The Reaction of Metmyoglobin with Strong Oxidizing agents

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In our previous papers (George & Irvine, 1951, 1952, 1953*a*) we showed that the intermediate compound, which is formed when metmyoglobin (MetMb) reacts with hydrogen peroxide, methyl or ethyl hydroperoxide, may be considered as one in which the iron has an effective oxidation number of +4. Its relationship to MetMb, represented by $\text{Fe}_p^{3+}(\text{H}_2\text{O})$, is given by the equation,

where Fe_p^{IV} stands for the intermediate compound. Since the reaction of MetMb with peroxides is an irreversible, single equivalent oxidation, rather than an equilibrium reaction of the type envisaged in the formation of an enzyme-substrate complex, it seemed possible that other oxidizing agents would also be capable of forming the compound.

Taube & Cahill (1951) showed that tetrasulphonated phthalocyanines (TSCP) can be oxidized by electron removal with the ceric ion

and so it was of interest to see whether electron transfer oxidizing agents would be effective in the case of the structurally similar MetMb. Reactions of MetMb with such oxidizing agents ought to be free from the complications inherent in its reactions with the peroxides. Of the two oxidizing equivalents of peroxides, one is transferred to MetMb while the second appears as a transient oxidizing entity. This entity has recently been shown to be involved in competitive reactions, some of which involve attack on the porphyrin ring (George & Irvine, 1953*a*).

Our attention has recently been drawn to the papers of Polonovski, Jayle, Glotz and Fraudet, published between 1938 and 1941 (full references are given later), who suggested, as we have done more recently, that quadrivalent iron compounds participate in the methaemoglobin and peroxidase reactions with peroxides. Since their publication, these papers have escaped notice in most discussions of the reaction mechanism, so we have briefly reviewed the experimental evidence, and the detailed mechanism proposed, at the end of this paper.

EXPERIMENTAL

Potassium molybdicyanide ($\text{K}_2\text{Mo}(\text{CN})_8$). This was prepared according to Willard & Thielke (1935). After three recrystallizations, titration with KMnO_4 showed the sample

to be 99% pure; stock solutions were therefore made up by weight. Experiments with $\text{K}_2\text{Mo}(\text{CN})_8$ were carried out in the dark because of the extreme sensitivity to light of the $\text{K}_2\text{Mo}(\text{CN})_8$ produced by its oxidation.

Potassium molybdicyanide ($\text{K}_2\text{Mo}(\text{CN})_8$). This was prepared according to Willard & Thielke (1935). Because it hydrolyses rapidly in the presence of light, both its preparation and the experiments with MetMb were carried out in the dark.

Potassium chloriridate (K_2IrCl_6). This was obtained from Messrs Johnson, Matthey and Co. Ltd. Fresh solutions were prepared immediately before use and standardized by spectrophotometric titration with AnalaR $\text{K}_4\text{Fe}(\text{CN})_6$. This precaution was necessary because of the slow hydrolysis of K_2IrCl_6 in aqueous solution.

Ruthenous tris-dipyridyl chloride ($\text{Ru}(\text{dipy})_3\text{Cl}_2 \cdot 6\text{H}_2\text{O}$). This was prepared from ruthenium trichloride and $\alpha\alpha'$ -dipyridyl according to the method of Burstall (1936). It was oxidized to the corresponding ruthenic compound by the addition of about 10 mg. of PbO_2 and 0.5 ml. of conc. HNO_3 to 10 ml. of a 10^{-3}M solution. The excess PbO_2 was removed by filtration through a fine sintered glass funnel.

Osmous tris-dipyridyl chloride ($\text{Os}(\text{dipy})_3\text{Cl}_2 \cdot 6\text{H}_2\text{O}$). This green compound was prepared according to Burstall, Dwyer & Gyrfas (1950). It was oxidized in solution to the red trivalent compound by HNO_3 . The concentration of the solution of the trivalent compound was determined by spectrophotometric titration with $\text{K}_4\text{Fe}(\text{CN})_6$.

Chlorine dioxide. A solution was obtained by heating a moist mixture of oxalic acid and KClO_3 and passing the gas into water.

Sodium hypochlorite was made by the double decomposition of $\text{Ca}(\text{OCl})_2$ (bleaching powder) and Na_2CO_3 .

All other inorganic reagents, such as KIO_4 and NaClO_2 , were of the highest grade analytical product. The methods described by George & Irvine (1952), were used in the preparation and standardization of MetMb and the buffer solutions. Spectrophotometric measurements were made with a Unicam quartz spectrophotometer. All reactions were carried out with dissolved air present in the solutions unless otherwise stated. Except where mentioned, experiments were conducted at room temperature, 18°.

RESULTS

Sodium chlorite, potassium periodate, potassium molybdicyanide and potassium chloriridate all react with MetMb under suitable conditions giving a product with the same spectroscopic characteristics as those of the intermediate compound produced by the peroxides.

Sodium chlorite. At pH 8.6 a concentration of sodium chlorite 20 times that of the MetMb was necessary to obtain 80% yield of the intermediate

compound. Under these conditions the formation was rapid, but it was also followed by fairly rapid destruction of the haemoprotein. Thus when sodium azide was added to the reaction product after reduction with potassium ferrocyanide, very little azide complex was obtained. At pH 10.0 a higher yield was obtained and the rate of haemoprotein destruction was slower.

Potassium periodate. The reaction at pH 8.6 was extremely slow, requiring about 30 min. for completion even with a 200-fold excess of potassium periodate. Fig. 1 shows the spectrum of the product. Comparison with the spectrum of the intermediate compound obtained from hydrogen peroxide and MetMb at this pH suggests that 80–85 % is formed in the periodate reaction. Reduction with potassium ferrocyanide followed by the addition of sodium azide showed that 80–85 % of the original MetMb was recoverable, indicating a destruction of 15–20 % of the MetMb. The extent of destruction was greater in more acid solutions. Thus the spectra of the CO-haemochromogen obtained from the products of the periodate reaction at pH's 8.6 and 8.2 showed that 85 % of the original MetMb was recovered at pH 8.6 but only about 60 % at pH 8.2. At pH 6.0 no CO-haemochromogen was obtained.

Potassium persulphate. The addition of potassium persulphate to MetMb, even in large excess, caused no perceptible change in its spectrum. If, however, after the addition of potassium persulphate, silver nitrate solution was added to give a 10^{-5} M solution, there was a rapid colour change from brown to reddish violet indicating the formation of the intermediate compound. This was followed by destruction of the MetMb and finally the formation of a precipitate, presumably of silver hydroxide. Because of this it was not possible to confirm spectrophotometrically the formation of the intermediate compound with this oxidizing agent.

Fig. 1. Visible spectrum of the compound formed when MetMb reacts with potassium periodate at pH 8.6. The dotted curve is that of the intermediate compound formed with hydrogen peroxide, shown for comparison.

Potassium molybdicyanide. Oxidation of MetMb occurred only in solution with the pH above 11.0. The spectrum of the compound obtained is shown in Fig. 2. At pH 10.2, and below, the addition of potassium molybdicyanide to the intermediate compound caused complete reduction. At pH 8.6 an equimolar concentration of molybdicyanide was required but at higher pH values an excess of molybdicyanide was necessary. The spectrum of the azide complex from the reduction product at pH 8.6 is given in Fig. 3. It agrees very well with that of the azide complex from fresh MetMb.

Fig. 2. Visible spectra of the compounds obtained in the reactions of metmyoglobin with hydrogen peroxide at pH 8.6, ethyl hydroperoxide (EtOOH) at pH 8.6, potassium chloridate at pH 8.6 and potassium molybdicyanide at pH 11.1. The ordinate scale refers to the spectrum of the compound formed by hydrogen peroxide, and it has been displaced upwards by 1.25 units in each case for the spectra of the compounds formed by the other oxidizing agents.

Fig. 3. Spectra of metmyoglobin-azide complex (650–500 mμ). Smooth curve is that from stock MetMb; O, that from the product of the reduction of the intermediate compound by potassium molybdicyanide at pH 8.6.

If after forming the intermediate at pH 11.1 with potassium molybdicyanide, the pH is then changed by the addition of dilute hydrochloric acid to about pH 6.0, the spectrum rapidly reverts to that of acidic MetMb. This reduction was found to be slower the more alkaline the final pH of solution.

Potassium chloriridate. Experiments at pH's 8.6 and 10.0 show that potassium chloriridate reacts very rapidly with MetMb, whether air is present or not, to give an intermediate compound whose absorption spectrum is identical with that obtained, under optimum conditions, using hydrogen peroxide or alkyl hydroperoxides (Fig. 2). Because of the instability of the intermediate compound formed under these conditions, it was necessary to make only a few optical density measurements on each of a series of solutions prepared identically. Complete regeneration of the MetMb occurred in about 3 hr., compared with about 48 hr. in the hydrogen peroxide system and about 12 hr. in the alkyl hydroperoxide system. This instability appeared to be due to the action of reducing substances produced by hydrolysis of potassium chloriridite. This explanation is supported by the slow reduction of the intermediate compound by potassium chloriridite, even at pH 8.6 where potassium chloriridate oxidizes MetMb.

With 5×10^{-6} M concentrations of both MetMb and potassium chloriridate at 18°, at least 97 % of the reaction had occurred within 20 sec. after mixing. The velocity constant must therefore be greater than 3.2×10^5 , or to the nearest power of ten, 10^6 l.mole⁻¹sec.⁻¹.

Spectrophotometric titrations of MetMb with potassium chloriridate at pH 8.6 were carried out by adding various amounts of stock chloriridate solution to 5×10^{-5} M MetMb solution and estimating the amount of Fe^{IV} from optical density measurements at 549 mμ. Fig. 4 shows that there is a linear relationship between the amount of Fe^{IV} formed and the chloriridate added; in this particular case a mole ratio (*r*) of chloriridate to

metmyoglobin of 1.37 was required for complete formation of the intermediate.

Since the intermediate compound is a single equivalent oxidation product of the MetMb a ratio of 1.0 would be expected, and values of *r* greater than 1.0 indicate that the reaction is accompanied by side reactions involving chloriridate. This could be either hydrolysis to an oxidant not powerful enough to oxidize the MetMb or reduction of the potassium chloriridate by other reducing groups, either on the protein or present as impurities in the preparation. Titrations carried out with potassium chloriridate made up in distilled water or in dilute hydrochloric acid, with and without addition of potassium chloride, were therefore compared; any hydrolysis should be suppressed in acid solution or by chloride ion, and lower values for *r* should be obtained. The results (Table 1) show that *r* varies only from 1.30 to 1.62, and the values for the solutions in which hydrolysis should have been suppressed, had it been an important factor, are only just significantly lower than the others. This test would not reveal a rapid hydrolysis occurring in the

Fig. 4. Plot of percentage oxidation against the ratio $[\text{IrCl}_6^{2-}]/[\text{MetMb}]$ for the reaction at pH 8.6. MetMb concentration is 4.73×10^{-5} M.

Table 1. *Titration of metmyoglobin with potassium chloriridate at pH 8.6*

Conditions	Ratio:
	K_2IrCl_6 consumed Intermediate compound formed
Aqueous solution of K_2IrCl_6	1.62
Aqueous solution of K_2IrCl_6 : fresh solution for each titration	1.53
K_2IrCl_6 in 2×10^{-3} M-HCl	1.30
K_2IrCl_6 in 4×10^{-3} M-HCl	1.43
K_2IrCl_6 in 5×10^{-3} M-HCl	1.46
K_2IrCl_6 in 5×10^{-3} M-HCl; 0.4 M-KCl present in titration mixture	1.48
K_2IrCl_6 in 5×10^{-3} M-HCl: MetMb titrated and allowed to stand. After spontaneous reduction of intermediate compound was complete (in about 3 hr.), the MetMb was again titrated	1.37

buffer solution used for the oxidation. However, a spectrophotometric comparison between solutions of potassium chloriridate made up in dilute hydrochloric acid and in the buffer gave no indication of any rapid reaction. The values of r greater than unity must therefore be due to reduction and not hydrolysis of the potassium chloriridate.

An attempt was made to confirm this by treating MetMb with potassium chloriridate in the molar proportion of 1.5:1 and then allowing the intermediate to decompose completely before carrying out the usual titration. The removal of reducing matter by this pre-treatment might have given lower titration values and a value of r nearer unity. The value 1.37 (Table 1) was not significantly different from those with fresh MetMb. However, this titration is complicated by the presence of a more powerful reducing agent formed by hydrolysis of the chloriridite ions remaining from the first treatment: this would tend to give a high value of r .

In more acid solutions, increasingly greater quantities of potassium chloriridate were required to give full formation of the intermediate compound; at pH 6.82 a fivefold excess yielded only about 50%. This might be due either to the simultaneous removal of the greater part of the chloriridate ion in side reactions, or to the setting up of an equilibrium

Estimates of the potassium chloriridate concentrations, after compound formation, were obtained from optical density measurements at 518 $m\mu$, an isosbestic wavelength for MetMb and the intermediate compound, where the chloriridite ion has negligible absorption. The measurements showed that nearly all the potassium chloriridate remained unreacted, which is evidence for an equilibrium reaction.

The equilibrium constant,

$$K = [\text{Fe}_p^{\text{IV}}] \times [\text{IrCl}_6^{3-}] / [\text{Fe}_p^3(\text{H}_2\text{O})] \times [\text{IrCl}_6^{2-}],$$

was determined from optical density measurements on a series of solutions that contained the same

initial MetMb concentration, $4.56 \times 10^{-5} \text{ M}$, and various potassium chloriridate concentrations. Measurements were made at two wavelengths, 518 and 580 $m\mu$, from which the amount of unreacted potassium chloriridate and the percentage formation of the intermediate could be obtained. The results are given in Table 2. The equilibrium constant cannot be calculated solely on the basis of reaction (2), since this does not allow for the possible reduction of some of the potassium chloriridate in side reactions, already observed at pH 8.6. From the data given in the Table it follows that for the initial concentrations of potassium chloriridate of 6.64, 9.96, 13.28 and $24.90 \times 10^{-5} \text{ M}$, 1.43, 1.52, 1.56 and $2.13 \times 10^{-5} \text{ M}$ respectively was reduced in this manner. Denoting this concentration by y , reference to reaction (2) shows that $[\text{IrCl}_6^{2-}]$ and $[\text{IrCl}_6^{3-}]$ at equilibrium are now given by $(b-x-y)$ and $(x+y)$. On this basis good agreement is obtained for the equilibrium constant

$$K = x(x+y)/(a-x)(b-x-y),$$

shown in the last column of Table 2, the mean value being 0.20 ± 0.01 .

The side reaction appears to be limited in extent and little affected by changing hydrogen-ion concentration. At pH 8.6 the results in Table 1 show that between 0.30 and 0.62 mole of potassium chloriridate is reduced/mole of MetMb oxidized to the intermediate compound. At pH 6.82 the above values for y give mole proportions of 0.31, 0.33, 0.34 and 0.47. Since this mole proportion is less than unity, and since the fraction is hardly altered as the concentration ratio $[\text{IrCl}_6^{2-}]/[\text{MetMb}]$ is increased, it is very likely that reducing matter present in the MetMb preparation is responsible rather than oxidizable groups on the MetMb itself. Even if there were only one such group, a mole proportion of at least 1.0 and probably 2.0 should be obtained since the chloriridate ion is a single equivalent oxidizing agent.

Other oxidizing agents. Chlorine dioxide and sodium hypochlorite did not give the intermediate compound, but caused considerable destruction of the MetMb as shown by the small amount of azide complex obtained after reduction by potassium

Table 2. Determination of the equilibrium constant K for the metmyoglobin-potassium chloriridate reaction

Phosphate buffer solution, pH 6.82 and $I=0.042$, 20.4° . Optical density measurements at 518 and 580 $m\mu$. All concentrations are in units 10^{-5} M ; initial MetMb concentration, $a=4.56$.

Initial IrCl_6^{2-} concn. (b)	% Fe_p^{IV} formed	Concn. of Fe_p^{IV} (x)	Concn. of IrCl_6^{2-} at equilibrium (b-x-y)	Equilibrium constant (K) $\frac{x(x+y)}{(a-x)(b-x-y)}$
6.64	23.9	1.09	4.12	0.19
9.96	31.0	1.41	7.03	0.19
13.28	37.6	1.72	10.00	0.20
24.90	49.8	2.27	20.5	0.21

Mean 0.20 ± 0.01 .

Fig. 5. Spectra obtained with the metmyoglobin-ruthenic tris-dipyridyl complex system. The dotted curve is the visible spectrum of the product of the reaction of MetMb with $\text{Ru}(\text{dipy})_3\text{Cl}_3$ at pH 8.6; the smooth curve is that of MetMb alone at pH 8.6, $\circ$ represents the curve obtained when the contribution of the absorption of $\text{Ru}(\text{dipy})_3\text{Cl}_3$, i.e. the ruthenous complex, is subtracted from the first curve.

ferrocyanide and addition of sodium azide. The ruthenic and osmic tris-dipyridyl complexes did not give the intermediate at any pH, although having oxidation-reduction potentials of 1.30 and 0.87 v (Dwyer, 1949; Dwyer, Gibson & Gyarfás, 1950) it was to be expected that they would, since potassium chloriridate and molybdicyanide are effective with oxidation-reduction potentials of 1.0 and 0.7 v respectively. However, the ruthenic complex, and presumably the osmic complex too, is reduced in the system, for the resultant spectrum corresponds to a mixture of MetMb and the ruthenous complex, as shown in Fig. 5. This behaviour suggests that other reducing groups in the system react with cationic oxidizing agents in preference to the haematin prosthetic group of metmyoglobin, in contrast to the anionic oxidizing agents which react preferentially to give the intermediate compound. Indirect evidence which supports this explanation was obtained in a series of experiments that showed that the amino acids tryptophan, tyrosine and cysteine reduce the ruthenic and osmic complexes rapidly, whereas the chloriridate ion is reduced much more slowly.

Although the osmic complex failed to oxidize MetMb, the osmous complex reduced the intermediate at all pH values below about 8.6.

DISCUSSION

Our observations show that the single equivalent oxidation of metmyoglobin occurs with oxidizing agents other than peroxides. This could be due to the direct action of the oxidizing agent; or it could still be attributed to the action of a peroxide,

formed, for instance, as a consequence of the attack of the oxidizing agent on a reducing group of the protein. Such an attack could give a radical as the primary product which, in the presence of molecular oxygen, could react to yield a peroxide. The latter mechanism is very unlikely because normal precautions to remove oxygen did not affect the production of the intermediate compound, and it is ruled out entirely in the case of potassium chloriridate and molybdicyanide by the speed of their reactions. Hydrogen peroxide and ethyl hydroperoxide react with acidic MetMb with velocity constants of about 1.7×10^2 and 1.7×10^3 l. mole⁻¹ sec.⁻¹ at 18° respectively (George & Irvine, 1953b), whereas the electron transfer reagents react with velocity constants greater than 10^5 l. mole⁻¹ sec.⁻¹, even with pH between 9 and 11. Under these conditions, where the MetMb is predominantly in its alkaline form, peroxides react more slowly than they do with acidic MetMb. Although peroxide formation might play a part in the action of some oxidizing agents, there is no doubt that the intermediate compound can be formed by direct oxidation, and that the special structural features of peroxides are not essential.

The very rapid formation with the electron transfer oxidizing agents, potassium chloriridate and molybdicyanide, strongly suggests that the atoms required for the structure of the intermediate compound are already present in metmyoglobin. The simplest interpretation is a reaction involving either electron or hydrogen atom removal, e.g.

where M or MH represent the metmyoglobin and M⁺ or M. the intermediate compound. The electron is then taken up by the IrCl_6^{2-} or $\text{Mo}(\text{CN})_8^{3-}$ ion. The observation that these oxidizing agents have certain threshold pH values, below which the intermediate compound is not formed, provides a means of obtaining a rough value of the effective oxidation-reduction potential of the $\text{Fe}_p^{\text{IV}}/\text{Fe}_p^{3+}$ couple, i.e. reaction (3) or (4). The oxidation-reduction potential of the chloriridate couple is 1.017 v according to Dwyer, McKenzie & Nyholm (1944), and that of the molybdicyanide couple is 0.74 v (Kolthoff & Tomsicek, 1936), a value which we confirmed for a pH of 11.0 by potentiometric titration. These potentials are independent of pH, since the reactions simply involve the transfer of an electron. Since potassium molybdocyane reduces the intermediate compound at pH 10.2 and molybdicyanide forms it from MetMb at pH values greater than 11.0, the effective potential (E'_p) of the couple $\text{Fe}_p^{\text{IV}}/\text{Fe}_p^{3+}$ may be fixed at about 0.7 v at pH 11.0. Its value at pH 6.82 may be calculated

from the equilibrium constant of the MetMb- K_2IrCl_6 reaction and the redox potential of the chloridate couple using the equation

$$E'_0 = 1.017 - \frac{RT}{nF} \ln K, \quad (5)$$

putting $n = 1$. This gives a value of E'_0 at pH 6.82, 20.4° and ionic strength (I) = 0.042, of 1.06 v. Thus the effective oxidation-reduction potential of the Fe_p^{IV}/Fe^{3+} couple is pH dependent, which would be the case if one or more hydrogen ions were formed along with the intermediate compound, as in the generalized reaction (4) above.

Fig. 6 shows the plot of E'_0 against pH. Two lines are shown in the figure, both having the point at pH 6.82 in common. One is for a single hydrogen-ion dependence and the other is for two hydrogen ions changing to a single hydrogen-ion dependence at pH 9.0, the pK of MetMb (Theorell & Ehrenberg, 1951; George & Hanania, 1952). The experimental values rule out consideration of any other type of dependence, and in fact it can be seen from Fig. 6 that even the single hydrogen dependence is not possible. The fact that the experimental data fit a variation of E'_0 with pH which changes its slope at the pK of MetMb is in accord with the general

chemical behaviour of reversible electrode systems where ionization occurs, e.g. the quinol-quinone system. It must be borne in mind, however, that this is merely the simplest interpretation of the data, for no consideration has been taken of haem-linked ionizing groups which if present could alter the slope of the E'_0 versus pH curve over a range of about half a pH unit on either side of their pK values.

Finally it is of interest to see what light these experimental results throw on the structure of the intermediate compound. George (1952) suggested four possible structures. Of these, however, we may rule out the simple ion of quadrivalent iron, Fe_p^{4+} , as well as the structure in which an electron is removed from the π -orbital of the conjugated ring system, since these oxidation reactions would not involve hydrogen ions. There are left two possibilities, derivatives of quadrivalent iron and radical structures. Measurements of the number of hydrogen ions produced when the intermediate compound is formed and consumed when it is reduced back to MetMb, favour the ferryl ion structure, Fe_pO^{3+} (George & Irvine, 1954); these experiments will be described in forthcoming papers.

The work of Polonovski, Jayle, Glotz and Fraudet

These authors were the first to consider in detail how a mechanism involving a higher oxidation state could explain certain haemoprotein reactions, just as in the earlier theories of Manchot and Job (see, for example, Hale, 1929) the formation of tetravalent and pentavalent iron compounds was suggested to account for the reactions of ferrous and ferric salts with peroxides and oxygen.

They discussed the formation and reactions of the methaemoglobin intermediate on the basis that it was a tetravalent iron derivative, and drew attention to the possibility that peroxidase action might also involve such a compound (Polonovski, Jayle & Glotz, 1938, 1939; Jayle, 1939; Polonovski & Jayle, 1939; Polonovski, Jayle & Fraudet, 1941a, b).

Although agreeing on the fundamental point that the intermediate compound is a higher oxidation state of MetHb (or MetMb), we have put forward a different explanation for the reaction with peroxides. Polonovski *et al.* (1939) confirmed that approximately 1.5 moles of peroxide are consumed in forming one mole of the intermediate compound per methaemoglobin (MetHb) iron atom, as had been reported by Keilin & Hartree (1935), and they proposed the following mechanism to account for it:

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Fig. 6. Two variations of E'_0 (v) with pH for the couple Fe_p^{IV}/Fe^{3+} , which are obtained on the basis of the equilibrium constant of the MetMb- K_2IrCl_6 reaction at pH 6.82, and the experimental observations that (i) $Os(dipy)_3^{3+}$ reduces Fe_p^{IV} at all pH values below about pH 8.6, (ii) $Mo(CN)_8^{3-}$ reduces it below pH 10.2, and (iii) $Mo(CN)_8^{3-}$ oxidizes MetMb above pH 11.0. (a) A line with a change in slope from -2×0.06 to -1×0.06 at the pK of acidic MetMb. (b) A line with a slope of -1×0.06 throughout.

For the corresponding reaction with oxyhaemoglobin (HbO₂) they suggested,

which accounted for their observation that there was a maximal colour change when the reactants were mixed in a 1:1 mole ratio. Both (A) and (B) denote the intermediate compound in these equations. Noting that the products from MetHb and HbO₂ had slightly different spectra, although the colours were similar, they proposed that (A) and (B) took part in an equilibrium reaction in which oxygen replaced the peroxide molecule bound to the tetravalent iron.

To explain the consumption of peroxide in excess of the one equivalent (half mole) required for oxidation, we suggested instead that a transient oxidizing entity X was first formed

and, depending on the extent to which it then reacted with endogenous reducing matter or with peroxide, a consumption ratio of unity, or greater, would then follow (George & Irvine, 1952, 1953*a*). In two respects this mechanism is more acceptable. Unlike reaction (6) it accounts for the high consumption ratios, greater than three, obtained with alkyl hydroperoxides in alkaline solution, and also reaction (9) is a simple bimolecular reaction. Reaction (6), on the other hand, expresses an overall reaction at a specified consumption ratio of 1.5 and is not a reaction capable of correlation with kinetic measurements since fractions of a mole participate.

A potentiometric titration was used to determine the number of equivalents involved per iron atom when the intermediate is formed from MetHb (Polonovski *et al.* 1941*a*). The results are given in Fig. 7: 10⁻³M MetHb was rapidly titrated with ethyl hydroperoxide in phosphate buffer of pH 6.5 at 1° to avoid as far as possible the rapid spontaneous decomposition of the intermediate compound that was very apparent at room temperature. The potential rose from 185 mV reaching a limiting value of 385 mV when 1.25 equivalents, i.e. 0.63 mole, of ethyl hydroperoxide had been added per mole of MetHb, as shown in curve *a*. On the addition of ascorbic acid the potential dropped and about 1.0 equivalent, i.e. 0.5 mole, per mole MetHb was needed for the potential to return to about its original value, as shown in curve *b*. Further addition of ascorbic acid only produced haemoglobin. At first sight these titrations appear to demonstrate

the one equivalent oxidation of MetHb to a higher oxidation state: but there are serious quantitative discrepancies. First, there is a difference between the potential values in curves *a* and *b* amounting to as much as 100 mV in the early stages of the reduction. This large difference casts doubt on the validity of the measurements, and if it arose through decomposition of some of the intermediate compound, then the results are not quantitative. Secondly, the end point of the titration suggests that 1 mole of the intermediate compound is produced by reaction with 1.25 equivalents, i.e. 0.63 mole, of peroxide, which is at variance with Keilin & Hartree's results, and their observations, which showed that more than 1 mole of peroxide is needed. It is unlikely, therefore, that under the conditions of the potentiometric titration full formation of the intermediate compound was obtained. For these reasons we do not believe that the titrations in Fig. 7 constitute a satisfactory proof of the single equivalent oxidation, although it might well be possible to find conditions where the method could be used.

In solutions of pH 6.5 it has recently been shown that additional reactions occur giving products that cannot be reduced to the original MetHb, or MetMb (Dalziel & O'Brien, 1952; George & Irvine, 1952). Whilst this further complicates the interpretation of the potentiometric titration, it offers a more likely explanation for the differences noted between the reaction of MetHb and of HbO₂ than the equilibrium reaction (8), in which an oxygen molecule is co-ordinated to the tetravalent iron atom. There was no direct evidence for the bonding of oxygen in this way, or to methaemoglobin as specified in reaction (6).

Fig. 7. Potentiometric titration of methaemoglobin with ethyl hydroperoxide (taken from Polonovski *et al.* 1941*a*). *a*, MetMb with ethyl hydroperoxide; *b*, reduction of the compound formed with ascorbic acid.

SUMMARY

1. Sodium chlorite, potassium periodate, potassium persulphate with silver ions present, potassium molybdiocyanide and potassium chloriridate all react with metmyoglobin to give compounds with the same spectroscopic characteristics as those of the intermediate compound produced by the peroxides, a one-equivalent oxidation product which can be denoted by the symbol Fe_p^{IV} .

2. The oxidizing agents containing oxygen, e.g. potassium periodate, react slowly with metmyoglobin, but the reactions with the electron transfer reagents are extremely rapid with velocity constants greater than $10^5 \text{ l. mole}^{-1} \text{ sec.}^{-1}$, even in alkaline solutions.

3. Potassium molybdiocyanide only oxidizes metmyoglobin above pH 11.0. At pH 8.6 and below, an equimolar concentration of potassium molybdocyanide reduces the intermediate compound.

4. In the reaction between potassium chloriridate and metmyoglobin at pH 8.6 the plot of percentage oxidation against the ratio $[\text{IrCl}_6^{2-}]/[\text{MetMb}]$ is linear, a mole ratio of about 1.5 being required for complete reaction.

5. In solutions of pH about 7.0 the intermediate compound is formed in an equilibrium reaction with potassium chloriridate, the constant (K) having a value of 0.20 ± 0.01 at pH 6.82, 20.4° and $I = 0.042$.

6. The cationic electron transfer oxidizing agents, ruthenic and osmic tris-dipyridyl complexes, do not form the intermediate compound with metmyoglobin. They appear to react preferentially with other groups rather than with the iron atom. However, the corresponding osmous complex reduces the intermediate compound at all pH values below about 8.6.

7. The variation of E'_0 with pH for the $\text{Fe}_p^{\text{IV}}/\text{Fe}_p^{3+}$ couple, inferred from the threshold pH values observed in the action of several of the oxidizing and reducing agents, is discussed together with its bearing on possible structures for the higher oxidation state.

8. The papers of Polonovski, Jayle, Glotz & Fraudet, who suggested between 1938 and 1941 that quadrivalent iron compounds participate in haemo-protein reactions, are critically examined.

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