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The Action of Anions on Catalase Peroxide Compounds

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Catalase, like other haemoproteins, shows two kinds of chemical behaviour in respect of its haem iron, namely complex-formation and oxidation-reduction. The role of these two types of reaction in the catalytic activity (both catalatic and peroxidatic) of this enzyme has been the subject of some controversy. According to Chance (cf. Chance & Fergusson, 1954), the intermediates involved in the catalytic reactions are essentially complexes (similar to the fluoride or cyanide derivatives);

according to George (1952, 1953*a*), they should rather be considered as higher oxidation states.

The chemical nature of these intermediates (catalase peroxide compounds I and II) can be most usefully investigated by examining the reactions they undergo. The best-known group of such reactions comprises those with hydrogen donors, the behaviour of which has been analysed in terms of six reaction types (Keilin & Nicholls, 1958; Nicholls, 1959). These six types of donor

each promote one or more of several reduction steps leading from the primary peroxide compound (compound I) back to the free catalase (ferric). The absorption spectra of the two chief catalytic intermediates, compound I (containing two oxidizing equivalents in excess of the ferric state) and compound II (with one such oxidizing equivalent) are markedly different. But the two one-electron reductions, that of compound I to II and that of compound II to the free ferric catalase, show certain similarities. For example, the effects of pH are identical, and the reactions with phenols are in each case second-order (Nicholls, 1959). The two processes are also very similar to the corresponding reactions in peroxidase (George, 1952; Keilin & Nicholls, 1958).

Of the reactions given in Fig. 1, the present paper is largely concerned with the two one-electron reduction steps. Unlike the other reactions (promoted by donors in groups D₃, D₅ and D₆), these reductions are also produced by a hydrogen donor endogenous to the catalase protein itself, as with peroxidase.

The question of the relation between the redox reactions of the enzyme and its ability to form reversible complexes in the ferric form with anionic ligands, will also be considered. It has long been recognized that the inhibition of catalase by such ligands can be interpreted in terms of complex-formation with the free ferric enzyme (Keilin & Hartree, 1935). Such spectroscopically distinct complexes are closely similar to those formed by methaemoglobin and peroxidase. For catalase, the proposed mechanism is (Chance, 1952*b*):

Nevertheless, the action of some anions on the catalytic activity of the enzyme cannot be ac-

counted for by this simple scheme. Agner & Theorell (1946) found that the inhibition of catalase activity by acetate does not correspond with the spectrophotometric dissociation curve for enzyme and inhibitor. Bonnichsen, Chance & Theorell (1947) demonstrated a progressive inhibition of catalase by acetate (but not by formate or phosphate) in the presence of peroxide. Ogura, Tonomura, Hino & Tamiya (1950) showed that such inhibition occurs in the presence of phenols and various complex-forming anions, except cyanide. Beers & Sizer (1954, 1956) confirmed these observations with respect to acetate, chloride, azide and sulphide, and suggested that rapid formation of the secondary peroxide compounds is involved.

Each of these scattered observations suggests that other reactions between anions and enzyme, besides reaction (1), occur during the catalytic reactions. They further suggest that the reactions concerned promote the formation of essentially irreversibly inhibited derivatives of catalase, in contrast with the typically reversible inhibition of reaction (1). These ideas are supported to some extent by an analogy with peroxidase. The existence of an endogenous hydrogen donor in peroxidase preparations is well known (Chance, 1949*a*; George, 1953*b*). It is responsible for the 'spontaneous' transition from compound I to compound II, and for the decomposition of compound II. Chance (1949*a*, 1952*c*) and George (1953*b*) have shown that the decomposition of compound II by this donor (although not the formation of compound II from compound I) is promoted by complex-forming ligands such as acetate, fluoride and cyanide. There is some doubt about the order of the reaction with respect to fluoride concentration (Chance, 1952*c*; George, 1953*b*). Nevertheless, the reaction shows that complex-forming anions can react with derivatives other than the free enzyme.

Fig. 1. Catalytic reactions of catalase. Hydrogen donors involved are expressed as follows: D₁, ascorbate and ferrocyanide; D₂, phenols; D₃, alcohols and formic acid; D₄, nitrous acid; D₅, hydrazoic acid and hydroxylamine; D₆, hydrogen peroxide.

The demonstration of the analogy between compounds II of catalase and peroxidase (George, 1952; Keilin & Nicholls, 1958) shows that the transitions producing and decomposing the secondary compound of catalase (Chance, 1950) must also be due to the 'endogenous donor'. As the secondary compound is catalytically inactive, its formation and steady state will influence markedly the observed kinetics of the catalytic reaction. It is thus possible that the anomalies observed in the inhibition of catalase are associated with an effect of anions on the redox reactions of the enzyme (i.e. the formation and decomposition of compounds I and II) and, in particular, those reactions in which the hydrogen donor is endogenous.

This paper shows that the reductions produced by this endogenous process are different in kind from the reductions by exogenous donors, and that the kinetics of the former reductions may be used to demonstrate a reaction between complex-forming ligands and the catalase peroxide compounds themselves.

METHODS AND MATERIALS

Catalase. Catalase was prepared from horse liver (method of Keilin & Hartree, 1945) or horse blood by a modification of the methods of Bonnichsen (1955) and Herbert & Pinsent (1948). In all experiments, the formation and destruction of the secondary peroxide compound was followed qualitatively by direct visual observations with a bench microspectroscope. Quantitative measurements were made with a spectrophotometer containing a glass-prism monochromator, giving high dispersion in the visible region of the spectrum. The kinetics of compound II were followed by fixing the wavelength at 565 $m\mu$, which is close to the absorption maximum of compound II and which is also effectively isosbestic for compound I and free catalase.

Manometry. Barcroft-Warburg microdifferential manometers were used, at 30°, with air as the gas phase.

Other materials. These included: solutions of notatin (glucose oxidase) prepared from a purified sample of the enzyme (supplied by Boots Pure Drug Co. Ltd.); ethyl hydroperoxide, made according to the method of Baeyer & Villiger (1901); sperm-whale metmyoglobin solutions were prepared from a crystalline sample of myoglobin kindly supplied by Dr J. C. Kendrew. All other reagents used were of AnalaR or equivalent quality.

RESULTS

Formation of catalase peroxide compound II and the endogenous donor

The addition of notatin (glucose oxidase) and glucose, followed by aeration, which generates H_2O_2 , or of ethyl hydroperoxide to a solution of catalase, results in the production of compound II, with absorption bands at 568 and 535 $m\mu$. Fig. 2 shows the changes in extinction that occur in acetate and phosphate buffers. Acetate but not

phosphate is a ligand forming a complex with catalase, and its effect on the system is considered in detail below. The true decomposition rate of compound II can be measured only if the steady-state compound I is removed, e.g. by the addition of ethanol, which decomposes compound I but not compound II (Keilin & Nicholls, 1958). Fig. 3 shows the effect of pH on the formation and decomposition of compound II by this method, and also its formation by ethyl hydroperoxide (cf. Chance, 1952a). The rate of formation is approximately four times as great in the latter case, because only one-quarter of the haems are bound as compound I by the hydrogen peroxide generated in the notatin system (Chance, 1950).

Table 1 shows the relationship between catalase concentration and the velocity constant for these one-electron transitions.

Unlike the results obtained by Fraser (1956) for the two-electron reduction of compound I to free catalase, the velocity of which depended on the square of the enzyme concentration, the transition from compound I to compound II is first-order with respect to catalase concentration. This indicates that the reducing substance involved is associated with the same protein molecule as the haem. Further, there is no consistent method of titrating the endogenous donor with an oxidizing agent to decrease the velocity constants for the

Fig. 2. Formation of catalase compound II by hydrogen peroxide generated in the notatin-glucose system, and its subsequent decomposition. Notatin (10 $\mu g.$) and 1 mg. of glucose were added to 2.7 ml. of aerated catalase solution (0.06 mm-liver-catalase haem). The formation of compound II is followed by spectrophotometric measurements at 565 $m\mu$: —, in 0.05 M-acetate buffer, pH 4.6; ---, in 0.1 M-acid phosphate, pH 5.0; . . . , concentration of oxygen calculated from manometric experiments in phosphate buffer, from the kinetic data of Keilin & Hartree (1948) and Laser (1952). 100% Oxygen indicates saturation with air (about 0.2 mm- O_2 at 20°). '100% Compound II' indicates E at 565 $m\mu$ in the presence of ferrocyanide, which reduces compound I to compound II without affecting the stability of the latter.

transitions. Incubation with ferricyanide had no effect whatever on the formation of compound II. Incubation with ethyl hydroperoxide affected the steady state of compound II produced in the

notatin system, but this effect was largely due to an increase in the rate of decomposition of compound II, and was accompanied by the destruction of part of the enzyme protein.

A similar decrease in the stability of compound II was found in certain samples of catalase that had undergone partial denaturation, such as 'para-catalase' (cf. Keilin & Hartree, 1951) and reducible catalase (cf. Dounce & Schwalenberg, 1950). The effect of ethyl hydroperoxide may therefore be due to alteration in the availability of the endogenous donor, rather than to its saturation with oxidizing equivalents. In respect of this lack of titratability, the donor resembles the endogenous donor in peroxidase (Chance, 1949*a*) and differs markedly from all added donors.

Action of ligands

The anions combining with free (ferric) catalase may be divided into three groups: (1) ligands forming ionic complexes (e.g. fluoride, acetate, formate, hypophosphite); (2) ligands forming ionic complexes, also inhibiting by donor reaction (e.g. azide, hydroxylamine); (3) ligands forming covalent complexes (e.g. cyanide, sulphide).

Normal ionic ligands. Fluoride and acetate were found to accelerate both the formation and decomposition of catalase compound II (see Fig. 1). In neither case is the effect directly proportional to anion concentration (cf. peroxidase; George, 1953*b*). The efficacy of fluoride proves that the reaction is a catalytic one, as fluoride is thermodynamically incapable of acting as a hydrogen donor itself (unlike acetate and formate).

In Fig. 4 the second-order velocity constants for the formation and decomposition of compound II have been plotted against the logarithm of the fluoride concentration. The results can be fitted to sigmoid curves which are consistent with Michaelis-Menten kinetics. The curves in Fig. 4 are compared with the dissociation curve for fluoride catalase obtained spectrophotometrically at the same pH. Table 2 summarizes the reaction parameters deducible from Fig. 4.

Change in pH has a marked effect on the action of fluoride towards compounds I and II. This is compounded of effects on the maximal velocity constant and on the apparent Michaelis constant. Table 3 illustrates this for pH values 5.0 and 6.3.

As in complex-formation with the free enzyme, the dissociation constants seem to be inversely proportional to H^+ ion concentration. As in the spontaneous transition (Fig. 2), the maximal velocity constants increase with H^+ ion concentration but are not directly proportional to it. We have here a relation between the action of catalase haem as complex-forming agent for anions and as oxidizing agent for hydrogen donors.

Fig. 3. Effect of pH on the rates of formation and decomposition of compound II. +, Rate of production of compound II from ethyl hydroperoxide compound I; x, rate of production of compound II from the compound I steady state in the presence of hydrogen peroxide generated by the notatin-glucose system; O, rate of decomposition of compound II induced by addition of ethanol to remove compound I. $20 \mu M$ -Catalase; 2μ moles of peroxide was added to 2.7 ml. of solution in ethyl hydroperoxide experiments; $10 \mu g$. of notatin and 1 mg. of glucose were added to 2.7 ml. of aerated solution in the notatin experiments; 10μ moles of ethanol was used to promote decomposition; phosphate buffers; 20° . k is in each case a first-order constant (with the dimensions sec.^{-1}).

Table 1. Velocity constants for compound II formation at different concentrations of catalase

The constants were obtained under the following conditions: $0.1 M$ -phosphate buffer, pH as indicated, at 20° . Notatin ($10 \mu g$.) with 1 mg. of glucose was added in some experiments, 2μ moles of ethyl hydroperoxide in others. The final reaction volume in all cases was 2.7 ml., and measurements were made at $565 m\mu$. The constant k , measured in reciprocal seconds, is the apparent first-order velocity constant for the formation of compound II from free catalase.

$10^5 \times$ Concn. of catalase (M)	Source of peroxide			
	Ethyl hydroperoxide		Notatin system	
	$10^3 k (\text{sec.}^{-1})$	pH	$10^3 k (\text{sec.}^{-1})$	pH
1.4	3.5	5.6	1.1	5.3
2.2	2.5	5.6	1.4	5.3
6.0	2.7	5.6	1.2	5.3

Fig. 4. Variation of velocity constants for the action of fluoride on catalase peroxide compounds compared with the dissociation curve of fluoride catalase. k_I is the second-order velocity constant for the transition between compound I and compound II catalysed by fluoride ($\text{M}^{-1} \text{sec}^{-1}$); k_{II} is the corresponding constant for the fluoride-catalysed decomposition of compound II ($\text{M}^{-1} \text{sec}^{-1}$). All measurements refer to phosphate, pH 5.0, at 20° . k_I values ($\times$) are given by ---, k_{II} values ($\circ$) by — and the fluoride-catalase dissociation curve by —. The dissociation curve was obtained by adding appropriate amounts of fluoride to catalase and measuring the appearance of fluoride catalase at $612 \text{ m}\mu$. The dissociation constant corresponds closely to that given by Chance (1952b).

Table 2. *Reactions of fluoride with catalase*

Compound II was formed by means of the notatin system or ethyl hydroperoxide in 0.1 M-phosphate buffer, pH 5.0, at 20° . The dissociation constants are obtained from Fig. 4.

Reaction	Velocity constant (sec^{-1})		Apparent dissociation constant (mM)
	Fluoride absent	Excess of fluoride	
Fluorocatalase formation	—	—	3.0
Formation of compound II	0.012	0.15	3.3
Decomposition of compound II	0.0009	0.08	4.4

Acetate, the action of which is shown in Fig. 1, behaves in a manner similar to fluoride. Formate acts likewise in the decomposition of compound II, but reacts with compound I as two-electron hydrogen donor (Keilin & Nicholls, 1958). Kinetic constants for these anions are given in Fig. 5 and Table 5.

That this effect is associated with the peroxide compound itself, and not a reactive equilibrium form in low concentration, is shown by the use of sodium hypophosphite. Unlike the other ionic ligands, its reaction with catalase is very slow,

although the resultant complex has a typical ionic spectrum.

$$k_9 = 0.35 \text{ M}^{-1} \text{sec}^{-1} \text{ (pH 4.8)}$$

$$k'_9 \text{ (undissociated acid, } pK 1.7) = 440 \text{ M}^{-1} \text{sec}^{-1}$$

$k_{10} \leq 10^{-6} \text{ sec}^{-1}$ (dialysis for 3 days removed only a small percentage of the hypophosphite complex).

Hypophosphite induces the decomposition of compound II, with the concomitant formation of hypophosphite-catalase. At low hypophosphite concentrations, the formation of bound enzyme from compound II in this way is as fast as from free enzyme. At high hypophosphite concentrations, the velocity reaches a limiting value of the same order of magnitude as with other anions. K_m corresponds to the ratio of the rates of formation and decomposition of a hypothetical anion-compound II complex spectroscopically indistinguishable from compound II itself, as shown in the following calculation.

If v represents the experimentally observable rate of disappearance of compound II (msec^{-1}),

$$v = k_4 [\text{compound II} \cdot \text{HA}] = \frac{k_4 [\text{HA}] [\text{II}]_{\text{total}}}{\frac{k_4 + k_3}{k_2} + [\text{HA}]} \quad (5)$$

where for fluoride, acetate etc. $k_3 \gg k_4$ and $K_m = k_3/k_2$, but for hypophosphite $k_3 \ll k_4$ and $K_m = k_4/k_2$. As described above, for hypophosphite, $k_2 = k_9 = 0.35 \text{ M}^{-1} \text{sec}^{-1}$ at pH 4.8;

$$k_4 = 4 \times 10^{-2} \text{ sec}^{-1}$$

at this pH; K_m can therefore be calculated to be 0.12 M , which corresponds with the value obtained experimentally.

Although anions thus have a marked effect on the reactivity of endogenous donor, no action in reductions by exogenous donors could be detected. The decomposition of compound II by a mixture of fluoride and *p*-cresol was no faster than was predictable on the basis of additive effect. Further, as shown in Table 4, when peroxide is generated by glucose oxidase, the percentage that reacts with hydrogen donors ('percentage utilization' in the

Table 3. *Effect of pH on catalysis by fluoride*

Compound II was formed by hydrogen peroxide generated in the notatin system, with 0.04M-phosphate buffer at 20°.

pK_d	Negative logarithm of the dissociation constant for fluoride catalase.
pK_m^I	Negative logarithm of the Michaelis constant for the decomposition of compound I by fluoride (i.e. formation of compound II, cf. Fig. 4 and Table 2).
pK_m^{II}	Negative logarithm of the Michaelis constant for the decomposition of compound II by fluoride (cf. Fig. 4 and Table 2).
$pk_{I \max.}$	Negative logarithm of the maximal velocity constant for the transition from compound I to compound II (cf. Fig. 4 and Table 2).
$pk_{II \max.}$	Negative logarithm of the maximal velocity constant for the decomposition of compound II (cf. Fig. 4 and Table 2).

K_d , K_m^I , K_m^{II} are expressed in terms of fluoride concentration (molar); $k_{I \max.}$, $k_{II \max.}$ in reciprocal seconds (sec.⁻¹).

pH	pK_d	pK_m^I	pK_m^{II}	$pk_{I \max.}$	$pk_{II \max.}$
5.0	2.5	2.5	2.4	0.8	1.1
6.3	1.2	1.3	1.0	1.4	1.6

Table 4. *Effect of anions on the peroxidatic utilization of hydrogen peroxide in the coupled oxidation system*

Notatin (20 µg.) and 10 µmoles of glucose were placed in 1.0 ml. (final volume) of 0.02M-phosphate buffer, pH 6.0, with the use of microdifferential manometers without alkali papers, at 30°. Catalase, donor and anion concentrations were as indicated. The percentage utilization of H₂O₂ was calculated from excess of oxygen uptake compared with the system in the absence of hydrogen donors.

Concn. of catalase (µM)	Concn. of donor (mM)	Concn. of anion (mM)	Utilization of H ₂ O ₂ * (%)	
			Anion absent	Anion present
18	Ferrocyanide 10	Acetate 20	16	22
2	Ethanol 20	Fluoride 1.0	65	66
4	Nitrite 10	Fluoride 1.0	77	76
10	<i>p</i> -Cresol 10	Fluoride 1.0	40	44
12	<i>p</i> -Cresol 10	Fluoride 10	51	49

* Percentage of H₂O₂ used peroxidatically rather than undergoing catalytic decomposition.

terminology of Keilin & Hartree, 1955) is unaffected by the addition of small quantities of anions.

Ionic ligands also inhibiting by a donor reaction. Both azide and hydroxylamine react with catalase in the presence of peroxide to give nitric oxide ferrocatalase (Keilin & Hartree, 1954). This is due to a reduction of compound I by the donor (Chance, 1947), thus (cf. Lemberg & Foulkes, 1948):

Hydroxylamine reduces compound II to free ferric catalase. At pH 5, the reaction is first-order with respect to hydroxylamine between 0.03 and 3 mM, the velocity constant being 280 M⁻¹ sec.⁻¹. Azide also reduces compound II to ferric catalase, but the kinetics are more complicated. As Fig. 5 shows, at low azide concentrations its effect re-

sembles that of other anions, but at high concentrations the decomposition velocity does not approach a limiting value. This suggests that azide reacts with compound II in two ways: (i) catalytically: $K_m = 1.1 \times 10^{-5}$ M, $k_{II \max.} = 0.1$ sec.⁻¹ (pH 5, 20°); (ii) stoichiometrically: $k = 1000$ M⁻¹ sec.⁻¹ (with respect to total azide present; pH 5, 20°). Hydroxylamine, with a much lower affinity, reacts only in the second way.

Covalent ligands. Cyanide was found by George (1953b) to react with peroxidase compound II in much the same way as fluoride, although the catalytic effect compared with the dissociation constant of cyanperoxidase was less than that with fluoride. Cyanide may, however, be added to a mixture of catalase and compound II in a concentration sufficient to convert all the catalase into cyanocatalase without noticeably affecting the stability of compound II (Chance, 1950).

This effect was also found in the course of the present investigation. If, on the other hand, cyanide is added in very high concentration, it also induces the decomposition of compound II, as shown in the relevant curve of Fig. 5. The maximal

velocity constant is of the order of 0.1 sec.^{-1} , and K_m at pH 5 is $7 \times 10^{-2} \text{ M}$, which is about 10^4 times the dissociation constant (K_d) for cyancatalase. The second-order velocity constant decreases with increasing pH, being $1.5 \text{ M}^{-1} \text{ sec.}^{-1}$ at pH 5.2 and $0.37 \text{ M}^{-1} \text{ sec.}^{-1}$ at pH 6.9.

Another ligand forming a covalent complex with the free enzyme is sulphide. The peculiar reaction of compound II with this reagent is described in the next paper (Nicholls, 1961).

Comparison with metmyoglobin

The present results with catalase were compared with those previously obtained by George and by Chance with peroxidase, and experiments with metmyoglobin to detect analogous reactions of anions with metmyoglobin peroxide were carried out. Fluoride has no significant effect on metmyoglobin peroxide either at pH 8.0 or at pH 5.6 (when the compound becomes spontaneously unstable). Assuming that the maximal decomposition rate of metmyoglobin peroxide in the presence of fluoride was $8 \times 10^{-3} \text{ sec.}^{-1}$, K_m for fluoride at pH 8 was estimated to be greater than 0.6 M , whereas $K_d = 2.7 \times 10^{-2} \text{ M}$. Cyanide at pH 8.0 produced a decomposition of the metmyoglobin peroxide compound. As with catalase peroxide compounds, the rate was not directly proportional to cyanide concentration; data indicated a maximal velocity of $8 \times 10^{-3} \text{ sec.}^{-1}$ and $K_m 4.4 \times 10^{-3} \text{ M}$ (cf. $K_d = 8 \times 10^{-7} \text{ M}$). The maximal velocity is only one-tenth of that found for the decomposition of catalase compound II.

Both K_m values are markedly greater than the K_d values; the greatest difference between catalase and peroxidase, on the one hand, and metmyoglobin, on the other, lies in the absence of marked effect of fluoride on the reactions of the latter.

DISCUSSION

The observations recorded here depend upon two properties of the catalase peroxide compounds, their reaction with complex-forming anions, and their instability towards the 'endogenous donor'. These two properties are reflected in the data collected in Fig. 5. In fact, as each reaction represents the formation of a complex with ligand by peroxide compound, each curve is sigmoid, with a slope corresponding to the dissociation of 1 mole of anion/mole of compound II; and, except with cyanide, all K_m values approximate to the K_d values for the corresponding anion-enzyme complexes. As each reaction also represents the activity of the endogenous donor, the velocity constants become zero-order at high anion

concentrations, except with azide; and the maximal values vary from about 0.04 to 0.10 sec.^{-1} with different anions.

Table 5 summarizes the constants from Fig. 5, together with the relevant data on the reactions with free enzyme.

Each curve therefore obeys an equation of the form of equation (5); the simplest theoretical system producing such an equation is given by the two postulated reactions (3) and (4). Theories which assume that the anions do not react with compound II itself are ruled out by the evidence that the reaction rate of hypophosphite with compound II is the same as with free catalase.

Comparison of these data for catalase with those obtained with peroxidase (George, 1953*b*) shows important differences, in particular a much greater affinity of the catalase peroxide compounds for the complex-forming ligands. These differences are summarized in Table 6.

Fig. 5. A comparison of the effects of several anions on catalase compound II: variation of the function k_s with anion concentration, plotted semilogarithmically. The variable k_s is defined as $k_{II} \times K_m$, where k_{II} is the apparent second-order velocity constant for compound II decomposition and K_m is the concentration of anion giving half the maximal rate of compound II decomposition. Theoretically, k_s is related to k_{max} (Table 5) by the equation $k_s = k_{max}/(1 + [HA]/K_m)$. Therefore, as the anion concentration approaches zero, k_s approaches k_{max} (the maximal first-order velocity constant for the decomposition of compound II in the presence of the anion). All curves refer to phosphate, pH 5.0, at 20° : $\times$, azide; $\circ$, formate; $+$, fluoride; $\square$, acetate; Δ , cyanide.

Table 5. *Equilibrium and rate constants for the reactions of anions with free catalase and catalase peroxide compound II*

Values obtained for acetate and formate were with a different set of catalase preparations from those used in the other cases.

Anion	Free enzyme reaction		Compound II reaction		
	Complex	K_d (M)	K_m (M)	K_m/K_d	$k_{\max.}$ (sec. ⁻¹)*
Fluoride	Ionic	3×10^{-3}	4.4×10^{-3}	1.5	0.08
Azide	Ionic	1.6×10^{-5}	1.1×10^{-5}	0.7	0.09†
Acetate	Ionic	1.6×10^{-2}	1.6×10^{-2}	1.0	0.05
Formate	Ionic	1.3×10^{-4}	1.3×10^{-4}	1.0	0.05
Cyanide	Covalent	4×10^{-6}	7×10^{-2}	1.7×10^4	0.10

* Values for pH 5.0, 20°.

† Excluding donor reaction.

Table 6. *Reactions of two anions, fluoride and cyanide, with catalase, peroxidase and metmyoglobin*

'Active' means active in catalysis of one-electron transition due to endogenous donor.

Haemoprotein	Reaction with fluoride			Reaction with cyanide		
	Free enzyme	Compound I	Compound II	Free enzyme	Compound I	Compound II
Catalase (pH 5)	Ionic (HF only)	Active $K_m = K_d$	Active $K_m = K_d$	Covalent (HCN only)	?	Active $K_m \gg K_d$
Peroxidase (pH 5.4)	Ionic (HF only)	Inactive	Active $K_m > K_d$	Covalent (HCN only)	Inactive	Active $K_m > K_d$
Metmyoglobin (pH 8.0)	Ionic (F ⁻ and HF react)	—	Inactive	Covalent (CN ⁻ and HCN react)	—	Weakly active $K_m > K_d$

The effects of anions on the catalase peroxide compounds, described above, are of the kind required to account for the anomalies found in the inhibition of catalase by such ligands, mentioned in the introductory paragraphs. Further evidence is thus supplied for the view that all the characteristics of the overall catalatic reaction can be interpreted in terms of reactions of the peroxide compounds. In the present case, such an interpretation is shown to be possible for the progressive and effectively irreversible inhibition occurring in the presence of anions during the decomposition of hydrogen peroxide.

The present results also suggest an answer to the question why the reaction of ethyl hydroperoxide with catalase to form compound II is not independent of the alkyl peroxide concentration (see Chance, 1949b) as it is with peroxidase (Chance, 1949a). Fraser (1956) proposes a reaction of the alkyl peroxide as a hydrogen donor towards compound I:

As the redox potential for the system $\text{RO}_2^\cdot/\text{ROOH}$ probably exceeds that of the system ROOH/ROH it is difficult to accept equation (7). Furthermore, as shown by Keilin & Nicholls (1958), no known donor reduces catalase compound I to II while being inactive towards peroxidase compound I.

The decrease in the second-order velocity constant for the reaction with increasing peroxide concentration (Chance, 1949b) indicates that the effect may be catalytic, following an equation similar to (5) above. In this case, K_m for ethyl hydroperoxide, acting as a ligand and not as a donor, would be about 2×10^{-2} M.

The reaction of formate and catalase is of interest in the analysis of the reaction mechanism. Chance (1952a) measured the rates of reaction for both free catalase and compound I and commented on their similarity. If the progressive inhibitions of catalase by peroxide in the presence of acetate and of formate are also compared (Bonnichsen *et al.* 1947), it can be seen that formate fails to accelerate this inhibition. Evidently the reaction mechanisms of compound I with formate as donor and acetate as inhibitor must be very similar. For if the reaction of anions as inhibitors with compound I was fundamentally different from the corresponding reaction of two-electron donors, then formate, despite its donor action, would, in the presence of an excess of peroxide, be as effective an inhibitor as acetate. Formate thus has a threefold action on catalase: (i) as a ligand for the free enzyme, (ii) as a two-electron donor for compound I, and (iii) as a catalyst for compound II decomposition. And at least part of the mechanism seems to be very similar in all three cases.

Another peculiarity in this set of reactions, the very low affinity of catalase compound II for cyanide (Table 6), can perhaps be correlated with the fact that in the binding of cyanide by free enzyme the bond type changes from ionic to covalent, and the absence of observable spectral change in compound II indicates that the bonding remains covalent with and without cyanide.

The variation in maximal velocity constant (Fig. 5 and Table 5) may well be due to differences between enzyme samples; it is possible that this constant takes on different values for different ligands, but the observed variation is not very large. The absence of catalytic effect of anions on one-electron reactions with added donors indicates that the endogenous donor reaction is fundamentally different from that with exogenous donors. The reducing equivalents involved are associated with the same enzyme molecule as the peroxide compound decomposing (Table 1), and their 'activity' is altered not by oxidation but by processes altering the protein configuration.

One interpretation of this is that the electrons required are introduced from the protein via the haem-linked group, the protein acting as an electron conductor (Evans & Gergely, 1949). The weak reducing power of such electrons may be offset by the powerful oxidizing capacity of the peroxide compounds.

Fig. 6 illustrates the reactions postulated in explanation of the present results, and summarizes some of the kinetic data. The formal simplicity of this Figure conceals many difficulties of chemical interpretation. Not only is it necessary to suppose that the haem iron in the higher-valency states of compounds I and II is able to form complexes, but also that the dissociation constants for these complexes are closely similar to those formed by the iron in the ferric state (Table 5). If similar structures be postulated for the corresponding peroxidase compounds, Fig. 6 does not indicate any reason for the much lower affinity of those compounds for the ligands (Table 6). We have only the empirical correlation between the ability of the two compounds of type I to form complexes

with anionic ligands, and their activity towards two-electron donors such as ethanol.

The evidence of the peroxide anion complexes is thus not compatible with the structures for the peroxide compounds themselves given previously either by Chance & Fergusson (1954) or by George (1953*a*), new chemical possibilities must be canvassed.

SUMMARY

1. The influence of pH and anions on the formation and decomposition of catalase peroxide compound II was examined.

2. The rates of formation of compound II produced by ethyl hydroperoxide or the hydrogen peroxide generated in the notatin system, and of the decomposition induced by ethanol, show a similar proportionality to pH ($\delta \log_{10} k = -0.6 \delta \text{pH}$).

3. The rates of reduction of compounds I and II by the endogenous donor concerned are first-order with respect to catalase concentration; they are not significantly affected by treating the enzyme with oxidizing agents, but are modified by processes which tend to denature the enzyme protein. It is concluded that the endogenous reductions are intramolecular reactions.

4. Anions accelerate both the reduction of compound I to II, and the reduction of compound II to free catalase, in the absence of added donors. The reactions show a Michaelis-Menten relationship with respect to anion concentration, from which a Michaelis constant (K_m) may be calculated.

5. For ionic ligands, K_m values associated with the decomposition of compound II closely approximate the dissociation constants for the corresponding enzyme-anion complexes. For the covalent ligand cyanide, K_m is much greater than the corresponding dissociation constant.

6. Azide reacts with compound II both catalytically and stoichiometrically as a hydrogen donor.

7. At low hypophosphite concentrations, the rates of formation of the catalase-hypophosphite

Fig. 6. Reactions of anionic ligands with catalase and its peroxide compounds. $k_7 = 1.2 \times 10^{-2} \text{ sec.}^{-1}$; $k_4 = 9 \times 10^{-4} \text{ sec.}^{-1}$; $k'_7 = 0.15 \text{ sec.}^{-1}$ (corresponding to the experimentally obtained constant $k_{\text{I max}}$); $k'_4 = 0.08 \text{ sec.}^{-1}$ (corresponding to the experimentally obtained constant $k_{\text{II max}}$); HA represents an acid ligand forming an ionic complex with the free enzyme. All numerical values refer to pH 5, 20°.

complex from compound II and from free catalase are identical.

8. The anions have no observable effect on the reduction produced by added donors.

9. The actions of anions on catalase, peroxidase and metmyoglobin peroxide compounds are compared, and the relevance of the phenomena to the questions of the mode of action of catalase, and the nature of the endogenous donor, is discussed.

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The Formation and Properties of Sulphmyoglobin and Sulphcatalase

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The preceding paper (Nicholls, 1961) dealt with the reactions of catalase peroxide compounds with various complex-forming ligands compared with the analogous reactions of peroxidase and metmyoglobin. Of covalent ligands, only cyanide was mentioned in that paper; the present paper describes the anomalous behaviour of another substance, hydrogen sulphide, which normally forms covalent complexes with haemoproteins.

The reactions of haemoglobin with hydrogen sulphide are complicated. At least two distinct derivatives occur: sulphaemoglobin and sulphide-methaemoglobin. The former is a green-purple (dichroic) compound produced when solutions of oxyhaemoglobin are allowed to stand in the presence

of hydrogen sulphide. Sulphide-methaemoglobin is a red compound produced by the reversible reaction of methaemoglobin and hydrogen sulphide. This latter reaction is of the usual kind involving replacement of a water molecule at the sixth co-ordination site of the iron (Keilin, 1938), and further reactions can occur only if the sulphide is displaced. Sulphaemoglobin can, however, undergo reactions in its own right; thus it forms a complex with carbon monoxide, and its absorption band can be destroyed by ferricyanide (Nijveld, 1943). These reactions suggest that sulphaemoglobin is a ferrous derivative; the compound produced by ferricyanide would then be metsulphaemoglobin. No distinct spectral bands have been reported for