RECOVERY IN STATIONARY-PHASE PARAMECIA FROM RADIATION EFFECTS LEADING TO MUTATION

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Recent studies\(^1\)\(^-\)\(^12\) of mutation induction by ionizing radiation and ultraviolet light have indicated some sort of reversibility in the mutation process and have established the important facts that alterations in postirradiation metabolism can change the amount of mutation and that a time limit exists for this change. This time limit probably occurs before the first postirradiation division in most, if not all, cases.

The following terminology will be used to make it easier to discuss these phenomena. The damage produced by the absorption of the radiation and the rapid reactions that immediately follow it will be called \textit{initial damage}. The term may include some mutation, i.e., inherited changes in the chromosomes, which are reversible only by further mutation if at all. It also includes damage that is potentially capable of leading to mutation but has not yet done so. This will be called \textit{premutational damage} in preference to such a term as "potential mutation" because we wish to include all forms of radiation damage that might eventually cause or facilitate mutation, e.g., diffusible mutagens and metabolic disturbances as well as local lesions in the chromosomes. The period between irradiation and the time limit during which premutational damage may be influenced by postirradiation treatments will be called the \textit{intermediate period}. We prefer this to Witkin's term\(^12\) "sensitive period" since the latter has also been used in describing stage sensitivity. The premutational damage that is still present at the end of the intermediate period may be converted to mutation during what will be called, following Witkin,\(^12\) the \textit{terminal event} and any other damage that is still present after this event no longer has any influence on the amount of mutation.

Earlier work\(^6\)\(^-\)\(^7\) has shown that recessive lethal and other deleterious mutations in \textit{Paramecium aurelia} conform to this general pattern. Posttreatment with various metabolic inhibitors decreases the amount of mutation; there is a time limit (the terminal event) for this modification about midway through the interdivision interval. Furthermore, the amount of detectable mutation increases the nearer to the terminal event the radiation is given. These findings were interpreted to mean that premutational damage is lost during the intermediate period and that posttreatment agents act by increasing the time available for loss. There was no direct demonstration, however, of loss during the intermediate period; it was simply inferred from the increase of mutation with decrease of the time between irradiation and the terminal event on the reasonable but untested assumption that the amount of initial damage is not dependent on the time of irradiation during interphase. This paper shows that loss of premutational damage really does occur and gives some information about its rate. This was accomplished by irradiating paramecia in the stationary phase and varying the period between irradiation and transfer to fresh culture medium.

Materials and Methods.—\textit{Paramecium aurelia} (variety 4, stock 51) individuals
undergoing the sexual process of autogamy were isolated and allowed to divide 10–12 times in limited quantities of culture medium (lettuce infusion inoculated with *Aerobacter aerogenes*). Five to seven days after isolation, samples were checked for autogamy and cultures with less than 1 per cent autogamy were selected; autogamy only rarely recurs so soon after a previous one.

The paramecia were irradiated in the exhausted culture medium in which they had been growing and kept en masse in this medium. At appropriate times after irradiation, animals were transferred to fresh culture medium, each to a separate container, and cultured thereafter by the daily isolation method.

Recessive lethal and other deleterious mutations were detected as in previous work. From each irradiated paramecium, 25 autogamous progeny were isolated 5–10 days after irradiation. The fraction of each group of 25 that survived with normal growth was recorded, and these fractions were transformed by a variance-equalizing transformation to give a variable that decreases as mutation increases. This variable was subtracted from 2.4, the mean of a large series of unirradiated controls, in order to obtain a quantity, *M*, that increases as mutation increases. *M* increases nearly linearly with dose and so is probably directly proportional to the number of mutations, though the proportionality factor is unknown.

The X-ray source was a General Electric Maxitron 250 operated at 250 kvp, 30 ma, and with 3 mm of Al added filtration, giving a half-value layer of 0.43 mm of Cu. Before each run, monitoring was done with a Victoreen thimble chamber. The intensity varied from 1.5 to 1.8 kr per minute, and the total dose was 4.5 kr.

**Results.**—Figure 1 shows the results of three series of experiments in which the time between irradiation and transfer to fresh culture medium was varied. The paramecia were 5 days old in some experiments and 7 days old in the others. No

![Figure 1](image-url)
differences that can be attributed to this variation in preirradiation starvation have been found in these or other experiments.

The data show a continuous decrease in mutation with duration of postirradiation starvation. The 24- and 48-hr points in the second series are significantly different at the 5 per cent level, showing that the decrease certainly continues for 24 hr, and there is no definite time at which the decrease stops. Instead, the curve appears to approach asymptotically to a lower limit of approximately 0.6.

Before the decrease in \( M \) in Figure 1 can be interpreted as a true loss of premutational damage, certain selection artifacts must be excluded. Since a dose of 4.5 kr is only 1–2 per cent of the dose needed to kill any vegetative paramecia, selective death during the period of holding en masse between irradiation and transfer to fresh medium is most improbable. Selective death could occur if autogamy took place during the postirradiation starvation since radiation-induced lethals could then become homozygous. This is an unlikely source of error, however, because (1) no increase in autogamy was observed, (2) death after autogamy usually occurs only after a postautogamous period of growth, and (3) only about 2 per cent of the paramecia that were transferred to culture medium failed to survive to produce autogamous progeny. This is too small a frequency to account for the decrease in detectable mutation; and, in any case, the percentage was nearly the same in the different time groups. Thus selective elimination of the more mutant animals by death before detection of mutation cannot be responsible for the results.

The existence of a real loss of premutational damage does not necessarily mean that the loss occurs during starvation. The additional starvation might change the conditions of the subsequent growth period in such a way that a greater loss would occur after transfer to fresh medium. If this were so, pre- and postirradiation starvation should be equally effective. The data in Table 1 show that an additional 2 days of preirradiation starvation had no effect. Therefore, the loss of

<table>
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<th>Table 1</th>
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<tr>
<td><strong>Lack of Effect of Additional Preirradiation Starvation on Mutation</strong></td>
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<tr>
<td>Preirradiation starvation time, days</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>5</td>
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premutational damage occasioned by postirradiation starvation must occur during the starvation period itself. This does not mean, however, that loss occurs only then. It is highly probable, from the earlier studies, that loss of damage also occurs after transfer to culture medium, but the data show that this latter loss is unaffected by variations in the period of starvation within the range used here.

Exposure of the animals to antibiotics after transfer to culture medium gave evidence that loss can indeed occur during the growth period. Irradiated, stationary-phase paramecia were transferred either shortly after irradiation or 24 hr later to culture medium containing 1 mg per ml of streptomycin (Lilly) or 0.5 mg per ml of chloramphenicol (Pfizer). The animals were left in the antibiotic for 3 hr before transfer to culture medium without the antibiotic. The data are shown in Table 2. The antibiotics decrease the amount of mutation just as they do for exponentially
TABLE 2

<table>
<thead>
<tr>
<th>Postirradiation starvation before antibiotic treatment, days</th>
<th>Antibiotic treatment</th>
<th>( M \pm \text{s.e.} )</th>
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<tr>
<td>0</td>
<td>-</td>
<td>1.01 ± 0.05</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>0.88 ± 0.05</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>0.88 ± 0.05</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>0.81 ± 0.05</td>
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growing paramecia. (The effect of chloramphenicol on paramecia has not been reported previously but is much the same as that of streptomycin.) There is a statistically significant effect for the group transferred immediately but not for that transferred after 24 hr. The difference is in the same direction, however; and the mean square for interaction between treatment and time is not significant. The effect at 24 hr would be expected to be small if a lower limit is being approached.

Discussion.—In our earlier papers on postirradiation treatments, the hypothesis was advanced that (1) loss of premutational damage occurs up to the terminal event, (2) this event is some feature in the normal development for division, and (3) posttreatments decrease detectable mutation by allowing more time for loss. The previous data made this hypothesis quite attractive, but none of its special features were completely demonstrated. The present experiments give strong support to the first two assumptions and give some information about the third.

The second assumption was based on the observation that posttreatments, to be effective, had to be started before the middle of the interdivisional interval. This moment was also marked by a sudden change in apparent "sensitivity" to mutation induction and has recently been shown to be the time at which DNA synthesis starts in the macronucleus. The time of DNA synthesis in the micronucleus, the nucleus in which mutations are detected, has not yet been ascertained. The present studies show that the period in which modification can occur does not come to a sudden end when the paramecia are not growing and makes the previous conclusion that the terminal event is a normal feature of development for division still more probable.

The first assumption is also confirmed by the present data. The main evidence for this in the previous studies was the observation that the earlier in the interdivisional interval the radiation was given, the less the mutation. This was interpreted to mean that the long intermediate period after early irradiation gave more time for loss of premutational damage, but a gradual increase during the interval in susceptibility to initial damage could not be excluded. The present data prove unambiguously that loss of premutational damage can occur during the intermediate period. This is shown, however, only for paramecia in the stationary phase, and final evidence for exponential growth will have to come from other experiments.

Suggestive evidence for an extension to exponential growth is provided by the present results, however. In the first place, the experiments with antibiotics show that irradiation of starved paramecia produces premutational damage that can be decreased by antibiotic treatment during subsequent growth. It seems probable that this is the same premutational damage that is lost during starvation since 1 day's starvation produces a very similar decrease in the amount of additional loss caused by either antibiotics or further starvation.
The second argument that supports loss during growth comes from a comparison of the maximum mutation produced by 4.5 kr to stationary and exponentially growing paramecia. Irradiation of the latter just before the presumptive terminal event gives a value for mutation of 1.7 $M$. Irradiation 2.5 hr earlier, at the beginning of the interdivision interval, gives 1.3 $M$. The maximum for irradiation in the stationary phase followed immediately by transfer to culture medium is 1.2 $M$. It is possible, of course, that starvation decreases the susceptibility to initial damage, but there is no a priori basis for predicting such an effect or its direction, and the physical state of the micronucleus, as far as it can be judged by microscopic observation, is not changed by starvation. The hypothesis of loss of premutational damage during growth predicts both the existence and direction of the effect since, even with immediate transfer to culture medium, a long intermediate period must intervene before the terminal event is reached.

The final feature of the original hypothesis that we wish to discuss is the assumption that decrease in mutation by postirradiation treatment is primarily the consequence of the increased time available for loss of premutational damage. The present data suggest, however, that the rate of loss of premutational damage is influenced by the metabolic state of the cell. The rate of loss during exponential growth can be estimated from the data in the previous paragraph to be about 0.4 $M$ in 2.5 hr, on the assumption that the difference between irradiating early and late in the interdivision interval is entirely caused by the difference in time available for loss. The rate of loss in stationary-phase paramecia is approximated by the initial slope in Figure 1 and is obviously much lower than during exponential growth. This leads to the conclusion that loss is a metabolic process proceeding more rapidly in growing than in stationary cells. Thus the original hypothesis must be modified to take into account metabolic influences on the rate of loss. This does not alter the original conclusion that decrease in mutation is caused by increase in the time available for loss, provided agents such as streptomycin decrease the rate of loss. It would mean that the two effects, increase in time and decrease in rate of loss, are often balanced in such a way that the amount of mutation is decreased. These problems are under further investigation.

The problem of determining the rate of loss of premutational damage requires a little more discussion. Most postirradiation procedures must be assumed to influence both the rate of loss and the time available for it, unless there is evidence to the contrary. Thus the slope of the curve relating mutation to the duration of a posttreatment cannot, in general, be separated into the two component effects unless additional information is provided. In the case of stationary-phase paramecia, evidence has been presented that additional starvation does not affect the duration of the poststarvation interval (Table 1). Even then, the damage lost during this interval is probably a function of the damage remaining at its beginning; i.e., of the duration of the postirradiation starvation. The initial slope of the curve in Figure 1 should provide a good approximation, however, to the rate of loss of premutational damage during the stationary phase since only a small change in $M$, and so presumably in premutational damage, is caused by several hours of starvation. Discussion of measurement of the rate of loss for other organisms and by other methods in Paramecium will be deferred until a later publication.

The present experiments demonstrate that at least some forms of premutational
damage undergo loss during the intermediate period and lead to mutation only during the terminal event. Other processes, conversion to mutation during the intermediate period or increase in premutational damage with time, would lead to an increase instead of a decrease in mutation with increase in postirradiation starvation. Such processes might occur and be overbalanced by the loss process, and in this case, the lower limit in Figure 1 could be accounted for by an eventual balance between loss and gain. It is quite probable, however, that some mutation is included in the initial damage, and this alone could account for a lower limit. Consequently, there is no need at present to assume any form of premutational damage other than that subject to loss during the intermediate period and conversion to mutation during the terminal event.

Two main suggestions have been made about the influence of metabolism on the mutational process, in addition to its effects on the duration of the intermediate period. On the one hand, synthesis of macromolecules has been postulated\(^1\)\(^2\) to convert premutational damage to mutation; on the other, synthesis of such molecules has been thought\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\) to repair or eliminate radiation damage. The *Paramecium* results are more in accord with the latter view because there is no direct evidence for conversion to mutation before the terminal event, and loss of premutational damage seems to occur at a higher rate in rapidly growing than in stationary cells. Metabolic processes could also be involved in the conversion to mutation during the terminal event, but no evidence for this is yet available in *Paramecium*.

**Summary.**—Previous work with exponentially growing paramecia led to the hypothesis that part of the recessive lethal and other deleterious mutations induced by radiation in *Paramecium aurelia* comes from premutational damage that is subject to loss up to a terminal event, about midway through the interdivision interval. At this time the remaining damage leads to mutation. The present studies show that, during the stationary phase, loss of radiation-induced premutational damage continues until a lower limit, possibly mutation induced at the time of irradiation, is reached. The data unequivocally demonstrate loss of premutational damage; this phenomenon had previously been inferred on somewhat equivocal grounds. They also support the earlier conclusion that the terminal event is something in the normal development of growing cells since there is no evidence for such an event in nongrowing cells. It is suggested that loss of premutational damage is a metabolic process, possibly involving repair, since the rate of loss is much lower in the stationary phase than in exponential growth.

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\(^*\) Operated by Union Carbide Corporation for the United States Atomic Energy Commission.


9 Newcombe, H. B., in *Brookhaven Symposia in Biology*, No. 8, pp. 88–102 (Brookhaven National Laboratory, Upton, L. I., 1956).

CHROMOSOME BREAKAGE PRODUCED BY TRITIUM-LABELED THYMIDINE IN TRADESCANTIA PALUDOSA*

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Introduction.—The original preparation of tritium-labeled thymidine by W. L. Hughes and its application by Taylor, Woods and Hughes in studies of chromosome duplication have led to wide-scale use of this labeled material in other areas of research. The fact that thymidine is a specific precursor of deoxyribonucleic acid (DNA) and that autoradiographs of very high resolution are obtainable using tritium have triggered most of these inquiries. Partial listings of the numerous investigations in which H³-thymidine has been used are given by Cronkite et al. and Hughes.

Most of these studies have been concerned with the synthesis of DNA, the duplication, behavior and structure of chromosomes and the kinetics of cell populations, but relatively little research has been devoted to the possible biological effects of the internal soft beta radiations emitted by tritium. Although most workers have never doubted that some damage occurs, the extent of the damage has not been satisfactorily determined. Recently Plaut has cautioned that use of H³-thymidine in experiments may lead to erroneous conclusions because of the endogenous radiation. Painter, Drew, and Hughes in a current paper have shown a marked inhibition of HeLa cell cultures grown on media containing 2.5 to 5.0 μc/ml of H³-thymidine. Taylor noted that fragments and interchromosomal exchanges were sometimes observed in squash preparations of Bellevalia romana roots that had been grown in nutrient solutions containing 2.5 to 5.0 μc/ml H³-thymidine. In the present work chromosome fragmentation and mitotic inhibition are shown to occur in an organism treated with H³-thymidine, and the degree of fragmentation will be related to amount of intranuclear irradiation measured by means of autoradiographs.

Materials and Methods.—Root tips of Tradescantia paludosa were used as experimental material. Plantlets were removed from the clonal parent (B2-2) and the bases were placed in aerated Hoagland’s solution under constant light (c. 400 ft. candles) and constant temperature (20° ± 1°C). After 72 hours most of the plantlets showed well developed roots.

Two procedures were used for labeling the nuclei. Some rooted plantlets were grown continuously in aerated nutrient solution containing 1 μc/ml H³-thymidine...