The Reactions of Azide with Catalase and their Significance

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The usual ('catalatic') activity of catalase, the decomposition of hydrogen peroxide to water and oxygen, can be represented as in eqn. (1) (cf. Chance & Fergusson, 1954), where $\text{catalase} \cdot \text{Fe}^{3+} \cdot \text{H}_2\text{O}_2$ represents compound I, the initial compound formed in the reaction with peroxide. Compound I contains both of the oxidizing equivalents of the original peroxide molecule, whereas compound II has only one (Keilin & Nicholls, 1958).

The precise chemical structure of compound I is unknown (see George, 1953; Brill & Williams, 1961; and the Discussion section in the present paper), and the formal representation as a complex with peroxide (eqn. 1) almost certainly requires some modification. However, it is most unlikely that the iron in compound I is in the ferrous state. On the other hand, in the presence of certain inhibitors (azide and hydroxylamine) and its substrate (hydrogen peroxide) catalase is reduced to the ferrous form. This was the basis for an earlier theory of catalase activity (Keilin & Hartree, 1938) before the correctness of eqn. (1) was recognized. Subsequently it was discovered that the reaction with azide involves a peroxidatic attack of compound I on azide (Chance, 1947) giving rise to a multiplicity of oxidation products including nitrous oxide and nitrogen (Theorell & Ehrenberg, 1952*b*) together with small amounts of nitric oxide (Keilin & Hartree, 1954).

Theorell & Ehrenberg (1952*b*) discussed the nature of the azide reactions, but their suggestions conflicted with the data of Foulkes & Lemberg (1949) and Keilin & Hartree (1954). In particular, although Theorell & Ehrenberg (1952*b*) claimed that the major product formed during the oxidation of azide is nitrous oxide, the latter groups of workers agree in postulating the production of sufficient quantities of nitric oxide to form the observed ferrous derivative of the enzyme which, according to Keilin & Hartree (1954), is nitric

oxide-ferrocatalase, $\text{catalase} \cdot \text{Fe}^{2+} \cdot \text{NO}$. It is therefore not clear which is the main reaction, or what side reactions may occur. The kinetics of the reactions as observed by the different workers are also different. Thus Theorell & Ehrenberg (1952*b*) claim that the rate of azide oxidation is independent of pH and azide concentration. On the other hand, the progressive inhibition of catalase by azide is dependent both on azide concentration and pH (Ogura, Tonomura, Hino & Tamiya, 1950*a*); and Chance's (1947) preliminary data suggested a bimolecular reaction between azide and compound I, similar to that found for the reaction with compound II (Nicholls, 1961*a*).

The reaction of azide with peroxidase in the presence of peroxide produces a ferric derivative of unknown composition (Keilin & Mann, 1937). As with the other classes of hydrogen donor considered by Keilin & Nicholls (1958), azide ('fifth group') reacts differently with the two primary peroxide compounds. But, unlike the other reactions with donors, those with azide give rise to identifiable intermediates in the reaction cycle between compound I and free enzyme. The azide reaction may, therefore, be of value in determining the nature of the two primary compounds. In particular the formation of ferrocatalase suggests that (a) the reaction of hydrogen donors is with the iron atom and not the porphyrin ring [cf. discussion of this point by George (1953) and Chance (1955)] and that (b) the overall three-electron reduction may require the preliminary formation of an enzyme-donor complex. These points are considered below with reference to recent views on the chemical structure of compound I.

The present experiments were carried out to determine the nature of the intermediates in the reactions of catalase with azide and peroxide and the stoichiometries of these reactions, in an attempt to reconcile some conflicting views in the literature. Two preliminary reports have been published (Nicholls, 1962*a, b*).

MATERIALS AND METHODS

Horse-liver catalase was obtained by a modification of the method of Keilin & Hartree (1945). At the end of the first stage of their technique (at which they claim 'the enzyme is pure enough for most purposes'), the preparation

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contained a quantity of enzymically-inert colourless protein which is precipitated at pH values below 5.5 and thus produces scattering changes in spectrophotometric experiments. The addition of acetate buffer, pH 4.5 (final concn. 0.1M), followed by standing at 4° overnight (Bonnichsen, 1955), removed this material, and the catalase was recovered on centrifuging and dialysis. A 45% saturation with $(\text{NH}_4)_2\text{SO}_4$ was found more satisfactory in the removal of ferritin than the 40% saturation recommended by Keilin & Hartree (1945).

Horse-blood catalase was a gift from Dr G. H. F. Fraser to Professor D. Keilin. Horse-radish peroxidase had been prepared by Dr E. F. Hartree. Notatin (glucose oxidase) was from the sample described by Keilin & Hartree (1955).

Sodium azide was obtained from British Drug Houses Ltd. The phenomena observed were constant from sample to sample. The azide-catalase complex formed at pH values between 5 and 6 gave a spectrum very similar to that published by Keilin & Hartree (1951). The affinity for catalase was markedly pH-dependent, as found by Chance (1952), and the equilibrium constant was the same as that given by Nicholls (1961*a*). [The much higher affinity obtained by Ogura, Tonomura, Hino & Tamiya (1950*b*) and their claim to have found a combined cyanide-azide-catalase complex could not be confirmed.]

Both AnalR hydroxylamine and a sample recrystallized from a cruder preparation were used; differences obtained with other samples are referred to below. Neutralized hydrazine (commercial sample) and AnalR hydrazine hydrate were used. Hydrogen peroxide was diluted from '100 volume' AnalR solution. Ethyl hydroperoxide was from the sample used by Nicholls (1961*a*). Other reagents were of AnalR or similar quality.

The formation of compound II was followed as described by Nicholls (1961*a*). The formation of ferrocatalase derivatives was followed in a similar way at 575 and 585 m μ by using a Hilger Uvispek spectrophotometer fitted with a glass prism. Those experiments carried out under nitrogen or carbon monoxide were done in Thunberg tubes with attached optical cells. All the experiments were carried out at or near room temperature (20°) with no special temperature control.

RESULTS

Effect of azide in the formation and decomposition of catalase peroxide compound II. Azide falls in the 'fifth group' of catalase donors (Keilin & Nicholls, 1958) because it reacts both with compound I and compound II, reducing the former to ferrocatalase derivatives and the latter to free (ferric) catalase (Nicholls, 1961*a*). The kinetics of these two reactions are not straightforward. In the presence of a low concentration of peroxide, maintained by the aerobic oxidation of glucose by glucose oxidase as described by Keilin & Nicholls (1958), catalase exists in the form of a steady-state mixture of free enzyme and compound I, from which compound II is gradually produced (Chance, 1950). The complexity of the interaction of azide with this system arises in two ways: (a) as a result of the reduction of compound II by endogenous donor and by azide directly (see Nicholls, 1961*a*) and (b) in conse-

quence of the suppression of compound II formation by azide.

At low concentrations (0.1 mM or less) the effect of azide on the notatin-catalase system at pH 5 resembles that of a donor such as ethanol or (more closely) formate ('third group' of Keilin & Nicholls, 1958). That is, when added before compound II has been formed, it inhibits the transition, the spectrum remaining that of ferric catalase ($\Delta E =$ zero at 565 or 585 m μ). On the other hand, at higher azide concentrations transition to a ferrocatalase complex occurs (Keilin & Hartree, 1936, 1945). Under none of the conditions used did azide accelerate the formation of compound II; and under no conditions in which excess of peroxide was not present were any but ferric derivatives (free catalase and its azide complex) produced during the reduction of compound II by azide (cf. Nicholls, 1961*a*). Hydroxylamine (see below) is the only other substance known to behave in this way. The low steady-state concentration of compound II is a feature also found in the hydrogen peroxide-dependent inhibition by 3-amino-1,2,4-triazole, but the explanation seems to be different (Nicholls, 1962*c*).

Spectra of derivatives formed at high azide concentrations compared with those of the hydroxylamine derivatives and ferrocatalase complexes formed directly. Fig. 1 shows the spectra of the derivatives formed in the presence and absence of carbon monoxide by liver catalase plus peroxide in concentrated solutions of sodium azide. Closely similar spectra are produced if: (a) ethyl hydroperoxide is substituted for hydrogen peroxide, (b) blood catalase is substituted for liver catalase (here slight differences, such as increased values of ΔE between maxima and minima of absorption, may be attributed to the absence of bile pigment in blood catalase), (c) hydroxylamine is substituted for sodium azide, or (d) any possible combination of these substitutions is carried out. Thus Fig. 2 shows the spectra of the corresponding derivatives produced by blood catalase, hydroxylamine and hydrogen peroxide. Some differences from the results of Keilin & Hartree (1936, 1945) and of Maehly (1961) may be noted. The sharp α -bands observed by Keilin & Hartree (1945) at 587 and 578 m μ for the derivatives produced in the absence and presence of carbon monoxide respectively, are here replaced by 'shoulders' (the sharp peaks were observed with the Hilger-Nutting visual spectrophotometer). This is in agreement with the spectrum of the derivative of bacterial catalase formed in the presence of azide and methyl hydroperoxide (Maehly, 1961), but the spectrum obtained by Maehly for the carbon monoxide compound seems to be appreciably different. Because of the otherwise close similarity

Fig. 1. Absorption spectra in part of the visible region of the spectrum of the two horse-liver catalase derivatives obtained by adding hydrogen peroxide to the azide-catalase complex (A), nitric oxide-ferrocatalase (B) and carbon monoxide-ferrocatalase (C). Part of the spectrum of nitric oxide-catalase (nitrosyl-ferrocatalase) (D) is also shown for purposes of comparison. The solutions of catalase were $30\mu\text{M}$ (with respect to haematin) in 1.0M -azide solution in 0.05M -phosphate buffer, pH 6.4, at room temperature. The addition of $600\mu\text{M}$ -hydrogen peroxide was made under either nitrogen (B) or carbon monoxide (C). The catalase used for the nitric oxide-catalase spectrum (D) was $45\mu\text{M}$ (with respect to haematin) in 0.15M -phosphate (giving a final pH of 5.0) to 3 ml. of which was added 0.05 ml. of an acid solution of 0.1M -sodium nitrite plus 0.1M -potassium ferrocyanide.

Fig. 2. Absorption spectra in part of the visible region of the spectrum of the derivatives of horse-blood catalase obtained by adding hydrogen peroxide to the azide complex with nitric oxide-ferrocatalase (A) and carbon monoxide-ferrocatalase (B), and the derivative obtained from catalase in hydroxylamine (C). The solutions of catalase used were $18\mu\text{M}$ (with respect to haematin) in 1.0M -azide solution in 0.05M -phosphate buffer, pH 6.4, at room temperature. Hydrogen peroxide ($600\mu\text{M}$) was added as in Fig. 1. The compound produced with hydroxylamine (3 mm) was obtained at pH 4.8 (phosphate) by using $20\mu\text{g.}$ of notatin (glucose oxidase) in 2.5 ml. of 5.5 mm -glucose solution (Keilin & Nicholls, 1958) to generate hydrogen peroxide continuously.

between blood and bacterial catalases this is rather puzzling. There seems to be no disagreement with the view that this derivative is simply the carbon monoxide derivative of the ferrous form of the enzyme (see discussion by Ehrenberg, 1962).

Keilin & Hartree (1936) stated that two derivatives may be observed, even in the absence of carbon monoxide, one with bands at 554 and $590\text{ m}\mu$ (with small amounts of hydrogen peroxide or with organic peroxides), and the other with bands at 547 and $588\text{ m}\mu$ (with excess of hydrogen peroxide). Only the former was subsequently investigated (Keilin & Hartree, 1945), although the positions of the absorption bands were variously reported to be between 548 and 559 , and 582 to $590\text{ m}\mu$ (Maehly, 1961). In the present investigation no difference could be detected between the initial spectra produced by hydrogen peroxide and by ethyl hydroperoxide. At high concentrations of hydrogen peroxide (5 mm or above), however, a slow decrease in the intensity of the absorption bands was seen, which could be duplicated in the catalase-azide-ethyl hydroperoxide spectrum by the subsequent addition of similar amounts of hydrogen peroxide. This apparently destructive action may be due to an oxidation of nitric oxide to nitrogen dioxide, or to a direct attack by hydrogen peroxide on the ferrohaem group (as in the formation of choleglobin from haemoglobin). No evidence for more than one ferrocatalase derivative was obtained.

Although normal catalase cannot be directly reduced to the ferrous form by dithionite, certain modified forms can be so reduced. One of these (a modified horse-blood catalase; cf. Nicholls, 1962*d*) was reduced with dithionite and then treated with carbon monoxide or with nitrite in acid solution (containing a certain amount of nitric oxide). Table 1 compares the positions of the absorption bands of these derivatives with their counterparts obtained with peroxides and azide. With carbon monoxide-ferrocatalase, catalase $\cdot\text{Fe}^{2+}\text{CO}$ (the most stable derivative by either method), the qualitative pattern of the two spectra is the same, but the bands are shifted some $5\text{ m}\mu$ further towards the blue for the compound obtained from reducible catalase.

Formation and decomposition of the azide derivatives. Owing to the rapidity of the reactions it was not possible to measure directly the rates of formation from compound I of the derivatives regarded as nitric oxide-ferrocatalase and carbon monoxide-ferrocatalase. Fig. 3 illustrates the formation of the two derivatives in carbon monoxide, in nitrogen and in air, under conditions such that the rate-limiting step is the formation of compound I of catalase and peroxide. Fig. 3 shows that: (a) the rate of formation of both carbon monoxide-

Table 1. *Absorption maxima for ferrocatalase complexes*

Details of the ferrocatalase complexes and methods of preparation are given in the text.

Derivative	Normal catalase		Reducible catalase	
	Method	Absorption bands (m μ)	Method	Absorption bands (m μ)
Catalase $\cdot$ Fe ³⁺ +H ₂ O	—	622, (500)	—	626, (500)
Catalase $\cdot$ Fe ²⁺ +NO	H ₂ O ₂ + HN ₃	585, 555	Na ₂ S ₂ O ₄ + NO	580, 545
Catalase $\cdot$ Fe ²⁺ +CO	H ₂ O ₂ + CO	575, 540	Na ₂ S ₂ O ₄ + CO	570, 535

Fig. 3. (a) Formation of carbon monoxide-ferrocatalase, with: A, 150 μ M-hydrogen peroxide in carbon monoxide; B, 15 μ M-hydrogen peroxide in carbon monoxide. (b) Formation of nitric oxide-ferrocatalase, with: C, 600 μ M-hydrogen peroxide in air; D, 150 μ M-hydrogen peroxide in nitrogen; E, 30 μ M-hydrogen peroxide in nitrogen. The conditions were as in Fig. 1 except for curve C, where an open unstirred cuvette was used. The other curves were obtained from measurements at 575 m μ and 585 m μ for carbon monoxide-ferrocatalase and nitric oxide-ferrocatalase respectively, by using the Thunberg tubes with attached optical cell.

ferrocatalase and nitric oxide-ferrocatalase is dependent on peroxide concentration (see eqn. 4 below); (b) at similar peroxide concentrations the rate of formation of carbon monoxide-ferrocatalase is about ten times that of nitric oxide-ferrocatalase; (c) the formation of carbon monoxide-ferrocatalase and of nitric oxide-ferrocatalase in nitrogen follow simple reaction curves, whereas the formation of nitric oxide-ferrocatalase in a cuvette open to the air is biphasic, with an initial slow rate and a final rapid rate. The biphasic pattern can be explained on the basis of the double reaction (giving both free ferrocatalase and nitric oxide-ferrocatalase) which is postulated below to explain the results with stoichiometric amounts of peroxide.

Once formed, both carbon monoxide-ferrocatalase and nitric oxide-ferrocatalase are stable in the absence of light and oxygen. In air, carbon monoxide-ferrocatalase remains comparatively stable provided that a reasonable partial pressure

Fig. 4. Photodissociation of carbon monoxide-ferrocatalase. The carbon monoxide compound of horse-liver catalase was formed under the same conditions as in Fig. 1. The Thunberg tube was then evacuated and filled with nitrogen (6 min. spectrum: curve A). The spectrum remained stable for at least 15 min. (15 min. spectrum: curve B). Light (shaking for 2 min. in front of a 100 w bulb) produced the progressive changes represented by curves C and D. Curve E represents the spectrum of the azide-catalase complex.

of carbon monoxide is maintained, but nitric oxide-ferrocatalase autoxidizes to give ferric catalase at a velocity of about 0.025 sec.⁻¹ at pH 6.4 and 20° [equivalent to *k* (bimolecular) of approx. 100 M⁻¹ sec.⁻¹]. This may be compared with the approximately tenfold lower rate of oxidation of ferrosulphocatalase (Nicholls, 1961b). If carbon monoxide-ferrocatalase is illuminated in the presence of an excess of peroxide and azide, it appears to give rise to the original compound (nitric oxide-ferrocatalase) (Maehly, 1961), but when illuminated in the absence of an excess of peroxide it gives only the spectrum of azide-catalase (Fig. 4). As 'nitric oxide-ferrocatalase' is stable under nitrogen (Fig. 3), it may be assumed that, contrary to the opinion expressed by Maehly (1961) and Ehrenberg (1962), the immediate product of the photolysis of carbon monoxide-ferrocatalase is not 'nitric oxide-ferrocatalase' or any similar derivative, but ferrocatalase itself. The latter is more vulnerable to oxidation, even

under nitrogen or carbon monoxide, and rapidly gives rise to the ferric derivative. That free ferrocatalase is much less stable with respect to oxygen or other oxidizing agents than is nitric oxide-ferrocatalase is in agreement with the views originally expressed by Keilin & Hartree (1945, 1954). Nitric oxide-ferrocatalase is in fact surprisingly stable, particularly against oxidation by hydrogen peroxide itself, the porphyrin ring being attacked as rapidly as the central iron atom. On the other hand, free ferrocatalase produced in the azide reaction can only be trapped as carbon monoxide-ferrocatalase (see below). Illumination of the latter gives only ferric derivatives, even in the presence of small quantities of sodium dithionite (which might have been expected to remove available oxidizing equivalents in residual oxygen, or elsewhere). As explained by Nicholls (1962*a*), Maehly's (1961) results were due to the presence of an excess of peroxide in his reaction mixture.

Titration of azide-catalase with peroxide. Keilin & Hartree (1945) showed that, in the presence of carbon monoxide, catalase could be inhibited by an amount of azide which is approximately stoichiometric with catalase haematin. Similarly, if azide-catalase is titrated with different amounts of peroxide, as in Fig. 3, it is found that in the presence of carbon monoxide only one molar equivalent of peroxide is required for the formation of each equivalent of ferrocatalase derivative. In the absence of carbon monoxide considerably more peroxide is required, although the presence or absence of oxygen does not markedly affect the amount of ferrocatalase (presumably the nitric oxide compound) initially formed. Oxygen does, of course, accelerate the subsequent decomposition, as described above. Fig. 5 shows the relationship between the percentage of ferrocatalase derivative and the [hydrogen peroxide]/[catalase] molar ratio for several cases.

If Theorell & Ehrenberg's (1952*b*) reaction (written in an adapted form in eqn. 2) is considered to be the major pathway of azide oxidation (80–90 %), while Foulkes & Lemberg's (1949) reaction (eqn. 3) contributes 10–20 % of the products (and all the nitric oxide), the relative rates of reduction (Fig. 3) and stoichiometries (Fig. 5) can both be accounted for. In eqn. 2, a reaction of azide with

compound I has been substituted for the originally proposed reaction of peroxide with azide-catalase (Theorell & Ehrenberg, 1952*b*). Such a reaction is indicated by the results of Chance (1947) and is

supported by the kinetic data of Fig. 3. Under the conditions employed (1.0 M-azide at pH 6 and at 20°) the catalase is 99.96 % in the form of the azide-catalase complex (Chance, 1952). The rate of formation of carbon monoxide-ferrocatalase from the catalase is 0.09 sec.⁻¹; i.e., the rate of attack of 30 μM-catalase (expressed in terms of haematin) on 15 μM-peroxide is given by eqn. (4):

$$k_1[3 \times 10^{-5}][4 \times 10^{-4}] = 0.09 \quad (4)$$

where k_1 is the rate of reaction with free catalase and 4×10^{-4} represents the proportion of free catalase present. Eqn. (4) gives a value of k_1 equal to

$$7.5 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}.$$

This is similar to the values obtained directly by Chance (1947, 1950) and is in accordance with conclusion (a) above, that the reaction with peroxide is rate-limiting.

The rate of combination of carbon monoxide with the ferrocatalase produced in reaction (2) is not rate-limiting. The velocity constant for this process must therefore be at least five times as great as that of eqn. (4), i.e. k_{CO} is equal to or greater than $0.45/[\text{carbon monoxide}]$ or $6 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$. The rate of replacement of nitric oxide by carbon monoxide in nitric oxide-ferrocatalase reported by Maehly (1961) as approx. $72 \text{ M}^{-1} \text{ sec}^{-1}$ is thus some tenfold lower. This is further evidence that the ferrocatalase derivative produced according to eqn. (2) differs from that which arises by eqn. (3).

Fig. 5. Titration of catalase with hydrogen peroxide. The conditions were the same as those in Figs. 1 and 3. Measurement of the percentage of nitric oxide-ferrocatalase was carried out at 585 mμ, that of the percentage of carbon monoxide-ferrocatalase at 575 mμ. □, Horse-liver catalase titrated under carbon monoxide; ○, horse-liver catalase titrated under nitrogen; △, horse-liver catalase titrated in air (open cuvette); ●, horse-blood catalase titrated under nitrogen; ▲, horse-blood catalase titrated in air (open cuvette). The three lines are drawn for the theoretical ratios 1:1, 5:1 and 10:1.

According to conclusion (b) above, the rate of formation of nitric oxide-ferrocatalase, though still peroxide-dependent, is lower by a factor of ten. This can only be explained in terms of the cyclic formation of ferrocatalase (eqn. 2) and its reoxidation, one molecule of nitric oxide being produced for each ten turns of the cycle. Thus the reoxidation of ferrocatalase must also be much faster than the reoxidation of nitric oxide-ferrocatalase, where the rate is as low as 0.025 sec^{-1} in air. Further, the kinetics of the formation of nitric oxide-ferrocatalase in air [conclusion (c) above] are consistent with the view that oxygen is being used up during the slow phase of nitric oxide production. For example, with $600 \mu\text{M}$ -hydrogen peroxide this phase lasts 17 sec., which allows $30 \mu\text{M}$ -catalase (expressed as haematin) to turn over 30 times ($30 \times 30 = 900 \mu\text{M}$ redox equivalents lost in ferrous-ferrocatalase, compared with $270 \mu\text{M}$ -oxygen in solution). With $300 \mu\text{M}$ -hydrogen peroxide, the phase lasts about 25 sec. ($23 \times 30 = 690 \mu\text{M}$ redox equivalents lost in ferrous-ferrocatalase transitions). What is the rate of reaction of azide with compound I? The data of Theorell & Ehrenberg (1952b) on the rate of gas evolution were obtained under conditions similar to the present ones, where the reduction of compound I is not rate-limiting. But from the results of Chance (1947) on the disappearance of compound I, it can be deduced that the value of $k_4(\text{azide})$ lies between 10^4 and $10^5 \text{ M}^{-1} \text{ sec}^{-1}$ (at pH 6-7 and at $20-25^\circ$). Such a rate would also be in agreement with the indirect data on the efficacy of azide as a substrate for coupled oxidation (Keilin & Hartree, 1955), the inhibition by azide of the catalytic reaction (Keilin & Hartree, 1945) and the rate of development of the latter inhibition (Foulkes & Lemberg, 1949).

From the present results the values of k_4^a and k_4^b (eqns. 2 and 3) may be given lower limits. The stoichiometry of Fig. 5 precludes the decomposition of more than 20% of compound I by the catalytic reaction. Hence:

$$k_4^a[\text{HN}_3] \geq 10 \times 2 \times 10^7 \times 3 \times 10^{-5} \quad (6)$$

which gives k_4^a equal to or greater than 6×10^3 , and k_4^b equal to or greater than $6 \times 10^2 \text{ M}^{-1} \text{ sec}^{-1}$, at pH 6.4 and at 20° . Calculated with respect to undissociated azide, this gives $k_4^a(\text{HN}_3)$ equal to or greater than $3 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$, and $k_4^b(\text{HN}_3)$ equal to or greater than $3 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$.

In the experiments of Theorell & Ehrenberg (1952b) the reaction rate was independent of the concentration of azide and peroxide. In this case the limiting reaction must have been the reoxidation of ferrocatalase to ferric catalase (eqn. 7),

permitting the cyclic oxidation of azide by peroxide. At pH 5.55, k_5 is equal to or greater than 0.25 sec^{-1} (in air). The stoichiometries of gas evolution under anaerobic and aerobic conditions (Theorell & Ehrenberg, 1952b) can also be accounted for in terms of a major contribution from the reaction indicated in eqn. (2) and a minor contribution from that of eqn. (3). Thus, at pH 5.5 in the presence of 0.05 M -azide, 0.8-0.9 mole of nitrous oxide and 0.2-0.3 mole of nitrogen were produced/mole of hydrogen peroxide decomposed, giving approximately 1 mole of gas/mole of hydrogen peroxide at high catalase concentrations both in nitrogen and in air (Expt. 1 of Theorell & Ehrenberg, 1952b). At low catalase concentrations under nitrogen, the gas evolved was proportional to catalase concentration, the [catalase haematin]/[gas] molar ratio being about 0.05. Under carbon monoxide the latter ratio was increased to about 0.3, and this indicates that 10-20% of reaction was producing a ferrous inhibitor as by eqn. (3). The excess of gas produced at high azide concentrations or at low pH can be attributed to the reaction of the N^0 radical or its equivalent (eqn. 2) with another molecule of azide. There is in fact some evidence for the destruction of a greater amount of azide than is stoichiometric with the peroxide generated (Keilin & Hartree, 1955). This explanation differs from that offered by Theorell & Ehrenberg (1952b) in that it does not require the postulation of either (a) the recombination of nitrogen atoms to give nitrogen gas, a chemically unlikely event, or (b) reactions involving hydrogen peroxide and azide-ferrocatalase, contrary to the observed kinetics and chemistry. Both nitrous oxide and nitrogen are produced by reactions of azide with compound I.

Reactions of catalase with hydroxylamine and hydrazine. Hydroxylamine, which can react in a fashion analogous to that of azide (eqn. 8), does not give rise to gases when oxidized (no reaction

analogous to eqn. 2). Unfortunately it has not been possible to carry out titrations, such as those of Fig. 5, with hydroxylamine, because high concentrations (1-2M) of hydroxylamine do not compete as well as azide for compound I. Most of the peroxide is therefore destroyed catalytically. By using peroxide generated by the glucose oxidase-glucose system, it was found that nitric oxide-ferrocatalase (cf. Fig. 2) could be produced at pH 4.5 (phosphate) at a rate about ten times lower than the rate of compound II production (see Nicholls, 1961a). The rate is independent of hydroxylamine concentration between 0.1 and 1 mM. Under these conditions hydroxylamine is

present in the acid form (hydroxylammonium ion, NH_2OH^+). Nevertheless, at such concentrations of hydroxylamine, the production of compound II is inhibited, indicating another reaction of hydroxylamine besides that of eqn. (8). Hydroxylamine therefore gives rise to a situation not necessarily simpler than that with azide.

Hydroxylamine, like azide, accelerates the decomposition of compound II, whether it be formed either in the glucose oxidase-glucose system or by ethyl hydroperoxide. The velocity constant of $280\text{--}300\text{M}^{-1}\text{sec}^{-1}$ (at pH 5 and at 20°) given previously (Keilin & Nicholls, 1958; Nicholls, 1961a) is in error. By using several different samples of recrystallized hydroxylamine (neutralized hydrochloride salt) at pH values from 4.8 to 6.8, overall velocity constants of $10\text{--}20\text{M}^{-1}\text{sec}^{-1}$ have now been found. Under some conditions an initial incomplete reaction with an apparent velocity of $200\text{--}900\text{M}^{-1}\text{sec}^{-1}$ may be observed. This suggests that the originally reported velocities were due to an impurity in the hydroxylamine.

Hydrazine has been considered to react with catalase in a way similar to that of eqn. (3) or eqn. (8), generating its own peroxide by autoxidation (Keilin & Hartree, 1936). With liver catalase at pH 5, however, hydrazine appears to give only a reversible ionic complex (α -band at $619\text{m}\mu$). The addition of ethyl hydroperoxide was required to give the spectrum with an α -band at $580\text{--}590\text{m}\mu$. Compound II formation was prevented and compound II decomposition accelerated by hydrazine. The destruction of catalase protein by hydrazine, and the possible formation of hydrazoic acid during hydrazine autoxidation, however, diminish the usefulness of this reagent in a study of ferrocatalase derivatives.

Reactions of azide with peroxidase and metmyoglobin. Only catalase compound I gives a ferrous compound with azide. When metmyoglobin peroxide is treated with a dilute solution of sodium azide free metmyoglobin is formed. Although Keilin & Hartree (1954) have stated that oxyhaemoglobin and nitric oxide-haemoglobin are found when methaemoglobin is treated with peroxide and azide, such ferrous derivatives only occur at high concentrations of peroxide and azide, and may result from a secondary attack by free radicals and other products of the initial oxidation of azide (a 'rapid destruction of the haem nucleus' was also observed).

Horse-radish peroxidase was shown by Keilin & Mann (1937) to give a ferric derivative on reaction with peroxide and azide. I have observed that this derivative (referred to below as 'peroxidase $\cdot\text{Fe}^{3+}\text{A}$ ') is a product of the reaction of azide with compound I of peroxidase. Peroxidase compound II, like catalase compound II, is reduced by azide

to the free ferric enzyme. At pH 4.5 (acetate buffer) the dissociation constant for the azide-peroxidase complex is large (approx. 0.6M), and therefore in the presence of 0.1M -azide $40\text{ }\mu\text{M}$ -peroxidase is mainly in the uncombined form. On the addition of 4mM -hydrogen peroxide a rapid and complete transformation into peroxidase $\cdot\text{Fe}^{3+}\text{A}$ occurs (A and B indicate the ligands of unknown nature occupying the sixth co-ordination site). Peroxidase $\cdot\text{Fe}^{3+}\text{A}$ has a spectrum closely similar to that of the cyanide or azide complex (Keilin & Hartree, 1951), with an α -band (shoulder) at $585\text{--}590$ (E_{mM} 8.5) and a β -band at $550\text{m}\mu$ (E_{mM} 11.0). The moiety A is firmly linked to the iron, the addition of cyanide or fluoride having no immediate effect on the spectrum. On standing, peroxidase $\cdot\text{Fe}^{3+}\text{A}$ gave rise to another compound 'peroxidase $\cdot\text{Fe}^{3+}\text{B}$ ' [$k(20^\circ)$ approx. $5 \times 10^{-3}\text{sec}^{-1}$]. This reaction is greatly accelerated by *p*-cresol and ferrocyanide, although nitrite has no effect; this suggests that B may be a reduction product of A. Peroxidase $\cdot\text{Fe}^{3+}\text{B}$ has a broad absorption spectrum with a peak at $545\text{m}\mu$ (E_{mM} approx. 10) and, unlike peroxidase $\cdot\text{Fe}^{3+}\text{A}$, rather strong end absorption in the red (E_{mM} approx. 7 at $610\text{m}\mu$). When cyanide and fluoride are added to peroxidase $\cdot\text{Fe}^{3+}\text{B}$, the corresponding ferric peroxidase derivatives are formed. It therefore seems that in place of eqns. (2) and (3), peroxidase compound I engages in a (two-electron?) reaction with azide to produce the ferric enzyme bonded to an unstable oxidation product of azide. No similar reaction can be seen with hydroxylamine.

Reversibility of catalase inhibition by azide. Although Blaschko (1935) had previously shown a partial reversibility of the inhibition of catalase by azide, Foulkes & Lemberg (1949) were unable to reverse by oxygenation the inhibition observed under their conditions. That this may have been at least in part due to the above described irreversible attack of hydrogen peroxide on nitric oxide-ferrocatalase is indicated by the following results.

The overall peroxidatic reaction with ethyl hydroperoxide when ethanol is used as hydrogen donor can be observed by following the decrease in extinction at $240\text{m}\mu$ (Chance, 1949). The corresponding reaction with pyrogallol as hydrogen donor can be followed by the initial rate of appearance of purpurogallin shown by the extinction at $520\text{m}\mu$ (Nicholls, 1959). The presence of azide ($14\text{--}140\text{ }\mu\text{M}$) produced an immediate and complete inhibition in all cases, the duration of which increased with increase in the azide concentration, followed by a peroxidatic reaction at the normal uninhibited rate. The period of complete inhibition was equivalent to the time calculated for the catalase to oxidize the azide via ferrocatalase and nitric oxide-ferrocatalase (a turnover rate per

catalase haematin of between 0.03 and 0.12 sec.⁻¹). The kinetic behaviour of catalase under these conditions (1.2 μ M-catalase, expressed as haematin, at 20° and at pH 6.8) is the same as that observed directly (30 μ M-catalase, expressed as haematin, at 20° and at pH approx. 6.4). Under aerobic conditions, the inhibition of the peroxidatic reaction by azide is completely reversible once the azide has been removed peroxidatically.

DISCUSSION

Peroxidatic oxidation of azide. The reactions between azide and catalase and catalase peroxide compounds described above may be represented as in Scheme 1. The available evidence on the stoichiometry of the overall reaction is collected in Table 2.

The oxidation of the hydrogen donor azide thus involves a ferrous-ferric cycle. Keilin & Hartree (1954) have suggested that such a cycle may also occur in the peroxidatic oxidation of other hydrogen donors, including hydrogen peroxide itself.

As pointed out by Nicholls (1962*a*), however, azide and hydroxylamine are unique among the donors oxidized by catalase in that their oxidation is a three-electron rather than a two-electron process. Further attempts to invoke a role for ferrocatalase (Wiesner, 1962; Westheimer, 1959) involve certain conflicts with the experimental data (see Nicholls, 1963). A major revision of eqns. (1*a*) and (1*b*) is thus unlikely to be necessary.

Some doubt has been expressed about the production of nitric oxide (Ehrenberg, 1962), on the grounds that a ferrous complex with nitric oxide should be paramagnetic (with one unpaired electron). Although the 'azide-peroxide-catalase' is usually described as diamagnetic, however, the original data reveal that accurate measurement of the susceptibility was difficult (Theorell & Ehrenberg, 1952*a*). The facts that: (a) the spectra of 'azide-peroxide-catalase' and nitric oxide-ferrocatalase are closely similar, (b) an identical compound is formed from hydroxylamine, (c) unlike carbon monoxide-ferrocatalase, 'azide-peroxide-catalase' is oxygen-labile, and (d) small amounts of

Scheme 1. Reactions of catalase with peroxide and azide. The values of the reaction velocity constants involving azide are all approximately proportional to $[H^+]$. At pH 6.4 and at 20°:

$k_1 = 6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, $k'_1 = 1.8 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$, $k_2 \geq 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, $k'_2 \geq 10^3 \text{ M}^{-1} \text{ sec}^{-1}$, $k_3 \geq 0.25 \text{ sec}^{-1}$ (in air, pseudo-first order), $k_4 \approx 0.025 \text{ sec}^{-1}$ (in air, pseudo-first order), $k_5 \approx 10^2 \text{ M}^{-1} \text{ sec}^{-1}$, $K_d = 4 \times 10^{-4} \text{ M}$. Details are given in the text and by Nicholls (1961*a*).

Table 2. *Estimated stoichiometries of the azide reactions in air and in nitrogen*

Reaction (a) catalase·Fe ³⁺ + H ₂ O ₂ + HN ₃ → 0.85 (catalase·Fe ²⁺ + N ₂ O + N ⁰) + 0.15 (catalase·Fe ²⁺ NO + N ₂)	
Reaction (b) 0.85 N ⁰ + HN ₃ (excess) → (≤ 0.85) N ₂	
Reaction (c) catalase·Fe ³⁺ + 0.25 O ₂ → catalase·Fe ³⁺ (in air)	
Reaction (d) catalase·Fe ³⁺ + 0.5 H ₂ O ₂ → catalase·Fe ³⁺ (in nitrogen)	

Stoichiometry of (a) + (b) + (c)

$$\begin{aligned}\Delta H_2O_2 &= -1.0 \\ \Delta O_2 &= -0.25 \\ \Delta (N_2O + N_2) &= +1.0 \text{ to } +1.85 \\ \Delta \text{gas}/\Delta H_2O_2 &= 0.75 \text{ to } 1.6^*\end{aligned}$$

Stoichiometry of (a) + (b) + (d)

$$\begin{aligned}\Delta H_2O_2 &= -1.5 \\ \Delta O_2 &= 0.0 \\ \Delta (N_2O + N_2) &= +1.0 \text{ to } +1.85 \\ \Delta \text{gas}/\Delta H_2O_2 &= 0.67 \text{ to } 1.2^\dagger\end{aligned}$$

* Experimental values of 1.1 to 1.7 were obtained by Theorell & Ehrenberg (1952*b*).

† Experimental values of 0.6 to 0.9 were obtained by Theorell & Ehrenberg (1952*b*).

nitrite are produced during the peroxidation of azide, all argue in favour of the idea of nitric oxide-ferrocatalase adopted in the present paper. The evidence presented tends to rule out the alternative idea put forward by Ehrenberg (1962), that the compound is a 'low-spin' ferrocatalase, because ferrocatalase is produced by the azide reaction, in addition to nitric oxide-ferrocatalase, and shows a different kinetic behaviour. The compound cannot be nitrosyl-ferrocatalase (nitric oxide-catalase) for the latter has a spectrum of different form.

It may be, however, that the nitric oxide involved is produced in a reaction other than that of eqn. (3) [Foulkes & Lemberg's (1949) equation]. The radical designated 'N^o' in eqn. (2) might be expected to engage in various reactions, including: (i) recombination to form molecular nitrogen, as in Theorell & Ehrenberg's (1952*b*) original formulation (probably its least likely fate); (ii) a reaction with excess of azide to produce nitrogen (as suggested in the balance sheet of Table 2); (iii) interaction with groups on the catalase protein; (iv) a reaction with water, to produce nitrogen oxyacids; (v) reactions with oxygen and hydrogen peroxide, to produce nitrous oxide or nitric oxide; and (vi) a reaction with nitrous oxide to produce nitrogen and nitric oxide. Eqn. (3) is retained here, however, because the available evidence suggests that the availability of nitric oxide in air and in nitrogen (Fig. 5) does not vary greatly (less than a factor of 2), indicating its production in the primary reaction. Eqn. (3) also provides the only reliable way of forming nitrogen gas in the reaction, a phenomenon consistently observed by Theorell & Ehrenberg (1952*b*).

Modes of reaction of catalase peroxide compounds. The reaction of compound I with azide provides a further example of a peroxide compound reacting in a manner other than a reduction by a hydrogen donor to ferric catalase (directly or via compound II). Reactions of this kind may be listed as follows: (1) The apparently reversible formation of complexes with anions such as fluoride and acetate, by both compound I and compound II (Nicholls, 1961*a*). (2) The reaction of compound II with hydrogen sulphide to give ferrosulphocatalase (Nicholls, 1961*b*). (3) The reaction of compound I with aminotriazole to give aminotriazole-inhibited catalase (Margoliash, Novogrodsky & Schejter, 1960; Nicholls, 1962*c*). (4) The dismutation of ethyl hydroperoxide compound I to give ferric catalase and acetaldehyde (unpublished work by G. R. Schonbaum, described briefly by Nicholls & Schonbaum, 1963). (5) The reaction of compound I with azide to give ferrocatalase and nitric oxide-ferrocatalase (the present paper).

These 'extra' reactions indicate that the formation of peroxide compounds involves more than

just 'activation' of the peroxide molecule. The reaction with azide shows that the third oxidizing equivalent is made available in compound I, and suggests the view that a complex between donor and peroxide compound occurs in the reaction (a three-electron collision process with a stoichiometry in excess of 90% as reported in the present paper would seem improbable). The occurrence of the two reactions [eqns. (2) and (3)] suggests that more than one form of this complex may be possible.

Structure of compound I. The primary peroxide compound (catalase·Fe³⁺·H₂O₂ in eqn. 1) has until recently been regarded as either a complex between iron and peroxide, analogous to other catalase complexes (Chance & Fergusson, 1954), or as a higher oxidation state (Fe⁶⁺ or FeO³⁺) of the iron (George, 1953). Both these views are based on 'models' thought to be appropriate. In the 'peroxide complex' theory, the model is the reaction of catalase with acidic ligands (eqn. 9).

This was regarded as appropriate because of its formal equivalence to the formation of a reversible Michaelis complex, in which the substrate becomes activated, and to the inhibition of such complex-formation by competitive inhibitors reacting with the 'active site'. In the 'higher oxidation state' theory, the model is the oxidation of haemoglobin to methaemoglobin and to 'methaemoglobin peroxide' (probably ferrylhaemoglobin), as in eqn. (10), where Hb indicates haemoglobin. This

point of view was considered appropriate because higher oxidation states of transition metals show powerful oxidizing capabilities similar to those shown by the primary peroxide compounds. It acquired considerable impetus when peroxidase compound II was shown to resemble ferrylmyoglobin and ferrylhaemoglobin in retaining only one oxidizing equivalent from the original peroxide (George, 1953).

Neither model is, however, capable of explaining all the features of the catalytic reaction. Disputes as to which is more nearly correct have, therefore, a somewhat artificial form. For example, the form in which the 'complex versus oxidation state' debate has most recently appeared involves the question of the reversibility of compound I formation (Chance & Schonbaum, 1962). Of the two possible formulations of eqn. (1*a*) indicated in eqns. (11*a*) and (11*b*), the first was shown to be the only one compatible with the evidence of an

equilibrium (a finite low value of k_{-1} at low enzyme and peroxide concentration). The value of k_{-1} for

bacterial catalase and methyl hydroperoxide is given as approx. $5 \times 10^{-3} \text{ sec.}^{-1}$. These, and other experiments on the release of ethanol during the reaction with alkyl hydroperoxide (Schonbaum, 1962), show that the ROH moiety is retained in the structure of compound I but leave many alternative structures equally probable. Reversibility of eqn. (1a) is also indicated by thermodynamic considerations. A minimum value for k_{-1}/k_1 of $1 \mu\text{M}$ is obtained if E'_0 for compound I is considered to be at least $+1.0 \text{ v.}$ On the other hand, a maximum value of about $1 \mu\text{M}$ can be calculated from the fact that concentrations of peroxide of this order give nearly complete formation of steady-state compound I (Chance, 1950). Thus we have (for hydrogen peroxide):

$$5 \times 10^{-6} \text{ sec.}^{-1} < k_{-1} < 5 \times 10^{-3} \text{ sec.}^{-1} \quad (12)$$

Reversibility does not indicate a simple complex, nor does irreversibility indicate something other than a complex. Sulphmyoglobin and sulph-catalase are formed 'reversibly', in some cases probably thermodynamically reversibly (Nicholls, 1961*b*), but are not complexes, and the iron remains capable of binding ligands. The formation of hypophosphite-catalase seems to be much less reversible than that of compound I (Nicholls, 1961*a*), but all its properties are those of an 'ionic' complex with the iron.

As a result of an analysis of the spectrum and magnetic susceptibility of catalase compound I, Brill & Williams (1961) have put forward a theory of its structure to account for the observed features in a more satisfying way. They propose that the observed spectrum represents a thermal mixture of two components, one ('POR') being a complex of the type envisaged by Chance, and the other ('ROX') a compound with a hydroxylated methene bridge similar to verdohaematin. If catalase[porph.²⁻]·Fe³⁺·H₂O represents normal catalase with [porph.²⁻] the doubly negatively charged porphyrin, then compound I is given by the equilibrium of eqn. (13). Although this theory

provides for a more reasonable understanding of spectrum and susceptibility than do the simpler 'complex' and 'oxidation state' theories, it is a rather static picture, and Brill & Williams (1961) do not attempt to indicate the mode by which either form oxidizes hydrogen donors. The action of anions and the formation of ternary complexes are difficult to incorporate into their picture. Moreover, if the search for model compounds is to extend into the bile-pigment haematin field, among derivatives with no catalytic activity, it is possible to find compounds which, unlike mesobiliverdin, imitate most if not all of the spectral characteristics of compound I, e.g. pyridine mesocholehaematin (Callaghan & O'Hagan, 1949).

Nicholls (1961*a, b*) has presented evidence in favour of the view that anionic ligands can form complexes with the iron of compound I, and that if the porphyrin ring is modified, as in the formation of sulphcatalase, all the usual derivatives of the enzyme can be formed, and the iron retains its usual pattern of reactivity except that 'analogue' peroxide compounds are not formed (unlike the otherwise analogous situation in metmyoglobin). The present paper shows that, in the peroxidatic oxidation of azide, the iron is reduced by a product of donor oxidation, and in some cases an oxidation product remains attached to it in the form of a complex. These reactions may be most readily explained by a structure with the peroxide attached to the porphyrin ring. A ROOH moiety may be added across either of two different kinds of double bond in a methene bridge or a C-N bond in a pyrrole ring [eqns. (14a) and (14b)]. Here the

possibility that the (OOR) moiety is linked to the N atom has been neglected because the large steric effects expected in such a system are not observed. In each of the other positions, a tetrahedral carbon atom occurs with two possible positions for the (OOR) moiety which may be stabilized by the presence of either HOH or HXOH as a ligand on the iron [formulae (I) and (II)]. Calculation based

on the bond distances in such structures in the porphyrin ring indicate that hydrogen bonds can

be formed with lengths of 2.5 and 2.9 Å in formulae (I) and (II) if C is a pyrrole carbon atom, or with lengths of 2.8 and 3.05 Å if C is a methene-bridge carbon atom, without distorting the C-O bond more than 5° from the tetrahedral configuration or the Fe-O bond more than 10° from the octahedral configuration.

Models made with a Courtauld atomic-model set show that the second configuration (II) is stereochemically possible without strain. In configuration (I), owing to a 'twist' imposed on the porphyrin ring by the saturation of the methene-bridge double bond, which was not taken into account in the above calculation, the space for a ligand (HOH in formula I) in the sixth co-ordination position is stereochemically restricted. It is therefore suggested that formula (II) may represent the correct configuration for both compound I (HOH in place of HXOH) and the compound I-donor complex. Scheme 2 shows the reaction cycle to be expected if such a 'bridge' operated during the catalytic reaction. A second hydrogen bond is shown here between a group Y on the protein and the acidic H of the iron ligand, in accordance with the theory put forward by Nicholls & Schonbaum (1963) to explain the binding of undissociated acids by catalase and peroxidase. The reaction mechanism, involving H⁻ ion transfer from the moiety X (X is CH₃ in methanol) to the second oxygen of the ROOH fragment, with loss of RO⁻ and H⁺ (from the donor), is of the type (III), where

X-Y is the oxidant and B is the base (Nicholls & Schonbaum, 1963). A mechanism of this kind can

be applied without modification to all donors of the 'third group' (alcohols) and the 'sixth group' (hydrogen peroxide) of Keilin & Nicholls (1958).

Hydroxylamine ('fifth group') can also give a complex of the form (II), producing a final ferric complex of the form Fe³⁺NOH, losing a H⁺ ion to form Fe³⁺NO⁻ or Fe²⁺NO. Azide, however, has only one available hydrogen atom [formula (IV)]. The other H atom, and the oxygen found in the products (nitrous oxide and nitric oxide) must come either from the peroxide group (ROOH) or from an intervening water molecule. Experiments with isotopically labelled alkyl hydroperoxide and water would be necessary to decide between these possibilities. The reactions involving azide are further complicated by the presence of two nitrogen atoms [formula (IV)] carrying partial negative charges and potentially capable of bonding with the haematin iron.

Scheme 2. The 'bridge' hypothesis. The first compound formed, with the peroxide moiety close to the iron atom, may be stereochemically hindered with respect to complex-formation at the sixth co-ordination site. Both compound I and Ogura's complex may therefore have the configuration of the second compound in the reaction cycle given here, the first compound representing an intermediate in the transfer of the peroxide from the iron to the porphyrin ring.

Several structures analogous to those of formulae (I) and (II) can therefore be written, and oxidation processes can be envisaged which leave either NO (or NOH) associated with the iron atom when molecular nitrogen splits off, or N^0 (or NH^0) associated with the iron atom when nitrous oxide splits off. A closer attempt at explanation would not be fruitful at the present time. The theory of Scheme 2, like those of Chance & Fergusson (1954), of George (1953) and of Brill & Williams (1961), attempts only an approximation to part of the truth.

Analysis of the overall catalatic reaction. The view that the peroxide moiety in compound I is primarily associated with the porphyrin ring, while the iron retains the ability to form complexes with donors and anionic ligands, may also permit an interpretation of several kinetic peculiarities of the catalase reaction. The acceleration of compound II formation by anions such as fluoride (Nicholls, 1961*a*) may be regarded as a mobilization of the 'endogenous donor' when HA replaces H_2O on the iron atom. The complex found by Ogura (1955) may be the one indicated in Scheme 2, limiting the breakdown of hydrogen peroxide at high concentrations of hydrogen peroxide. The acceleration of compound II formation at such peroxide concentrations (George, 1949) can then be attributed to the effect of the bound hydrogen peroxide on the activity of the endogenous donor, as with those ligands that cannot act as hydrogen donors. As compound III will also be formed from compound II at such high concentrations, the reversible inhibition by excess of substrate (George, 1949) is also comprehensible.

The main difficulty with the present mechanism is that it does not provide for an easy one-electron reduction to compound II (normally regarded as an FeO^{2+} or $FeOH^{3+}$ complex). The similar behaviour of compound II towards anionic ligands (Nicholls, 1961*a*) has, however, already suggested that more complicated structures for this derivative should also be considered.

SUMMARY

1. The rates and stoichiometries of the reactions of mammalian catalase with azide and peroxide have been studied.

2. Azide diminishes the rate of compound II formation in the glucose oxidase-glucose-catalase system, reacting with compound I as a hydrogen donor like alcohols.

3. The final product of the reaction with azide and peroxide in air or nitrogen is nitric oxide-ferrocatalase; under carbon monoxide, the product is carbon monoxide-ferrocatalase. Similar products are formed whether (a) hydrogen or ethyl hydro-

peroxide is used to form compound I, (b) azide or hydroxylamine is present as hydrogen donor, or (c) liver or blood catalase is used. The spectra of the nitric oxide and carbon monoxide derivatives of the ferrous form of a reducible catalase are closely similar to those produced indirectly.

4. The rates of formation of carbon monoxide-ferrocatalase and nitric oxide-ferrocatalase under anaerobic conditions are proportional to peroxide concentration, indicating compound I formation to be the rate-limiting step; but the rate of formation of nitric oxide-ferrocatalase is only one-tenth that of carbon monoxide-ferrocatalase formation. The formation of nitric oxide-ferrocatalase in air is a diphasic reaction. Nitric oxide-ferrocatalase is vulnerable to oxidation by oxygen, but carbon monoxide-ferrocatalase is stable; however, carbon monoxide-ferrocatalase is dissociated by light, the first detectable product being ferric catalase.

5. One molar equivalent of hydrogen peroxide is required to form one equivalent of carbon monoxide-ferrocatalase in the presence of an excess of azide; five to twelve molar equivalents of hydrogen peroxide are required to form one equivalent of nitric oxide-ferrocatalase, the precise ratio depending on the state of aerobiosis and the catalase sample used.

6. Hydroxylamine and hydrazine also give rise to nitric oxide-ferrocatalase in the presence of peroxide, but in neither case is the reaction simpler than that with azide.

7. Horse-radish peroxidase compound I reacts with azide to give a firmly bound ferric derivative, reducible to a second ferric derivative from which peroxidase may be regenerated.

8. The reactions of catalase are interpreted in terms of two reactions of compound I with azide, one (80–90 % of the total turnover) giving ferrocatalase, nitrous oxide and a nitrogen (N^0) radical the other (10–20 % of the total turnover) giving nitric oxide-ferrocatalase and nitrogen. These correspond to reactions suggested by Theorell & Ehrenberg (1952*b*), and by Foulkes & Lemberg (1949) respectively.

9. The reactions with azide support the view that the oxidation of 'multi-electron' donors by catalase compound I involves the formation of a complex between the donor molecule and the haematin iron. A structure for compound I is put forward in which the peroxide moiety is associated with the porphyrin ring, and some consequences for reaction mechanisms are examined in the light of this hypothesis.

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Condensed Tannins

18. STEREOCHEMISTRY OF FLAVAN-3,4-DIOL TANNIN PRECURSORS: (+)-MOLLI-SACACIDIN, (-)-LEUCO-FISETINIDIN AND (+)-LEUCO-ROBINETINIDIN*

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The concomitance of flavan-3,4-diols and related polymeric leuco-anthocyanidins in a variety of heartwoods and barks (Roux, 1958, 1959) may be regarded as evidence of the biogenesis of 'condensed' tannin polymers from flavan-3,4-diols (Roux & Evelyn, 1958, 1960; Roux & Paulus, 1961b, 1962a,b). This is supported by the progressive transformation of flavan-3,4-diols into tannins in one instance (Roux & Evelyn, 1958).

The tentative 2,3-*trans*-3,4-*cis* configurations previously assigned to the precursors of wattle-

bark and -heartwood (*Acacia mearnsii*) and quebracho-heartwood (*Schinopsis* spp.) tannins, (+)-mollisacacidin (Clark-Lewis & Roux, 1959; Clark-Lewis & Katekar, 1960), (-)-leuco-fisetinidin (Clark-Lewis & Roux, 1959) and (+)-leuco-robinetinidin (Roux & Paulus, 1962b) have been revised recently to 2,3-*trans*-3,4-*trans* assignments (Clark-Lewis & Katekar, 1962; Drewes & Roux, 1963; Lillya, Drewes & Roux, 1963a). Details of the syntheses, comparative oxidation and nuclear-magnetic-resonance studies which led to these revised and now unambiguous assignments are presented below.

* Part 17: Roux & de Bruyn (1963).