Role of Externally Produced Hydrogen Peroxide in Damage to *Paramecium aurelia* by X Rays.* (19679)

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It has been suggested repeatedly that the effects of X-rays on cells are due, at least in part, to hydrogen peroxide produced by the irradiation of water. Not all investigators agree that it plays a prominent role, but it seems clear that this substance must be considered carefully in any investigation of radiation damage. We have been able to show that it is of major importance in the production of certain kinds of nongenetic effects in *Paramecium aurelia* but only under certain circumstances. We have not been able to demonstrate any effect upon the genetic mechanism.

Methods. A General Electric Maxitron 250 machine was used at 250 kvp and 30 ma. The machine had an inherent filtration of 1 mm of Al and no filtration was added. Measurements of intensity were made in air with a nylon Victoreen thimble chamber. This method cannot be considered to give more than a rough approximation to the intensity because of the large soft component. Intensities measured in this way were 70 and 15 kr/minute at the 2 positions used. The animals were irradiated in thin liquid preparations by methods to be discussed elsewhere(1). Stock 90 of variety 1 of *P. aurelia* was used throughout and all animals were selected to be in the first half of the interval between successive divisions.

After exposure, the irradiated animals, usually 30 for any one treatment group, were isolated individually in depression slides. The next day the number of animals in each depression was recorded. From these records, the percentage of survival and the average number of divisions among the survivors were calculated. These 2 quantities are considered to be measures of nongenetic effects for reasons discussed by Kimball and Gaither(2). Genetic effects were investigated by allowing the descendants of some of the irradiated animals, usually 20 from each treatment group, to undergo autogamy and isolating 25 autogamous animals from the descendants of each irradiated animal. At low doses, the percentage of autogamous animals that produced a large population in 4 days was used as a measure of genetic effect; whereas, at high doses, the percentage surviving at 4 days was a more convenient measure. The procedure for low doses was described by Kimball(3).

The culture medium (C) used in all these experiments was a lettuce infusion inoculated with *Aerobacter aerogenes* one day before use. In a number of investigations, irradiation and other treatments were carried out in a medium (D), consisting of 3 parts of boiled C to 97 parts of distilled water or 0.001 M KH_2PO_4 adjusted to pH 7. Just before treatment, the paramecia were transferred through 6 changes of this medium by capillary pipette, and groups of about 50 washed animals were transferred into separate depressions of D. The dilution of the original culture medium was at least 1:50,000 and usually greater. All exposures to H_2O_2 were made in this medium.

Effect of D medium and its various modifications. A comparison between animals irradiated in C and in D medium is given in Fig. 1. In each experiment, a group of paramecia in C medium and one in D medium were exposed simultaneously to the same dose. In part of the experiments, more than one dose was given. Points in Fig. 1 are averages for similar doses over all experiments (an average of 3 per point). Considerable variation was found from experiment to experiment in the response to a given dose, but consistent dose curves were obtained within experiments in which more than one dose was given. It is clear from the figure that there was a greater nongenetic effect in animals irradiated in D than in C medium. However, there was no appreciable influence of the medium upon

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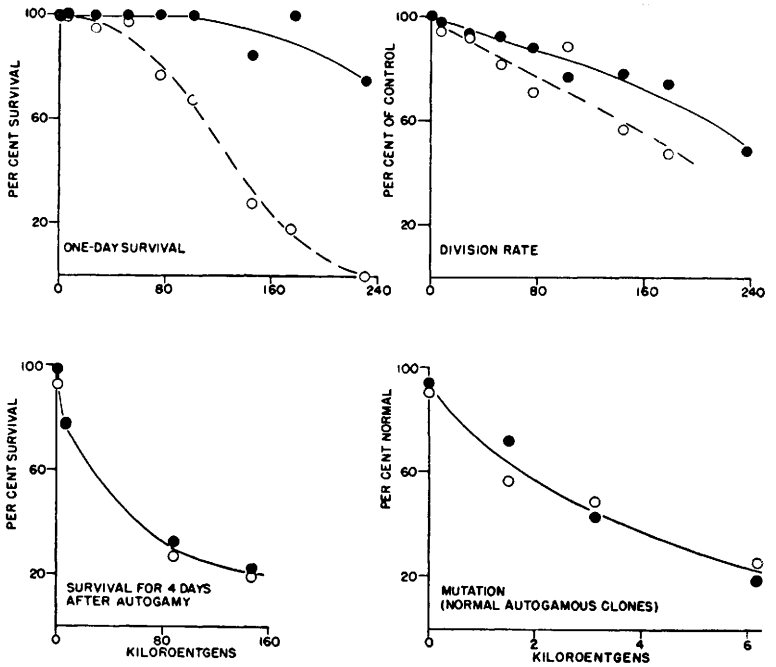

FIG. 1. Effect of culture medium upon action of X rays measured by % survival for one day, divisions per day as a % of the control, % survival for 4 days after autogamy, and % normal clones 4 days after autogamy. Part of the irradiations were at 70 kr/min and part at 15.5 kr/min. ○ = D medium. ● = C medium.

genetic effects either at high or low doses.

The protective action of C was destroyed either by filtration through an ultrafine sintered glass filter or by boiling with no filtration. Catalase (either a solution of crystalline enzyme or a partially purified commercial preparation) added to D medium before irradiation afforded nearly the same protection as C medium. Boiled catalase was without effect as was also crystalline bovine albumin. These results suggest that the protective action of C medium may be due to the catalase content of the bacteria which it contains. Organic ingredients in the lettuce infusion and in the boiled, "bacterized" medium were certainly much less effective than catalase. The hypothesis that the protection is due to the catalase content of the bacteria is supported by the fact that H_2O_2 added to C medium is fairly rapidly destroyed, while little, if any, destruction was brought about by boiled C medium.

Effect of irradiated D medium and of hydrogen peroxide. The results of the preceding section suggest that a major part of the non-genetic damage to *Paramecium* irradiated in D medium is due to H_2O_2. To test this conclusion, exposures were made to H_2O_2 in D medium and to irradiated D medium. All irradiations were made at 15.5 kr/minute. Just before each experiment, the concentration of the analytical grade "30%" peroxide was determined by titration with $KMnO_4$. A series of dilutions were used as standards for the determination of the amount of H_2O_2 in the irradiated medium (D), using the colorimetric method of Savage(4) which measures the iodine liberated from KI. Fig. 2 shows the results of the tests for peroxide at several different doses of X-rays.

Paramecia were exposed to the various concentrations of H_2O_2; and, at the same time, others were exposed to the irradiated fluid. Both had a marked effect on survival and division rate as shown in Fig. 3. While much variability was found, there seems little question that this substance can account for a considerable portion of but not all the effect of irradiated fluid. It can be estimated from Fig. 3 that the concentration of H_2O_2 needed

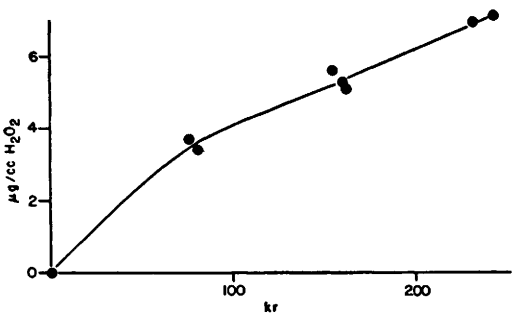

FIG. 2. Concentration of H_2O_2 produced by various doses of X rays to 20 cc of D medium in 10 cm petri dishes. Unfiltered beam. Measured intensity, 15.5 kr/min.

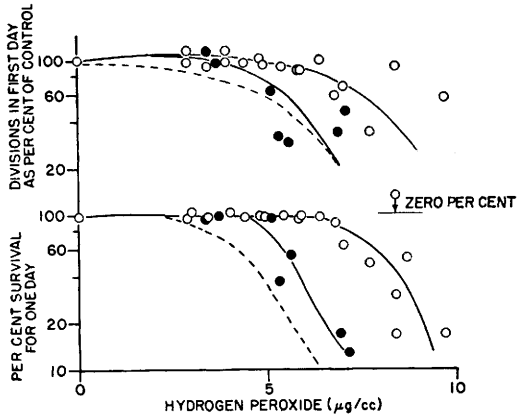

FIG. 3. Divisions in the first day as % of the control and % survival for one day plotted against the concentration of H_2O_2. The data for peroxide and irradiated fluid were obtained from 3 experiments in which the 2 were used simultaneously. The peroxide concentration for the irradiated fluid was obtained by colorimetric comparison with the peroxide solutions, using the KI-starch method The curve for direct irradiation is taken from Fig. 1. The dose in kr was converted to μg/cc of H_2O_2 using the curve in Fig. 2. ○ = H_2O_2 in D. ● = Irradiated D. – – – = Direct irradiation in D.

to produce the same effect as the irradiated fluid was about 1.3-1.5 times that found in the latter. In other words, two-thirds to three-quarters of the effect could be accounted for by the H_2O_2 which was formed. It is of interest in this connection that irradiation but not H_2O_2 destroyed the faint yellow color of the D medium. The change was not detectable at the wave length (660 mμ) used for the colorimetric determinations and so could not have produced a serious discrepancy in the readings. However this observation, together with the results in Fig. 3, shows that X-irradiation produces changes in the medium which cannot be accounted for by the H_2O_2 which it produces.

A comparison between direct irradiation and exposure to irradiated medium is also made in Fig. 3. The curves from Fig. 1 were replotted after converting kr to μg/cc of H_2O_2 using Fig. 2. The comparison is subject to errors of at least 3 kinds. The data in Fig. 1 were obtained by exposing the animals in small drops; whereas, the fluid was irradiated by exposing 20 cc in a Petri dish of 10 cm diameter covered with a thin piece of mica. Thus the actual dose to the animals was probably greater than that to the fluid. The time of exposure to irradiated fluid was variable in the case of directly irradiated paramecia and was probably greater on the average than the 10 minutes used in the experiments in Fig. 3. Both these factors would make the effect of a given dose measured in air greater upon directly irradiated animals. However, directly irradiated animals were exposed to gradually increasing concentrations of the active substances; whereas, those exposed to irradiated fluid were put immediately into the final concentration. Thus, for equal exposure times, the latter would be more effective. Considering these opposing sources of uncertainty in the comparison, it can be concluded that there is no convincing evidence that directly irradiated animals are affected to a greater extent than would be expected from the action of irradiated medium alone. However, direct absorption of energy within the paramecia is not excluded as a small contributing factor.

Further evidence that H_2O_2 was, in considerable part, responsible for the activity of irradiated medium and peroxide-treated medium was furnished by the demonstration that the addition of catalase destroyed the detectable activity in both cases. However, the possibility remains that organic peroxides formed from H_2O_2 and destroyed by catalase were involved.

Neither the irradiated medium nor H_2O_2 produced any detectable genetic effect as measured by the percentage of normal clones after autogamy even at doses at which only about 10% of the animals survived the first day. This is in good agreement with the fact,

as shown in Fig. 1, that the medium in which irradiation is carried out is without appreciable influence on genetic effects. Thus the marked genetic effects produced by the high doses used must have been due entirely to energy absorbed directly in the cell.

Discussion. A number of investigators have demonstrated that substances produced in the medium are responsible for part of the effects of X-rays. Some of these investigators(5) have implicated H_2O_2, while others(6) have suggested other substances, stable or unstable. In particular, Barron *et al.*(6) have presented rather strong evidence that H_2O_2 could not be of much importance in the inhibition of the respiration of sea urchin sperm by irradiated sea water. The work of Wyss *et al.*(7), and Dickey *et al.*(8) suggests that H_2O_2 may not, in itself, be a mutagen for microorganisms, but that organic peroxides formed by its reaction with organic compounds are mutagenic. However, Wagner *et al.*(9), and Demerec *et al.*(10) have obtained evidence which suggests that H_2O_2 itself may be a mutagen. In addition Thoday and Read(11), and Giles and Riley(12) have suggested that H_2O_2, produced locally in the cell, may be responsible for chromosome breakage by ionizing radiations.

The data in the present paper are most easily interpreted on the hypothesis that H_2O_2 and at least one other fairly stable injurious substance are formed on irradiation of D medium and are responsible for most of the effect upon paramecia directly irradiated in this medium. It is possible that the H_2O_2 formed by irradiation reacts with organic ingredients in the medium to form organic peroxides which, in turn, produce the effect. In this case, we would have to assume, with Wyss *et al.*(8), that catalase destroys organic peroxides. Even granting that this is so, it should not invalidate the comparison between irradiated medium and medium to which peroxide was added, since organic peroxides should be formed in the same amounts in the latter. The difference in effectiveness which was found can be attributed only to some action of the X-rays which does not involve H_2O_2 as an intermediate step. One form that this action could take is the production of another injurious substance. However, it is also possible that the medium was altered in such a way that it protected the paramecia against the action of H_2O_2, not by the destruction of the peroxide, but by altering the paramecia to make them more resistant to it.

The failure to find a postautogamous effect of medium with H_2O_2 added and of irradiated medium could be interpreted to mean that this substance was not mutagenic. However, in the light of the evidence on other organisms cited in a previous paragraph, it seems more probable that H_2O_2 was unable to penetrate to the micronuclei in sufficient concentration to produce an effect. *Paramecium* is known to contain catalase (Holter and Doyle)(13), and the micronuclei are usually situated some microns below the surface. Thus it seems quite possible that the H_2O_2 or organic peroxides would have more difficulty in penetrating to them than to the nuclei of the microorganisms upon which positive tests have been obtained. The corollary to this hypothesis is that the materials whose alteration leads to death in one day and also to division delay are located in the surface layers of the *Paramecium* cell. In any case, it is clear that the high rate of mutation (80% or more of exautogamous clones not normal) induced by direct irradiation with doses of the order used in these studies could not be due, except to a negligible extent, to substances produced in the medium.

In assessing the importance of H_2O_2 produced outside the cell in the genesis of radiation damage within it, one must bear in mind that radiation damage to cells can, potentially, be brought about by a variety of mechanisms. The production of H_2O_2 is only one of these, and its relative importance is probably quite different under different circumstances of irradiation and with different effects of the radiation. It is not surprising, then, that estimates of its importance have varied so widely.

Summary. The production of nongenetic death and division delay by X-rays is greatly enhanced if the paramecia are irradiated in a dilute medium with very few bacteria rather than the usual lettuce infusion with many bacteria. This effect is shown to be accounted for almost entirely by relatively stable sub-

stances produced by irradiation of this medium. About two-thirds of the total effect can be attributed to H_2O_2 or its reaction products, while the rest results from an action on the medium not due to H_2O_2. On the other hand, the frequency of genetic changes is not affected by the medium in which the organisms are irradiated nor does irradiated medium or medium to which H_2O_2 was added induce such changes. The failure to find an effect upon the genetic mechanism is interpreted as probably the result of the failure of the substances produced in the medium to reach the micronuclei which are situated some microns below the cell surface. The corollary to this is that the nongenetic changes are due to alterations in the superficial layers of the cell.

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Spectrophotometric Studies on Mixtures of Purified Diphtherial Toxin and Ferriprotoporphyrin IX.*† (19680)

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Pappenheimer(1-3) has proposed that diphtherial toxin is the protein moiety of diphtherial cytochrome b. Direct evidence for this concept would be available if it could be demonstrated that purified diphtherial toxin interacts with iron-porphyrins in a limiting molar ratio of 1:4 to form complexes spectrophotometrically and enzymatically related to cytochrome b. In other porphyrin-protein systems, specific interactions occur *in vitro* under a variety of experimental conditions. In the case of hemoglobin synthesis, the heme-globin linkage is not affected by considerable alteration of the protohemin‡ structure(4). Thus, mesohemoglobin and hematoglobin have been prepared by Hill and Holden(5) and synthetic hemoglobins from rhodohemin, diacetyldeuterohemin and pheohemin-b by Warburg and Negelein(6). All of these compounds combined reversibly with oxygen. Experiments on the recombination of the protein moiety of horseradish peroxidase with various hemins have been summarized by Maehly(7). The formation of methemalbumin has been shown by Rosenfeld and Surgenor(8) to occur over a wide pH range and to be unaffected by small changes in ionic

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‡ The nomenclature and terminology of Lemberg and Legge(4) is used in this paper when designating porphyrin derivatives and complexes.