With a larger excess of peroxide some compound I is also formed. The way in which this reaction occurs excludes an explanation based on shifting the position of an equilibrium in the system; instead, peroxide must be used up in irreversible reactions.

4. The peroxide reactions of metmyoglobin, peroxidase and catalase are discussed in terms of the redox properties of the intermediate compounds and the way they are formed.

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The Reaction between Metmyoglobin and Alkyl Hydroperoxides

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In a previous paper, George & Irvine (1952) showed that the reaction between metmyoglobin and hydrogen peroxide was inconsistent with an equilibrium of the enzyme-substrate type, i.e. $E+S \rightleftharpoons ES$. In this reaction the metmyoglobin (MetMb) is oxidized by the peroxide, and the resultant compound is one in which iron is in the effective oxidation state of +4.

Since it was known that alkyl hydroperoxides behaved similarly to hydrogen peroxide in forming a red intermediate compound with methaemoglobin (Keilin & Hartree, 1935), the interactions of these peroxides and MetMb were investigated to see whether the MetMb underwent oxidation of the same type.

In this paper it is shown that spectroscopically the intermediate compounds formed by two alkyl hydroperoxides are the same, and identical with that produced by hydrogen peroxide. The experimental evidence supports a reaction mechanism similar to that suggested for the hydrogen peroxide reaction.

Nomenclature

In the previous papers (George & Irvine, 1951, 1952) the intermediate compound was referred to as a 'complex' partly to preserve continuity with the widespread use of this term for the intermediate compounds formed by peroxides in peroxidase and catalase systems, and partly because such compounds, independent of their chemical structure, may be regarded as co-ordination complexes of iron in the sense that more groups are bound to the iron atom than can be accounted for by the primary valencies of 2 and 3 in its ferrous and ferric states.

In enzyme chemistry however the term complex is usually identified with the enzyme-substrate complex of the Michaelis and Menten theory of enzyme action,

$$E+S \rightleftharpoons ES,$$
$$ES \rightarrow \text{products} + E,$$

or with the modification in which the final reaction is written

$$ES + \text{another reactant} \rightarrow \text{products} + E.$$

The formation and subsequent reactions of the intermediate compound formed from metmyoglobin and peroxides do not correspond to the scheme of chemical

reactions implicit in these generalized equations. The combining proportions are different and the same intermediate is formed by different substrates. For these reasons the purely descriptive term 'intermediate compound' will be used in this and forthcoming papers instead of the term 'complex', and in chemical equations it will be represented by Fe_p^{IV}, following the convention of representing the formula of a metallic compound, in which the oxidation state of the metal is known but not the precise chemical structure of the compound, by its usual symbol combined with the appropriate Roman numeral.

EXPERIMENTAL AND MATERIALS

Methyl and ethyl hydroperoxides were prepared by the action of H_2O_2 on dialkyl sulphates according to the method of Baeyer & Villiger (1901). Following the procedure of Stern (1936) the temperature was kept below 10° during the reaction. The crude products obtained on distillation were redistilled three times and the fraction between 96 and 100° was collected. This was neutralized with disodium hydrogen phosphate and stored at 0°. Tests for H_2O_2 by the titanium method showed that in both preparations there was less than 1%.

Before each experiment the stock solution was standardized iodometrically and was then diluted to the desired concentration.

The metmyoglobin was prepared as described previously and its concentration was determined on the basis of its haematin iron, following the procedure of Keilin & Hartree (1952). In our previous paper the concentration had been determined by using as a standard de Duve's (1948) carboxymyoglobin spectrum. His optical densities corresponded to a concentration of 1 g./l. based on a molecular weight of 16 200. In converting his concentration to molar concentration we however used the value 17 000 for the mol. wt. of MetMb. The molar concentration of our sample was therefore too low by about 4·5% and the molar extinction coefficients recorded were correspondingly too high.

Similar buffer solutions were used as in the previous investigation.

Spectrophotometric measurements were carried out with a Unicam quartz spectrophotometer.

RESULTS

Both methyl and ethyl hydroperoxides were found to give similar results in the following experiments. They form intermediate compounds with MetMb which are spectroscopically identical with that produced by H_2O_2, as shown in Figs. 1 and 2. In contrast, however, to the reaction with H_2O_2, the formation of the intermediate compounds by these hydroperoxides when the pH is greater than 9·0 is unattended by side reactions which lead to the destruction of MetMb. However at pH 10·0 a considerable excess of hydroperoxide, 15 to 20 times the concentration of MetMb, is necessary for complete formation of the intermediate.

If the pH is less than 8·0, the same type of behaviour is observed as in the H_2O_2 reaction, namely the appearance in the spectrum of the reaction product of a pronounced band at 590 mμ. in place of a shoulder and of an additional band at 615 mμ. in the CO-protohaemochromogen. These are shown in Figs. 1 and 3. The addition to the MetMb of a reducing agent, such as iodide ion, before the hydroperoxide, greatly reduces the side reaction, as can be seen in Fig. 3. The same effect is obtained by

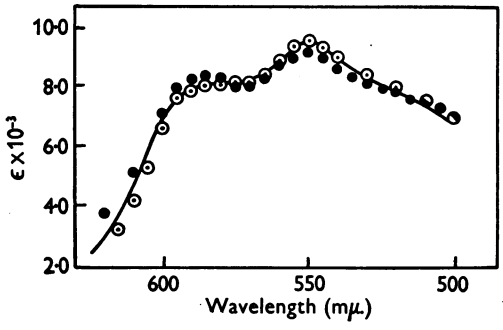

Fig. 1. Visible spectra of the reaction product of MetMb with H_2O_2 and alkyl hydroperoxides, region 650–500 mμ.; smooth curve, using H_2O_2 at pH 8·6; ⊙, using alkyl hydroperoxides at pH 8·6; ●, using alkyl hydroperoxides at pH 6·8.

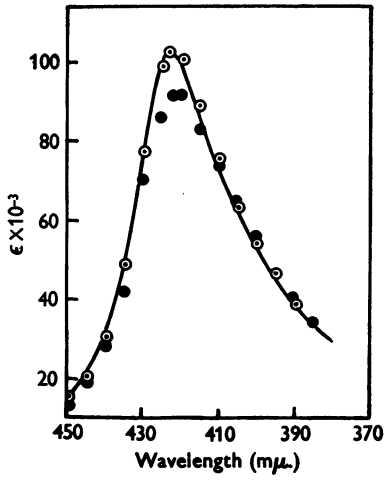

Fig. 2. Soret spectra of the reaction product of MetMb with H_2O_2 and alkyl hydroperoxides; smooth curve, intermediate compound from H_2O_2 and MetMb at pH 8·6; ⊙, alkyl hydroperoxides and MetMb at pH 8·6; ●, alkyl hydroperoxides and MetMb at pH 6·8.

using a large excess of hydroperoxide, e.g. a 25–50 molar ratio of peroxide to MetMb. The reaction product, which is formed very rapidly under these conditions, is identical with that formed at pH 8·6. On standing, however, the haemoprotein is slowly destroyed. This diminution of side reaction was also observed once when an old sample of MetMb was used, when the peroxide present was only just

sufficient to give complete formation of the intermediate compound at these pH values namely, a 3–4 molar excess. This should have represented the optimum condition for the side reaction in view of its suppression by excess peroxide. The reason for this behaviour is unknown, but is possibly due to the production of reducing matter in the stored sample, as will be shown later.

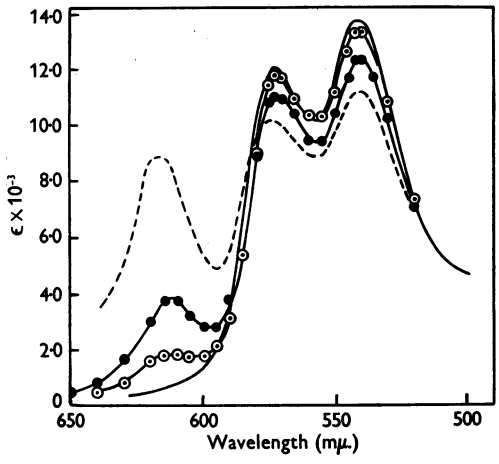

Fig. 3. Spectra of CO-haemochromogen obtained from the reaction product of MetMb and alkyl hydroperoxides under different conditions; smooth curve, from MetMb alone; dotted curve, from product formed at pH 6·8 from methyl hydroperoxide and fresh sample of MetMb; ●, from product formed at pH 6·8 but using an old sample of MetMb; ⊙, from product formed at pH 6·8 from methyl hydroperoxide and fresh sample of MetMb in the presence of iodide ions.

Although a 3–4 molar excess of hydroperoxide (ROOH) is necessary for complete compound formation at pH's about 8·0–9·0, and an even larger excess in more alkaline solutions, this does not result from the existence of an equilibrium. This was shown by changing the absolute concentration of MetMb while keeping constant the ROOH/MetMb ratio. The results are shown in Fig. 4. It will be seen that a tenfold change in MetMb concentration hardly affects the percentage compound formation. This would not have been the case if an equilibrium existed.

Further confirmation of the absence of an equilibrium was given by adding to MetMb just sufficient methyl hydroperoxide for full compound formation. When the reaction was complete, a further quantity of MetMb was added. If there were any equilibrium, peroxide should have been available for reaction with this additional quantity of MetMb and this would have resulted in an optical density change corresponding to more compound formation.

If a calculation is made on the basis of an equilibrium system and that therefore CH_3OOH is available for reaction with the additional MetMb, the intermediate would have been formed in accordance with a CH_3OOH/MetMb ratio of $\frac{1{\cdot}59 \times 10^{-4}}{8{\cdot}7 \times 10^{-5}}$, i.e. 1·84, (from c in Table 1). The concentration of intermediate on this basis can be calculated from the percentage formation corresponding to this ratio, as read off from the appropriate graph in Fig. 4 (⊙—⊙), which refers to the

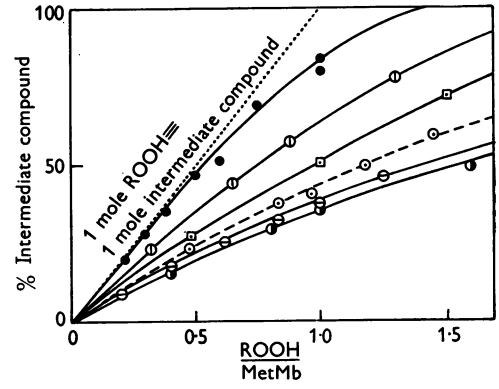

Fig. 4. Percentage formation of the intermediate compound for different ratios of ROOH/MetMb. ●, H_2O_2 and MetMb, $5{\cdot}2 \times 10^{-5}$ M, at pH 8·6. ⓘ, CH_3OOH and an old sample of MetMb, $5{\cdot}2 \times 10^{-5}$ M, at pH 8·6; ⊡, CH_3OOH and fresh MetMb, $5{\cdot}2 \times 10^{-5}$ M, at pH 8·0; ⊖ and ◑, CH_3OOH and fresh MetMb, $5{\cdot}2 \times 10^{-5}$ M and $5{\cdot}2 \times 10^{-6}$ M, respectively, pH 8·6; ⊙, CH_3OOH and MetMb, from a different preparation, at pH 8·6, MetMb concentration $5{\cdot}2 \times 10^{-5}$ M.

same sample with which these experiments were made. The percentage formation is 68 %, and this corresponds to an optical density of 0·711, calculated from the measured optical densities of free MetMb and intermediate compound given in (*a*) and (*b*).

This value of 0·711, recorded in (*d*) of Table 1, corresponds, on a concentration basis, to a further formation of $1{\cdot}0 \times 10^{-5}$ M intermediate compound or approximately 26 % of the added MetMb. The observed optical density after addition of MetMb is 0·670, which is in agreement with the calculated value of 0·671 based on the assumption that the additional MetMb did not react. This experiment proves that even though there was initially a threefold excess of ROOH over MetMb all the peroxide was used up during the formation of the intermediate compound. This points to a reaction mechanism involving irreversible competition for the peroxide and not one involving an equilibrium.

The way in which the percentage formation of the intermediate compound depends on the ratio of

peroxide to metmyoglobin is shown in Fig. 4. Whereas for H_2O_2 a ratio of H_2O_2/MetMb = 1 produces about 80 % of the intermediate, only about 40 to 50 % is obtained for a similar ratio of ROOH/MetMb. This value was reproducible provided fresh samples of MetMb were used. One experiment however, which was carried out on a sample that had been standing at 0° for about 4 months, gave about 60 % compound formation for ROOH/MetMb = 1. Hydrogen-ion concentration was also found to affect the percentage formation. As mentioned before, a considerable excess of alkyl hydroperoxide is necessary for 100 % formation at pH 10·0. In addition, at pH 8·0 the percentage compound formation is greater than that at pH 8·6 for the same ratio of ROOH/MetMb. This is also shown in Fig. 4.

Table 1. *The effect of adding MetMb to a solution of MetMb and* CH_3OOH, *the latter being present at a concentration which was just sufficient to give* 100 % *formation of the intermediate compound, within experimental error*

(pH 8·6; MetMb stock soln. $4·15 \times 10^{-4}$M; CH_3OOH stock soln. $1·75 \times 10^{-3}$M.)

Composition of solution	Optical density at 549 mμ.
(*a*) 1·0 ml. MetMb + 9·0 ml. buffer	0·232 (observed)
(*b*) 1·3 ml. MetMb + 7·7 ml. buffer + 1·0 ml. CH_3OOH. (This gives just 100 % formation of intermediate compound)	0·506 (observed)
(*c*) 1·3 ml. MetMb + 7·7 ml. buffer + 1·0 ml. CH_3OOH: + further addition of 1·0 ml. MetMb	0·670 (observed)
(*d*) As in (*c*) assuming equilibrium and CH_3OOH available for reaction with additional MetMb	0·711 (calculated)
(*e*) As in (*c*) assuming additional MetMb does not react	0·671 (calculated)

Under similar conditions the rate of formation of the intermediate compound from the alkyl hydroperoxides is about 10 times as fast as its rate of formation from H_2O_2. The rate, however, shows the same type of dependence on pH, becoming slower in alkaline solutions. As was found with H_2O_2, the compound on standing reverts to MetMb, but the change is faster than in the H_2O_2 system, about 10–15 % in 30 min. as compared with about 2 % in the same time in the latter system. This regeneration of MetMb is also pH dependent and is faster in acid solutions.

The relative instability of the intermediate in the hydroperoxide systems made quantitative reduction experiments more difficult to carry out than in the H_2O_2 system. In the reduction experiments with ferrocyanide, shown in section (*a*) of Table 2, it was therefore necessary to run a blank and make a correction for the amount of intermediate which disappeared spontaneously. The values of (ferrocyanide ion)/(compound) which are obtained this way are slightly greater than 1·0, but they represent an upper limit for this ratio since this correction for the spontaneous reaction neglects the concurrent ferrocyanide reduction.

It was found that the addition of a little H_2O_2 to the compound after its formation increased its stability, and some reduction experiments were carried out with ferrocyanide under these conditions. Since ferrocyanide reacts only very slowly with

Table 2. *Titration of the intermediate compound formed from MetMb and* CH_3OOH *with ferrocyanide by allowing an excess to react with a limited amount of ferrocyanide at* 18°

(The last three results in section (*a*) are from experiments carried out at pH 6·3, where the intermediate was formed at pH 8·6 and then introduced into strong buffer at pH 6·3. Corrections have been made in column 4 of this section for the spontaneous decomposition of the intermediate. The other experiments in this section and those in section (*b*) were carried out at pH 8·6. No correction for spontaneous decomposition was necessary in section (*b*) as the intermediate compound was stabilized by the addition of a little H_2O_2, the volume of which was small enough to be neglected. All concentrations are in μM.)

	Concentration of ferrocyanide present initially	Concentration of intermediate compound present initially	Measured concentration of intermediate compound left after ferrocyanide reacted	Calculated concentration of intermediate compound reacting with ferrocyanide	Ratio: $Fe(CN)_6^{-4}$: intermediate compound
	(1)	(2)	(3)	(4)	(5)
(*a*)	20·0	40·0	12·0	19·0	1·05
	30·0	38·0	6·0	23·0	1·30
	10·0	32·0	19·0	8·0	1·25
	30·0	32·0	2·0	25·0	1·20
	20·0	27·0	5·0	14·0	1·43
(*b*)	15·0	42·0	28·0	14·0	1·07
	22·5	36·0	15·0	21·0	1·07
	30·0	31·0	1·0	30·0	1·00
	24·0	35·0	10·0	25·0	0·96

H_2O_2, the amount used up is a direct measure of the oxidizing equivalent of the intermediate compound. The values of (ferrocyanide ion)/(compound) which are obtained from these experiments are given in section (*b*) of Table 2. In this table, column 1 gives the molar concentration of added ferrocyanide before reaction with the intermediate compound, the necessary correction having been made for dilution effects, e.g. 1 ml. of ferrocyanide added to 9·0 ml. of intermediate. Columns 2 and 3 are the molar concentrations of the intermediate before and after reaction with the added ferrocyanide, the effect of dilution by ferrocyanide having been taken into account in the case of column 2. Column 4 is the calculated concentration of intermediate which reacts with the ferrocyanide. This is obtained by subtracting from the difference between columns 2 and 3 a correction for the spontaneous decomposition of the intermediate over the same reaction time, as determined from control experiments.

The results in Table 2 show that the intermediate compound has one oxidizing equivalent relative to its reduction to MetMb. It may therefore be considered as a compound in which the iron has an effective oxidation number of +4. Hence the reaction of MetMb with the alkyl hydroperoxides is analogous to the H_2O_2 reaction. In this case it was shown, in addition, that a transient oxidizing entity was produced during the formation of the intermediate according to the equation

$$\underset{\substack{\text{2 oxidizing}\\\text{equivalents}}}{\text{MetMb} + H_2O_2} \rightarrow \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{Fe_p^{IV}} + \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{X.} \qquad (1)$$

Similar experiments were carried out to establish whether a transient oxidizing entity was also formed in alkyl hydroperoxide systems. When a little luminol was added to the MetMb, a bright flash was observed on addition of the hydroperoxide, and this was followed by a dull luminescence. The addition of luminol after the hydroperoxide gave only a dull luminescence. The addition of ferrocyanide before hydroperoxide produced the same type of behaviour as in the H_2O_2 reaction, although there was a quantitative difference. Thus when 1 mol.prop. of ferrocyanide was added to MetMb and 1 mol.prop. of H_2O_2 introduced, no reduction of the intermediate was observed. In the case of the hydroperoxides, however, reduction was observed whenever more than 0·5 mol.prop. of ferrocyanide was present for 1 mol.prop. each of MetMb and ROOH. With less than 0·5 mol.prop. ferrocyanide no reduction occurred and the amount of intermediate produced was the same as that produced in the absence of ferrocyanide.

DISCUSSION

The above results show that the reactions between the alkyl hydroperoxides or H_2O_2 and MetMb are essentially the same. The identical nature of the spectrum of the intermediate compound in both systems, as well as the fact that in each case the intermediate is one oxidizing equivalent above MetMb, suggests that the same intermediate is produced. In both reactions there is no evidence of any equilibrium, the intermediate being produced in an irreversible reaction given by equation (1).

In the presence of added reducing agent the side reaction, which occurs when the peroxides react with MetMb at pH's less than 8·0, is greatly reduced. This reduction of the side reaction is also brought about by excess peroxide. Two conclusions may be drawn from these experiments, first that the peroxide molecule itself cannot be responsible for these side reactions, and, secondly, that the entity responsible is capable of reacting both with reducing agents and peroxide.

In the previous paper it was shown that the intermediate compound does not react with MetMb. The side reaction which occurs in acid solution may therefore be attributed to the transient oxidizing entity which is produced during the formation of the intermediate compound, and the slight differences which are revealed between the reactions of H_2O_2 and the alkyl hydroperoxides to competitive reactions involving this entity.

A reaction scheme which fits the observations and accounts for the differences between the reactions of H_2O_2 and the alkyl hydroperoxides is given by the following equations

$$\underset{\text{2 oxidizing equivalents}}{Fe_p^{III} + ROOH} \rightarrow \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{Fe_p^{IV}} + \underset{\substack{\text{1 oxidizing}\\\text{equivalent}}}{X,} \qquad (2)$$

$$X + ROOH \rightarrow \text{chain reaction leading to destruction of ROOH,} \qquad (3)$$

$$X + AH_2 \rightarrow HX + AH\cdot, \qquad (4)$$

$$X + \text{MetMb} \rightarrow \text{side reaction products,} \qquad (5)$$

where Fe_p^{III} and Fe_p^{IV} represent myoglobin in which the iron is in the effective oxidation states of +3 and +4 respectively; AH_2 represents a reducing agent, which may either be reducing groups on the haemoprotein or reducing matter associated with the preparation; AH· is the half-oxidized form of AH_2; X is a transient oxidizing entity which may be the OH radical or OR radical.

On the basis of these equations, the formation of 1 mole of the intermediate from 1 mole of H_2O_2 at low H_2O_2/MetMb ratios can be explained if reaction (3) is unimportant with respect to (2) and (4).

In the case of the alkyl hydroperoxides, at low ROOH/MetMb ratios less of the intermediate is formed than would be expected if only reaction (1) occurred, and in fact a three to four molar ratio of the peroxide to metmyoglobin is required for its full formation.

This behaviour, which is illustrated in Fig. 4, would follow from the above equations if reaction (3), leading to peroxide destruction, occurs much more readily with ROOH than H_2O_2 and so plays a significant role in the alkyl hydroperoxide system. Since the rate of reaction (3) will depend on the peroxide concentration, it also follows that this reaction will be relatively more important with high as opposed to low peroxide concentrations. Consequently, in a series of experiments in which the peroxide concentration is varied at constant metmyoglobin concentration, much less of the intermediate compound would be expected at high ratios of peroxide to metmyoglobin than would be the case if only reaction (1) occurred.

If the percentage compound formation is then plotted as ordinate against peroxide/MetMb ratio this would result in the bending of the curve towards the abscissa. Fig. 4 shows that this feature is present in both H_2O_2 and ROOH systems, although it is more marked in the latter in agreement with the above indication that in this system reaction (3) occurs more readily.

As mentioned before the side reaction, which occurs if the pH is less than 8·0 in both the H_2O_2 and ROOH reactions, may be attributed to the attack of X on the haemoprotein. This could occur when reaction (5) in the above scheme becomes important relative to (3) and (4). By adding a reducing agent before the peroxide, or by increasing the peroxide concentration, the importance of reactions (4) and (3) may respectively be increased, resulting in the observed diminution of the side reaction.

Above pH 9·0 the ROOH reaction differs from the H_2O_2 reaction in that no side reaction occurs which involves haemoprotein destruction. The fact that in the H_2O_2 system the side reactions in acid and alkaline solutions are different suggests that it is possibly the attack of H_2O_2 itself on the haemoprotein which is responsible for the latter. Whatever the mechanism operating, the absence in the ROOH system in alkaline solution of a side reaction similar to that observed in the H_2O_2 system may be explained by the importance of reaction (3). The destruction of ROOH as a consequence of this reaction would also explain why such a large excess of ROOH, between 15 and 20 times the metmyoglobin concentration, is required to give full formation of the intermediate compound in alkaline solution.

In conclusion, reaction (4) can account for the rapid disappearance of reducing agents when these are added before peroxide, and the scheme as a whole is consistent with the difference observed in these experiments between the H_2O_2 and ROOH systems. Thus, at pH 8·6, a mol.prop. of ferrocyanide as high as 1 was almost entirely consumed whilst the intermediate compound was being formed in a mixture of 1 mol.prop. metmyoglobin and 1 mol.prop. H_2O_2 to which the ferrocyanide had been added before the peroxide (George & Irvine, 1952), whereas, in the experiments with ROOH reported above, if the mol.prop. of ferricyanide exceeded 0·5 not all of it was consumed in this way, but some remained in the solution as shown by the subsequent reduction of the intermediate compound. In the H_2O_2 system, reactions (2) and (4) and not (3) appear to be the important steps under these conditions, whereas with the alkyl hydroperoxides reaction (3) is also important, so that the amount of added reducing agent which is removed would therefore depend in this case on the relative importance of reaction (3) with respect to reaction (4).

The observations on the aged sample of MetMb can be explained in a similar way. For, if reducing matter was formed in the MetMb on standing, the increased importance of reaction (4) by preventing some of the peroxide destruction in reaction (3) would result in an increase in the formation of the intermediate, and, by removing a greater proportion of X, would result in a diminution of the side reaction which occurs in acid solution.

SUMMARY

1. The intermediate compounds which result from the reaction of alkyl hydroperoxides with metmyoglobin are spectroscopically the same, and identical with that produced by hydrogen peroxide.

2. The compound is not formed in an equilibrium reaction but irreversibly according to the equation

$$\text{MetMb} + \text{ROOH} \rightarrow \text{compound} + \text{X},$$

where X is a transient oxidizing entity.

3. At pH's less than 8·0 a side reaction is produced similar to that in the hydrogen peroxide system. Above pH 9·0 no side reaction, which destroys the haemoprotein, is observed; however a large excess of hydroperoxide is required for complete formation of the intermediate.

4. Addition of a reducing agent to metmyoglobin before hydroperoxide reduces the side reaction which otherwise occurs at pH's less than 8·0. The same effect is brought about by using a large excess of peroxide.

5. Reduction with ferrocyanide shows the intermediate to have one oxidizing equivalent relative to metmyoglobin, and the intermediate may thus be considered as a compound in which iron has an effective oxidation number of +4. Its formation and reactions may thus be written in stoicheio-

metric equations using the symbol Fe_p^{IV}, which indicates its general oxidation-reduction behaviour and not a particular chemical structure.

6. A mechanism is proposed for the overall reaction of hydrogen peroxide and hydroperoxide with metmyoglobin involving competition reactions of the transient oxidizing entity, which is produced during the formation of the intermediate compound.

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A Spectrophotometric Study of Ionizations in Methaemoglobin

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Whereas Gamgee (1868) was the first to observe a reversible change between two coloured forms of methaemoglobin, it was Hartridge (1920) and Haurowitz (1924) who showed that an acid-base indicator relationship was involved in this equilibrium. Austin & Drabkin (1935) used canine-blood methaemoglobin (MetHb) and a spectrophotometric method for the measurement of its equilibrium, or ionization, constant. They concluded that the ionization was single, that is it involved one H^+ per Fe atom, and that it had a pK′ value of 8·12 at ionic strength $I = 0{\cdot}10$ (and an unspecified temperature).* They also concluded, although this is not shown directly in their data, that at ionic strengths below 0·15 the variation of pK′ with $\sqrt{I}$ is linear with a slope of about $-0{\cdot}6$. Coryell, Stitt & Pauling (1937) titrated bovine MetHb magnetometrically at higher ionic strengths and stated that the linear slope of pK′ with $\sqrt{I}$ is about $+0{\cdot}6$. In this paper it is shown that these results are not contradictory, for the addition of neutral salts favours the ionization of acid MetHb up to $I \simeq 0{\cdot}1$, but beyond this the effect is reversed. A similar behaviour has been described in a previous paper (George & Hanania, 1952, subsequently referred to as Paper 1) which dealt with the ionization of acidic metmyoglobin (acid MetMb) and the evaluation of its approximate heat and entropy of ionization.

No value for the corresponding heat of ionization of acid MetHb is reported in the literature, though Wyman & Ingalls (1941) have taken it to be 13·0 kcal./mole on the assumption that its ionization would be very similar to that of water. This assumption is not borne out by the value $\Delta H = 3{\cdot}91 \pm 0{\cdot}49$ kcal./mole obtained in the present investigation.

* Following the usual convention the symbol pK′ is used here to refer to an experimentally determined equilibrium constant at a finite ionic strength, whereas pK denotes a value obtained by extrapolation to zero ionic strength.

MATERIALS AND METHODS

Preparation and storage of MetHb. Samples of crystalline oxyhaemoglobin (HbO_2) were prepared from the blood of horse, ewe and ram, following the method of Keilin & Hartree (1935). MetHb was prepared by the oxidation of HbO_2 with an excess of $K_3Fe(CN)_6$ not exceeding two equivalents, and was freed from inorganic salts by dialysis. Next, it was salted out by adding $(NH_4)_2SO_4$ to about 65% saturation and was redissolved to make the required stock solution. It was found best to keep haemoglobin as crystallized HbO_2 at about 0°, but MetHb could also be successfully stored for long periods in a 2% salt solution (w/v) kept frozen at −10°. Freeze-dried MetHb appeared to keep indefinitely, but its solutions showed some loss of resistance to alkali denaturation (cf. Keilin & Hartree, 1952). Samples of MetHb prepared simply from salted-out HbO_2 did not keep so well and slight opalescence was observed in dilute solutions at the lowest ionic strengths when the pH was around the isoelectric point.

Except where otherwise indicated, all results quoted in this paper refer to horse-blood MetHb prepared from HbO_2 which had been crystallized once from 20% (v/v) ethanol. MetHb was invariably stored in 2% (w/v) NaCl or 2% (w/v) $(NH_4)_2SO_4$ at low temperatures. Concentrations of MetHb solutions are expressed in terms of their haematin content (1 M≡17 000 g./l.). This was measured as the pyridine haemochromogen according to the method of Keilin & Hartree (1951). The method gave concentrations which accorded well with those based on the standard absorption curve for human carboxyhaemoglobin (de Duve, 1948), discrepancies not exceeding 1·5%.

It should be mentioned that in paper 1, metmyoglobin concentrations were based on the latter standard but a molecular weight of 17 000 was assigned to MetMb instead of the 16 200 used by de Duve. Concentrations determined in