

## Spectrophotometric, Magnetic and Titrimetric Studies on the Heme-linked Groups in Myoglobin

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Hemoglobin (Hb) and its derivatives have been subjected to a great deal of chemical and physical studies. Myoglobin (Mb), however, has not been so extensively investigated. This is partly due to the fact that Mb was not isolated and definitely characterized as different from Hb until 19 years ago. Its similarity to Hb in many respects, such as iron content, light absorption in the visible, and biological function, has probably induced a temptation to think that only minor differences could exist between Mb and Hb.

There are, however, some facts that make this assumption doubtful. Mb has a much greater affinity for oxygen and the pH-dependence of this is much smaller<sup>1</sup>. The molecular weight of Mb is only one fourth that of Hb<sup>2</sup>. One Mb-molecule thus contains just one iron atom.

The brown ferriHb (metHb, Hb<sup>+</sup>) shows a change in absorption spectrum in alkaline solution giving a red colour. This change is reversible and follows a monovalent dissociation curve. On the basis of spectrophotometric measurements by Austin and Drabkin<sup>3</sup> and magnetic measurements by Coryell, Stitt and Pauling<sup>4</sup> the pK of this transition was found to be 8.12. The latter workers found that the magnetic moment changed from  $\mu_{\text{eff}} = 5.80$  Bohr magnetons for the neutral ferriHb to  $\mu_{\text{eff}} = 4.47$  for the alkaline form (HbOH). The first value corresponds closely to the theoretical one for 5 unpaired electrons, while the second can be ascribed to 3 unpaired electrons only if a rather big orbital contribution is assumed. It was also observed that the paramagnetic susceptibility decreased somewhat with increasing acidity. This was interpreted as the effect of a heme-linked histidine group with pK = 5.3 (Coryell and Pauling<sup>5</sup>).

The corresponding transition from neutral (Mb<sup>+</sup>) to alkaline ferriMb (MbOH) has been known since 1934, when one of us<sup>6</sup> pointed out that the change

in colour of  $\text{Mb}^+$  takes place at a higher pH than for  $\text{Hb}^+$ . Bowen has recently made measurements of the light absorption of ferriMb in the visible and at different pH<sup>7</sup>. However, he did not attempt to find the  $pK$  of this transition but simply stated that "at pH-values above 7... the absorptions are so affected by variations of pH that they are difficult to duplicate". Thus no comprehensive absorption data are available.

Taylor<sup>8</sup> in 1939 measured the magnetic susceptibility of ferriMb and ferroMb, assuming CO-ferroMb to be diamagnetic. He found  $\chi_m = 14\,200$ , resp.  $12\,400 \times 10^{-6}$  cgs. "A very dilute solution of ferriMb" at high, but undefined pH, gave the value  $\chi_m = 8\,000 \times 10^{-6}$ . Taylor's results checked so closely with the corresponding susceptibilities in the case of Hb that he concluded "that magnetic interactions do not occur between the four hemes of hemoglobin". In view of the uncertainty of some of Taylor's values a more detailed investigation of the magnetic properties of myoglobin seemed to be of interest. He made no attempts to study the  $pK$  of the transition from  $\text{Mb}^+$  to  $\text{MbOH}$  or to determine whether the dissociation constant found for Hb at low pH ( $pK^1 = 5.3$ ) likewise obtains for Mb. Magnetic and spectrophotometric measurements on the fluoride compound of ferriHb has given valuable information on its hemelinked groups and similar studies for  $\text{Mb}^+$  are indicated.

#### TECHNIQUE

The spectrophotometric determinations were made with a Beckman apparatus. An optical depth of 1 cm was employed for the dilute solutions and a 0.016 cm cell for the more concentrated solutions that were subjected to both magnetic and spectrophotometric study.

Most of the pH-values were determined with a Pt- $\text{H}_2$ -electrode in a volume of about 1 ml with bubbling hydrogen gas and one drop of octanol to avoid foaming. The glass electrode was used in some cases for dilute  $\text{Mb}^+$  solutions.

The magnetic measurements were performed with the micro-apparatus designed by the authors and described elsewhere<sup>9</sup>. The apparatus was calibrated with a nickel chloride solution ( $\chi_{\text{NiCl}_2, 20^\circ\text{C}} = 4434.10 \cdot 10^{-6}$  cgs), so that the constant  $k$  of the equation

$$\Delta\kappa = kp \tag{1}$$

was found to be equal to  $2.154 (\pm 0.008) \cdot 10^{-11}$  cgs/g $\mu$ . To obtain  $p$  from the readings  $s$ , the specific gravities of the protein solution and buffer must be known. The molecular constant can be calculated from  $\Delta\kappa$  if the concentration of heme-iron is known. These values are easily obtained from the amounts

of stock solutions mixed, the partial specific volume of Mb ( $V_{sp} = 0.743$  ml/g) and the tabulated specific gravities of the solutions employed.

The molecular susceptibility of the iron is obtained in the following way:

A sample containing  $a$  g Mb/ml (dry weight) and  $c$  g iron/ml is subjected to magnetic measurement at the temperature  $T^\circ K$  against a buffer containing the same concentration of salt and acid or base as the sample. The measurement gives  $p$ . The susceptibility of the buffer is found to be  $\kappa_b$  by a measurement relative to water.

A diamagnetic compound of Mb (MbCO is convenient) in a water solution containing  $d$  g Mb/ml is also measured. This gives  $p_{MbCO}$  and according to equation (1)

$$\kappa_{MbCO, H_2O} - \kappa_{H_2O} = k \cdot p_{MbCO} \quad (2)$$

Assuming that Wiedemann's law is applicable we get

$$\kappa_{MbCO} = \frac{k \cdot p_{MbCO}}{V \cdot d} + \kappa_{H_2O} \quad (3)$$

where  $V$  is the specific volume of Mb. If the Mb in the original sample ( $a$  g Mb/ml) was made diamagnetic and the sample measured in this state, the value  $p_{diam}$  would be obtained. This should give

$$\kappa_{Mb, diam} = \frac{k \cdot p_{diam}}{V a} + \kappa_b \quad (4)$$

Since the diamagnetism is independent of the temperature the results of equations (3) and (4) are equal. Hence

$$p_{diam} = \frac{a}{d} p_{MbCO} + \frac{Va}{k} (\kappa_{H_2O} - \kappa_b) \quad (5)$$

$p_{diam}$  is the diamagnetic correction and it is the difference  $p_T = p - p_{diam}$  that is due to the paramagnetism of the iron ( $c$  g/ml). This paramagnetism is assumed to follow Curie's law. As all of our measurements have been made within the temperature range 17–23°C the value is corrected to 20°C,

$$\Delta\kappa_{20^\circ C} = k \cdot p_T \frac{T}{293} \quad (6)$$

The molar susceptibility of the heme-iron at 20° C is obtained by

$$\chi_{\text{Fe}, 20^{\circ}\text{C}} = k \frac{55.85}{c} (p - p_{\text{diam}}) \frac{T}{293} \quad (7)$$

#### MATERIAL

The Mb was prepared with slight modifications according to the method described by Theorell<sup>10</sup>. 65 kg of horse muscle were employed and the material was recrystallized three times, the last time in fractions. The crystals formed at 73 % saturated ammonium sulfate were taken as fraction 1 and those at 80 % as fraction 2.

Fraction 1 weighed 90 g and  
 » 2 » 100 g

The iron content of both fractions was 0.285 %. This value is much lower than the 0.34 % found by Theorell in 1932<sup>10</sup>. More recently Bowen<sup>7</sup> has reported values of the iron content of crystallized horse Mb varying between 0.30 and 0.34 %. Electrophoretic study of our material at pH 6.8 showed two uncoloured fractions in addition to Mb. These impurities were eliminated by electrophoresis on a preparative scale and 100 mg of purified Mb were thus obtained. This sample had an iron content of 0.34 % in agreement with the value found earlier by Theorell. Fractions 1 and 2 were, however, utilized for the magnetic and spectrophotometric measurements without purification. The uncoloured impurities could not influence the adsorption measurements in the visible but the magnetic measurements, however, could be influenced by the impurities if they contained iron. Since the spectrophotometric determination of hemin (as pyridine hemochromogen) gave, within the limits of error, the same values as those calculated from the iron content, we concluded that no foreign iron was present. The molar data for the susceptibilities and the extinctions have been calculated from the hemin content.

#### PRELIMINARY SPECTROPHOTOMETRIC MEASUREMENTS ON FERRIMYOGLOBIN \*

Studies of the absorption of ferri-Mb in the visible at different pH-values were carried out after dissolving the protein in buffers prepared according to Clark<sup>21</sup>. The pH was defined within one tenth of a unit in this way. The ionic strength ( $\mu$ ) varied from 0.02 to 0.35.

\* The experiments in this paragraph were carried out in collaboration with dr Margit Béznak during her stay in Stockholm 1949.

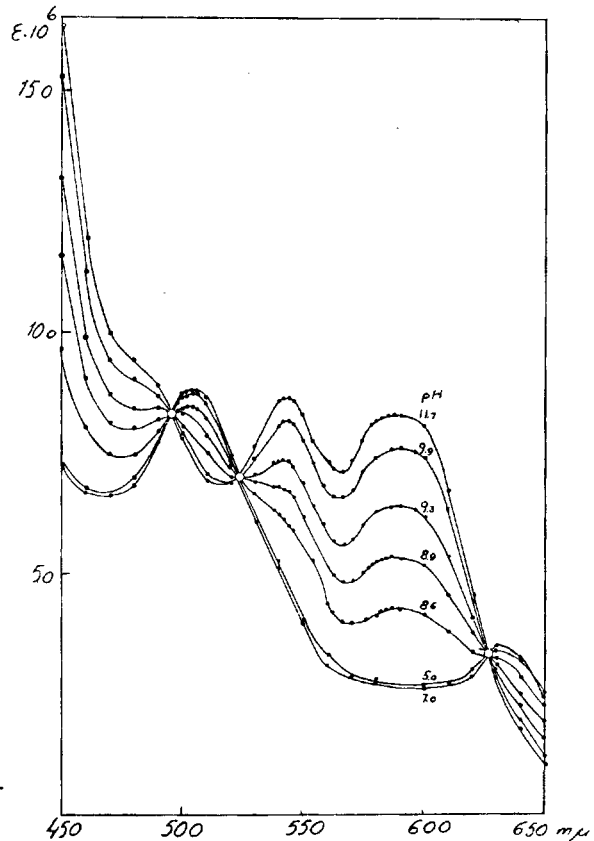


Fig. 1. Absorption curves for ferrimyoglobin at different pH.

Contrary to Bowen's<sup>7</sup> experience we found that the variations of light absorption with pH could be readily reproduced. The curves of Fig. 1 show the molar extinction coefficients in the region 450—640  $m\mu$  with pH as parameter. All the curves intersect at three isosbestic points with  $\lambda = 495, 523$  and  $626 m\mu$ . The pH dependence is especially large at  $590 m\mu$ , where a flat minimum for the neutral form corresponds to a flat maximum for the alkaline form. This wavelength is thus very suitable for quantitative studies of the transition, and all of the measurements for determination of  $pK$  have been made at this wave length.

Below pH 7 practically only  $Mb^+$  and above pH 11 only  $MbOH$  are present. If the extinction coefficients at  $590 m\mu$  are taken from Fig. 1, and the expressions  $\log \frac{\epsilon - \epsilon_{pH5.0}}{\epsilon_{pH11.7} - \epsilon}$  calculated and plotted with pH as abscissa, a straight line of unit slope is obtained. This shows that the transition has the character

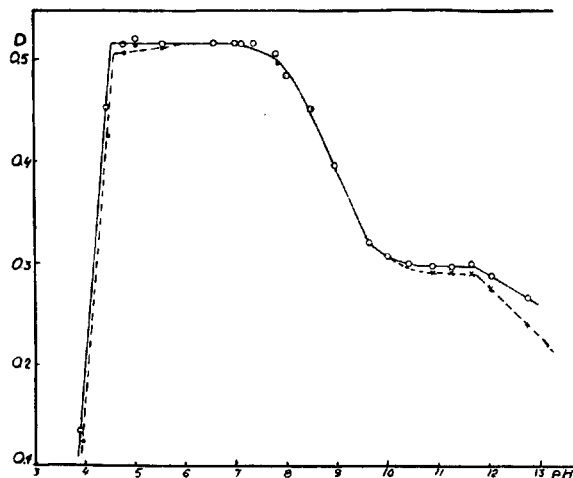


Fig. 2. Stability of ferrimyoglobin at different pH values.  
 Extinction,  $D$ , at  $409\text{ m}\mu$  measured  
 immediately  $\circ$   
 after 1 hour  $\bullet$   
 after 2 hours  $\times$

of a monovalent dissociation. From the graph the  $pK$  is found to be about 9.

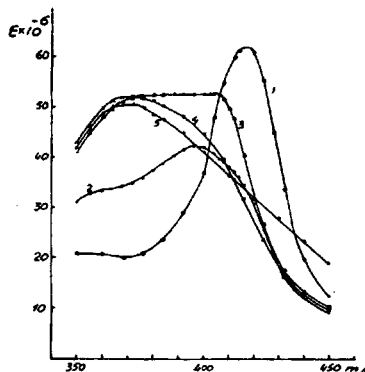
The limits of stability of ferri Mb in alkaline and acid solutions could also be determined spectrophotometrically. It was found convenient to make these measurements in the region of the Soret-bands,  $410\text{ m}\mu$  for acid and  $414\text{ m}\mu$  for alkaline Mb. Fig. 2 illustrates how the extinction coefficient at  $409\text{ m}\mu$  varies with pH and time. The discontinuity of the slope of the curves shows that there is a sudden change in the stability at pH 4.60 and 11.75. It is also seen that a slow change occurs below pH 6 and above pH 10.

All measurements on native ferri Mb thus must be made within the pH-region 4.60—11.75. Mb can be stored for a long time in a neutral solution but only for a few hours near the limits of stability.

The data of Fig. 3 for the spectrophotometric studies on solutions outside of the pH stability range indicate that a profound change in the hematin-protein bonds occurred. It seems very interesting that the pH range of stability can be defined so exactly. The explanation for this phenomenon may be the following: If we assume that the first steps of splitting or denaturation processes are reversible with speeds dependent on pH, stability will obtain as long as the destructive processes are slower than the reverse ones. Increas-

Fig. 3. Absorption curves of ferri-myoglobin in the Soret region of different pH.

- Curve 1. pH 12.9 immediately ○  
 2. pH 12.9 2 days ●  
 3. pH 4.1 immediately △  
 4. pH 3.4 ×  
 5. N/10 HCL +



ing the concentration of the hydrogen or hydroxyl ions will increase the velocity of destruction. As soon as this velocity exceeds the reverse by an infinitesimal amount the first step product will accumulate and be destroyed in a second step by a following irreversible reaction or reactions. It seems probable to us that analogous processes may be operating in the heat denaturation of proteins.

#### DETERMINATION OF THE DIAMAGNETISM OF THE PROTEIN

This determination was made on Mb from fraction 2 that had been transferred into diamagnetic ferrous Mb carbonmonoxide (MbCO). Two samples of Mb in aqueous solution were saturated with carbon monoxide. They were reduced with a tenfold excess of sodium hydrosulphite in an atmosphere of CO whereupon MbCO was rapidly formed. Two samples of water were treated in exactly the same way and all four samples were measured against water in the magnet. The difference between the two mean values of  $p$  gave  $p_{\text{MbCO}} = -192 \text{ g}\mu$ , which together with the dryweight of Mb ( $d = 0.0735 \text{ g/ml}$ ) has been used to obtain the diamagnetic corrections from equation (5).

The mass susceptibility of the protein is calculated by means of equation (3) multiplied by the specific volume of the protein.

$$\chi_{\text{MbCO}} = V \cdot \kappa_{\text{MbCO}} = -0.591 \cdot 10^{-6} \text{ cgs}$$

This value is about what can be expected for a protein and thus confirms our previous conclusion that foreign iron was essentially absent.

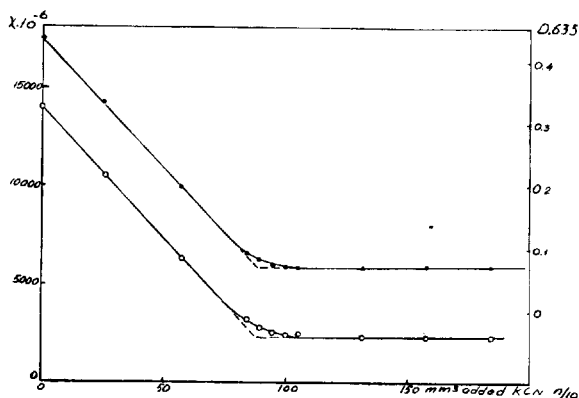


Fig. 4. Magnetic (○) and spectrophotometric (●) titration of  $Mb^+$  with KCN at pH 6.93.

#### FERRIMYOGLOBIN CYANIDE

When cyanide is added to  $Mb^+$  the solution becomes bright red. The absorption at  $630 m\mu$  disappears and a single band appears with its maximum at  $540 m\mu$ ,  $\epsilon = 9.25 \cdot 10^6 \text{ cm}^2/\text{M}$ . A solution of  $Mb^+$ ,  $8.51 \cdot 10^{-3} \text{ M}$  according to spectrophotometric determination of the pyridine hemochromogen, was titrated at pH 6.93 (Phosphate buffer 0.1  $M$ ) both magnetically and spectrophotometrically ( $635 m\mu$ ) with KCN 0.1  $N$ . The results are seen in Fig. 4. The concentration of  $Mb^+$  found by this titrations agreed with the pyridine hemochromogen value within 2 %.

The dissociation constant

$$K_{Mb, CN} = \frac{[Mb^+][CN^-]}{[MbCN]}$$

could be estimated from the values obtained near equimolarity. The ionization constant of HCN was taken as  $2 \times 10^{-9}$ . Two points on the magnetic curve gave  $4.6 \times 10^{-7}$  and  $3.0 \times 10^{-7}$  one point on the spectrophotometric curve  $3.2 \times 10^{-7}$ , average =  $3.6 \times 10^{-7}$ . This value is 10 times higher than the value ( $3.6 \times 10^{-8}$ ) found by Coryell, Stitt and Pauling for ferrihemoglobin cyanide<sup>4</sup>.

The paramagnetic molar susceptibility of MbCN was found to be  $2340 \cdot 10^{-6}$  cgs.



SIMULTANEOUS SPECTROPHOTOMETRIC AND MAGNETIC MEASUREMENTS  
ON FERRIMYOGLOBIN

In order to ascertain whether the transition in light absorption and the expected change in magnetism are parallel phenomena, measurements on portions of the same samples were carried out simultaneously in the spectrophotometer and in the magnet. A solution containing 0.0828 g/ml of Mb from fraction 1 was prepared and divided into portions of 2.5 ml each. Then 0.25 ml of mixtures of solutions of hydrochloric acid or sodium hydroxide in sodium chloride were added in order to obtain different pH-values at a constant ionic strength ( $\mu = 0.10$ ). The buffer capacity of the concentrated Mb-solution was sufficiently large to obviate pH changes. In the calculation of the ionic strength the contribution of Mb has not been regarded.

Each sample was divided into three portions, which were used for magnetic, spectrophotometric and pH measurements respectively. The extinction coefficient was determined just before and after the magnetic measurement and the mean value employed. A small drift was observed in only a few cases. These results are tabulated in Table 1.

Table 1. Measurements on fraction 1. Ionic strength  $\mu = 0.1$ .

pH	$\chi_{Fc}$ , 20°C 10 <sup>-6</sup> cgs	$\epsilon_{590}$ m $\mu$ 10 <sup>6</sup> cm <sup>2</sup> /mol
6.55	13 590	2.66
6.92	13 760	2.71
7.96	13 710	3.23
7.97	13 710	3.03
8.09	13 800	3.44
8.22	13 320	3.56
8.36	12 950	3.90
8.47	12 800	4.49
8.48	12 920	3.97
8.83	12 510	4.91
8.94	12 500	5.08
8.96	12 190	5.28
9.07	11 870	6.05
9.13	12 490	6.11
9.63	11 700	7.10
9.78	11 390	7.25
10.06	11 260	7.60
10.21	11 110	7.73
10.23	11 190	7.76
10.40	11 060	7.87
10.79	11 030	8.00
11.30	11 150	8.11
11.80	11 150	8.11
9.35		6.55

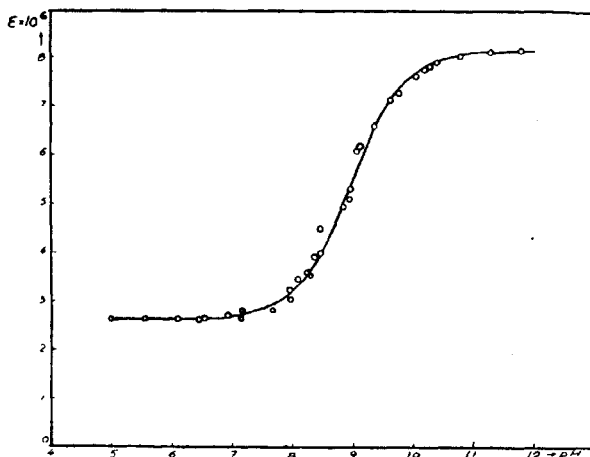
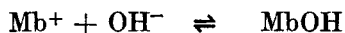


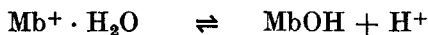
Fig. 5. Transition  $Mb^+ \rightleftharpoons MbOH$ . measured spectrophotometrically at 590  $m\mu$ .

The curve of Fig. 5 shows how the molar extinction coefficient varies with pH from  $\epsilon_{590} = 2.66 \cdot 10^6$   $cm^2/mole$  for  $Mb^+$  to  $\epsilon_{590} = 8.11 \cdot 10^6$   $cm^2/mole$  for  $MbOH$ . The  $pK$  of the dissociation has been found graphically to be equal to  $8.95 (\pm 0.01)$ . A theoretical curve for this  $pK$  has been drawn in the figure.

The magnetic susceptibility values are plotted against pH in Fig. 6. Since they are less reliable than the absorption data, the most probable values of  $pK$  and the two asymptotic susceptibilities have been calculated in a manner similar to that employed by Coryell, Stitt and Pauling in the case of  $Hb^4$ . If we write  $Mb^+$  for the neutral form and  $MbOH$  for the alkaline form of  $Mb$ , the equilibrium can be illustrated by the formula



or



The following equation is obtained for the equilibrium constant

$$pK = pH + \log \frac{x}{1+x} \quad (8)$$

where  $x$  is the actual mole fraction of  $Mb^+$  and is related to the measured susceptibility ( $\chi$ ) by the equation

$$\chi = x \cdot \chi_{Mb^+} + (1-x) \chi_{MbOH} \quad (9)$$

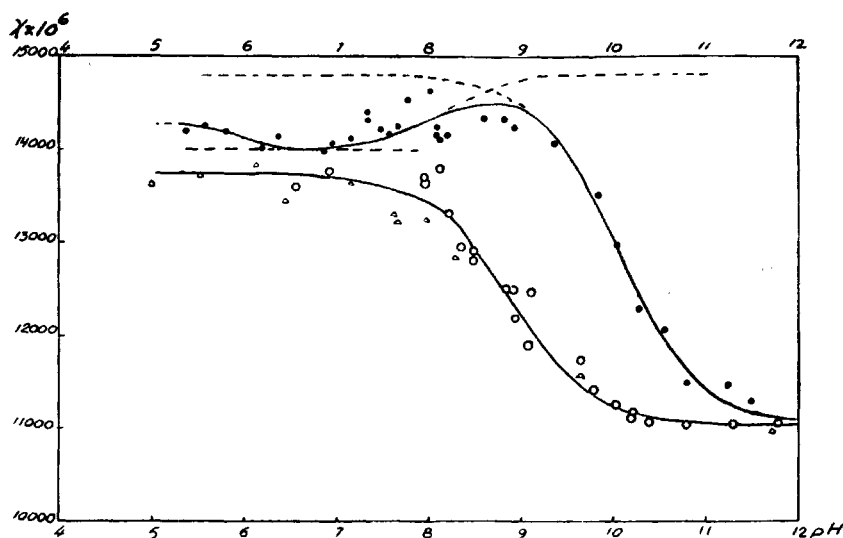


Fig. 6. Paramagnetic susceptibilities at different pH of ferrimyoglobin, fraction 1 (O) and fraction 2 ( $\Delta$ ), and of ferrimyoglobin with N/1 fluoride ( $\bullet$ ).

where  $\chi_{\text{Mb}^+}$  and  $\chi_{\text{MbOH}}$  are the asymptotic susceptibility values. From table 1 the approximative values  $\chi_{\text{Mb}^+} = 13\,670 \cdot 10^{-6}$  cgs and  $\chi_{\text{MbOH}} = 11\,090 \cdot 10^{-6}$  cgs are calculated. With these as starting point and by means of equations (8) and (9) and the method of least square the parameters  $pK$ ,  $\chi_{\text{Mb}^+}$  and  $\chi_{\text{MbOH}}$  are then determined by successive approximations.  $pK = 8.90 (\pm 0.04)$ ,  $\chi_{\text{Mb}^+} = 13\,740 (\pm 90) \cdot 10^{-6}$  cgs, and  $\chi_{\text{MbOH}} = 11\,040 (\pm 40) \cdot 10^{-6}$  cgs, where all the errors are estimated errors.

The behaviour at low pH-values was studied on a solution containing 0.0657 g/ml of  $\text{Mb}^+$  from fraction 2. A very small amount of  $\text{Mb}^+$  was denatured when the acid was added to obtain the three points below pH 6.2. A few control magnetic measurements were also taken on this sample at higher pH. The results are shown in Table 2, and the corresponding points have been plotted in Figs. 5 and 6. Both susceptibilities and extinctions are constant within the limits of error between pH 5 and 7.5. As mean values we obtained  $\chi_{\text{Mb}^+} = 13\,640 (\pm 60) \cdot 10^{-6}$  cgs and  $\epsilon_{\text{Mb}^+} = 2.63 \cdot 10^6$  cm<sup>2</sup>/mole, in good agreement with the above results. The two sets of experiments for  $\text{Mb}^+$  give as mean values at low pH  $\chi = 13\,690 (\pm 90) \cdot 10^{-6}$  cgs and  $\epsilon_{590} = 2.64 \cdot 10^6$  cm<sup>2</sup>/mole.

Table 2. Measurements on fraction 2. Ionic strength  $\mu = 0.1$ .

pH	%Fe, 20°C 10 <sup>-6</sup> cgs	$\epsilon_{590} \text{ m}\mu$ 10 <sup>6</sup> cm <sup>2</sup> /mol
5.00	13 650	2.59
5.54	13 690	2.62
6.11	13 830	2.62
6.43	13 440	2.62
7.16	13 610	2.64
7.64	13 290	2.67
7.66	13 230	2.81
7.96	13 240	3.01
8.27	12 820	3.53
9.63	11 550	
11.73	10 970	

## THE INFLUENCE OF IONIC STRENGTH UPON pK

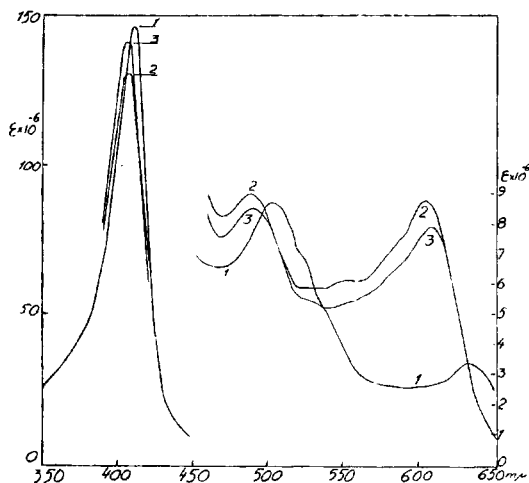
Three sets of absorption measurements were performed on dilute solutions of Mb<sup>+</sup> from fraction 2. The different pH-values were obtained by means of buffers containing glycine and sodium hydroxide and were measured for each solution. The lowest pH-values, where glycine alone was used, are approximate since the potentials were not stable. The ionic strengths were adjusted by additions of sodium chloride. Table 3 shows the experimental data. The values of pK obtained for the three sets of experiments are

$\mu = 0.02$	pK = 8.92
0.10	8.95 (from the preceding paragraph)
0.20	9.01
2.0	9.17

Table 3. The variation of  $\epsilon_{590} \text{ m}\mu$  with pH at different ionic strengths.

$\mu = 0.02$		$\mu = 0.20$		$\mu = 2.0$	
pH	$\epsilon \cdot 10^{-6}$	pH	$\epsilon \cdot 10^{-6}$	pH	$\epsilon \cdot 10^{-6}$
5.48	2.73	6.36	2.67	5.13	2.66
7.60	2.91	8.17	3.45	5.97	2.71
8.09	3.10	8.83	4.92	7.97	3.08
8.81	5.11	9.73	7.35	8.73	4.26
9.35	6.63	10.63	7.99	9.05	4.97
10.23	7.96	11.51	8.19	9.26	5.78
11.61	8.13			9.70	6.90
				11.37	8.28
				11.39	8.19

Fig. 7. Light absorption curves for  
 ferrimyoglobin pH 7.0 (1)  
 » fluoride pH 6.9 (2)  
 » » pH 5.4 (3)



$pK$  as a function of  $\mu$  is quite well approximated by the equation

$$pK = pK_0 + a \frac{\sqrt{\mu}}{1 + \sqrt{\mu}} \quad (10)$$

with  $a = 0.56$  and  $pK_0 = 8.84$ . If instead one employs

$$pK = pK_0 + a \sqrt{\mu}$$

the equation found by Austin and Drabkin<sup>3</sup> to be valid for ferri Hb, one obtains  $a = 0.19$  and a poorer approximation. A comparison between this value and  $a = 0.6$  for Hb<sup>+</sup> thus shows that the  $pK$  of Mb<sup>+</sup> is far less dependent on  $\mu$  than is Hb.

#### MEASUREMENTS ON THE FLUORIDE COMPOUND OF FERRIMYOGLOBIN

The addition of fluoride to a neutral solution of Mb<sup>+</sup>, gives rise to a pronounced change in the absorption spectrum, as is seen from Fig. 7. This indicates the formation of Mb<sup>+</sup> fluoride, with absorption bands in the visible at 495 and 610  $m\mu$ .

A series of samples containing Mb<sup>+</sup> from fraction 2 and potassium fluoride (1  $M$ ) was measured in the magnet. The results are given in Table 4. The first five values (pH 5.36—6.35) were obtained by adding acetate buffers containing

Table 4. Magnetic measurements with added fluoride,  $c_F = 1.00 M$ .

pH	$\chi_{Fe}, 20^\circ C$ $10^{-6}$ cgs	pH	$\chi_{Fe}, 20^\circ C$ $10^{-6}$ cgs
5.36	14 220	8.08	14 230
5.54	14 270	8.08	14 130
5.80	14 160	8.19	14 150
6.20	14 020	8.34	14 510
6.35	14 130	8.62	14 270
6.85	13 980	8.81	14 310
6.93	14 090	8.93	14 240
7.16	14 110	9.36	14 050
7.36	14 400	9.84	13 530
7.36	14 330	10.03	12 980
7.47	14 210	10.28	12 270
7.56	14 190	10.53	12 020
7.66	14 240	10.81	11 680
7.77	14 520	11.23	11 420
8.02	14 640	11.50	11 290
8.06	14 150		

sodium chloride to maintain constant ionic strength in the sample ( $\mu = 1.25$ ). If only acetic acid or hydrochloric acid was added instead of the buffer a small amount of the myoglobin was precipitated. In the other samples the pH was adjusted with acetic acid or sodium hydroxide. Under these conditions the ionic strength was, within a few per cent, equal to 1.03.

The susceptibility values have been plotted in Fig. 6. The curve has a maximum at pH about 8.7 and a minimum at pH about 6.8, which suggests the possibility of three different MbF compounds to be designated as I, II and III, with  $pK$ -values in the regions 6, 8 and 10 respectively.

Between pH 9.3 and 12 there are practically only  $MbF_{III}$  and  $MbOH$  present. The data in this pH-range are treated in the same way as those without added fluoride. This gives  $pK_{III} = 10.06 (\pm 0.03)$ ,  $\chi_{MbOH} = 11\ 040 (\pm 20) \cdot 10^{-6}$  cgs, in excellent agreement with the value found without fluoride, and  $\chi_{MbF_{III}} = 14\ 790 (\pm 30) \cdot 10^{-6}$  cgs, which is very close to the theoretical value for 5 unpaired electrons.

The  $pK$  of the equilibrium between  $MbF_{II}$  and  $MbF_{III}$  is evaluated from the data between pH 6.8 and 8.9. Corrections are made for the  $MbOH$  present. This gives  $pK_{II} = 8.11 (\pm 0.15)$  and  $\chi_{MbF_{II}} = 14\ 000 (\pm 130) \cdot 10^{-6}$  cgs.

The  $pK_I$  is estimated from magnetic measurements to be  $6.0 \pm 0.5$ , and  $\chi_{MbF_I} = 14\ 240 \cdot 10^{-6}$  cgs.

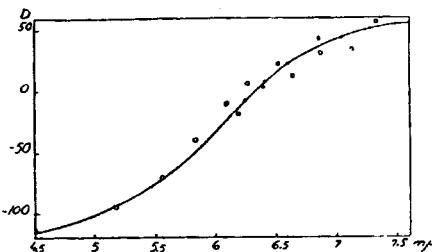


Fig. 8. Change in optical density of ferri-myoglobin fluoride at 610  $m\mu$  with change in pH.

It is worth noting that the spreading of the susceptibility values (both with and without added fluoride) is less than 1 % below pH 6 and above pH 10, but somewhat larger (less than 4 %) between these pH values.

Light absorption measurements have been made to verify the above results. No difference in absorption spectrum has been found between samples with and without added fluoride at pH 11.8. Samples at pH 7.1 and 8.7 showed identical absorption spectra in the visible. However, between pH 5.4 and 6.9 there is, as shown by Fig. 7, a pronounced difference. The transition from neutral to acid solution causes the whole absorption curve to be depressed and the maxima displaced a few  $m\mu$  toward longer wave lengths. The steep parts to the right of the maxima thus coincide. The maximum in the red is displaced from 610  $m\mu$  at pH 6.8 to 614.5  $m\mu$  at pH 5.5. The Soret band is altered too but in the opposite direction, from 407 to 406  $m\mu$  and the maximum absorption is increased by 10 %. The transition is reversible: When an acid sample is neutralised the spectrum changes in the reverse manner.

The  $pK$  of the transition between pH 7 and 5 has been determined spectrophotometrically in the following way. Samples containing  $Mb^+$  ( $c=0.132\text{ mM}$ ) and  $KF$  ( $c=0.90\text{ M}$ ) in buffers of various pH were prepared. Phosphate buffers were employed above pH 5.8 and acetate buffers below this value. The total ionic strength varied between 1.0 and 1.2. As these solutions in a 1 cm layer have optical densities at 610  $m\mu$  larger than 1, and the change to be studied was about 0.15 it was convenient to compare them with a neutral solution of pure  $Mb^+$  with a density of about 1. Two sets of measurements were carried out. The results are shown in Fig. 8. The  $pK$  was found to be 6.03 ( $\pm 0.03$ ). The theoretical curve for this  $pK$  and  $n=1$  is also shown in the latter figure.

The  $pK_I$  and the  $pK_{III}$  are thus both magnetically and spectrophotometrically operable. The  $pK_{II}$ , on the contrary, is found to be spectrophotometrically inoperable.

A spectrophotometric shift corresponding to our  $pK_I$  for  $MbF$  has been observed by Haurowitz for  $HbF$ <sup>12</sup>. He found that  $HbF$  behaves like an

indicator that changes its colour between pH 5.4 and 6.2. Coryell, Stitt and Pauling <sup>4</sup> in their investigation of the magnetic properties of the HbF did not observe any changes in the region from pH 5.4 to 6.9. However, no determinations were made between these pH values, so that nothing can be said about the possibility of a slight decrease of the susceptibility in this region. Moreover, two single determinations at pH 5.2 and 5.4 both gave the value of 5.89 Bohr magnetons, a value that cannot be said with certainty to be different from our value 5.80 at pH 5.4. It seems probable from the close analogy in the spectral behaviour of MbF and HbF that a slight shift in magnetic susceptibility is likely to occur around pH 6 in HbF as well as in MbF.

The dissociation constant  $K_{\text{MbF}}$  is calculated from

$$pK_{\text{Mb, OH}} = 8.84 (= pK_0) \text{ and } pK_{\text{III}} = 10.06 (c_{\text{F}^-} = 1 M)$$

and from the following equation:

$$K_{\text{Mb, F}} = \frac{K_{\text{Mb, OH}} \cdot [\text{F}^-]}{K_{\text{III}} - K_{\text{Mb, OH}}} = 10^{-1.19} = 0.065 \quad (11)$$

This value is at least 4 times higher than  $K_{\text{Hb, F}}$  according to Lipmann <sup>13</sup> and Coryell, Stitt and Pauling <sup>4</sup>. Some spectrophotometric titrations of Mb<sup>+</sup> with fluoride at pH between 7.99 and 5.9 were carried out (at the isobestic point 626 m $\mu$  for Mb<sup>+</sup> and MbOH in experiments above pH 7). (See Table 5 and Figs. 9, 10 and 11.) The ionic strength was close to 0.15 in all these experiments. From the straight lines in Fig. 9 and 10 the  $K_{\text{app}}$  is obtained by the equation  $K_{\text{app}} = \frac{m}{b}$  where  $m$  is the slope and  $b$  the intercept on the ordinate, according to Lewis <sup>14</sup>. Three more experiments were carried out between pH 7 and 8 and  $\mu$  approximately = 1. The correction term for the influence of ionic strength was calculated from the data obtained to be  $\frac{0.44 \sqrt{\mu}}{1 + \sqrt{\mu}}$ . This

Table 5. Ionic strength = 0.

pH	$-\log K_{\text{app, Fe, F}}$
5.91	2.26
6.42	2.02
6.90	1.86
7.32	1.90
7.57	1.87
7.99	1.78



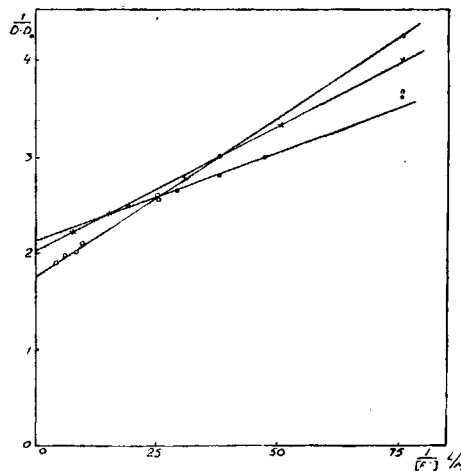


Fig. 9. Equilibrium data of  $Mb^+ + F^- \rightleftharpoons MbF$  measured at 610  $m\mu$ . Ionic strength  $\sim 0.15$   
 pH = 6.90 ○  
 pH = 6.42 ×  
 pH = 5.91 ●

value is not far from the correction term found for the  $pK_{Mb, OH^-}$ . In Fig. 11 and Table 5 all values are corrected to ionic strength 0.

Lewis<sup>14</sup> has made an attempt to calculate the values of the dissociation constants of methemoglobin fluoride from the known heme-linked groups in methemoglobin with  $pK$  5.3, 6.65 and 8.0 and spectrophotometric experiments with fluoride + methemoglobin. Since he seems to have overlooked Haurowitz' paper, no use was made of the spectrophotometric transition in HbF that occurs around pH 6. Rather complicated formula were used by Lewis. This seemed unnecessary in the present investigation, where the value of this  $pK$  was determined experimentally.

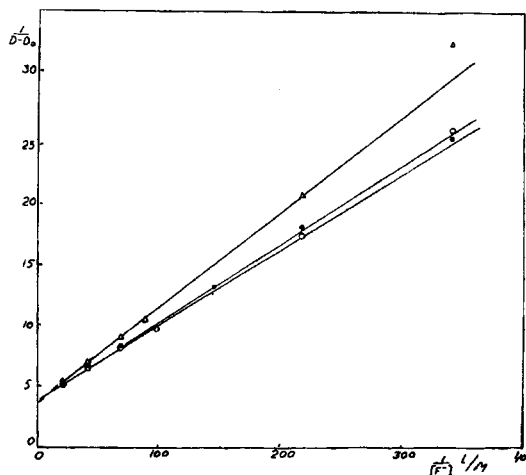


Fig. 10. Equilibrium data of  $Mb^+ + F^- \rightleftharpoons MbF$  measured at 626  $m\mu$   
 pH = 7.32 ○  
 pH = 7.51 ●  
 pH = 7.99 △

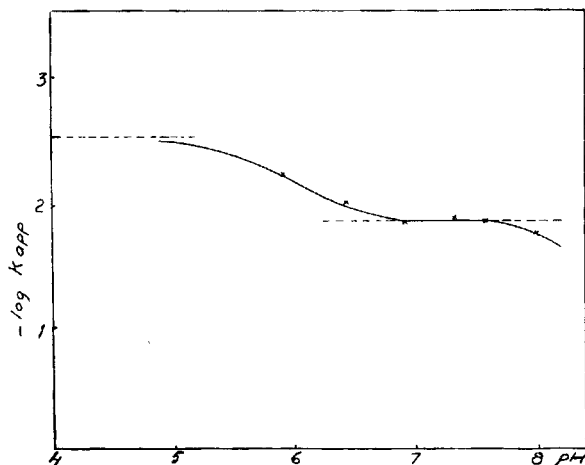


Fig. 11.  $-\log$  of apparent dissociation constants for myoglobin fluoride at different pH.

Our case, however, was complicated by the absence of any detectable transition in  $\text{Mb}^+$  corresponding to  $\text{p}K = 5.3$  in  $\text{Hb}^+$ , and by the inaccuracy in the value of the  $\text{p}K$  around 8.1 in  $\text{MbF}$ . Furthermore, experimental data on the redox potential in the system ferro-ferrimyoglobin are still lacking outside the pH region 5.9—7.4 (Taylor and Morgan<sup>15</sup>). For these reasons it seemed premature to apply the method of Lewis to the data hitherto available. The use of simplified methods nevertheless permits us to draw certain conclusions concerning the  $\text{p}K$ :s of the heme-linked groups in ferrimyoglobin.

By equation (11) we have calculated:

$$K_{\text{Mb, F}} = \frac{[\text{Mb}^+][\text{F}^-]}{[\text{MbF}]} = 10^{-1.19} \quad (12)$$

It is seen from Fig. 11 that another level with constant values of  $\text{p}K_{\text{app, MbF}}$  is reached in the pH-region 6.9—7.6. This seems to indicate that a  $K_{\text{HMb, F}}$  is operating essentially alone in this pH region

$$K_{\text{HMb, F}} = \frac{[\text{HMb}^+][\text{F}^-]}{[\text{HMbF}]} = 10^{-1.83} \quad (13)$$

$$K_{\text{H, MbF}} = \frac{[\text{MbF}][\text{H}^+]}{[\text{HMbF}]} = 10^{-8.11} \quad (14)$$

is determined magnetically. From equations (12)—(14) we can compute

$$K_{\text{H, Mb}} = \frac{[\text{Mb}^+][\text{H}^+]}{[\text{HM}^+]} = \frac{K_{\text{H, MbF}} \cdot K_{\text{Mb, F}}}{K_{\text{HMb, F}}} = 10^{-7.42} \quad (15)$$

Towards lower pH-values the  $pK_{\text{app, MbF}}$  (Fig. 11) is increasing again, but because of the spectrophotometrically determined

$$K_{\text{H, HMbF}} = \frac{[\text{HMbF}][\text{H}^+]}{[\text{H}_2\text{MbF}]} = 10^{-6.03} \quad (16)$$

the next level should not be reached until below pH 5.5. At these low pH values the etching effect of HF prevents accurate spectrophotometric determinations. If we assume that the second proton exerts the same influence as the first an approximate value is obtained for

$$K_{\text{H, Mb, F}} = \frac{[\text{H}_2\text{Mb}^+][\text{F}^-]}{[\text{H}_2\text{MbF}]} = 10^{-2.5} \quad (17)$$

in analogy with equation (15) thus

$$K_{\text{H, HMb}} = \frac{[\text{HMb}][\text{H}^+]}{[\text{H}_2\text{Mb}]} = 10^{-5.3} \quad (18)$$

The three dissociation constants  $pK_{\text{H, HMb}} = 5.3$ ,  $pK_{\text{H, Mb}} = 7.4$  and  $pK_{\text{Mb, OH}} = 8.84$  could possibly be determined by redox potential measurements. Unfortunately Taylor and Morgan<sup>15</sup> confined their work to the region pH 5.9—7.4, where according to our present results no change in the slope  $\frac{dE_0}{dpH}$  could be expected.

#### TITRATION EXPERIMENTS

(In collaboration with Å. Åkeson)

It was shown in 1934<sup>1</sup> that the oxygen and carbon monoxide equilibria with myoglobin are very little affected by changes in pH. This fact indicates a profound structural difference between myoglobin and hemoglobin, the latter giving a very strong "Bohr effect".

Therefore it was of great interest to carry out titrations on ferromyoglobin, with the iron held by essentially ionic bonds, and CO-myoglobin, with essen-

tially covalent bonds, in analogy with the differential titrations German and Wyman<sup>16</sup> made on hemoglobin and oxihemoglobin. We preferred to work with the CO-derivative rather than with oximyoglobin, since ferromyoglobin is more easily autooxidized than hemoglobin.

Ferromyoglobin was prepared by reducing 4 to 5 ml of an 8 to 10 % solution of Mb in cellophane tubes by the addition of sodium dithionite in slight excess. The cellophane tubes were sealed immediately and put into a large flask containing 5 liters of water that was freed of oxygen by alternating evacuation and flushing with oxygen-free hydrogen gas. The flask was sealed by a stopper containing an outlet and inlet tube, and the air rinsed out by letting hydrogen through the system. After two days dialysis, during which the myoglobin became diluted two-fold, 3 ml of the solution were transferred to the titration vessel through a hole in the stopper. During this procedure a rapid stream of hydrogen was passed through the vessel. The hole was stopped, and after all oxygen had disappeared (no O<sub>2</sub>Mb-bands visible in the spectroscopie) 0.1 *N* hydrochloric acid was added from a microburette with magnetic stirring to pH around 5.0. The titration with 1 *N* NaOH was initiated when the potentials of the glass and hydrogen-Pt-electrodes were perfectly stable. The hydrogen was washed free of oxygen by passing it through Fiesers solution (sodium anthraquinone- $\beta$ -sulphonate + sodium hyposulphite) and silver sulphate.

The titrations of CO-myoglobin were carried out in the same way with the exception that 2 % CO was added to the stream of hydrogen gas. In this way it was possible to avoid the formation of ferrimyoglobin. In Fig. 12 the results of one set of experiments with ferromyoglobin (dots) and CO-myoglobin (full drawn line) are shown. It is seen that the titration curves for the two compounds coincide exactly. A control experiment with Hb and HbCO gave the same differences as were observed by German and Wyman for titrations on Hb and HbO<sub>2</sub>. The lack of a Bohr effect in myoglobin thus coincides, as expected, with the similarity in titration curves for ferro- and CO-myoglobin.

Some attempts were made to titrate ferro-versus ferrimyoglobin. These, however, were not entirely successful. It is necessary to let an aliquot of the ferro-myoglobin autoxidize to ferrimyoglobin in order to get strictly comparable results. A pH of around 5 and comparatively long time, in one case up to six days, were required in order to reach complete oxidation. Under these conditions the ferrimyoglobin is not entirely stable. The transition of a *pK* 8.9 could always be nicely demonstrated, but conclusions about other heme-linked groups could not be drawn.

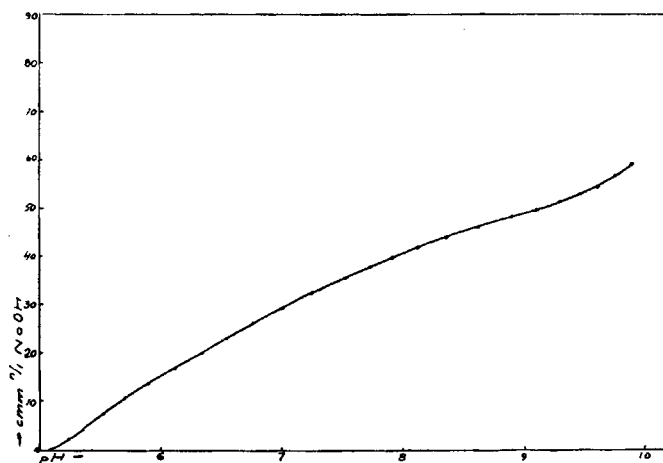


Fig. 12. Titration of 6.41  $\gamma$ -equivalents of ferromyoglobin (●) and CO-myoglobin (full drawn curve).

## DISCUSSION

### 1. Magnetic and spectral properties

A comparison of the magnetic data now available for ferriperoxidase (from horseradish, "P.O.OH")<sup>17</sup>, ferrihemoglobin<sup>4</sup> and ferrimyoglobin in their alkaline form (see Fig. 13 and Table 6) reveals striking differences. Column II, table 6, gives the number of Bohr magnetons calculated from the experimental susceptibility values by the equation

$$\mu_{\text{exp}} = 2.84 \sqrt{\chi_{\text{Fe}} \cdot T} \text{ Bohr magnetons}$$

Column IV gives the differences between the experimental and the theoretical values for the numbers of unpaired electrons indicated in column III  $\mu_{\text{teor}} = \sqrt{n(n+2)}$ . The values found are nearest to 1 odd electron for P.O.OH, 3 for HbOH and 5 for MbOH, but the differences are still too large to allow for definite conclusions. A strong connection between magnetic and spectral properties would seem very probable, and has in general been found to obtain in the case of the hemoproteins. The three compounds discussed here show in spite of the differences in magnetic properties very similar spectra (see Fig. 14). One difference, however, should be pointed out. Both P.O.OH and HbOH have absorption maxima at 575  $m\mu$ , but in addition to this HbOH has an inflection at 595–600  $m\mu$ . MbOH has a broad band in the whole region 575–

Table 6.

I	II	III	IV	V
	$\mu_{\text{exp}}$ Bohr magnetons	Possible numbers of unpaired electrons	$\mu_{\text{exp}} - \mu_{\text{theor}}$ Bohr magnetons	$p k_{\text{Fe, OH}}$ ionic strength = 0
P. O.+	5.44	5	- 0.48	
P.O.OH	2.66	3,1	- 1.11, 0.93	~ 11
Hb+	5.80	5	- 0.12	
HbOH	4.77	5,3	- 1.45, 0.60	7.88
Mb+	5.68	5	- 0.24	
MbOH	5.11	5,3	- 0.81, 1.24	8.84

600  $m\mu$  that seems to be formed through the confluence of bands around 575 and 600  $m\mu$ . The absorption at 600  $m\mu$  thus for the alkaline ferric compounds increase parallel to the ionic character of the bond.

Coryell and Stitt<sup>18</sup> found that the paramagnetic susceptibility of alkaline ferriHb increased when ethanol was added to the solution. The asymptotic change was  $3\,800 \cdot 10^{-6}$  cgs (highest ethanol concentration used  $\leq 20\%$ ). This would bring the total paramagnetic susceptibility up to the neighborhood of our value of alkaline ferriMb (without ethanol!). It is interesting to note that Coryell and Stitt observed a broadening towards the red of the 575  $m\mu$  absorption band of alkaline ferriHb upon the addition of ethanol.

The addition of ammonia to alkaline ferriHb caused a decrease in paramagnetic susceptibility down to  $3\,700 \cdot 10^{-6}$  cgs<sup>18</sup>. This value is very close to the  $\chi_M$  of P.O.OH<sup>17</sup>. Coryell and Stitt could see no change in the spectroscopy but we found in spectrophotometric measurements that the addition of ammonia to HbOH causes the inflection at 600  $m\mu$  to disappear and lowers the 575  $m\mu$  band so that the spectrum becomes very similar to that of P.O.OH. The correspondence between magnetic and spectral properties is thus very striking in these cases. We hope to be able to give more details on this item in the near future.

The spectrum of HbOH in the region mentioned is so strictly intermediate between those of P.O.OH and MbOH that one would be inclined to think that all four iron atoms in the HbOH are not necessarily alike and held by bonds with three odd electrons. For instance two could be held by covalent bonds with one odd electron and two by ionic bonds with five odd electrons. The magnetic data<sup>4</sup> would be well compatible with such an assumption.

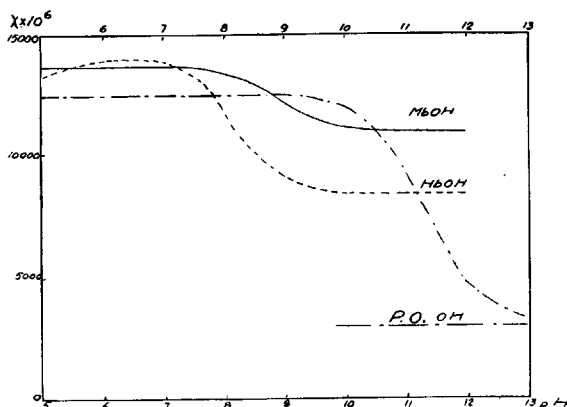


Fig. 13. Paramagnetic susceptibility at different pH of ferrimyoglobin, ferrihemoglobin and ferriperoxidase.

## 2. Heme-linked groups

In Table 7 the available data for the heme-linked groups in ferrihemoglobin, ferrimyoglobin and their fluoride compounds are summarized. The first row of values shows close analogies between hemoglobin and myoglobin. In fact the small differences are far from significant. Lewis' value  $pK$  for HbF appears to be too low. Haurowitz found the spectrophotometric shift between pH 5.4 and 6.2, indicating a value of 5.8 to be more likely. Furthermore, the attachment of the fluoride would probably increase the value of the  $pK$  more than 0.05—0.20.

According to Coryell and Pauling<sup>5</sup> this heme-linked group in hemoglobin is ascribed to a histidine residue which "is restrained by the configuration of the hemoglobin molecule to a relatively unfavorable position for electrostatic coordination with the iron atom" as suggested by Conant<sup>20</sup>. This assumption may need some further confirmation; but we can say from our present data that the same chemical configuration as in hemoglobin seems to be present in myoglobin on one side of the heme disc. This is the same side where oxygen is supposed to be attached to the iron, thus substituting the loosely bound histidine. Perhaps this mechanism is of importance in explaining the peculiar ability of ferrous iron in hemoglobin and myoglobin to attach oxygen reversibly without being oxidized to the ferric state.

The second row of values in Table 7 shows considerable differences between the  $pK$  of ferrihemoglobin and ferrimyoglobin and their fluoride compounds. The  $pK_2$  for hemoglobin is explained by a histidine residue being attached to

Table 7.

p <i>K</i> -values of heme-linked groups in ferrimyoglobin, ferrihemoglobin and their fluorides.								
	Hb <sup>+</sup>		Mb <sup>+</sup>		HbF		MbF	
p <i>K</i> <sub>1</sub>	5.3—5.45 (5, 22)	Mo	5.3	Mi	5.5—5.8 (14, 12)	Mi?	6.0	Mo
		Pi		Pi?		So		So
		Si		Si				
p <i>K</i> <sub>2</sub>	6.65 (21, 22)	Mi	7.4	Mi	6.9 (14)	Mi?	8.1	Mo
		Po		P?		Si?		Si
		Si		Si				
p <i>K</i> <sub>3</sub>	7.88 (3, 4)	Mo	8.84	Mo	—		—	
		So		So				
		To		To				

M = magnetically

o = operable

P = potentiometrically

i = inoperable

S = spectrophotometrically

( ) = references

T = titrimetrically

the iron on the other side of the hemin by covalent bonds in oxyhemoglobin and by ionic bonds in Hb and Hb<sup>+</sup>. This group is responsible for the "Bohr effect"<sup>5</sup>. The absence of this effect in myoglobin and the above mentioned differences in p*K* seem to exclude the possibility of histidine being the analogous heme-linked group in myoglobin. The chemical nature of this group in myoglobin is still obscure. It can be said, however, that this group seems to be more negative in character than histidine, since the dissociation constants of the component between iron and the negative ions OH<sup>-</sup>, CN<sup>-</sup> and F<sup>-</sup> are all larger by a factor of ten:

$$K_{\text{Mb, OH}} = 10^{-5.16} \text{ and } K_{\text{Hb, OH}} = 10^{-6.12}, K_{\text{Mb, CN}} = 10^{-6.44} \text{ and}$$

$$K_{\text{Hb, CN}} = 10^{-7.44}, K_{\text{Mb, F}} = 10^{-1.19} \text{ and } K_{\text{Hb, F}} = 10^{-2.33}$$

(the last value is taken from reference (4)). The dissociation constants of the hydrogen ions (p*K*<sub>2</sub> in Table 7) are accordingly lowered by the same factor in myoglobin compared with hemoglobin.



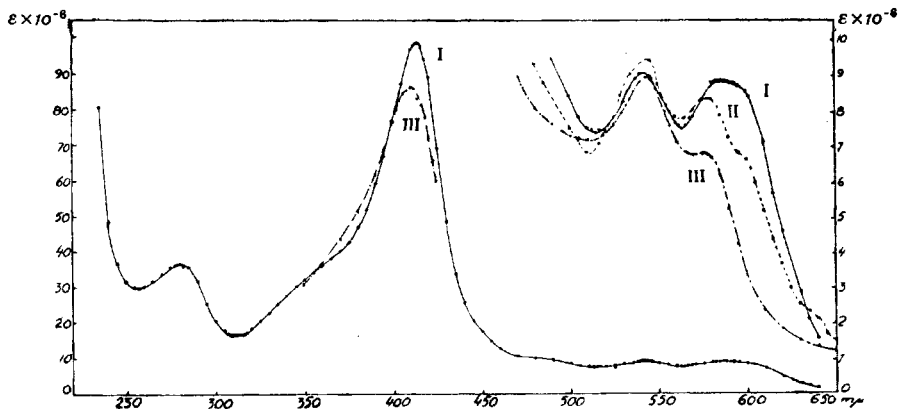


Fig. 14. Lightabsorption curves of MbOH (I, —), HbOH (II, ----) ref. (19) and P.O.OH at pH 12.0 (III, - · - · -).

#### SUMMARY

1. Ferrimyoglobin has a well-defined pH-stability range. Rapid destruction occurs at pH < 4.60 or > 11.75.

2. The transition from neutral to alkaline ferrimyoglobin follows a monovalent dissociation curve. Its p*K* value, extrapolated to zero ionic strength, has been determined spectrophotometrically (p*K* = 8.84) and magnetically (p*K* = 8.77 ± 0.04), thus one unit higher than for ferriHb. The dependence of p*K* of the ionic strength is three times less than in the case of ferriHb.

3. The paramagnetic susceptibility of ferrimyoglobin changes from 13 690 · 10<sup>-6</sup> cgs in neutral solution to 11 040 · 10<sup>-6</sup> cgs in alkaline. In contrast to ferriHb, ferriMb gives constant values between pH 7.5 and 5.

4. The ferriMb-fluoride gives three magnetically operable transitions around pH 6, 8 and 10. The first and last one are spectrophotometrically operable and could thus be determined with high accuracy. The transition at pH 8 is spectrophotometrically inoperable. The dissociation constants of the corresponding heme-linked groups were calculated.

5. Titration experiments revealed that ferromyoglobin, with ionic bonds, and CO-myoglobin, with covalent bonds, give identical titration curves. This explains the nearly complete absence of Bohr effect in myoglobin. The conclusion is drawn that the iron in myoglobin cannot be firmly linked to a histidine residue, as is assumed to be the case in hemoglobin.

6. The nature of the heme-linked groups in myoglobin is discussed. An unknown group of more negative character than a histidine residue must be

present, as judged from the values of the dissociation constants for  $\text{OH}^-$ ,  $\text{CN}^-$  and  $\text{F}^-$  that are all ten times higher than in ferriHb.

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