X-RAY DOSE RATE AND DOSE FRACTIONATION STUDIES ON MUTATION IN PARAMECIUM

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PREVIOUS evidence has shown (KIMBALL, GAITHER and PERDUE 1957, 1961; KIMBALL, GAITHER and WILSON 1959) that most of the X-ray induced mutations detected by inviability and slow growth after autogamy in *Paramecium aurelia* originate from reparable premutational lesions. These lesions are subject to repair until the chromosomes duplicate, and only then are they fixed as final mutation. Chromosomal breakage is a well-known form of lesion that is subject to repair by restitutional rejoining, but in the best-studied cases, rejoining of breaks appears to be independent of duplication. The earlier evidence for *Paramecium*, however, did not exclude the possibility that the mutational events in this organism arise mainly from breaks that can only reconstitute before duplication and can unite with independently produced breaks only at duplication. This alternative would be more plausible if the aberrations were really not the result of breakage and subsequent rejoining of the breaks, but came about by an exchange process between reparable lesions (REVELL 1959).

Whatever the mechanism of interaction—lesion and exchange or breakage and rejoining—if chromosomal aberrations contribute to mutations, some of them should arise from two or more independent radiation events. This contribution should be decreased by fractionating the dose or by lowering the dose rate. Thus dose-rate and fractionation experiments should allow us to detect the mutagenic contribution by two-hit aberrations, to estimate its magnitude, and to measure the rate of disappearance of breaks by restitutional rejoining (or of exchange-inducing lesions by repair). It should then be possible to compare this rate with the previously measured rate of disappearance of premutational damage (KIMBALL *et al.* 1961), and from this comparison to decide whether the bulk of the premutational lesions could be the same as those producing two-hit aberrations.

Such dose-rate and fractionation experiments have now shown that two-hit aberrations contribute to some extent to postautogamous inviability and slow growth. The bulk of the reparable premutational lesions, however, are repaired more slowly than those producing two-hit aberrations and must, therefore, be a different kind of lesion, quite possibly a precursor of true point mutation. A brief report of these findings along with a summary of our earlier findings on pre-mutational damage has been given (KIMBALL 1963).

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METHODS

The method of detecting mutations in *Paramecium aurelia* has been described previously in some detail, especially by KIMBALL and PERDUE (1962), and will be reviewed rather briefly here. The method is based on the circumstance that the two old micronuclei and the old macronucleus are replaced during autogamy by derivatives of a single, completely homozygous synkaryon formed by the union of the products of the mitotic division of a single haploid meiotic product of one of the two micronuclei. Each treated specimen is allowed to multiply vegetatively to form a small clone. This clone is caused to undergo autogamy, and 25 autogamous specimens are isolated from it, each into a separate container. The frequency (p) of surviving clones with normal growth in this group of 25 is ascertained. This frequency is converted by a variance equalizing transformation to a quantity y , and this is converted to a quantity M by subtraction from the mean y for the unirradiated controls. It has been shown empirically that the variance is effectively equalized over a wide range of values of M and that M increases approximately linearly with dose, though there may be somewhat less than a linear increase at the highest doses. Thus M can be taken as roughly proportional to the amount of mutation, though with an unknown constant of proportionality. Well-established theoretical expressions relating either p or M to dose are not available; consequently, the form of the dose curve cannot be used to test for the presence of a two-hit component, and we have avoided, wherever possible, conclusions that depend on this form.

Synchronized groups of paramecia were obtained, as previously described, by picking dividing specimens out of small log phase cultures, and allowing the group to reach the desired stage in the cell cycle before irradiation. Stationary phase cultures were checked by staining a sample to assure that autogamy was not occurring.

The X-ray source was a General Electric Maxitron 250, operated at 250 kvp, with 3 mm aluminum added filtration to give a half-value layer of 0.44 mm of copper. Different dose rates were obtained by varying the tube current from 0.8 ma to 30 ma.

In the fractionation experiments, the output of the tube was monitored in air immediately before each fraction with a Victoreen 250 r thimble chamber substituted for the container of paramecia.

In the experiments in which dose rates of approximately 1.0 and 0.03 kr/min were to be compared, the following monitoring procedure was used. The output at each beam current was monitored as above before and after the whole set of exposures. During the exposures, the beam was monitored with a Victoreen Radocon integrating dosimeter in air next to the container of paramecia and with the Victoreen 250 r thimble chamber placed in such a position that it accumulated considerably less than the maximum reading. The dose was estimated from (1) the mean dose rate from the *before* and *after* monitorings multiplied by the duration of the exposure, (2) the total dose from the Radocon integrator, and (3) the thimble-chamber reading empirically corrected for its position in the

beam. These three estimates differed from each other by a few percent, and were averaged to give the final estimate of the dose. In one experiment in which dose rates of 1.6 and 0.03 kr/min were compared, only the first method was used because the Radocon integrator was unable to measure the higher rate.

A somewhat different procedure was used in the series of experiments with dose rates of approximately 4.6, 1.6, and 0.4 kr/min. The beam was monitored at the two lower dose rates before and after the whole set of exposures by substituting the Victoreen 250 r chamber for the container of paramecia. The highest dose rate was estimated from these readings, using a correction factor for tube current obtained at a greater distance from the target, since the thimble chamber was unable to measure the highest rate directly. This tube-current factor was obtained separately for each set of monitor readings. The beam was monitored during the exposure of the paramecia with a Radocon integrator and with the 250 and 25 r thimble chambers all placed in such positions that they reached less than their maximum readings. Not all the monitors were read at every exposure; consequently, the doses were calculated only from the pre- and postirradiation thimble-chamber reading, and were equal on this basis for all dose-rate groups. The mean values of the readings during the exposures suggested, however, that the dose at the lowest rate may have been more than that at the other two by about five percent. Thus, the amount of mutation at the lowest dose rate may have been overestimated by five percent relative to the other two.

RESULTS

Fractionated dose experiments with log phase paramecia: The results of two kinds of fractionated dose experiments are summarized in Table 1. In one kind, half the dose was given in the early part of G1 (preduplication interphase), and half in the late G1 of the same cell generation. In the other kind, half the dose was given in one cell generation, and half either one (set B) or four (sets C and D) cell generations later. In this second kind of experiment, both fractions were given in the early part of the G1 period of the respective cell generations, and the paramecia were synchronized before the second as well as the first cell generation by picking out specimens in division. In both kinds of experiments there were two single-exposure groups for comparison with the fractionated one. One of these groups was given the whole dose at the time of the first fraction; the other at the time of the second fraction. In set C both kinds of fractionations were used.

The difference (ΔM) between the mean amount of mutation in the two single-exposure groups, and the amount in the fractionated group should be zero if there were no fractionation effect. The ΔM 's were calculated separately for each experiment, and the variances of these values were calculated from the within-group variances of the transformed variable by the formula for the variance of a difference. An analysis showed that there was no statistically significant heterogeneity between the ΔM 's of the different experiments (variance ratio 0.8 for 12 and 2001 degrees of freedom). Therefore, the variances of the mean differences were calculated from the pooled within-group variances. It is obvious that neither

TABLE 1

Fractionated-dose experiments with log phase paramecia

Set	Number of experiments in set	Number of paramecia per group	Group*	Dose (kr)	Dose rate (kr/min)	Time between fractions (hr)	Mutation	
							<i>M</i>	$\Delta M \pm S.E.$
A	1	80	1,0,0	4.63	1.63		1.424	
			0,1,0	4.57	1.61		1.600	
			1/2,1/2,0	4.60	1.62	1.5	1.548	-0.036 ± 0.078
B	1	80	1,0,0	4.60	1.62		1.506	
			0,0,1	4.60	1.61		1.493	
			1/2,0,1/2	4.60	1.61	6.3	1.481	0.019 ± 0.062
C	4	160	1,0,0	3.00	1.67		1.092	
			0,1,0	3.01	1.65		1.196	
			0,0,1	3.00	1.67		1.130	
			1/2,1/2,0	3.01	1.66	1.4	1.086	0.058 ± 0.041
			1/2,0,1/2	3.00	1.67	23.7	1.075	0.036 ± 0.047
D	1	80	1,0,0	3.00	1.53		1.178	
			0,0,1	3.00	1.67		1.123	
			1/2,0,1/2	3.00	1.59	23.7	1.164	-0.014 ± 0.069
E	2	120	1,0,0	2.52	0.97		0.889	
			0,1,0	2.50	0.96		1.121	
			1/2,1/2,0	2.55	0.96	1.7	0.948	0.057 ± 0.059
Total A,C&E	7	720	{1,0,0 0,1,0	3.19	1.42		1.179	
		360	1/2,1/2,0	3.21	1.42	1.5	1.143	0.036 ± 0.034
Total B,C&D	6	640	{1,0,0 0,0,1	3.40	1.64		1.218	
		320	1/2,0,1/2	3.40	1.64		1.199	0.019 ± 0.033
Grand total	13	1360	{1,0,0 0,1,0 0,0,1	3.29	1.52		1.198	
		680	{1/2,1/2,0 1/2,0,1/2	3.30	1.52		1.169	0.029 ± 0.023

* The numeral in the first position means that a whole (1), half (1/2), or no (0) dose was given early in the first G1 period; in the second position, late in the first G1; and in the third position, early in a later G1 period.

kind of fractionation had a significant effect nor was there a significant effect when the mean of the whole set of experiments was used. The difference is in the right direction, however, for a small two-hit component. This essentially negative result agrees with earlier, smaller scale experiments (KIMBALL 1949, and unpublished).

Dose-rate experiments with log phase paramecia: The effect of irradiation at various dose rates is summarized in Table 2. The six experiments in sets A to E were designed, much like the fractionated dose experiments, to compare the amount of mutation in a low dose-rate group with the mean of the amount in

TABLE 2

Dose-rate experiments with log phase paramecia

Set	Number of experiments in set	Number of paramecia per group	Group*	Dose (kr)	Dose rate (kr/min)	Mutation	
						<i>M</i>	$\Delta M \pm S.E.$
A	1	60	H1	4.47	1.01	1.109	0.145 $\pm$ 0.078
			H2	4.47	1.04	1.040	
			L	4.33	0.036	0.930	
B	1	60	H1	3.46	1.04	1.240	0.126 $\pm$ 0.078
			H2	3.44	1.05	1.209	
			L	3.49	0.037	1.099	
C	1	60	H1	3.30	0.97	1.117	-0.052 $\pm$ 0.095
			H2	3.30	0.97	0.834	
			L	3.20	0.029	1.027	
D	1	80	H1	2.51	1.57	0.740	0.207 $\pm$ 0.080
			H2	2.53	1.58	0.871	
			L	2.52	0.034	0.599	
E†	2	120	H1	2.52	0.97	0.889	0.140 $\pm$ 0.059
			H2	2.50	0.96	1.121	
			L	2.55	0.030	0.865	
Total A,B,D & E	5	640	H1	3.05	1.14	1.009	0.155 $\pm$ 0.036
		320	H2				
			L		0.033	0.854	
F	4	320	1	4.48	4.63	1.445 $\pm$ 0.026	1.394 $\pm$ 0.027
			2	4.48	1.56	1.394 $\pm$ 0.027	
			3	4.50	0.36	1.305 $\pm$ 0.027	

* H1 is the high dose-rate group irradiated before the low dose-rate group (L); and H2 the high dose-rate group irradiated after the low dose-rate group.

† These are the same experiments as set E in Table 1. Each experiment consisted of four groups, namely, 1,0,0=H1; 0,1,0=H2; $\frac{1}{2},\frac{1}{2},0$; and L. L was irradiated in the interval between the two half-dose fractions of $\frac{1}{2},\frac{1}{2},0$.

two high dose-rate ones, the first being exposed just before and the second just after the low dose-rate group. The low dose-rate irradiation occupied a large fraction of the G1 period.

The difference between the amount of mutation in the low dose-rate group and the mean of the amount in the two high dose-rate ones should be zero if there were no dose-rate effect, as can be seen from the following consideration. It is known (KIMBALL *et al.* 1961) that more mutation is produced by irradiating late in G1 than early in G1, and this increase is essentially linear. Thus the mean of the amount of mutation produced by irradiating at high dose-rate just before and just after the low-rate exposure should give a good estimate of the amount of mutation expected from the latter, provided the radiation itself does not affect the rate of progress through G1. Such an effect is unlikely, since doses of X rays of this magnitude have very little effect in *Paramecium* either on the time of division (KIMBALL and PERDUE 1962) or on the time of DNA synthesis in the micronucleus (unpublished data).

A complication can arise from the circumstance that there is a very great (10-fold) decrease in sensitivity to mutation induction during or immediately after the very brief S period following G1 (KIMBALL and PERDUE 1962). Thus the group irradiated at high dose-rate immediately after the low-rate exposure might have included, because of imperfect synchrony, some very resistant G2 cells. Indeed, this may have been the case in sets A and B, since the H2 group did not show the expected increase of mutation over the H1 one. In these two experiments, therefore, the mean of the high dose-rate groups probably was an underestimate of the amount to be expected in the low dose-rate ones, and ΔM , therefore, is an underestimate. It almost certainly was so in set C, in which H2 gave a markedly lower value of M than H1 and showed a distinctly bimodal distribution, some irradiated individuals producing mostly normal exautogamous clones, some mostly not normal. (The distribution at this dose is usually more nearly unimodal because the average number of mutations per micronucleus is considerably greater than one.) The temperature in the X-ray room was higher for set C than for sets A and B; consequently, the G1 period was probably shorter. Set C is omitted from the rest of the calculations. It should be emphasized that all groups in all experiments were taken to the X-ray room and kept there during the complete set of exposures; thus all were at the same temperature throughout.

Just as in the fractionation experiments, there was no statistically significant heterogeneity between the ΔM 's of the five experiments in sets A, B, D, and E (variance ratio 0.3 for four and 945 degrees of freedom). Consequently, they were averaged, and the standard error of this average was calculated from the pooled within-group variances. It is obvious that significantly less mutation was produced by the low dose-rate exposures than expected on the null hypothesis.

The effect of dose rate was explored further in the four experiments shown as set F in Table 2. Three different dose rates were used and the mean values of M and their standard errors were calculated. The total time required to give the three exposures was about 20 min, and they were given early in G1. The order of exposures was varied among the four experiments in such a way as to eliminate the effects on mutation of irradiating at slightly different times during G1. The decrease of mutation with decrease in dose rate is statistically significant (variance ratio 7.1 for two and 957 degrees of freedom).

Figure 1 shows these data in graphical form with the exposure time, rather than the dose rate, plotted on the abscissa. The upper curve shows the results of fitting the points to the equation $M = A + BG$, where A is the dose-rate independent component of M ; B , the dose-rate dependent component of M ; and G , a function given by LEA (1947) for this kind of data. G is given by $2(\tau/T)^2(T/\tau - 1 + e^{-1T/\tau})$ where T is the duration of the exposure and τ is the time for 63 percent of the breaks to rejoin in the absence of radiation. G approaches unity as T approaches zero and approaches zero as T becomes very large. It was assumed by LEA (1947) that the number of breaks decreases in the absence of radiation according to the exponential function, $e^{-t/\tau}$. The best fit to the three points is given by $\tau = 1.8$ min, $A = 1.15 M$ units, and $B = 0.23 M$ unit. The upper curve in Figure 1 is drawn from these values. In order to establish limits for the value

FIGURE 1.—Relation between the amount of mutation produced by 4.5 kr and the duration of the X-ray exposure. The solid circles are the data from Table 2, set F; the open circle point is from Table 2, set A, group L corrected for the low M of the H1 group. The 95 percent confidence limits are shown. Each curve is labeled with the value of τ assumed in constructing it (see text).

of τ , group L in set A of Table 2 was used. This group received nearly the same dose as set F. Unfortunately the average value of M in the H1 group of set A was obviously much lower than would have been predicted from set F. Such variations in M from experiment to experiment are not uncommon, presumably because the life of a clone is brief and different exautogamous clones must be used in each experiment. A correction was made, therefore, by multiplying the value of M for group L by the factor needed to bring group H1 to its proper position on the curve in Figure 1. This corrected value of group L is the point at 120 minutes. The curves of best fit to the first three points with $\tau = 10$ min and $\tau = 16$ min also are shown. The 10-minute curve falls near the lower 95 percent confidence limit of the 120-minute point; the 16-minute one, near the uncorrected value of the 120-minute point. Finally, if the uncorrected lower 95 percent confidence limit, 0.805 M unit, for group L of set A, is used, the best fit is given by $\tau = 25$ min. It seems very probable, therefore, that the true value of τ lies somewhere between one and ten minutes, and it is essentially certain that it is no greater than 25 minutes. The value of τ may be even smaller than these calculations indicate if M was overestimated for group 3 of set F, as suggested by some of the monitor readings (see METHODS).

Experiments with stationary phase paramecia: The results of two sets of experiments with stationary phase paramecia are summarized in Table 3. The first set essentially repeats for stationary phase the dose-rate experiments listed under sets A to E in Table 2. In one of the three experiments, the paramecia from the

TABLE 3

Dose-rate and dose-fractionation experiments with stationary phase paramacia

Set	Number of experiments in set	Number of paramacia per group	Group*	Dose (kr)	Dose rate (kr/min)	Mutation	
						<i>M</i>	$\Delta M \pm \text{S.E.}$
A	3	380	H1 & H2	3.33	1.08	0.500	
		220	L	3.41	0.031	0.489	0.011 $\pm$ 0.033
B	2	160	S	4.53	1.65	0.944	
		160	F	4.53	1.65	0.774	0.170 $\pm$ 0.056

* H1, H2, and L have the same meaning as in Table 2. S is the mean of the four single-exposure groups; F, of the fractionated-dose group. See text for further details.

two high dose-rate groups were mixed, and a single group of 60 paramacia was obtained from the mixture for comparison with the 60 obtained from the low dose-rate group. In the other two experiments, groups of 80 paramacia were obtained separately from each high dose-rate group as well as from the low dose-rate ones. There is no significant heterogeneity among the ΔM 's of the three experiments (variance ratio 1.91 for 2 and 592 degrees of freedom); therefore, the pooled data were used and show, unlike the case for the log phase paramacia, that dose-rate differences of this magnitude had no detectable influence.

The second set of experiments was designed to look for a fractionation effect. The dose was given to one group in four equal fractions in a period of slightly less than two days with six to 17 hours between the fractions. At the time of each fraction, a group was given the whole dose in a single exposure. In each experiment, 20 paramacia were obtained from each single-exposure group, and 80 from the fractionated-dose groups, and the data from the single-exposure groups were averaged for presentation in the table. All groups were kept in stationary phase until all the irradiations were completed, and then transferred to fresh medium simultaneously. The two experiments were not identical in the arrangement of long and short intervals between fractions nor in the time between the final fraction and transfer, but this difference did not result in appreciable heterogeneity in the ΔM 's (0.190 and 0.150 *M* unit). Clearly, fractionation of the dose into four equal parts spread out over about two days has reduced appreciably the amount of mutation.

DISCUSSION

The results of the fractionation and dose-rate experiments can be explained by a small but measurable contribution of two-hit chromosomal aberrations to post-autogamous lethality and slow growth. It is necessary to assume that the breaks responsible for the aberrations rejoin rapidly in log phase but more slowly in stationary phase. The evidence for rapid rejoining in log phase is of two main kinds: (1) the dose-rate experiments plotted in Figure 1 show that the half-life of breaks must be small, probably no more than a few minutes, and (2) the fractionation experiments lead to a smaller estimate of two-hit aberrations than do

the dose-rate experiments, suggesting that rejoining is partially completed within the minute or so required to give a half dose. These two points will be discussed in some detail.

As was shown in the results section, the lifetime of the breaks can be calculated from the dose-rate data by using the relation $M = A + BG$ where A is the one-hit or dose-rate independent component, B the two-hit or dose-rate dependent component, and G is a function (LEA 1947) of the ratio between the duration (T) of the exposure and the time (τ) for 63 percent of the breaks to rejoin. The assumption that the G function can be used for aberrations expressed in M units cannot be rigorously proved, but on this assumption, τ can be shown in all probability to lie between 1 and 10 mins, and it is most unlikely to be as high as 25 minutes. Whatever function may be appropriate, if G is not, it is clear from Figure 1 that the lower asymptote, A , cannot be much less than the value of M when the dose is given in about two hours. It is also clear that the time constant must be such that when $T = 15$ min, BG is not more than half the value obtained when T approaches zero. This in conjunction with the shape of the curve makes it probable that no more than one-third the maximum value of M , quite possibly less, can be attributed to two-hit aberrations, and that the breaks must have a half-life in the order of minutes, not hours.

These conclusions are supported by a comparison between the fractionation and dose-rate experiments. At the higher dose rate, the two-hit component, BG , of M should be equal to ΔM in Table 2, since at the lower dose rate, the value of M should be close to that of the dose-rate independent component, A . The two-hit component should be equal to twice ΔM in Table 1 if the following assumptions are correct: (1) The number of two-hit aberrations, N , is directly proportional to its contributions to M —i.e., $M = A + bN$; (2) the number of two-hit aberrations increases as the square of the dose—i.e., $N = cD^2$; and (3) no interaction occurs between the breaks produced by different dose fractions, but complete interaction occurs between the breaks produced by the same dose fraction—i.e., $N = n(cD/n)^2$, where n is the number of equal dose fractions. If these assumptions are true, then ΔM in the fractionation experiment should be $\Delta M = bcD^2 - 2bc(D/2)^2 = bcD^2/2$. On this basis, the two-hit component, bcD^2 , can be calculated from Table 1 to be $0.058 \pm 0.046 M$ unit ($2 \times 0.029 \pm 0.023$), and from Table 2 to be $0.155 \pm 0.036 M$ unit. The difference between these two estimates is significant at the ten percent but not the five percent level. When considered in conjunction with the small value of τ from the dose-rate data, however, the difference between the estimates strongly suggests that fractionation has less effect than expected because rejoining is so rapid that interaction between breaks is incomplete in single exposures to X rays lasting as long as a minute or so. In this case, ΔM would be better represented by $\Delta M = bcG(T)D^2 - 2bcG(T/2)(D/2)^2$, and this would be appreciably less than $bcD^2/2$ if $G(T)$ were appreciably less than unity. The arguments of this paragraph thus support the view that τ is of the order of minutes, not hours. The arguments are less convincing than those of the preceding paragraph, both because the evidence is less compelling statistically, and because it has been neces-

sary to make specific assumptions about dose relations, assumptions that cannot be rigorously defended.

No matter what assumptions are made to interpret them, the fractionation and dose-rate experiments taken together show that slowly rejoining breaks can contribute few, if any, mutations. If, despite the arguments just made, the half-life of most of the breaks were an hour or more, then the statistically significant decrease in mutation when T is increased from one to 13 minutes must mean that two-hit aberrations make a large contribution to M . The fractionation experiments, however, exclude a large contribution by such slowly rejoining breaks. If there were a class of slowly rejoining breaks in addition to the rapidly rejoining ones, then the small value of ΔM in the fractionation experiments, in which one or more cell generations intervened between the fractions, shows that such slowly rejoining breaks cannot make an appreciable contribution, since even the most slowly rejoining breaks would be expected to rejoin within one cell generation. In particular, the idea is excluded that there is an appreciable contribution to M by breaks or lesions that can only reconstitute or repair before the chromosomes duplicate, but then can rejoin with independently produced breaks or exchange with independently produced lesions to form two-hit aberrations.

WOLFF and LUIPPOLD (1956a) found two classes of breaks in *Vicia*, a slowly rejoining class with a lifetime of the order of hours, and a rapidly rejoining class with a lifetime of less than a minute. We are inclined to identify our presumptive breaks with their slowly rejoining class despite the greater rapidity of rejoining in *Paramecium*, and to think that our dose rates have been too low to detect their rapidly rejoining class. However this may be, it is clear that we do not have a class of breaks with a lifetime of the order of hours.

The data in Table 3 suggest that rejoining is much slower in stationary phase than in log phase. There is little if any rejoining within the 110 minutes required to give the dose at low rate, but appreciable rejoining within the two days over which the fractionated dose was spread. A slow rate of rejoining in stationary phase is in accord with WOLFF and LUIPPOLD's evidence (1956b) that the rate of rejoining depends on energy from metabolism and on protein synthesis.

The experiments with stationary phase *paramecia* also provide a check on the validity of the dose measurements for high and low dose-rates. The same methods of dosimetry were used as for the experiments with log phase *paramecia*. Therefore, the lack of a significant ΔM in the stationary phase experiments makes errors in dosimetry unlikely as an explanation for the statistically significant values of ΔM in the log phase experiments.

The number of two-hit aberrations produced by complete interaction between breaks appears to be the same in stationary and log phase. If complete rejoining occurred between fractions, then the total contribution of two-hit aberrations to M , that is, bcD^2 , should be calculable from the data in Table 3 as $4 \Delta M/3$ because $\Delta M = bcD^2 - 4bc(D/4)^2 = 3 bcD^2/4$, using the same assumptions made in the discussion of the log phase fractionation experiments. This gives an estimate of 0.23 M unit, which is a minimum estimate, since rejoining between

fractions may well have been incomplete. This estimate is identical with the estimate of the two-hit component, 0.23 M unit, from the best fit to set F in Table 2. The latter appears to be a minimal estimate also, judging from Figure 1. Therefore, it seems quite possible that the contribution of two-hit aberrations to postautogamous inviability and slow growth at a dose of 4.5 kr is greater than 0.23 M unit, perhaps as great as 0.5 M unit, but there is no reason to think that it is different in log and stationary phase, provided the dose could be given rapidly enough in the former to allow complete interaction between breaks.

In order to compare the rate of removal of breaks by rejoining and of pre-mutational damage by repair, the latter must be calculated as a fraction of the total reparable damage. The total reparable damage can be estimated in two ways: postirradiation treatments with different concentrations of streptomycin have reduced the amount of mutation produced by 4.5 kr by 0.4 M unit (KIMBALL *et al.* 1961). This is probably not a lower limit for the streptomycin effect; consequently, it is a minimal estimate of reparable premutational damage. A second estimate comes from the comparison between the maximum amount of mutation produced by irradiating in log phase and the minimum amount produced by irradiating in stationary phase. Irradiation with 4.5 kr at 1.6 kr/min in log phase just before chromosome duplication produces about 1.7 M units of mutation (KIMBALL *et al.* 1961). This procedure should allow nearly maximum conversion of reparable premutational damage to mutation, because the time for repair is very short. Irradiation with 4.5 kr at 1.6 kr/min in stationary phase followed by a postirradiation stationary phase period of several days produces a lower limiting value of about 0.6 M units (KIMBALL, GAITHER and WILSON 1959). In this case the time for repair is so long that probably little, if any, of the premutational damage is available for conversion to mutation when the chromosomes duplicate. The difference between these two values, 1.1 M units, is thus an estimate of the maximum contribution to M by reparable premutational damage. Thus it seems probable that the true value is 1 M unit or a little more, but it could not be less than the 0.4 M unit estimated from the streptomycin data.

The rate of repair of premutational damage has been estimated by KIMBALL *et al.* (1961) to lie between 0.1 and 0.2 M unit per hour after a dose of 4.5 kr in log phase. Thus no more than half the premutational damage is lost per hour (0.2 M units per hour/0.4 M unit), and a more probable estimate is 15 percent per hour (0.15 M unit per hour/1 M unit). Consequently, the half-life of pre-mutational damage under log phase conditions cannot be less than one hour, and is probably much longer.

A long half-life is also indicated, though less precisely, by the observation (KIMBALL *et al.* 1961) that mutation can be decreased by 0.2 to 0.3 M units by a post-treatment with streptomycin begun an hour after irradiation. Thus at least this much of the premutational damage must remain unrepaired after an hour. This is a minimum estimate, since the streptomycin treatment was insufficient to produce a maximum effect. These calculations taken together show quite unambiguously that the half-life of the bulk of the premutational damage

under log phase conditions is much longer than the half-life of breaks. The former is of the order of hours, the latter of minutes. It follows that the chromosomal lesions involved must be different.

The lesions making up the bulk of the premutational damage have the following properties: (1) they do not interact with one another to produce two-hit events; (2) they are repaired at a relatively slow rate during log phase, and still more slowly during stationary phase or in the presence of various metabolic inhibitors (KIMBALL *et al.* 1959, 1961); and (3) they are fixed as mutation at the time of DNA synthesis (KIMBALL *et al.* 1961; KIMBALL 1962). These properties are clearly compatible with the initial lesion, being enzymatically reversible alterations of the DNA bases. Various other possibilities cannot be excluded, but a contribution by breaks in the DNA helices or in protein linkers could be involved only if these breaks were unable to react with others to produce two-hit mutational events.

To avoid possible misinterpretation, it should be pointed out that our attribution of dose-rate effects in *Paramecium* to two-hit chromosome aberrations does not imply a belief that the same attribution could be made for the superficially similar dose-rate effects for specific locus mutations in the spermatogonia and oocytes of the mouse (RUSSELL, RUSSELL and KELLY 1958; RUSSELL 1961). The dose-rate effects are very much larger in the mouse than in *Paramecium*, and neither the dose curves nor the infrequent occurrence of detectable deficiencies in the spermatogonia would encourage the view that such a large fraction of the specific locus mutations in the mouse could arise from two-hit events. As a matter of fact, the *Paramecium* data give general support to RUSSELL's interpretation of the mouse data in terms of reparable premutational damage, since a large fraction of all one-hit events in *Paramecium* do arise from such reparable damage. The lack of a dose-rate effect on this class of mutational events in *Paramecium* may result from the circumstance that the mutagenic doses of X rays probably produce little, if any, effect on the over-all metabolism of this organism, judging by the very small effects on cell growth and generation time. Since mammalian cells are in general much more sensitive to radiation than protozoa, larger effects on metabolism might be produced by mutagenic doses in the mouse and, consequently, larger effects of the radiation on repair.

There has been a lively discussion in the past on whether ionizing radiations produce only chromosomal aberrations or point mutations as well (see, for example, STADLER 1954; MULLER 1956, 1958). In *Paramecium* it seems clear that at least two kinds of initial damage are produced, one having the properties expected of the precursor of chromosomal aberrations—i.e., breaks—and the other having properties entirely compatible with a precursor of point mutations. It may be possible eventually to show genetically that the events produced by the first kind of damage are chromosome aberrations. Identification of the events produced by the second kind of damage as point mutations can never be more than a presumption in *Paramecium*, since fine structure analysis is probably beyond the genetic potentialities of this organism. The *Paramecium* studies, however, can serve as a guide to what to look for in organisms better suited for

fine structure studies, but less well suited for analyzing premutational processes. Thus the chance of producing point mutations by X rays should be greatest if the time to DNA synthesis is very short; least, if it is very long. The chance of producing two-hit aberrations with a given dose rate should differ greatly from one cell type to another, depending on the rate of rejoining. It will be high when the rate of rejoining is low relative to the dose rate, low when it is high. It is of interest in this connection that a higher ratio of demonstrable chromosomal aberrations to apparent point mutations is found among the mutations induced in sperm and in the late stages of spermatogenesis than in spermatogonia for both *Drosophila* (WARD and ALEXANDER 1957) and the mouse (RUSSELL, BANGHAM and GOWER 1958; RUSSELL and RUSSELL 1959, 1960). Moreover, there is some evidence for maize that apparent point mutations can be induced in the earlier stages of microsporogenesis (CASPER and SINGLETON 1957) although they have not been detected in pollen (STADLER and ROMAN 1948; STADLER 1954). The evidence is somewhat fragmentary and subject to various interpretations. Nevertheless, when considerable time must elapse before the chromosomes next duplicate and the rate of metabolism, and so the rate of rejoining, may be low, there is evidence for a high ratio of aberration to apparent point mutations. On the other hand, when chromosome duplication is recurring regularly and the rate of metabolism, and so the rate of rejoining, may be high, this ratio is lower. These findings are at least suggestive that similar relations to those found in *Paramecium* may exist in other organisms.

SUMMARY

The contribution of two-hit mutational events to X-ray induced lethal and slow-growth mutation in *Paramecium aurelia* has been estimated by dose-rate and dose-fractionation studies. The half-life of the lesions producing the two-hit events is of the order of minutes under log phase conditions, whereas our earlier work shows that the half-life for one-hit, reparable premutational lesions is of the order of hours. Both types of lesions have longer half-lives in stationary phase, as shown in our earlier work for one-hit damage, and in the present study for two-hit.

At least two kinds of reparable lesions are induced in chromosomes by X rays: (a) a lesion with a short half-life in log phase that can interact with similar lesions to produce two-hit events, and (b) a lesion with a longer half-life that is unable to interact to produce two-hit events, and produces final mutation only when the chromosomes duplicate. Lesion (a) is presumed to be a chromosomal break that can interact to produce chromosomal aberrations. Lesion (b) is presumed to be the reparable precursor of point mutations. Genetic evidence for this identification is not available, however.

The relative contribution of these two kinds of lesions to the final mutation will depend on the dose, on the relation of the dose rate to the rate of rejoining of the breaks, and on the rate and time available for repair of the precursor of point mutation. This latter type of lesion can contribute most of the mutations under some conditions, essentially none under others.

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