Effects of Radiations on Catalase Solutions

1. KINETIC STUDIES OF INACTIVATION

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In his original studies of the inactivation of catalase solutions by X-rays, Forssberg (1945, 1946) showed that this system possessed somewhat peculiar kinetic properties. In this work the effects of various radiations on catalase solutions have been explored in some detail, in an attempt to explain these peculiarities. Some of the results have been reported briefly elsewhere (Sutton, 1955).

EXPERIMENTAL

Preparation of catalase. Crystalline catalase was prepared from fresh beef liver essentially as described by Sarkar & Sumner (1951), but without any alteration of the pH of the first catalase filtrate until the addition of the phosphate gel, which was previously adjusted to pH 5.7. The catalase was recrystallized in the needle form and was stored in suspension in ammonium sulphate solution at 0°. For use, a portion was dialysed against several changes of twice-distilled water, and a strong (approx. 10 μ M) solution of the dialysed crystals was made up in 0.05 M phosphate buffer. Such stock solutions were stored at 0° for some weeks without loss of activity. The dilute solutions used in irradiation studies were less stable, and were made up daily from this stock. Two preparations of crystals were used in these studies. The Kat.f. (Bonnichsen, Chance & Theorell, 1947) of the first preparation was 49 600 at 21° and 39 700 at 2°. The ratio ϵ (275 m μ)/ ϵ (405 m μ) was 1.08. If one assumes a molecular weight of 225 000 then the enzymic rate constant for decomposition of hydrogen peroxide (s) was 1.71×10^7 l. mole⁻¹ sec.⁻¹ at 2°, and 2.14×10^7 l. mole⁻¹ sec.⁻¹ at 21°. ϵ (405 m μ) = 2.85×10^4 . The properties of the second preparation were Kat.f. = 40 100 at 21°, $s = 2.16 \times 10^7$ l. mole⁻¹ sec.⁻¹ at 21°, ϵ (275 m μ)/ ϵ (405 m μ) = 1.11, and ϵ (405 m μ) = 2.99×10^4 .

Measurement of catalase activity. The substrate used was 0.007 M-H₂O₂ buffered to pH 7.0, or in some cases to 7.4. In early experiments the decomposition of this substrate was followed by the rapid-titration method of Bonnichsen, Chance & Theorell (1947) at 1–3°, and with sufficient catalase to obtain a half-reaction time of the order of 30 sec. In later work the reaction was followed at room temperature in 4 cm. cells in a Unicam spectrophotometer (model SP. 500) by the method of Beers & Sizer (1952). Temperature could not be controlled in these measurements but it did not vary significantly throughout any one determination. When required, corrections for temperature variation were made with predetermined data. Deviations between duplicate measurements on the same catalase sample were less than 3% by either method, but the second was much more convenient. The initial concentration of catalase in an untreated sample was calculated from activity measurements with the use of the rate constants reported above.

X-irradiation technique. The X-ray source used in most of this work was a Westinghouse Quadracondex therapeutic X-ray machine (model 220), operated manually at 200 kv and 16 ma without external filtration. The half-value layer of this unfiltered radiation is 0.50 mm. of copper. Solutions were irradiated in Pyrex vessels placed in an ice or water bath at a point on the axis of the X-ray beam. The position of this point was varied according to the dose rate required. The ferrous sulphate chemical dosimeter was employed in measurements of dose rate in the following manner: A solution of 10⁻³ M ferrous ammonium sulphate in 0.8 N-H₂SO₄ containing 10⁻³ M-NaCl was irradiated in the same vessel and under the same conditions as those used in experimental runs. The concentration of ferric sulphate produced by the irradiation was determined by measurements of the optical absorption of the ferric sulphate at 303 m μ . In calculating the dose rate from such measurements, a yield of 16 mol. of ferric sulphate/100 ev (equivalent to 16 μ moles l.⁻¹/kr.) was assumed in conformity with recent measurements of this quantity. The yield is possibly slightly lower with the radiation employed, but this was ignored.

Intermittent radiation. In experiments with intermittent radiation a Philips Contact therapy X-ray machine operating at approximately 40 kv and 2 ma was employed. The unfiltered output of this machine (half-value layer = 0.14 mm. of aluminium) was directed vertically downwards on to the catalase solution, which was contained in a shallow uncovered vessel seated in an ice or water bath. Catalase solutions (10⁻³ M) were placed 0.57 cm. deep in a vessel of 4.7 cm. diameter at 8.8 cm. from the X-ray focal spot. The dose rate varied by a factor of five between extreme points of the irradiated solution, but this does not affect results based only on comparisons since the same dose rate distribution was maintained in each experiment. Catalase solutions (5 $\times$ 10⁻³ M) were placed 0.21 cm. deep in a vessel of 2.8 cm. diameter at 4.3 cm. from the focal spot. In this instance variations of dose rate did not exceed a factor of two. The average dose rate in both cases was determined with ferrous sulphate dosimetry, again with the assumption of a yield of 16 mol./100 ev.

Intermittent irradiation was obtained by chopping the X-ray beam with a metal sector of 30 cm. diameter, rotating in a horizontal plane 2.5 cm. below the X-ray focal spot, and of such design that any point in the irradiation vessel was exposed to the X-rays for one-quarter of each half revolution of the sector. For the remainder of each half revolution the vessel was screened from the X-rays by 1.1 mm. of brass plus 1.0 mm. of aluminium. The screen reduced the intensity of the X-ray beam by a factor of more than 10⁴, and the radiation transmitted through the screen had no observable effect on catalase solutions. The rotation of the sector was controlled by a motor and gearing system.

Irradiations with ultraviolet rays. A low-pressure mercury 'resonance' lamp (type T/M5/544 A, supplied by Thermal Syndicate Ltd.), operated at 16 ma and approximately 300 v, was used as the source of radiation. Such lamps emit virtually all their ultraviolet radiation at the two wavelengths 253.7 and 184.9 m μ . In most of the experiments the unfiltered output of this lamp was directed on to solutions contained to a depth of 0.17 cm. at room temperature in uncovered glass vessels, mounted at 6 cm. below the lamp. No attempt at actinometry was made, since most of the results are based on comparative measurements at constant incident intensity. Known fractions of this intensity were obtained with a grid placed immediately below and touching the lamp. The lamp was always warmed up for more than 5 min. before use, and measurements of voltage and current were made during irradiations to check the constancy of running conditions.

α -Irradiations. Radon and its decay products were used as the source of α -radiation. The technique employed is described below:

A 5 ml. glass syringe was coated with a monolayer of the organosilicon compound known as Desicote (Beckman Instruments Inc.). This syringe was partially filled with 2.5 ml. of radon solution in the manner described by Gray & Scholes (1951), but with the use of glass joints instead of rubber connecting tubing. The syringe was left for 2–3 hr. in order to obtain secular equilibrium between the radon and its decay products, and its radon content was checked immediately before use by comparison of its γ -activity with that of a known amount of radon. The syringe was then coupled with a ground-glass joint to a second syringe of 20 ml. capacity, also treated with Desicote, and containing 12 ml. of catalase solution. At zero time the two solutions were mixed, and the mixture was left in the 20 ml. syringe for the desired exposure time. The 5 ml. syringe was then removed, a glass U-tube leading to an air supply was connected in its place, the plunger was removed from the 20 ml. syringe, and at time t air was bubbled through the catalase and radon solution for 3 min. All operations up to this stage were performed in a fume cupboard with a strong draught. Portions of the catalase solution (now free of radon) were removed with a syringe for determinations of catalytic activity in the open laboratory at intervals of 10 min. The activity at time t was obtained by extrapolation from these measurements.

The α -radiation emitted during these operations is completely absorbed in the solutions in which it is generated, and is derived from radon (half life of 3.8 days) and two of its decay products, namely RaA (half life of 3.05 min.) and RaC (half life of 19.7 min.). The rate of dissipation of α -ray energy in the catalase solution is readily calculated from the known energy of each of these radiations, provided that the concentrations of radon, RaA, and RaC are known. Information on this point was obtained from measurements at various intervals of the γ -activity of portions of the apparatus and samples of the solutions; a well-shielded Geiger counter responding chiefly to RaC was used for this purpose. Amounts of radon were determined by similar measurements on samples which had been sealed for some hours in order to attain secular equilibrium. From these measurements it was shown that (a) all the radon present in the 5 ml. syringe remained in solution and was mixed with the catalase solution, (b) the air-bubbling procedure removed 99% of the radon in 1 min. and more than 99.9%

in 3 min., and (c) approximately 20% of the RaC present in the 5 ml. syringe remained adsorbed on its walls and was not injected with the radon into the catalase solution. Approximately 35% was retained on the walls of syringes which were not treated with Desicote.

Since the active deposit is formed initially in solution, one factor which determines the fraction adsorbed on the syringe walls is the time available for the process. One might therefore expect that the fraction of RaA adsorbed would be less than that of RaC, owing to the former's smaller half life. This was verified by studies of the α -activity of a glass plunger immediately after removal from a syringe of radon solution in secular equilibrium. If the number of millicuries of RaA adsorbed on the plunger is as large or comparable with that of RaC then the α -activity would decay initially with a half life of 3 min. No evidence of such behaviour was observed; the decay of α -activity during the first hour of measurement could be expressed approximately by a half life of 40 min.

In estimating concentrations of decay products in the catalase solution it was assumed, therefore, that of the known amounts present in secular equilibrium with the radon in the 5 ml. syringe, all of the radon, all of the RaA, and 80% of the RaC were mixed with the catalase solution.

Experiments with added hydrogen peroxide. The final experiments on this subject were performed as follows. A glass reservoir connected to a fine glass nozzle was used to direct a steady stream of hydrogen peroxide solution at the rate of 0.46 ml./min. on to the inside wall of an uncovered irradiation vessel, containing a known volume (generally 4 ml.) of catalase solution. Addition of hydrogen peroxide was begun 45 sec. before irradiation, and was continued until the end of the exposure. The solution was stirred constantly with a slowly rotating magnetic stirrer during this addition, and the final volume was determined by weight.

This procedure by itself caused a small loss of activity of approximately 5% in 3 min., and allowance was made for this in assessing the inactivation due to irradiation. This mechanical inactivation was not due to the added hydrogen peroxide since the same result was obtained when water was added.

Determination of hydrogen peroxide. Concentrations of hydrogen peroxide in irradiated water were measured colorimetrically with the titanium sulphate reagent at 410 m μ .

Materials. The hydrogen peroxide used in catalase activity measurements was diluted from AnalaR reagent stock. That used for addition to irradiated solutions was diluted from inhibitor-free hydrogen peroxide, kindly supplied by British Drug Houses Ltd. The water used in irradiated solutions was redistilled from alkaline potassium permanganate solution in an all-glass still.

RESULTS AND DISCUSSION

Nature of inactivation curve

Fig. 1 illustrates the manner in which the activity of an aerated catalase solution is diminished by X-irradiation at constant dose rate. The activity of each sample was measured less than 10 min. after irradiation, and is recorded as a percentage of the activity of the diluted stock solution from which it was taken. The activity of irradiated solutions was

not entirely constant, in some cases a partial recovery of activity was observed, particularly in concentrated solutions made from freshly prepared crystals. In general this recovery was slow, and in all reported experiments it was negligible within 10 min.

Inspection of Fig. 1 shows that the initial portion of the inactivation curve follows the exponential relation $A/A_0 = \exp(-kt)$, where A/A_0 denotes the fraction of initial activity remaining after an exposure of t sec., and the coefficient k denotes the rate of inactivation. When the exposure time is increased above 5 min. this relation is no longer observed, and the behaviour with still longer exposures was somewhat erratic. The effects of short exposures were quite reproducible with samples of any one catalase solution, and to a lesser extent with solutions of the same concentration made up on successive days. This is illustrated in Fig. 1 by the behaviour of three solutions of identical initial activity, one of which was prepared from catalase crystals kindly supplied by Professor J. B. Sumner.

It must be emphasized that reproducible results could be obtained only with due attention to cleanliness, and with solutions which were freshly diluted each day from the concentrated stock. Thus a dilute solution would occasionally display anomalously low radiation sensitivity if kept for a day or more before irradiation; this behaviour was accelerated by shaking the solution in glass vessels or by bubbling gases through it, and the effects of irradiation were markedly reduced if the solution was allowed to touch the hand. Such procedures had

virtually no effect on the activity of the solution. Somewhat similar difficulties have been mentioned by McDonald (1955) in her studies with solutions of crystalline trypsin.

Effect of variables on the kinetics of inactivation

A study of the inactivation under various conditions revealed the following features: with irradiation times less than 4 or 5 min. the inactivation curve in all cases was of the exponential form shown in the initial portion of Fig. 1, though in concentrated solutions and with high dose rates this exponential behaviour was maintained for longer exposures. The variation of the rate of this initially exponential inactivation with concentration (C) and dose rate (R) at 0° and pH 7.4 is shown in Fig. 2. Inactivation rates are expressed in k units, defined by $k = \{\ln(A_0/A)\}/t$. Evidently k is a somewhat complex function of both C and R , as Forsberg observed in his original studies of this system in 1945. If we express k as proportional to $C^m R^n$, then at 642 r./min. m has the value of -0.16 over the concentration range $2 \times 10^{-9} \text{M}$ – 10^{-8}M , and decreases to -0.2 over the concentration range 10^{-8}M – $5 \times 10^{-8} \text{M}$. At 29 r./min. the values of m observed over the same concentration ranges were -0.34 and -0.53 respectively. The variation of m with C suggests that a limiting value might be attained at large concentrations. Smith (1954), using impure catalase solutions, has in fact observed the limiting value of $m = -1$ at concentrations much higher than those studied here.

The most striking feature of these results lies in the magnitude of the dose-rate exponent, n . Average values of n over the 22-fold variation in

Fig. 1. Inactivation of catalase in aerated aqueous solutions at 0° and pH 7.4 by X-irradiation at 642 r./min. The points (●) relate to 10^{-8}M catalase, (○) to 10^{-8}M catalase in $1.36 \times 10^{-4} \text{M}$ sodium chloride, and (▲) to 10^{-8}M catalase, prepared from crystals supplied by Professor J. B. Sumner.

Fig. 2. Variation of initial inactivation rate with catalase concentration and dose rate. Catalase solutions at 0° and pH 7.4 were irradiated with X-rays at 642 r./min. (●), and at 29 r./min. (○).

dose rate shown in Fig. 2 range from 0.35 in $0.2 \times 10^{-8} \text{ M}$ solution to 0.61 in $5 \times 10^{-8} \text{ M}$ solution. The data of Forssberg (1945), when expressed in this form, yield the value $n = 0.21$ over a 1000-fold variation in dose rate in solutions $0.32 \times 10^{-8} \text{ M}$ irradiated at 20° and pH 6.8. A similar value may be calculated from Forssberg's (1946) later publication, but under unstated conditions. The fact that n is lower than 1, and indeed even lower than 0.5 under certain conditions, is thus well established.

The inactivating process must, therefore, involve an intermediary product of finite life, and experiments were made with intermittent irradiation to determine the magnitude of this lifetime.

Studies with intermittent irradiation

The apparatus described in the experimental section was used to irradiate catalase solutions through a rotating sector with an opening of one-quarter of a revolution. The average rate of catalase inactivation (k_i) observed under these conditions is defined by the relation $k_i = \{\ln(A_0/A)\}/f$, where f is the total time of treatment (i.e. $f = 4r$, where r denotes the total time in which radiation is transmitted by the sector to the solution).

Let the lifetime of the intermediary be τ sec., and the time taken by the open portion of the rotating sector to traverse a point in the X-ray beam be t_1 sec. Then the rate of inactivation (k_i) observed at a sector speed corresponding to t_1 should vary from a value denoted as k_r at fast speeds such that $t_1 \ll \tau$, to the value $k_r (4^{n-1})$ at slow speeds such that $t_1 \gg \tau$. Further, k_r should coincide with the rate (k_c) observed with continuous irradiation of one-quarter of the dose rate obtained during open portions of each cycle of the sector.

Fig. 3. Inactivation of 10^{-8} M catalase by intermittent X-irradiation. Aerated solutions at 0° and pH 7.4 were irradiated at 75 r./min. (O), at 300 r./min. (Δ), and at an average dose rate of 75 r./min. obtained by chopping the 300 r./min. X-ray beam with a sector with an opening of one-quarter revolution, rotating at $t_1 = 0.005$ sec. (●).

Preliminary experiments showed that k_i coincides with k_c under the conditions studied when t_1 is less than 0.1 sec. Fig. 3 shows the results of such an experiment in which catalase solutions, as specified in the legend, were irradiated (a) with continuous irradiation at 75 r./min., and (b) at 300 r./min. through the rotating sector run at $t_1 = 0.005$ sec. The effect of continuous irradiation at 300 r./min. is shown for comparison. Evidently the effects of (a) and (b) are identical within the limits of experimental error. In subsequent experiments exposures were limited to 3 or 4 min. in order to obtain the values corresponding to the exponential portion of the inactivation curve. A series of measurements of k_i at a desired value of t_1 was alternated with measurements of k_r at $t_1 = 0.08$ sec. in order to obtain reliable values of k_i/k_r with the same catalase solution. The results of such measurements are shown in Fig. 4. Owing to the restriction in exposure time it has not been possible to attain limiting values of k_i/k_r at large values of t_1 , but these have been calculated from the experimental value of n and are included in Fig. 4 for comparison.

In the absence of detailed knowledge of the mechanism of inactivation a precise evaluation of τ from the data of Fig. 4 is not possible. Approximate

Fig. 4. Inactivation of aerated catalase solutions at 0° by X-irradiation through a rotating sector with an opening of one-quarter revolution. The ordinates are in units of k_i/k_r , where k_i denotes the inactivation rate observed when the sector is run with an 'on' period of t_1 sec., and k_r denotes the inactivation rate observed when $t_1 < 0.1$ sec. Other conditions are (where I_0 denotes the instantaneous dose rate in the irradiated solution during 'on' periods of the sector): A, 10^{-8} M catalase at pH 7.4, $I_0 = 300$ r./min.; B, $5 \times 10^{-8} \text{ M}$ catalase at pH 7.4, $I_0 = 1700$ r./min.; C, $5 \times 10^{-8} \text{ M}$ catalase at pH 6.0, $I_0 = 1700$ r./min.

values, correct within at least an order of magnitude, have been assessed as one-quarter of t_1 at the point where k_i/k_r attains a value midway between its two extremes. On this basis we conclude that: (a) τ is of the order of 1 sec. in 5×10^{-8} M catalase solutions at 0° and pH 7.4; (b) τ varies approximately inversely as the catalase concentration in the range 10^{-8} – 5×10^{-8} M; (c) τ is independent of pH between 6 and 7.4.

Role of hydrogen peroxide

These values of τ at once suggest that the intermediary may be hydrogen peroxide. Hydrogen peroxide is produced in all aerated solutions during X-irradiation, and in 5×10^{-8} M catalase solutions it is decomposed at a rate corresponding to a mean lifetime of 1.15 sec. The magnitude of its lifetime varies inversely as the catalase concentration and is independent of pH between 6 and 8. From this equality of τ with the lifetime of hydrogen peroxide one must conclude that hydrogen peroxide is involved in a rate-determining step of the inactivation process.

Hydrogen peroxide itself is found to inactivate catalase solutions only when maintained at concentrations of 10^{-4} M or higher, whereas the concentrations attained in radiation experiments do not exceed 10^{-7} M. One could envisage some type of effect similar to that observed by Alper (1952) with bacteriophage suspensions, in which their sensitivity to hydrogen peroxide was increased by other products of X-irradiation. To test this possibility hydrogen peroxide was added to catalase solutions before, during, and after a short exposure to X-irradiation at 825 r./min. The small degree of inactivation caused (generally about 10%) was compared with that observed in control experiments in which water was added to the catalase solutions during irradiation, instead of hydrogen peroxide. The addition of hydrogen peroxide did not increase the inactivation observed in control experiments, nor was any increase observed when the hydrogen peroxide so added was made by irradiating water with doses much higher than those used in the subsequent irradiation of the catalase. Evidently the hydrogen peroxide produced during irradiation does not cause inactivation of catalase.

On the contrary, when the above experiments were repeated with longer exposures it was found that the effect of the X-irradiation was somewhat reduced by the addition of hydrogen peroxide. This protection by hydrogen peroxide is illustrated in Fig. 5 by the results of a typical experiment using the methods described in the experimental section. Hydrogen peroxide (2×10^{-3} M, inhibitor-free) was placed in the reservoir, and was added at the average rate of 2×10^{-6} mole l.⁻¹/sec. to each portion of catalase solution before and during each exposure

to irradiation. This rate of addition of hydrogen peroxide exceeds by about 100 times the rate at which hydrogen peroxide is generated in the solution during irradiation at 825 r./min. The effects of irradiation under such conditions at two dose rates are shown in Fig. 5B and are to be compared with those observed without external addition of hydrogen peroxide as shown in Fig. 5A. The protection is evidently most marked at low dose rates; in fact the dose-rate exponent (n) has been increased from 0.5 to nearly unity under the conditions illustrated. Hydrogen peroxide was found to protect the catalase only when it was added during irradiation. Solutions treated with peroxide an hour before irradiation were not protected.

Further experiments have shown that the magnitude of n may be correlated with the equilibrium concentration of hydrogen peroxide attained during irradiation. After irradiation for the first few seconds, hydrogen peroxide builds up to a concentration given by the relation $[H_2O_2]_{\text{equil.}} = a/s$ [catalase], where a denotes the total rate of addition of hydrogen peroxide to the system both externally and by radiation, and s is the catalytic-rate constant, which is assumed to remain unchanged even at the low concentrations of hydrogen peroxide which are considered. Chance (1947) has shown that s is at least approximately independent of hydrogen peroxide concentration over very wide ranges. The results of several experiments have been assessed on this basis, and are summarized in Table 1. Inspection of these results shows that the protection afforded by hydrogen peroxide increases with the hydrogen peroxide concentration. Since, in the absence of externally added peroxide, the concen-

Fig. 5. Effect of hydrogen peroxide on the inactivation of catalase solutions by X-rays. The graphs in A show the effect of irradiation at 191 r./min. (○), and at 825 r./min. (●) on 3×10^{-8} M aerated catalase solutions at 18° and pH 7.0, without external addition of hydrogen peroxide. The graphs in B show the effect of irradiation at 191 r./min. (△), and at 825 r./min. (▲) on similar solutions to which hydrogen peroxide was added continuously at an average rate of $2.5 \mu\text{moles l.}^{-1}/\text{sec.}$ during irradiation.

Table 1. *Effect of added hydrogen peroxide on the X-ray inactivation of 3×10^{-8} M catalase solutions*

Expt. no.	Rate of addition of hydrogen peroxide		Dose rate (r./min.)	$[H_2O_2]_{\text{equilib.}}$ ($\times 10^8$ M)	Rate of inactivation (relative units)	Dose-rate exponent
	By radiation ($\times 10^8$ M/sec.)	Externally ($\times 10^8$ M/sec.)				
1	2.3	0	825	3.54	1.0	0.56 ± 0.06
2	0.53	0	191	0.82	0.44	
3	2.3	10*	825	22.8	0.93	0.75 ± 0.15
4	0.53	10*	191	19.4	0.31	
5	2.3	250	825	460	0.57	0.95 ± 0.07
6	0.53	250	191	460	0.14	

* Hydrogen peroxide in this experiment was made by irradiation of water.

tration of peroxide attained during irradiation is proportional to the dose rate, it follows that the radiation sensitivity of a catalase solution decreases with increase in dose rate, and it is suggested that this is the explanation of the low values of the dose-rate exponent reported above. On this view the radiation sensitivity of a catalase solution is determined by the hydrogen peroxide concentration, so that the lifetime as determined with intermittent irradiation should be that of hydrogen peroxide, as observed. The values of n recorded in Table 1 accord reasonably well with this interpretation, since n should, and does, approach unity as the rate of addition of hydrogen peroxide to the system is increased above that due to irradiation.

Additional evidence for the above interpretation was found in studies with short-wave ultraviolet radiation, as described in the next section.

Effects of ultraviolet radiation

An exposure of 90 sec. to the output of the mercury resonance lamp under the conditions specified in the Experimental section reduced the activity of 4×10^{-8} M catalase solutions to 40% of their initial value. When a quartz-walled filter containing a layer of 1.3 cm. of aerated water was placed in the path of the radiation, no inactivation of catalase solutions could be detected after exposure for some minutes. Since this filter transmits 80% of incident radiation of wavelength 253 m μ ., and practically 100% of the visible output of the lamp, it is obvious that the inactivation caused by unfiltered radiation is not due to either of these components. The effective radiation must, therefore, be the remaining component emitted by such lamps, namely that of 184.9 m μ ., wavelength. This 184.9 m μ ., radiation is strongly absorbed in water, forming hydrogen peroxide as a reaction product. In confirmation of the identity of the effective wavelength, it was found that the unfiltered radiation produced hydrogen peroxide in aerated water at the rate of 4×10^{-8} mole l.⁻¹/sec. under conditions similar to those used above, and that this

rate was reduced to an undetectable value when the above filter was placed in the path of the radiation.

With the use of various depths of water and solutions in the filter, estimates of their absorption coefficients at 184.9 m μ ., were obtained from measurements of relative initial rates of peroxide formation in the vessel below the filter, on the assumption that this rate is proportional to the incident intensity of 184.9 m μ ., radiation. The following values were obtained in this way: water, decadic linear co-efficient (α) = 1.5 cm.⁻¹; quartz, α = 0.07 cm.⁻¹; catalase, decadic molar coefficient (ϵ) = 10^7 l. mole⁻¹ cm.⁻¹; hydrogen peroxide, ϵ = 150; phosphate buffer of pH 7.0, ϵ = 350. These values are approximate but are considered to be of the correct order of magnitude. On the basis of these values it appears that catalase solutions as irradiated above absorbed approximately 89% of the incident 184.9 m μ ., radiation, of which 58% was absorbed by the buffer (0.01 M), 25% by the water, and only 6% by the catalase.

It seems possible, therefore, that the catalase inactivation was due to energy absorbed by the water and transferred to the catalase via certain products of water decomposition. The mechanism may in fact be analogous to that occurring in X-irradiated solutions. Direct action due to energy primarily absorbed by the catalase is not excluded since a small fraction of the incident energy certainly is so absorbed, but certain kinetic evidence has been obtained in support of the former view.

This kinetic behaviour is illustrated in Fig. 6. In these experiments 1.5×10^{-8} M catalase solutions were irradiated (a) as specified above and (b) under similar conditions but through the rotating sector used in X-ray experiments. The sector was rotated at 90 rev./min., corresponding to $t_1 = 0.083$ sec. Results show that the shape of the inactivation curve is similar to that observed with X-rays, though exponential inactivation is maintained for smaller times of irradiation. More striking is the fact that the initial inactivation rate in (b) is 0.42 ± 0.05 of that in (a). Since this value is greater

than 0.25 by a margin well outside experimental error, one concludes that the dose-rate exponent in this system is less than unity, and the lifetime of the intermediary involved is greater than 0.1 sec. Measurements of the dose-rate exponent in $4.8 \times 10^{-8} \text{ M}$ catalase solutions with continuous irradiation yielded a mean value of 0.64 ± 0.2 . This is subject to large error because of the small range of exponential behaviour, and the same reason precluded a more accurate estimate of the lifetime of the intermediary. Nevertheless, the kinetic analogy with X-irradiated systems is well established, and was further confirmed in studies of the effect of added hydrogen peroxide. When the concentration of peroxide present during irradiation was raised from its normal value (of the order of 10^{-8} M) to $1.7 \times 10^{-5} \text{ M}$ by external addition, the rate of inactivation of $4.8 \times 10^{-8} \text{ M}$ catalase solutions was very approximately halved. This protection is not due to an inner-filter effect since the above concentration of peroxide absorbs a negligible fraction of 184.9 m μ . radiation.

Thus it is established that the production of hydrogen peroxide in water by 184.9 m μ . radiation as well as by X-rays is accompanied by low values of the dose-rate exponent for catalase inactivation, and further that hydrogen peroxide protects against

inactivation in both systems. This observation lends further support to the conclusions of the previous section.

Effect of α -radiation

The effect on catalase solutions of the α -, β - and γ -rays emitted by radon and its decay products was determined by the method outlined above. The mechanical treatment of the catalase involved in this method caused some loss of activity, and estimates of the effect due to radiations could be obtained only by comparisons with control experiments in which the same procedure was followed with radon solutions of negligible activity, prepared from very old radon seeds. Two further control experiments were also performed. In the first, a catalase solution was treated with a radon solution of negligible activity in the usual manner. Half of this preparation was irradiated in the 20 ml. syringe with X-rays for 10 min. at approximately 500 r./min., air was then bubbled through for 3 min., and it was assayed as usual. The other half was left in a similar syringe, air was subsequently bubbled through, and then it was assayed at the same times as the first portion. The activity of the X-irradiated sample was 35% of that of the other sample. This is the degree of inactivation expected of such a dose of X-rays on the basis of previous data, so that the treatment with radon solutions has not rendered the catalase insensitive to radiation. In the second experiment an approximate check of the α -ray dose rate was made by observing the rate of oxidation of ferrous sulphate by radon solutions under irradiation conditions identical with those used in catalase experiments. An exposure of 76 min. at an α -ray dose rate estimated by the usual procedure as $1.01 \times 10^{14} \text{ ev/ml. sec.}$ produced $7.61 \times 10^{-5} \text{ M}$ ferric sulphate. This corresponds to 9.9 molecules of iron oxidized/100 ev, as compared with the value of 5.9 reported by Miller & Wilkinson (1954) for α -rays. Owing to the presence of β - and γ -radiation and to other uncertainties the two values are not strictly comparable, but the result suggests nevertheless that the actual dose rate is at least as high as that estimated.

The results obtained in the main series of experiments are shown in Table 2. The second column

Fig. 6. Inactivation of aerated catalase solutions by 184.9 m μ . radiation. Catalase solutions ($1.5 \times 10^{-8} \text{ M}$) at 18° and pH 7.0 were irradiated through a quarter-open sector rotating at $t_1=0.083 \text{ sec.}$ (○), and without the sector but under otherwise similar conditions (●).

Table 2. *Effect of α -radiation on catalase solutions*

Expt. no.	Amount of radon (mc)	α -Ray dose rate ($\times 10^{13} \text{ ev/ml. sec.}$)	Exposure time (min.)	Catalase concn. ($\times 10^8 \text{ M}$)	Residual activity (%)
1	0.02	0.07	26	1.9	77
2	0.06	0.26	2	1.1	90
3	0.96	4.26	7	0.8	82
4	3.75	16.15	13	1.9	85
5	5.36	23.3	6.5	1.7	77
6	23.0	90.5	10.5	1.7	79

shows the amount of radon mixed with the catalase solution, the third shows the estimated rate of dissipation of α -ray energy in the catalase solutions, and the final column shows the residual activity of the catalase at the end of the exposure to radon. Inspection of these results shows that the inactivation observed is independent of the intensity of α -irradiation over the range studied, and must be attributed to the mechanical effects involved in the treatment. It is difficult to assess the limits of error in this statement, but if one plots the observed inactivation as a function of time of treatment and takes Expts. 1 and 2 as controls, it appears that the inactivation due to irradiation for 10.5 min. in the extreme case of Expt. 6 is less than 10%, and is probably zero. X-irradiation at 90×10^{13} ev/ml.sec. (900 r./min.) would cause 10% inactivation in 45 sec. under similar conditions, so that on this basis the inactivating efficiency of radon radiation is less than 7.5% of that of X-rays. Since a small fraction (probably less than 4%) of the energy dissipated by radon radiation in these solutions is due to β - and γ -rays with inactivating efficiency comparable to that of X-rays, it is evident that the inactivating efficiency of the α -rays is even lower than the above estimate.

Alpha radiation produces hydrogen peroxide in aerated water in approximately the same yield per unit energy dissipation as is observed with X-rays. A yield of 1.7 molecules of hydrogen peroxide/100 ev is reported by Anta & Lefort (1954) with the α -rays from polonium, as compared with a yield of 2.2 similar units observed by Sutton (1952a) with X-rays. It follows from the low inactivating efficiency of α -rays relative to that of X-rays that inactivation is not due to hydrogen peroxide. This conclusion conforms with that obtained above by independent methods.

Mechanism of inactivation

Forssberg (1945) concluded that the inactivation of catalase in aqueous solution by X-irradiation proceeds by an indirect mechanism in which the energy absorbed by the water is transferred to the catalase via certain products of water decomposition. The results of these studies conform fully with this conclusion and for similar reasons, namely, (a) an inactivation yield as high as 0.02 molecule inactivated/100 ev absorbed in the solution was observed under certain conditions, and still higher values would be obtained at lower dose rates; (b) the dose-rate exponent was found to be less than unity, and (c) evidence of suppression of the X-ray effect by small concentrations of iodide ions and other agents was found in the course of this work. The observed dependence of inactivation rate on catalase concentration also conforms with this view if one assumes that the fraction of available inter-

mediates which react with the catalase increases with the catalase concentration over the range studied.

Among the established products of X-irradiated water are the radicals OH and H, and the molecular products hydrogen and hydrogen peroxide. It has been shown that hydrogen peroxide acts as a rate-determining intermediary in this system but that its role is one of protection and not inactivation. The inactivation must therefore be attributed to other primary products and most probably to either or both of the radicals OH and HO₂, since the H atom is known to be oxidized to HO₂ in aerated solutions. The hydrogen peroxide produced in water by α -rays is also accompanied by a small yield of these radicals. From the literature reviewed by Hart (1954) and Allen (1954) it appears that this yield per unit energy dissipation is in the range of 7–14% of that obtained with X-rays, so that one might expect the inactivating efficiency of these two radiations in catalase solutions to be in the same proportion. Results indicate that α -rays are somewhat less effective than this proportion would require. This may be due to differences in distribution of the radicals obtained from the two radiations, but in any case the small discrepancy is scarcely outside the limits of experimental error, and does not invalidate the assumption that the radicals OH and/or HO₂ are responsible for inactivation.

Other investigators (e.g. Dainton & Rowbottom, 1953) have shown that the HO₂ radical may have a lifetime of the order of seconds under certain conditions, and it is therefore of interest to note that while the results obtained with intermittent radiation show that the rate of inactivation is largely controlled by an intermediate with the lifetime of hydrogen peroxide, they do not exclude the possibility that a second intermediate of longer lifetime such as HO₂ may play a minor role. This could be decided only by exploring larger values of t_1 than were possible in these studies.

Sutton (1952b) concluded from studies of protection by hydrogen that the OH radical is the inactivating agent in this system. It must be pointed out that this conclusion does not necessarily apply to the present investigation, since the hydrogen protection experiments were performed with an impure sample of catalase and the results could not be wholly confirmed in this work.

In view of the observed similarities of the effects of 184.9 m μ . radiation and of X-rays, and of the energy-absorption data relating to the former radiation, the question arises whether the primary intermediates in the two systems may be similar. One primary product common to both systems is hydrogen peroxide, but this, alone, does not explain the similarity since the peroxide acts only as a protecting agent. The primary products of

184.9 m μ . irradiation of water are not well established. The absorption coefficient of aerated water which was reported above is about what one might extrapolate from Kreusler's (1901) measurements of the absorption coefficient of water at 186 and 193 m μ ., but it is more than 10^8 times higher than could be attributed to dissolved oxygen. The true light acceptor therefore appears to be water, and possible primary products of this radiation are hydrogen and excited oxygen atoms. Professor F. S. Dainton (private communication) suggests that hydrogen peroxide formation by this radiation may be due to reaction of the oxygen atom with water. No definite conclusion regarding the nature of the inactivating intermediary is possible from the data available, but the similarities of the effects when compared with those of X-rays strongly suggests that the two radiations may act by a common mechanism. If this conclusion is confirmed it may well prove to be of general application.

With regard to the secondary reactions of this evidently complex inactivation mechanism, it appears from these studies that the low values of the dose-rate exponent observed with both X-rays and 184.9 m μ . radiation, the lifetime measurements with intermittent radiation, and the relative efficiencies of X- and α -rays may all be satisfactorily correlated with the protecting action of hydrogen peroxide. The mechanism of this protecting action in relation to the inactivation process will be the subject of a further paper.

SUMMARY

1. The inactivation of dilute aqueous solutions of catalase by small doses of X-rays proceeds exponentially at a rate proportional to a power (n) of dose rate, where n is less than unity.

2. Studies with intermittent X-irradiation have shown that the lifetime of the intermediary responsible for this low value of n is of the order of seconds under particular conditions, and is equal to the lifetime of hydrogen peroxide in the irradiated solutions.

3. Addition of hydrogen peroxide to catalase solutions during X-irradiation reduces the inactivating effect of the radiation, and also increases n up to a limiting value of unity attained at high rates of addition.

4. It is suggested that protection by hydrogen peroxide produced by radiation is the cause of the low values of n .

5. Similar kinetic properties are exhibited by catalase solutions irradiated with 184.9 m μ . radiation. This is attributable in part to protection by hydrogen peroxide produced by radiation, but there is also evidence that the primary products of 184.9 m μ . radiation on water may be similar to those produced by X-rays.

6. The inactivating efficiency of α -rays on catalase solutions is less than 7 % of that of X-rays.

7. The primary reactions of the inactivation mechanism are considered. In the case of X-rays, the radicals OH and/or HO₂ are probably the primary inactivating agents.

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