THERMODYNAMICS OF THE REACTION OF FERRIMYOGLOBIN WITH IMIDAZOLE

BY

ARMENAG H. BAGHDOYAN

submitted in partial fulfillment for the requirements
of the degree Master of Science
in the Chemistry Department of the
American University of Beirut
Beirut, Lebanon
June 1965

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ABSTRACT

The present work is a detailed thermodynamic study of the reaction between sperm whale ferrimyoglobin and imidazole. The reaction involves the replacement of a water molecule by imidazole at the sixth - the only available - coordinating position of the hematin iron(III) atom in ferrimyoglobin.

The equilibrium constant for this ligand-bonding reaction was measured by a spectrophotometric micro-titration at constant ionic strength (I = 0.10M) but covering a wide range of myoglobin concentrations (1.0 to 60 μM), imidazole concentrations (1 to 20 x 10^{-3}M), pH (5 to 9) and temperature (14 to 35^{0}C).

Both the equilibrium constant and the enthalpy of reaction showed a marked and complicated variation with pH.

To obtain thermodynamic parameters for the reaction, equations were derived taking account of theoretical variation of the equilibrium constant with pH on the basis of three ionizations which are known to be involved, as well as a residual "heme-linked" ionization from acidic groups on the protein.

Mathematical analysis of the results showed that the pH variation of the equilibrium constant could be accounted for adequately over the entire range of pH 6 to 9. In more acidic solutions, multiple prototropic equilibria appear to be involved. The above model also

accounts satisfactorily for the pH variation of the enthalpy of reaction.

The results of the work are compared with corresponding data on similar systems and their possible conformational significance is discussed.

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INTRODUCTION

The present work is a thermodynamic study of the reaction between sperm whale ferrimyoglobin and imidazole in dilute aqueous solution, and of the various acid-base equilibria involved, with special reference to the question of heme-linked ionizations in myoglobin.

Physiologically, ferromyoglobin is important as the muscle pigment responsible for oxygen storage. However, because of its rapid autoxidation into its stable iron(III) derivative - ferrimyoglobin - the latter is more conveniently used in studies of structure and reactivity, even though it has no physiological significance. Chemically, ferrimyoglobin may be regarded as an unsymmetrical octahedral complex of iron. The iron atom is incorporated in the center of a porphyrin molecule whose four central nitrogen atoms are bound to the iron. The metallo-porphyrin (heme) is very nearly planar. The remaining two bonds are directed at right angles from the plane of the disk. One of these sites is bound to the protein component through an imino nitrogen of histidine and the sixth is loosely attached to a water molecule which is also hydrogen bonded to a histidine residue in the single polypeptide chain which consists of 153 amino-acid residues.[1]

The relatively simple and stable structure of myoglobin makes it an ideal model for investigating more complex and labile macro-

molecules. The study of myoglobin has gone beyond the interest in its physiological role as oxygen store. However, a comparison of the work of Hanania,[2] 1953, with the recent review by Rossi Fanelli et al.,[3] shows that not much attention has been given to detailed kinetic and thermodynamic investigations of ligand bonding reactions. In particular, there remains the question of interpreting the observed second-order effects of ionizations in the protein and their influence on the reactivity of the hematin iron atom in myoglobin and other hemoproteins.

This problem arises from the observation that the equilibrium constant for the oxygenation of ferrohemoglobin, a reaction which does not involve explicitly H^+, still shows a pH dependence.[4] It has also been observed that in the formation of the cyanide (and fluoride) complexes of ferrimyoglobin[5] this kind of pH variation is superimposed on the usual mass law effect, due to the liberation of H^+ and the H^+-dependent concentrations of hydrogen cyanide and cyanide.

This effect of pH, which is present in both classes of reaction, is usually attributed to the ionization of acidic groups in the neighbourhood of the heme, affecting the affinity of the hematin iron for the ligand. Such groups are referred to as heme-linked ionizing groups, the term "linkage" being used in two senses, thermodynamic and chemical. Thermodynamically, it is implied that the affinity of iron for a ligand depends on the extent to which the heme-linked group is ionized, just as the acid strength of the group depends on the nature of the ligand attached to the iron. In the chemical sense, it is generally assumed that the ionizing group is in the amino acid residues of the protein to which the heme is attached.

In the following section the origin and development of this concept are outlined.

Development of the Concept of Heme-Linked Ionization

In 1904, Bohr[6] discovered that the oxygen equilibrium of blood was influenced by changes in the partial pressure of carbon dioxide. Ten years later, Christiansen, Douglas, and Haldane[7] reported the reciprocal effect, viz. that oxygenated blood absorbed less carbon dioxide than deoxygenated blood. It was not recognized at the time that this was a thermodynamic consequence of the earlier result. It was Hendersen[8] who in 1920 first assumed that there was an oxygen-linked acid group in the hemoglobin molecule, and that the acid strength of the group increased as a result of oxygenation, pK shifting from 7.6 in hemoglobin to 6.7 in oxyhemoglobin, the stronger acid. The investigation of van Slyke and coworkers[9] also supported the idea of an "oxylabile" group in hemoglobin whose dissociation in the region of pH 7 increased when the pigment combined with oxygen.

It was not until the advent of the glass electrode that more reliable data could be obtained by potentiometric acid-base titration techniques. German and Wyman[10] made the first attempt of a direct differential titration on recrystallized horse ferrohemoglobin and oxyhemoglobin at 25° in the presence of 1.33M sodium chloride and also at several lower salt concentrations. Their results showed that in the physiological range oxyhemoglobin was a stronger acid than hemoglobin, but in the more acid range, pH < 6, the situation was reversed.

Wyman and Ingalls[11] interpreted the above results, and similar data obtained by other workers, by assuming that there are in hemoglo-

bin four identical sets of two oxygen-linked acidic groups, one of which is rendered stronger, and the other weaker upon oxygenation. They obtained the best fit of all data available at the time for horse hemoglobin at 25° at I = 0.16M with:

	Hb	HbO_2
pK_1	7.93	6.68
pK_2	5.25	5.75

Wyman[12] next analyzed the data on enthalpy of ionization of the acidic groups in oxyhemoglobin and the variation of ΔH with pH. The heats of dissociation found at pH $<$ 4.5 were characteristic of carboxyl groups.[13] Between pH 6 and pH 8 a heat of dissociation of 6200 cal.mole^{-1} was observed; this is of the right order for the ionization of imidazolinium $=NH^+$ in imidazole and some derivatives.[13] Beyond pH 9.5 the heat of dissociation, 11500 cal.mole^{-1} corresponded to that expected for the terminal $-NH_3^+$ amino groups of lysine.

Wyman[14] also investigated the variation of the heat of the oxygenation reaction with pH. Since at any pH this heat must include the heat of dissociation of the oxygen-linked acid groups, its variation with pH provides a means of obtaining the heat of dissociation of just those groups. For this purpose Wyman derived the equation

$$(\partial \Delta H / \partial \Delta \bar{X})_T = -2.303RT^2 \, (\partial pH / \partial T)_{\Delta \bar{X}}$$

where ΔH denotes the measured increase in enthalpy, and $\Delta \bar{X}$, the number of protons dissociated, as the result of introducing one molecule of oxygen into hemoglobin at constant pH. It also represents the change in

the heat of oxygenation per unit change in the number of protons dissociated on oxygenation; it therefore represents the heat of oxygenation of oxygen linked acid groups. Wyman obtained $(\partial\Delta H/\partial\Delta\bar{X})_T$ = 6500 cal. This value is characteristic of imidazole, and since it was found to be applicable to the whole range of the Bohr effect, it was taken as evidence that both oxygen-linked acid groups were imidazolinium groups of histidine.

The structural interpretation of the above findings started with the suggestion by Conant[15] in 1933 that in hemoglobin the heme might be bound to two "hemaffine" groups in the protein, one of these groups being displaced upon oxygenation. Conant pointed out the improbability of the entry of oxygen affecting the dissociation of the carboxyl groups of the porphyrin and suggested that the oxylabile hydrogen ion might originate from the displaced "hemaffine" group, which would probably be a histidine residue on the protein.

In the light of Conant's suggestion, Wyman[14] interpreted his results by assuming that the iron is situated between two imidazole groups, one of which is displaced when oxygen enters the molecule, with consequent changes in the ionization of the imidazoles. He attributed the opposite effects, which he found the introduction of oxygen to exert on the ionizations, to the shift of one histidine pK from 5.25 to 5.75 and the other from 7.81 to 6.80, both changes being toward the pK value of free histidine, 6.0.

Based on Wyman's investigation, Coryell and Pauling[16] have given an interesting structural interpretation of the manner in which change of bond type (from ionic in hemoglobin to covalent in oxyhemoglobin)

affects the ionization of "oxylabile" groups. A modification of Conant's structure is taken, the heme being assumed to be between two imidazoles and, after Wyman, closer to one imidazole than to the other. It has been customary to refer to these as proximal and distal imidazoles respectively. From a consideration of the relative stabilities of resonance structures for hemoglobin and oxyhemoglobin, Coryell and Pauling "predict with certainty that the change of bond type for the iron atom accompanying removal of the oxygen molecule must be accompanied by a decrease in the acidity of the attached (proximal) imidazole group."[16] For the other (distal) imidazole, the interpretation of the heme-linked effect invokes configurational factors and is not precise.

This subject has been reviewed by several workers, notably by Theorell[17], Wyman[4] and Lemberg and Legge.[18] More recently, Wyman has given a detailed theoretical treatment of linked functions.[19] In this connection it is noteworthy that the recent crystal structure determination of sperm whale ferrimyoglobin by Kendrew and coworkers[1] has shown that the environment of the heme is in close agreement with the picture of proximal and distal histidines used in Pauling's interpretation.

However, several lines of investigation by other workers have shown the need for a wider interpretation of the heme-linked concept. Thus, George and Hanania have shown from measurements of the ionization constant for the iron-bound water molecule in ferrihemoglobin[20] and ferrimyoglobin[21] that the effective charge on the hematin iron varies with pH. This effect is screened out by salt ions at high ionic strength, which suggests that it is an electrostatic effect due to non-

specific interaction with charged groups in the protein.

Hanania and Irvine have considered the role of the metal ion in their studies of the effect of coordination on ionization in selected chelates of iron[22,a and b] and cobalt[23], the latter being vitamin B_{12} derivatives. Their results showed that the acid strength of an ionizing group in a ligand molecule can increase more than 1000-fold as a result of coordination of the ligand to iron(II) even though charge types are formally the same in free ligand and the complex. Moreover, the effect was reflected mainly in a more favorable enthalpy change, the entropy of ionization remaining substantially unaltered after coordination. In cases where the complex carried extra positive charge the effect was even greater. It was therefore concluded by the authors that both electrostatic and conjugative factors contribute to the net effect of coordination on ionization in the chelates studied.

Beetlestone and Irvine[24] made a comparative thermodynamic study of the ionization of the iron-bound water in vertebrate ferrihemoglobins. Large variations were observed in the enthalpy and entropy of ionization, but pK changed only slightly due to compensation of the changes in ΔH and $T\Delta S$. The authors concluded that the variations in ΔH and $T\Delta S$ reflect differences in electrostatic interactions originating from different charge configurations in each of the hemoglobins. Thus they found no need to invoke specific ionizations of the heme-linked groups.

Similarly, Awad and Badro[25] were able to account for the marked shoulder in the pH profile of the forward rate constant for the reaction of cyanide with sperm whale ferrimyoglobin; their proposed mechanism involved a prototropic transition in myoglobin without reference to

classical heme-linked groups. Hanania's early work[5] had also shown a shoulder effect around pH 6 in the pH profiles of the rate and equilibrium constants for the reaction of cyanide with horse ferrimyoglobin, but in this case a classical heme-linked approach had been adopted in the treatment of the results.

The influence of pH on protein structure and its prototropic groups was demonstrated by the acid-base titration and other studies of Steinhardt, Ona and Baychok[26] on free and masked imidazoles in ferrohemoglobin-carbon monoxide and ferrihemoglobin-cyanide, and by the work of Breslow and Gurd[27] on imidazoles in sperm whale ferrimyoglobin. In the former system, 22 imidazoles out of the total 36 histidyl residues were shown to be masked in the native molecule. In the case of myoglobin 6 of the 12 histidyls were free; the other imidazole groups being masked. The masked groups were shown to be released upon denaturation of the protein. These results have a direct bearing on the interpretation of heme-linked effects, especially since recent crystallographic evidence has confirmed the old idea that protein-to-heme linkage is via a proximal histidine and, through the iron-bound water, via distal histidine.

In this connection, the study of ionizations in the ligand imidazole of ferrimyoglobin imidazole complex would be particularly important. For, being a neutral molecule, the ligand imidazole introduces minimal charge effects at the binding site, the hematin iron atom, and could, therefore, be used in an investigation of non-specific electrostatic effects in such a system. Moreover, the imino =NH group of the ligand imidazole in the ferrimyoglobin-imidazole complex is an unequi-

vocal example of a group on a ligand directly bonded to the metal, forming an artificially heme-linked ionizing group in the complex.

A preliminary thermodynamic treatment of this reaction was applied by Abu-Isa[28] to whale ferrimyoglobin, horse ferrimyoglobin and horse ferrihemoglobin. However, the data obtained were not extensive or precise enough to permit mathematical analysis of the results except in the pH range 10 to 12. From this region it was possible to obtain thermodynamic data for the imino $=NH^+$ ionization in the ferrimyoglobin-imidazole complex.[29] Nevertheless, there still remained the need for a more extensive study of the system both in alkaline solutions as well as in the region of the Bohr effect.

The present work was undertaken in the light of the above discussion. It is a thermodynamic study of the reaction of imidazole with sperm whale ferrimyoglobin in the pH region 5 to 9. Its primary object is to obtain precise and reliable thermodynamic data for the reaction and apply the results to test the validity of the assumptions regarding heme-linked ionizations.

THEORY

The ligand-bonding reactions of hemoproteins are in general divided into two classes:

1.- Ferrohemoproteins tend to combine strongly with neutral ligands like O_2, NO, CO. Such reactions are generally considered to involve replacement of H_2O by the neutral ligand molecule at the sixth (the only available) octahedral coordinating position on the iron(II) atom of the heme which carries a formal charge of zero. These reactions can be represented by the general equation:

$$Fe(H_2O) + X = Fe\text{-}X + H_2O \qquad (1)$$

2.- Ferrihemoproteins have strong affinity towards anionic ligands such as CN^-, F^-, OH^-, CNS^-, N_3^-. These reactions also involve replacement of H_2O by ligand anion at the sixth coordinating position. In this case, however, the hematin iron(III) atom carries a formal charge of +1, and the reaction can be represented by the general equation:

$$Fe^+(H_2O) + Y^- = Fe\text{-}Y + H_2O \qquad (2)$$

The present work is concerned with the uncommon bonding of a neutral ligand imidazole to the hematin iron(III) atom of ferrimyoglobin. Although this reaction is formally similar to both of the above classes, it differs in the charge types involved, as shown in the following equilibrium:

$$Fe^+(H_2O) + :\text{imidazole} = Fe\text{-}\text{imidazole}^+ + H_2O \qquad (3)$$

$$Fe^{+}(H_2O) + ImH \quad = \quad Fe^{+}\text{-}ImH + H_2O$$

By analogy with reactions of nitrogen chelates with inorganic ions in aqueous solutions, this reaction is expected to involve the lone pair of electrons on one of the nitrogen atoms of the imidazole molecule, forming a low-spin iron-nitrogen bond. Two further lines of evidence support this argument:

1.- In the presence of excess imidazole, the spectra of ferrimyoglobin and ferrihemoglobin develop a single absorption band in the visible (530 - 540 mμ) with a shoulder (560 - 570 mμ). This change is similar to that observed with other nitrogen ligands, such as azide[30] and pyridine[31], and may therefore be taken to characterize the same type of reaction (replacement of H_2O). In the course of the present work, the absorption spectrum for the imidazole complex of sperm whale ferrimyoglobin was taken in the visible and near U.V. region. It is shown in Figure 1, and the molar absorbancies are listed in Table 1.

2.- The one-to-one stoichiometry of the reaction is in accord with its chemical nature (single reactive site) and is also supported by the constancy of equilibrium constants calculated on this basis over a wide range of ferrimyoglobin and of imidazole concentrations as shown in the Experimental chapter.

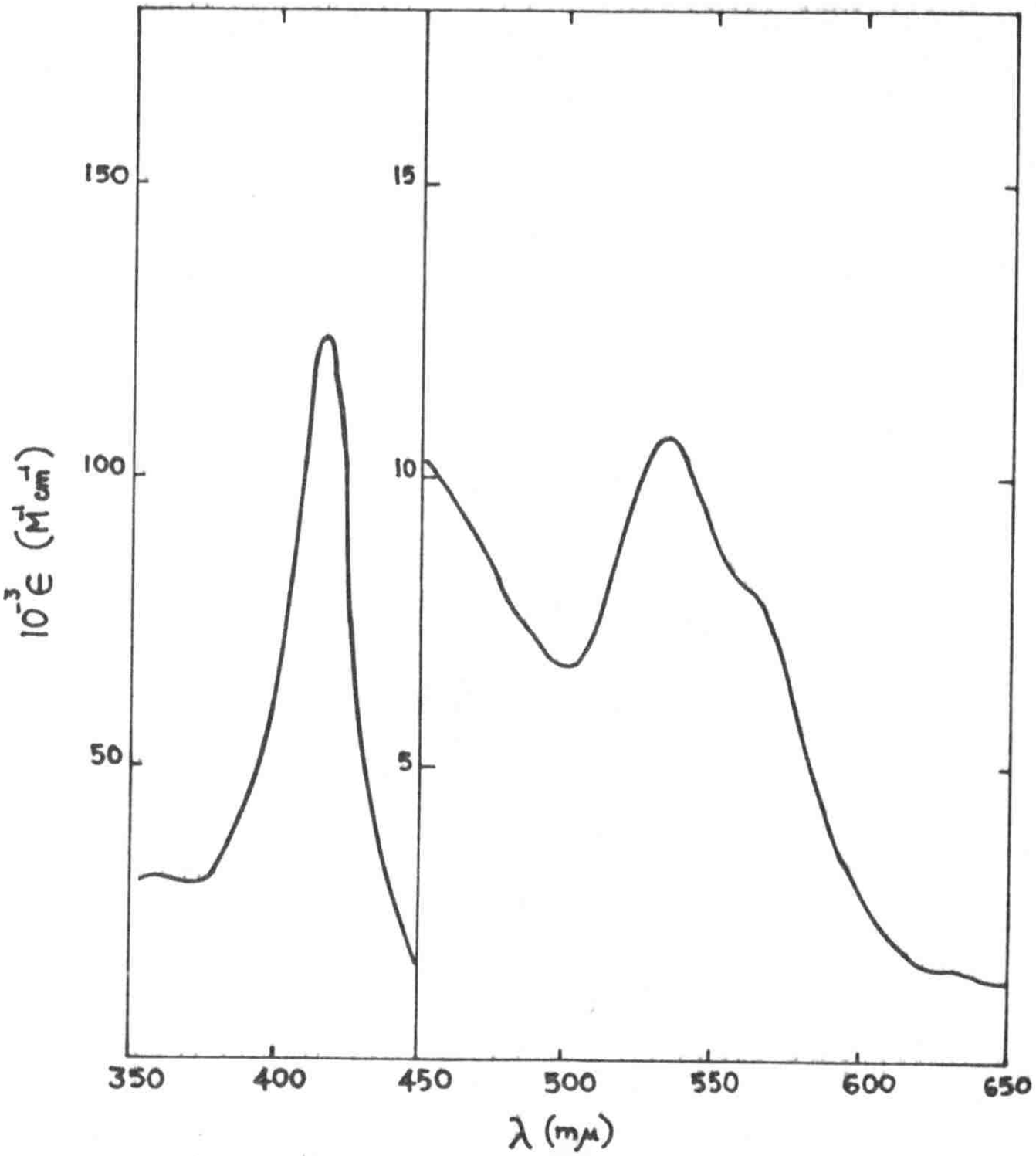


Fig. 1.- Absorption spectrum, visible and near U.V., of the sperm whale ferrimyoglobin-imidazole complex. Borate buffer pH ~ 8.2; I ~ 0.1M; imidazole ~ 0.5M corresponding to approximately 99% complex formation.

Table 1

Molar absorbancies of the ferrimyoglobin-imidazole complex for the Spectrum in Figure 1. Borate buffer, ionic strength 0.10M and pH $\sim$ 8.2. Imidazole $\sim$ 0.5M giving approximately 99% complex formation. Myoglobin concentration determined as the cyanide complex using ϵ (540 - 542 mμ) = 10.7 x $10^3 M^{-1}cm^{-1}$ and ϵ (423 mμ) = 110 x $10^3 M^{-1}cm^{-1}$.

(mμ)	10^{-3} ϵ	(mμ)	10^{-3} ϵ	(mμ)	10^{-3} ϵ
349	30.4	424	89.4	540	10.38
354	30.7	429	57.9	545	9.67
359	31.0	439	26.3	550	8.93
364	30.9	449	17.8	555	8.43
369	30.4	470	8.84	560	8.14
374	30.2	480	7.79	565	7.95
379	31.5	490	7.23	570	7.47
384	35.2	495	6.92	575	6.69
389	41.8	498	6.80	580	5.79
394	51.0	500	6.79	585	4.82
399	62.1	502	6.80	590	4.04
404	78.9	505	6.93	600	2.80
409	100.7	510	7.41	610	2.08
412	114.8	520	9.16	620	1.67
413	118.3	525	9.97	625	1.56
414	120.8	530	10.59	630	1.48
415	122.5	531	10.65	635	1.40
416	123.2	532	10.68	640	1.32
417	123.2	533	10.72	650	1.13
418	121.3	535	10.72		
419	119.1	538	10.57		

The thermodynamic equilibrium constant K for the reaction in equation 3 is given in terms of activities:

$$K = a_{Fe^+\text{-ImH}} \,/\, a_{Fe^+(H_2O)} \cdot a_{ImH} \qquad (4)$$

The present work was carried out entirely at constant ionic strength I = 0.10M. Since the reactant and product being considered (equation 3) have the same formal charge, and since the protein solutions are very dilute ($\sim 5 \times 10^{-6}$M), it may be assumed that the activity coefficients for $Fe^+(H_2O)$ and Fe^+-ImH are equal, and that the activity coefficient of the neutral ligand imidazole (concentration $\sim 10^{-2}$M) is about unity. Consequently, as a first approximation, the activities may be replaced by concentration terms, so that the equilibrium constant defined in equation 4 is also given by equation 5 below:

$$K = (Fe^+\text{-ImH}) \,/\, (Fe^+(H_2O)).(ImH) \qquad (5)$$

where brackets indicate molar concentrations.

Now, this equilibrium constant K refers to the reaction in equation 3. However, in practice one measures an "observed" equilibrium constant K_{obs}. This is because the reactant, ligand and the product molecules, all undergo acid-base equilibria such that they do not exist as single species within the experimental pH range. Consequently, one obtains in effect

$$K_{obs} = \Sigma(\text{Complex}) / \Sigma(\text{Ferrimyoglobin}).\ \Sigma(\text{Imidazole}) \qquad (6)$$

where Σ's are used to indicate total molar concentrations. The relative contribution of the various ionic forms to the total concentration of the species will, in each case, depend on pH; and K_{obs} will therefore be a

function of pH.

The variation of K_{obs} with pH will now be considered from the point of view of each of the following factors: (a) ionization of ferrimyoglobin; (b) ionization of imidazole; (c) ionization of the complex; (d) heme-linked ionizations.

(a) Effect on K_{obs} of Ionization of Iron-bound H_2O

The acid ionization of the iron-bound water molecule at the sixth coordination position in ferrimyoglobin is represented by the equation:

$$Fe^+(H_2O) \quad = \quad Fe\text{-}OH \quad + \quad H^+ \qquad (K_{Fe}) \qquad (7)$$

with an ionization constant[32] $K_{Fe} \sim 10^{-9}$. The fact that rates and equilibria for ferrimyoglobin reaction fall markedly beyond pH 9 suggests that the form Fe-OH is non-reactive and that reaction occurs with the form $Fe^+(H_2O)$. The variation of $Fe^+(H_2O)$ concentration with pH affects K_{obs} in the following manner:

From equation 7

$$K_{Fe} = (Fe\text{-}OH).h/(Fe^+(H_2O)) \qquad (8)$$

in which brackets indicate molar concentrations and h the hydrogen ion activity obtained from pH on the basis $pH = -\log a_{H^+}$. Rearrangement of equation 8 gives

$$(Fe^+(H_2O))/\ (Fe^+(H_2O)) + (Fe\text{-}OH) \quad = \quad h/(K_{Fe} + h) \qquad (9)$$

Writing $\sum Mb = (Fe^+(H_2O)) + (Fe\text{-}OH)$, equation 9 becomes

$$Fe^+(H_2O) = \sum Mb \ . \ h/(K_{Fe} + h) \qquad (10)$$

Thus the single effect on K_{obs} due to the ionization of H_2O can be seen by comparing equations 5, 6 and 10, leading to

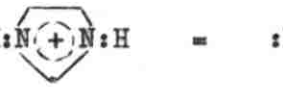
$$K^1_{obs}\ /K \ = \ h \ / \ (K_{Fe} + h) \qquad (11)$$

where K^1_{obs} refers to the observed formation constant of equation 6 assuming that the ferrimyoglobin-imidazole complex and the ligand imidazole both exist in single forms.

Equation 11 shows that the pH variation of K^1_{obs} is represented by a titration curve with an inflexion point at $pK_{Fe} = pH \sim 9$, as is shown in Figure 2a.

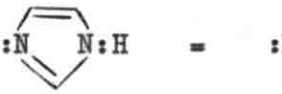
(b) Effect on K_{obs} of Ionization of Imidazole

The ligand imidazole ionizes in two stages:[29]



$$Im^+H_2 = ImH + H^+ \qquad (K_1) \qquad (12)$$

which has an ionization constant of $K_1 \sim 10^{-7}$, and



$$ImH = Im^- + H^+ \qquad (K_2) \qquad (13)$$

with an ionization constant $K_2 \sim 4 \times 10^{-15}$. For present purposes the latter ionization may be neglected as the experimental work extends from pH 5 to 9 only. Under these circumstances the only reactive form of imidazole will be ImH and it can be shown, by argument similar to that used above that

$$K^2_{obs} / K = K_1 / (K_1 + h) \qquad (14)$$

where K^2_{obs} is the formation constant on the basis that ferrimyoglobin and the complex both exist as the single species indicated in equation 3. Equation 14 shows that the effect of imidazole ionization, unlike that of the water, is to favor reaction at higher pH, and that the variation of K^2_{obs} with pH follows a titration curve with an inflexion point at

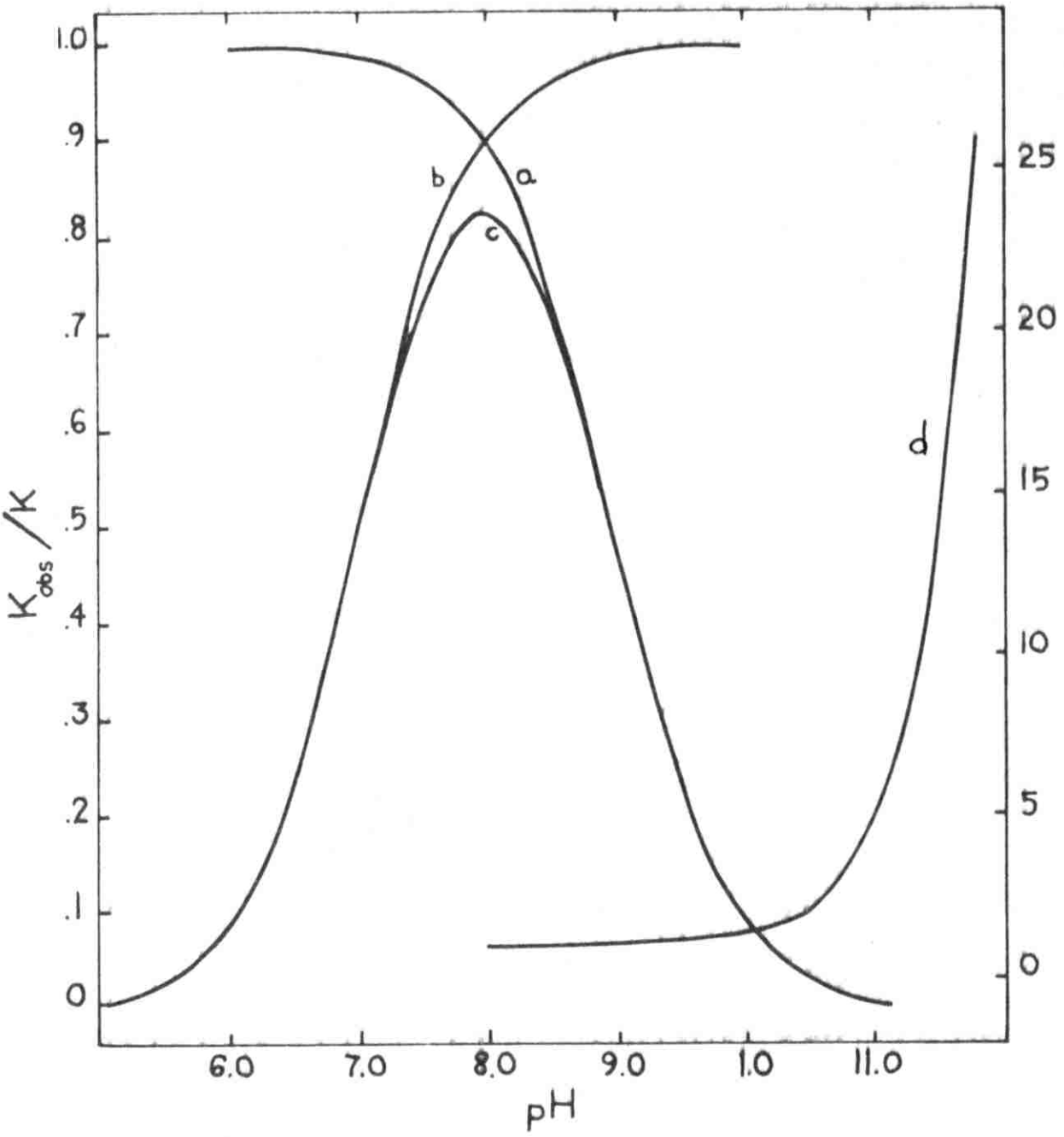


Fig. 2 - Effect of various acid-base equilibria on the measured equilibrium constant K_{obs} for the reaction of imidazole with sperm whale ferrimyoglobin. (a) iron-bound water (equation 7); (b) imidazole (equation 12); (c) combined effect of curves a and b; (d) ligand imidazole in the complex (equation 16). Temperature 25°C.

$pK_1 = pH \sim 7$.

Reference to Figure 2 shows the separate effects on the measured equilibrium constant of the acid ionizations of ferrimyoglobin and imidazole. The combined effect will produce a nearly symmetrical bell-shaped curve with a maximum around pH 8 arising from the balancing of the effects of $pK_1 \sim 7$ and $pK_{Fe} \sim 9$. The variation of K_{obs} with pH will now follow the equation:

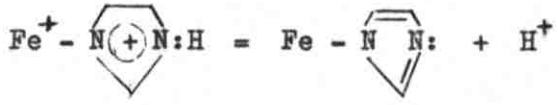
$$K^3_{obs} / K = (h / (K_{Fe} + h)) \cdot (K_1 / (K_1 + h)) \qquad (15)$$

where K^3_{obs} is the formation constant under the influence of the ionizations of the iron-bound water and of imidazole, the complex being assumed as a pH independent single species.

The measured formation constant, however, varies in a more complicated way not only beyond pH 10 where the complex ionizes, but also below pH 7 where other prototropic equilibria occur.

(c) Effect on K_{obs} of the Ionization of the Complex

It has been shown[23] that the acid strength of the imino =NH group in imidazole (an extremely weak acid with $pK_2 > 14$) becomes very much greater as a result of coordination to a metallic cation, and that in the case of sperm whale ferrimyoglobin the corresponding ionization has $pK_3 = 10.4$. This refers to the equilibrium



$$Fe^+\text{-}ImH = Fe\text{-}Im + H^+ \qquad (K_3) \qquad (16)$$

which introduces another factor into the above discussion. From equation 16,

$$K_3 = (Fe\text{-}Im) \cdot h / (Fe^+\text{-}ImH) \qquad (17)$$

which can be rearranged to give

$$(Fe^{+}\text{-ImH}) \;/\; (Fe^{+}\text{-ImH}) + (Fe\text{-Im}) \;=\; h \;/\; (K_3 + h) \qquad (18)$$

Comparison of equations 5 and 6 and substitution from equation 18 leads to

$$K^{4}_{obs} \;/\; K = (K_3 + h) \;/\; h \qquad (19)$$

where K^{4}_{obs} is the formation constant under the influence of the acid ionization of the imino group in the imidazole ligand of the complex, the reactants ferrimyoglobin and imidazole being considered pH independent as they appear in equation 3.

The pH variation resulting from the above effect (equation 19) is illustrated in Figure 2d. The combined effect of three ionizations (that of ferrimyoglobin, imidazole and the complex) on K_{obs} will therefore lead to a function F, given by

$$F = K^{5}_{obs} \;/\; K = (h \;/(K_{Fe} + h))\,(K_1/(K_1 + h)).((K_3 + h)/h) \qquad (20)$$

The pH variation of F describes an unsymmetrical bell-curve (Figures 3,4).

(d) Effect on K_{obs} of Heme-Linked Ionization

On the basis of experimental evidence in the present study, we shall further assume the possibility of existence on the protein of an ionizing or acidic group thermodynamically linked to the iron-ligand bonding reaction. We shall consider three possibilities with respect to the group in question:

(i) Heme-linked interaction occurs in the reactant molecule but not in the product. That is, although the iron-bound water and the ionizing group are mutually effective, the replacement of the water by

the ligand imidazole will break the linkage. If this effect involves a single acid-base equilibrium, with ionization constant K_r, it may be represented as follows:

$$H^+ \ldots Fe^+(H_2O) \quad = \quad Fe^+(H_2O) \; + \; H^+ \tag{21}$$

for which

$$K_r = (Fe^+(H_2O)) . \; h \; / (H^+ \ldots Fe^+(H_2O)) \tag{22}$$

and, if the complex exists only in the form defined in equation 3, one can define a "linked" function L_r as

$$L_r = K^6_{obs} \; / \; K \; = \; K_r \; / \; (K_r + h) \tag{23}$$

so that the combined influence of the ionizations already considered together with one heme-linked ionization on reactant (but not product) is given by

$$F.L_r \; = \; F.K_r \; / \; (K_r + h) \tag{24}$$

The net effect is illustrated in Figure 3, with hypothetical ionization constants pK_r 6.6 and also the case pK_r 7.0 (curves 2 and 3 respectively).

(ii) Heme-linked interaction occurs in the product, the complex, but not in the reactant molecule. In this case it is assumed that interaction occurs through the bonded ligand in the complex or as a result of a configurational change which accompanies reaction.

The heme-linked ionization of the complex may be represented as follows:

$$H^+ \ldots Fe^+\text{-ImH} \; = \; Fe^+\text{-ImH} + H^+ \tag{25}$$

for which the ionization constant is

$$K_p \; = (Fe^+\text{-ImH}) . \; h \; / (H^+ \ldots Fe^+\text{-ImH}) \tag{26}$$

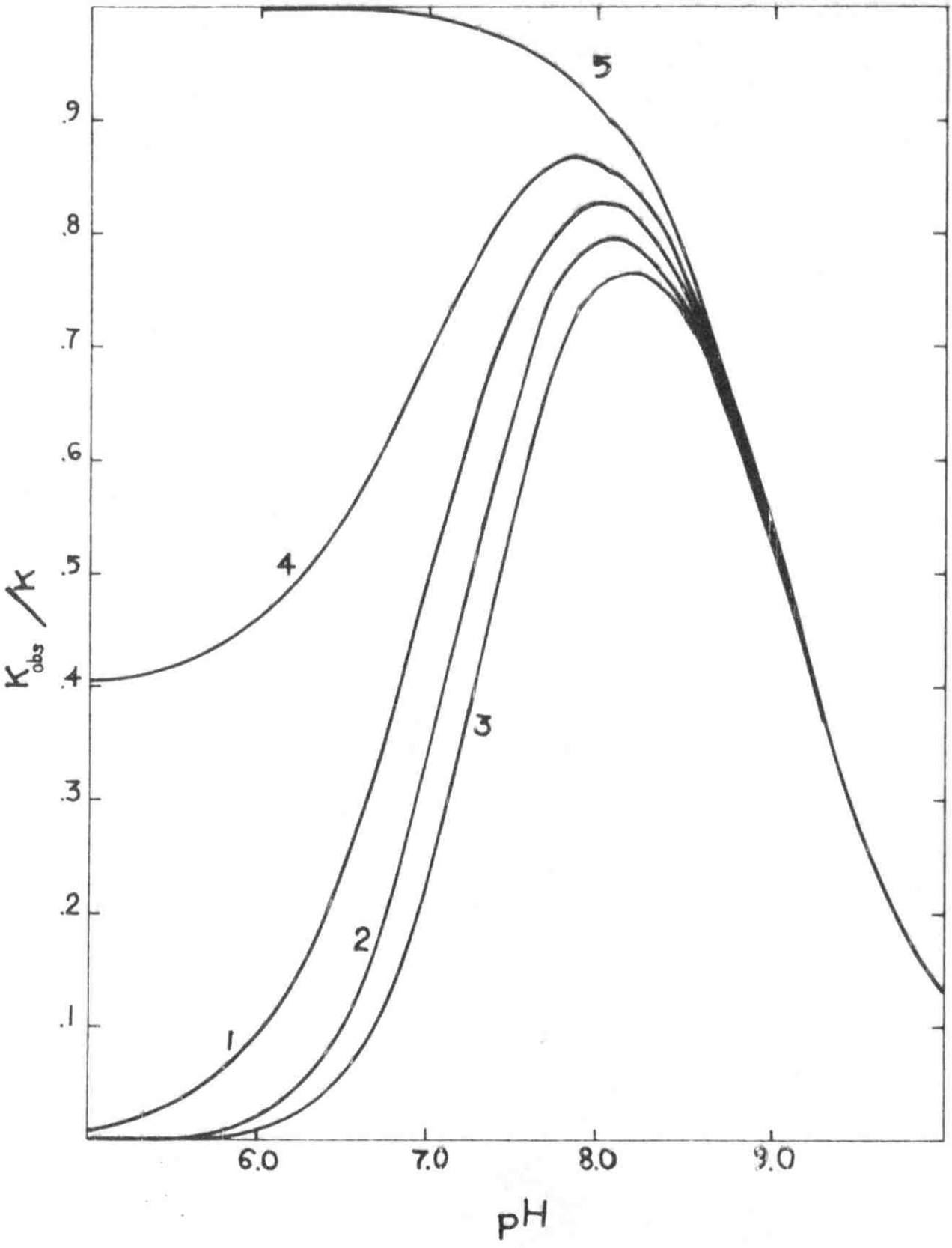


Fig. 3 - Heme-linked ionization effects on K_{obs}. (1) Total effect of non heme-linked ionizations (curves a, b and d combined from Figure 2). The other curves show the additional effect of heme-linked ionizations on either reactant or product. For reactant: (2) pKr=6.6; (3) pKr=7.0; For product: (4) pKp=6.6; (5) pKp=7.0. Temp. 25°C

Here again we can define a "linked" function L_p,

$$L_p = K'_{obs} / K = (K_p + h) / K_p \qquad (27)$$

so that the combined effect in this case is given by

$$F.L_p = F.(K_p + h) / K_p \qquad (28)$$

which corresponds to equation 24, and is illustrated in Figure 3 with two hypothetical constants pK_p 6.6 and pK_p 7.0 (curves 4 and 5 respectively).

(iii) Heme-linked interaction occurs in both reactant and product (equations 21 and 25). The "linked" function L in this case involving reactant and product is given by

$$L = (K_r / (K_r + h)) \cdot ((K_p + h) / K_p) \qquad (29)$$

and consequently,

$$F.L = F\ (K_r/(K_r + h)).((K_p + h) / K_p) \qquad (30)$$

Figure 4 illustrates the pH effect on K_{obs} resulting from heme-linked ionizations in reactant and product, and shows that the type of effect depends on the relative magnitudes of pK_r and pK_p. It is also noteworthy that if $pK_r = pK_p$ then no heme-linked effect is observed, and equation 30 reduces to equation 20.

Combining all the pH effects considered above (a, b, c and d), it follows that the observed equilibrium constant of the reaction of ferrimyoglobin with imidazole is related to the various parameters in its final form by the equation

$$K_{obs} = K \cdot F \cdot L \qquad (31)$$

where K is the thermodynamic equilibrium constant for the reaction (equation 4), and F and L are the functions defined above. Thus the

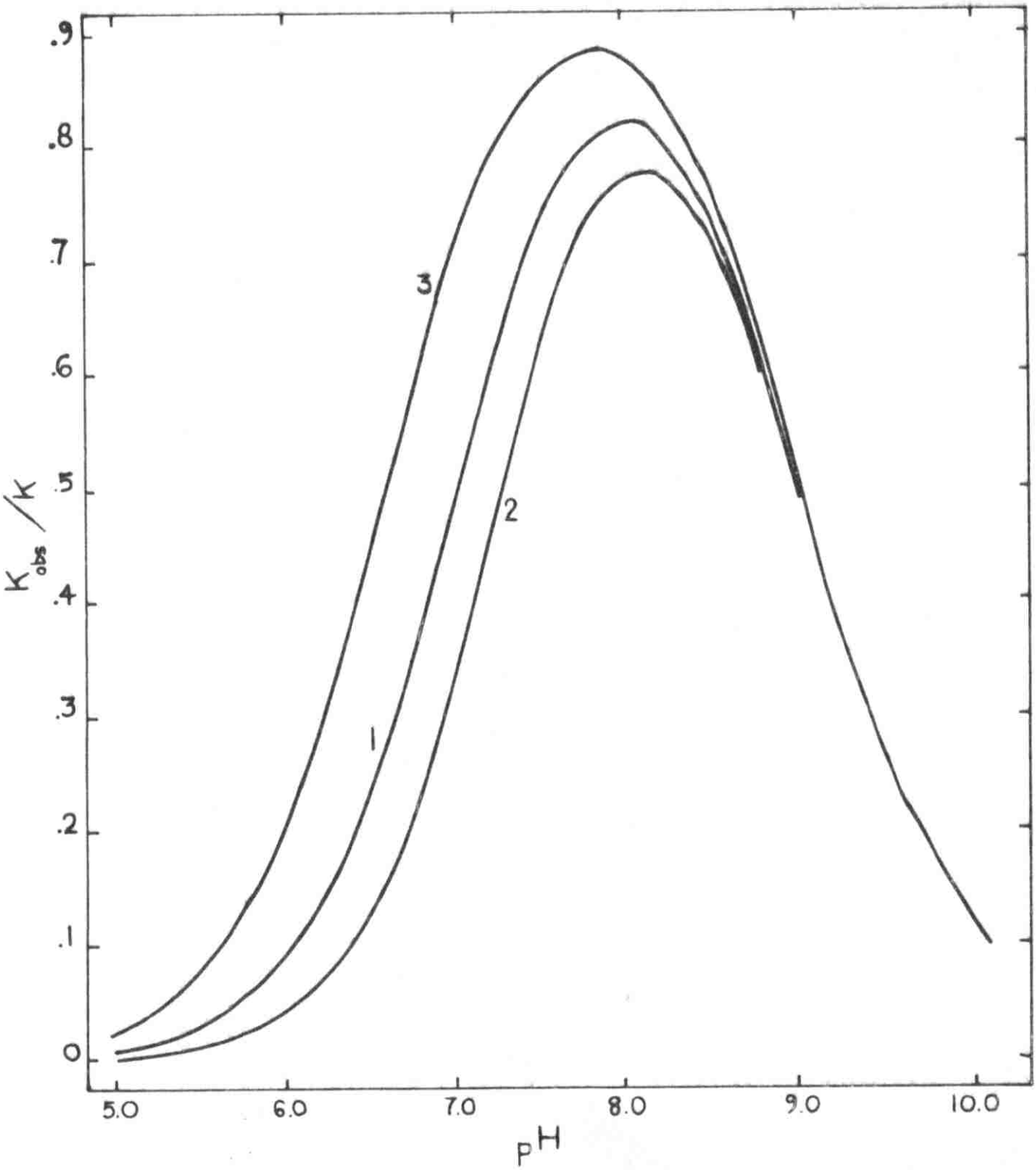


Fig. 4 - Heme-linked ionization effects on K_{obs}. (1) Total effect of non heme-linked ionizations (curves a, b and d combined from Figure 2); (2) additional effect of heme-linked ionizations in reactant and product with pKr 7.0 and pKp 6.6; (3) same with pKr 6.6 and pKp 7.0.

thermodynamic (pH independent) formation constant of the reaction in equation 3 is

$$K = K_{obs} / F \cdot L \qquad (32)$$

In the above consideration of heme-linked ionizations it was assumed that K referred to species in which the heme-linked groups were in conjugate base form. There is no a priori reason for selecting this as standard, and a reconsideration of the equilibrium is possible on the basis of conjugate acid form participating in the reaction. Thus,

$$H^+ \ldots Fe^+(H_2O) + ImH = H^+ \ldots Fe^+\text{-}ImH + H_2O \qquad (33)$$

The equations derived in sections (a), (b) and (c) would still be valid. However, introduction of heme-linked ionizations into this equilibrium leads to

$$K^8_{obs} / K = h / (K_r + h), \qquad (34)$$

$$K^9_{obs} / K = (K_p + h) / h \qquad (35)$$

where the constants are defined as in the previous case. Furthermore, using the function L' corresponding to L (equation 29), we obtain

$$F.L' = F. (h/(K_r + h)).((K_p+h)/h) = F. ((K_p+h)/(K_r+h)) \qquad (36)$$

corresponding to equation 23, 27 and 30.

Comparison of equation 30 with equation 36 shows that these two relations are identical except for the factor K_r/K_p which does not appear in the latter. This constant factor, however, does not affect the analysis of experimental data as described above.

Calculation of Thermodynamic Parameters

ΔF^0, the standard free energy change, is related to the equilibrium constant by the equation

$$\Delta F^0 = -RT\ln K = -4.57T \log_{10} K \qquad (37)$$

where K is the equilibrium constant in M^{-1} and T the temperature (^0K).

The value of ΔH, the overall enthalpy change in the reaction can be calculated from the change of the equilibrium constant with temperature, according to the van't Hoff isotherm

$$\Delta H = \frac{RT^2 d\ln K}{dT} = -4.57d \log_{10} K / d\,(1/T) \qquad (38)$$

and consequently, at given temperatures

$$\Delta S = (\Delta H - \Delta F) / T \qquad (39)$$

Since the reaction (equation 3) involves replacement of H_2O by neutral ligand molecule, the free energy of reaction would not be expected to vary much with ionic strength. Furthermore, since the reaction is carried out/in dilute solutions, it may be assumed, as a first approximation, that the above equilibrium data are equal to the corresponding thermodynamic data K^0, ΔH^0 and ΔS^0 at zero ionic strength.

EXPERIMENTAL

I. Materials

Myoglobin: Sperm whale ferrimyoglobin was purchased from Seravac Laboratories (Pty. Ltd., Colnbrook, England) as lyophilized and salt free sample (batch 2) prepared from skeletal muscle. This commercial product has been shown by Awad[33] to be chromatographically identical with the material used by Edmundson and Hirs[34] in their studies of amino acid sequence, and by Kendrew and coworkers[1] in their crystallographic work. It was used in the present work without chromatographic fractionation.

Imidazole: AnalaR grade imidazole ($C_3N_2H_4$) was purchased from Light and Co., Colnbrook, England. It was kept in a desiccator over calcium chloride and was used throughout without further purification.

Water: Deionized redistilled water was used in the preparation of all the solutions and buffers. Tap water was passed through a two-bed ion-exchanger (Deminerolit Mack 4E, United Water Softeners Ltd., London), redistilled from a commercial glass still (Laughborough Glass Co., England) and stored in polyethylene containers.

All the other chemicals used for buffer preparations and pH adjustments were of AnalaR or Pro-Analysi grade.

Buffers: The pH range in the present study extended from 5.0 to 9.0. Three different sets of buffers were used to cover this range:

KHPhtalate / NaOH for the pH range 5 - 6

$NaH_2PO_4.2H_2O$ / NaOH for the pH range 6 - 8

H_3BO_3 / NaOH for the pH range 8 - 9

The ionic strength of each solution was adjusted to 0.10M by the addition of the requisite amount of NaCl. For calibrating the pH scale standard solutions were prepared according to the specifications of the U.S. National Bureau of Standards.[35]

II. Apparatus

The apparatus used in the present work was the following:

Spectrophotometer: All absorbancy measurements were made on a Unicam SP. 500 instrument (Unicam Instruments Ltd., Arbury Works, Cambridge, England) fitted with a thermostated cell holder with water circulating from a constant temperature bath. This allowed temperature control in the spectrophotometer cell to $\pm$ 0.1° or better.

pH Meter: All pH measurements were made on a Radiometer, pH Meter model PHM4c (Radiometer Co., Copenhagen, Denmark) with precision $\pm$ 0.005 pH and reproducibility $\pm$ 0.01 pH.

Microburette: In all experiments, addition of reactant imidazole was made in a plunger type microburette (Strohlein and Co., Dusseldorf) with dial indicator, readable minimum delivery 0.0002 ml and a capacity of 2 ml.

Temperature Control: A thermostat (WACO Lo-Temp Bath, Chicago, U.S.A.) was used for control of temperature from 14° to 35°. Water from this thermostat was circulated through the pH measurement cell and the spectrophotometer. A telethermometer thermistor probe calibrated

against standard thermometers was used in measuring the temperature of the solutions in the spectrophotometric cells. The calibrated thermometers had been certified by the National Physical Laboratory, London, in 1961.

III. Procedure

A. Preparation of Solutions

Two solutions were used in every experiment: one of ferrimyoglobin in buffer at a particular pH, ionic strength and temperature, and the other containing an appropriate concentration of imidazole in water adjusted to these conditions. The main problem was the matching of pH, keeping to a fixed concentration of imidazole. The most practical way of achieving this was found to be the following:

A number of buffer solutions covering a narrow pH range were prepared and placed in the thermostat. About half an hour was allowed for temperature equilibration. The pH of each solution was then measured. The appropriate amount of ferrimyoglobin (estimated to give absorbancies of about .600 - .800) was dissolved in each test tube.

The formation constant for the ferrimyoglobin-imidazole complex being pH dependent, it was necessary to have a constant pH during the titration of myoglobin with imidazole. The following procedure was adopted in preparing imidazole solutions of given pH and concentration: titration curves were obtained for 10.0 ml of 0.50 M imidazole and 10.0 ml of 0.20 M imidazole using 5.5 N HCl (Figure 5). A stock solution of 1.00 M imidazole in water was prepared and used later for the preparation of 0.500 M and 0.200 M imidazole solutions. 2.00 or 5.00 ml of stock solution were pipetted into a 10 ml volumetric flask. The

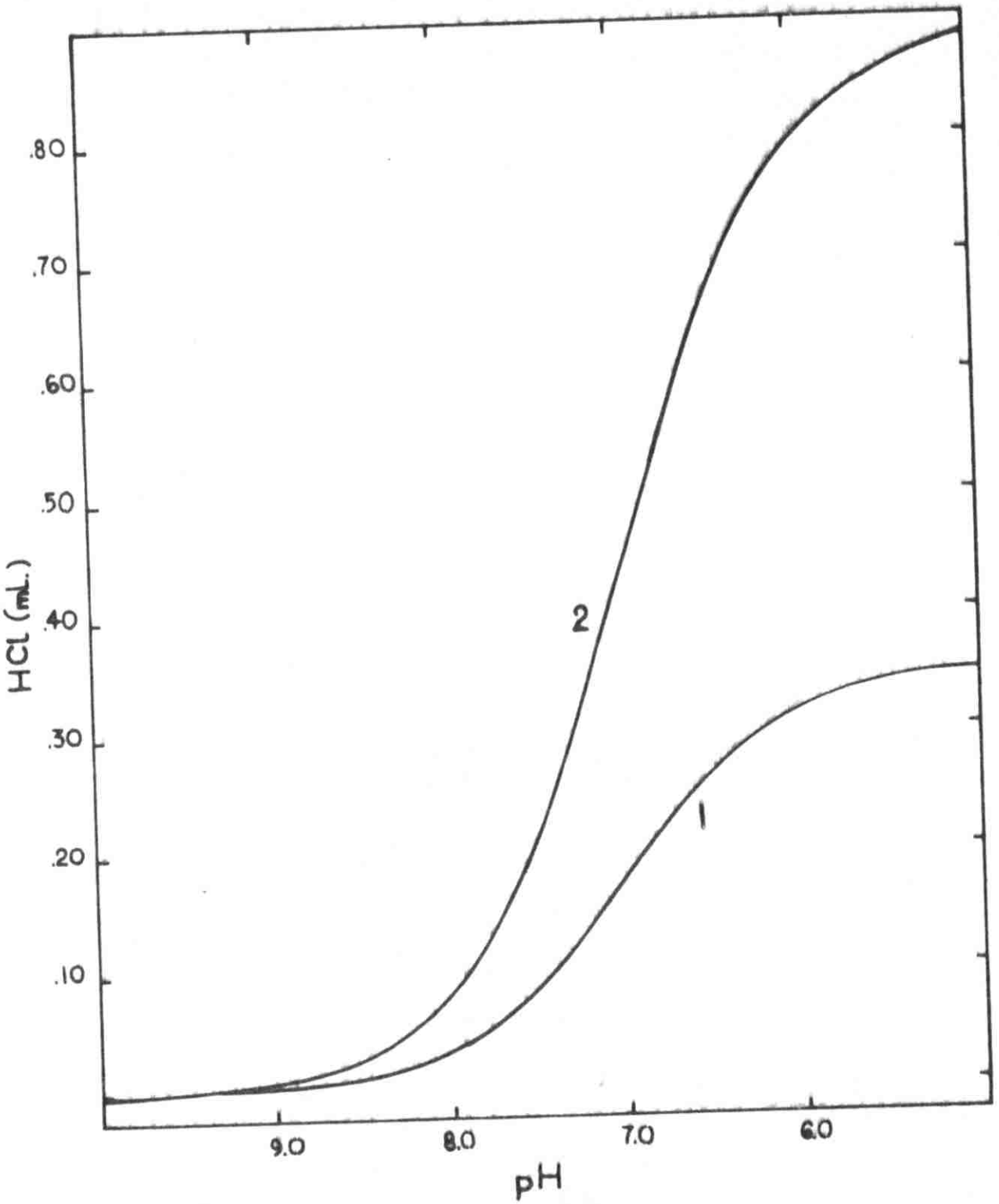


Fig. 5 - Titration curves of imidazole with 5.5N HCl. Original volume of imidazole 10.0 ml. T ~ 25°. Curve (1): 0.20M imidazole. Curve (2): 0.50 M. imidazole.

volume of HCl needed to give the desired pH - that of the buffer - was found by interpolation from the titration curves of Figure 5, and was added into the flask. The solution was then made up to 10.0 ml with water. At this point, however, one would not expect exact agreement between pH of the buffer and that of the imidazole solution.

Accurate matching of pH was achieved as follows: imidazole solutions were left in the water bath for temperature equilibration. The pH of the myoglobin solution was again measured. The pH of the corresponding imidazole solution was also taken. In case they did not match exactly, minute amounts of 5.5 N HCl or 6N NaOH were added until matching was obtained within 0.02 pH. In no case the volume of NaOH or HCl added exceeded 0.02 ml. This would still leave the concentrations of the solutions practically unaffected.

B. Equilibrium Measurements

3.00 ml of myoglobin solution were pipetted into a dry spectrophotometric cell and a few minutes were allowed for the solution to attain constant temperature. The microburette was filled with the appropriate imidazole solution and its delivery tube was connected through a narrow polyethylene tube of diameter < 1 mm. The absorbance of the myoglobin solution at the wavelength of measurement (410 mμ or 565 mμ) was taken. The polyethylene extension tube was next dipped into the cell containing the solution, and reactant added in aliquots of 0.01 to 0.10 ml. After each addition the tube was removed and the solution mixed with a polyethylene plunger. The absorbance of the mixture was then taken. The temperature of the solution in the cell would not be expected to change appreciably as the volumes of imidazole

solution added were usually small. In cases where it was felt necessary (especially when large volumes were added at 14^0 and 35^0) a certain lapse of time was allowed for equilibration. Each run comprised 8 to 11 additions of titrant.

It is noteworthy that the total molar ionic strength of the mixtures varied during the titration as a result of the net effect of addition of reactant imidazole and dilution of the solution. Calculation shows that the mean ionic strength was $I = 0.10 \pm 0.004M$ for the experiments at pH 6.0 to 8.5 and up to $0.10 \pm 0.01M$ beyond these limits.

C. pH Measurement

A thermostated cell was used for pH measurements. Contact between the calomel half cell (satd. KCl) and the solution the pH of which was to be measured was established through a saturated KCl salt bridge.

The pH was measured at the start and at the end of titration for each experiment. In every case the initial and final pH values are reported. All pH measurements were made following the calibration procedure of Bates.[35]

D. Calculation of K_{obs}

The formation constant K_{obs} of the ferrimyoglobin-imidazole complex was calculated from the measured absorbancies in the above titration. The principle of the method can be summarized as follows:

From equation 6 written in the form

$$K_{obs} = C / Mb.Im \qquad (40)$$

where C, Mb and Im are the equilibrium molar concentrations of complex,

ferrimyoglobin and imidazole respectively, one obtains

$$Mb = C \,/\, K_{obs}.Im \qquad (41)$$

The ratio C/Mb is related to absorbancy readings. It can be shown that

$$C \,/\, Mb = (A_0 - A) \,/\, (A - A_\infty) \qquad (42)$$

where A_0 stands for the absorbancy of the ferrimyoglobin solution before the addition of any imidazole, A for the absorbancy of the solution after the addition of the ligand, and A_∞ for the absorbancy of the solution at 100% complex formation. Replacing Mbin equation 42 by its value given in equation 41 and rearranging we get

$$A = (A_0 - A) \,/ Im.K_{obs} + A_\infty \qquad (43)$$

The linear plot of A versus $(A_0 - A) \,/\, Im$ has slope = $1/K_{obs}$ and intercept = A_∞.

In the experimental procedure employed the volume of the solution increased as imidazole was added. Thus the absorbancies read (A') were corrected for this dilution effect to give A values.

A typical run is reproduced below for illustration.

Sample Run

Stock Solutions

1. Ferrimyoglobin $\sim 5 \times 10^{-6}$M in buffer (KHPhthalate-NaOH, I = 0.10M)

2. Imidazole 0.500M in water (pH matched against ferrimyoglobin)

Conditions:

T = 25.0 $\pm$ 0.1°C I = 0.10M pH 6.11 - 6.17

pH of ferrimyoglobin in buffer = 6.11

pH of ferrimyoglobin solution after titration with imidazole = 6.17

3.00 ml of ferrimyoglobin pipetted in a 10 mm cell and absorbancies taken at λ = 410 mμ against water as blank.

Ml Im. added	A'	A (corrected for dilution)	$A_0 - A$	$10^4(Im)^*$	$(A_0 - A)/(Im)$
.00	.658	.658			
.05	.608	.619	.039	82	4.76
.10	.574	.593	.065	161	4.04
.15	.545	.572	.085	238	3.59
.20	.524	.558	.099	312	3.19
.25	.505	.547	.111	385	2.88
.30	.488	.537	.121	455	2.66
.40	.460	.522	.136	588	2.31
.50	.438	.511	.147	715	2.06
.60	.420	.504	.154	834	1.85

Plot of A vs $(A_0 - A)/(Im)$ is shown in Figure 6

K = 1/slope = 25 $\pm$ 0.5 M^{-1}

* Concentration of imidazole in the cell solution.

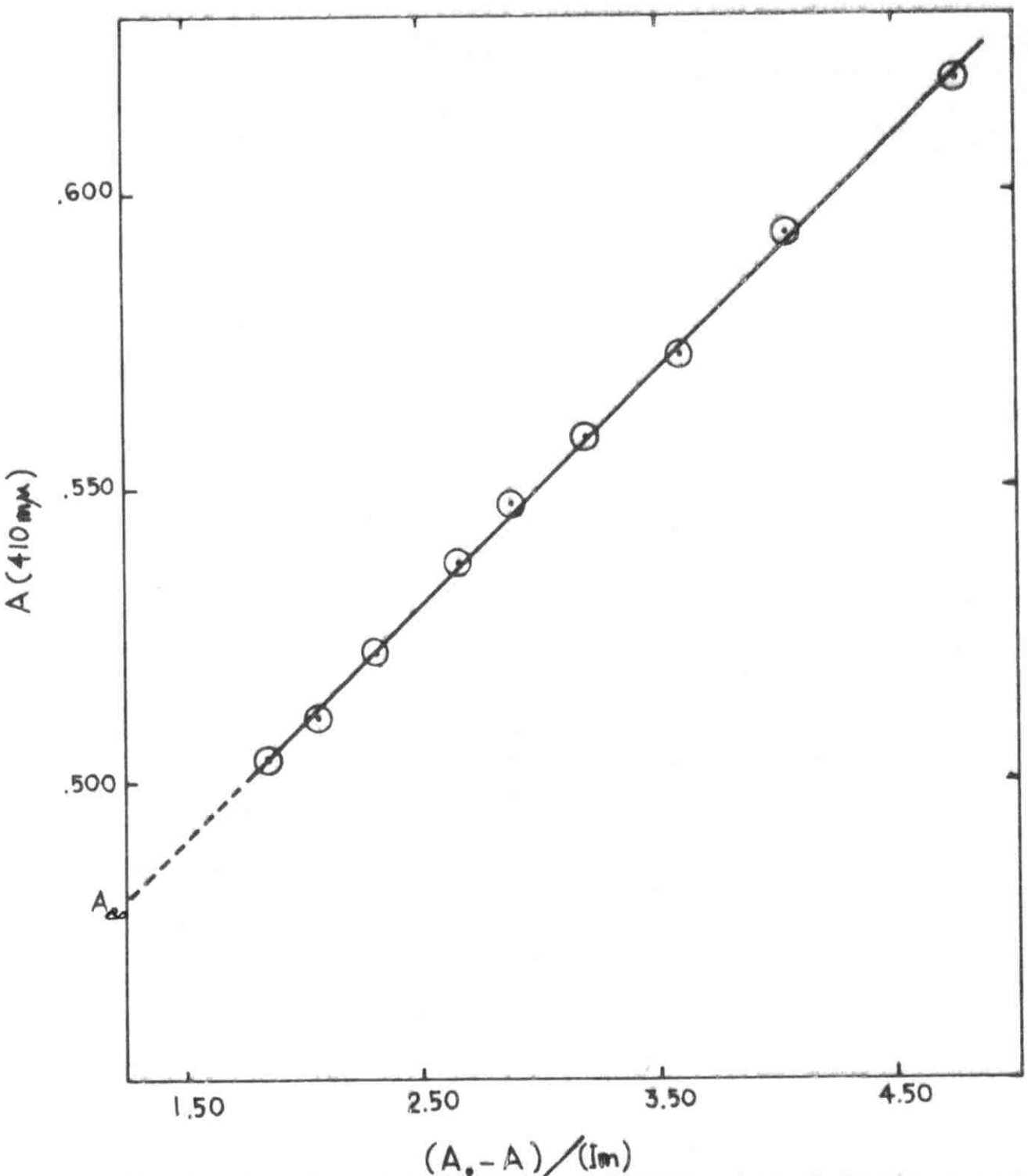


Fig. 6 - Determination of equilibrium constant. Plot of absorbancy A vs. the function $(A_0-A)/(Im)$ for the sample run described in the text. A_0 refers to ferrimyoglobin, A to mixtures of ferrimyoglobin and imidazole and A_∞ to 100% complex (obtained by linear extrapolation). Linear slope = $1/K_{obs}$.

RESULTS

Following the above procedure, the formation constant K_{obs} for the reaction of ferrimyoglobin with imidazole was measured over a wide range of myoglobin concentration, pH and temperature, all at I 0.10M.

Effect of Myoglobin Concentration on K_{obs}

Measurements were first made covering the range 1.0 to 60 x 10^{-6}M ferrimyoglobin at constant pH 7.6, temperature approximately 25°C and I = 0.10M. The same buffer (phosphate) was used, and 10 or 40 mm cells were employed to fit the absorbancy requirements.

Table 2

Mb conc. (M)	Method	K_{obs} (equ.6)
$1x10^{-6}$	40mm cell; λ =410 mμ	133 ± 2
$6x10^{-6}$	10mm cell; λ =410 mμ	132 ± 2
$10x10^{-6}$	40mm cell; λ =565 mμ	130 ± 2
$60x10^{-6}$	10mm cell; λ =565 mμ	133 ± 2
	mean K_{obs} =	132 ± 2

The results in Table 2 show that the equilibrium constant is independent of myoglobin concentration over the experimental range normally employed in spectrophotometry. This leads to the conclusion that no aggregation equilibria of myoglobin occur in this range of

concentrations.[36] It seems unlikely that such equilibria exist but so balance out as to lead to an apparent constancy of the formation constant.

Effect of pH and Temperature on K_{obs}

Having established the fact that the measured equilibrium constant is independent of protein concentration, the measurements were repeated to study the pH variation at constant temperature and ionic strength.

At the lower limit of pH 5 the very small value of K_{obs} necessitated use of large imidazole concentrations; this in turn had two effects: (a) it made impossible the control of ionic strength; (b) excess imidazole tended to cause protein denaturation.

At the upper limit, there was no need to extend measurements beyond pH 9 as the data for the region are available[29] at ionic strength 0.20 M.

The results at 25.0°C are listed in Table 3 together with the corresponding values for K_{obs} at 14.0°, 20.0° and 35.0°. In each case, the table also gives the calculated values of K' (equations 20 and 44) and of K (equations 29 and 45) which are obtained by the analysis of the data as discussed below.

Table 3

Variation of K_{obs} with pH for the reaction of sperm whale ferrimyoglobin with imidazole, I 0.10M.

Temp. °C.	Buffer	pH Range	K_{obs} equ. (31)	K' eq. (44)	K equ. (32)
14.0	Phosphate	5.92-6.00	14 ± 1	335 ± 24	228 ± 16
"	"	6.19-6.24	18.5 ± .5	257 ± 7	184 ± 5
"	"	6.37-6.44	27.0 ± .5	252 ± 5	189 ± 4
"	"	6.77-6.80	50.0 ± .5	220 ± 2	183 ± 2
"	"	6.98-7.05	71 ± 1	214 ± 3	187 ± 3
"	"	7.18-7.30	100 ± 1	222 ± 2	203 ± 2
"	"	7.34-7.39	113 ± 1	217 ± 2	203 ± 2
"	"	7.53-7.62	130 ± 3	207 ± 5	197 ± 5
"	"	7.72-7.81	145 ± 1	204 ± 1	197 ± 1
"	Borate	8.03-8.08	150 ± 2	191 ± 3	188 ± 3
"	"	8.29-8.32	150 ± 2	188 ± 3	185 ± 3
"	"	8.50-8.54	148 ± 2	192 ± 3	192 ± 3
"	"	8.66-8.77	138 ± 2	193 ± 3	193 ± 3
"	"	8.89-8.91	123 ± 2	192 ± 3	192 ± 3
20.0	"	7.99-8.05	145 ± 1	182 ± 1	180 ± 2
"	"	8.19-8.24	147 ± 1	183 ± 1	181 ± 2
"	"	8.39-8.44	144 ± 1	187 ± 1	185 ± 1
"	"	8.60-8.65	132 ± 1	185 ± 2	184 ± 2

Table 3 cont'd.

Temp. °C.	Buffer	pH Range	K_{obs} equ. (31)	K' equ.(44)	K equ. (32)
25.0	Phthalate	4.97-5.03	4.5 ± 1	572 ± 127	362 ± 80
"	"	5.18-5.24	5.5 ± 1	433 ± 79	277 ± 51
"	"	5.44-5.45	7.5 ± 1	351 ± 47	228 ± 30
"	Phosphate	5.43-5.45	7.5 ± 1	351 ± 47	228 ± 30
"	Phthalate	5.65-5.65	10 ± 1	292 ± 29	194 ± 19
"	Phosphate	5.75-5.77	13 ± 1	298 ± 23	200 ± 15
"	"	5.74-5.85	13 ± 1	273 ± 21	185 ± 14
"	"	5.83-5.83	15 ± 1	295 ± 20	200 ± 14
"	Phthalate	5.90-5.90	16 ± 1	270 ± 17	185 ± 12
"	Phosphate	5.95-6.01	19 ± 1	270 ± 14	188 ± 10
"	"	6.10-6.14	24.5 ± .5	259 ± 5	187 ± 4
"	Phthalate	6.11-6.17	25.0 ± .5	253 ± 5	183 ± 4
"	Phosphate	6.15-6.17	25.0 ± 1	243 ± 10	176 ± 7
"	"	6.37-6.38	38 ± 1	238 ± 6	182 ± 5
"	"	6.46-6.49	42 ± 1	217 ± 5	171 ± 4
"	"	6.52-6.52	44 ± 1	212 ± 5	169 ± 4
"	"	6.61-6.61	51 ± 2	210 ± 8	171 ± 7
"	"	6.68-6.71	61 ± 2	216 ± 7	180 ± 6
"	"	6.72-6.79	61 ± 1	199 ± 3	168 ± 3
"	"	6.78-6.81	67 ± 1	202 ± 3	172 ± 3
"	"	6.98-7.00	86 ± 2	199 ± 5	178 ± 4
"	"	7.17-7.20	109 ± 2	203 ± 4	186 ± 4
"	"	7.24-7.33	105 ± 2	179 ± 3	168 ± 3
"	"	7.35-7.40	125 ± 3	196 ± 5	181 ± 5
"	"	7.39-7.42	120 ± 3	185 ± 5	175 ± 5
"	"	7.72-7.79	143 ± 3	186 ± 4	181 ± 4
"	"	7.72-7.80	142 ± 3	185 ± 4	180 ± 4
"	"	7.79-7.81	138 ± 2	177 ± 3	172 ± 3
"	"	7.84-7.94	146 ± 3	184 ± 4	181 ± 4
"	"	7.87-7.94	140 ± 3	177 ± 4	175 ± 4

Table 3 cont'd.

Temp. °C.	Buffer	pH Range	K_{obs} equ. (31)	K' equ. (44)	K equ. (32)
25.0	Borate	7.99-8.02	140 ± 2	175 ± 3	173 ± 3
"	"	8.14-8.19	137 ± 2	172 ± 3	170 ± 3
"	"	8.37-8.39	135 ± 2	178 ± 3	178 ± 3
"	"	8.60-8.61	113 ± 2	165 ± 3	165 ± 3
"	"	8.80-8.80	109 ± 2	184 ± 5	184 ± 5
"	"	9.01-9.02	87 ± 3	180 ± 6	180 ± 6
35.0	Phosphate	5.70-5.75	16.5 ± .5	266 ± 8	183 ± 6
"	"	5.85-5.90	23 ± 3	269 ± 35	191 ± 25
"	"	6.06-6.10	26 ± 1	202 ± 8	150 ± 6
"	"	6.28-6.32	40.0 ± .5	204 ± 3	159 ± 2
"	"	6.91-6.93	90 ± 2	180 ± 4	162 ± 4
"	"	7.50-7.50	123 ± 2	164 ± 3	159 ± 3
"	Borate	7.65-7.67	129 ± 2	163 ± 3	160 ± 3
"	"	7.67-7.77	130 ± 2	161 ± 2	158 ± 2
"	"	7.88-7.95	129 ± 2	159 ± 2	157 ± 2
"	"	8.09-8.14	127 ± 3	161 ± 4	159 ± 4
"	"	8.32-8.37	117 ± 1	163 ± 1	163 ± 1
"	"	8.54-8.57	103 ± 2	163 ± 3	163 ± 3
"	"	8.71-8.76	85 ± 2	156 ± 4	156 ± 4

Calculation of the Equilibrium Constant K

Equation 31 gives the theoretical dependence of the measured equilibrium constant K_{obs} (defined in equation 6) on the two pH functions: L (heme-linked ionizations, equation 29) and F (the other ionizations, equation 20). The pH independent formation constant K for the ferrimyoglobin imidazole complex (first defined in equation 5) can now be calculated using equation 32.

Analysis of the data was carried out in two stages:

(i) Expressing the effect of non-heme-linked ionizations on K_{obs} as

$$K' = K_{obs}\,(1/F) \qquad (44)$$

K' was calculated using the measured K_{obs} values and the appropriate values of K_1, K_{Fe} and K_3. A summary of the latter is given in Table 4, which also gives their references.

Table 4

Ionization Constants at I = 0.10M Used in Analysis of Results (equation 20)

Temp.	pK_1	pK_{Fe}	pK_3
14.0°	7.32	9.16	10.7
20.0°	7.19	9.05	10.5
25.0°	7.10	8.96	10.4
35.0°	6.90	8.79	10.1
Ref.	(37)	(32)	(29)

The calculated values of K' are listed in Table 3 and the 25° results are shown in Figure 8. From these it can be seen that K' is

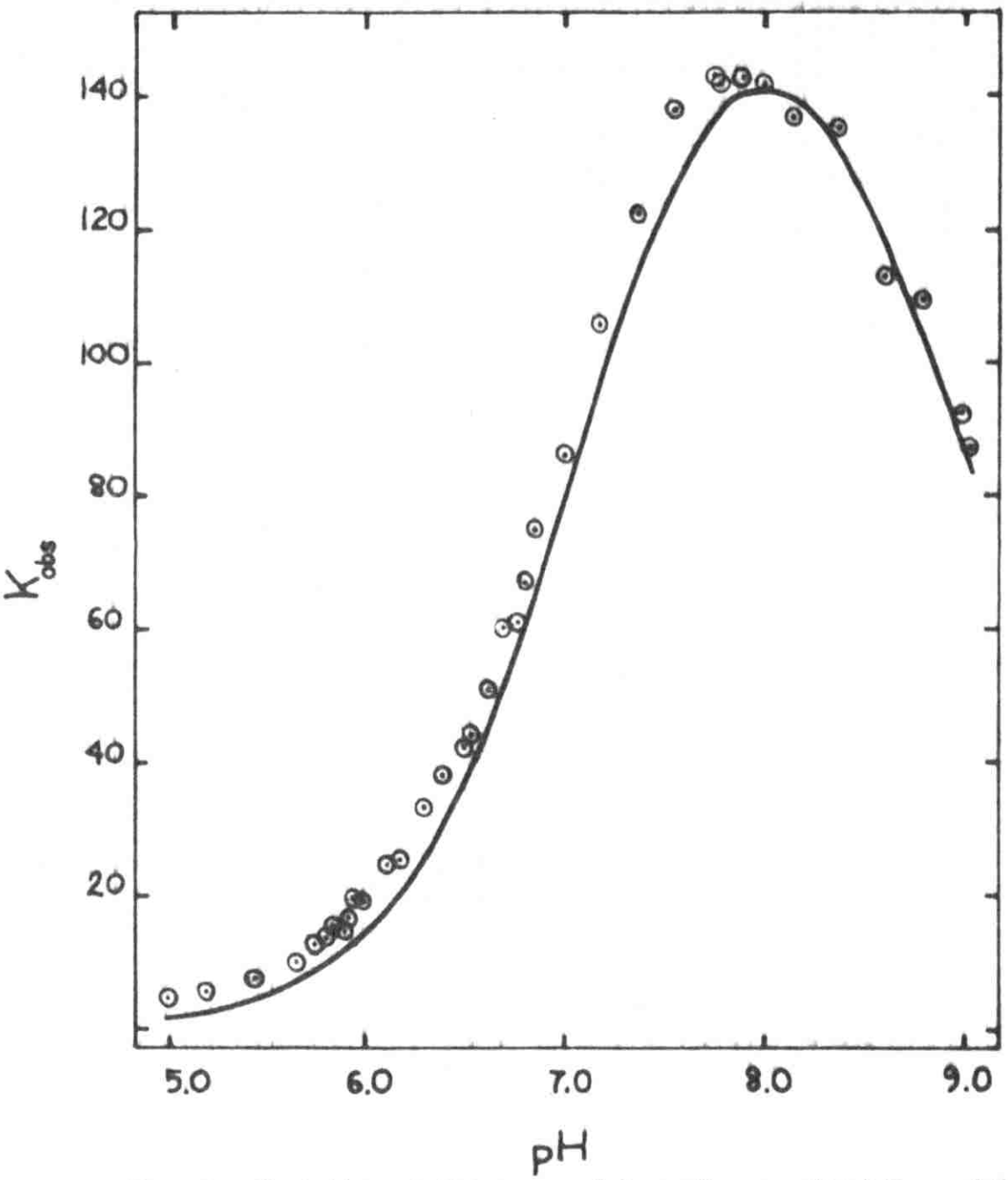


Fig. 7 - Variation of the measured formation constant K_{obs} with pH for the reaction of sperm whale ferrimyoglobin with imidazole (25.0°, I 0.10M). Data from Table 3 without limits of uncertainty. Curve shows theoretical variation of K^{5}_{obs} with pH calculated from equation 20 which does not include heme-linked effects. K = 175 (other contents in Table 4). Position of experimental points indicates pKp $>$ pKr (Figure 4).

approximately constant from pH 9 down to about pH 7, but that it rises sharply at pH $<$ 7 to more than twice the constant value at pH $>$ 8. It may be concluded on this basis that heme-linked effects cannot be ignored in the region of pH $<$ 7.

(ii) The combined effect of non-heme-linked ionizations (F factor) and that of heme-linked ionizations (L factor) is given in equation 32 which can be also written in the form

$$K = K_{obs}.(1/F).(1/L)$$
$$= K'.(1/L) \qquad (45)$$

In this case, the values of L (equation 29) have to be obtained from the best fit of data.

An approximate idea of the values of pK_r and pK_p (equation 30) can be formed with reference to Figure 4. In that theoretical plot, the function FxL is seen to be shifted with respect to the plot of F, the shift depending on the relative magnitudes of pK_r and pK_p. In Figure 7, K_{obs} values at 25° are plotted against pH together with the corresponding F function; the asymmetry in the curve is seen to be of the typefor F.L curve in Figure 4 with $pK_p > pK_r$. Furthermore, the difference $pK_p - pK_r$ could not be much different from 0.2.

On the above basis, an analysis of the data was carried out trying first various ΔpK values (0.3, 0.2, 0.1). Concordant results were obtained with $\Delta pK = 0.2$. Subsequently, several alternative combinations of pK_r and pK_p were attempted, all with $\Delta pK = 0.2$. A sample calculation for the 25.0° data is shown in Table 5, from which it can be seen that the set of K values computed on the basis of pK_r 6.4 ---> pK_p 6.6 yields a mean value which is in closest agreement

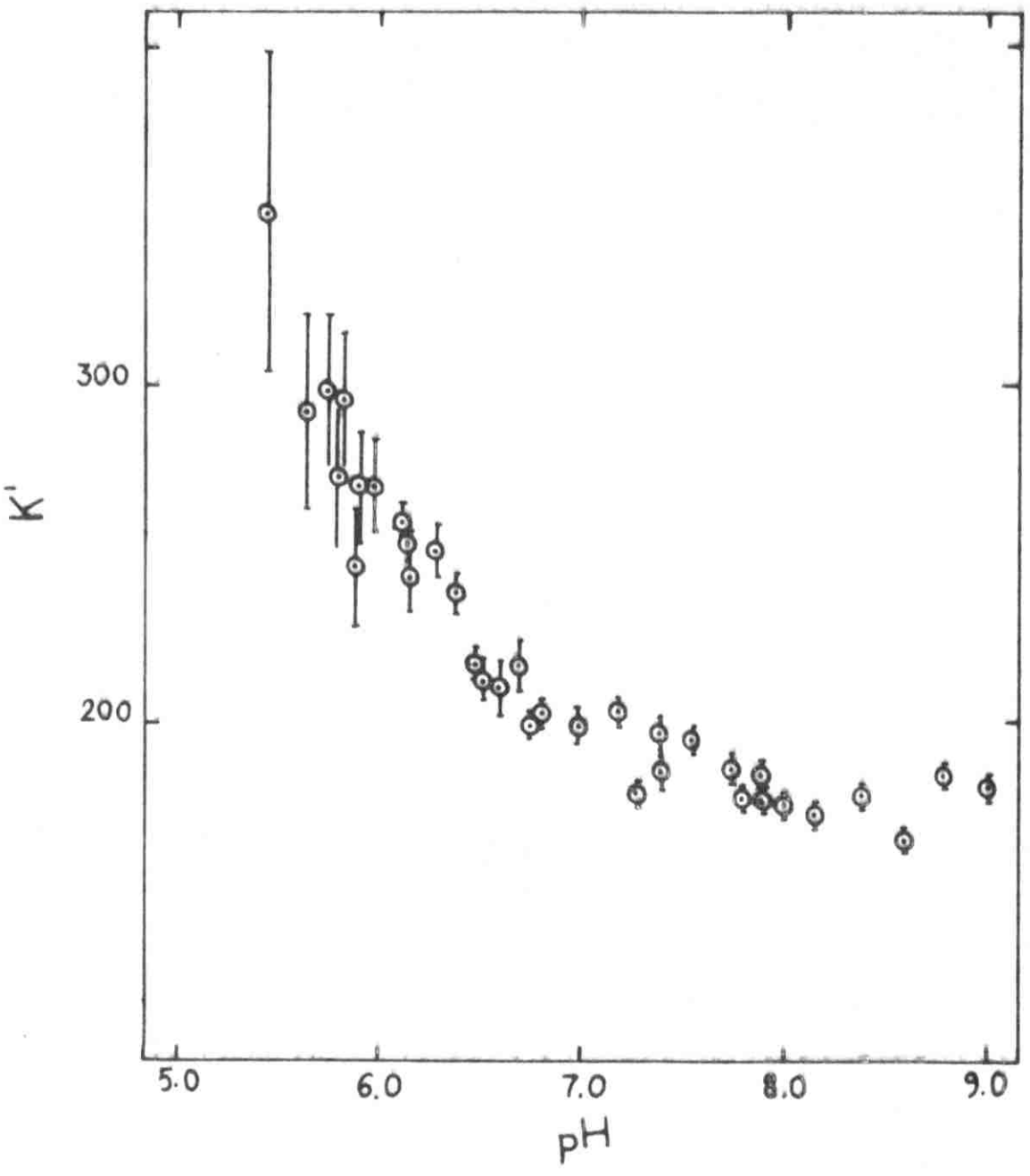


Fig. 8 - Equilibrium constant k', calculated from equation 44. Variation with pH indicates a residual effect of heme-linked interactions. Data at 25.0° and I = 0.10M taken from Table 3. Factor F includes the effect of all non heme-linked ionisations (equation 20) constants are given in Table 4.

Table 5

Analysis of data in terms of single heme-linked ionizations on reactant and product. Several pK_r ---> pK_p values are shown. Data from Table 3; T = 25.0°, I = 0.10M. Mean value from pH range 8 - 9, where K is independent of heme-linked ionizations, is 175 ± 5. Analysis shows that best fit is given by pK_r 6.4 --> pK_p 6.6.

			K				
pH	K_{obs}	K'	6.2 → 6.4	6.3 → 6.5	6.4 → 6.6	6.5 → 6.7	6.6 → 6.8
6.12	24.5	259	195	190	186	183	179
6.14	25.0	253	192	187	183	180	175
6.16	25.0	243	185	180	176	174	169
6.38	38.0	238	192	186	182	179	173
6.48	42	217	180	175	171	168	162
6.52	44	212	178	173	169	166	160
6.61	51	210	179	174	171	167	161
6.75	61	199	175	170	168	164	158
6.80	67	202	179	175	172	169	163
6.99	86	199	183	179	178	175	168
Mean			184 ± 6	179 ± 6	175 ± 5	172 ± 6	167 ± 6

with the mean K value 175 ± 5 obtained from the pH > 8 region (where heme-linked ionizations are not involved). However, the value 175 ± 5 is seen to cover within its limits also the values obtained from the analysis at 6.3 --> 6.5 as well as 6.5 --> 6.7. Accordingly, the result of the analysis at 25.0° must be taken as

$$pK_r\ (6.4 \pm 0.1) \text{ -----} \rightarrow \ pK_p\ (6.6 \pm 0.1)$$

Following the same detailed procedure, this type of analysis was repeated on the data at the other temperatures. The results of these calculations are summarized in Table 6. The values of the equilibrium constant K for the pH independent reaction (equation 3) have been calculated on the basis of equation 32 using the data on pK_r and pK_p,

Table 6

Ionization constants for the heme-linked group in sperm whale ferrimyoglobin (pK_r, equation 22) and its imidazole complex (pK_p, equation 26) obtained by analysis of the pH dependence of K_{obs} (equation 31). Details as in text. All pK values are uncertain within ± 0.1 unit.

T (°C.)	pK_r	pK_p
14.0	6.5	6.7
25.0	6.4	6.6
35.0	6.3	6.5

and are listed in the last column of Table 3. The values at 25.0° have also been plotted in Figure 9, where it is seen that K is constant over the entire pH range 6 to 9, but that at pH < 6 it assumes an abrupt rise,

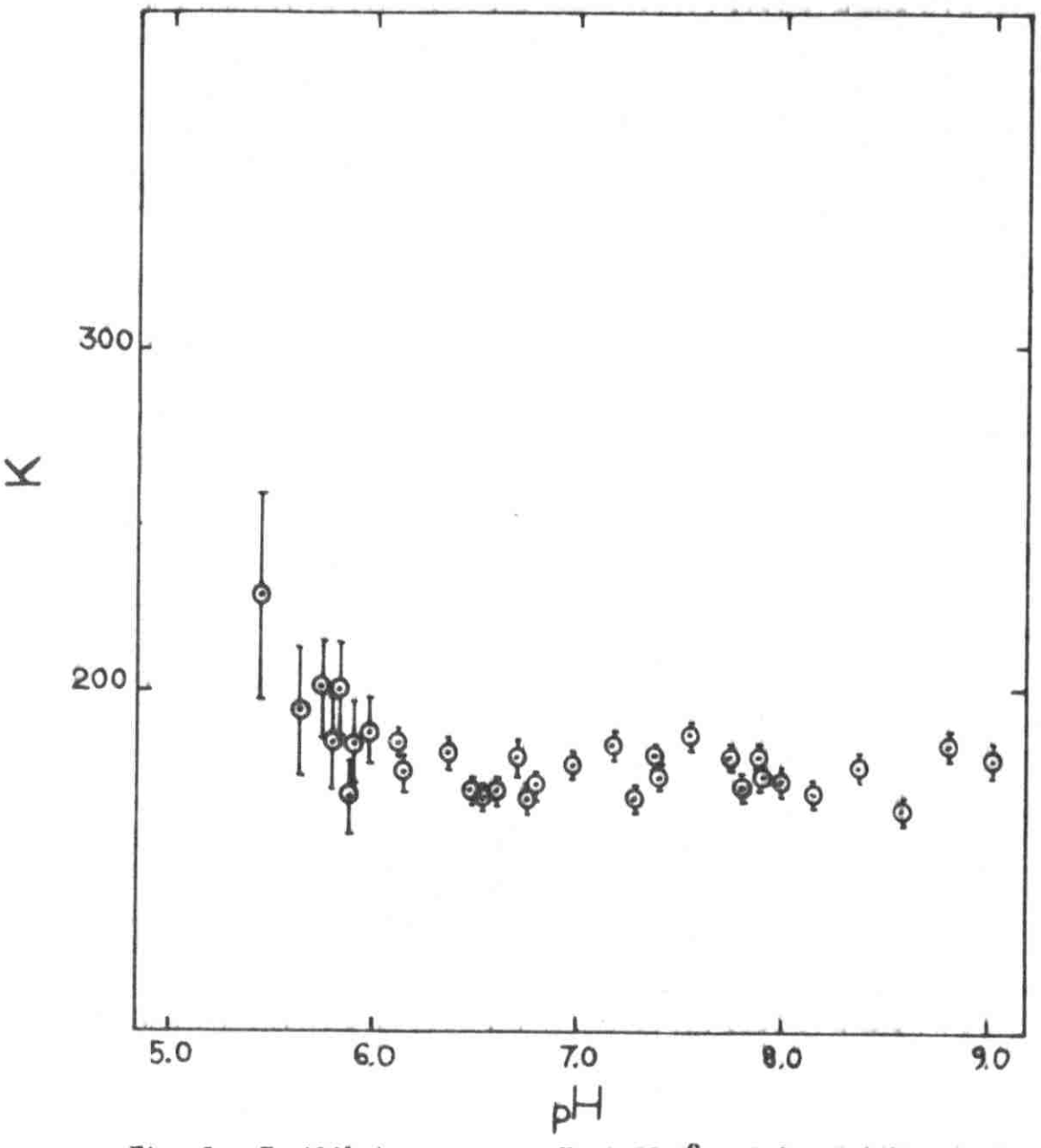


Fig. 9 - Equilibrium constant K at 25.0° and I = 0.10M calculated from equation 32, using values for the measured formation constant K_{obs} (Table 3) and the various ionization constants (Table 4) including heme-linked ionizations pKr 6.4, pKp 6.6. Plot shows constancy of K over the pH range 6 to 9. Region pH $<$ 6 is not accounted for.

thus suggesting that in more acidic solution other prototropic effects are involved. A possible explanation of the latter phenomenon is considered in the Discussion.

Calculation of Thermodynamic Quantities

Since the reaction (equation 3) involves the replacement of neutral H_2O by neutral imidazole molecule, it may be assumed that the equilibrium constant is approximately independent of ionic strength up to 0.10M. Hence for 25.0°

$$K^0 \simeq K = 175 \pm 5$$

and so,

$$\Delta F^0 = -RT\ln K^0 = -3.06 \pm 0.02 \text{ Kcalmole}^{-1}$$

The corresponding values of K^0 at the other temperatures are: 192 ± 4 (14.0°); 183 ± 3 (20.0°); 157 ± 3 (35.0°). A plot of log K vs. $1/T$ (°K) is linear yielding $\Delta H^0 = -2.20 \pm 0.70$ (equation 38). Hence at 25.0°

$\Delta S^0 = +2.9 \pm 2.4$.

A summary of the thermodynamic data is given in Table 7, the last column of which shows the enthalpy change of the heme-linked ionizations.

Table 7

T	K^0	ΔF^0	ΔH^0	ΔS^0 (25°)	ΔH (I = 0.10M) pK_r	pK_p
14.0	192±4	-3.00±0.01				
20.0	183±3	-3.04±0.01	-2.20±0.70		3.5±3	3.5±3
25.0	175±5	-3.06±0.02		+2.9±2.4		
35.0	157±3	-31.0±0.01				

DISCUSSION

As the present work is a quantitative study of a reversible chemical reaction and its allied acid-base equilibria, it is necessary to establish the degree of reliability of the results obtained.

In the first place, it may be concluded from the above results that the overall 1 to 1 stoichiometry of the reaction is well established. By thermodynamic criteria, the experimental ranges of protein concentration (1 to 60 x 10^{-6}M) and of imidazole concentration (1 to 20 x 10^{-3}M) are adequate for testing the stoichiometry. The constancy of the measured equilibrium constant K_{obs} (at given pH, ionic strength and temperature) precludes the involvement of polymerization equilibria by the protein, a complication which may conceivably be encountered in more concentrated protein solutions or in the analogous reaction of ferrihemoglobin.

Although the reaction has been shown to be simple, there are two main factors which limit the precision of the results: the relatively small magnitude of K_{obs} (the free energy of reaction), and the presence of several overlapping acid-base equilibria involving both reactants as well as the product, the complex.

Because of the small magnitude of K_{obs}, relatively large concentrations of imidazole had to be used in the equilibrium mixtures (approxi-

mately 1000 times the protein concentration). This in turn had two effects: (a) it limited the range of experimental measurements to pH $>$ 5 because of the tendency of the protein to denature by the combined effect of high imidazole and H^+ concentrations; (b) it imposed a serious limitation on the control of ionic strength, which could not be made less than 0.10M. This was especially the case at pH $<$ 6, where there are appreciable contributions from the imidazolinium cation and its counter ions (see Experimental section IIIB). Accordingly, it was not possible to carry out the measurements at a series of progressively lower ionic strength with a view to obtaining thermodynamic constant K (equation 5) by proper extrapolation. However, since the reaction involves replacement of H_2O by a neutral ligand in dilute solutions, it was assumed in the calculations that the data at I = 0.10M give, to a first approximation, the thermodynamic quantities corresponding to zero ionic strength.

It can be seen with reference to the experimental procedure that K_{obs} is obtained with an uncertainty of about 2% at pH $>$ 6, and that the errors increase progressively in the more acidic solutions reaching about 20% at pH 5. The reason for this is again the small value of K_{obs} which makes it impossible to cover more than a small span of the extent of reaction.

The main complication in the analysis of results arises from the pH variation of K_{obs}. As was shown above (Theory), at least two types of pH effects are involved. One group comprises the ionizations of the iron-bound water in ferrimyoglobin (equation 7, $pK_{Fe} \sim 9$), that of imidazole (equation 12, $pK_1 \sim 7$), and that of the imino nitrogen of the

ligand in the complex (equation 16 $pK_3 \sim 10.4$). Another group includes "heme-linked" ionizations from acidic groups in the protein, the chemical nature of which is uncertain.

When the first group of ionizations was applied in analysis of the data on the observed equilibrium constant, the resulting K' values (equation 44) were constant only at pH $>$ 8 (Table 3, Figure 8). This shows that the effect of the second group of ionizations is negligible in this region where K' equals K, the true equilibrium constant (equation 5).

Using the value of K thus obtained as a guide for the analysis in the region pH $<$ 8, and assuming only one heme-linked ionization in reactant (K_r) and in product (K_p), it was also shown (Table 3, Figure 9) that the best fit of the 25° data could be obtained with pK_r 6.4 ---→ pK_p 6.6. Thus it was possible to account adequately for the residual pH effect over the entire pH range 6 to 9. However, the resulting uncertainty of $\pm$ 0.1 in pK values reflects 25% maximum error in the derived ionization constants for this postulated heme-linked group.

Figure 9 also shows that the region pH $<$ 6 remains unaccounted for, with K increasing rapidly at lower pH. By extending the above method of analysis, it can be shown that even if a second set of heme-linked ionizations is assumed to be operative in this region, it is still not possible to fit the data into a constant value for the equilibrium constant at pH $<$ 6. It may thus be concluded that in these acidic solutions multiple prototropic equilibria and / or conformational changes in the protein are responsible for this trend.

Another way of illustrating the effect of pH on this equilibrium is

to examine the enthalpy of reaction as a function of pH.

The apparent $\triangle H$ at any given pH is obtained from the experimental data at the three temperatures listed in Table 3. This has been done at half pH units and the values of $\triangle H$ are plotted in Figure 10 over the range 6 to 9 (with the corresponding data[29] at I = 0.20M also shown for pH 10 - 11). The curve in Figure 10 gives the theoretical variation of $\triangle H$ with pH. The latter is obtained by summing contributions to $\triangle H$ from each of the three known ionizations (pK_1 = 7.10, $\triangle H$ = 7.7 Kcal-mole^{-1}; pK_{Fe} = 8.96, $\triangle H$ = 7.2 Kcal-mole^{-1}; pK_3 = 10.4, $\triangle H$ = 10.5 Kcal-mole^{-1}) at 25^0 and given pH, and neglecting the minor contribution from the heme-linked ionizations. The curve is made to pass through the experimental point at pH 8.0, viz. $\triangle H$ = -1.7 Kcal-mole^{-1}. The curve represents the theoretical variation of $\triangle H$ of reaction with pH relative to the enthalpy of reaction at pH 8.0. Thus, with increase in pH, the reaction becomes more exothermic as a result of the successive ionizations on the two reactants, but the trend is reversed by the opposite effect of the strongly endothermic ionization on the product at higher pH. The general agreement between the experimental points and the curve further supports the hypothesis concerning the three ionizations involved.

The results obtained in the present study with sperm whale ferrimyoglobin may be compared with work which has been carried out on analogous systems.

Abu-Isa[28] made a comparative study of the reaction of imidazole with several hemoproteins (horse ferrimyoglobin, horse ferrihemoglobin and whale ferrimyoglobin) and observed minor species differences but an overall pattern for the pH profile which is similar to the one obtained

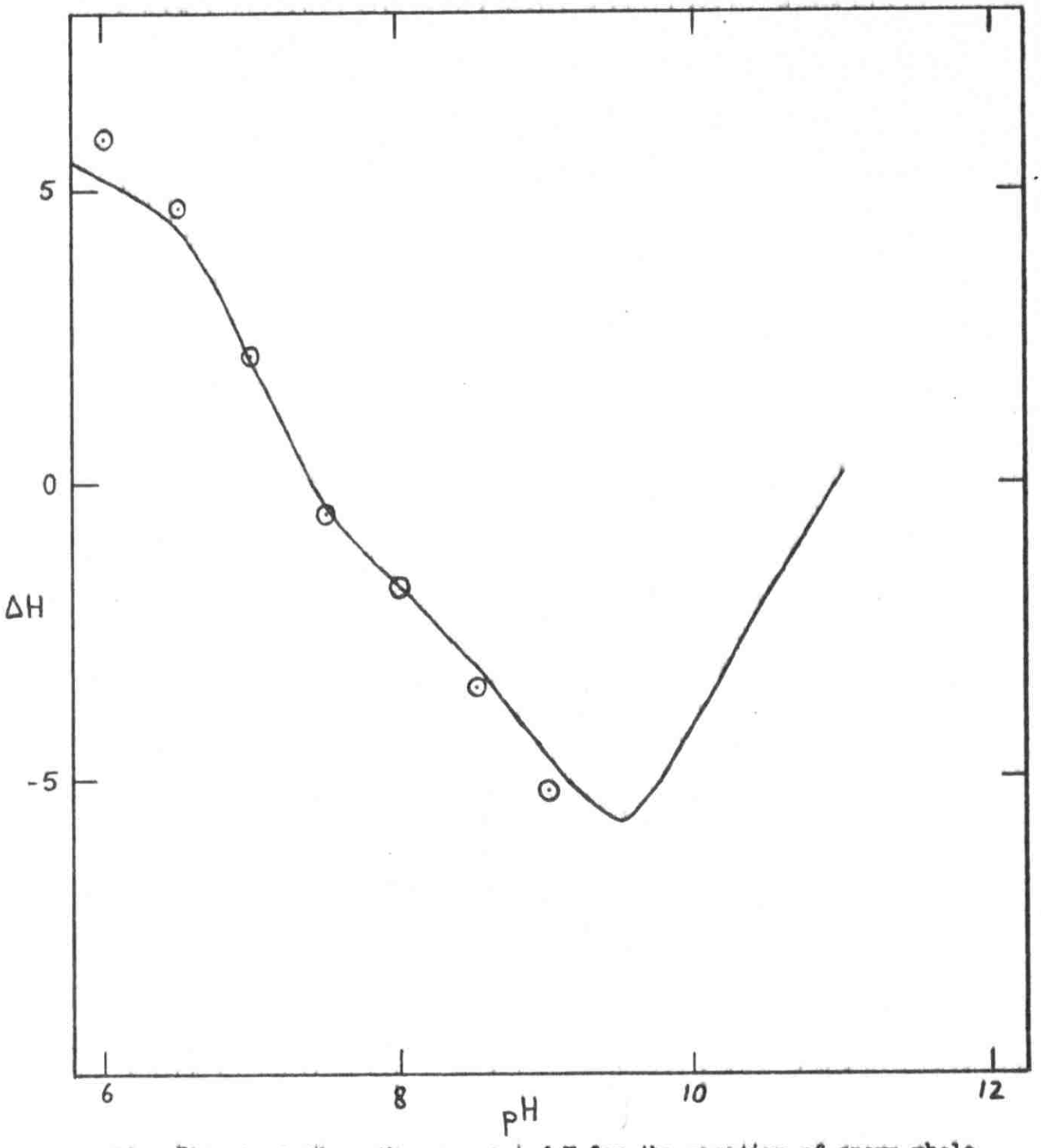


Fig. 10 - Effect of pH on the apparent ΔH for the reaction of sperm whale ferrimyoglobin with imidazole at I = 0.10M. Circles indicate ΔH values obtained from the experimental data (Table 3, 25.0° to 35.0°). Theoretical curve obtained by summing contributions from the known non heme-linked ionizations (see Text).

in the present work. The early results, however, were not extensive enough for a thorough mathematical analysis. Later measurements on sperm whale ferrimyoglobin[29] at I = 0.20M, in the pH region 10 to 12, enabled the calculation of more reliable data which may be compared with the present results (Table 8). The parallelism is evident, although the difference in the enthalpy of reaction seems to exceed the limits of uncertainty.

Scheler[38] has made a study of the corresponding reaction of imidazole with ferrihemoglobin from horse blood. His results again confirm the general pH profile and the order of magnitude of equilibrium data, although in this case the equilibrium constant is twice as large as for myoglobin. Unfortunately, however, the data are not extensive enough for a test of the heme-linked hypothesis in the Bohr region (pH 6 - 7). Clearly, the need remains for more extensive and precise thermodynamic data on such systems.

Table 8

Comparison of thermodynamic data on imidazole complexes of ferrimyoglobin and ferrihemoglobin. K at 25°(equation 5)

References	Species	Experimental Conditions	K (M^{-1})	ΔH (Kcalmole^{-1})	ΔS (e.u.)
(29)	sperm whale ferrimyoglobin	I 0.2M pH 10-12	160±10	-4.0±0.6	-3±2
(this work)	" "	I 0.10M pH 5-9	175±5	-2.2±0.7	+3±2.5
(38)	horse ferrihemoglobin	I 0.02M pH 8.0	330	-4.3	-3

Two questions concerning the reaction may be asked at this stage:

1. Is the "heme-linked" effect electrostatic or electronic in nature?

2. Can the ionizing group which is responsible for the effect be identified with imidazole?

Regarding the nature of the "heme-linked" effect, several lines of evidence seem to favor an essentially electrostatic picture. The results obtained by George and Hanania[21], Hanania and Irvine,[22] Beetlestone and Irvine,[24] Awad and Badro[25] among others, all indicate the importance of electrostatic charge effects (see Introduction). Furthermore, recent work[39] has shown that the "heme-linked" effect in the ferrimyoglobin-imidazole reaction is considerably weakened in the presence of high salt concentrations. This again suggests an electrostatic phenomenon.

On the above basis, it may be concluded that this "heme-linked" effect cannot be attributed to the heme-linked imidazole group of histidine which binds the heme to the protein at the fifth coordinating position in both myoglobin and hemoglobin. This conclusion is also in accord with the evidence from the thermodynamics of ionizations involved. For, the imino =NH group of the heme-linked imidazole is known to be hydrogen bonded to a carbonyl group in the polypeptide chain[1], and this is expected to weaken the acidity and increase the endothermicity of ionization to well beyond the experimental pK 6.4 and ΔH 3.5 Kcal-mole^{-1}.

Since the effect of coordination to the hematin iron on the ionization of neighbouring acidic groups can vary depending on the nature of the linkage, no definite conclusions can be made regarding the chemical identity of such groups. There are, however, three possible situations which may explain such an effect:

1. The imidazolinium group of a histidine residue other than the one directly linked to iron, but still in the environment of the heme.

2. A salt-like ion-pair of the carboxyl-amino type which reversibly forms a cation as a result of protonation of the carboxyl group or its equivalent.

3. Non-specific electrostatic interaction between the iron atom and the charged groups on the protein surface.

It is clear from the above discussion that more extensive work and different approaches are needed before conclusive results can be drawn. Nevertheless, it is interesting that despite all these difficulties reliable thermodynamic data were obtained for such systems for comparative study of various metal complexes and hemoproteins.

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