a check on such accidents, some of the Curly F_3 offspring were always crossed to *cn bw* stock to be sure that they contained the *cn bw* chromosome before the culture was scored as a lethal.

All " brother " lethal cultures, *i.e.* those derived from the same kind of second chromosome (*cn bw* or *cn bw sp*) of the same treated P_1 male, are tested for allelism, in order to determine whether the series represents just one lethal, or two or more of independent origin. This is done by crossing virgins from one lethal culture to males of each of the others. Any cross which produces no whites is considered one in which both brother cultures contain the same lethal. If now the entire series of crosses between brother cultures produces no whites, then all members of the series, being allelic to the same lethal, are considered as allelic to each other and derived from the same mutant polar cap cell. On the other hand, if a cross between two members of the series produces whites, then the parents contain lethals at different loci and hence are of independent origin (having arisen in different cells of the polar cap of the treated male). When more than one lethal non-allelic to that in the culture providing the virgin females are found, they are tested similarly for allelism with each other.

In the *sifter* stock there is some crossing over between the left and right *Cy* inversion. Though this is rare, it occasionally leads to the loss of *Cy* and the left *Cy* inversion from the *sifter* chromosome. Crossing over would therefore not be suppressed in the left arm of the second chromosome of any females that happened to get this chromosome and one of treated origin in the F_2 or later generations. As a result, such females might produce white offspring, even though the chromosome of treated origin originally contained a lethal. Thus a culture that was really lethal might occasionally be scored as non-lethal. The *sifter* stock as used in later experiments was somewhat modified to take care of this situation by the introduction of additional lethal markers, as explained in the separate paper on the *sifter* technique.

4. SOURCES OF ERROR IN THE SCORING OF LETHALS

It is evident that if there is a pre-existing lethal (one present at or before the time of fertilisation) in either second chromosome of a P_1 male (*cn bw sp*/*cn bw*) then that lethal will be contained in the given (*cn bw* or *cn bw sp*) chromosome of all the F_1 " brother " males,

and not just in some or one of the males, as happens when the mutation to a lethal gene takes place in one of the several pole cells of the P_1 male. Hence, when the same lethal was found in an entire series, or " complete run ", as it was called, of " brother " P_3-F_3 cultures (all derived from the same kind of chromosome, or genome, of a given P_1 male), it was regarded as a " pre-existing lethal ", one present before treatment (probably in a parent of a P_1 male), and was not counted as having been induced. An occasional induced lethal is thereby lost sight of, however. This happens when the mutation occurred in a pole cell which managed to give rise to all the offspring (F_1) that were bred to test the given genome—an event that may have been caused by destruction of all the other germ cells by the ultraviolet, or merely by the disproportionately great amount of multiplication of the given cell.

A mutation was classed as a lethal or sublethal, in the Rice Institute experiments, when flies homozygous for the *cn bw* (*sp*) chromosome containing it had a viability of less than about one-thirtieth the viability of their heterozygous Curly sibs, thus changing the ratio of the former to the latter from the expected 1 : 2 to less than 1 : 60. Now the P_3-F_3 cultures in that work usually contained, when counted, some 20 to 30 flies, of which number some 7 to 9 homozygous *cn bw* (*sp*) (either with or without the Pale insertion, P^i) were to be expected, in the absence of a mutation in the *cn bw* (*sp*) chromosome. In case there was a sublethal mutation such that, on the average, only one out of every 30 expected *cn bw* (*sp*) developed, the homozygous type would as a rule not make its appearance at all in a given culture, and so the culture would be tentatively classed as a suspected lethal, tested further, and finally scored in the combined lethal-sublethal category. But, if a homozygote did happen to appear in such a culture, particularly in one containing few other flies, and if this were the first sublethal culture of a series of brother cultures examined, so that the investigator was not forewarned that it might be a sublethal, the culture might inadvertently be scored as nonmutant and discarded. This error would be unlikely, however, where there were sublethal brother cultures and where, as in the later work, all brother cultures were kept together in a group in the same portion of the box of cultures, to be recognised as such and scored in close succession.

Insofar as this source of error existed it would tend to result in a lower recorded mutation rate than the actual one, except in cases in which all of a group of brother cultures had the same sublethal, derived from a pre-existing mutation, but in which one or more of them, that had been spatially separated from the rest, had been erroneously scored as nonmutant with the result that the rest might be considered as of later origin and therefore induced. As pre-existing lethals would appear as often in the controls these would furnish a measure of the frequency of such errors ; they turned out to be few. Nevertheless, further to guard against them, for experiments in which

the brother cultures had not consistently been kept in groups, two methods of calculation were employed in those series in calculating the mutation rate, and both results are presented. According to one of these methods (*a*) the mutations in question—those which had been recorded as appearing in a large but not entire series of brother cultures (*i.e.* the large but not " complete runs ")—were counted as induced, while according to the other method (*b*) they were classed as pre-existing sublethals and therefore not counted as having arisen in the experiment proper.

In the work done at Indiana University the brother cultures were always kept in groups, so that the given error could hardly arise unless a culture had been wrongly grouped. In the Indiana University work, however, the dividing line for classifying cultures as sublethal or nonlethal was at one-tenth instead of one-thirtieth of the expected number of homozygotes. Here it was the practice to retest, by breeding another generation, not only apparently lethal cultures but also those giving unusually low frequencies of homozygotes. The difference in the position of the dividing lines used by the two groups of investigators would not be the cause of much difference in the results, because tests have shown that only a comparatively small proportion of suspected lethals actually falls within the range between these two lines.

Somewhat more important, in the work at both places, was the error caused by the fact that in some cases a series of brother P_3-F_3 cultures was very small, with just a few cultures that proved fertile instead of the desired 10 (or more) derived from the same treated chromosome. In such cases, when all the cultures of a genomal sibship contained the same lethal, it was much less certain that the mutation was a pre-existing one. Had the series been larger, a nonlethal culture might have been found in some of these cases and this would have proved that the mutation should have been counted as having occurred after treatment. We have therefore followed the practice of not counting any all-lethal sibships that consisted of fewer than 5 (or, in some Rice Institute series, fewer than 4) fertile cultures. This has made it necessary to reject, in addition, all other series of fewer than 5 fertile cultures, whether or not they contained any mutations ; for if we had included these and rejected the all-lethal sibships we would have created a bias in favour of a lower lethal mutation rate.

5. STATISTICAL TECHNIQUE OCCASIONED BY MUTATIONAL CLUSTERS

The fact that many of the induced mutations occurred in clusters necessitated the development of a new statistical method for the calculation of the error of sampling. This allows for the fact that each such cluster must be treated as a single event, with a correspondingly high error, in relation to its effect on mutation frequency,

even though in the calculation of that frequency each mutant F_1 must be counted separately, for obtaining the sum of the mutants divided by the sum of mutants plus nonmutants. The formula for the error of the mutation frequency so obtained, worked out by Muller (1952) * is as follows :

$$e_p = \frac{\sqrt{q \cdot \Sigma(c_m \cdot m^2)}}{n}.$$

In this formula e_p is the standard error of p, the observed mutation frequency based on all the given material ; q is $1-p$; n is the number of chromosomes tested ; m is the number of mutants of common origin found in any given F_1 sibship (*i.e.* the size of the cluster) ; and c_m is the number of clusters of the given size (m), found in all the material. In other words, in order to get the standard error by the above formula we square all of the lethal clusters (m^2), get their sum, *i.e.* $\Sigma(c_m \cdot m^2)$, multiply this sum by the percent of nonlethals (q), take the square root of this product, and divide it by the total number of chromosomes tested (n).

This formula is still defective in assuming that there is as much variation in mutation frequency (apart from that caused by clustering) between F_1 derived from different pole cells of the same P_1 cap as between those derived from different P_1 caps, so that the actual error of sampling would be slightly larger than that reckoned. However, results recently obtained by Meyer and Muller, and not included in this report, indicate that polar caps of the same dosage series differ little from one another in regard to the amount of ultraviolet they receive (or in their sensitivity to it), since those caps in which mutations had been produced in the second chromosome were not appreciably more likely than the others to have had mutations in their third chromosome. This indicates the substantial correctness of the error given by the above formula.

6. RESULTS

Table 2 gives the results of two series of experiments, carried out at the Rice Institute laboratory, in which second chromosome lethals were looked for by the *sifter* method above described. The polar caps were irradiated from the side (at an angle of about 15° to the plane of the polar cap) and only the intensity of the radiation in each series was varied, not the time, one series receiving a treatment of 4 minutes, the other one of 8 minutes. In each series, two lots of eggs were irradiated, one lot at 25 cm. from the lamp, the other at 45 cm., the intensity, and therefore the dose, at the near distance being about 2·9 times that at the far.

* In that article (an abstract which explained how the formula was arrived at), the symbol r was used in place of m, I_r in place of c_m, and s_p in place of e_p. The value there given was that of the square of the error instead of the error itself, but by a typographical error n in the denominator was represented without the exponent 2.

TABLE 2

Series designation	Time exposed (min.)	Distance (cm.)	Relative intensity	Relative dose*	No. of chrom. tested	Av. no. chrom. tested per F_1 genome	Per cent. new lethals (incl. large "runs")	Stand. error, this per cent. new lethals	Calc. per cent. induced lethals with st. error	Relat. freq. of ind. lethals*	Per cent. new lethals (excl. large "runs")	Stand. error, this per cent. new lethals	Calc. per cent. induced lethals with st. error	Rel.* freq. of ind. lethals
1A	4	45	1·0	1·0	1091	7·9	9·0	2·0	6·8±2·1	1·00	7·7	1·8	5·5±1·9	1·00
2A	4	25	2·9	2·9	914	8·5	12·6	2·9	10·4±2·9	1·53	11·3	2·6	9·1±2·7	1·66
3A	0	...	0	0	650	6·4	2·2	0·6	0	0	2·2	0·6	0	0
4B	8	45	1·0	1·0	372	6·2	9·9	3·3	9·1±3·4	1·00	6·7	2·3	5·9±2·4	1·00
5B	8	25	2·9	2·9	199	5·9	13·6	6·8	12·8±6·9	1·39	10·9	5·0	10·1±5·1	1·70
6B	0	...	0	0	185	7·1	0·8	0·8	0	0	0·8	0·8	0	0

* The "relative doses" and the "relative frequencies of induced lethals" are expressed as multiples of the doses and frequencies obtaining at the lower dosage intensity, in the series of the same letter (same dosage time), taking the values at the lower intensity as 1. The series designated with the letter A were done at a different time from those designated B. It is for this reason that they have separate controls (3A and 6B, respectively) and that, being not strictly comparable, the relative doses and frequencies for the two groups have not been expressed in terms having a common standard of reference. In all cases the eggs were irradiated from the side (at approximately 90° to the egg axis).

If, for a first consideration, complete runs are excluded but all incomplete runs, even the large ones, are included in the calculation of mutation rates, then in the case of the series given the 4-minute treatment the rate for the near distance proves to be only one and a half times that for the far distance, although the former dose was nearly three (2·9) times the latter. Similarly, for the 8-minute treatment, the rate at the near distance was 1·4 times that at the far, despite the 2·9-fold greater dose of the former. Thus the ultraviolet at the higher dose was only half as efficient in giving rise to observed lethals. When large but incomplete runs are excluded from the calculation, on the assumption that they represent pre-existing sublethals, the relationships are not significantly changed in either the 4-minute or 8-minute treatments, although in both cases the apparent fall in efficiency is somewhat less marked. The latter feature of the results would indicate that the large but apparently incomplete runs probably were, in the main, pre-existing, since of course the pre-existing lethals would not tend to rise at all with dose, but the absence of such cases from the controls would argue for these lethals having been induced. Even with these large runs excluded, when both the series at 4 and 8 minutes are considered together, the fall in mutagenic efficiency with dose turns out to be significant, if the calculation of significance is based on the errors obtained by the formula previously presented.

If now, in table 2, the results from flies treated with different durations of ultraviolet, but with what was intended to be the same intensity, are compared, it is found, similarly, that doubling the time increased the mutation frequency by a much smaller factor than 2 (by only about 1·1, when the lethals of large runs are assumed to be pre-existing). This conclusion is reached by comparing the results of the upper line of the 4-minute treatment with the upper line of the 8-minute treatment, and the lower line of the former with the lower line of the latter. Thus, whichever way the dose was increased, the increase failed to produce anything like a proportionate rise in mutation frequency, at these dosage levels. The comparison of the effect of different durations is not nearly as reliable as that of different intensities in this case, however, since the lots differing in duration were done at different times of the year, and not so strictly parallel in other respects.

Table 3 shows the results of several series of treatments carried on at the Indiana University laboratory in the summer (S) and fall (F) of 1948. In this work, as previously mentioned, the ultraviolet formed an angle of 45° with the longitudinal axis of the egg and of some 60° with the plane of the polar cap. At this higher angle much less ultraviolet was required for attaining the same mutation rate than when it shone from the side ; this is a main reason why the longer distances here used gave higher rates than those shown in table 2. Cases of complete runs, signifying pre-existing lethals, are

TABLE 3

Series designation*	Time exposed (min.)	Distance (cm.)	Relative intensity†	Relative dose‡	No. of chrom. tested	Av. no. chrom. tested per P_1 genome	Per cent. of tested eggs forming larvæ	Per cent. of larvæ forming adults	Per cent. of adults fertile	Size of "run" (av. per cent.)	Per cent. of new lethals found, with st. error	Calc. per cent. induced lethals, with st. error
1 (S)	6	150	1·8	3·6	337	8·9	55·4	37·1	81·2	23·3	17·2±4·1	16·6±4·1
2 (S)	6	100	4	8	335	8·8	43·1	27·9	42·3	29·4	20·0±4·4	19·4±4·4
3 (S)	3	100	4	4	354	10·1	41·8	58·8	70·3	22·5	15·0±3·5	14·4±3·5
4 (S)	3	50	14	14	183	9·6	28·9	20·7	42·5	33·0	18·0±5·8	17·4±5·8
5 (S)	0	...	0	0	1387	9·1	54·0§	85·3	95·4	16·6	0·6±0·4	0
6 (F)	3	200	1	1	807	9·6	76·4	80·0	92·1	22·4	12·0±2·0	11·1±2·0
7 (F)	3	100	4	4	543	9·4	62·0	57·4	79·4	25·8	19·1±3·2	18·2±3·2
8 (F)	10½	200	1	3·5	427	9·5	61·3	62·4	82·4	24·6	21·8±3·9	20·9±3·9
9 (F)	0	...	0	0	639	9·5	47·2§	78·5	95·6	11·0	0·9±0·4	0

* (S) refers to series of experiments carried out in summer, (F) to those in fall, of 1948, at Indiana University. Incident ultraviolet formed an angle of 45° to the egg axis.

† 1 unit of relative intensity represents 0·61 ergs/sec./mm².

‡ 1 unit of relative dose represents 111 ergs/mm².

§ These controls were defective in so far as survival of the eggs was concerned since they were subject to too much heat and drying after dechorionation. This happened because the handling of the control eggs was here postponed until all the treated ones had been attended to. Such differences in handling also affect the other figures in this column to some extent. However, they have very little effect on the results in the next two columns which therefore afford a more reliable index of the physiological effects of ultraviolet.

excluded from table 3. However, long runs, when incomplete, are included, since in all this work special procedures were followed to avoid recording a sublethal culture as nonlethal. These procedures included the grouping of brother P_3-F_3 cultures together in the container and the testing of those cultures which in F_3 had given a low number of homozygotes, until at least 100 flies of F_3 type had been obtained, the upper limit of viability for homozygous sublethals here being taken as 10 per cent. of the viability of the Curly heterozygotes.

The data have been grouped so as to show in juxtaposition those which involved treatments of the same duration but different intensity. It will be seen that the results of changing the dose by changing the intensity, as recorded here, are entirely in accordance with those of table 2, which were obtained independently by the Rice Institute workers during the same year. In the case in which the dose was a little more than doubled by changing the intensity (lines 1 and 2) the induced mutation rate (after the control value has been subtracted) was raised by only about 17 per cent. of its lower value, while in the cases in which it was nearly quadrupled (lines 3 and 4) or quadrupled (lines 6 and 7 and lines 6 and 3) the rate was raised by only between 22 and 64 per cent.

It is also to be observed that when the dose was doubled by increasing the time only (lines 3 and 2) the mutation rate was raised by only about 35 per cent., and when it was nearly quadrupled by raising the time (lines 6 and 8) the rate was raised by only about 87 per cent. It should be mentioned in this connection that in the work reported in table 3, unlike that in table 2, lots differing in duration of treatment were (when of the same series, S or F) irradiated on the same occasion, just as were those differing in intensity, and that they therefore present as valid a parallel with one another, for comparison of the results of altering the dose, as do the series differing in intensity.

Although, on account of the relatively large sampling errors, we remain very uncertain as to the precise relations, nevertheless it is clear that a rise in the dose, no matter whether achieved by an increase in intensity or time, brings about nothing like a proportionate increase in mutation rate. Another way of expressing this relation is to say that, at low levels of dosage, a given rise in dose must produce a greater increase of mutation frequency than at most of the levels used in these experiments, *i.e.* the curve expressing the relation of dosage, as abscissa, to mutation rate, as ordinate, must on the average have gone up more steeply between its origin and the lowest dose here used than after that point. Study of table 3 also suggests that, between the different doses here studied, the curve continues to become more nearly horizontal, until possibly it may even decline somewhat.

In the same experiments counts were made of the percentage of treated embryos which hatched to form larvæ and of the percentage

of these larvæ which survived to the imaginal stage. It will be seen that survival at both stages is affected markedly by dose, although later studies have indicated larval survival to be a more reliable guide to dose than the egg hatching rate. At the higher doses, even though the mutation rate shows little if any increase, the survival rates are, if anything, more affected by a given factorial rise than at lower doses. The fertility rate is seen to vary similarly to the survival rate.

We have listed in addition the average size of cluster or length of " run ", calculated as explained on p. 155. The trend of these figures shows clearly the influence of higher doses in increasing the size of cluster. This is an expression of the reduction, by the ultraviolet, of the number of pole cells which proliferate to give rise to gametes. This index, like larval survival, seems to have the curve of its rise less subject to flattening out, at higher doses, than that for mutation rate.

TABLE 4

Irradiation parallel to egg axis

Series designation	Time exposed (min.)	Distance (cm.)	Relative intensity	Relative dose	No. of chrom. tested	Av. no. chrom. tested per P_1 genome	Per cent. new lethals with stand. error	Calc. per cent. induced lethals with st. error	Relat. freq. of ind. lethals
1*P*	½	45	1·0	1·0	783	16·3	4·2±1·4	3·4±1·5	1·0
2*P*	½	25	2·9	2·9	889	22·2	9·0±2·4	8·2±2·5	2·4
3*P*	0	...	0	0	629	13·1	0·8±0·5	0	0

Table 4 gives the results of a later series of experiments, carried out at the Rice Institute laboratory, in which the polar cap was treated from above. Here the incidence of the ultraviolet to the egg axis was 0° and to the plane of the polar cap about 75°. In this table, as in table 3, the more nearly vertical illumination has resulted in a higher mutation rate, for a given exposure, than that, shown in table 2, which resulted from a side illumination. Thus, according to table 4, a ½-minute treatment from above at the 25 cm. distance gave a mutation rate of 8 per cent., almost as high as the rate of 10 per cent. which according to table 2 was given by a 4-minute treatment from the side at the same distance. Moreover, one or more recovered lethals were induced in as many as 17 out of 20 treated polar caps when the treatment was from above, as compared with a proportion of only 37 out of 54 when treatment was from the side. Yet despite the relatively high mutation rate the number

of lethals in a run was smaller from polar caps treated from above than from the side. This effect, which had the advantage of reducing the error of sampling, was doubtless caused by the greater evenness of the illumination from above. For with this more even illumination fewer cells would be so heavily exposed as to be inactivated, while those not inactivated would nevertheless receive more ultraviolet than if they had been shaded by the others.

In table 4, as in 1, 2 and 3, there appears to be a falling off of the mutation rate relative to the dose, as the dose is increased, for the higher dose is 2·9 times that of the lower, while the mutation rate at the higher dose is only about 2·4 times that at the lower. Inspection shows, however, that the difference between these ratios is less than its own standard error, and it is also evident that the approach to a linear relation is significantly closer than in any of the previous experiments. The closer approach to linearity in this series of experiments is to have been expected on two grounds. First, a lower range of dosage, giving a lower range of mutation rates, was here being used. That is, the more steeply rising portion of the mutation rate-dosage curve was dealt with. Second, the more vertically impinging ultraviolet here used, with its more even distribution, minimises the selective effect that tends to reduce the frequency with which lethals are recovered at high doses (see discussion in the following section).

7. INTERPRETATIONS

The results of the present experiments show that the frequency of recovered mutations induced by ultraviolet light does not increase proportionately with rise in dose, for the doses used by us, when the dose is varied by changing either the intensity or the time. That is to say, the induced rate found, per unit dose of ultraviolet, decreases with increase in dose, when either the time or the intensity is held constant. Thus, the rate-dosage curve (with rate as the ordinate) at first rises and then tends to level off until it reaches a point where further increase in dose causes no further appreciable increase in rate.

The results thus agree, so far as they go, with those first obtained by Hollaender and his co-workers (1939 *et seq.*) for visible mutations in fungi of various kinds after exposure of the spores to ultraviolet, and by Stadler and his co-workers (1939 *et seq.*) for endosperm deficiencies caused by chromosome breakage after ultraviolet treatment of maize pollen. However, we have not in the present experiments found a significant drop in the rate-dosage curve at the highest doses. This was first definitely found in the work with the fungi, and was later found by Sell-Beleites and Catsch (1942) for lethals in *Drosophila* spermatozoa which were exposed to ultraviolet through the body wall of the male.

The results obtained in *Trichophyton* (Hollaender and Emmons, 1941) and later in some other fungi show that the decrease in mutagenic effectiveness of ultraviolet with rise in dose is caused at least in part

by its physiologically damaging effects being exerted with greater force, at higher doses, against those cells which have had mutations induced in them, as compared with nonmutant cells, on account of the lower viability of the mutants. In other words, the physiological damage acts synergistically with the genetic damage, in reducing viability. The existence of this selective effect was shown by the finding that post-irradiation treatments which favoured recuperation of the fungus spores from the ultraviolet allowed relatively more of the mutant cells, as compared with the nonmutants, to be recovered. At very high doses the selection against the mutants may even become so strong that the surviving cells include a smaller percentage of mutants than at lower doses. Further evidence for these conclusions is afforded by the finding by Swanson, McElroy and Miller (1949) that when spores of *Neurospora* are treated with ultraviolet the mutation rate-dosage curve of the morphological mutants shows a levelling off and drop with increase in the dose, while that of the biochemically deficient mutants fails to do so, inasmuch as the latter mutants, unlike the former, are protected, by the complete nutrient medium used, from the detrimental effects on survival which their abnormalities would otherwise have occasioned in them.

In our *Drosophila* material, a similar protective action against the effects of the mutations on the cells is, in the case of autosomal genes, exerted by the presence of the unmutated allele in the homologous chromosome. As most of the gene mutations we have dealt with are, except to a slight degree, recessive, even in their action on the individual as a whole, and would usually have less effect still on the life of mutant cells surrounded by a nonmutant body, there could have been very little direct selective effect of the ultraviolet against cells with autosomal mutations of the sort studied by us. This possibility then can be ruled out as a direct explanation of the marked levelling out of the curve of autosomal mutation frequency shown in our data.

It was recognised by Hollaender (1939) that inequalities in ultraviolet illumination could also lead to a flattening of the mutation rate-dosage curve, by killing off, at higher doses, a larger proportion of the spores which had been more exposed. However, he considered it unlikely for this situation to hold for the minute spores of *Trichophyton*. Stadler, on the other hand, noted that among his maize pollen there would be marked inequalities in illumination of the chromosomes of different spores, due to the eccentric positions of their nuclei, and he calculated that the resulting selective effect was sufficient to account for the convexity of the rate-dosage curve. Later, evidence was adduced by Hollaender, Sansome, Zimmer and Demerec (1945) that there were inequalities in illumination and/or " response " to illumination among *Neurospora* spores. In any situation involving uneven illumination, when the dose rises high enough to kill off or retard the multiplication of some of the cells, the frequency of recovered (*i.e.* found) mutants will be lower than that of actually arising mutants.

For the more illuminated cells, in which there is a higher mutation rate, will have been selectively killed off or retarded in multiplication. And the higher the dose rises the stronger will this effect be, so that the recovered rate will become lower and lower in proportion to the actual rate, with progressive flattening of the rate-dosage curve.

Differences in illumination alone would not cause an actual drop in the curve at high doses, however, unless the cells were in two or more rather discontinuous groups, in respect to the amount of illumination received by them, with comparatively few cells receiving intermediate amounts. That is, the frequency distribution of illumination would have to follow a bimodal or polymodal rather than a unimodal curve or a straight line. For if the curve were not bi- or poly-modal, then as the dose was raised and selection killed off the cells with the highest mutation rate, those in the next lower category of illumination would now be getting as much light as the most highly illuminated had at the previous dose and their mutation rate would be as great. As this would apply to the cells all along the line the over-all recovered mutation rate would actually be slightly higher, with any rise in dose, since with each increment the cells in even the very lowest category of illumination would have moved up. On the other hand, with a polymodal curve or—what amounts to the same thing—a fairly sharp discontinuity in the amount of illumination received by different groups of cells, there could be a drop in rate at higher doses. For in that event when the dose was raised, the most affected group might be virtually eliminated at a dose at which there was not yet sufficient illumination of the remainder to bring their mutation rate up to the previously existing average rate. It is to be noted, however, that a rise in dose beyond this would again bring the rate up, supposing that it was practicable to increase the dose further. Thus, there would tend to be a rate-dosage curve the modes of which corresponded with those of the illumination-categories. Such findings have not yet been reported.

There is no doubt that in our *Drosophila* material there must be some inequalities in the amounts of illumination received by different polar caps. For it is impossible to place them all in just the same position relative to the source of light or to shade them in precisely the same way. They also differ somewhat from one another in regard to their stage of development and the number, size, shape, arrangement and opacity of their contained cells and cell parts. All these differences are probably very minor, however, compared with those differentiating the cells of the same polar cap. Certainly, with side illumination, the far cells must be much more shaded than those nearest the source of light. But even with vertical or 75° illumination, although the cells in the same layer can shade each other very little, those on the surface must receive much more ultraviolet than the underlying ones.

It can be calculated from measurements of protoplasmic penetrability which have been reported for other material (see Lea, 1946)

that light of the wave length here used by us (2537 Å) might be reduced to as little as about a tenth its intensity by passing through a cell the size of a pole cell (about 5 micra in diameter). A slightly different absorption coefficient (such as the present material might have) could change this figure considerably. Now a reduction to about 1/18 the intensity would suffice to explain our finding that a 14-fold rise in dose occasioned a rise of only about 50 per cent. in the mutation rate. This can be reckoned as follows. With a difference of 18 times between the illumination of the layers, if we assumed that at the 14-fold dose *all* the top-layer cells had been killed, the lower-layer cells now remaining would be getting 14/18, or 0·78 times, as much ultraviolet as the top-layer cells received at the lesser dose. Since at the lesser dose the lower cells would be getting 1/18 as much as the upper cells, the average illumination of both layers at this dose would be $(1/18+1) \div 2$, or 0·53, of the amount received by the top-layer cells alone at the lesser dose. Therefore the figure 0·78/0·53, or 1·5, would represent the ratio between the amounts of ultraviolet received by the actually functional germ cells of the polar caps at the greater as compared with the lesser doses. It is further to be observed that since the cells are to a large extent arranged in two layers (with perhaps an occasional cell still lower down), even that condition of comparative discontinuity of grouping is provided which might lead to a drop of the rate with increase in dose.

That in our material ultraviolet does have a damaging effect, varying with dose, on cell multiplication and/or survival is evident from the figures we have given on the dose-dependent lowering of the hatchability of eggs, viability of larvæ and fertility of the imagos which emerge. Moreover we have found (observations of Meyer) a pronounced delay in development, of both embryonic and larval stages, which also varies with dose. There is little reason to doubt that such effects would likewise differentiate cells of the same embryo which had been illuminated to different extents. That the different pole cells of the same cap are subject to different degrees of damage has been shown by our finding of the increase in the relative size of mutant clusters (*i.e.* of the reduction in the number of germinally functional germ cells) with rise in dose.

The fact that inequality of illumination of different cells of the same cap is an important factor in determining which cells are to be inactivated (*i.e.* that the damage is selective) is shown by the larger cluster size, implying a larger proportion of pole cells inactivated, in connection with the production of a given mutation rate, when the illumination is from the side than when it is more nearly vertical. It is shown, secondly, by the fact that a larger total dose is needed to attain a given frequency of recovered mutants with side illumination as compared with more vertical illumination. And it is shown, thirdly, by the fact that, within dosage ranges of not very different mutagenic effectiveness for both kinds of treatment, vertical illumination allows

the mutation frequency to rise more nearly in proportion to increase in the dose than does side illumination. The conclusion is thereby indicated that in our material the inequality in the amount of ultraviolet reaching different cells, through its selective action against the survival of the more illuminated ones, is the chief factor, or at least a major factor, responsible for the falling off of the mutation rate relative to dose at comparatively high doses.

It is evident that if cells of the same or of different polar caps should differ from one another in their sensitivity to the action of ultraviolet in retarding their multiplication and in killing them, and if these differences were positively correlated with differences in their sensitivity to the mutagenic action of ultraviolet, we should have a situation the effect of which would be like that of differential ultraviolet illumination of the cells. Like the latter influence, therefore, it would lead to a levelling off of the mutation rate-dosage curve with rise in dose, and could also lead to one or more drops in the curve, if the cells were grouped more or less discontinuously in regard to their sensitivities. It seems unlikely however that the *Drosophila* pole cells, either of the same or different embryos, would differ greatly in their sensitivity to ultraviolet. It is true that at an early period of the polar cap the polar granules within the cells gradually fade and at the same time the nucleus assumes a more typically " resting " configuration. However, the cells change little in appearance thereafter and have evidently settled down into a long-lasting interphase of considerable stability. In this condition we could hardly expect some of them to be some 18 more times as sensitive as others—to correspond with the 18-fold differences in illumination which we found, on p. 172, to be necessary for explaining, on that interpretation, the pronounced levelling off of the curve at high doses. Since, on the other hand, there is reason to believe that such differences in illumination may exist, it is not necessary to suppose that any considerable role is played by differences in sensitivity.

Granted either differences in illumination or sensitivity or both, with resultant selection on the basis of " physiological damage " against the cells in which more mutations had been induced, this effect would be very slightly enhanced by genetic selection against the same cells. For the unevenness in illumination and/or sensitivity would cause the mutations to have a positive correlation with one another in their distribution among the cells. Although mutations in the autosomes, being so nearly recessive, would very seldom have an appreciable effect on cell survival, many of those occurring in the single *X*-chromosome in cells of males would detrimentally affect their survival or proliferation, as studies on X-ray induction of mutations have shown. These inactivations would reduce the frequency of recoverable mutations in the autosomes as well, because of the correlation of mutations with one another. However, it can be calculated that this effect would be of too low an order of magnitude

to account for the levelling off of our curve of second chromosome lethals, or for the reduction in pole-cell number shown by our results on cluster size. For, whereas the latter effects require the elimination of 50 per cent. or more of the pole cells, the induced lethal rate in the *X*-chromosome is far lower than this, and even with a high correlation of mutations with one another there would be a still lower proportion of autosomal lethals thereby eliminated. Hence this selective influence is not comparable in magnitude with that caused by the selective " physiological " (*i.e.* nongenetic) damage attendant upon the unevennesses of illumination.

Ultraviolet is peculiarly subject to selective effects because of (1) its low penetration (high absorption) in cells, coupled with (2) its extremely harmful action on the physiological (including the mitotic) systems of the cell. This applies especially to ultraviolet as short as that used by us. Visible light would be subject to negligible inequalities in distribution within a tissue so thin and transparent as the polar cap, while ordinary X-rays would of course be distributed with virtual uniformity throughout the entire body of so small an organism as an insect.

Selection is not the only mechanism which could result in a tendency of the mutation rate-dosage curve of ultraviolet to level off, and on occasion even to drop, at high doses. The phenomenon commonly referred to as " photoreactivation ", which we now prefer to term " photorepair " (Muller, 1954), could also produce such an effect, provided the material treated with higher doses of the mutagenic ultraviolet also received sufficiently higher doses of reparative light (that which interfered with mutagenesis). This possibility arises from the fact that the amount of repair or interference effected by a given amount of the reparative light is not fixed but is proportional to the amount of damage to be repaired (*i.e.* to the potential mutagenic effect of the mutagenic light). This was shown, for instance, in the experiments of Novick and Szilard (1949) on *E. coli.* Thus if the amount of reparative light rose along with increase of the dose of mutagenic light, a larger and larger *proportion* of the potential mutagenesis would, as it were, become sidetracked, and the slope of the rate-dosage curve would become increasingly depressed (except for the limitation mentioned in the second paragraph below) as the curve proceeded to the right.

It is true that about 90 per cent. of the output of the Germicidal lamp used in our own experiments was of the mutagenic wave length 2537 Å, and part of the rest was also in the mutagenic range. Nevertheless the amount of nonmutagenic reparative light may have been enough to affect our results appreciably, and this amount must have increased along with the rise of dose of the mutagenic light. Filtering had not been resorted to because the experiments were performed prior to the discovery of photorepair as a general phenomenon. Moreover, the possibility cannot be dismissed that even the mutagenic

light itself may in this material have some reparative effect in addition, despite Dulbecco's (1950) finding that this is not true in phage, since the action spectrum for repair is not the same in all organisms (Kelner, 1949, 1952).

Several considerations, however, indicate that most of the decline in slope of our rate-dosage curve at higher doses was not the result of photorepair. One of these considerations concerns itself with the fact that there is a limit to the amount of this effect, set by the circumstance that only a fixed proportion of the potential ultraviolet damage is reparable at all by the reparative light. Suppose for instance that only one-fifth of it was irreparable. Then under no circumstances (provided photorepair was the only factor causing the curve to decline from linearity) would a ten-fold rise in dose produce less than a doubling of the mutation rate, or a fifteen-fold rise in dose less than a tripling of the mutation rate. Thus the fact that in table 2 a fourteen-fold rise in dose produced a mutation rate only one and a half times as great, and that the other data are in harmony with this, indicates that photorepair alone could not have lain at the basis of this effect, inasmuch as in other experiments, in which very strong doses of reparative light were intentionally administered (Meyer, 1951 ; L. and E. Altenburg, 1952), the portion of the mutation rate which remained uninterfered with was about a quarter. A further limitation on the repair effect is that, when the mutagenic and reparative light are increased *pari passu*, a near-maximum level of effectiveness of the latter is after a while attained (provided so high a dose can be tolerated), after which the *proportion* of potential damage repaired remains virtually constant. Thereafter, then, further increases of dose must give the same rate of increase of observed effect as if no reparative light were present at all.

Those features of our data which have already been mentioned as evidences of a selective effect based on inequalities of illumination are not explicable as effects of photorepair. These include the much lower mutagenic efficiency of side as compared with more vertical illumination and the much greater influence of the former type of exposure both in depressing the rate-dosage curve and in increasing cluster size, when the same level of mutagenic effectiveness is maintained in the two lots. Another telling fact in favour of selection is that, at the high doses at which the rate-dosage curve for mutations is practically flat, both the damaging effect on larval survival and the cluster size are continuing to increase with increase of dose. This would not be expected if photorepair lay at the basis of the decline of slope of the rate-dosage curve for mutations since others (first among them, Novick and Szilard in 1949) have reported that photorepair affects survival and mutagenesis in parallel fashion, as though by changing the amount of a substance upon which both depend. Selection, on the contrary, would at higher doses produce an ever more adverse effect on survival, both of larvæ and of individual pole

cells, but by that very means would tend further to reduce the frequency of observed mutants, below the frequency with which they had actually been induced. All in all, then, we are led back to the conclusion that most of the depression of the slope of the mutation rate-dosage curve has been caused by inequalities of illumination.

At the same time, it should be recognised that a part of the explanation of this phenomenon might be even more complicated. The prolonged plateau exhibited by the curve suggests some sort of saturation effect, such as might be caused by the using up of materials which take part in the process of mutagenesis. In bacteria, for instance, as Stone *et al.* have shown, a photon of ultraviolet can often produce a mutation indirectly, through first producing a mutagenic substance in the medium. Now although the correspondence between the absorption spectrum of ultraviolet by nucleic acid and its mutagenic action spectrum in varied plant materials has indicated that most of the mutagenesis in these cases results from quanta absorbed by chromatin itself, the possibility is not thereby excluded that even under these circumstances the process may involve a number of steps. Photorepair, in fact, gives evidence of this. If, now, there were a chain of reactions intervening between quantum absorption and mutation, the probability of occurrence of such a mutation might depend upon the concentration of some substance necessary for the formation of a given one of the links in this chain. The amount of that substance which could be formed might be limited by limitations of the substrate for it, or (what amounts to the same thing) the rate at which it was used up might outrun its rate of formation, with the result that the rate of mutagenesis tended to approach a limit at high doses. Nevertheless, we do not as yet need this additional hypothesis to arrive at a reasonable interpretation of our results.

As the dosage is reduced, both the selective effect and the photorepair, as well as, if it should exist, the effect postulated in the preceding paragraph, must of course become gradually less pronounced, and finally negligible. Only at these low doses could it be determined whether what might be called the primary rate of mutagenesis had a linear relation to ultraviolet dose, as it would have if a mutation was caused by a single " lucky " quantum absorption, or whether several quanta cooperated or interfered with one another. Since our results indicate that, at moderate doses at any rate, most of the hindrance to our observance of the primary rate of mutagenesis is caused by selection, the question arises as to how the dosage level may be recognised at and below which selection plays only a negligible role. The answer, theoretically, is that this must be a level at and below which there is no longer any influence of dose on mutant cluster size. It is however difficult to accumulate a sufficient number of mutations for such a study at such low doses, and the task has not yet been carried through on a sufficient scale to arrive at a definite answer to the question in our material.

It is true that, in such of the work herein reported by us as was done with relatively low doses, administered more nearly vertically, as in table 4, a comparatively linear rate-dose relation was shown. Nevertheless, the selective factor may not yet have been negligible here and it therefore remains possible that the primary rate of mutagenesis varies as a higher power of the dose than one, or follows an even more complicated course. That is, the apparently linear relation may have resulted from our observing a portion of the rate-dose curve which was in the region of transition from a concave to a convex shape. Although the matter cannot be regarded as settled, direct support for this interpretation has very recently been provided by another series of experiments (L. and E. Altenburg and Baker, 1952), involving rather low doses, in which the relative rise in induced mutation rate exceeded, by an amount greater than the sampling error, the relative increase in dose.

In other material, the reported results differ with the organism used. The frequency of chromosome breaks was found by Swanson (1940, 1942) to vary linearly with dose of ultraviolet in *Tradescantia* microspores. Likewise, in the work of Stadler and Uber (1942) on endosperm deficiencies caused by chromosome breaks induced by ultraviolet treatment of maize pollen, it was deduced that the relation would have appeared linear had the nuclei not been eccentric. By analogy, one might expect point mutations also to have a linear rate-dose relation. Such a relation has in fact been found by Kaplan (1948, 1949*a*, *b*) in *Bacterium prodigiosum* for the phenotypically defined mutation to stunted colony induced by ultraviolet.

On the other hand, the results published by Demerec and Laterjet (1946) and by Novick and Szilard (1949) on ultraviolet mutations in *E. coli* give curves the slopes of which would indicate that, over the wide range of dose used by them, two to three hits cooperated in producing a mutation. Similarly, although the earlier results on the frequency of mutations induced by ultraviolet in various fungi appeared capable of interpretation as representing a linear relation at low doses, a definite decision on this point could not be made because of the paucity of the mutations found at such doses, and recent work of Swanson (1952) on *Aspergillus* shows a concavely rising curve, indicative of a multi-hit effect.

Kaplan's further observations on *B. prodigiosum* and on *E. coli* are of special interest in their bearing on the problems here at issue. He found that although the mutation rate-dose relation was linear when ultraviolet was used, it exhibited a two-hit curve when the mutagen was visible light in the presence of erythrosine (Kaplan, 1950*a*). As the rate was at the same time simply proportional to the amount of erythrosine (at low concentrations of the latter) he concluded that a mutation required two quanta of visible light to be accumulated on the same particle of erythrosine but that one quantum of ultraviolet, being larger, sufficed for a mutation

(even without erythrosine, since it could be absorbed directly by chromatin).

On the other hand, Kaplan (1950*b*) found that a special strain of *E. coli*, histidineless, having unusually long cells, showed a multi-hit mutation-rate curve (of back mutations to histidine-independence) even when ultraviolet was used. The latter results are similar to the already mentioned findings of Novick and Szilard on *E. coli*. However, Kaplan found that whereas his long-celled strain also showed a multi-hit survival curve (as had that of Novick and Szilard), another strain (" B ") of *E. coli* gave a one-hit (linear) survival curve with ultraviolet. Although strain B was not tested for mutation rate it seems not unlikely, in view of the difference in survival and in length of cells, that its mutation rate also would have been found to be one-hit. In that case the multi-hit curves might possibly represent a derived condition, caused by a compoundness of the long cells, which allowed only multiple changes to give observable effects.

Although it is hardly possible that a condition similar to the one in the histidineless strain of *E. coli* exists in *Neurospora* or *Drosophila*, it is conceivable that it did in the strain of *E. coli* used by Novick and Szilard. Moreover, it must be conceded that other biological differences might influence a result of this kind. Even more telling is the fact that mutations in the same organism could be produced by either one or two hits, according to the size of the quantum. This indicates that a different rate-dose curve might be obtained by a relatively slight change of wave length, if the transition zone of wave length could be determined. The latter, in turn, is one of the factors which might vary with the biological material. At the same time, the fundamental mechanism of mutagenesis would probably remain much the same in these cases.

8. RECOMMENDATIONS FOR FUTURE WORK ON THE SUBJECT

In view of the various considerations advanced above, taken together with the experience gained and the results here reported, certain specifications for further work on the primary mutation rate-dosage relation in cells of the *Drosophila* polar cap can now be recommended. The work should be conducted, so far as possible, with doses so low as to avoid intercellular selection, as shown by the fact that the size of the " run " has reached its final minimum throughout (with due precautions to avoid a possible false minimum before the final one, as explained on p. 155). In place of light of 2537 Å there should be used the longest mutagenic wave lengths practicable—if possible, those of 2900 to 3100 Å, in order to get a more nearly equal penetration of the ultraviolet among the pole cells in different positions and thus to reduce the selective effect. The illumination should be at an angle of not more than 45° to the egg axis, preferably less,

again in order to give more uniform illumination. To avoid the destruction of too much somatic tissue by penetrating light entering in such a direction, a special shielding method, such as the pinhole technique used by the Rice Institute group, is advisable.

Nonmutagenic light of photoreparative wave lengths, except of an intensity equal to that which is continuously present in the room anyway before and after treatment, should be excluded during treatment, or else should be present in constant amount. One way of meeting this requirement is to vary only the duration, not the intensity, of the treatment with mutagenic light, and to do this removing, for the desired interval, by a filter which rather sharply cuts off the ultraviolet shorter than about 3200 Å but leaves the longer light virtually unchanged ; the exposure to the nonmutagenic light would be equally long and equally intense for all the embryos. Alternatively, a monochromatic beam derived from a spectrum could be employed, but it is less convenient to obtain this at the desired intensity and size of field. There would be an advantage in intentionally exposing all embryos to a considerable and equal dose of nonmutagenic reparative light, since this would tend to swamp out any differences in this respect attendant upon the treatment with mutagenic light. In fact, it would be desirable to use so high a reparative dose as to attain practically maximum repair. For this would even nullify any possible reparative effect of the mutagenic light itself (provided only that the same portion of the damage is reparable by different wave lengths of reparative light, a matter subject to verification). A comparison of the rate-dose curves obtained with and without the nonmutagenic reparative light would in fact disclose whether or not the mutagenic light also acts reparatively.

The attainment of the arrangements above suggested is facilitated by making time, not intensity, the variant in changing the dose. That the effect on mutation rate in the pole cells is the same regardless of which of these two factors is varied has recently been shown, for a nineteen-fold time-intensity range, by E. Altenburg, Bergendahl and L. Altenburg (1952). It might be thought that this result proves mutation to result from a single quantum absorption, but this is not the case. For the finding that both photorepair (Meyer, 1951 ; L. and E. Altenburg, 1952) and thermal repair (Edmondson and Meyer, 1952 ; Meyer and Muller, 1952) of potential mutagenesis can be effected in *Drosophila* pole cells subsequently to the exposure to the mutagenic ultraviolet shows that an interval of the order of several minutes at least (*i.e.* as long as the duration of the treatments themselves) commonly intervenes between quantum absorption and finished mutation. In the meantime there would be opportunity for effects of different quantum absorptions to interact (if that occurs) in the production of a mutation.

A further procedure to be recommended is that, if the males derived from the treated embryos are used for the mutation rate

studies, preference be given to carrying out the tests on the sperm which they release during their first week of imaginal life, since these sperm are derived from a larger number of pole cells than are those released later, and the sampling error is therefore smaller for them. It might be better, however, to have a genetic scheme which would allow the investigation of treated females. For their primordial gonads are composed of approximately twice as many primary germ cells (former pole cells) than those of males, and it is probable that these multiply more equally in giving rise to the later germ cells, since separate egg strings are established rather early. This procedure would allow the testing of a larger number of pole cells per treated individual investigated. Moreover, in females any genetic selection occasioned by correlated mutations in the *X*-chromosome would be minimised. It is true that fewer offspring can be obtained from one female than from one male, but this might be more than compensated for since even the offspring derived from eggs laid in the later weeks of life should be suitable for testing. Unfortunately, however, only one autosome of a pair can by present genetic techniques be investigated in females, since there are pre-existing lethals accompanying the inversions used heterozygously to prevent the production of crossovers.

Chiefly, then, the present experiments must be regarded as doing the heavy work of breaking the ground for more definitive attacks, in showing the nature of the disturbing influences to be encountered, and methods of circumventing them. It is believed that, now this has been done, it should be possible to determine, with a lesser amount of effort than already expended, the primary mutation-rate dosage relation. The choice of this material is justified by the fact that the types of genetic change involved can be determined with comparative exactitude. The problem is one which bears on virtually all fields of radiation genetics since ultraviolet, unlike ionising radiation, gives opportunity for determining the effectiveness of individual excitations.

9. SUMMARY

Studies were made of the rate at which lethals are induced in the second chromosome of *Drosophila melanogaster* by ultraviolet light, predominantly of 2537 Å wave length, applied to the germ cells at the time when they are present in the " polar cap " of the early embryo. Lethals induced at this stage undergo multiplication by cell division and therefore occur in clusters among the gametes. In order to determine the sampling error of the mutation frequency based upon results in which such clusters of mutants of identical origin are present, a new error formula had to be devised. For the detection of the lethals, a new genetic technique, designated as the " *sifter* " method, was employed.

It was found that the frequency of lethals increases much less than

proportionately to rise in dose of ultraviolet, at doses large enough to give the readily detectible frequency of 10 per cent. or more. Thus an increase in dose to nearly 3-fold was found in two series of experiments in this range to cause an increase in frequency to only about 1½-fold, while in another experiment, utilising doses that gave somewhat higher frequencies, an increase in dose to 14-fold caused an increase in frequency to only about 1½-fold. This falling off in mutagenic efficiency with increase in dose appeared both when intensity alone and when time alone were the factors used in changing the dose. However, in experiments in which somewhat lower doses were used than any of those in the experiments above referred to, and in which the radiation was more efficiently and uniformly applied to the polar cap cells, by causing its incidence to be more nearly vertical, the frequency proved to be much more nearly proportional to the dose (a rise in dose to 2·9-fold its former value giving a rise in frequency to 2·4-fold, with the difference between these two ratios less than its own standard error).

There is a series of facts showing that ultraviolet, because of its low penetrating power, is far from evenly distributed among the cells of the polar cap, and that the cells receiving more of it tend to be selectively killed or inactivated. Among these facts are

(1) the far greater mutagenic efficiency of the dose when vertically or nearly vertically applied than when applied from the side ;

(2) the fact that vertical application causes less departure of the mutation frequency-dosage curve from linearity ;

(3) that there is also less departure of the curve from linearity at lower doses than at higher ; and

(4) that a less even application or higher doses are both accompanied by considerable reduction in the effective number of surviving germ cells in the polar cap, as judged by the increase in the relative sizes of clusters of identical mutants.

As the selective damaging of the more illuminated cells—which are the very ones in which more mutations have been induced—would be greater at higher doses, it must result in a part at least of the observed tendency to levelling off of the frequency-dosage curve at higher doses. It could even result in an actual decline of that curve (such as has been noted in some other material) if there were two or more rather discontinuous groups of germ cells, receiving different amounts of illumination. The arrangement of most of the polar cap cells in two layers might provide sufficient discontinuity for this.

If the germ cells differ both in their sensitivity to having their multiplication checked by ultraviolet, and to having mutations induced in them by this agent, and if these two sensitivities are positively correlated, the frequency-dosage curve would be affected

thereby in a manner similar to the effect of the unequal illumination. It does not seem likely however that the polar cap cells differ nearly as much in their sensitivities as in the amount of illumination they receive, when light of 2537 Å is used.

Another circumstance which could result in a continued falling off of the slope of the curve with rise in dose, and its eventual decline, even in case both the degree of illumination and the sensitivities of all the germ cells were identical, would be the existence of a significant amount of photoreparative effect by the ultraviolet in addition to the mutagenic effect of the same wave length. A similar result would be produced if the mutagenic light were accompanied by a sufficient amount of nonmutagenic photoreparative light, the dose of which varied *pari passu* with that of the mutagenic light. It does not seem likely however that there was sufficient difference in the amount of photorepair, in the present experiments, to produce a major share of the observed effects on the mutation frequency-dosage relations.

In the absence of quantitative information concerning the influence of unequal illumination and of photorepair, or of data wherein they are excluded, the present experiments do not decide whether in this material the primary mutagenic action of the ultraviolet is usually produced by one or by more than one activation. However, recent low dosage studies by L. and E. Altenburg and Baker indicate that activations act in combination. Recommendations for further tests are presented.

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