SUMMARY

1. Washed suspensions of *Pseudomonas fluorescens* grown on media containing nicotinic acid oxidize nicotinic acid, 5-fluoronicotinic acid and 2-fluoronicotinic acid at approximately the same rate; 5-chloronicotinic acid is oxidized at a slower rate whilst neither 5-bromonicotinic acid nor 6-fluoronicotinic acid are oxidized.

2. No intermediates in the oxidation were detected when nicotinic acid or the 5-fluoro and 2-fluoro analogues were substrates. An intermediate was detected during the oxidation of 5-chloronicotinic acid; chromatographic and spectroscopic evidence suggested that the pyridine ring was still intact and that substitution of the α -carbon atom had occurred.

3. Washed suspensions of cells grown with asparagine oxidize nicotinic acid after a lag period of 90–120 min. During the later stages of the lag period, 6-hydroxynicotinic acid was isolated and identified by mixed melting point, chromatographic and spectrographic properties.

4. Cells grown on nicotinic acid oxidize 6-hydroxynicotinic acid without a lag period; 2-hydroxynicotinic acid and α -hydroxy pyridine are not oxidized. It is suggested that the formation of 6-hydroxynicotinic acid is the first step in the oxidative attack.

5. Cells grown on asparagine adapt to oxidize 6-hydroxynicotinic acid or 5-fluoronicotinic acid. They do not adapt to the other halogen analogues nor to 2-hydroxynicotinic acid, 2:6-dihydroxynicotinic acid or α -hydroxy pyridine.

6. Extracts prepared from cells broken in a bacterial press converted nicotinic acid into 6-hydroxynicotinic acid; 6-hydroxynicotinic acid was not oxidized.

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Catalase, Peroxidase and Metmyoglobin as Catalysts of Coupled Peroxidatic Reactions

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According to Chodat & Bach (1903) an oxidase is a system composed of oxygenase, an autoxidizable component which on reaction with oxygen produces a peroxide, and peroxidase, which uses this peroxide for oxidation of different substances. This concept, which they believed to be supported by their experiments on fractionation and reconstruction of

Lactarius oxidase, would in their view explain both the function of peroxidase and the mechanism of action of oxidases. The concept of an oxygenase-peroxidase system was further developed by Thurlow (1925), who grouped under Chodat & Bach's term oxygenase, autoxidizable substances such as cysteine or glutathione and oxidizing

systems such as xanthine oxidase plus either hypoxanthine or aldehyde, all of which on reaction with molecular oxygen reduce it to H_2O_2 . The latter could then be utilized by peroxidase for oxidation of a variety of substances. The oxidizing system which generates H_2O_2 and that in which H_2O_2 is utilized were later described by Harrison & Thurlow (1926) as 'primary' and 'secondary' oxidizing systems. The secondary, peroxidatic or coupled oxidation was found to be catalysed not only by peroxidase but to a lesser degree by haemoglobin and its haematin derivatives as well as by other iron compounds. The only reaction known to be catalysed by catalase, however, was the decomposition of H_2O_2 to oxygen and water.

The fact that catalase can under certain conditions function peroxidatically and bring about a coupled oxidation was first demonstrated with H_2O_2 generated by a primary oxidizing system such as uricase, D-amino acid oxidase, xanthine oxidase or glucose oxidase. Although the addition of catalase to such systems decomposes the H_2O_2 as fast as it is generated into oxygen and water (i.e. catalytically), the addition of catalase and ethanol results in a quantitative utilization of the H_2O_2 for the coupled oxidation of ethanol to acetaldehyde (Keilin & Hartree, 1936*a*, 1945*b*). If the source of H_2O_2 is the glucose oxidase (notatin) system, the oxidation of one molecule of glucose to gluconic acid yields one molecule of H_2O_2 , which in presence of catalase will be used for peroxidatic oxidation of one molecule of ethanol to acetaldehyde. In absence of catalase the H_2O_2 accumulates, whether or not ethanol is added. These reactions are summarized in Table 1 (from Keilin & Hartree, 1945*b*).

progressively smaller increases and *n*-pentanol is virtually unoxidized. Ethylene glycol is also oxidized but we have been unable to confirm our previously reported coupled oxidation of 2-amino-ethanol.

Although the addition of a solution of H_2O_2 to aqueous ethanol containing catalase gives rise solely to a catalatic decomposition of H_2O_2 , we were able by very slow addition of H_2O_2 to bring about a slight oxidation of ethanol (Keilin & Hartree, 1945*b*). Since then Anan (1951), working at very high dilutions, has been able to show that as much as 7% of added H_2O_2 may escape catalatic decomposition and be utilized for oxidation of ethanol. More recently, by an ingenious manometric method, wherein H_2O_2 diffuses in through a cellophan membrane, Laser (1954) was able to demonstrate that in presence of $20 \mu M$ catalase up to 80% of added H_2O_2 could be used for the peroxidatic oxidation of ethanol.

Since 1945 several other substances have been found to undergo coupled oxidations in presence of catalase, i.e. to act as hydrogen donors. Chance (1948) found that formaldehyde and formic acid could be utilized in such reactions, although his claim that ascorbic acid could act as a donor (Chance, 1947) was later withdrawn (Chance, 1950). Heppel & Porterfield (1949) found that catalase could also catalyse a coupled oxidation of nitrite. The possibility that ferrocyanide, dithionite and certain phenolic substances can act as donors in peroxidatic oxidation by catalase was indicated in spectrophotometric experiments by George (1952*a*), who found that these substances can react with catalase-methyl hydroperoxide compounds, either

Table 1. *The oxidation of glucose in presence of notatin. Utilization of the H_2O_2 in presence of ethanol and catalase (from Keilin & Hartree, 1945*b*)*

Reaction system	End product of primary reaction	Secondary reaction induced by catalase
1. Notatin + glucose + O_2 *	Gluconic acid† + H_2O_2	None
2. Notatin + glucose + ethanol + O_2		None
3. Notatin + glucose + catalase + O_2		$H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$
4. Notatin + glucose + catalase + ethanol + O_2		$H_2O_2 + C_2H_5OH \rightarrow CH_3CHO + 2H_2O$

* $C_6H_{12}O_6 + O_2 + H_2O = C_6H_{12}O_7 + H_2O_2$.

† See the text, p. 312.

Thus the net O_2 uptake in reactions 1, 2 and 4 is one mole O_2 , and in reaction 3 one-half mole O_2 per mole of glucose oxidized. In other words, the addition of ethanol to the primary oxidation system already containing catalase leads to a doubling of the total O_2 uptake. Notatin is an exceptionally useful oxidase for quantitative demonstration of these relationships, since it is not inhibited by accumulated peroxide. Whereas methanol gives a similar increase of O_2 uptake, higher alcohols give rise to

by accelerating the change from compound I to II or by liberating catalase from compound II. Finally, it has been shown recently by Theorell & Ehrenberg (1952) that azide can also undergo a peroxidatic oxidation in presence of catalase. We were able to confirm this reaction and to show that hydroxylamine was similarly oxidized (Keilin & Hartree, 1954). These two reactions are unique inasmuch as the hydrogen donor is also an inhibitor of catalase.

Peroxidase is known to catalyse by means of H_2O_2 the oxidation of a great variety of aromatic amines, phenols and leucodyes as well as of ascorbic acid, nitrite and cytochrome c. In the case of amines and phenols the same, or very similar, peroxidatic reactions are catalysed by haemoglobin and its haematin derivatives, although the levels of activity are very much lower. Such reactions have for long been used in the detection of blood and haematin compounds in biological materials, since highly coloured products are formed. Recently the specificity of different peroxidases towards hydrogen donors was studied manometrically in presence of H_2O_2 generated by the notatin system and of a minimal concentration of catalase (Knox, 1954).

The main object of the present study was to assess the relative catalytic activities of catalase, peroxidase and metmyoglobin in the coupled oxidation of different hydrogen donors under comparable conditions.

METHODS

General. Oxidations were carried out in a volume of 3.0 ml. in Barcroft differential manometers. All experiments with glucose oxidase were performed at pH 5.9 and 20°; those with xanthine oxidase at pH 7.8 and 30°. Unless otherwise stated, no alkali was placed in the centre wells.

Glucose oxidase (notatin) + glucose was chosen as the standard primary oxidation system generating H_2O_2 . It has been shown that the actual substrate for notatin is β -D-glucopyranose (Bentley & Neuberger, 1949; Keilin & Hartree, 1951b), which is oxidized to δ -gluconolactone, the latter hydrolysing spontaneously to gluconic acid (Bentley & Neuberger, 1949). At equilibrium aqueous glucose contains 64% of β -D-glucopyranose and the initial rates of oxidation will thus not be limited by mutarotation. Nevertheless, although at 20° mutarotation may become limiting towards the end of a manometric experiment, the final O_2 uptake will not be affected. It was, however, decided to carry out the present experiments at 20°, since catalase readily became denatured at pH 5.9 when shaken at higher temperatures for the experimental periods (1.5–2 hr.).

Since it was necessary to reduce the concentrations of heavy metals to a minimum when working with systems generating H_2O_2 , especially in presence of ascorbic acid, the laboratory distilled water was redistilled in a Pyrex apparatus. All glassware was cleaned with chromic-sulphuric acid followed by 6N-HCl.

Phosphate buffer. This consisted (unless otherwise stated) of 40 ml. 0.5M- KH_2PO_4 plus 4.7 ml. 0.5N-KOH diluted to 100 ml. (pH 5.9). The KH_2PO_4 had been freed from heavy metals by repeated extraction of a molar solution with a $CHCl_3$ solution of 8-hydroxyquinoline until the extracts were colourless. After removal of excess 8-hydroxyquinoline with $CHCl_3$ the aqueous solution was evaporated and crystallized. The KOH solution had been purified by passage through a column of sulphonated polystyrene resin kindly supplied by Dr S. M. Partridge. The resin had previously been extracted exhaustively by alternate washings with hot π solutions of HCl and KOH. After a final KOH washing the column was well washed with distilled water and the KOH solution was passed through it.

Materials

Notatin was the same material as previously described (Keilin & Hartree, 1945b). It had been preserved at -15° as a dry powder for 8 years without appreciable loss of activity. Catalase was obtained from horse-liver (Keilin & Hartree, 1945a). It has been found that the addition of 1% NaCl to this preparation greatly increases its stability both at 5° and -15° . Peroxidase had been prepared from horse-radish root (Keilin & Hartree, 1951a) and had a purpurogallin number (PN) of 1220 when fresh. When the present experiments started it had been in store for about 4 years at -15° and the PN was ~ 1000 . Xanthine oxidase was kindly supplied by Professor F. Bergel. It consisted of an approximately 0.9% solution of crystalline material. Spectrophotometric measurements showed $E_{480} = 10.0$ and $E_{450} = 1.80$. The activity was 94 units/l. (see Avis, Bergel, Bray & Shooter, 1954). Of this solution 0.2 ml. was used/manometer flask.

Metmyoglobin. Myoglobin had been obtained from horse heart by a method similar to that of Theorell (1932) and recrystallized from 3M phosphate buffer, pH 6.3, by addition of more buffer (in the form of solid phosphates) until a slight opalescence appeared. During these manipulations the pigment underwent oxidation and the final crystalline material was pure metmyoglobin.

Haemin. This was prepared from horse blood by the method of Schalfjew (1885).

Glucose and sodium nitrite were analytical grades, *p*-cresol was redistilled *in vacuo* and quinol was recrystallized from water. Ethylenediaminetetraacetic acid (EDTA) was a very pure sample of the disodium salt. Aqueous solutions were brought to pH 5.9 with redistilled HCl.

Estimations

Estimations of haematin in catalase, peroxidase and metmyoglobin were made by a haemochromogen method involving colorimetric comparison with standard solutions (Keilin & Hartree, 1951a). Concentrations of these haemoproteins are expressed throughout this paper in terms of haematin.

Estimation of ascorbic acid was carried out in the manometer flasks at the end of an experiment by addition of starch and titration with 0.1N- I_2 .

Estimation of nitrite. The contents of a manometer flask were washed into a 100 ml. volumetric flask, treated with 14 ml. 0.1N-Ba(OH) $_2$ and diluted to the mark. When the precipitate had settled, 0.2 ml. was diluted to 9 ml. with water, treated with 1 ml. Griess reagent (Feigl, 1937), heated for 5 min. at 70–80°, cooled and the colour intensity measured in a Hilger absorptiometer using Ilford 604 (transmitting 500–540 m μ) filters and 1 cm. cells. Standard nitrite solutions for calibration purposes were prepared from $AgNO_3$ and excess NaCl.

Estimation of formate. For this purpose *Escherichia coli* (N.C.T.C. no. 86) was grown in Roux flasks on a glucose-peptone-Lemco-NaCl-agar medium for 24 hr. at 37°. The collected cells were washed 4 times with water, suspended in the pH 5.9 buffer, aerated for 2 hr. at 37°, centrifuged down and resuspended in the buffer. The suspension contained 8.9 mg. dry wt. of cells/ml. The liquid from a manometer flask was heated for 3 min. at 100° and any precipitate formed was centrifuged off. To another manometer flask were added 2.0 ml. of the clear liquid and 0.75 ml. 0.016M

2,4-dinitrophenol in buffer. NaOH (0.3 ml. 10%) was placed in the centre well with a roll of filter paper, and a dangling cup containing 0.25 ml. *Esch. coli* suspension was introduced. The O_2 uptake, in which one atom O_2 corresponds to one molecule of formic acid, was followed to completion (120–140 min.). The dinitrophenol suppresses the slow oxidation of gluconate by this organism and also most of the residual endogenous respiration. In control experiments with known amounts of formate the error was found to be <5%.

Estimations of azide and glucose in experiments on the coupled oxidation of azide. At the conclusion of a set of experiments exemplified in Table 2 (H-d. = 10 or 50 μ moles NaN_3) the flask contents were deproteinized and freed from phosphate. The flask contents were sucked into a test tube and the following solutions were used as rinsings to effect a quantitative transfer to the tube: 0.75 ml. 0.8M-BaCl₂; 0.75 ml. 0.44N-NaOH. The mixtures were shaken, heated for 5 min. at 100°, treated hot with 0.75 ml. 0.33M-Na₂SO₄, cooled in ice and filtered.

Glucose was estimated in the filtrate by treating 1 ml. with 3 ml. water and 8 ml. anthrone reagent according to the procedure of Morris (1948).

For determination of azide the filtrate was diluted 1:20 (50 μ moles NaN_3) or 1:4 (10 μ moles NaN_3), treated with 2 vol. 0.075M-FeCl₃, and the extinction measured at 463 m μ . (Wehrle & Fried, 1952). In preparing calibration curves, known amounts of NaN_3 were added to 3 ml. phosphate buffer and taken through the deproteinization procedure. Two such curves were prepared corresponding to the two filtrate dilutions. These precautions are essential since some loss of azide occurs during deproteinization.

Procedure for the study of coupled oxidation reactions

Manometric study of the oxidations of different hydrogen donors catalysed by haemoproteins was carried out in two stages. The first, which was of an exploratory nature aiming at a general survey of hydrogen donors, was carried out in presence of high concentrations of haemoproteins, e.g. 30 μ M or 0.1 μ mole/manometer flask. The second stage consisted in the comparative study of the catalytic efficiency of haemoproteins in concentrations varying between 30 and 0.00003 μ M or 10^{-1} to 10^{-7} μ mole/manometer flask. The activities of haemoprotein catalysts can be conveniently expressed as pC, the negative logarithm of the haematin molarity necessary to cause 50% of H_2O_2 generated under standard conditions to be utilized in the coupled oxidation.

The standard manometric procedure is summarized in Table 2. Notatin (0.1% aqueous solution stored at -15°) was diluted tenfold with buffer as required. Of this solution 0.5 ml. was taken for each manometer with 0.2 ml. (3.0 mg., 16.7 μ moles) aqueous glucose in a dangling cup. The concentrations of haemoproteins were varied within the limits given above, while the quantity of hydrogen donor under test was 50 μ moles. At the end of the experiment (100–120 min.) catalase (0.1 μ mole) was introduced in a second dangling cup to experiments *a*, *b*, *e*, *f*, *g* and *h* in order to determine the accumulated H_2O_2 . When very small quantities of catalase (<10⁻⁵ μ mole) were present initially in experiments *c* and *d*, a little H_2O_2 was sometimes found to accumulate. In such experiments a second addition of catalase was made. The reaction mixtures were in certain cases analysed for residual glucose and hydrogen donor.

The theoretical total O_2 uptake for the oxidation of 3 mg. glucose without accumulation of H_2O_2 (*c*) is 187 μ l. Although in each set of experiments catalase was dropped simultaneously in all flasks at the conclusion of O_2 uptake, the additions are shown in the plotted results to occur at slightly different times in order to avoid confusion from superimposition of the curves.

Table 2. *Manometric experiments for testing the peroxidatic oxidation of various hydrogen donors (H-d.) by the notatin system in presence of catalase, peroxidase or metmyoglobin*

50 μ g. notatin; 3.0 mg. glucose; 50 μ moles H-d.; 10^{-1} to 10^{-7} μ mole haemoprotein. The glucose solution was made up in water: other ingredients were dissolved in phosphate buffer (pH 5.9) which was also used to make up the volume to 3.0 ml. Temp. = 20°.

- a* Notatin + glucose
- b* Notatin + glucose + H-d.
- c* Notatin + glucose + catalase
- d* Notatin + glucose + catalase + H-d.
- e* Notatin + glucose + peroxidase
- f* Notatin + glucose + peroxidase + H-d.
- g* Notatin + glucose + metmyoglobin
- h* Notatin + glucose + metmyoglobin + H-d.

RESULTS

Catalatic decomposition by haemoproteins of H_2O_2 generated in the glucose oxidase system

It was found previously (Keilin & Mann, 1937) that a strong solution of peroxidase in absence of a hydrogen donor catalyses the liberation of O_2 from H_2O_2 . To a lesser extent this applies also to methaemoglobin (Keilin & Hartree, 1950). However, the three haemoproteins, catalase, peroxidase and methaemoglobin differ markedly in the magnitude of their catalatic activities towards H_2O_2 . We have used metmyoglobin rather than methaemoglobin in the present experiments because it is more resistant to degradation by H_2O_2 and can be more easily obtained completely free of catalase. The variations in the catalatic activities of different concentrations of the three haemoproteins towards H_2O_2 generated in the notatin system are shown in Fig. 1. Thus 50% of the generated H_2O_2 is decomposed by 2.3×10^{-10} M catalase, 1.3×10^{-6} M peroxidase and $\sim 10^{-4}$ M metmyoglobin: expressed as pC the values are 9.6, 5.9 and ~ 4 respectively. Uncertainty in the value for metmyoglobin arises from the fact that it is partly oxidized by H_2O_2 formed in the primary oxidation (see p. 318). In similar experiments it was found that the activity of free protohaematin lies between those of peroxidase and metmyoglobin, but precise figures are not obtainable owing to gradual precipitation of protohaematin. Since peroxidase shows an appreciable catalatic activity, it is not surprising that in the

experiments to be described the presence of peroxidase at a final concentration of $30 \mu\text{M}$ abolishes the accumulation of H_2O_2 and so leads to the uptake of one atom of O_2 per molecule of glucose oxidized. (This is illustrated in Figs. 2, 4 and 6.)

Fig. 1. Decomposition, in the presence of catalase, peroxidase and metmyoglobin, of H_2O_2 produced by the glucose oxidase system. Effect of varying haemoprotein concentrations on the percentage of generated H_2O_2 split catalytically. 100% utilization indicates an O_2 uptake of 0.5 mole O_2 /mole glucose (i.e. all H_2O_2 decomposed); zero utilization indicates an uptake of 1 mole O_2 /mole glucose (H_2O_2 accumulating). These reactions are summarized in Table 1 (lines 3 and 1 respectively). The experimental conditions are given in Table 2 (a, c, e, g), no hydrogen donor being present. Catalase, $\square$ --- $\square$; peroxidase, $\bullet$ — $\bullet$; metmyoglobin, $\circ$ — $\circ$.

Oxidation of nitrite

The experimental results on the coupled oxidation of nitrite are given in Fig. 2. The amount of nitrite oxidized was obtained from estimation of residual nitrite by the Griess reaction (Table 3). At the initial stages of the reaction it is apparent that nitrite has an inhibitory action on glucose oxidase both when H_2O_2 is allowed to accumulate (Fig. 2b) and when the H_2O_2 is utilized (d and f). The expected O_2 uptakes, however, are eventually achieved. In confirmation of earlier work (see Introduction) it has been found that both catalase (d) and peroxidase (f) can bring about a coupled oxidation of nitrite. In presence of these haemoproteins the amount of nitrite disappearing is greater than the expected 16.7 μmoles and in fact a slow oxidation of nitrite occurs in absence of haemoproteins. Thus in experiment b the final O_2 uptake slightly exceeds twice the theoretical volume for gluconic acid formation and the net uptake after addition of catalase is greater in b than in a. These results indicate a small spontaneous oxidation of nitrite which is independent of H_2O_2 . The formation of free nitrous acid would be expected at pH 5.9 and can indeed be detected by shaking 50 μmoles sodium nitrite in 3 ml. buffer in a manometer flask containing Griess reagent in the centre tube. The reagent progressively develops a magenta colour under these conditions, while no colour is observed in a parallel experiment at pH 7.3.

Since nitric oxide will arise from decomposition of nitrous acid an extra O_2 uptake will occur.

The catalytic efficiencies of the three haemoproteins in the coupled oxidation of nitrite can be expressed as $pC = 5.1$ for catalase, 6.2 for peroxidase and 4.5 for metmyoglobin (Fig. 3).

Fig. 2. Coupled oxidation of nitrite in presence of catalase and peroxidase by H_2O_2 generated in the glucose oxidase system. 50 μg . notatin; 3.0 mg. glucose (16.7 μmoles); 50 μmoles sodium nitrite; 0.1 μmole catalase; 0.05 μmole peroxidase; pH = 5.9; temp. = 20° . All flasks contained glucose and glucose oxidase. Additional components as follows: a, none; b, nitrite; c, catalase; d, catalase + nitrite; e, peroxidase; f, peroxidase + nitrite. The dotted lines indicate the evolution of O_2 on addition of 0.01 μmole catalase at the conclusion of oxygen uptake.

Fig. 3. Coupled oxidation, in presence of catalase, peroxidase and metmyoglobin, of sodium nitrite (50 μmoles) by H_2O_2 generated in the glucose oxidase system. Effect of varying haemoprotein concentrations on the percentage of generated peroxide that is used for oxidation of nitrite. In contrast to Fig. 1, 100% utilization indicates an O_2 uptake of 1 mole O_2 /mole glucose oxidized since an equimolecular quantity of nitrite is simultaneously oxidized ($\text{C}_6\text{H}_{12}\text{O}_6 + \text{NaNO}_2 + \text{O}_2 = \text{C}_6\text{H}_{12}\text{O}_7 + \text{NaNO}_3$). Zero utilization indicates a net O_2 uptake, after decomposition of any accumulated H_2O_2 (see Fig. 2), of 0.5 mole O_2 . The experimental conditions are given in Table 2. Catalase, $\square$ --- $\square$; peroxidase, $\bullet$ — $\bullet$; metmyoglobin, $\circ$ — $\circ$.

Oxidation of formate

Since formate is oxidized to CO_2 , 10 % NaOH and filter paper were placed in the centre tubes of the manometer flasks. These experiments, which are summarized in Figs. 4 and 5 and Table 3, show that formate has no effect upon glucose oxidase itself, the initial rate of O_2 uptake in presence of formate (Fig. 4, curve *b*) being slightly higher than in its

Fig. 4. Coupled oxidation of formate in presence of catalase and peroxidase by H_2O_2 generated in the glucose oxidase system. 50 μmoles sodium formate; experimental conditions otherwise as in Fig. 2. For explanation of dotted lines see Fig. 2.

Fig. 5. Coupled oxidation in presence of different concentrations of catalase, peroxidase and metmyoglobin of sodium formate (50 μmoles) by H_2O_2 generated in the glucose oxidase system. See legend to Fig. 3.

absence. The coupled oxidation of formate by catalase at high concentration utilized only about 80 % of generated H_2O_2 ; the remaining 20 % was decomposed catalytically (*d*). Estimation of residual formate showed in fact that only 13.4 μmoles underwent coupled oxidation instead of the maximum possible 16.7.

Experiments on the catalytic efficiency of the three haemoproteins in the coupled oxidation of formate (Fig. 5) showed that only catalase has an appreciable catalytic activity, the *pC* value being 5.1. Peroxidase is completely inactive, even at 10^{-4}M , while the activity of metmyoglobin is only just significant. Although extrapolation suggests that for the latter *pC* is approximately 2, such a value has no meaning since it corresponds to a 17 % (w/v) solution of the haemoprotein.

Table 3. Coupled oxidation of nitrite and formate in the standard manometric test (see Table 2)

0.1 μmole haemoprotein; 50 μmoles nitrite or formate. Theoretical figure for complete utilization of H_2O_2 generated in the glucose oxidase system = 16.7 μmoles (equivalent to the glucose added).

	μmoles hydrogen donor oxidized	
	Sodium nitrite	Sodium formate
Notatin + glucose + donor	3.1	0
Notatin + glucose + donor + catalase	19.0	13.4
Notatin + glucose + donor + peroxidase	21.6	1.9

Oxidation of aldehydes

All attempts to confirm directly that formaldehyde can undergo coupled oxidation by catalase were unsuccessful. The addition of 50 μmoles formaldehyde in the standard test caused a complete inhibition of glucose oxidase, while as little as 5 μmoles gave rise to a progressive inhibition which was nearly complete after 1 hr. D-Amino acid oxidase from pig kidney (Negelein & Brömel, 1939) was equally sensitive to formaldehyde. Indirect evidence for a coupled oxidation of formaldehyde by the glucose oxidase system and catalase was obtained as follows: we had already established (Keilin & Hartree, 1945b) that methanol, like ethanol, undergoes a rapid oxidation in this system. If the oxidation of methanol were to stop at the formaldehyde stage, the formaldehyde produced should have a marked inhibitory action long before the theoretical 16.7 μmoles are formed. The absence of inhibition suggests that formaldehyde is oxidized further, and experiments in which the final formaldehyde concentrations were determined by the phloroglucinol method (Rona, 1929) showed that this was indeed the case. A doubling of O_2 uptake in presence of methanol and catalase was accompanied by the formation of much less than 50 % of the expected quantity of formaldehyde. It is therefore justifiable to conclude that less than one mole of methanol is oxidized per mole of glucose and that the doubling of O_2 uptake is achieved by further oxidation of the resulting formaldehyde. It is of interest that in our first paper on coupled oxidation

of alcohols it was shown that the oxidation of methanol in the uricase system led to a progressive inhibition of the rate of O_2 uptake: an effect which was not noticeable with ethanol and *n*-propanol (Keilin & Hartree, 1936*a*, Fig. 2).

Acetaldehyde underwent slight coupled oxidation in presence of catalase but not in presence of peroxidase, while salicylaldehyde showed appreciable oxidation only in presence of peroxidase. However, this is probably due to its phenolic character, since neither piperonal nor benzaldehyde was attacked under the same conditions.

Oxidation of ascorbic acid

The results of experiments on the coupled oxidation of ascorbic acid (AA) (Fig. 6 and Table 4) show that catalase does not promote this reaction (curve *AC*); on the contrary it protects AA from the considerable oxidation that occurs in its absence (*A*). Since coupled oxidation of AA takes place in absence and in presence of peroxidase (*Ap*) the true catalytic activity of the latter could not be ascertained. However, very different results were obtained in presence of ethylenediaminetetraacetic acid (EDTA). While the coupled oxidation of AA in absence of peroxidase is virtually abolished by EDTA so that H_2O_2 accumulates (*AE*), the more complete coupled oxidation that is obtained in presence of peroxidase is unaffected by EDTA (curves *Ap* and *ApE*). Furthermore, the slight oxidation induced by catalase (Table 4) was also completely abolished by EDTA. The concentration of this chelating agent in all the above experiments was sufficient to abolish the oxidation of AA by traces of catalytic metals present in the solutions.

These experiments also show that EDTA abolishes the inhibition by AA of the initial rate of oxidation of glucose (*A* and *AE*). This inhibition is in fact due to a partial destruction of notatin during the metal-catalysed oxidation of AA. This was demonstrated by experiments in which glucose was oxidized in presence of AA and/or EDTA and the residual notatin activity then determined by

addition of a second 3 mg. portion of glucose together with catalase, in the presence of which AA has no inhibitory effect. Details of the manometric fillings are shown in Table 5 (A, i-vi) while the curves

Fig. 6. Coupled oxidation of ascorbic acid in presence of catalase and peroxidase by H_2O_2 generated in the glucose oxidase system. Effect of ethylenediaminetetraacetic acid (EDTA). In all experiments 3 mg. glucose was oxidized in presence of 50 μ g. notatin. Additional components as follows: *A*, 50 μ moles ascorbic acid; *E*, 2 mM EDTA; *C*, 0.1 μ mole catalase; *P*, 0.01 μ mole peroxidase; *p*, 0.001 μ mole peroxidase; (*O* indicates notatin and glucose only); pH = 5.9; temp. = 20°. The dotted lines indicate the evolution of O_2 on addition of 0.01 μ mole catalase.

Table 4. Coupled oxidation of ascorbic acid (AA; 50 μ moles) in presence and in absence of ethylenediaminetetraacetic acid (EDTA; 2 mM)

Experimental conditions as in Table 3.

	μ moles AA oxidized	
	Without EDTA	With EDTA
Notatin + glucose + AA	16.4	1.1
Notatin + glucose + AA + catalase	2.6	0
Notatin + glucose + AA + peroxidase	16.4	16.5

Table 5. Order of filling manometers to test conditions under which ascorbic acid irreversibly inhibits notatin

The results are plotted in Fig. 7, where curves are numbered to correspond with the experiments enumerated here. 50 μ g. notatin; 3 mg. glucose in 0.2 ml.; 50 μ moles ascorbic acid (AA) in 0.2 ml. (neutralized); 2 mM ethylenediaminetetraacetic acid (EDTA); 0.001 μ mole catalase; phosphate buffer, pH 5.9; temp. = 20°.

A		B	
Added at zero time		Added at 120 min. (taps opened)	Added at 130 min. (taps closed)
i Notatin + buffer to 2.8 ml.	Glucose	Catalase	Glucose
ii Notatin + buffer to 2.6 ml.		Catalase	Glucose + AA
iii Notatin + AA + buffer to 2.6 ml.		Catalase	Glucose
iv As i + EDTA		Catalase	Glucose
v As ii + EDTA		Catalase	Glucose + AA
vi As iii + EDTA		Catalase	Glucose
vii Notatin + AA + catalase + buffer to 3.1 ml.		—	—
viii As vii + EDTA		—	—

of O_2 uptake, numbered to correspond with the table, appear in Fig. 7A. As would be expected from previous experiments, only curve iii shows any significant variation from the control curve i or from the latter experiment with added EDTA (iv). Experiments vii and viii show that in presence of catalase there is no inhibition of the primary reaction by AA. After 120 min. O_2 uptake had virtually ceased and the right-hand flasks of the manometers were then removed and catalase was added.

Fig. 7. O_2 uptakes of systems listed in Table 5. Curves A correspond to section A of the table; curves B indicate the additional O_2 uptake on adding the components of section B.

AA and glucose were then introduced as indicated in Table 5 B, the glucose being placed in dangling cups. After equilibration of the manometers the dangling cups were dislodged. The O_2 uptakes recorded in these experiments are given in Fig. 7B. Since in presence of catalase AA does not inhibit notatin, the marked fall in activity shown in experiment iii (B) (representing $\sim 67\%$ of the initial rate as compared

with experiment i) must be due to an irreversible loss of enzyme activity occurring during the first 120 min.

Oxidation of ferrocyanide

Since ferrocyanide has been shown to react as a hydrogen donor with metmyoglobin-peroxide (George & Irvine, 1952, 1953), peroxidase-peroxide (Kenten & Mann, 1951; George, 1952b) and catalase-methylhydroperoxide (George, 1952a), it was of interest to follow manometrically the coupled oxidation of ferrocyanide catalysed by the above haemoproteins. The results of these experiments, which are shown in Fig. 8, can be summarized

Fig. 8. Coupled oxidation of ferrocyanide in presence of catalase, peroxidase and metmyoglobin by H_2O_2 generated in the glucose oxidase system. $50 \mu\text{moles } K_4Fe(CN)_6$; $0.1 \mu\text{mole catalase}$; $0.1 \mu\text{mole peroxidase}$; $0.3 \mu\text{mole metmyoglobin}$. Conditions otherwise as in Fig. 2. All flasks contained glucose and glucose oxidase. Additional components as follows: a, none; b, ferrocyanide; c, peroxidase + ferrocyanide; d, peroxidase or catalase; e, catalase + ferrocyanide; f, metmyoglobin; g, metmyoglobin + ferrocyanide; pH = 5.9; temp. = 20° . The dotted lines indicate the evolution of O_2 on addition of $0.01 \mu\text{mole catalase}$.

as follows: (i) A certain amount (about 25%) of H_2O_2 generated by glucose oxidase is utilized in the oxidation of ferrocyanide in absence of haemoproteins. This is shown by comparing the descending portions of curves a and b indicating the liberation of O_2 following the addition of catalase. (ii) Peroxidase and metmyoglobin at a concentration of not less than $30 \mu\text{M}$ catalyse well the coupled oxidation of ferrocyanide (c and g). Catalase on the contrary scarcely oxidizes it (e). (iii) As already shown,

metmyoglobin can act as a feeble catalase, but a true estimate of its activity is difficult to assess by the present method, as it may itself undergo a coupled oxidation. Thus curve *f* indicates an O_2 uptake well in excess of one atom (187 μ l.) /mole glucose, yet very little residual H_2O_2 was found on addition of catalase. Furthermore, the flask contents showed a distinctly greenish tinge indicating that metmyoglobin had been partly oxidized to a bile-pigment derivative (see also Keilin & Hartree, 1945*b*). Under these experimental conditions the metmyoglobin acts partly as a catalase decomposing about 50 % of generated H_2O_2 and partly as a hydrogen donor undergoing coupled oxidation. In fact the presence of ferrocyanide protects metmyoglobin from partial peroxidatic degradation.

The catalytic efficiency of the three haemo-proteins in the coupled oxidation of ferrocyanide is shown in Fig. 9, the values of *pC* being 8.2 for

Fig. 9. Coupled oxidation in presence of different concentrations of catalase, peroxidase and metmyoglobin of potassium ferrocyanide (50 μ moles) by H_2O_2 generated in the glucose oxidase system. See legend to Fig. 3.

peroxidase and 5.5 for metmyoglobin. There is no evidence that catalase can bring about a coupled oxidation of ferrocyanide: in fact it protects ferrocyanide from oxidation.

Oxidation of azide

The reaction between catalase-azide and H_2O_2 has been extensively investigated, and it is now established that during this reaction the iron of catalase undergoes reduction to the bivalent level (Keilin & Hartree, 1936*b*, 1945*a*, 1951*a*). This reaction was recently reinvestigated by Theorell & Ehrenberg (1952), who confirmed our results as to the valency changes of catalase iron and found also that azide undergoes oxidation to nitrous oxide and nitrogen. We were able to corroborate their observations, but since nitrogen and nitrous oxide are the main products of this reaction the coupled oxidation could not be followed manometrically in the same way as that of other hydrogen donors. Nevertheless, the reactions were carried out in manometer flasks, which were shaken in the usual way, in order to simulate the conditions of the previous tests. Estimations of the amounts of glucose and azide disappearing were made after deproteinization of the reaction mixtures. The results of these experiments, which are summarized in Table 6, show that although a certain small amount of azide appears to be oxidized by peroxide itself, it is mainly catalase and not peroxidase or metmyoglobin that is capable of catalysing its coupled oxidation. In this reaction the formation of a very small amount of nitrite was readily detected. The significance of this coupled reaction will be discussed later.

Table 6. *Coupled oxidation of azide in Barcroft manometers by the glucose oxidase system. Effect of haemoproteins*

n: 50 μ g. notatin; *g*: 3 mg. glucose (16.7 μ moles); *c*: catalase (0.1 μ mole); *p*: peroxidase (0.05 μ mole); *m*: metmyoglobin (0.1 μ mole). 2 ml. buffer; final vol: 3 ml. At the end of the experiment the solutions were deproteinized and analysed for glucose (anthrone) and azide (ferric chloride). Experiments 1*a*, 2*a*, 1*c*, 2*c* were repeated and when O_2 uptakes had ceased the solutions were treated with 4 ml. 0.11*N*-Ba(OH)₂, filtered and tested for nitrite with Griess' reagent. Only 2*c* gave a positive result.

Expt. no.	Enzyme system	Azide added (μ moles)	Duration of expt. (min.)	Glucose oxidized (μ moles)	Azide disappearing (μ moles)
1 <i>a</i>	<i>n g</i>	0	100	16.7	—
2 <i>a</i>	<i>n g c</i>	0	100	16.7	—
3 <i>a</i>	<i>n g p</i>	0	100	16.7	—
4 <i>a</i>	<i>n g m</i>	0	100	16.7	—
1 <i>b</i>	<i>n g</i>	10	50	11.2	2.1
2 <i>b</i>	<i>n g c</i>	10	50	13.0	10
3 <i>b</i>	<i>n g p</i>	10	50	11.5	1.8
4 <i>b</i>	<i>n g m</i>	10	50	11.4	0.5
1 <i>c</i>	<i>n g</i>	50	100	14.3	1.1
2 <i>c</i>	<i>n g c</i>	50	100	12.0	19.5
3 <i>c</i>	<i>n g p</i>	50	100	13.8	3.1
4 <i>c</i>	<i>n g m</i>	50	100	14.2	2.5

Oxidation of phenols

The ability of haemoproteins to catalyse the coupled oxidation of different phenols was assessed manometrically in presence of 50 μ moles phenol and 0.1 μ mole haemoprotein. At such concentrations all three haemoproteins were found to catalyse to varying degrees the oxidation of most of the phenols tested (Table 7). When using pyrogallol it is necessary to introduce NaOH to absorb CO_2 .

haemoprotein concentration. The two methods of expressing activities are, however, related in as much as 50% in Table 7 corresponds to $pC=4.5$. The pC values for the oxidation of pyrogallol, *p*-cresol, guaiacol and adrenaline are given in Table 8. In the case of pyrogallol (see also Fig. 10) the values are 5.5, 9.3 and 4.8 for catalase, peroxidase and metmyoglobin respectively. Here the activity of catalase is exceptionally high in comparison with its effect on other phenols (see Table 7).

Table 7. *Coupled oxidation of phenolic hydrogen donors by H_2O_2 generated through the oxidation of 3 mg. glucose in presence of notatin*

The catalytic effects of the three haemoproteins at a constant concentration (30 μM) are compared in terms of the percentage utilization of H_2O_2 . The experiments were carried out as described in Table 2 and at the conclusion of O_2 uptake catalase was added, where not already present, in order to determine accumulated H_2O_2 (as in Fig. 2). The oxidation of glucose accounts for 187 μl . O_2 and the figures given below are the percent excess of the net recorded uptakes above 187 μl .

Hydrogen donor	Percentage utilization of peroxide in presence of haemoproteins		
	Catalase	Peroxidase	Metmyoglobin
Phenol	14	44	53
Catechol	28	33	38
Quinol	33	20	28
Resorcinol	14	98	80
Pyrogallol	94	96	66
Guaiacol	6	100	83
3:4-Dihydroxyphenylalanine	0	81	87
Adrenaline	14	107	100
<i>m</i> -Cresol	3	66	47
<i>p</i> -Cresol	47	94	50
<i>o</i> -Cresol	0	62	0

Fig. 10. Coupled oxidation in presence of different concentrations of catalase, peroxidase and metmyoglobin of different amounts of pyrogallol by H_2O_2 generated in the glucose oxidase system. See legend to Fig. 3. NaOH was present to absorb CO_2 . Figures in brackets indicate μ moles pyrogallol added. Catalase [50], $\square$ --- $\square$; catalase [500], $\square$ --- $\square$; peroxidase [50], $\bullet$ — $\bullet$; peroxidase [25], $\circ$ — $\circ$; peroxidase [10], $\ominus$ — $\ominus$; metmyoglobin [50], $\circ$ — $\circ$.

Under standard metal-free conditions no significant coupled oxidations were observed in absence of haemoprotein. This method of comparing activities does not provide quantitative relationships between the activities of the three haemoproteins, as do pC determinations, since the ratio of the three values for the oxidation of each phenol will vary with

A cyclic oxidation reaction

Under the term cyclic oxidation we described a coupled oxidation reaction whereby H_2O_2 generated by xanthine oxidase (= Schardinger enzyme) oxidizing acetaldehyde to acetic acid is used by catalase for peroxidatic oxidation of ethanol to acetaldehyde, the secondary oxidation thus generating the substrate for the primary reaction (Keilin & Hartree, 1936*a*). With purified catalase and crude xanthine oxidase (caseinogen preparation) the amount of ethanol that could be oxidized to acetic acid was four to five times larger than the amount of acetaldehyde added at the beginning of the experiment (Keilin & Hartree, 1945*a*). We were recently able to repeat these experiments using pure catalase and crystalline xanthine oxidase. The complete system consisted of xanthine oxidase, catalase, ethanol (25 or 50 μ moles) and acetaldehyde (3.5 μ moles) made up in phosphate buffer to 3.0 ml. The aldehyde, as a solution in 0.2 ml. buffer, was placed in a dangling cup. The cup was not dislodged after the manometer taps had been closed but the aldehyde was left to diffuse into the enzyme solution. While the O_2 uptake in absence of ethanol corresponded closely to the theoretical value of 39 μl ., for

Table 8. *Catalytic efficiencies, in terms of pC, of catalase, peroxidase and metmyoglobin in coupled oxidations of various hydrogen donors under the conditions described in the legends to Figs. 1 and 3*

A: Phenols; B: other donors discussed in the present paper.

	Catalase	Peroxidase	Metmyoglobin
A Pyrogallol	5.5	9.3	4.8
<i>p</i> -Cresol	4.5	6.5	4.5
Guaiacol	~0	8.3	5.0
Adrenaline	~0	7.9	5.3
B Hydrogen peroxide	9.6	5.9	~4*
Ethanol	5.5	0	0
Formate	5.1	0	~0
Nitrite	5.1	6.2	4.5
Ferrocyanide	~0	8.2	5.5
Ascorbic acid	0	7.7	†
(in presence of EDTA)			

The symbol ~0 indicates that slight activity was detected but that 50% peroxide utilization could not be achieved owing to practical limitations of haemoprotein concentration. Absence of activity at all experimental concentrations is denoted by 0.

* This value is approximate since some destruction of metmyoglobin occurs.

† Reduction to myoglobin occurs. In this form it exhibits no catalytic activity.

the aldehyde added, the presence of 25 μ moles ethanol increased the final O_2 uptake to 600 μ l., i.e. the expected figure for the oxidation of both aldehyde and ethanol to acetic acid (Fig. 11). An almost theoretical O_2 uptake was obtained in presence of 50 μ moles ethanol. In absence of aldehyde there was of course no oxidation. The only limits to the amount of alcohol that can be oxidized would thus appear to be the stability of the two enzymes xanthine oxidase and catalase and in particular the protective action of catalase towards xanthine oxidase which, unlike notatin, is readily inactivated by H_2O_2 if accumulation of the latter is not prevented.

DISCUSSION

Peroxidatic reactions catalysed by catalase, peroxidase and metmyoglobin can be followed (a) by the disappearance of hydrogen donor or the formation of its oxidation product, (b) by changes in the absorption spectra of haemoproteins in presence of H_2O_2 and donors and (c) by disappearance of substrate (H_2O_2) determined manometrically.

The manometric study of peroxidatic reactions described in the present paper was carried out in presence of the primary oxidation system composed of glucose oxidase (notatin) and glucose generating H_2O_2 which was utilized by haemoproteins for catalysis of coupled or peroxidatic oxidation of different donors. Since in our experiments the concentrations of notatin and glucose, as well as other experimental conditions, were fixed, the formation of H_2O_2 was standardized and the coupled oxidation of different donors by haemoproteins could be followed manometrically under strictly comparable conditions.

Fig. 11. Cyclic oxidation of ethanol by the Schardinger enzyme system (=xanthine oxidase+aldehyde) in presence of catalase. 0.2 ml. xanthine oxidase (0.9% solution of crystals); 0.1 μ mole catalase; 3.5 μ moles acetaldehyde; ethanol as indicated; 0.1M phosphate buffer, pH 7.8; temp. = 30°. The aldehyde, as 0.2 ml. solution in buffer, was allowed to diffuse by vaporization from the dangling cups which were not dislodged. Figures in brackets indicate the theoretical O_2 uptakes in μ l. for complete oxidation to acetate. (1): xanthine oxidase + aldehyde + catalase [39]; (2): as 1 + 25 μ moles ethanol [600]; (3): as 1 + 50 μ moles ethanol [1160].

Following the demonstration that catalase can use H_2O_2 for peroxidatic oxidation of alcohols (Keilin & Hartree, 1936*a*, 1945*b*) the concept of the peroxidatic activity of catalase was extended to the oxidation of other substances including H_2O_2 itself which thus acts both as substrate and hydrogen donor (Chance, 1948, 1949*b*). The catalatic decomposition by haemoproteins of H_2O_2 generated by the glucose oxidase system can therefore be considered as a coupled or peroxidatic oxidation of H_2O_2 in the same sense as the coupled oxidation of other donors such as ethanol. However, the experimental conditions for oxidation of H_2O_2 differ from those for any other donor in two respects: (1) While H_2O_2 is present only in the amounts generated by glucose oxidase at the initial rate of about 7.6×10^{-6} mole litre⁻¹ sec.⁻¹ and reaching a final concentration, if accumulation is not prevented, of $16.7 \mu\text{moles}$ per manometer flask, other donors are added at an initial concentration of $50 \mu\text{moles/flask}$. (2) While coupled oxidation of generated H_2O_2 brings the final net O_2 uptake by glucose oxidase down to half mole O_2 /mole glucose oxidized, the coupled oxidation of other donors doubles this O_2 uptake (see Figs. 1 and 3). Moreover, in coupled oxidation reactions a haemoprotein-peroxide compound may react with and oxidize, with different efficiencies, two hydrogen donors present simultaneously: the continuously generated H_2O_2 , available at a very low concentration, and added donor present in great excess.

The range of donors susceptible to oxidation by a haemoprotein will increase as its concentration is increased and therefore any survey of donors by manometric methods involving fixed haemoprotein concentrations (as in Table 7) will not give information on the relative efficiencies of the haemoproteins as catalysts. By carrying out the manometric determinations at varying haemoprotein concentrations it is possible to obtain a measure of catalytic efficiency in terms of the molar concentration of haemoprotein that brings about utilization of 50% of generated H_2O_2 for the oxidation of the donor in question. We have expressed the negative logarithm of this critical concentration by the term pC .

The results of our comparative studies of coupled oxidations of different donors catalysed by catalase, peroxidase and metmyoglobin can be summarized as follows:

(1) Peroxidatic oxidation of H_2O_2 (in other words its catalatic decomposition) is a property common to all haemoproteins as well as to free haematin and iron. However, on the basis of pC the activities of peroxidase and metmyoglobin are respectively 5000 and 400 000 times lower than that of catalase. There is considerable uncertainty in the figure for metmyoglobin since in presence of H_2O_2 it becomes reduced to the ferrous forms haemoglobin and oxyhaemoglobin and these are to a considerable extent

oxidized destructively with fission of the haem nucleus.

(2) Contrary to the view previously expressed that catalase iron is accessible only to small molecules (Chance, 1949*a*), catalase was found able to catalyse coupled oxidations of phenols such as catechol, quinol, *p*-cresol and especially pyrogallol. It is true that the standard methods for measuring peroxidatic activity, which involve the addition of a solution of H_2O_2 , would reveal no activity since all H_2O_2 is then decomposed catalatically. The low concentration of H_2O_2 present in our experiments provides favourable conditions for the detection of peroxidatic activity of catalase. However, peroxidase has a much higher activity in the oxidation of pyrogallol than has catalase. In fact the relative activities of catalase and peroxidase towards pyrogallol are exactly the reverse of those towards H_2O_2 (Fig. 12).

Fig. 12. Comparison of the activities of catalase, peroxidase and metmyoglobin in the coupled oxidation of three hydrogen donors; hydrogen peroxide, pyrogallol and ethanol. Catalase, ---; peroxidase, —; metmyoglobin, ····; *h*, hydrogen peroxide (curves taken from Fig. 1); *p*, pyrogallol (curves taken from Fig. 10); *e*, $50 \mu\text{moles}$ ethanol (experimental conditions as in Fig. 3). With this donor peroxidase and metmyoglobin are completely inactive.

The activity of peroxidase remains unchanged ($pC=9.3$) even when the concentration of pyrogallol is lowered to $10 \mu\text{moles}$ per flask (in which case it is quantitatively oxidized) and the activity is not affected by the presence of $1.5 \times 10^{-9} \text{M}$ catalase which alone would be capable of decomposing catalatically about 80% of the generated H_2O_2 (Fig. 1). This confirms the results previously obtained by Knox (1954). Catalase present at higher concentrations: 1.5×10^{-8} , 1.5×10^{-7} , $1.5 \times 10^{-6} \text{M}$ lowers the peroxidase efficiency in the coupled oxidation of pyrogallol from $pC 9.3$ to 8.8 , 8.2 and 6.9 respectively.

The activities of metmyoglobin towards H_2O_2 and pyrogallol, i.e. its catalatic and peroxidatic activities, are about the same. One must, however, always remember the marked differences in the concentrations of these two donors and the fact that metmyo-

globin partly undergoes an irreversible destruction in absence of added donor.

(3) Catalysis of the coupled oxidation of alcohols by catalase is remarkable in two respects. First, the reaction carried out in presence of H_2O_2 generated in the glucose oxidase system is catalysed specifically by catalase—the other haemoproteins being completely inactive—and secondly ethanol was the first example of a donor which was not in itself an oxygen-sensitive substance. All previously known 'peroxidase substrates' (donors) as well as H_2O_2 were readily autoxidizable in presence of trace heavy metals and the peroxidatic activity of haemoproteins could therefore be regarded as an acceleration of more or less appreciable spontaneous reactions.

The catalatic decomposition of H_2O_2 is, on the contrary, a property common to all haematin compounds, and to many other substances, although the activity of catalase in this reaction is by far the highest. It was no doubt the impressively high, almost explosive, decomposition of H_2O_2 in presence of catalase that led to a tacit assumption that this reaction must represent the physiological function of the enzyme. The discovery of the coupled oxidation of ethanol, however, has led to the generally accepted view that the function of catalase is mainly peroxidatic. The efficiency of catalase in the coupled oxidation of H_2O_2 is about 12 500 times greater than in the oxidation of ethanol. This agrees reasonably with the ratio of the velocity constants (k_4) of the reactions of the primary catalase peroxide compounds with H_2O_2 and with ethanol respectively, which Chance, Greenstein & Roughton (1952) estimated to be about 35 000. The lower value obtained in our manometric experiments may be related to the very great difference between the concentration of added ethanol and the low steady-state concentration of H_2O_2 , i.e. between the concentrations of H-donor in the two types of reaction.

An interesting development in the study of the coupled oxidation of ethanol was the discovery of cyclic oxidation in which the substrate for the primary, H_2O_2 -producing, reaction is the product of the secondary peroxidatic reaction. Thus H_2O_2 generated in the oxidation of acetaldehyde to acetate in presence of xanthine oxidase will, in presence of catalase, oxidize ethanol to acetaldehyde. In this way a continuous supply of aldehyde is made available for the primary oxidation, and O_2 uptake will continue until all ethanol has been oxidized to acetate. In such a reaction it should be possible to replace acetaldehyde by any system which produces H_2O_2 and, in fact, a cyclic mechanism can be induced if aldehyde is replaced, in the experiments of Fig. 11, by 1 μg . notatin plus 0.5 mg. glucose. In cyclic oxidations the steady-state concentrations of H_2O_2 and aldehyde may well be so

low that they cannot be detected: furthermore, while the oxidation will be sensitive to inhibitors of both catalase and xanthine oxidase, such inhibitors will not cause accumulation of either H_2O_2 or aldehyde. These facts emphasize the difficulties that could arise in attempts to analyse, by means of specific inhibitors, an *in vivo* oxidation which happened to be of the cyclic type. That such reactions may occur in nature is indicated by the coexistence, e.g. in liver, of catalase with xanthine oxidase and other peroxide-producing systems.

(4) While the coupled oxidation of lower alcohols does not proceed beyond the aldehyde or ketone in presence of catalase, methanol may be oxidized completely to CO_2 . It has not been possible to establish directly the coupled oxidation of formaldehyde by our manometric method in view of its high toxicity. It can nevertheless be inferred that, in the coupled oxidation of methanol, the resulting formaldehyde can itself undergo peroxidatic oxidation. Of the three haemoproteins only catalase is markedly effective in the coupled oxidation of formate but the efficiency here is somewhat lower than in the case of ethanol ($pC = 5.1$ against 5.5). The pH dependence of the reaction between the primary catalase- H_2O_2 compound and formate revealed, according to Chance (1952*a*), that the undissociated acid is the true hydrogen donor. Thus, while the measured velocity constant of this reaction at pH 7.2 was found to be about $470\text{M}^{-1}\text{sec}^{-1}$, he derives a constant as high as 10^6 for the undissociated acid.

(5) A coupled oxidation of nitrite was first demonstrated by Thurlow (1925), who used the xanthine oxidase-hypoxanthine system, as a source of H_2O_2 , together with milk peroxidase. Elliott (1932) and later Dixon (1934) compared the activities of milk and horse-radish peroxidases towards a number of donors but used a solution of H_2O_2 instead of H_2O_2 generated enzymically. However, they could obtain peroxidatic oxidation of nitrite only with the milk enzyme. Since this earlier work was carried out with crude enzyme preparations it is not possible to decide whether the reported inactivity of horseradish peroxidase towards nitrite was a property of the particular preparation or of its inactivation by components of the reaction. It has now been established that horseradish peroxidase catalyses the coupled oxidation of nitrite, but attempts to carry out the same reaction with a solution of H_2O_2 in place of generated H_2O_2 have revealed that a considerable loss of peroxidase activity occurs. Thus, when a mixture of peroxidase and nitrite in buffer equivalent to experiment *f* of Fig. 2 was treated with 50 μmoles H_2O_2 instead of notatin plus glucose, an initial oxidation of nitrite was detected but this soon ceased and further additions of H_2O_2 brought

about little or no oxidation. That milk peroxidase is about 14 times more active than horseradish peroxidase in the coupled oxidation of nitrite has recently been shown by Knox (1954).

The catalytic efficiencies of the three haemoproteins in the coupled oxidation of nitrite are not very different but catalase is the most active ($pC=6.3$). The reactions of nitrite with catalase-peroxide compound I and peroxidase-peroxide compounds I and II are pH dependent, the velocity constants increasing about tenfold per unit of pH decrease, which indicates (Chance, 1952*a, b*) that free nitrous acid is probably the actual donor. Thus, the experimental value of the velocity constant $k_4 = 2.5 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ of the rate-determining reaction between peroxidase peroxide II and nitrite becomes $2.4 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ on the basis of free nitrous acid.

(6) The study of ascorbic acid is complicated by its marked autoxidizability in presence of traces of heavy metals. The activity of haemoproteins could be determined only by experiments carried out with metal-free reagents in specially cleaned glassware and in presence of ethylenediaminetetraacetic acid (EDTA). Whereas either careful purification of reagents and cleaning of glassware or the addition of EDTA is sufficient to reduce the autoxidization of ascorbic acid to negligible levels, it was found necessary to take both precautions in order to render insignificant the coupled oxidation by H_2O_2 that otherwise occurs in absence of haemoproteins (Table 4 and Fig. 6). Under such conditions it was possible to establish that catalase does not promote the coupled oxidation of ascorbic acid, whereas peroxidase catalyses this reaction very effectively, the pC reaching a value of 7.7. Metmyoglobin also catalyses the peroxidatic oxidation of ascorbic acid but this reaction is complicated by reduction of metmyoglobin to haemoglobin followed by oxygenation and irreversible modification of the haem nucleus. The presence of EDTA has an additional advantage in that it prevents a destruction of glucose oxidase that occurs, even under the best obtainable metal-free conditions, when the oxidation of glucose takes place in presence of ascorbic acid (Fig. 7).

(7) Ferrocyanide was found to undergo efficient coupled oxidation promoted by peroxidase, to a lesser extent by metmyoglobin but not at all by catalase. In fact, catalase abolishes the slight oxidation of ferrocyanide that occurs in absence of haemoprotein. In this reaction peroxidase shows a very high catalytic efficiency, with pC about 8.3 while the figure for metmyoglobin is only 5.5.

(8) Studies of the peroxidatic oxidation of azide have been fruitful in throwing light upon the problem of the mechanism of decomposition of H_2O_2 by catalase.

Azide is well known as a strong inhibitor of cellular respiration and of metallo-protein enzymes (Keilin, 1936). With haemoproteins it gives spectroscopically well-defined compounds, the formation of which requires one molecule of azide per iron atom of haematin. Thus with brown acid methaemoglobin it forms a red compound with absorption bands at 575 and 543 $m\mu$. On the other hand, catalase when combined with azide does not change its colour or the general pattern of its absorption spectrum. Its characteristic band in the red however becomes sharper and moves slightly towards the blue end of the spectrum (Keilin & Hartree, 1936*b*, 1945*a*). Whereas methaemoglobin and catalase combine with azide over a wide range of pH, peroxidase reacts with azide only between pH 4 and 4.5, forming a red compound with a prominent band at 534 $m\mu$. (Theorell, 1942; Keilin & Hartree, 1951*a*).

When catalase-azide is treated with H_2O_2 , the colour of the solution turns from greenish brown to red and the methaemoglobin-like absorption spectrum is replaced by two bands at 587 and 554 $m\mu$. That the iron of this red derivative of catalase is bivalent clearly follows from the facts that it readily autoxidizes to the original catalase-azide and that it forms a CO derivative with bands at 580 and 545 $m\mu$. (Keilin & Hartree, 1945*b*). These findings led us to postulate the view that the catalytic activity of free catalase towards H_2O_2 , like that of catalase-azide, is accompanied by valency changes of the iron, which is reduced by peroxide molecules undergoing catalatic decomposition and reoxidized by oxygen. Inhibition of the catalatic reaction by azide was ascribed by us to stabilization of ferrocatalase; that is, to a slowing down of its reoxidation.

That the reaction between catalase azide and H_2O_2 is accompanied by valency changes of catalase iron was recently confirmed by Theorell & Ehrenberg (1952). However, they found that in this reaction azide undergoes peroxidatic oxidation to nitrous oxide and nitrogen, and they suggested that the ferrous derivative of catalase is either an azide or a nitrous oxide complex of ferrocatalase. While we were able to corroborate these observations our experiments revealed that a small amount of nitric oxide is also formed in the reaction, and it is this that combines with the ferrocatalase and stabilizes it as the NO-ferrocatalase complex (Keilin & Hartree, 1954). These results, together with those of our earlier study of the reaction between methaemoglobin and H_2O_2 (Keilin & Hartree, 1950), can now be summarized as follows.

Catalase peroxide (compound I) oxidizes azide peroxidatically undergoing at the same time reduction to ferrocatalase which is stabilized as the less autoxidizable NO-ferrocatalase.

Methaemoglobin peroxide also oxidizes azide peroxidatically and is itself reduced to haemoglobin which is stabilized as oxyhaemoglobin and NO-haemoglobin.

Catalase peroxide on reacting with H_2O_2 oxidizes it to O_2 and water (catalatic reaction) but this violent reaction is too rapid for observation of intermediate catalase compounds.

Methaemoglobin peroxide also oxidizes H_2O_2 , liberating oxygen, and at the same time undergoes reduction to haemoglobin which is stabilized as oxyhaemoglobin.

On comparing these peroxidatic activities of methaemoglobin and catalase, especially towards H_2O_2 itself, it is not surprising that reduction of the iron can so easily be recorded in methaemoglobin but cannot be observed in catalase. In fact this reaction provides in the vicinity of the iron atoms a high localized concentration of O_2 which will oxygenate the bivalent iron of haemoglobin, stabilizing it as oxyhaemoglobin, and thus inhibiting the reaction, whereas it will reoxidize the iron of ferrocatalase as fast as it appears and so promote the overall reaction. Thus in a peroxidatic reaction catalysed by a haemoprotein the chances of detecting it in the ferrous state depend upon the appearance in the vicinity of the iron of a substance capable of combining with the ferrous form of the haemoprotein and so making it less autoxidizable.

Of the few substances which combine with the bivalent iron of haemoproteins, only oxygen and nitric oxide are so far known to be generated by peroxidatic reactions. While oxygen can combine with and stabilize haemoglobin, nitric oxide can combine with and stabilize both haemoglobin and ferrocatalase and thus inhibit their reoxidation. The reduction of catalase iron is thus readily observed when azide or hydroxylamine, but not hydrogen peroxide, act as donors in a peroxidatic reaction catalysed by this enzyme.

SUMMARY

1. A manometric method is described for the study of peroxidatic activities of haemoproteins.

2. The catalytic activities of catalase, peroxidase and metmyoglobin are compared in coupled or peroxidatic oxidations of different hydrogen donors by means of H_2O_2 generated by the glucose oxidase (notatin) system under standardized conditions.

3. The catalytic efficiencies of the three haemoproteins are expressed as pC : the negative logarithm of the molar concentration at which they utilize 50% of generated H_2O_2 for coupled oxidation of a given donor.

4. The following donors have been examined: H_2O_2 , ethanol, aldehydes, formate, nitrite, ascorbic acid, ferrocyanide, phenols and azide.

5. All three haemoproteins are able to catalyse the coupled oxidation of H_2O_2 , i.e. the catalatic decomposition of generated H_2O_2 to O_2 and water. However, in this reaction the efficiencies of peroxidase and especially of metmyoglobin are much lower than that of catalase.

6. Ethanol is oxidized peroxidatically only by catalase. At no concentration are peroxidase, metmyoglobin or free haematin able to promote this reaction which appears to be the only absolutely specific catalytic property of catalase.

7. Cyclic oxidation of ethanol to acetic acid is described, whereby H_2O_2 generated by pure xanthine oxidase and a little acetaldehyde is used by pure catalase to oxidize ethanol to acetaldehyde thus regenerating the substrate for xanthine oxidase. In this reaction both H_2O_2 and acetaldehyde are utilized as fast as they are generated.

8. An almost specific requirement for catalase was demonstrated for formate although a very low activity could be detected at high metmyoglobin concentrations.

9. Coupled oxidation of pyrogallol and certain other phenols is catalysed not only by peroxidase, and to a much lesser degree by metmyoglobin, but also by catalase.

10. The peroxidatic activity of catalase towards pyrogallol, although about 10 000 times lower than that of peroxidase, is somewhat higher than the activity of metmyoglobin and is of the same magnitude as its peroxidatic activity towards nitrite, formate and ethanol. This militates against the view that catalase iron is accessible only to very small donor molecules.

11. Study of the coupled oxidation of ascorbic acid must be carried out under the best attainable metal-free conditions as well as in the presence of a chelating agent such as ethylenediaminetetraacetic acid.

12. Peroxidase shows marked activity in catalysis of coupled oxidations of ascorbic acid and ferrocyanide, metmyoglobin is about 1000 times less active, whereas catalase is completely inactive.

13. Observations that azide in presence of catalase and peroxide undergoes peroxidatic oxidation are confirmed and their bearing upon the mechanism of catalatic decomposition of H_2O_2 is discussed.

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Phosphorylcholine in Rat Tissues

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During a recent investigation on the role of glyceryl-phosphorylethanolamine and glycerylphosphorylcholine in rat-liver lipid metabolism, a large phosphorus-containing spot was observed on two-dimensional chromatograms of the water-soluble phosphorus-containing compounds of liver, in a position which was different from that of the known major components of this fraction. This substance has been isolated and identified as phosphorylcholine. It is present in rat liver at a fairly high concentration and accounts for approximately 9% of the water-soluble phosphorus in the tissue. Evidence has also been obtained that phosphorylcholine is present in the testes, spleen, intestines, kidney and brain of the animal, while only trace amounts occur in muscle, heart and blood.

Inukai & Nakahara (1935) have isolated the picrate of phosphorylcholine from ox liver, but it has been suggested that because of the minute yield obtained (1×10^{-4} %) this might have been formed from lipid material during the drastic procedures used in the isolation (Lundquist, 1947). The latter author has found a high concentration of the ester in fresh human semen, where it is thought to provide a substrate for seminal phosphatase.

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EXPERIMENTAL

Isolation of phosphorylcholine from rat liver. The livers from ten rats were quickly removed, and homogenized in 500 ml. of 10% (w/v) trichloroacetic acid solution. The precipitate was centrifuged down and washed with a further 100 ml. of trichloroacetic acid solution. The combined supernatants were extracted 3 times with equal volumes of ether, and ethanol was added until the mixture contained 70% (v/v). After standing 1 hr. at 0° the precipitate of glycogen was filtered off under reduced pressure using a filter aid. The filtrate was concentrated under reduced pressure to 15 ml., and ethanol again added to 70% (v/v). After standing, a further precipitate of glycogen was removed by filtration and the filtrate concentrated to 10 ml. The solution was then passed through a column of Amberlite IR-100 H⁺ resin (10 cm. $\times$ 0.8 cm.²) and washed through with a further 25 ml. of water. The effluent was concentrated to a small volume and applied as starting strips on two Whatman no. 3 filter-paper chromatograms (40 cm. wide). The chromatograms were developed for 18 hr. with a phenol-NH₃ solvent, the solvent removed in a drying cabinet and marker strips sprayed with acid molybdate (Hanes & Isherwood, 1949) and irradiated with ultraviolet light. The phosphorus-containing substances moving near the edge of the solvent front (R_f approximately 0.8-1.0) were eluted from the paper by extracting it 3 times with 100 ml. of water and the extract was filtered and concentrated to a small volume. It was then applied as the starting