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The Reaction between Metmyoglobin and Hydrogen Peroxide

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Transient complexes with well defined spectroscopic characteristics play an important role in the reaction of the haemoprotein enzymes, catalase and peroxidase, with hydrogen peroxide. Two and sometimes three different complexes can be observed depending on the experimental conditions. For example, Keilin & Mann (1937) obtained two red complexes with peroxidase and hydrogen peroxide, and later Theorell (1941) showed that a green complex preceded the formation of the first red complex.

This system has been studied intensively by Chance (1949*a, b*) who extended the observation to systems of catalase and hydrogen peroxide or alkyl hydroperoxides (1949*c*). There is, however, very little information from which structural differences accounting for the various complexes can be inferred. The transient nature of the complexes leads to experimental difficulties.

These difficulties were absent in the case of the complex formed with metmyoglobin (MetMb) and hydrogen peroxide. The existence of a well defined red complex in this system and with haemoglobin too is well established. Keilin & Hartree (1935) found that the formation requires 1 molecule of hydrogen peroxide per haematin iron atom. With reducing agents the complex is reduced back to metmyoglobin or methaemoglobin. As far as is known, only this single complex is formed and because it shares certain properties with the secondary complexes of peroxidase and catalase, a detailed knowledge of its behaviour is valuable for the further elucidation of enzyme reaction mechanisms.

In this paper experiments are described on the formation of the metmyoglobin-hydrogen peroxide complex over a wide pH range which reveal that only between pH 8 and 9 is the formation unattended by oxidative side reactions affecting the porphyrin ring. Reduction of the complex with

ferrocyanide shows it to have 1 oxidizing equivalent, and magnetic measurements suggest that it may be a compound containing quadrivalent iron. A preliminary account of these experiments has been published (George & Irvine, 1951).

MATERIALS AND METHODS

Metmyoglobin. The metmyoglobin (MetMb) used in these experiments was obtained from horses' hearts, the method of preparation following that of Theorell (1932). Briefly, the minced hearts are extracted with water at 0° and muscle globulins removed by addition of basic lead acetate to the extract, the pH of which has been previously adjusted to 7.0 with 1*N*-NaOH. The excess Pb is removed as phosphate with Na₃PO₄, and haemoglobin precipitated by adding (NH₄)₂SO₄ to 75% saturation. MetMb is precipitated when the solution is saturated to 100% with (NH₄)₂SO₄ and is taken up in the minimum quantity of 3*M*-phosphate buffer, pH 6.8. Phosphate is removed by dialysis against distilled water and MetMb again salted out. It is then taken up in 0.6*M*-phosphate buffer, pH 5.7, passed through a Seitz filter and stored at 0°.

Quantities of this stock solution were thoroughly dialysed against distilled water before use and the concentration determined following the method of de Duve (1948).

Hydrogen peroxide. Pure 97% (w/w) H₂O₂, free from inhibitors, was used. This was kindly supplied by Laporte Chemicals Ltd. Before each experiment a stock solution (approx. 0.1*N*) was standardized against KMnO₄ and then diluted to the desired concentration.

Buffer solutions. In the pH range 5.8–7.0 phosphate buffers (Green, 1933) were used. For the pH range 7.8–10.0 borate buffers were used. Experiments at pH 11.1 were carried out in a glycine buffer, while at higher pH values dilute alkali was used. The pH values of all buffer solutions were measured on a Cambridge pH meter.

Methods

Colorimetric or spectrophotometric determinations of optical density were made in the majority of the experiments. Because of its easier and more rapid application, the

former method was used wherever possible (pH range 5.8–9.0). Yellow filters were found to give the most suitable wavelength region. In the more alkaline regions and also in experiments requiring a complete analysis of spectra in the visible and Soret regions, spectrophotometric measurements were made using a Unicam quartz spectrophotometer.

Owing to the relative slowness of the formation and reduction of the H_2O_2 -MetMb complex at the concentrations used and especially in the alkaline region, it was necessary to follow the reactions kinetically in order to ascertain when full formation or reduction had taken place.

The percentage complex formation or reduction was calculated by interpolation using Lambert and Beer's law. The optical density of a solution of MetMb and H_2O_2 was found to reach a maximum value when a slight excess of H_2O_2 was present, e.g. a molar ratio between 1.5 and 2.5 to 1 depending on the pH of the solutions. This optical density was taken to be that corresponding to 100% complex formation. The procedure is justified by the experiments of Keilin & Hartree (1950), who found that H_2O_2 concentrations in great excess (100 to 1) were required before appreciable reaction of H_2O_2 and the complex itself occurred.

For the magnetic measurements a modification of the Theorell (1942) microbalance was used. The calibration was carried out with ferrous ammonium sulphate solutions.

RESULTS

Qualitative experiments indicated that a reddish violet complex was formed throughout the pH range 5.0–12.0 when H_2O_2 was added to MetMb. The

Fig. 1. Recovery of MetMb from complex. Azide spectra; smooth curve represents MetMb + azide; $\circ$, reduced complex at pH 8.4 + azide; $\bullet$, reduced complex at pH 11.1 + azide.

complex is comparatively stable. Between pH 8 and 9 slight decomposition could be detected at the end of half an hour after formation and complete destruction was apparent after about 48 hr. standing at room temperature. The end product was mainly MetMb. The rate of destruction was more rapid in acid solution.

The complex is a good oxidizing agent and will oxidize ascorbic acid, quinol, *p*-phenylenediamine, leuco-malachite green, iodide ion (but not bromide) and ferrocyanide ion, itself being reduced to MetMb. The rate of oxidation is dependent on pH and is faster in more acid solutions.

Fig. 2. Effect of pH on complex. Visible region 650–500 $\text{m}\mu$. $\bullet$, complex at pH 8.4; $\times$, complex at pH 10.0; $\bullet$, complex at pH 6.8; $\circ$, complex at pH 5.8.

Fig. 3. Effect of pH on complex. Soret region; (a) smooth curve represents complex at pH 8.4; $\circ$, MetMb, pH 6.8; $\bullet$, MetMb, pH 11.1. (b) $\circ$, complex at pH 8.4; ∇ , complex at pH 10.0; $\blacktriangle$, complex at pH 11.1; $\bullet$, complex at pH 6.8; $\circ$, complex at pH 5.8.

Experiments show that between pH 8.0 and 9.0 the reaction between H_2O_2 and MetMb is straightforward in the sense that the minimum amount of side reactions occur. Thus it is possible within this region to recover 90–100% of MetMb from the complex by the addition of mild reducing agents. By comparison only about 65% MetMb is recoverable at pH 11.1 (Fig. 1). Below pH 8.0 and especi-

ally at pH values <7.0 side reactions occur producing one or more compounds with strong absorption in the red. Above pH 9.0 the products of side reactions have no such absorption. Thus in Figs. 2 and 3, which show the spectra of the complex in the visible and Soret region, it will be seen that the difference between the spectrum of the complex at pH 10.0 and at pH 8.4 is mainly a difference in intensity. Table 1 gives the millimolar extinction coefficients of the complex at 549 and 423 m μ . at pH values 8.3, 10.0 and 11.1.

On the other hand, there is a definite shift of the band in the Soret region when the complex is formed at a pH <8.0; at pH 6.8 the band is at 420–423 m μ . and at pH 5.8 it is at 413 m μ . (Fig. 3*b*). In the visible region the difference is borne out by the appearance of a new band at 590 m μ . (Fig. 2).

Table 1. Millimolar extinction coefficient ($\epsilon \times 10^{-3}$) of complex at 549 and 423 m μ . at different pH values

pH	$\epsilon \times 10^{-3}$ at 549 m μ .	$\epsilon \times 10^{-3}$ at 423 m μ .
8.4	9.8	107.0
10.0	8.3	87.0
11.1	6.5	62.0

Table 2. Effect on extinction and position of peak of changing the pH after complex formation at a given pH

(a) Complex formed at pH 6.8		
pH	Position of peak (m μ .)	$\epsilon \times 10^{-3}$
6.8	420–423	93.0
9.0	420–423	94.0
11.1	420–423	94.0
(b) Complex formed at pH 9.0		
pH	Position of peak (m μ .)	$\epsilon \times 10^{-3}$
9.0	423	102.0
6.8	423	100.0
11.1	423	101.0

An interesting observation on this effect of pH is that once the complex is formed changing the pH seems to have little influence on the spectrum. Table 2*a, b* shows some of the results obtained from measurements in the Soret region. This shows that the influence of different hydrogen-ion concentration is exerted during the formation reactions and the effect is not due to a change in spectrum of the complex as the pH is varied.

The nature of the side reactions is not fully understood. It would seem that the reduction of the intensity, which is observed when the complex is formed at a pH > 9.0, is due to varying amounts of cholemyoglobin formed. Thus the carbon monoxide-haemochromogen (obtained by reducing the complex at pH 11.1 with Na₂S₂O₄ in the presence of carbon monoxide and adding 20 % alkali, cf. Lemberg, Legge & Lockwood (1941)) shows, in

addition to the bands at 542 and 573 m μ . of the true haemochromogen, a band at 630 m μ . There is also a considerable reduction of the bands at 542 and 573 m μ . (see Fig. 4). At pH 6.8 and 5.8,

Fig. 4. Haemochromogen spectra, 650–500 m μ ., from complex formed at pH >8.0; smooth curve is haemochromogen from MetMb; ○, from complex formed at pH 8.6; ●, from complex formed at pH 8.0; △, from complex formed at pH 11.1.

Fig. 5. Haemochromogen spectra, 650–500 m μ ., from complex formed at pH <8.0. Smooth curve is haemochromogen from MetMb; ○, from complex formed at pH 6.8; ●, from complex formed at pH 5.8.

however, a different band appears at 615 m μ ., and there is very little reduction in the bands of the true haemochromogen (Fig. 5). In addition, the Soret spectrum of the haemochromogen obtained from the complex formed at pH 5.8 is even more intense than that of the true haemochromogen

(Fig. 6). Substitution of the porphyrin ring without ring fission seems a likely explanation.

The following observations show that these side reactions occur during the actual formation of the complex. If MetMb is allowed to stand with the preformed complex for about 1 hr. at either pH 5.8 or 11.1, the haemochromogen spectrum obtained from the mixture is the same as that produced when

Fig. 6. Haemochromogen spectra. Soret region; ●, from MetMb; ○, from complex formed at pH 5.8.

MetMb is present with the complex for a few minutes. This suggests that an oxidizing entity more powerful than the complex itself is present during the formation of the complex. Qualitative experiments with luminol support this. If luminol is added to the complex a dull luminescence is obtained, whereas if luminol and MetMb are mixed and H_2O_2 then added (under conditions where the complex is formed in a few seconds) a bright flash is observed followed by a dull luminescence.

Quantitative measurements on the formation of the complex

The formation of the complex between pH 8.0 and 9.0 is characterized by the fact that a 1:1 ratio of H_2O_2 to MetMb does not produce 100% complex formation even in a concentration of MetMb as high as 10^{-4}M . At this concentration 80–90% of complex is produced, the percentage varying between these limits for different samples of MetMb.

The effect of concentration on the formation is only very slight over a tenfold change of absolute

concentration and rules out the possibility of an equilibrium of the type

The results of the experiments are shown in Fig. 7.

Fig. 7. Effect of concentration on complex formation. ○, MetMb $1.0 \times 10^{-4}\text{M}$; □, MetMb $4.9 \times 10^{-5}\text{M}$; ●, MetMb $8.8 \times 10^{-6}\text{M}$. Broken line is theoretical curve based on a 1:1 ratio of H_2O_2 to MetMb.

Fig. 8. Effect of pH on complex formation. Broken line is theoretical curve based on a 1:1 ratio of H_2O_2 to MetMb. ●, pH 8.0, MetMb $4.7 \times 10^{-5}\text{M}$; □, pH 8.4, MetMb $5.0 \times 10^{-5}\text{M}$; ○, pH 9.0, MetMb $4.8 \times 10^{-5}\text{M}$.

Fig. 8 shows the effect of pH on complex formation which is small within the pH region 8.0–9.0. Although in the figure the maximum complex formation occurs at about pH 8.0–8.4, the pH at which maximum formation is observed appears to differ to a small extent for different samples of MetMb. In none of the samples used, however, was this pH outside the range 8.0–9.0.

Reduction of the complex

Experiments with ferrocyanide ion as the reducing agent show that 1 mole of ferrocyanide will reduce 1 mole of complex completely to give MetMb, within the pH region 8.0–9.0. Typical results of these experiments are shown in Table 3a, b.

If, instead of adding ferrocyanide to the pre-formed complex, the ferrocyanide is first added to MetMb in varying amounts and hydrogen peroxide

Table 3. *Reduction of the metmyoglobin-hydrogen peroxide complex by ferrocyanide*

(a) pH 8.0				
Initial complex (μM)	Ferrocyanide (μM)	Observed complex left (μM)	Complex reduced (μM)	Ratio: $\frac{\text{Fe(CN)}_6^{4-}}{\text{Complex}}$
24	10	14	10	1.00
24	15	10	14	1.07
24	24	1	23	1.04
40	30	9	31	0.97

(b) pH 8.4				
Initial complex (μM)	Ferrocyanide (μM)	Observed complex left (μM)	Complex reduced (μM)	Ratio: $\frac{\text{Fe(CN)}_6^{4-}}{\text{Complex}}$
12	10	2	10	1.00
30	30	2	28	1.07

Table 4. *Reaction of metmyoglobin with hydrogen peroxide in the presence of ferrocyanide at pH 8*

(In these experiments ferrocyanide was added before hydrogen peroxide.)

(a) Initial MetMb and H_2O_2 concentrations $1.04 \times 10^{-4}\text{M}$ and $1.01 \times 10^{-4}\text{M}$ respectively

Fe(CN)_6^{4-} (μM)	Complex formed (μM)	Oxidizing equivs. of H_2O_2 accounted for (μM)*	(%)
0	91	91	45.0
120	72	192	95.0
140	56	196	97.0
160	38	198	98.0

* On the basis 1 mole complex = 1 mole Fe(CN)_6^{4-} = 1 equiv. H_2O_2 . 202 equivs. present initially.

(b) Initial MetMb and H_2O_2 concentrations $5.2 \times 10^{-5}\text{M}$ and $5.0 \times 10^{-5}\text{M}$ respectively

Fe(CN)_6^{4-} (μM)	Complex formed (μM)	Oxidizing equivs. accounted for (μM)*	(%)
0	37	37	37.0
60	29	89	89.0
70	23	93	93.0
80	14	94	94.0
90	5	95	95.0

* On the basis 1 mole complex = 1 mole Fe(CN)_6^{4-} = 1 equiv. H_2O_2 . 100 equivs. present initially.

then introduced, complex formation is observed even when the amount of ferrocyanide present is in excess of the amount of complex expected. Table 4a, b gives the results of two typical experiments of this nature at different absolute concentrations.

Magnetic properties of the complex

Preliminary measurements of the molar magnetic susceptibility of the complex at pH 8.6 indicate that it is about $11\,000\text{--}11\,800 \times 10^{-6}$ c.g.s. units at room temperature. This corresponds to a magnetic moment of 5.1–5.3 Bohr magnetons (μ_B) and suggests the presence of four unpaired electrons. These

figures may be compared with those quoted by Hartree (1946): reduced peroxidase $\chi_m \times 10^{-6}$ c.g.s. units = 11 410; haemoglobin $\chi_m \times 10^{-6}$ c.g.s. units = 12 290, $\mu_B = 5.43$; myoglobin $\chi_m \times 10^{-6}$ c.g.s. units = 12 400, $\mu_B = 5.46$. (See footnote p. 516.)

DISCUSSION

The first significant fact which emerges is the effect of pH on the reaction between hydrogen peroxide and metmyoglobin. The reaction can be said to be 'normal' within the pH range 8.0–9.0. Outside this region there is the peculiarity on the alkaline side of a reduction of the intensity of the complex, and on the acid side the production of a different spectrum (Figs. 2 and 3). The haemochromogen spectrum obtained from the complex formed at pH 11.1 (Fig. 4) shows a peak at 630 $\text{m}\mu$, and a reduction of the intensities of the peaks at 542 and 573 $\text{m}\mu$ of the true haemochromogen. This is typical of the behaviour which Lemberg *et al.* (1941) associate with choleglobin formation. Moreover, the formation of choleglobin, more specifically in this case of cholemyoglobin, would account for the absence of any marked absorption in the visible and Soret regions other than that attributed to the complex.

On the acid side, however, this explanation will not hold. The haemochromogen spectra obtained from the complex at pH 6.8 and 5.8 show a peak at 615 $\text{m}\mu$, not at 630 $\text{m}\mu$, and there is little reduction of the peaks at 542 and 573 $\text{m}\mu$. (Fig. 5). This fact, together with the observation that in the Soret region there is not only no decrease, but an actual rise in the intensity of the haemochromogen spectrum obtained from the complex at pH 5.8 above the true haemochromogen (as shown in Fig. 6), suggests that a different type of oxidative attack on the porphyrin ring has occurred. This type of attack presumably does not affect the conjugated double-bond system to the same extent that cholemyoglobin formation does, for the intensity of light absorption in the Soret region is believed to be a characteristic of the conjugated system.

The absence of any effect of pH on the spectrum of the complex once it has been formed and the fact that the complex does not apparently react either with itself or added metmyoglobin to give the side reactions, indicate clearly that these side reactions occur during the formation of the complex. The experiments with luminol suggest that a transient oxidizing agent, more powerful than the complex, is produced during the formation reaction.

The data of the other experiments reveal three important features:

(1) The complex is not formed in any set of equilibrium reactions involving metmyoglobin and hydrogen peroxide, either in their ionized or unionized forms, e.g.

In this respect its formation is unlike that proposed by Chance (1949*b*, 1951) for the peroxidase and catalase complexes, i.e.

This suggests that neither H_2O_2 nor HO_2^- is present in the metmyoglobin complex.

(2) Full formation of the complex is not obtained for a 1:1 ratio of hydrogen peroxide to metmyoglobin. In view of the fact that the system is not an equilibrium system this result must mean that hydrogen peroxide or metmyoglobin or complex (or more than one of these) is disappearing during the reaction. The disappearance of metmyoglobin can be ruled out since increasing the peroxide ratio increases complex formation. Furthermore, within the pH region 8.0–9.0 nearly all the metmyoglobin can be recovered, even though in this region 100% formation is not observed for 1:1 ratio. If complex is being destroyed, the destruction must take place while formation is occurring, since once the complex has been formed no destruction is detectable for about half an hour (within the pH region 8.0–9.0). In the absence of an equilibrium the destruction of complex could only be interpreted as producing some new entity which would be both colourless and reducible to metmyoglobin, to account, first, for the lack of absorption and, secondly, for the complete recovery of metmyoglobin at certain pH values. This does not seem likely.

(3) The oxidizing equivalent of the complex is unity (Table 3). Hence the full oxidizing equivalent of the peroxide can only be accounted for by assuming that some other oxidizing entity also with 1 oxidizing equivalent is produced during the reaction of hydrogen peroxide and metmyoglobin. Evidence supporting this is obtained by adding a reducing agent to the metmyoglobin before introducing hydrogen peroxide when the full oxidizing capacity of the peroxide is realized (see Table 4).

On the basis of this last feature, the equation for the formation of the complex can be written in its simplest form

2 oxidizing equivs.

$\rightarrow$ 1 oxidizing equiv. + 1 oxidizing equiv.

A tentative mechanism accounting for the complex and X each with 1 oxidizing equivalent is the formation of an OH $\cdot$ radical and ferryl myoglobin in a reaction of the type

where Fe^+ represents the haematin iron atom of metmyoglobin. Ferrylmyoglobin by analogy with the hypothetical ferryl ion FeO^{2+} and the well known vanadyl ion VO^{2+} would be a compound of quadrivalent iron and would thus have 1 oxidizing equivalent when reduced to a compound in the ferric state. The identification of the complex with ferrylmyoglobin would be in accordance with magnetic measurements which show the presence of four unpaired electrons.* The electronic structures of the Fe^{2+} , Fe^{3+} and Fe^{4+} ions are given in Fig. 9.

	3d	4s	4p	Unpaired electrons
Fe^{2+}	$\otimes \otimes \otimes \otimes \otimes$	$\bigcirc$	$\bigcirc \bigcirc \bigcirc$	4
Fe^{3+}	$\otimes \otimes \otimes \otimes \otimes$	$\bigcirc$	$\bigcirc \bigcirc \bigcirc$	5
Fe^{4+}	$\otimes \otimes \otimes \otimes \bigcirc$	$\bigcirc$	$\bigcirc \bigcirc \bigcirc$	4

Fig. 9. Electronic structures of Fe^{2+} , Fe^{3+} , Fe^{4+} .

It can be seen that both Fe^{2+} and Fe^{4+} ionic compounds would have four unpaired electrons. But in the case of the $\text{MetMb-H}_2\text{O}_2$ complex, this clearly cannot be a ferrous compound for it is a strong oxidizing agent which on reduction yields a ferric compound.

The identification of X with an OH radical fits in with the observations that (1) X is an oxidizing agent. (2) X is transient; only during the actual formation of the complex is its oxidizing ability observed. (3) During the formation of the complex a flash is observed if luminol is present. No flash is

* Note added in proof: Theorell & Ehrenburg (1952) have reported a susceptibility value of 3800×10^{-6} c.g.s. units: our more recent experiments show that the high value of about 11000×10^{-6} c.g.s. units quoted above is in error on account of decomposition of the complex during the measurements. We find the correct value to be $< 8400 \times 10^{-6}$ c.g.s. units. A covalent instead of an ionic structure containing quadrivalent iron would be in accord with these new values, for d^2sp^3 covalent bond formation of Fe^{4+} results in two unpaired electrons ($\chi_m = 3390 \times 10^{-6}$ c.g.s. units) as can be seen from Fig. 9.

obtained if luminol is added after the complex has been formed.

The side reactions could be accounted for because of the high oxidizing activity of the OH radical.

SUMMARY

1. Metmyoglobin forms a complex with hydrogen peroxide throughout the pH range 5.0–12.0. Only within the pH range 8.0–9.0 is this reaction unattended by oxidative attack on the porphyrin ring.

2. On the alkaline side the attack on the ring seems to give cholemyoglobin, for a regular decrease in the intensity of the absorption bands is observed both in the visible and Soret regions of the spectrum. In solutions more acid than pH 8.0 a different type of oxidative attack occurs, for no regular decrease in intensity is observed, but there is a shift of the band maxima. These side reactions are not due to the reaction of the complex itself with metmyoglobin,

but arise by some other mechanism during the formation of the complex.

3. The system $\text{MetMb} + \text{H}_2\text{O}_2$ is not an equilibrium system. The effect of concentration on the formation of the complex is only very small over a tenfold change of absolute concentration.

4. The oxidizing equivalent of the complex is unity. By adding a reducing agent to the metmyoglobin before peroxide it is possible to account for the two oxidizing equivalents of the peroxide. This indicates the reaction to be of the type

5. A tentative mechanism is proposed identifying the complex with ferrylmyoglobin, a compound of quadrivalent iron, and X with the OH radical. The high oxidizing activity of the OH radical could account for the oxidative side reactions.

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The Ionization of Acidic Metmyoglobin

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The problem of establishing thermodynamic constants for the ferric complexes of haemoproteins is important in at least two respects. In the first place such constants offer a workable criterion where a parallelism is sought between the behaviour of simple ferric iron and that of the iron atom in the porphyrin ring of haemoproteins. Secondly, a knowledge of such data would offer additional evidence for elucidating hitherto uncertain structures, for instance those of haemoprotein-peroxide complexes, by comparing approximate thermodynamic constants for the formation of these complexes with corresponding data for the formation of, say, the hydroxide, fluoride or cyanide complexes where the structure is better known.

Some equilibrium data on the ionization of acidic methaemoglobin in water, which is equivalent to the formation of the hydroxide complex, have been published by several workers but no values were obtained for ΔH or ΔS . Furthermore, there appears to be an interesting anomaly in the effect of neutral salts on this ionization constant. An inspection of the results of Austin & Drabkin (1935) which were based on spectrophotometric studies of canine methaemoglobin, and those of Coryell, Stitt & Pauling (1937), derived from magnetic measurements on bovine methaemoglobin, shows that this ionization constant falls with increasing ionic strength, i.e. pK moves away from the isoelectric point of the protein, whereas the effect of neutral