Properties of Catalase. Catalysis of Coupled Oxidation of Alcohols

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It was previously shown (Keilin & Hartree, 1936) that on addition of ethanol and catalase to a primary oxidation system, such as xanthine oxidase and hypoxanthine, the rate of oxygen uptake is markedly increased and the final uptake is doubled. Similar results were obtained using other oxidizing enzymes, e.g. uricase and *d*-amino-acid oxidase and their respective substrates, in other words systems which react directly with molecular O_2 and reduce it to H_2O_2 . The increased O_2 uptake was shown to be due to the secondary oxidation of ethanol to acetaldehyde by H_2O_2 formed in the primary oxidation reaction. It was a matter for some surprise to find that this secondary oxidation of ethanol took place only in presence of catalase, which catalyzes this reaction instead of preventing it by the usual catalatic decomposition of H_2O_2 into O_2 and water. Since H_2O_2 formed in the primary oxidation reaction could be replaced by barium or cerium peroxide, but not by a solution of free H_2O_2 , it appeared that the H_2O_2 oxidizing ethanol was in a 'nascent state'.

In this paper we propose to deal with three aspects of this reaction: (1) the catalysis of the reaction by pure catalase in order to eliminate the possible presence of other catalytic systems reacting with alcohols, (2) the comparison of the rate of oxidation of different alcohols, and (3) the nature of the peroxide required for this oxidation reaction. This study will be concluded by a critical examination of the possible function of catalase.

METHODS

Oxidation systems. We have used three primary oxidation systems which reduce O_2 to H_2O_2 :

(1) *Xanthine oxidase* in the form of a crude caseinogen preparation was obtained by precipitating milk by half saturation with $(NH_4)_2SO_4$, drying the precipitate between filter papers and defatting it with pure ether. Its substrate hypoxanthine is oxidized via xanthine to uric acid.

(2) *d*-Amino-acid oxidase was used in the crude form as an acetone dried mince of pig's kidney and also in a pure form prepared according to Negelein & Brömel (1939). Its substrate *d*(-)-alanine is oxidized to pyruvic acid.

(3) *Glucose oxidase* was obtained from *Penicillium notatum* in a highly purified state (notatin) and was kindly supplied by Prof. Raistrick and Dr Birkinshaw. Its substrate glucose is oxidized to gluconic acid.

Catalase used in these experiments was either a highly purified enzyme preparation obtained from horse liver (Keilin & Hartree, 1945) or a crystalline ox-liver catalase prepared according to Sumner & Dounce (1937).

The experiments were carried out as previously described (Keilin & Hartree, 1936) in Barcroft manometers at 39° in 0.1M-phosphate buffer of pH 7.8 for xanthine oxidase, 7.3 for crude amino-acid oxidase, and 6.0 for glucose oxidase. For the purified amino-acid oxidase a pyrophosphate buffer was used (0.07M, pH 8.3).

Estimation of acetaldehyde. The method used was based on the Schiff reaction. 1–5 ml. of the solution to be analyzed containing $>100\mu g$. acetaldehyde were added to 5 ml. 5N- H_2SO_4 in a boiling tube. The tube was warmed to 80° and a slow current of air, purified by passage through charcoal, was passed through the solution at a rate of 100–150 ml./min. The air-stream was then bubbled through 1 ml. of Schiff's reagent in a small tube 6 × 1 cm. kept at 0°. The entire apparatus was made up with ground-glass joints. After 10 min. aeration the reagent was transferred to another small tube and, after a further 20 min. at 0°, was compared with a series of freshly made standards. 5 μg . acetaldehyde are just detectable. The reagent is prepared from a 0.1% solution of pure fuchsin treated with SO_2 until almost colourless: on short standing the remaining colour disappears. 20 min. aeration under the standard conditions should give no coloration. The reagent should be decolorized freshly each day. The error for a single determination is of the order of 10%, but the method has the advantage of simplicity.

EXPERIMENTS

Oxygen uptake by the primary oxidation system

Since the above oxidizing systems reduce O_2 to H_2O_2 , the O_2 uptake should be double the amount calculated for the oxidation of the substrate. However, this does not apply generally to all three systems. Thus in the xanthine oxidase system the total O_2 uptake differs according to the experimental conditions. It may be below the theoretical amount in the case of a purified enzyme preparation which is easily inactivated by the peroxide formed in the reaction (Dixon, 1925). It may reach the theoretical amount in the case of the caseinogen preparation which is more stable, the H_2O_2 formed during the oxidation of the substrate acting as a hydrogen acceptor for the oxidation of more of the same substrate (Dixon, 1926). It may be above the theoretical amount owing to a secondary oxidation of impurities by means of H_2O_2 and peroxidase present in the crude preparations.

With a crude preparation of *d*-amino-acid oxidase the total O_2 uptake usually reaches the theoretical amount, which is due to the presence of a sufficient concentration of catalase for the decomposition of H_2O_2 . In a purified catalase-free preparation the

O₂ uptake is double the theoretical amount (see below).

In the glucose oxidase system the total O₂ uptake is double the theoretical amount, owing to the complete absence of catalase and to the resistance of the enzyme to H₂O₂.

In all the control experiments referred to in this paper, the theoretical O₂ uptake with any of the three oxidizing enzyme systems was invariably obtained by carrying out experiments in presence of pure catalase.

Oxidation of alcohols in presence of the xanthine oxidase system plus catalase

We repeated the experiments which we have previously described, this time using purified catalase.

The experiments were carried out in four Barcroft manometers, the right-hand flasks of which received about 100 mg. oxidase preparation (caseinogen) and a dangling tube containing 1 mg. hypoxanthine in 0.3 ml. dilute NaOH. In addition to oxidase the flasks of the second, third and fourth manometers received respectively catalase (≈ 0.016 mg. haematin), ethanol (10 mg.) and catalase plus ethanol. The solution in the flasks was made to 3 ml. with 0.1M-phosphate buffer pH 7.8. In other series of similar experiments the central tubes of the flasks received 0.3 ml. 10% KOH and a roll of filter paper.

A typical result of such experiments (Fig. 1) shows that the H₂O₂ formed in the primary oxidation reaction is utilized by purified catalase for the

Fig. 1. Coupled oxidation of ethanol by the xanthine oxidase system in presence of purified horse-liver catalase. Xanthine oxidase = 100 mg. caseinogen preparation of milk, 1 mg. hypoxanthine ($\approx 167 \mu\text{l. O}_2$), 10 mg. ethanol, 0.3 ml. catalase. Temp. 39°; pH 7.8. Similar results were obtained using recrystallized ox-liver catalase.

oxidation of ethanol to acetaldehyde. The formation of aldehyde is easily recognized not only by smell but also by yellowing of the potash papers due to resin formation.

Cyclic oxidation of ethanol to aldehyde

We have shown previously (Keilin & Hartree, 1936) that when the primary oxidizing system is composed of xanthine oxidase and aldehyde (i.e. the Schardinger system), the H₂O₂ formed, being used by catalase for the oxidation of ethanol to acetaldehyde, increases the supply of substrate for the primary oxidation reaction. The reaction will therefore proceed as long as ethanol is present and the enzymes involved are still active. This reaction, in which the product of the secondary oxidation

Fig. 2. Cyclic oxidation of ethanol by the Schardinger system and purified catalase: 300 mg. caseinogen preparation of milk, 1 mg. acetaldehyde, 5 mg. ethanol, 0.3 ml. catalase. Experimental conditions as in Fig. 1.

supplies the substrate for the primary oxidation, was described by us as a 'cyclic oxidation'. We have been able to obtain this reaction with a highly purified horse-liver catalase (Fig. 2) as well as with the crystalline catalase prepared from ox liver.

Coupled oxidation of ethanol by purified d-amino-acid oxidase

It was mentioned above that when a purified catalase-free preparation of amino-acid oxidase catalyzes the oxidation of *d*(-)-alanine the O₂ uptake is double the theoretical amount calculated for oxidation of the substrate. This is due, as was shown by Negelein & Brömel (1939), to a spontaneous reaction between the two products of this oxidation, namely, H₂O₂ and pyruvate, which yield acetic acid and CO₂. In this case the reactions proceed as follows:

Following the description given by these workers, we prepared their enzyme fraction *D*, which is free

Table 1. *Effect of catalase and ethanol on the system purified amino-acid oxidase + d(-)-alanine*

d-Amino-acid oxidase is composed of the enzyme protein + alloxazine adenine dinucleotide. The oxidation of 1.5 mg. *d*(-)-alanine (or 3 mg. *dl*-alanine) to pyruvate requires 188 μ l. O_2 : $CH_3CH(NH_2)COOH + \frac{1}{2}O_2 = CH_3CO.COOH + NH_3$.

Manometer	Reaction system	Primary reaction products	Final products of secondary reaction	Final O_2 uptake (μ l.)
1	Oxidase + alanine + O_2	$CH_3CO.COOH$ $+ NH_3 + H_2O_2$	$CH_3CO.COOH + NH_3 + CO_2$	340
2	Oxidase + alanine + catalase + O_2		$CH_3CO.COOH + NH_3 + \frac{1}{2}O_2$	190
3	Oxidase + alanine + ethanol + O_2		$CH_3CO.COOH + NH_3 + CO_2$	362
4	Oxidase + alanine + ethanol + catalase + O_2		$CH_3CO.COOH + NH_3 + CH_3CHO$	350

from catalase, and with this preparation we carried out a series of manometric experiments.

In the right-hand flasks of four Barcroft manometers were placed 0.5 ml. of enzyme fraction *D* and excess (50 μ g.) of flavine adenine dinucleotide, while 0.3 ml. 1% *dl*-alanine (equivalent to 188 μ l. O_2) was put in a dangling tube in each flask. Manometer 2 received 0.2 ml. of strong catalase solution, manometer 3 received 0.1 ml. 10% ethanol, while the fourth manometer received both ethanol and catalase. 0.3 ml. 10% KOH and a roll of filter paper were placed in the central tubes to absorb CO_2 . The volumes were made up to 3 ml. with 0.07M-pyrophosphate pH 8.3.

The results of these experiments, summarized in Table 1, show that the addition of catalase to the primary oxidation system halved its O_2 uptake owing to the decomposition of H_2O_2 and liberation of O_2 . Catalase thus protects the pyruvate from oxidation and brings the O_2 uptake down to the theoretical amount. When, however, both catalase and ethanol are added to the primary oxidation system the O_2 uptake again becomes double. In this case ethanol and not pyruvate is oxidized by the H_2O_2 , the latter being preferentially utilized by catalase for catalysis of the coupled oxidation of ethanol.

It may be mentioned here that, according to Sevag & Maiweg (1934), pyruvate as well as catalase will protect pneumococci from the toxic effect of the H_2O_2 formed when these micro-organisms are exposed to air.

Coupled oxidation of other alcohols

Although the oxidation of other alcohols was investigated with each of the three primary oxidizing systems, we shall refer now only to results obtained with *d*-amino-acid oxidase and glucose oxidase. These experiments were carried out in the same manner as those with xanthine oxidase.

In the case of crude amino-acid oxidase the right-hand flasks of each manometer received a phosphate buffer extract of 50 mg. of pig's kidney powder and catalase, while 0.3 ml. 1% *dl*-alanine was put into the dangling tube. With the exception of the control the flasks received 5–20 mg. of different alcohols. In the case of glucose oxidase, the right-hand flasks received 50 μ g. glucose oxidase and the dangling tube contained 0.3 ml. 1% glucose, the other ingredients being added as before.

Fig. 3. Coupled oxidation of alcohols by crude *d*-amino-acid oxidase system (=extract of 50 mg. kidney powder + 3 mg. *dl*-alanine). Temp. 39°; pH 7.3. In addition to oxidase system + catalase the following substances were present: (1) none (control); (2) 5 mg. methanol; (3) 5 mg. ethanol; (4) 10 mg. *n*-propanol; (5) 10 mg. *isopropanol*; (6) 15 mg. *n*-butanol; (7) 15 mg. *isobutanol*; (8) 20 mg. amyl alcohol.

Fig. 4. Coupled oxidation of alcohols by the glucose oxidase (notatin) system: 50 μ g. notatin + 0.3 ml. 1% glucose. Temp. 39°; pH 6.0. In addition to oxidase system + catalase the following substances were present: (1) none (control); (2) 5 mg. methanol; (3) 5 mg. ethanol; (4) 10 mg. *n*-propanol; (5) 10 mg. *isopropanol*. Higher alcohols were not oxidized.

The results of these experiments, given in Figs. 3 and 4, show that the alcohols which most readily undergo coupled oxidation in presence of catalase and a primary oxidation system are methanol and ethanol whereas *n*-propanol and *isobutanol* are much less readily attacked. Similar results have been obtained with uricase (Keilin & Hartree, (1936). Ethylene glycol and β -amino-ethanol are oxidized as readily as ethanol (Fig. 5), but since

Fig. 5. Coupled oxidation of β -amino-ethanol and ethylene glycol by the *D*-amino-acid oxidase system. Experimental conditions as for Fig. 3. 5 mg. ethylene glycol, 5 mg. β -amino-ethanol.

glycollic aldehyde is not oxidized, this substance appears to be the oxidation product of ethylene glycol. Glycerol and choline, however, are not oxidized. In Table 2 are listed the substances which have been tested and found not to undergo coupled oxidation.

Table 2. *Substances which do not undergo coupled oxidation in presence of catalase*

<i>n</i> -Octanol	Dulcitol	Glycollic aldehyde
Benzyl alcohol	Pentaerythritol	Glycollic acid
Choline	Xylose	Dihydroxymaleic acid
Glycerol	Glucose	Lactic acid
Erythritol	Fructose	Malic acid
Mannitol	Galactose	Tartaric acid
Inositol		

Relative concentrations of catalase required for the decomposition of H_2O_2 and for its utilization in the coupled oxidation of alcohol

We have shown above that catalase added to a primary oxidation system can catalyze two distinct

reactions: (1) ordinary catalytic decomposition of H_2O_2 formed in the primary reaction, and (2) utilization of this peroxide for a secondary oxidation of alcohols. In a primary oxidation system like glucose oxidase, the former reaction halves the total O_2 uptake, bringing it down to the theoretical level for the oxidation of the substrate, whereas in the second reaction the O_2 uptake is again double the theoretical value (Table 3).

It was of interest to determine the minimum concentrations of catalase which are required for promoting these reactions (3 and 4 in Table 3). For this purpose a series of manometric experiments was carried out, using glucose oxidase as the primary oxidation system with and without ethanol and with different concentrations of purified catalase.

The results of these experiments, summarized in Table 4, clearly show that the concentration of

Table 4. *Effect of varying concentrations of catalase on the system glucose oxidase (enzyme) + glucose $\pm$ ethanol*

(Theoretical O_2 uptake (3 mg. glucose) = 187 μ L. Catalase concentration expressed in terms of haematin.)

Contents of flasks	Total O_2 uptake (μ L.)
Enzyme + glucose $\pm$ ethanol	345
Enzyme + glucose + catalase (2×10^{-10} M)	250
Enzyme + glucose + catalase (2×10^{-9} M)	187
Enzyme + glucose + ethanol + catalase (2×10^{-8} M)	240
Enzyme + glucose + ethanol + catalase (2×10^{-7} M)	302
Enzyme + glucose + ethanol + catalase (2×10^{-6} M)	348

catalase required for oxidation of alcohols by means of the H_2O_2 formed in the primary oxidation is about 1000 times greater than that required for its catalytic decomposition into O_2 and water.

Nature of the peroxide required for oxidation of ethanol by catalase

We have shown previously that ethanol could be oxidized by H_2O_2 deriving either from a primary oxidation reaction or from decomposition of barium and cerium peroxides, but not by H_2O_2 added as a solution (Keilin & Hartree, 1936). It appeared therefore that the peroxide acting in this reaction was in a specially active state which we have described as a 'nascent state'. It is, however, possible that it is not the supposed higher level of activity of the 'nascent peroxide' which enables catalase to

Table 3. *Analysis of the effect of ethanol and catalase on the system glucose oxidase + glucose*

	Reaction system	Primary reaction products	Secondary reaction induced by catalase	Net O_2 uptake*
1	Oxidase + glucose + O_2	Gluconic acid + H_2O_2	None	2n
2	Oxidase + glucose + ethanol + O_2		None	2n
3	Oxidase + glucose + catalase + O_2		$H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$	n
4	Oxidase + glucose + catalase + ethanol + O_2		$H_2O_2 + C_2H_5OH \rightarrow CH_3CHO + 2H_2O$	2n

* n = theoretical O_2 uptake for oxidation of glucose to gluconic acid: $C_6H_{12}O_6 + \frac{1}{2}O_2 = C_6H_{12}O_7$.

utilize it for the oxidation of ethanol, but rather the presence of the peroxide in a low concentration throughout the reaction. If this is the case ethanol should undergo oxidation in presence of catalase by free H_2O_2 provided the latter is supplied slowly and continuously. The slow delivery of H_2O_2 into a mixture of catalase and ethanol can be assured by at least three different methods: (1) volatilization at 39° of a solution of H_2O_2 , (2) addition of H_2O_2 drop by drop combined with efficient stirring, and (3) addition of H_2O_2 in the form of a fine mist produced by a suitable atomizer.

(1) Experiments with the addition of H_2O_2 by volatilization were carried out in manometer flasks. For this purpose three differential manometers were used and their right-hand flasks were filled as follows: (i) 0.3 ml. catalase; (ii) 0.3 ml. catalase + 0.1 ml. 20% ethanol; (iii) 0.1 ml. 20% ethanol. The volumes in the flasks were made up to 3 ml. with 0.1M-phosphate buffer pH 6.0 and 0.3 ml. 30% hydrogen peroxide (perhydrol) was put into the central tube of each flask. The manometers were shaken for 7 hr. in a bath at 39° with the taps open. In these experiments peroxide gradually diffuses as vapour into the solution in the flasks. This H_2O_2 may be partly decomposed catalatically and partly used for oxidation of ethanol. The latter reaction is very slow except in presence of catalase as is shown by estimations of acetaldehyde (Table 5, a).

Table 5. *The catalytic oxidation of ethanol by gradual addition of H_2O_2 in presence of catalase:*

(a) Diffusion of H_2O_2 vapour. (b) Dropwise addition of H_2O_2 . (c) Addition of H_2O_2 as mist.

	Contents of flasks	Acetaldehyde formed ($\mu\text{g.}$) after slow addition of H_2O_2		
		200	400	100
(a)	Catalase + ethanol	200	400	100
	Catalase	10	15	0
	Ethanol	40	50	5
(b)	Catalase + ethanol	1050	.	
	Ethanol	175		
(c)	Catalase + ethanol	2500		
	Ethanol	80		

(2) Experiments with a continuous and slow addition of a solution of H_2O_2 were carried out at room temperature. For this purpose 20 ml. 0.7N- H_2O_2 were added slowly during 35 min. to a mixture of 40 ml. 0.1M-phosphate buffer pH 6.0, 10 ml. 10% ethanol and 1 ml. catalase ($\equiv$ 0.18 mg. haematin) in a 150 ml. flask. The H_2O_2 was introduced drop by drop while the mixture was stirred very rapidly. In a control experiment without catalase 1 drop of the latter was added at the end of the experiment in order to decompose the accumulated H_2O_2 . At the completion of the experiments the aldehyde was estimated and the results are shown in Table 5, b.

(3) Experiments with the addition of H_2O_2 solution in the form of a mist were carried out as follows: N_2 from a cylinder, after being heated to 40° , was passed via active charcoal to an atomizer containing 2.4 g. perhydrol. After trapping the larger droplets, the mist of H_2O_2 was passed into a 500 ml. flask containing 5 ml. 0.1M-phosphate buffer

pH 6.0, 5 ml. 5% ethanol and 1 ml. catalase ($\equiv$ 0.18 mg. haematin). The issuing gas escaped via a liquid air trap. The apparatus was set up in a shaker and the experiment was run for 1 hr. A control experiment was carried out in absence of catalase. At the end of the experiment the excess of H_2O_2 in the control was decomposed by the addition of 1 drop of catalase solution and the contents of the flask plus liquid air trap were analyzed in both cases for acetaldehyde (Table 5, c).

All these experiments clearly show that in presence of catalase ethanol can be oxidized to aldehyde not only by 'nascent peroxide' but also by a solution of free H_2O_2 . There is, however, a very marked difference in the efficiency of these two processes. Whereas in the coupled oxidation of ethanol almost the entire amount of H_2O_2 formed in the primary reaction is used for the secondary oxidation process, thus oxidizing an equivalent amount of ethanol, in the oxidation of ethanol by a solution of H_2O_2 and catalase only a small fraction of H_2O_2 is utilized for this purpose, the main portion being decomposed catalatically. It does, therefore, appear to be impossible to devise an efficient method of continuous delivery of free H_2O_2 , the concentration of which throughout the solution and during the whole course of the experiment could be maintained at the optimum low value.

The possible functions of catalase

It is generally believed that catalase by catalyzing the decomposition of H_2O_2 protects cells and tissues from its toxic effect. That catalase is capable of protecting an enzyme such as xanthine oxidase from destruction by H_2O_2 formed during the catalytic oxidation of hypoxanthine was well demonstrated by Dixon (1925) and confirmed by other workers (see also Fig. 2). In this connexion it is interesting to note that liver and kidney, which are rich in catalase, are also rich in enzyme systems such as uricase, amino-acid oxidase, tyraminase and others which, *in vitro*, are capable of reacting directly with O_2 , reducing it to H_2O_2 . On the other hand, the non-nucleated erythrocytes of mammals, which also contain a very high concentration of catalase, have hardly any respiratory or oxidative activity, whereas nucleated erythrocytes of certain birds which show a marked respiratory activity contain very little catalase.

It has been suggested by Bingold (1933) that the function of catalase within corpuscles is the protection of haemoglobin against oxidative destruction by H_2O_2 formed in different tissues, especially in the kidney. That endoerythrocytic catalase, under certain experimental conditions, is capable of protecting haemoglobin from oxidation by H_2O_2 to methaemoglobin can easily be demonstrated. Thus, on treating mammalian red blood corpuscles with a solution of H_2O_2 , the latter undergoes a

violent decomposition accompanied by the liberation of O_2 ; the oxyhaemoglobin, however, remains almost completely unchanged. Only when the endoerythrocytic catalase is inhibited by cyanide, azide or hydroxylamine can the oxyhaemoglobin be oxidized to methaemoglobin by H_2O_2 . On treating bird erythrocytes with H_2O_2 the latter undergoes a very slow catalatic decomposition while oxyhaemoglobin is rapidly oxidized.

At first sight these experiments seem to support Bingold's view that endoerythrocytic catalase protects oxyhaemoglobin from oxidation by H_2O_2 . However, further study of this problem revealed several points at variance with this view. First of all there is a marked difference between the experimental conditions described above and the conditions within a living organism. Thus, in our experiments *in vitro* H_2O_2 was used in fairly high concentrations, whereas in cells or tissues of living organisms H_2O_2 , if it is formed, must be present in a very low concentration. In fact, the H_2O_2 may derive only from oxidizing systems which, like xanthine oxidase, can react directly with O_2 , reducing it to H_2O_2 . Since the affinities of these systems for O_2 are distinctly smaller than that of the cytochrome system, and as the tension of O_2 within cells and tissues is usually very low, little peroxide may be expected to derive from this source. The best way of testing the protective function of endoerythrocytic catalase under conditions approaching those *in vivo* is to bring intact mammalian erythrocytes in contact with a primary oxidation system reducing O_2 to H_2O_2 .

For this purpose 40 μ g. glucose oxidase and 0.3 ml. 6% glucose are mixed with 2.7 ml. isotonic phosphate buffer pH 7.3 and 0.3 ml. washed horse erythrocytes. The mixture is shaken at 39°. After 5 min. shaking about 75%, and after 15 min. 100% of the haemoglobin is found to be oxidized to methaemoglobin. Experiments carried out with laked corpuscles gave similar results, the amount of oxyhaemoglobin converted to methaemoglobin after 5, 15 and 25 min. shaking being approximately 50, 90 and 100% respectively. The oxidation of haemoglobin was not affected by the addition of ethanol, which simultaneously underwent coupled oxidation to aldehyde. Similar experiments were carried out with oxyhaemoglobin which had been recrystallized several times until free from catalase. In this case not only was oxyhaemoglobin oxidized to methaemoglobin, but a portion of the pigment underwent an irreversible oxidative destruction of its tetrapyrrolic ring accompanied by denaturation of the protein and the formation of green verdohaemochromogen.

These experiments show that endoerythrocytic catalase does not protect haemoglobin from oxidation to methaemoglobin by very small concentrations of H_2O_2 formed in a primary oxidation reaction. In other words, under natural conditions the H_2O_2 , if formed in such reactions, should be capable of oxidizing the divalent iron of haemoglobin in

spite of the large concentration of endoerythrocytic catalase. If such an oxidation does not take place it is certainly not due to the protective action of catalase but rather to the fact that H_2O_2 does not come in contact with the haemoglobin.

We may now ask how catalase can protect haemoglobin from oxidation by a great excess of H_2O_2 if it does not protect it against a very small concentration of H_2O_2 such as may be formed *in vivo*. On addition of a large amount of H_2O_2 to a suspension of red blood corpuscles the H_2O_2 is violently decomposed into O_2 and water. The solution becomes saturated with O_2 and the equilibrium reaction $HbO_2 \rightleftharpoons Hb + O_2$ is shifted towards the left, thus reducing the concentration of Hb. Since it is the unoxxygenated haemoglobin (Hb) that reacts with H_2O_2 and thus undergoes oxidation, the removal of it will reduce the rate of formation of methaemoglobin. This is analogous to the autoxidation of haemoglobin to methaemoglobin by O_2 . In fact, it was shown by Neill & Hastings (1925) and by Brooks (1929, 1935) that the optimum rate of oxidation of oxyhaemoglobin by O_2 is at small tensions of the gas. According to Brooks (1935) the rate of autoxidation of Hb at different O_2 pressures is proportional to the concentration of Hb and to a function of the O_2 pressure. In this reaction O_2 has a triple function: (1) the determination of the concentration of Hb, (2) the oxidation of Hb, and (3) the inhibition of this oxidation by forming oxyhaemoglobin.

If we assume that H_2O_2 is formed in cells as a result of certain oxidation reactions, it is unlikely that cells are protected from its toxic action only by catalatic decomposition of this H_2O_2 . In fact we have already seen that endoerythrocytic catalase is unable to protect haemoglobin from oxidation by small amounts of H_2O_2 , yet neither haemoglobin nor myoglobin are oxidized within the living organism. Moreover, the formation of H_2O_2 and its catalatic decomposition would be a very uneconomic process. It is more likely that the H_2O_2 , if formed, is utilized in coupled oxidations. That the main function of catalase may be the catalysis of such oxidative reactions by means of H_2O_2 is supported by another consideration. We have seen that the concentration of catalase required for the oxidation of ethanol by means of H_2O_2 formed in a primary oxidation reaction is about $2 \times 10^{-6} M$ (estimated in terms of haematin) which is about 1000 times greater than the concentration ($2 \times 10^{-9} M$) required for the catalatic decomposition of this H_2O_2 . The concentration of catalase in liver and in non-nucleated erythrocytes of mammals, which is of the order of $4 \times 10^{-6} M$, would be adequate for oxidative utilization of the H_2O_2 produced by these tissues on the assumption that all the O_2 utilized during respiration is reduced to H_2O_2 . At present coupled oxida-

tion may be considered as a much more likely biological function of catalase than the decomposition of H_2O_2 into O_2 and water. This, however, does not exclude the possibility that catalase may have yet another function.

DISCUSSION

Oxidizing enzymes such as xanthine oxidase, uricase, *d*-amino-acid oxidase, tyraminase, glucose oxidase and others, which catalyze the oxidation of their respective substrates by molecular O_2 , reduce the latter to H_2O_2 . The total O_2 uptake by such systems should therefore be double the amount calculated for the oxidation of the substrate. This can be demonstrated experimentally provided that (a) the reaction is carried out in the complete absence of catalase, (b) the peroxide formed in the reaction does not inactivate the enzyme, and (c) the peroxide formed in the oxidation of one molecule of the substrate does not act as hydrogen acceptor in the oxidation of a further molecule of the same substrate. However, on addition of a small amount of catalase to these systems, the velocities of their reactions are reduced and the total O_2 uptake is brought down to the theoretical amount, since H_2O_2 formed in the primary reaction is decomposed catalytically, liberating O_2 .*

Addition of ethanol to primary oxidizing systems already containing catalase doubles the total O_2 uptake by these systems since H_2O_2 formed in the primary reaction is utilized for the secondary or coupled oxidation of ethanol. The oxidation of ethanol by H_2O_2 takes place only in presence of catalase which catalyzes this reaction instead of abolishing it by splitting H_2O_2 into O_2 and water. The fact that the coupled oxidation of ethanol is catalyzed by a highly purified or crystalline catalase shows that no other enzyme is involved in this reaction.

In a system composed of the Schardinger enzyme, acetaldehyde, catalase and ethanol, H_2O_2 formed in the primary oxidation of aldehyde to acid is utilized by catalase for the oxidation of ethanol to acetaldehyde. Thus the product of the secondary oxidation supplies the substrate for the primary oxidation system. This 'cyclic oxidation' reaction, which proceeds until all ethanol is used up, can also be catalyzed by highly purified catalase.

In addition to ethanol, purified catalase promotes the oxidation of other alcohols such as methanol, ethylene glycol, β -aminoethanol, and to a lesser degree *n*-propanol and isobutanol.

* This point must be borne in mind in calculating the activities of enzymes during purification. In fact, the apparent increase in the activity of uricase (Holmberg, 1939) and of amino-acid oxidase (Negelein & Brömel, 1939) during their purification is partly due to the removal of catalase.

For the catalytic oxidation of ethanol by catalase, H_2O_2 formed in a primary oxidation reaction can be replaced by barium or cerium peroxide or even by free H_2O_2 provided that the latter is added so slowly and continuously that its concentration remains very low. However, the primary oxidation reaction is by far the most efficient mechanism of supply of H_2O_2 since nearly every molecule of H_2O_2 thus formed is utilized in the oxidation of the ethanol. In all other methods of addition of H_2O_2 only a small fraction of added H_2O_2 is used in this reaction, the main portion being decomposed catalytically. Such methods show nevertheless that the high efficiency of H_2O_2 formed in the primary oxidation reaction is not due to a 'nascent' or active form of this peroxide but to a more suitable manner of its supply to the catalase-ethanol system.

The concentration of catalase which is required for catalytic decomposition of H_2O_2 formed in a primary oxidation reaction is about 1000 times lower than that which is required for the coupled oxidation of ethanol. This explains why some crude enzyme preparations containing traces of catalase give with their substrates theoretical O_2 uptakes and yet are unable to catalyze the coupled oxidation of ethanol.

As to the function of catalase, it is far from being elucidated in spite of the facts that it is one of the most widely distributed intracellular enzymes, that it can easily be obtained in a pure crystalline state, and that its structure as well as the kinetics of the catalytic decomposition of H_2O_2 are well determined. In this respect the position of catalase resembles that of peroxidase and phenol oxidases which can also be obtained in pure states. Although the structure and the intimate mechanism of the reactions they catalyze are well understood, very little is known as to the part they play in cellular metabolism.

Since the work of Thénard (1811), Schönbein (1863 *a*, *b*) and Loew (1901) it has been considered that the main if not the only function of catalase is protective and consists in the decomposition of H_2O_2 into O_2 and water ($2\text{H}_2\text{O}_2 = 2\text{H}_2\text{O} + \text{O}_2$). The fact that this reaction is violent and is readily detected and measured directly with cells and tissues has tended to rule out any inquiries into other possible functions of the enzyme.

This idea of a protective function of catalase tacitly assumes that H_2O_2 is formed *in vivo* as a result of intracellular oxidation reactions. That a small amount of H_2O_2 may be formed within cells is supported by the following few considerations:

(a) The formation of H_2O_2 has been demonstrated *in vitro* as a result of oxidation reactions catalyzed by several intracellular oxidizing enzymes which react directly with O_2 . In certain of these cases it has even been shown that catalase protects the enzymes against the toxic effect of H_2O_2 .

(b) Catalase and peroxidase, which are the only enzymes reacting *in vitro* with H_2O_2 , to some extent exclude each other. Thus catalase, although universally distributed, reaches the highest concentration in animals, whereas peroxidase is widely distributed in plants, and in animals is known only in milk and in leucocytes.

(c) H_2O_2 can easily be detected in culture media of certain bacteria, devoid of catalase, when they are exposed to air, as is the case with pneumococci (see p. 295) and with lactic acid bacteria (*B. delbrückii*, *acidophilus* or *bulgaricus*). It has also been found in culture media of certain moulds such as *Penicillium* and *Aspergillus*.

Contrary to the view that H_2O_2 is generally formed in cells and tissues during respiratory processes are the following two facts: (1) although H_2O_2 can be detected in certain isolated enzyme systems, it has not, with very few exceptions, been detected in highly respiring cells of strictly aerobic organisms even when the catalase is inhibited by hydroxylamine, and (2) enzyme systems which *in vitro* reduce O_2 to H_2O_2 usually have small affinities for O_2 , and as the tension of O_2 within tissues of higher organisms is very low it is conceivable that these systems do not react directly with O_2 but through other systems which do not give rise to H_2O_2 .

If, however, we accept the idea that H_2O_2 is formed within cells of aerobic organisms, it is unlikely that the main function of catalase would consist in splitting this peroxide into O_2 and water in order to protect cells and their enzymes from its toxic effect, as was shown for the xanthine system *in vitro* (Dixon, 1925). That the function of catalase is not merely a protective one is supported by the following considerations:

(a) A high concentration of catalase in mammalian red blood corpuscles does not protect haemoglobin against oxidation to methaemoglobin by H_2O_2 formed in a primary oxidation reaction.

(b) The presence of catalase in *Bacillus subtilis*, *Proteus vulgaris* and *Escherichia coli* does not protect these micro-organisms against the toxic effect of very small concentrations of H_2O_2 (1 in 4,000,000) added directly to the media or formed as the result of the autoxidation of ascorbic acid (Lwoff & Morel, 1942). Neither does the presence of catalase in *Staphylococcus aureus* afford protection against very low concentrations of H_2O_2 formed during the oxidation of glucose (Coulthard *et al.* 1942; Birkinshaw & Raistrick, 1943) or of hypoxanthine (Lipmann & Owen, 1943) catalyzed by their respective enzymes.

(c) If we assume that the rate of H_2O_2 formation in cells of liver is equivalent to the rate of O_2 uptake, the concentration of catalase in these cells is at least 1000 times higher than is necessary for an

efficient catalytic decomposition of this peroxide but is of the right order for catalysis of coupled oxidation.

(d) Catalytic decomposition of H_2O_2 , liberating O_2 for further oxidation reactions, is a wasteful process which can hardly be considered as a general mode of utilization of H_2O_2 formed in some primary oxidations.

The catalysis of coupled oxidation by catalase appears therefore to be a more likely biological property of this enzyme than the decomposition of H_2O_2 into O_2 and water. It is conceivable that in addition to alcohols catalase may promote a peroxidatic oxidation of other biologically important substances. On the other hand, the fact that catalase is present in very high concentrations in the non-nucleated and hardly respiring erythrocytes of mammals suggests that it may have yet another physiological function not necessarily connected with H_2O_2 .

SUMMARY

1. Certain oxidizing enzymes, in catalyzing the oxidation of their substrates by molecular O_2 , reduce the latter to H_2O_2 , which in presence of catalase is decomposed as follows:

Pure catalase may alternatively utilize the H_2O_2 formed in such primary oxidation reactions for catalysis of the secondary or coupled oxidation of alcohols to aldehydes:

2. For catalysis of this coupled oxidation (2), H_2O_2 formed in the primary oxidation can be replaced by H_2O_2 resulting from the slow decomposition of barium or cerium peroxides, or by free H_2O_2 provided the concentration of the latter is kept low by very gradual addition. However, H_2O_2 deriving from a primary oxidation is much more efficient in this reaction, since every molecule of H_2O_2 thus formed can be used in the coupled oxidation of alcohols, whereas in all other forms of addition of H_2O_2 reaction (1) greatly predominates.

3. In presence of xanthine oxidase and aldehyde, purified catalase also promotes a cyclic oxidation of ethanol to acetic acid, which proceeds until all alcohol is oxidized.

4. In a system composed of purified *D*-amino-acid oxidase and alanine, the H_2O_2 formed is utilized in a non-catalyzed coupled oxidation of pyruvate to acetate and CO_2 . If, however, catalase and ethanol are added to this system, H_2O_2 is utilized for the coupled oxidation of the ethanol, leaving the pyruvate unattacked.

5. The high concentration of endoerythrocytic catalase does not protect the divalent iron of haemoglobin from oxidation by H_2O_2 formed in a primary

oxidation system. Similarly the presence of catalase in micro-organisms does not protect them from the toxic effect of H_2O_2 added as such or formed in the medium as a result of certain oxidation reactions. It is therefore unlikely that the main function of catalase consists in the decomposition of H_2O_2 into O_2 and water in order to protect cells from its toxic effect.

6. The catalysis of coupled oxidation by means of H_2O_2 formed in a primary oxidation is a more likely biological property of catalase. In fact, the

concentration of catalase in liver tissues appears to be about 1000 times higher than that required for the catalytic decomposition of H_2O_2 , and is of the same order as is found to be necessary for catalysis of coupled oxidation.

7. So far only methanol, ethanol, *n*-propanol, isobutanol, β -amino-ethanol and ethylene glycol have been shown to undergo coupled oxidation catalyzed by catalase *in vitro*. It is possible that *in vivo* other biologically important compounds may undergo similar oxidations.

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The Urinary Partition of Sulphur in Rats Treated with Aromatic Hydrocarbons, with Special Reference to Growth Retardation

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Haddow, Scott & Scott (1937) found that certain carcinogenic hydrocarbons produced immediate, constant, and long-continued reduction in the growth rate of young rats, while some non-carcinogenic hydrocarbons did not show this effect. White & White (1939) produced growth retardation in rats by the incorporation of methylcholanthrene, 3:4-benzpyrene, or pyrene in the diet. Later the same workers (1940) observed no difference between the growth effects produced by single intraperitoneal injections of carcinogenic or non-carcinogenic hydrocarbons; both types retarded growth.

White & White concluded, on the basis of their own results and from a consideration of data available in the literature for bromobenzene, naphthalene, and anthracene, that the growth retardation produced by the hydrocarbons is due to a specific deficiency of the sulphur-containing amino-acids

imposed on the animals by an increased requirement for organic sulphur, in the form of cystine or methionine, for detoxication mechanisms that involve the formation of mercapturic acids. They emphasized the desirability of actual isolation of the postulated mercapturic acids in order to demonstrate conclusively the relation between growth effects and sulphur metabolism.

Stekol (1943) showed that the addition of bromobenzene to the diet of rats produces, in effect, a decrease in available protein proportional to the mercapturic acid excreted. Growth retardation does not occur on a high protein diet but increases as the protein content is progressively lowered. Bromobenzene, composition of diet, and the degree of growth retardation are quantitatively related.

The mechanism of the growth retardation produced by these hydrocarbons is important, since