151. *The Action of Ionizing Radiations and of Radiomimetic Substances on Deoxyribonucleic Acid. Part V.* Some Experiments on the Action of X-Rays and Free Radicals.*

By B. E. Conway and J. A. V. Butler.

Experiments have been made to determine the nature of the agent which causes the slow continued change of viscosity of thymus deoxyribonucleic acid after irradiation with *X*-rays in oxygen-containing solutions. Although the change resembles that brought about by hydrogen peroxide when suitably activated, the actual amount of hydrogen peroxide formed is too small to account for the phenomenon. It is suggested that the initial agent is the radical HO_2.

When deoxyribonucleic acid solutions are irradiated with *X*-rays, the immediate effect of the irradiation is followed by a slow fall of viscosity which continues for many hours (Taylor, Greenstein, and Hollaender, *Biochem. Arch.*, 1948, **16**, 19). It was shown (Part II, *J.*, 1950, 3418) that this " after-effect " does not occur if the irradiation is carried out in oxygen-free solutions. The effect was therefore ascribed to the reaction of a primary product of the radiation with molecular oxygen. The most likely product of this reaction appeared to be the radical HO_2, formed by $H + O_2 \longrightarrow HO_2$, which, it was suggested, could then react with nucleic acid forming a compound of a peroxidic nature, which slowly decomposes giving rise to the observed loss of viscosity. There are two distinct problems: (*a*) the nature of the product which causes the effect; (*b*) its action on the nucleic acid. In this paper we report experiments designed to establish more fully the nature of the agent, and particularly to distinguish the action observed from that of hydrogen peroxide. It is well known that hydrogen peroxide is formed by the *X*-irradiation of pure water in the presence of oxygen (Bonet-Maury and Lefort, *Nature*, 1948, **162**, 381, *J. Chim. phys.*, 1950, **47**, 624; Lefort, *ibid.*, p. 624, 776; Gray, *Progress in Biophysics*, Vol. II, 1951) by reactions such as $H + O_2 \longrightarrow HO_2$; $HO_2 + H \longrightarrow H_2O_2$; $2HO_2 \longrightarrow H_2O_2 + O_2$. The effect of added substances is complex. Substances which can react with HO_2 will decrease the yield of hydrogen peroxide, and it has been suggested (Allen, *J. Phys. Coll. Chem.*, 1948, **52**, 473) that reducing agents may increase it.

It was found (Part II, *loc. cit.*) that the effect of added hydrogen peroxide on nucleic acid was complicated in that some samples were degraded and others not. Since (i) samples of nucleic acid which are not sensitive to hydrogen peroxide can be made so by addition of small quantities (10^{-5}M) of ferrous salts, and (ii) samples which are sensitive can be made less sensitive by reprecipitation from aqueous solution by alcohol, it was concluded that hydrogen peroxide has no apparent effect on purified nucleic acid but that small quantities of ferrous salt have an activating effect. It has been found that other reducing agents, such as ascorbic acid and cysteine, are effective in this way (see Fig. 1).

It has also been found (see Fig. 2) that a sample of nucleic acid which is not affected by added hydrogen peroxide still shows the " after-effect." It might perhaps be argued that the added hydrogen peroxide contains an inhibitor, while that formed by the action of *X*-rays does not. However, since the action of hydrogen peroxide on different samples of nucleic acid, some sensitive and some not, is not changed by the distillations, it was concluded that the presence of an inhibitor was not a determining factor.

It is also possible that the action of *X*-rays on the nucleic acid produces an activating agent, which is capable of initiating the decomposition of hydrogen peroxide. There is evidence that this is the case (see Fig. 3), since hydrogen peroxide added to an insensitive nucleic acid preparation, soon after the finish of an irradiation in the absence of oxygen, is able to produce a change similar to the " after-effect." It is to be noted that when the hydrogen peroxide is not added until 24 hours after the finish of the irradiation this effect

* Part IV, *J.*, 1952, 626.

was not observed. The activating agent formed by *X*-rays thus disappears when the solution is kept.

It is evident from this experiment that hydrogen peroxide, if it were formed in the *X*-irradiation in sufficient amount, would be capable of causing the " after effect." The con-

FIG. 1.

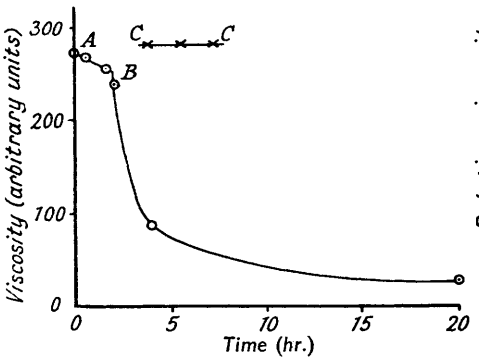


FIG. 2.

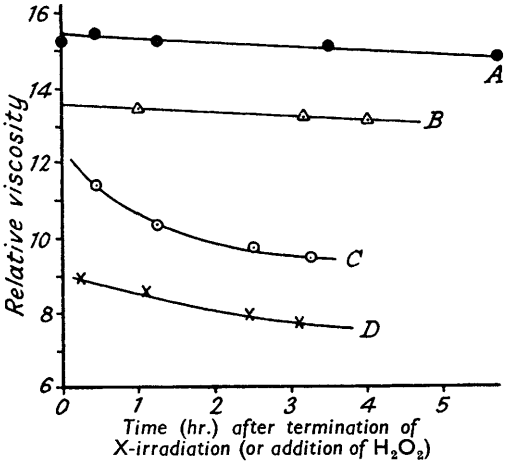


FIG. 1. *Effect of ascorbic acid* (10^{-3}M) *on the hydrogen peroxide* (5×10^{-3}M) *degradation of thymonucleic acid in* 0·1N-*sodium hydrogen carbonate.*

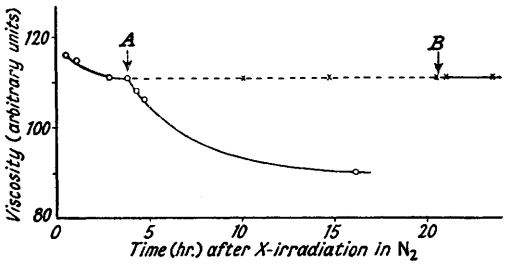
AB shows action of hydrogen peroxide alone; at *B* the ascorbic acid was added; *CC* shows effect of ascorbic acid without hydrogen peroxide.

FIG. 2. *Showing* (1) *that the " after-effect " still occurs with a preparation not directly sensitive to hydrogen peroxide ;* (2) *that the primary effect of* X-*irradiation is increased in the presence of added hydrogen peroxide.*

A, 10^{-3}M-H_2O_2; *T.N.A. is insensitive.*
B, 7000 r. X-*irradiation in* N_2.
C, 7000 r. X-*irradiation in* O_2.
D, 7000 r. ,, *in* $O_2 + 3 \times 10^{-3}$M-H_2O_2.

centration required to produce the observed effects is of the order of 10^{-3}M. The table on p. 836 shows the concentrations of hydrogen peroxide formed with *X*-ray doses of the order used in the experiments, both in oxygenated water and in the presence of oxygenated nucleic acid solution. It can be seen that the concentrations are smaller, by a factor

FIG. 3. *Effect of hydrogen peroxide on* X-*irradiated thymonucleic acid after two periods of time.*



A, H_2O_2 *added* (10^{-3} *mole/l.*). *B*, H_2O_2 *added* (10^{-3} *mole/l.*) *after* 20 *hours : no effect.*

of at least 30, than those which would be required to produce the " after-effect " if hydrogen peroxide were the active agent. The figures for water are in reasonable agreement with the observations by Bonet-Maury and Lefort (*loc. cit.*).

A direct comparison was made by determining (*a*) the concentration of hydrogen peroxide formed in the *X*-irradiation (7000 r.) of an oxygenated solution of nucleic acid and (*b*) the effect of this concentration (*ca.* 4×10^{-5}M) when added to the same solution

irradiated in nitrogen with the same dose of *X*-rays. The change of viscosity produced by the latter was only 2—3% in 5 hours, whereas the "after-effect" under these conditions is a 20—50% change.

Formation of hydrogen peroxide (in moles/l. × 10^3) in oxygenated water and nucleic acid solutions.

Concn. of nucleic acid (%)		0	0·005	0·01	0·05	0·1
X-Ray dose (r.) (206 r./min.)	3 000	0·1	0·04	0·06	0·04	0·04
	10 000	0·08	0·04	0·04	0·04	0·06
	20 000	0·06	0·04	0·04	0·04	0·06

It might be argued that, when nucleic acid is present, larger amounts of hydrogen peroxide are actively formed which become adsorbed on the nucleic acid. If this were the case a similar phenomenon would be expected with added hydrogen peroxide, but when this is added to nucleic acid solutions at a concentration of 10^{-3}M, the amount adsorbed is not more than equivalent to 10^{-4}M.

Further evidence that the degradation of nucleic acid is not caused by an agent which

FIG. 4.

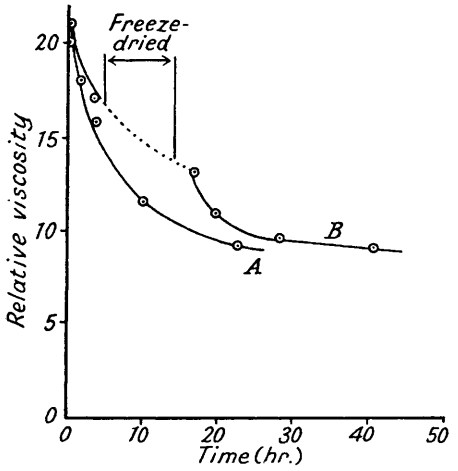


FIG. 5.

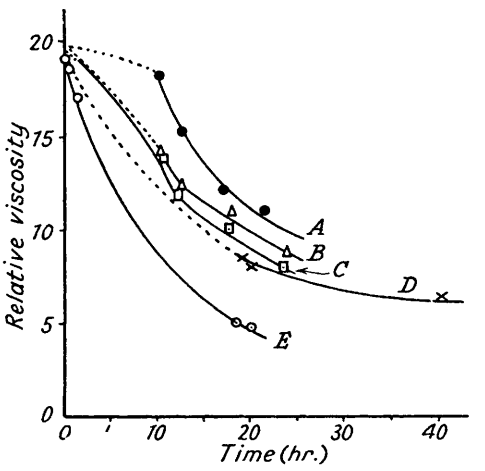


FIG. 4. *Effect of freeze-drying on the "after-effect" in thymonucleic solution X-irradiated* (7000 r.) *in oxygen.*

A, Control, not freeze-dried.
B, Freeze-dried for period shown and reconstituted to same concentration with water.

FIG. 5. *Effect of freeze-drying of thymonucleic acid treated with hydrogen peroxide under various conditions.*

A, Frozen at −20° *but not dried.*
B, Freeze-dried for 10 *hr.* (99% *water removed*), *reconstituted.*
C, Freeze-dried for 2 *hr.* (60% *water removed*), *reconstituted.*
D, Freeze-dried for 20 *hr.* (100% *water removed*), *reconstituted.*
E, Control at 25° *without freezing or drying.*

remains in the solution is given by experiments in which the solution was frozen as soon as possible after the completion of the irradiation and the volatile components were removed by sublimation in a freeze-drying apparatus. It was found (Fig. 4) that (*a*) after the substance had been kept for a limited time in the solid state and then redissolved, the degradation process continued, (*b*) degradation also appeared to occur during the time the substance was in the solid and in the dried solution.

These observations support the view that the slow degradation process produced both by *X*-rays in oxygenated solutions and by activated hydrogen peroxide is caused by an agent which has become attached to the nucleic acid. The radical HO_2 is indicated because it is formed both by the action of a primary product of *X*-rays on molecular oxygen and by the action of ferrous ions (and possibly other reducing agents) on hydrogen peroxide,

as in the following scheme (Haber and Weiss, *Proc. Roy. Soc.*, *A*, 1934, **147**, 332; Humphrey and Weiss, *Nature*, 1949, **163**, 691; Barb, Baxendale, George, and Hargrave, *ibid.*, p. 692):

$$H_2O_2 + Fe^{++} \longrightarrow OH + Fe^{+++} + OH^- \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (1)$$

$$H_2O_2 + OH \longrightarrow H_2O + HO_2 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

$$HO_2 + H_2O_2 \longrightarrow O_2 + H_2O + OH \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (3)$$

$$Fe^{+++} + HO_2^- \longrightarrow Fe^{++} + HO_2 \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad (4)$$

With small concentrations of ferrous ions, a chain reaction involving (2) and (3), initiated by (1), can occur, but it is evident that if a substance which removes HO_2 is present, (3) will be inhibited and the continuation of the formation of HO_2 requires the regeneration of Fe^{++} by some such process as (4).

An experiment was also made to find if irradiation with *X*-rays increased the "after-effect" (see Fig. 2) when hydrogen peroxide was present. It was found that the *primary* effect of the *X*-rays is greatly increased, *e.g.*, 1000 r. in the presence of 3×10^{-3}M-hydrogen peroxide has a greater effect than 7000 r. in nitrogen. There is some "after-effect," but not more than could be attributed to traces of oxygen formed from the hydrogen peroxide. It therefore appears likely that the *X*-irradiation of hydrogen peroxide gives rise mainly to OH radicals, which produce a primary effect, and that the decomposition as $H_2O_2 \longrightarrow H + HO_2$ is not sufficient to produce a large secondary effect.

Experimental

The materials and methods of measurements were similar to those described in Part II (*loc. cit.*).

Determination of Hydrogen Peroxide.—The peroxide formed during the *X*-irradiation of the oxygenated nucleic acid solution was determined colorimetrically with titanic sulphate solution. 1 Ml. of 0·6% aqueous titanic sulphate in 20% aqueous sulphuric acid was added to 1 or 5 ml. of the nucleic acid solution, and the mixture made up to 10 ml. The precipitated nucleic acid was centrifuged off, and the optical density of the solution was measured in a Unicam spectrophotometer at 4300 Å and compared with that obtained with suitable standards. When 5 ml. of nucleic acid with known concentrations of hydrogen peroxide are used, the accuracy is $\pm 2 \times 10^{-5}$M-H_2O_2.

Effect of Freeze-drying.—*X*-Irradiated solutions in oxygen were freeze-dried as quickly as possible after the irradiation. In the similar experiments carried out in the presence of hydrogen peroxide and ferrous salts the solution was frozen as soon as possible after the addition of the reagent. After being in the dry state for 16—20 hours, the solution was reconstituted, and a much degraded product was obtained. This could have been obtained either by the reaction's occurring in the dry frozen state, or as a reaction of hydrogen peroxide accelerated by concentration of the solution, during the actual stages of the freezing. The freezing process only occupies a few seconds. Experiments with hydrogen peroxide solutions showed that no significant concentration occurs during partial freezing or during the evaporation; *i.e.*, the hydrogen peroxide is evaporated at a rate which is at least not greatly different from that of the water. Experiments in which the thymonucleic acid solutions containing hydrogen peroxide were frozen and dried for various times so as to remove different amounts of water, and an experiment in which the solution was merely frozen and remelted after 10 hours indicated that the viscosity-decay curves for the reconstituted solutions from *partly* and *completely dried* solids were largely superimposable (Fig. 5). However, mere freezing of the solution without removal of the water greatly diminishes the rate of change in the solid state.

Temperature Coefficient of the Degradation Process.—It is difficult to deal with the kinetics of the change when the relation between the viscosity and the underlying chemical changes is unknown. The relation $(\eta_0 - \eta_t)/(\eta_t - \eta_\infty) = k_2 t$, where η_0 and η_∞ are the initial and the final viscosity and η_t is that at time t, was found to be in good agreement with the observed results in most cases. This relation can be derived on certain assumptions from a second-order law, the initial concentrations of reactants being assumed to be the same. The following values of k_2 were obtained:

Temp.	5°	15°	25°	37°
H_2O_2 + nucleic acid	0·16	0·45	0·92	0·96
X-Irradiation of nucleic acid oxygen (7000 r.)	(3°) 0·073	0·100	0·155	0·58

From these temperature coefficients it can be concluded that in aqueous solution the rate of change at 0° is only about 0·25 of that at 25° and will be still less at the lower temperatures which prevail at least during the early stages of the freeze-drying. It would appear from this that the rates of change observed during the drying process and in the dry state are at least as great as, and possibly greater than, those to be expected in solution at similar temperatures.

Action of Catalase.—Since hydrogen peroxide is decomposed by catalase, it was thought that its effect on nucleic acid containing hydrogen peroxide would be of interest. Catalase alone has no effect on the viscosity of nucleic acid, but catalase and hydrogen peroxide produced an abrupt decrease of viscosity, after which the viscosity remained constant. This is evidence that catalase liberates hydroxyl radicals in its action on hydrogen peroxide, but the amount is relatively small and is insufficient to produce measurable chemical changes, such as can easily be observed in the case of hydroxyl radicals produced by the photochemical decomposition of hydrogen peroxide. The liberation may, however, not be connected with the specific catalase decomposition of hydrogen peroxide, but a consequence of a secondary activation of hydrogen peroxide, caused by catalase acting as a reducing agent and so producing an effect similar to that noted with ascorbic acid (Fig. 1). The addition of catalase to *X*-irradiated nucleic acid in oxygen had very little effect.

Effect of Substances added after the Irradiation.—The effect of oxygen on the results of *X*-irradiation is of considerable importance in radiobiology, since it has been found in a variety of experiments that radiation damage is increased by an increased concentration of oxygen (*e.g.*, Thoday and Read, *Nature*, 1947, **160**, 608; 1949, **163**, 133; Hollaender, Stapleton, and Morton, *ibid.*, 1951, **167**, 103) and is much reduced by exclusion of oxygen (Evans, *Radiobiology*, 1942, **38**, 29). Since oxygen exclusion is difficult to achieve with many living organisms, alternative methods of protection have been sought, *e.g.*, the addition of cysteine or other substances (Patt *et al.*, *Science*, 1949, **110**, 213; *Proc. Soc. Exp. Biol.*, 1950, **73**, 18). To be effective, these substances have to be present during the irradiation. They exert this effect by competing for the primary radicals produced by *X*-rays. It appeared to us that it might be possible to prevent the " after-effect " of nucleic acid by adding *after the irradiation* substances which could reduce or decompose the suggested nucleic acid peroxide, and that this experiment might indicate substances which would be effective *in vivo*. None of the substances added after the irradiation (cysteine, ascorbic acid, ferrous ions, catalase, peroxidase) was effective in preventing the occurrence of the after-effect. On the contrary most of them accelerated it, and it would appear that reaction such as $XO_2H + R \longrightarrow XO\cdot + R(OH)$ may be possible. It is noteworthy in this connection that Kharasch has shown (*Science*, 1951, **113**, 392) that alkoxy-radicals are formed by the action of ferrous ions on alkyl peroxides.

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