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

this way were therefore too low by about 4%, and the extinction coefficients were correspondingly too high.

Buffer solutions. The main pH region, 8·0–10·2, was covered by borate buffers made from H_3BO_3-NaOH mixtures. In the pH range 5·8–8·0, phosphate buffers were made from the appropriate NaH_2PO_4, $2H_2O$-NaOH mixtures, and for lower pH values phthalate buffers were used. All solutions were prepared using A.R. chemicals and water which had passed through a column of exchange resin (Bio-Deminrolit FF, Permutit Co. Ltd.), and made up to the same total ionic strength, usually 0·05, with NaCl. It will be shown below that the contribution to I from solutions of MetHb about 5×10^{-5}M is considerably less than 0·001.

Spectrophotometric measurements. Optical densities were measured with a Unicam quartz spectrophotometer, temperature being controlled to $\pm 0{\cdot}2°$ by means of a circulating water device. In the usual type of experiment the total MetHb concentration was about 5×10^{-5}M, and optical densities were measured at 578 mμ. using 1 cm. glass cells. Fig. 2 shows that at this wavelength alk. MetHb has a sharp band with an extinction coefficient $\epsilon = 8610 \pm 80$ while acid MetHb has $\epsilon = 3180 \pm 30$. As a check, the MetHb concentration was varied in a series of experiments by a factor of 200. At the lowest concentrations, $2{\cdot}5 \times 10^{-6}$M, measurements were made in the near ultraviolet region, at the sharp Soret band, 405 mμ., of acid MetHb. Solutions of the order $5{\cdot}0 \times 10^{-4}$M were measured at 578 mμ. using 1 mm. optical paths. Some experiments were also made using the 501 mμ. band of acid MetHb.

Measurement of pH was done as described in Paper 1. The pH standard of 0·05M-borax was taken as 9·21 at 20°, 9·15 at 28° and 9·09 at 37°, and of 0·05M-potassium hydrogen phthalate as 4·01 at the three temperatures. No further corrections were applied for the change in liquid junction potentials with the changing ionic strength of the solutions.

RESULTS

Spectrophotometric characteristics. The absorption spectra of the acidic and alkaline forms of MetHb have been recorded by several workers, but the extinction coefficients reported are not always in satisfactory agreement. Reference may be made to Horecker (1943) for the infrared and visible, to Austin & Drabkin (1935) for the visible, and to Hicks & Holden (1929) for the ultraviolet region of the spectrum.

A more thorough study of these spectra reveals a subtle dependence, hitherto unobserved, of the spectrum of acid MetHb on ionic strength. Two minor changes in the absorption spectrum of the brown acid MetHb are in fact detectable at sufficiently low ionic strength, and they can be attributed to the following ionizations:

$$H_2Pr.Fe^+(H_2O) = HPr^-.Fe^+(H_2O) + H^+ \qquad pK_1' \simeq 5{\cdot}1, \quad (1)$$

$$HPr^-.Fe^+(H_2O) = Pr^{2-}.Fe^+(H_2O) + H^+ \qquad pK_2' \simeq 6{\cdot}4, \quad (2)$$

which precede the main ionization

$$Pr^{2-}.Fe^+(H_2O) = Pr^{2-}.FeOH + H^+ \quad pK_3' \simeq 8{\cdot}4. \quad (3)$$

This last equilibrium involves the iron-bound water molecule and is responsible for the colour change from brown to red (Keilin & Hartree, 1949; Haurowitz, 1949). The symbols used above were chosen arbitrarily and the charges indicated are purely formal. Pr stands for the globin moiety, $H_2Pr.Fe^+(H_2O)$ and $HPr^-.Fe^+(H_2O)$ being respectively, the conjugate acid and base associated with pK_1'. pK_2' and pK_3' are defined in a similar manner. The location of the ionizable H^+ in Eqns. 1 and 2 is unknown though, presumably, they must be near the iron atom. The haematin-linked property of these two ionizations is operative, spectrophotometrically, at low ionic strength only. At all higher values of I the spectrum of acid MetHb corresponds to that of the species $HPr^-.Fe^+(H_2O)$ alone.

This complicated behaviour is illustrated in Figs. 1–3. Fig. 1 gives some absorption spectra, in the visible range 650–470 mμ., for acid MetHb at pH values below 5·8 and $I = 0{\cdot}02$. A small but definite and reversible change can be seen, in accordance with Eqn. 1. Unfortunately, the onset of irreversible denaturation at pH values below about 4·5 prevents the attainment of a clear spectral definition of the species $H_2Pr.Fe^+(H_2O)$.

Fig. 2 covers the same wavelength region at higher pH values. These spectra are given at $I = 0{\cdot}02$. The one at pH 5·6 represents the species $HPr^-.Fe^+(H_2O)$ and is obtained by extrapolation using the pK′ values which are given below. The absorption curve at pH 6·6 corresponds to about 80% formation of the intermediate species $Pr^{2-}.Fe^+(H_2O)$. The curve labelled $Pr^{2-}.FeOH$ represents the red alk. MetHb at pH 10·2.

The effect of neutral salts is to mask the two ionizations with the constants K_1' and K_2' so that at high salt concentrations, independently of the nature of the salt, the species $Pr^{2-}.Fe^+(H_2O)$ can no longer be detected, and the change identified with the appearance of $H_2Pr.Fe^+(H_2O)$ is much smaller. These two effects are of opposite sense, for it is the spectrum of the conjugate acid of pK_1', and that of the conjugate base of pK_2', which are suppressed. That is, only one species of acid MetHb, that corresponding to $HPr^-.Fe^+(H_2O)$, is operative in the pH range 4·6–7·0 at sufficiently high ionic strength. This point is illustrated in Fig. 3 which gives a composite spectrophotometric titration curve of MetHb at 578 mμ. and where the differentiation between the various ionized forms can be seen clearly.

Determination of pK_3', pK_2' *and* pK_1'. It is convenient to measure an equilibrium constant K_3' which refers to the ionization in Eqn. 3 which may be defined by the equation

$$pK_3' = pH + \log \frac{(\text{acid MetHb})}{(\text{alk. MetHb})}, \quad (4)$$

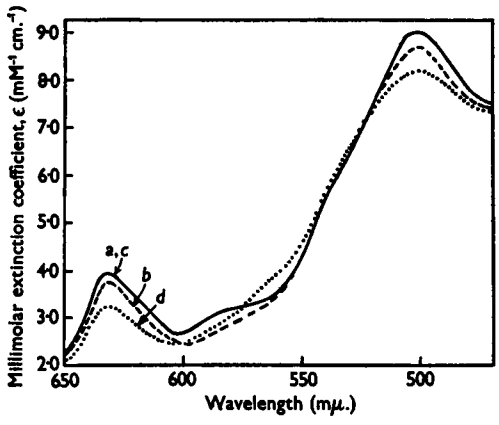

Fig. 1. Absorption spectra of acidic methaemoglobin in the range 650–470 mμ. The solutions are in phthalate buffers at 20° and $I=0{\cdot}02$. (*a*) pH = 5·72 (continuous line). (*b*) pH = 4·70 (broken line). No change is observed after 1 hr. The spectrum corresponds roughly to the species $H_2Pr.Fe^+(H_2O)$ of Eqn. 1. (*c*) Solution (*b*) diluted with buffer of pH 6·2 so that the pH of the resulting solution is 5·68: when corrected for dilution the spectrum coincides, within experimental error, with that of (*a*). (*d*) pH 4·39, the spectrum was taken within 20 min. and a slow irreversible change in the absorption was observed.

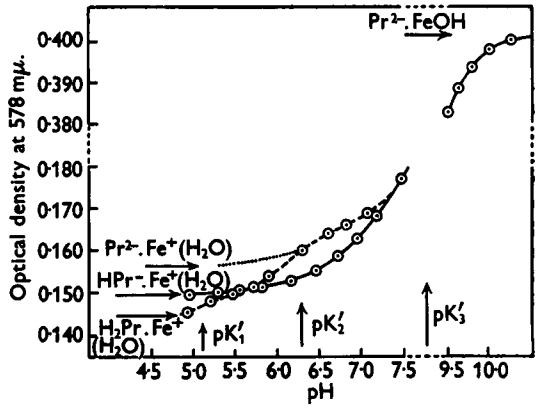

Fig. 3. Two titration curves of $4{\cdot}1\times10^{-5}$ M-MetHb plotting optical density at 578 mμ. against pH, at 20°. The continuous curve is at $I=0{\cdot}26$ and shows the equilibrium between acid MetHb, the species $HPr^-.Fe^+(H_2O)$, and alk. MetHb, $Pr^{2-}.FeOH$. The dashed curve is at $I=0{\cdot}01$ and shows the various species that appear below pH 7; it coincides with the other curve above pH 7·3. The dotted curve shows the extrapolation to the optical density corresponding to the species $Pr^{2-}.Fe^+(H_2O)$ calculated by the method described in the text.

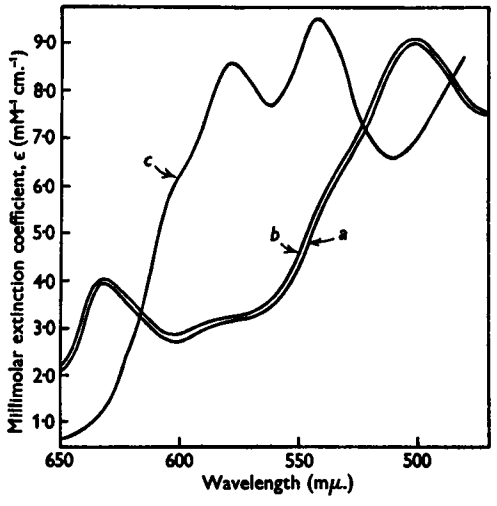

Fig. 2. Absorption spectra of MetHb at low and high pH, in the range 650–470 mμ. $T=20°$, $I=0{\cdot}02$. (*a*) Solution in phthalate buffer of pH 5·60. The curve was plotted after applying a slight extrapolation based on the known values of pK_2' and pK_3', and it corresponds to the species $HP^-.Fe^+(H_2O)$ of Eqns. 1 and 2. (*b*) Solution in phosphate buffer of pH 6·60. The curve represents maximum formation, about 80%, of the species $Pr^{2-}.Fe^+(H_2O)$ of Eqns. 2 and 3. (*c*) Solution in borate buffer of pH 10·2. The curve represents almost complete formation of alk. MetHb, which is the species $Pr^{2-}.FeOH$ of Eqn. 3.

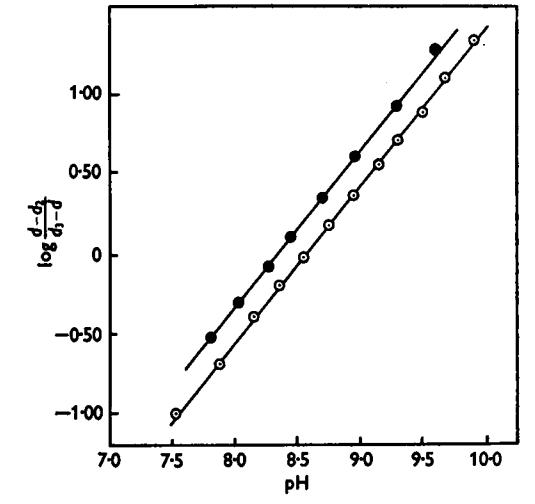

Fig. 4. Two tests of Eqn. 5 carried out at 20° using $5{\cdot}0\times10^{-5}$ M-MetHb in borate buffers. Values of $\log\frac{d-d_2}{d_3-d}$ are plotted against pH, the slopes of the lines being the values of n in Eqn. 5. Optical densities, d, were measured at 578 mμ.; d_3 is the corresponding optical density of 100% alk. MetHb and d_2 that of 100% acid MetHb obtained from an appropriate extrapolation. ⊙—⊙, test (1), $I=0{\cdot}02$, $n=1{\cdot}00$; ●—●, test (2), $I=0{\cdot}22$, $n=0{\cdot}99$.

where the brackets indicate molar concentration and where pH is measured against an arbitrary standard. However, the MetHb molecule contains four haematin groups which can ionize in this way, and if interaction occurs between these groups such that the ionization constant of one group is dependent on the degree of ionization of the others this would show itself by the experimental data fitting an equation of the type

$$\text{Const.} = n.\text{pH} + \log \frac{(\text{acid MetHb})}{(\text{alk. MetHb})}, \qquad (5)$$

where the value of n at 50 % reaction may be > 1 or < 1 depending on whether the ionization of one haematin group is favoured or hindered by the ionizations of the other haematin groups (cf. Wyman, 1948). Behaviour of this kind would be similar to that found in the combination of oxygen and carbon monoxide with reduced haemoglobin. Fig. 4 shows two runs which were carried out to test this point at two ionic strengths. The value of n was invariably found to be unity, which implies that no such interaction occurs and that the various haematin groups ionize independently. Eqn. 5 therefore reduces to Eqn. 4, and it was on this basis that pK_3' values have been calculated. A different complication was encountered at low ionic strengths due to the appearance of three spectroscopic species of acid MetHb; a method of analysing the results at these low ionic strengths is described later.

It follows from the account given above that at ionic strengths which are conveniently high ($I > 0{\cdot}2$) the main ionization constant K_3' may be calculated directly according to Eqn. 4. Details of the procedure are essentially the same as described in Paper 1. This method was found to be quite simple and accurate; mean deviations did not usually exceed ± 1 %. Several parallel runs also established that there were no specific effects due to the buffer or, excepting ammonium salts, to the added salt. Ammonium salts had a definite and reversible effect on the 578 mμ. band of alk. MetHb, similar to the effect of ammonia on alk. MetMb described by Theorell & Ehrenberg (1951).

The complications encountered at lower ionic strengths can be seen in Fig. 3. The shape of the curve suggests that there are three species involved in two overlapping ionizations with constants K_2' and K_3', as a consequence of which the middle species $Pr^{2-}.Fe^{+}(H_2O)$ is never completely formed; in fact it can be shown by analysing the data that its maximum formation corresponds to about 80 %. Similar considerations lead to the conclusion that the ionizations with constants K_1' and K_2' also overlap and hence that the species $HPr^{-}.Fe^{+}(H_2O)$ is also never fully formed. In view of these facts the determination of the three pK′ values requires the analysis of the data by a series of approximations. There is, however, another practical difficulty. The span, in units of optical density, of the pK_2' ionization is only 4–5 % that of the main pK_3' ionization, and so is that of the pK_1' ionization. It is consequently difficult to obtain data accurate enough for the precise calculation of pK_1' and pK_2'. On the other hand, pK_3' can be obtained fairly easily by applying two successive approximations to the analysis of the results obtained in the usual type of run. This method of calculation was used extensively and the limits of error on pK_3', even at the lowest ionic strengths, did not exceed ± 2 %. Fig. 5 shows the variation of pK_3' with $\sqrt{I}$ at three temperatures, 20, 28 and 37°. The region of low ionic strengths has been investigated at 20° and 37° only.

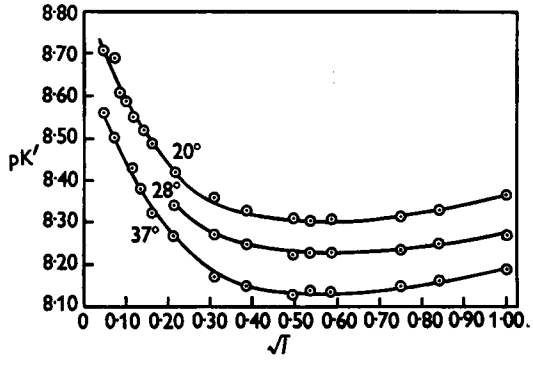

Fig. 5. The variation of pK_3' for (horse) acid MetHb with ionic strength, I, and temperature. I is calculated from the contributions of the various buffer ions.

An alternative and more elegant method for the analysis of overlapping equilibria has been described recently (Thamer & Voigt, 1952). This method leads to the following relation between pK_2' and pK_3':

$$pK_2' + pK_3' = 2pH_m - \log \frac{d_m - d_2}{d_m - d_3}, \qquad (6)$$

in which d_m is the maximum optical density in the titration curve of Fig. 6 and pH_m is the corresponding pH value, d_2 and d_3 are the optical densities corresponding to 100 % species $HPr^{-}.Fe^{+}(H_2O)$ and $Pr^{2-}.FeOH$ (alk. MetHb) respectively. Of the terms in Eqn. 6 only pK_3' and d_3 can be obtained with any certainty. Despite this severe limitation it was possible to obtain rough values of pK_2' and to show that as the ionic strength increased from $I = 0{\cdot}01$ to $I = 0{\cdot}06$ the value of pK_2' rose from about 6·33 to 6·40 at 20°. This effect is smaller and in the opposite sense to the effect of ionic strength on pK_3'. Fig. 6 shows one run at 22° and $I = 0{\cdot}016$ used for the calculation of a pK_2' value together with the theoretical curve computed from the various parameters given in the legend.

No accurate method could be devised for the determination of pK_1', not only because of the difficulties outlined above but also due to the onset of irreversible changes below pH 4·6. Approximate estimates place pK_1' around 5·1 at 20° and $I = 0{\cdot}01$.

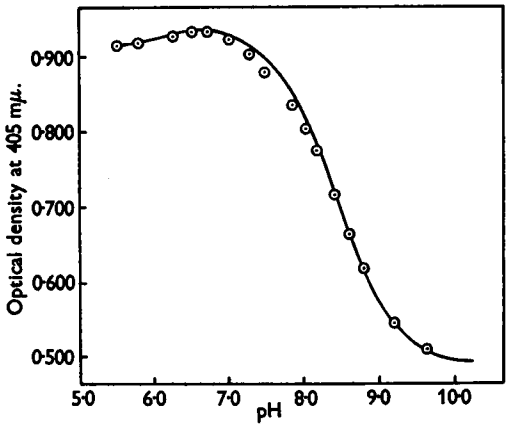

Fig. 6. Method of calculating pK_2' from the measured optical density at 405 mμ. as a function of pH. The experimental points are shown in circles. $T = 22°$, $I = 0{\cdot}016$. A theoretical curve, drawn as the continuous line, was calculated from the following parameters: $d_2 = 0{\cdot}916$, $d_m = 0{\cdot}933$, $d_3 = 0{\cdot}492$, $pH_m = 6{\cdot}70$, $pK_3' = 8{\cdot}48$, $pK_2' = 6{\cdot}34$ (after Thamer & Voigt, 1952).

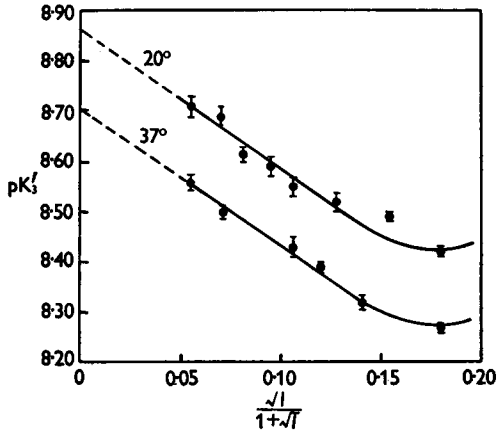

Fig. 7. Approximate thermodynamic ionization constants at 20° and 37° obtained from the data which are shown in Fig. 5 by a linear extrapolation of pK_3' values plotted against $\sqrt{I}/(1+\sqrt{I})$, where the ionic strength I includes the contributions of the buffer and salt ions as well as the contribution from MetHb estimated at the upper limit to be 0·001.

Approximate thermodynamic constants. Values for the approximate thermodynamic ionization constant K_3 were obtained by linear extrapolation to zero ionic strength. Fig. 7 shows the extrapolation at 20 and 37° made from $\sqrt{I}/1+\sqrt{I}$ plots. The two lines are approximately parallel with slopes of about $-2{\cdot}7$.

$$pK_3\,(20°) = 8{\cdot}86 \pm 0{\cdot}02, \quad pK_3\,(37°) = 8{\cdot}70 \pm 0{\cdot}02.$$

From these two values of pK_3 the corresponding heat of ionization ΔH may be calculated directly using the integrated van't Hoff isochore if one assumes that the value of ΔH is constant over the temperature range 20–37°. This gives

$$\Delta H° = 3{\cdot}91 \pm 0{\cdot}49 \text{ kcal./mole.}$$

The corresponding free energy and entropy changes at, say, 20° may now be obtained:

$$\Delta G° = -RT \ln K_3 = 11{\cdot}9 \pm 0{\cdot}03 \text{ kcal./mole,}$$

$$\Delta S° = \frac{\Delta H° - \Delta G°}{T} = -27{\cdot}2 \pm 1{\cdot}8 \text{ cal./mole/degree.}$$

Methaemoglobin from different species. The results quoted so far have been those of studies made on horse MetHb. In view of the possibility that, at very small concentrations of the haemoprotein, neutral salts may cause the dissociation of horse MetHb molecules into smaller fragments (Gutfreund, 1949; see, however, Adair, 1949) extensive parallel studies were carried out on ewe MetHb, the molecules of which may have greater resistance to such dissociation. Substantially the same results were obtained, and the various spectral characteristics were present. The actual values of the ionization constant K_3' differed slightly, but consistently, from the values for horse MetHb. This behaviour is illustrated in Table 1 which gives some

Table 1. *A comparison of the ionization constant* K_3' *in horse and ewe MetHb over a range of ionic strength*

($T = 20°$. Method of calculating pK_3' is given in the text. I = ionic strength calculated from the contributions of buffer and salt ions. The estimated probable errors are shown in brackets.)

I	pK′ horse (±0·01)	pK′ ewe (±0·02)	K′ ewe / K′ horse (±0·08)
0·006	8·66	8·62	1·10
0·029	8·47	8·41	1·15
0·050	8·41	8·34	1·18
0·123	8·34	8·28	1·15
0·155	8·33	8·26	1·18
0·304	8·30	8·24	1·15

pK_3' values at 20° for both species of haemoglobin. No difference in behaviour attributable to protein dissociation could be detected in this comparative study.

DISCUSSION

A comparison of the results with those of Paper 1 shows that the main ionization of (horse) acidic methaemoglobin, with ionization constant K_3', is similar in many respects to the ionization of (horse)

acidic metmyoglobin. The first important conclusion is the confirmation that the ionizations of the four haematin-bound water molecules in MetHb are single and independent. There are two lines of evidence for this. The similar behaviour of horse and sheep MetHb, with their varying degrees of dissociation into subunits, supports the view that the ionizations of the haems within the molecule are independent. Moreover, the value of n in Eqn. 5 was invariably found to be unity. This shows that the ionization involves one H^+ per Fe atom, and that pK_3' is constant over the pH range 7·8–10·0 even at a low ionic strength. There is no 'tailing' of pK_3' values, as was encountered in the case of the MetMb ionization, and hence there is no reason to suppose that any other equilibria affect the ionization of the iron-bound water molecule in acid MetHb above pH 9.

It has also been shown that both ionizations are endothermic processes, on which neutral salts have the same marked effect, though with MetHb the reversal of this effect is more easily observed because it occurs at a relatively higher ionic strength ($I \simeq 0{\cdot}1$ for MetHb and 0·01 for MetMb). Further, the limiting slope of the plots of pK_3' against $\sqrt{I}/(1+\sqrt{I})$, is about $-2{\cdot}7$ which, according to a simple Debye-Hückel model, indicates that acid MetHb has the charge type -2 and alk. MetHb -3. Hence the effective negative charge on MetHb, influencing the ionization of the iron-bound water, must be small even in solutions 3 pH units on the alkaline side of the isoelectric point. It is for this reason that its contribution to ionic strength has been taken at the upper limiting value of 0·001. A similar conclusion about MetMb was arrived at in Paper 1.

It is interesting to note that other workers have also arrived at similar conclusions. Thus Stadie (1928) examined the effect of haemoglobin on the activity coefficient of HCO_3^- ions and concluded that its 'ionic strength valence' was about unity. Barnard (1944) carried out acid-base titrations of canine haemoglobin and concluded that its ionic strength valence was zero at the isoelectric point, and that it rose on either side of this point, reaching a maximum of -3 in alkaline solutions. Clearly, these charge types can only accord with a Debye-Hückel model in solutions of extremely low ionic strength. However, Theorell & Ehrenberg (1951) as well as Coryell *et al.* (1937) and Wyman & Ingalls (1941) have all used linear extrapolation of pK' values from the region of high ionic strength with the result that their 'extrapolated' ionization constants are as much as 8 times the value of the true thermodynamic constants. For instance the above results give pK_3 (25°) = 8·80, compared with the value 7·89 obtained by Coryell *et al.* (at, presumably, $24 \pm 2°$).

The approximate thermodynamic constants given in this paper are subject to the same limits of uncertainty mentioned in Paper 1. When the two sets of data are compared (at, say, 20°) a curious compensation of the heat and entropy terms can be seen. Thus for MetHb, $\Delta H = 3{\cdot}91 \pm 0{\cdot}49$ kcal./mole and $\Delta S° = -27{\cdot}2 \pm 1{\cdot}8$ cal./mole/degree, for MetMb $\Delta H° = 5{\cdot}75 \pm 0{\cdot}67$ kcal./mole and

$$\Delta S° = -21{\cdot}7 \pm 2{\cdot}4 \text{ cal./mole/degree.}$$

Consequently, the corresponding free energies, and hence the thermodynamic ionization constants, are close to each other, the values of pK at 20° being $8{\cdot}86 \pm 0{\cdot}02$ for MetHb and $9{\cdot}04 \pm 0{\cdot}03$ for MetMb.

In these spectrophotometric studies the main difference between the behaviour of MetHb and that of MetMb has been the detection, in the former, of two ionizations possessing a haematin-linked property and characterized by equilibrium constants K_1' and K_2'. The term 'haematin-linked property' has been used rather than 'haematin-linked group' because the present data only reveal a change in the spectroscopic properties of the molecule. The very nature of this chemical reaction of the iron atom precludes a direct determination of its equilibrium constant K_3' in the pH range 5–7 where the ionizations indicated in Eqns. 1 and 2 occur. No evidence can therefore be obtained to show whether the affinity of the haematin iron for a proton depends on the extent of ionization of these groups which would then justify the use of the term 'haematin-linked group' (Wyman, 1948).

The presence of two haematin-linked ionizations in horse MetHb has been deduced previously from other studies. Coryell & Pauling (1940) concluded from a magnetic titration of MetHb that $pK_1' = 5{\cdot}3$; Theorell (1943) gave the value 5·45 at 20° and low ionic strength based on an acid-base titration of MetHb, and Wyman & Ingalls (1941) obtained 5·17 at 30° and $I = 0{\cdot}16$ from an analysis of a differential acid-base titration of MetHb and HbO_2 and a consideration of other related data. In this paper the approximate value of pK_1' is estimated to be 5·1 at 20° and $I = 0{\cdot}01$ and that of pK_2' to be 6·34 at 20° and $I = 0{\cdot}016$. The latter value presumably corresponds to $pK' = 6{\cdot}65$ at 30° and $I = 0{\cdot}2$ deduced by Taylor & Hastings (1939) from the effect of pH on the oxidation-reduction potential of the Hb-MetHb system, and to $pK' = 6{\cdot}50$ at 20° and low ionic strength obtained by Theorell (1943) from the acid-base titration of MetHb. In view of the uncertainties about the heats of ionization and the effects of neutral salts on pK_1' and pK_2' no thorough comparison between the various results can be made, but it is worth noting that in these spectrophotometric studies the value of pK_2' has been shown to rise with increasing ionic strength, and so the discrepancies between the results are probably much less than they appear to be.

The structural interpretation of the relation between these two ionizations and the haematin iron must now also explain the observation that neutral salts can modify their haematin-linked properties in such a way that, at high ionic strengths, the absorption spectrum of acid MetHb is also that of the conjugate base of the ionization in Eqn. 1 (with equilibrium constant K_1') and that of the conjugate acid of the ionization in Eqn. 2 (with constant K_2').

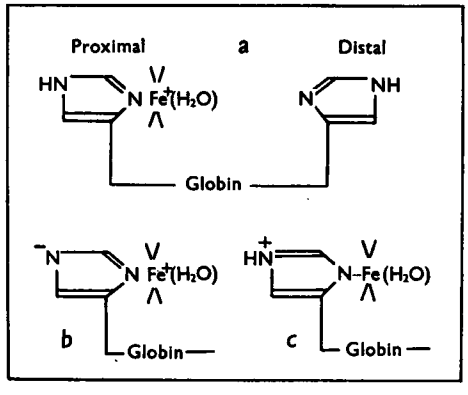

Fig. 8. Configurations of the haematin group in MetHb chosen to illustrate the possible effect of neutral salts on haematin-linked ionizations. (*a*) Schematic representation in which the iron atom is situated between the glyoxalinium N atoms of two histidine groups, with a strong bond to the proximal group and a weak bond to the distal group (after Coryell & Pauling, 1940), but the bonds are not specified in the diagram. (*b*) Proximal histidine with the imino N in the form of its conjugate base, representing one particular electronic configuration of the glyoxaline ring. (*c*) An alternative electronic configuration of the glyoxaline ring with the imino N in the form of its conjugate acid.

The type of interaction which can account, in part, for this neutral salt effect may be illustrated with reference to the structural model developed by Wyman (1939) and Coryell & Pauling (1940). In this model, which is shown in Fig. 8 (*a*), the iron atom is firmly bound to the glyoxalinium nitrogen atom of a histidine on one side of the haematin plane and is near enough for weak interaction to occur with another histidine group on the other side of the plane. The haematin-linked ionizations are attributed to the imino nitrogen of the first, or proximal, glyoxaline group and to the glyoxalinium nitrogen of the second, or distal, group. If the proximal group has the electronic configuration shown in Fig. 8 (*b*), then the clustering of cations, from a neutral salt, could reduce the electrostatic effect of the negatively charged base such that the spectrum resembles that of the conjugate acid. Conversely, if this group has the electronic configuration in Fig. 8 (*c*), then the clustering of salt anions could reduce the electrostatic effect of the positively charged acid group such that the spectrum resembles that of the conjugate base. This ionization can therefore be in accord with the salt effect on either pK_1' or pK_2'. However, the ionization of the distal group can accord with the salt effect in one sense only. The interaction between this group and the iron occurs only when the group is in its basic form and is prevented when salt cations shield the glyoxalinium nitrogen atom, in which case the spectrum would resemble that of its conjugate acid.

Coryell & Pauling (1940) attributed pK_1' to the glyoxalinium nitrogen of the distal group and pK_2' to the imino nitrogen of the proximal group. The above discussion shows that the effect of neutral salts on these two ionizations is in agreement with either pK_1' or pK_2' being attributed to the proximal group but not with pK_1' involving interaction between the iron and the distal group. In any structure where bonding to the iron involves an atom which is a proton acceptor this disagreement persists. Further, a water molecule must be incorporated in the methaemoglobin structure, with possibly an O—H...N hydrogen bond to the distal glyoxaline group. Although such a model is more acceptable than that in Fig. 8 (*a*) it does not help to resolve the difficulty.

It is structurally possible for the propionic acid side chain to be near enough to any proximal group or to the iron-bound water molecule for some type of weak bonding to occur, but there appears to be no particular reason for choosing such structures. Besides, arguments similar to those given above also lead to uncertainties about the identification of a pK′ value with a particular dissociating group. Independent knowledge of the charge types involved in these ionizations would help enormously to clarify this problem, as would a knowledge of the chemical nature of these groups.

SUMMARY

1. The spectrophotometric acid-base character of horse methaemoglobin has been investigated and compared with that of horse metmyoglobin.

2. All the results are consistent with the view that the ionizations of the four haematin-bound water molecules in methaemoglobin are single and independent. There is no evidence for interaction effects between the various haem groups and, unlike the case of metmyoglobin, no other ionizing groups appear to affect this ionization above pH 9.

3. The ionization of acid methaemoglobin, like that of acid metmyoglobin, shows a marked neutral-salt effect which also indicates that its 'ionic strength valence' per haem does not exceed -3 even at pH 10.

4. Approximate thermodynamic constants for this ionization have been determined by extrapolation from the data obtained at the lowest ionic strengths. The ionization constant

$pK_3\,(20°) = 8{\cdot}86 \pm 0{\cdot}02$,
$\Delta H = 3{\cdot}91 \pm 0{\cdot}49$ kcal./mole,
$\Delta S°\,(20°) = -27{\cdot}2 \pm 1{\cdot}8$ cal./mole/degree.

The values of pK_3 which previous workers obtained by extrapolation from the high ionic strength region are considerably different from the values given in this paper.

5. The spectral characteristics of ewe methaemoglobin are very similar to those of horse methaemoglobin. In a comparative study of the ionization the values of the ionization constant K_3' were found to be substantially the same in the two species, and no differences attributable to possible dissociation of the haemoprotein into subunits could be detected.

6. Small reversible changes in the absorption spectrum of acid methaemoglobin, not hitherto observed, have been attributed to two ionizations, probably of haematin-linked groups. At 20° and $I = 0{\cdot}01$ the following approximate values for these ionization constants have been obtained: $pK_1' = 5{\cdot}1$, $pK_2' = 6{\cdot}33$.

7. Spectroscopically, these two ionizations are remarkably sensitive to neutral salts. At comparatively high ionic strengths ($I > 0{\cdot}2$) no change can be detected in the spectrum of acid methaemoglobin over the pH range where these ionizations occur. The effect is such that, at all values of $I > 0{\cdot}2$, the spectrum of acid methaemoglobin corresponds to the conjugate acid of pK_2', which is identical with the conjugate base of pK_1'.

8. The effect of neutral salts on the haematin-linked ionizations is discussed with reference to possible structures for methaemoglobin.

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