Effects of Radiations on Catalase Solutions

2. THE PROTECTING ACTION OF COMPLEXING AGENTS

By H. C. SUTTON

Hugh Adam Department of Cancer Research, University of Otago Medical School, Dunedin, N.Z.

(Received 17 February 1956)

Previous studies of the inactivation of catalase solutions by X-rays (Sutton, 1956) have shown that hydrogen peroxide exerts a protecting action. The question arises whether this protection is associated with the known complexing action of hydrogen peroxide on the iron centres in catalase. This possibility is explored in the present work, together with the effects of other iron-complexing agents on the radiation sensitivity of catalase. The results throw some light on the mechanism of inactivation induced by radiation.

EXPERIMENTAL

Irradiation technique. The apparatus and method used for irradiations with X-rays and 184.9 m μ . radiation were the same as those described previously (Sutton, 1956).

Determination of catalase. Measurements of catalytic activity were performed at room temperature by spectrophotometric observations of the rate of decomposition of 0.007 M hydrogen peroxide at pH 7, with due allowance for the optical density of the fully decomposed solutions.

Inhibition of catalase. Measurements of catalytic inhibition were made by adjusting substrate and catalase to the desired concentration of inhibitor before mixing, and comparing the rate of peroxide decomposition so observed with that in inhibitor-free solutions. In the case of hydrogen cyanide the inhibition was so quickly reversible that the degree of inhibition was determined only by the final concentration of cyanide in the mixture, regardless of initial concentrations in the substrate and catalase.

Inactivation studies. The general procedure was as follows: Catalase solutions, generally 2×10^{-8} M, were made up to such a concentration of inhibitor as was determined by the degree of inhibition required. The activity of dilutions of such solutions was measured before and after irradiation for 2-3 min. at 800 r./min. by injecting a portion (generally 0.5 ml.) of catalase solution into 10 ml. of inhibitor-free substrate, and observing the rate of peroxide decomposition. In this way the inhibiting action of hydrogen cyanide was largely reversed during activity measurements, so that strongly inhibited solutions could still be assayed.

Spectroscopic determinations. A Unicam spectrophotometer (model SP. 500) was employed for these measurements. In order to minimize the concentration of catalase required for optical absorption measurements, cells of 10 cm. length were employed in measurements of the spectra of solutions before and after irradiation.

Materials. Catalase solutions were prepared from the crystalline catalase described previously (Sutton, 1956). Hydrogen peroxide and potassium cyanide were of AnalaR

reagent quality. Solutions of hydrogen cyanide were freshly prepared each day by dissolving potassium cyanide in buffer of pH 7. Hydroxylamine solutions were made from AnalaR hydroxylamine hydrochloride. Sodium azide was of commercial grade.

RESULTS

Dissociation constant of catalase cyanide

Since the magnitude of this quantity depends on the nature of the catalase it was necessary to determine it for the enzyme studied. In agreement with Chance (1949) it was found that measurements of catalytic inhibition in hydrogen cyanide at pH 7 could be expressed satisfactorily by the relation

$$K_{\text{inhib.}} = x/(A_0/A_x - 1),$$

where $K_{\text{inhib.}}$ denotes the dissociation constant as determined by this method, A_0 denotes the uninhibited activity, and A_x denotes the activity observed when the hydrogen cyanide concentration is x moles/l. The results yielded the value

$$K_{\text{inhib.}} = 3.0 \pm 0.25 \times 10^{-6} \text{ M.}$$

A second determination of the dissociation constant was made by measurements of the optical absorption of 3×10^{-7} M catalase solutions in 4 cm. cells at 425 m μ . (the peak of the Soret band of catalase cyanide) in various concentrations of hydrogen cyanide. The dissociation constant was obtained from these measurements with the relation

$$K_{\text{spect.}} = [\text{HCN}] [\text{free catalase}] / [\text{catalase cyanide}] \\ = x (\Delta D_{\text{max.}} / \Delta D_x - 1),$$

where $\Delta D_{\text{max.}}$ denotes the difference in optical densities at 425 m μ . observed between solutions of free catalase and of catalase saturated with hydrogen cyanide, and ΔD_x denotes the value of ΔD observed when the hydrogen cyanide concentration is x moles/l. The spectroscopic data were well fitted by this equation and yielded the value

$$K_{\text{spect.}} = 2.93 \pm 0.25 \times 10^{-6} \text{ M,}$$

in close agreement with $K_{\text{inhib.}}$. Chance (1949) has observed somewhat higher values of these constants, namely,

$$K_{\text{inhib.}} = 4.7 \times 10^{-6} \text{ M, and } K_{\text{spect.}} = 4 \times 10^{-6} \text{ M,}$$

but the results obtained by the two methods were in mutual agreement.

Effect of hydrogen cyanide on the inactivation of catalase induced by radiation

It has been shown in the previous paper of this series (Sutton, 1956) that the inactivation of catalase solutions by X-rays proceeds exponentially during the first 2 or 3 min. of irradiation. The same type of behaviour was observed when hydrogen cyanide was added to the catalase solutions, so that we may define the rate (k_x) of this X-ray induced inactivation by the relation $k_x = \{\ln (C_0/C_x)\}/t$, where $(C/C_0)_x$ denotes the fraction to which the initial activity of a catalase solution in hydrogen cyanide of molarity x is reduced, after irradiation for t sec. The method of determining the activities of catalase plus cyanide solutions before and after irradiation, and hence of evaluating the ratio $(C_0/C)_x$, was explained in the Experimental section. The activity actually measured is that of a diluted sample of the original solution, so that the inhibition caused by the cyanide is reversed to a known degree.

In a series of experiments with $2 \times 10^{-8} \text{ M}$ catalase solutions at pH 7, containing various concentrations of hydrogen cyanide, it was found that the rate of inactivation at constant dose rate was diminished by hydrogen cyanide in a manner directly related to the inhibiting action of this agent (Fig. 1). A constant time of irradiation (2.25 min.) was employed in these experiments such that $(C/C_0)_0$ was 0.57. In Fig. 1 the ordinates denote the ratio (k_x/k_0) of the inactivation rate observed in hydrogen cyanide of molarity x to the rate when $x = 0$; and the abscissae denote the fraction (A_x/A_0) to which the activity of catalase is inhibited by the same molarity, x , of hydrogen cyanide. It was shown in control experiments that X-irradiation had no effect on solutions of hydrogen cyanide of concentration 10^{-4} M under the conditions used in catalase irradiations.

The effect of hydrogen cyanide on the inactivation caused by 184.9 m μ . radiation was also observed in a few experiments. It was found that 10^{-4} M hydrogen cyanide completely suppressed the inactivation, and 10^{-7} M hydrogen cyanide had no measurable effect.

Effects of radiation on the absorption spectrum of catalase

Fig. 2 shows the absorption spectrum of $7 \times 10^{-8} \text{ M}$ catalase at pH 7 before and after X-irradiation with 5200 r. at 650 r./min. Two effects of radiation are apparent, namely (a) the absorption in the protein portion of the molecule at wavelengths less than 300 m μ . is increased, and (b) the absorption associated with the iron centres at 405 m μ . is decreased. Dale & Russell (1955) have reported similar effects of X-irradiation on more concentrated solutions of catalase. The decrease of absorption in

Fig. 1. Effect of hydrogen cyanide on the inactivation of catalase solutions by X-rays. Catalase solutions ($2 \times 10^{-8} \text{ M}$) containing various concentrations of hydrogen cyanide were irradiated for 2.25 min. at 800 r./min. The ordinates denote the ratio (k_x/k_0) of the inactivation rate observed in cyanide of molarity x to that observed in cyanide-free solutions, and the abscissae denote the inhibiting power of hydrogen cyanide expressed as the fraction (A_x/A_0) to which the activity of catalase is inhibited by cyanide of the same molarity x . The interrupted curve is discussed in the text.

Fig. 2. Effect of X-irradiation on the absorption spectrum of catalase solutions. The spectrum of $7 \times 10^{-8} \text{ M}$ catalase at pH 7.0 is shown before (A), and after (B), irradiation with 5200 r. at 650 r./min. The irradiated solution possessed 20% of its initial activity.

the Soret band at 405 m μ . was found to be partially reversible after small doses, and this reversible effect is probably due to the formation during irradiation of the catalase-hydrogen peroxide complex I, as described by Chance (1947). This complex has a somewhat smaller absorption coefficient at 405 m μ . than catalase, and slowly reverts to catalase after removal of the hydrogen peroxide.

Similar changes in the catalase absorption spectrum were produced by 184.9 m μ . radiation.

The effects of X-irradiation on the absorption spectrum are reduced in the presence of hydrogen cyanide, in the manner shown in Fig. 3. The results of these experiments are expressed as optical density changes in three selected regions of the absorption spectrum, as observed after a fixed dose of 5200 r. at 650 r./min. Hydrogen cyanide itself has practically no influence on the absorption of catalase at wavelengths less than 300 m μ ., but it moves the peak of the Soret band to 425 m μ ., and reduces its intensity by a factor of 0.8. In order to compare radiation effects in the Soret band in catalase solutions containing hydrogen cyanide with those observed in free catalase, the changes of optical density at 405 and 425 m μ . are expressed in Fig. 3 as the quantity ΔD_{Soret} , defined by the relation $\Delta D_{\text{Soret}} = \Delta D_{405 \text{ m}\mu} + 1/0.8 \Delta D_{425 \text{ m}\mu}$.

Fig. 3. Effect of hydrogen cyanide on the radiation sensitivity of catalase solutions. The lower three curves show the change of optical absorption observed in the Soret band (●), at 275 m μ . (◐), and at 255 m μ . (○), after irradiating 7×10^{-8} M catalase solutions at pH 7.0 in hydrogen cyanide. The ordinates denote the change of optical density observed in 10 cm. cells after a fixed dose of 5200 r. at 650 r./min. The method of expressing ΔD_{Soret} is explained in the text. The upper two curves show the inhibiting effect of hydrogen cyanide on catalase activity expressed as A_x/A_0 (■), and the protecting effect of hydrogen cyanide against inactivation of catalase solutions by small doses of X-rays expressed as k_x/k_0 (▲).

For comparison with these results Fig. 3 also shows the effects of hydrogen cyanide on (a) inhibition of enzymic activity, expressed as A_x/A_0 and (b) inactivation of catalase solutions by small doses of X-rays, expressed as k_x/k_0 .

Influence of other inhibiting agents

Hydroxylamine. The reversible inhibition of catalase by hydroxylamine was found to occur with a dissociation constant of approximately 4.8×10^{-7} M. However, hydroxylamine was not found suitable for studies of the type illustrated above, since it is itself affected by X-irradiation. Thus when a catalase solution in 10^{-4} M hydroxylamine was irradiated with 5200 r. at 650 r./min. its activity had apparently increased after irradiation when measured by the methods described for hydrogen cyanide. This was shown to be due to a decrease in the inhibiting power of the solution owing to radiation effects on the hydroxylamine. Curiously, the apparent activity of an irradiated catalase-hydroxylamine solution varied somewhat on standing.

Sodium azide. The inhibition of catalytic activity caused by sodium azide was not fully reversible, and the influence of azide on the catalase absorption spectrum is difficult to interpret. Azide was therefore rejected as a suitable inhibitor for these studies.

DISCUSSION

The inhibition of catalytic activity by hydrogen cyanide is due to the reversible formation of a complex of hydrogen cyanide with the iron atom in each haem group of catalase. The quantity A_x/A_0 as defined above may be equated to $(1 - F)$, where F denotes the fraction of haem groups so 'complexed'. The results of the protection experiments shown in Fig. 1 clearly demonstrate that there is a definite relation between k_x/k_0 and A_x/A_0 , which can be expressed approximately by the equation

$$k_x/k_0 = A_x/A_0.$$

It follows that $k_x/k_0 \approx (1 - F)$.

A reasonable explanation of this result is that those haem groups which are complexed with hydrogen cyanide are protected from inactivation. On this view the fraction F of the catalase is unaffected by radiation, while the fraction $(1 - F)$ is inactivated at the same rate (k_0) as is observed in the absence of cyanide. Hence the fraction of the initial activity remaining after irradiation for t sec. is given by

$$A/A_0 = (1 - F) \exp(-k_0 t) + F. \quad (1)$$

This fraction A/A_0 corresponds to the experimentally measured quantity C/C_0 , since the same cyanide inhibition factor applies to both initial and irradiated solutions. The shape of the inactivation curve

predicted by equation (1) for solutions of constant cyanide concentration is not quite exponential. However, it approximates very closely to the observed exponential form when the factor $\exp(-k_0 t)$ is greater than 0.55, and the experimental observations were restricted to this range. As the cyanide concentration is varied in experiments with constant times of irradiation, equation (1) requires that

$$k_x/k_0 = \ln \{(1-F) \exp(-k_0 t) + F\} / \ln \{\exp(-k_0 t)\}. \quad (2)$$

From this relation, the predicted variation of k_x/k_0 with the factor $(1-F)$ has been calculated for the conditions studied, and the result is shown as an interrupted curve in Fig. 1. The predicted variation is not quite linear, but it agrees well with the observations within the limits of experimental error. It has been assumed in this treatment that uncomplexed catalase is inactivated at the same rate as in the absence of hydrogen cyanide. In fact the rate may increase slightly as the concentration of free catalase is reduced by cyanide, but this would have only a very small influence on k_x/k_0 . Thus the experimental results wholly conform with the view that the haem groups in catalase are protected from X-ray inactivation when they are complexed with hydrogen cyanide.

In the course of its catalytic decomposition, hydrogen peroxide also combines with catalase, and probably at the same point as does hydrogen cyanide. The question arises whether this could account for the protection by hydrogen peroxide observed in X-irradiated catalase solutions, and the kinetic properties of this system which are believed to be associated with this protection (Sutton, 1956). Evidence of a similar type of protection against enzyme inactivation whilst the enzyme is acting on its substrate has been obtained by Dale (1940), in his studies of the inactivation of solutions of carboxypeptidase induced by X-rays. The careful studies of Dr Chance and others on the mechanism of catalase action have been summarized by Chance, Greenstein & Roughton (1952) in terms of consecutive reactions of hydrogen peroxide (a) with catalase to form the catalase-hydrogen peroxide complex, and (b) with this complex to form decomposition products and regenerate free catalase. A reaction of the complex with oxidizable substrates such as alcohol is also included. On this basis, it is shown in agreement with observation that the fraction (Q) of catalase iron bound to hydrogen peroxide during its catalytic decomposition increases with increase in the concentration of hydrogen peroxide added to the system, and ultimately attains a limiting value independent of peroxide concentration. In a particular sample of catalase Q attains a maximum value of 0.25 at

hydrogen peroxide concentrations above $1 \mu\text{M}$. This statement relates to the initial concentration of hydrogen peroxide added to a catalase solution, but the same type of behaviour would be observed in catalase solutions in which hydrogen peroxide is continuously produced by X-irradiation. In the latter case, the magnitude of Q would be determined by the equilibrium concentration of hydrogen peroxide, and would attain the same limiting value at large concentrations. If it is assumed that peroxide-bound catalase is insensitive to X-rays, then the radiation sensitivity of catalase solutions would be proportional to $(1-Q)$, and would decrease with increase in equilibrium peroxide concentration over a limited range. The protection by hydrogen peroxide follows this pattern, and is observed in the range 10^{-9} – 10^{-6} M hydrogen peroxide, which would be expected on the basis of the data of Chance *et al.* (1952). There are, however, two objections to a quantitative treatment of the observed inactivation kinetics based solely on this assumption:

(1) The range of radiation sensitivities required to account for all the data is such that Q_{max} would have to be assigned a value at least as large as 0.9. Values of Q_{max} reported by Chance *et al.* (1952) are 0.25 for erythrocyte catalase and approximately 0.33 for some liver catalases. An estimate of Q_{max} relating to the enzyme studied in this work has been obtained from a comparison of spectroscopic and inhibition data relating to hydrogen cyanide. If it is assumed that (a) hydrogen peroxide and hydrogen cyanide combine with the same site in the catalase molecule, and (b) that the activity of catalase in the presence of cyanide is proportional to the fraction of cyanide-free (but not necessarily peroxide-free) catalase, then starting from the equations of Chance *et al.* (1952) relating to cyanide inhibition it can be shown that

$$Q_{\text{max}} = 1 - K_{\text{spect.}}/K_{\text{inhib.}}$$

Substituting the values reported above, we obtain

$$Q_{\text{max}} = 0.025 \begin{cases} +0.15 \\ -0.025. \end{cases}$$

Evidently, either the above assumptions are not justified, or Q_{max} is less than 0.2 and is therefore too small to account for the kinetic data.

A further consequence of the assumptions (c) that catalase hydrogen peroxide is protected from inactivation as well as catalase cyanide and (a) that cyanide and peroxide combine with the same site is that A_x/A_0 cannot equal k_x/k_0 unless Q_{max} is very small, or is nearly equal to the value of Q attained during X-irradiation.

(2) The dose-rate exponent for catalase inactivation in X-irradiated solutions varies with the catalase concentration in such a manner that no self-consistent set of values of Q can be assigned to fit all the data on the assumption that Q is dependent

only on hydrogen peroxide concentration. One has to assume that the radiation sensitivity of catalase decreases not only with increase in concentration of hydrogen peroxide, but also with decrease in catalase concentration.

It is concluded, therefore, that the mechanism discussed above can account for only part of the observed protection by hydrogen peroxide. The variation of radiation sensitivity with catalase concentration suggests that a second mechanism operates; this may be a competition of peroxide with catalase for the radicals produced by irradiation.

Regardless of the mechanism of peroxide protection there is good evidence that catalase is protected from inactivation when its iron atoms are bound to hydrogen cyanide. This suggests that the inactivation process is localized on the iron centre, and the question arises whether the chemical effects of radiation are restricted to this centre. Fig. 2 clearly shows that this is not so. The protein portion of the molecule is affected at the same time as the iron centre, in a manner similar to that observed by Barron & Flood (1952) with cytochrome *c*, and with other proteins containing tyrosine residues. Similar effects are also observed by McLean & Giese (1950) and by other workers, after ultraviolet irradiation of protein solutions, and it seems possible that these effects may be due to the 184.9 m μ . radiation which would accompany the 253.7 m μ . radiation used in their studies. Previous studies by the writer (Sutton, 1956) have shown that the effects of this 184.9 m μ . radiation on catalase solutions are closely akin to those produced by X-rays, and further observations made in the course of this work emphasize this analogy.

One would expect these effects on the protein to have some influence on catalytic activity, since the specificity and rate of reaction of an enzyme depends on its protein structure. If so, then hydrogen cyanide should suppress these radiation effects as well as those concerned with the iron centre. Experiments have shown that while this is indeed the case, the concentration of hydrogen cyanide required to suppress the protein effects is higher than that required for suppression of the effects on the iron centre. Thus from Fig. 3 we see that 3×10^{-6} M hydrogen cyanide will half inhibit the inactivating action of small doses of X-rays and also the effect of 5200 r. on absorption by the iron centre, while it has practically no influence on the effect of 5200 r. on absorption by the protein at 255 and 275 m μ . At higher concentrations (of the order of 10^{-4} M) all radiation effects are suppressed, so that a second

mechanism of protection (possibly a radical-scavenging process) must be operating in this region. A clearer distinction between these two effects was sought with other inhibiting agents, but no other such agent was found more suitable for these studies.

It is concluded, therefore, that radiation effects on the protein do not necessarily cause inactivation, while effects on the iron centre certainly do. Two interpretations are still possible, namely, (a) the inactivation induced by radiation is due solely to effects on the iron centre, and (b) energy absorbed in the protein may normally be transferred to the iron centre, but this process is blocked by hydrogen cyanide. The evidence does not distinguish between these two, though (a) appears the more likely.

SUMMARY

1. Catalase solutions are protected against X-ray inactivation when the iron centres in catalase are complexed with hydrogen cyanide.

2. Some, but not all, of the evidence relating to protection by hydrogen peroxide can be explained on a similar basis. A second mechanism of protection by hydrogen peroxide must predominate over that due to its complexing action.

3. Spectroscopic studies in the presence of hydrogen cyanide have shown that the protein portion of catalase may be damaged by radiation without loss of catalytic activity.

4. It is concluded from (1) and (3) that the major inactivating effect of X-rays on catalase solutions is localized on the iron centres.

The author wishes to express his thanks to Dr J. Read for his interest and helpful discussions, and to acknowledge the assistance of the staff of the Radiotherapy Department of the Dunedin Public Hospital, in which the X-irradiations were carried out.

This work was financed by the N.Z. Branch of the British Empire Cancer Campaign.

REFERENCES

- Barron, E. S. G. & Flood, V. (1952). *Arch. Biochem. Biophys.* **41**, 203.
Chance, B. (1947). *Acta chem. scand.* **1**, 236.
Chance, B. (1949). *J. biol. Chem.* **179**, 1299.
Chance, B., Greenstein, D. S. & Roughton, F. J. W. (1952). *Arch. Biochem. Biophys.* **37**, 301.
Dale, W. M. (1940). *Biochem. J.* **34**, 1367.
Dale, W. M. & Russell, C. (1955). *Nature, Lond.*, **175**, 33.
McLean, D. J. & Giese, A. C. (1950). *J. biol. Chem.* **187**, 537.
Sutton, H. C. (1956). *Biochem. J.* **64**, 447.