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 MetMb + H_2O_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

$$\text{MetMb} + H_2O_2 \rightarrow \text{complex} + X.$$

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

salts on titration curves of several proteins, both simple and conjugated, is known to cause the reverse trend (Sørensen, Linderstrøm-Lang & Lund, 1926; Cohn, Green & Blanchard, 1937; Cannan, Palmer & Kibrick, 1942).

This paper deals with the corresponding ionization on the haem iron of metmyoglobin. Metmyoglobin contains only one ferric protoporphyrin (haematin) group per molecule, and it therefore presents a system which is similar to but simpler than that of methaemoglobin. The variation of its ionization constant with ionic strength and temperature is investigated in this paper and values are obtained for pK, ΔH and ΔS. Corresponding work on methaemoglobin is in progress.

Theoretical formulation

Myoglobin is stable in the pH range 6·0–13·0 (Theorell, 1934). Around pH 9·0 the reversible equilibrium between acidic (brown) and alkaline (red) forms of metmyoglobin may be represented by a single ionization of the type

$$HP \rightleftharpoons P + H^+,$$

where HP stands for a molecule of acidic metmyoglobin and P for its conjugate base, the alkaline form. The net electrical charges on these molecules are unspecified, but P will clearly have a charge which is one integral unit less than that of HP. The ionization involves a water molecule occupying the sixth co-ordination position of the octahedral ferric complex (Keilin & Hartree, 1949; Haurowitz, 1949). It may therefore also be represented by

$$Fe^+(H_2O) \rightleftharpoons FeOH + H^+,$$

or by the equivalent relation

$$Fe^+(H_2O) + OH^- \rightleftharpoons FeOH + H_2O.$$

For such an equilibrium

$$K_{HP} = \frac{a_P\, a_{H^+}}{a_{HP}} = a_{H^+} \frac{c_P}{c_{HP}} \frac{f_P}{f_{HP}}, \qquad (1)$$

where for a given solvent and at a given temperature K_{HP} is a true thermodynamic constant. The symbols a, c, f stand for activity, molar concentration and activity coefficient respectively.

Experimentally it is convenient to measure an equilibrium constant K' which is defined by the equation

$$pK' = pH + \log \frac{c_{HP}}{c_P}, \qquad (2)$$

where pH values are measured against an arbitrary standard in a pH meter and the ratio c_{HP}/c_P is measured spectrophotometrically. This empirical constant is related to the true thermodynamic constant by the following equation:

$$pK' = pK_{HP} + (pH + \log a_{H^+}) + \log \frac{f_P}{f_{HP}}. \qquad (3)$$

pK′ is therefore subject to primary salt effects and in the limit of infinite dilution, where all activity coefficients approach unity, the value of pK′ becomes that of the extrapolated quantity pK'_0 such that

$$pK'_0 = pK_{HP} + (pH + \log c_{H^+}).$$

K'_0 may be described as an approximation to the thermodynamic ionization constant, but it cannot be precisely identified with K_{HP} because pH cannot be identified with $-\log a_{H^+}$ or $-\log c_{H^+}$ (Guggenheim, 1930; Bates, 1948). Nevertheless, it has been assumed throughout this work that the measured changes in pH give reasonable values for the corresponding changes in $\log a_{H^+}$. An estimate of the uncertainties involved is made below in the Discussion.

MATERIALS AND METHODS

Preparation of metmyoglobin. Metmyoglobin (MetMb) was prepared from horses' hearts following a modification of the method of Theorell (1932). Details of the procedure are the same as used by George & Irvine (1952). The concentration of a given solution of MetMb was estimated, when required, following the procedure of de Duve (1948), which involves the conversion of MetMb into the carboxymyoglobin complex in a 0·067 M-Na_2HPO_4 buffer. de Duve's absorption spectrum for this complex was used as a standard.

Buffer solutions. Borate buffers were used throughout except in the few cases shown in Table 1 and Fig. 1 where veronal buffers were used to investigate specific salt effects. To cover the pH range 7·8–10·2 borate buffers were made from mixtures of boric acid and NaOH, and veronal buffers over the pH range 7·8–9·2 from sodium diethylbarbiturate and HCl. All solutions were prepared from A.R. materials. Ionic strengths were calculated from the contribution of Na^+ and $H_2BO_3^-$ ions (or Na^+ and barbiturate ions) and the buffer solutions were adjusted to the same total ionic strength with NaCl. Normally, these buffer mixtures were made from 0·1 M solutions and had ionic strength (I) adjusted to 0·041. However, for work at very low salt concentrations, stock buffers were made with CO_2-free water and adjusted to $I = 0{\cdot}018$; for lower ionic strengths these were further diluted with the same water. The contribution to I by the haemoprotein and traces of associated salt is discussed later in this paper.

Reference solutions of acidic and alkaline metmyoglobin were made, respectively, in a KH_2PO_4-NaOH buffer of pH 6·0 and a glycine-NaOH buffer of pH 11·1 and, as was known from preliminary measurements, these were taken to represent 99·5% conversion into acidic or alkaline form respectively.

Spectrophotometric measurements. In a given buffered solution of MetMb, usually about 5×10^{-5} M, the fraction present as alkaline (alk.) MetMb was determined by comparing the optical absorption of the solution with the corresponding absorptions of the reference solutions of acid MetMb and alk. MetMb containing the same concentration of MetMb. A Unicam quartz spectrophotometer was used for measuring optical densities. This method of analysis assumes the validity of both Lambert's and Beer's Laws and also neglects the specific effects of ions in the solution on the absorption of light. The applicability of this method at ionic

strengths below 0·01 has been verified by previous workers, for instance Halban & Körtum (1934). It is also known that the molecular extinction coefficient of an absorbing ion is not constant at high salt concentrations (Halban & Eisenbrand, 1928), but as the measurements reported in this paper refer to differences and not absolute quantities this effect could be neglected, especially so because the region investigated had $I < 0{\cdot}7$. The question of specific salt effects on the equilibrium is dealt with elsewhere in this paper.

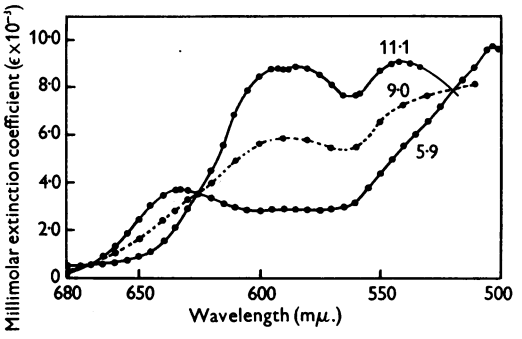

Fig. 1. Absorption spectra of metmyoglobin (MetMb) in the visible range 680–500 mμ. at 20°. Concentration, $5{\cdot}0 \times 10^{-5}$ M. $I = 0{\cdot}01$. Spectrum at pH 5·9 represents nearly 100% acid MetMb, that at pH 11·1 nearly 100% alk. MetMb and the intermediate one at pH 9·0 nearly 50% of each.

The absorption spectra for the brown acid MetMb and red alk. MetMb in the visible range 680–500 mμ. are quite similar to the corresponding ones of MetMb. In Fig. 1 three such spectra are shown for a $5{\cdot}0 \times 10^{-5}$ M solution of MetMb at $I = 0{\cdot}01$; the one at pH 5·9 represents the acidic form, that at pH 11·1 represents the alkaline form, and the intermediate one is at pH 9·0. The general shape of this intermediate spectrum and particularly the fact that it passes through three isobestic points strongly support the original assumption that the system consists of two species only (acidic and alkaline forms). At the wavelength 582 mμ. acid MetMb has a millimolar extinction coefficient $\epsilon \times 10^{-3} = 2{\cdot}9_2$ while that of alk. MetMb is $8{\cdot}7_1$. This wavelength was chosen for comparing optical densities and all measurements were made using 1 cm. glass cells, the solutions being read against distilled water as a blank.

During the measurement of optical density it was possible to control temperatures to $\pm 0{\cdot}2°$ using the following simple methods. For measurements above room temperature the spectrophotometer cell-holder with the cells and solution in it was warmed slowly on an electric hotplate to the temperature required, and it was found that subsequent cooling during the actual measurement of optical density did not exceed the limit indicated above. For measurements below room temperature water was circulated first through an ice-water mixture and then through two brass boxes fitted tightly in the cell-holder on either side of the cells.

Measurement of pH. pH was measured directly with a Cambridge pH meter using a glass electrode and a 0·05 M solution of A.R. borax in CO_2-free water as standard. To test the reliability of the instrument the pH of this standard (9·18 at 20°) was checked against that of a 0·05 M-potassium hydrogen phthalate buffer (3·99 at 20°). The discrepancy did not exceed 0·01 unit of pH; the degree of uncertainty involved in the measurement of pH by this method is referred to in the discussion. In the early stages of the investigation the pH of buffer alone was measured. A study of the salt effect on the pH of borate buffers at 20° showed that in the low ionic strength region the results were in reasonable agreement with a Debye-Hückel limiting law, and it was therefore assumed that the method of measurement was good enough. All later measurements were done on the actual buffered solution of MetMb directly after the measurement of optical density. In the case of very dilute buffers, where CO_2 effects become significant, the simultaneous measurement of optical density and pH was carried out with the help of an assistant immediately after the preparation of the solutions. Invariably it took under 2 min. for electrodes and solution to come to equilibrium. In this time the contribution to I made by KCl diffusing from the bridge, as assessed from conductimetric measurements, is inappreciable and was therefore ignored. Further, this KCl bridge was frequently renewed in order to ensure a constant potential at the boundary junction with the solution. During an actual measurement of pH the temperature of the solution was accurately controlled in a thermostat.

Sample run. Temp. $= 37{\cdot}0 \pm 0{\cdot}2°$. Stock solution of MetMb in water $= 4{\cdot}0 \times 10^{-4}$ M. Reference solutions: (i) Acid MetMb containing 2·5 ml. stock solution + 18·0 ml. buffer solution (KH_2PO_4 + NaOH, pH 6·0). (ii) Alk. MetMb containing 2·5 ml. stock solution + 18·0 ml. buffer solution (glycine + NaOH, pH 11·1). Solutions or measurements made in H_3BO_3-NaOH buffers of pH values 8·6, 8·8, 9·0, 9·2, 9·4 (all adjusted to $I = 0{\cdot}041$) containing 2·5 ml. stock MetMb + 3·0 ml. of 2 M-NaCl + 15·0 ml. buffer solution. Calculated $I = 0{\cdot}32$. For results see Table 1.

Table 1. *Preliminary experiment to demonstrate the validity of the optical method to determine* pK′

	pH (measured)	Optical density (measured at 582 mμ.)	Alk. MetMb (%)	pK′ using eqn. 2
Reference acid MetMb	—	0·143	Taken as 0·5	—
Reference alk. MetMb	—	0·427	Taken as 99·5	—
Solution in pH 8·6 buffer	8·29	0·210	24·1	8·79
Solution in pH 8·8 buffer	8·49	0·242	35·1	8·76
Solution in pH 9·0 buffer	8·69	0·267	44·0	8·79
Solution in pH 9·2 buffer	8·90	0·302	55·9	8·80
Solution in pH 9·4 buffer	9·11	0·333	67·0	8·80

Mean pK′ $= 8{\cdot}79 \pm 0{\cdot}01$.

RESULTS

The above procedure was used in determining pK′ over a range of ionic strengths and temperature. The results are given in Table 2, every pK′ value being the arithmetic mean of some four determinations. The region of very low ionic strengths was investigated at two temperatures only, 20 and 37°. Fig. 2 shows the variation of pK′ with $\sqrt{I}$ and temperature; the important point it illustrates is the reversal of the salt effect below $\sqrt{I}=0{\cdot}1$. Table 2 and Fig. 2 also show that pK′ values obtained with veronal buffers fall within the limits of experimental error on pK′ obtained with borate buffers. Although this was taken to suggest that no marked specific salt effects interfered with our measurements it does not preclude the possibility of specific interaction between electrolyte ions and the protein (cf. Adair & Adair, 1934; Sidwell, Munch, Barron & Hogness, 1938).

Table 2. *Effect of buffer, neutral salts and temperature on the ionization of acidic metmyoglobin (acid MetMb) in water,* $Fe^{+}(H_2O)=FeOH+H^{+}$, *measured within the* pH *range* 7·8–9·2

(I = ionic strength calculated from contribution of buffer alone. $pK'=pH+\log\frac{(\text{acid MetMb})}{(\text{alk. MetMb})}$ as in eqn. 2.)

Temperature (°)	Buffer	I (calc.)	pK′
7·5±0·2	Borate	0·037	9·12±0·01
		0·117	9·14±0·01
		0·284	9·18±0·00
20·0±0·2	Borate	0·001	8·95±0·01
		0·002	8·93±0·02
		0·003	8·91±0·02
		0·004	8·89±0·01
		0·009	8·89±0·01
		0·027	8·90±0·00
		0·080	8·92±0·00
		0·173	8·95±0·01
		0·224	8·96±0·01
		0·399	8·98±0·01
		0·556	9·01±0·01
		0·698	9·03±0·01
	Veronal	0·044	8·93±0·02
	Veronal	0·286	8·99±0·02
28·0±0·2	Borate	0·037	8·80±0·01
		0·088	8·85±0·01
		0·166	8·87±0·01
		0·315	8·92±0·01
	Veronal	0·044	8·81±0·02
	Veronal	0·286	8·90±0·03
37·0±0·2	Borate	0·001	8·72±0·01
		0·002	8·69±0·02
		0·004	8·68±0·01
		0·005	8·65±0·01
		0·009	8·65±0·02
		0·037	8·67±0·01
		0·088	8·72±0·01
		0·166	8·75±0·01
		0·315	8·79±0·01

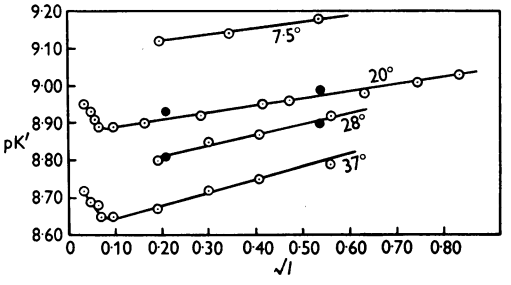

Fig. 2. Variation of pK′ with $\sqrt{I}$ and temperature taken from the data of Table 1. ⊙, runs using borate buffers; ●, runs using veronal buffers.

The values given in Table 3 for the approximate thermodynamic ionization constant pK'_0 were obtained by linear extrapolation in the low ionic strength region after a correction term 0·001 was added to the calculated ionic strengths. This, as will be shown below, is an estimate of the contribution to I from the effective charge on the haemoprotein molecule. Now the limits of uncertainty in the results are such that the plots of pK′ against I, $\sqrt{I}$, $\frac{\sqrt{I}}{1+\sqrt{I}}$ and $\frac{\sqrt{I}}{1+2\sqrt{I}}$ all appear tolerably linear in this low ionic strength region. Four sets of pK'_0 values are therefore given in Table 3, and one of these extrapolations is illustrated in Fig. 3. In all these cases it was found that the lines at 20 and 37° are approximately parallel, their slopes, excluding that of the plot against I, ranging from $-2{\cdot}3$ to $-2{\cdot}9$.

In the higher ionic strength region it was found that pK′ plots were also linear with $\sqrt{I}$ as well as with I. Linear extrapolations of these plots give respectively pK′ ($\sqrt{I}\to 0$) and pK′ ($I\to 0$) which have no thermodynamic significance, but it is interesting to note that their temperature coefficients, shown in Fig. 4, are both linear.

Table 3. *Values for the approximate thermodynamic ionization constant* pK'_0 *of acidic metmyoglobin obtained by linear extrapolation of* pK′ *plots against* I, $\sqrt{I}$, $\frac{\sqrt{I}}{1+\sqrt{I}}$ *and* $\frac{\sqrt{I}}{1+2\sqrt{I}}$ *in the region of* $I<0{\cdot}01$

(One of the plots is illustrated in Fig. 3.)

Temperature (°)	$I\to 0$	$\sqrt{I}\to 0$	$\frac{\sqrt{I}}{1+\sqrt{I}}\to 0$	$\frac{\sqrt{I}}{1+2\sqrt{I}}\to 0$
20·0±0·2	8·995	9·020	9·065	9·070
37·0±0·2	8·765	8·790	8·825	8·830

The results referred to above were all obtained from measurements within the pH range 7·8–9·2 and, as can be seen from Table 2, pK′ was invariably found constant with an experimental scatter not exceeding ± 4 %. However, when solutions of metmyoglobin in buffer outside this pH range were

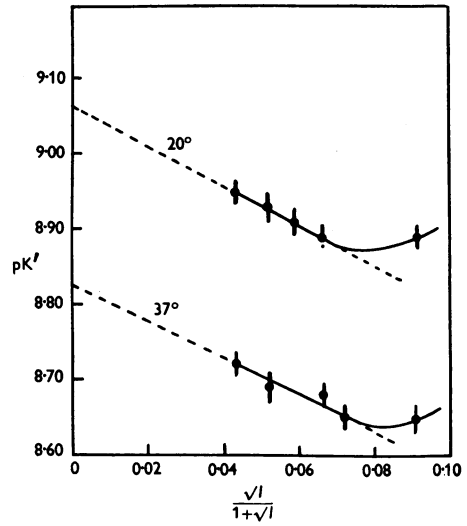

Fig. 3. Illustrating the method of obtaining the extrapolated constant pK'_0. Plots at 20 and 37° of the measured ionization constant, pK′, against the function $\frac{\sqrt{I}}{1+\sqrt{I}}$, using corrected values of I and showing the limits of experimental scatter in the results.

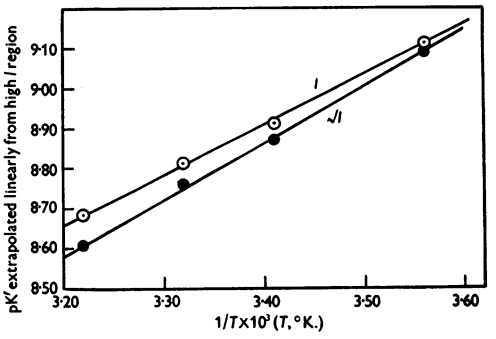

Fig. 4. Temperature coefficients of the 'hypothetical' ionization constants obtained by linear extrapolation of the plots of pK′ against $\sqrt{I}$, ●, and I, ⊙, from the region corresponding to $I > 0{\cdot}01$.

used pK′ was found to be no longer constant and its value rose steadily with increasing pH. The effect was observed both below pH 7·8 and above pH 9·2 and is illustrated in Fig. 5 where $\log \frac{\text{(alk. MetMb)}}{\text{(acid MetMb)}}$ is plotted against pH over the range 8·2–10·2. For a 'normal' run below pH 9·2 this plot is linear and with a slope of unity. The plots above pH 9·2 are very slightly curved and have mean slopes which are evidently values of n in the equation:

$$\log \frac{\text{(alk. MetMb)}}{\text{(acid MetMb)}} = \text{const.} + n.\text{pH}. \qquad (4)$$

These values of n are fractional and approach unity with increasing ionic strength of solution. Further comment on this result is made below in the discussion.

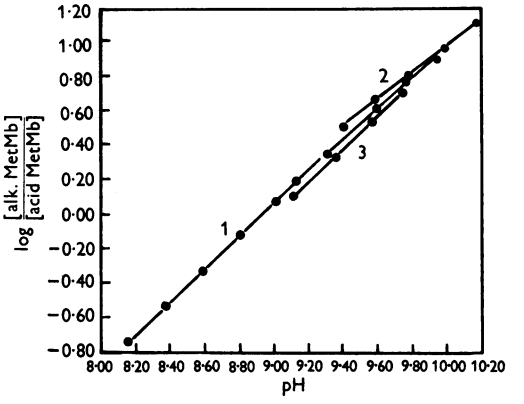

Fig. 5. Plots of $\log \frac{\text{(alk. MetMb)}}{\text{(acid MetMb)}}$ against pH, the linear slopes of which are the values of n in eqn. 4. Experimental details as described in the text, borate buffers being used. Temp. 20°. Run (1): $I = 0{\cdot}11$; section below pH 9·2 gives $n = 1{\cdot}00$ (normal run), section above pH 9·2 gives $n = 0{\cdot}80$. Run (2): $I = 0{\cdot}02$; pH range 9·4–10·2, $n = 0{\cdot}75$. Run (3): $I = 0{\cdot}32$; pH range 9·1–9·8, $n = 0{\cdot}92$.

Calculation of $\Delta H°$ and $\Delta S°$

An approximate value for the change in heat content accompanying the ionization can be obtained by integrating the van't Hoff equation

$$\left(\frac{\mathrm{d} \ln K}{\mathrm{d}T}\right)_p = \frac{\Delta H°}{RT^2},$$

if it is assumed that $\Delta H°$ is constant over the range of temperature investigated. On this assumption the values for pK'_0 at 20 and 37° given in Table 3 lead to a mean $\Delta H° = 5{\cdot}75 \pm 0{\cdot}67$ kg. cal./mole. It is interesting to compare this result with other estimates of ΔH. Thus the minima of the pK′/$\sqrt{I}$ plots at 20 and 37° give ΔH roughly as 6·1 kg. cal./mole, while the temperature coefficients shown in Fig. 4 lead to ΔH values of 6·5 and 5·7 kg. cal./mole.

The entropy change accompanying this ionization may now be obtained at, say, 20°. Table 3 gives mean pK'_0 at 20° as $9{\cdot}038 \pm 0{\cdot}030$, therefore the free energy change for the process is

$$\Delta G° = -RT \ln K = 12{\cdot}13 \pm 0{\cdot}04 \text{ kg. cal./mole},$$

therefore

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

DISCUSSION

The results outlined above indicate that the ionization of acid metmyoglobin, which probably involves the releasing of a proton from the water molecule attached to the haem iron, is an endothermic process which is markedly influenced by neutral salts. The addition of sodium chloride was found to favour this ionization up to ionic strengths of about 0·01, but further addition of salt repressed the ionization. In view of the uncertainty of the term $(pH + \log a_{H+})$ in eqn. 3 it is not possible to discuss this effect adequately in terms of the general effect of salts on activity coefficients, though it is easy to interpret the reversal of the salt effect qualitatively in terms of a Debye-Hückel extended equation.

It was stated above that in the region with $I < 0{\cdot}01$ our data fitted linear plots against four functions of I all equally well. Previous workers in the allied fields of solubility and dissociation curves of proteins have used a variety of Debye-Hückel equations as well as the zwitterion model of Scatchard & Kirkwood (1932) in discussing activity coefficients of protein ions. This may be illustrated by referring to the discussions of Cohn & Prentiss (1927), Green (1932), Cannan (1938) and Grönwall (1942). We have given our results in Table 2 in four sets corresponding to four modes of extrapolation without committing ourselves on the question of the theoretical model. Taking $-2{\cdot}6$ as the mean slope of the linear plots, and assuming that a Debye-Hückel limiting law is applicable to such dilute solutions of haemoprotein and electrolyte, the ionization is found to correspond roughly to the charge types $z_{HP} = -2$ and $z_P = -3$ (if these charge types are considered tolerably constant over this linear range). The result implies that as far as this ionization is concerned the molecule behaves as though it had a small effective negative charge. Since the net negative charge on a metmyoglobin molecule around pH 9 is almost certain to be large, the above result suggests that most of the charged groups in the molecule are, as it were, frozen at large distances from the haem iron atom. This argument also illustrates how difficult it is to assess the contribution of metmyoglobin to the total ionic strength of the solution. We have reckoned that our solutions, usually about 5×10^{-5} M-metmyoglobin, do not contribute more than 0·001 to I, i.e. 20 equiv. per mole (cf. Cannan, 1938).

Further to this ambiguity in assigning values to I, the following sources of error in our results may be mentioned. The effect of variation in liquid junction potential on the calibration of the pH scale cannot be estimated with confidence, but our pH measurements are probably precise to ± 3 or 4% (Edsall, 1943; Bates, 1948) and the spectrophotometric measurements to ± 1%. The error in pK′ is therefore ± 4 or 5% which is seen to cover all experimental scatter around the mean pK′ values of Table 2, and due to the uncertainty about the mode of extrapolation ΔH° should be uncertain to about ± 12% and this leads to a $\Delta S^\circ = -21{\cdot}7 \pm 2{\cdot}4$ cal./mole/degree.

This entropy change could arise not only from changes in charge and structure involving the haem Fe, H_2O and OH^- but also from structural changes within the protein molecule. For instance, Eley (1943) suggested that in the dissociation of oxyhaemoglobin such changes occurred within the 17 000 molecular weight sub-units of the molecule. Actually the value obtained above for ΔS accompanying the ionization of acidic metmyoglobin can be roughly accounted for in terms of contributions from Fe, H_2O and OH^- without taking into consideration any contribution from the protein. Despite the great measure of uncertainty of such a calculation we consider it justifiable to conclude that the ionization of acidic metmyoglobin is a normal equilibrium which does not involve structural changes within the protein.

As mentioned previously, the results quoted in Tables 2 and 3 were all obtained from measurements done within the pH range 7·8–9·2. Since the net charge on a metmyoglobin molecule depends on the pH it was rather surprising to find pK′ constant. In terms of eqn. 2, this means that no appreciable interaction between this equilibrium and those of, say, other acidic groups in the molecule occurs within this pH range. Fig. 5 illustrates the behaviour at higher pH in the region which corresponds to over 80% alkaline metmyoglobin. The value of n in eqn. 4 is fractional and approaches unity as the ionic strength increases. Although the theoretical interpretation of the phenomenon remains somewhat uncertain (cf. Linderstrøm-Lang, 1924; Cannan, 1938) this could be a case of interacting equilibria caused, for instance, by the ionization of ϵ-amino groups of lysine residues which form a considerable fraction of the protein in the molecule (Tristram, 1949) and which start ionizing about pH 9·4 (Cohn & Edsall, 1943). The extent of this interaction, which is given quantitatively by the value of n, is small when compared with that which occurs in the haemoglobin-oxygen reaction (cf. Roughton, 1949) and there is the further difference that in the metmyoglobin ionization interaction leads to a decrease in the equilibrium constant, that is the groups interact in such a way as to weaken one another (Wyman, 1948), whereas with haemoglobin the effect of interacting haems is to increase the apparent overall equilibrium constant. This is the physical significance of the fractional values of n in the metmyoglobin case. The effect of increasing ionic strength may be thought of

either in the terms of a general salt effect, or in terms of specific interaction between the ions in solutions and the charged groups in the protein, with subsequent suppression of this effect.

SUMMARY

1. The equilibrium constant for the ionization of (horse) acid metmyoglobin which may be represented as $Fe^+(H_2O) = FeOH + H^+$ has been measured over a range of pH, ionic strength and temperature.

2. The effect of salts on this equilibrium is marked. Thus addition of sodium chloride favours the ionization up to an ionic strength of about 0·01 but beyond this the effect is reversed. Extrapolation to zero ionic strength gives $pK'_0 = 9·038 \pm 0·030$ at 20° and $8·803 \pm 0·025$ at 37°. From the equilibrium constant at two temperatures the ionization is found to be endothermic to the extent of $5·75 \pm 0·67$ kg. cal./mole. $\Delta S^\circ_{20} = -21·7 \pm 2·4$ cal./mole deg.

3. It is only possible to measure this ionization constant within the pH range 7·8–9·2. In more alkaline or acidic solutions significant interaction takes place between this equilibrium and, presumably, other ionizations in the molecule, and the value of the ionization constant falls steadily with increasing pH. This effect is suppressed at high ionic strengths which suggests specific interaction between electrolyte ions in solution and the charged groups on the protein.

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The Estimation of Vitamin E

2. QUANTITATIVE ANALYSIS OF TOCOPHEROL MIXTURES BY PAPER CHROMATOGRAPHY

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The chemical methods originally used for estimating the vitamin E potency of natural products, notably those of Emmerie & Engel (1938) and of Furter & Meyer (1939), did not distinguish between the different tocopherols. Since α-tocopherol is the only member of the group which possesses marked vitamin E activity, and since the tocopherol mixtures present in natural products differ widely in composition, failure to differentiate between the individual tocopherols is a considerable disadvantage. The fact that γ- and δ-tocopherols couple with diazonium salts, whereas the α and β compounds do not, has, however, been used to achieve a partial analysis of the individual tocopherols (Quaife, 1944; Weisler, Robeson & Baxter, 1947). More recently, Quaife (1948) has described a method