AMERICAN UNIVERSITY OF BEIRUT

Thesis Title:

EQUILIBRIUM STUDY OF THE REACTION OF FERRIMYOGLOBIN WITH CYANIDE

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FERRIMYOGLOBIN WITH CYANIDE

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EMILE T. NAKHLEH

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Using sperm whale ferrimyoglobin IV (Awad 1968), the equilibrium constant for this reaction was measured by a spectrophotometric method under the following conditions: ferrimyoglobin concentration from 0.80 to 1.30 x 10^{-6} M; cyanide concentration from 0.50 to 5.0 x 10^{-6} M; total ionic strength I = 0.010 M and 0.100 M; temperatures 15.0°, 25.0°, and 35.0°C; pH range 6 to 8 (phosphate buffer), 7 to 9 (TRIS buffer) and 8 to 10 (borate buffer). The results show the reaction to be highly favored, with K~10^{8}, and also markedly dependent on pH and sensitive to ionic strength.

Analysis of data, with respect to theoretical curves obtained from the known ionization parameters of reactant ferrimyoglobin and ligand cyanide, confirmed the expected pH profile; but in addition several other pH effects were observed. Of these, a major perturbation appears in the range of pH 8 to 9, at I = 0.010 M, but the effect is considerably diminished at I = 0.100 M, and is

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Analysis of data, with respect to theoretical curves obtained from the known ionization parameters of reactant ferrimyoglobin and ligand cyanide, confirmed the expected pH profile; but in addition several other pH effects were observed. Of these, a major perturbation appears in the range of pH 8 to 9, at I = 0.010 M, but the effect is considerably diminished at I = 0.100 M, and is

therefore probably a general electrostatic effect of the heme environment.

The data were also analyzed after incorporation of the effect of the two known ionizations. The resulting values of the derived pH-independent equilibrium constant again showed the additional pH effects. So did the corresponding enthalpy of reaction, which was found to vary with pH in a complicated manner. Two main features of this variation were noted :
(1) the exothermicity of the reaction rises to a maximum (- 18 kcal/mole) at pH 8 which is near the isoionic point of the protein; (2) the exothermicity of the reaction drops to about - 4 kcal/mole at pH > 9, while the corresponding entropy of reaction rises from - 26 e.u. at pH 8 to + 20 at pH > 9. The free energy of reaction remains substantially constant. These features are probably interrelated, and may be attributed to general electrostatic effects, the protein being assumed to undergo a major conformational transition between pH 8 and 9, with concomitant changes in charge distribution and hydration on the protein surface.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I N T R O D U C T I O N

This work is a preliminary equilibrium study of some aspects of the reaction between ferrimyoglobin and cyanide. In particular, the influence of two ionizations and of other charge effects is investigated, with reference to the general problem of heme-linked effects in myoglobin.

Physiologically, ferromyoglobin is important as the muscle pigment responsible for oxygen storage. However, due to its rapid autoxidation into its stable iron (III) derivative, ferrimyoglobin, the latter is more conveniently used in structure and reactivity studies even though it has no physiological significance. From the point of view of the present work, there are two main reasons for this special interest in ferrimyoglobin studies. In the first place, the structure of the molecule has been established in detail (Kendrew et al, 1961), and its physical and chemical properties are being extensively studied by a variety of modern techniques (Chance et al, 1966). Secondly, the myoglobin molecule has a single site at the iron atom available for chemical bonding, and its derivatives have sharp and characteristic absorption spectra. These properties enable one to make very precise and detailed physico-chemical measurements, particularly the kinetic and equilibrium study of ligand bonding reactions.

Myoglobin Structure;

Myoglobin is a hemoprotein, with molecular weight about 18,000. It contains the iron-protoporphyrin-IX complex in conjugation with a protein, globin. Details of its crystal structure are well known (Kendrew et al, 1961; and later papers); only some aspects are noted here in so far as they relate to the present work.

The heme (protoporphyrin IX) is a tetrapyrrolic ring with an iron at the center and coordinated to the four nitrogens. The ring system is nearly planar, the iron atom being displaced out slightly towards the epsilon nitrogen of histidine E7 on the globin (Figure 1). The fifth coordination site of the iron atom is occupied by a nitrogen of the imidazole of the histidine residue F8. The sixth coordination site is normally occupied by a water molcule which is presumably held by weak ion-dipole forces, and is readily displaced by ligands. The heme has a polar end consisting of two propionic acid groups which lie outside the molecule (one is linked to Argenine CD3, and the other possibly to histidine FG3 (Breslow, 1962). The non-polar end consists of vinyl and methyl groups which are buried within the molecule.

Myoglobin's protein, globin, consists of a single polypeptide chain of 153 amino acids (Figure 1), which starts with an N-terminal valine and ends with the carboxylic acid glycine. Table I lists the amino acids of the globin chain in myoglobin together with side-chain functional groups and their ionization constants, and the number of units (residues) of each amino acid. The secondary structure of

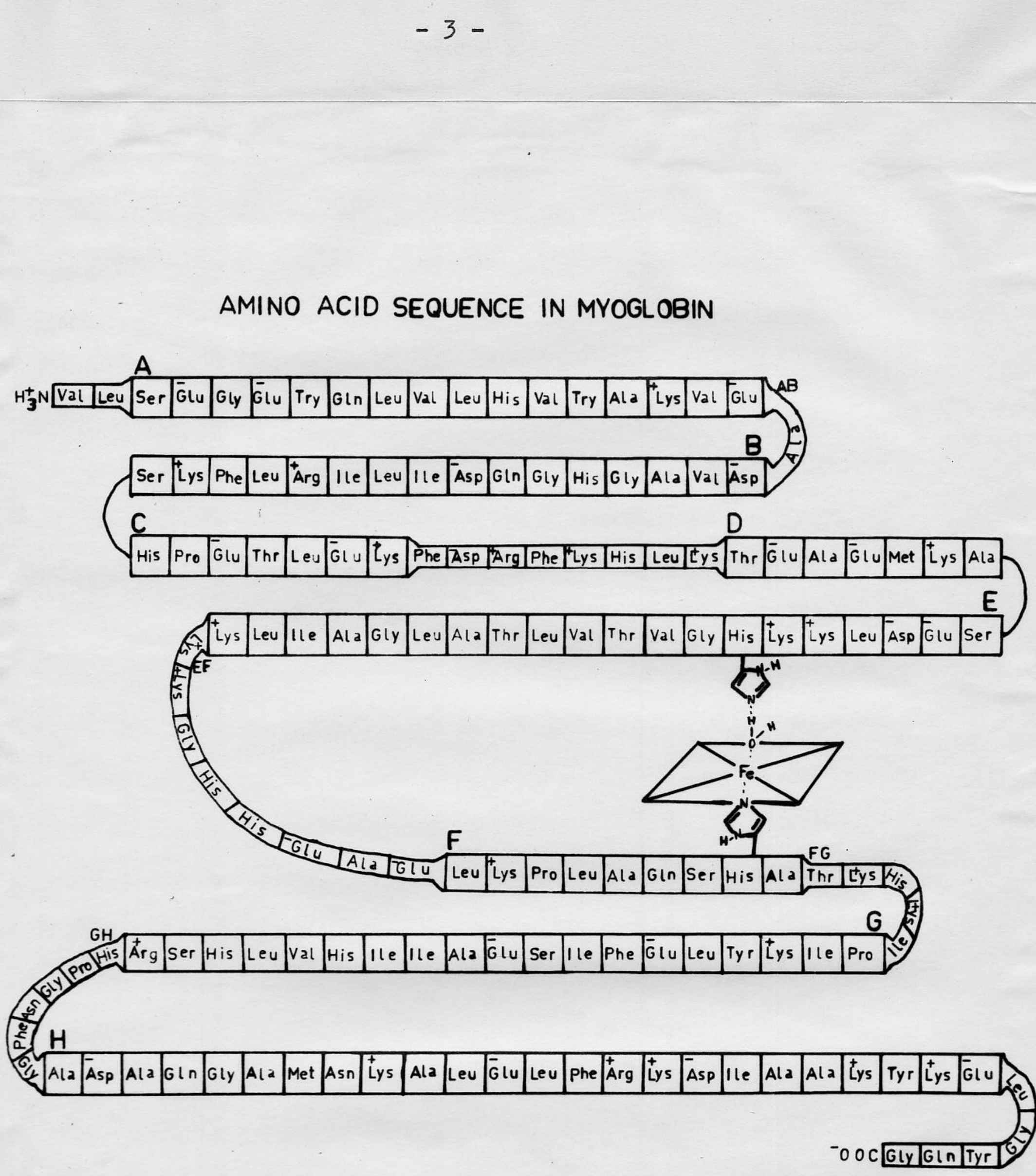


Figure 1 Sperm whale ferrimyoglobin IV, amino acid sequence.

(Edmundson, 1965)

Table I

Amino Acid Composition of Sperm Whale Ferrimyoglobin IV

Amino Acid	Abbreviation	Number of Residues[a]	Acidic Functional group	pK^b
Aspartic	Asp	6	β -COOH	4.6
Asparagine	Asn	2		
Glutamic	Glu	14	δ -COOH	4.6
Glutamine	Gln	5		
Serine	Ser	6	-OH	
Threonine	Thr	5	-OH	
Methionine	Met	2		
Proline	Pro	4		
Glycine	Gly	11		
Alanine	Ala	17		
Valine	Val	8		
Leucine	Leu	18		
Isoleucine	Ile	9		
Phenylalanine	Phe	6		
Tyrosine	Tyr	3	-OH	9.8-10.4[c]
Tryptophan	Try	2		
Histidine	His	12	imidazole	5.6-7.4[c]
Lysine	Lys	19	ϵ -NH_2	10.2
Argenine	Arg	4	guanidyl	>12.0
Total		153		

a) Edmundson (1965)

b) Tanford (1965), pK values expected from data on small molecules.

c) Cohn and Edsall (1943)

myoglobin is formed mainly through hydrogen bonding and consists of eight right handed α-helices with non-helical segments between them. The polypeptide chain is folded in a complex and unsymmetrical manner forming a flattened, roughly triangular prism about 45 x 35 x 25 Å. The whole structure is compact; there are practically no channels and the volume of the internal space is small. The heme group is disposed almost normally to the surface of the molecule, and the edge which contains the propionic acid groups is at the surface, and the rest buried deeply within. This structure gives rise to interactions of different types between the heme and the globin, and these interactions determine to a large extent the properties of the protein as a whole. In particular, hydrophobic forces between the heme and the aliphatic and aromatic side chains of the amino residues (around 90 van der Waals' contacts) account for the stability of the molecule (Kendrew, 1962). In addition, two propionic carboxylic groups form hydrogen bonds between the heme and the protein, one of which is with the nitrogen atom of the guanidinium group of argenine CD3. However, these hydrogen bonds are probably not important in stabilizing the molecule, since in other proteins the corresponding amino acids often have hydrocarbon side chain (Watson, 1966).

X-ray and amino acid sequence studies reveal the presence on the surface of the molecule of most of the amino acids with ionizable functional groups in their side chain. Acid-base titration curves and studies on reactivity towards certain reagents (Breslow and Gurd,1962) show that these groups are responsible for charge distribution on the

surface of the molecule. However, certain groups are not free, being involved internally in hydrogen or chemical bonding, or otherwise involved in molecular conformations which sterically hinder interaction of solvent with the group. Specifically, of the twelve histidine residues only six are free, and only two of the three tyrosines react similarly (Breslow and Gurd, 1962). Sperm whale ferrimyoglobin is a positively charged protein with isoionic point at pH 8.07 (Sabri,1968).

The Cyanide Reaction

Although sperm whale ferrimyoglobin has been available for only two decades, the cyanide reaction of other species of myoglobin and hemoglobin was studied much earlier. In fact ferrihemoglobin cyanide was discovered by Haldene in 1899 and was first crystallized by von Zeynek in 1901. Lemberg and Legge (1949) have reviewed the early history of the reaction.

The main characteristics of the cyanide complex of any hemoprotein are its red color and low magnetic moment (2.2 B.M. as compared with 5.9 for F^- and 5.8 for H_2O complexes of ferrimyoglobin). The absorption spectrum develops a wide characteristic band in the visible at 540 nm. Figures 2&3 shows pen recordings of the absorption spectra of ferrimyoglobin: acidic, alkaline, and cyanide. The relation between nuclear, electronic, optical and magnetic properties of ferrimyoglobin derivatives is being explored in increasing detail (Gouterman and Zerner,1966; Williams,1966).

It is generally agreed, on theoretical and experimental grounds, that bonding of cyanide to the iron occurs through its carbon not nitrogen atom.

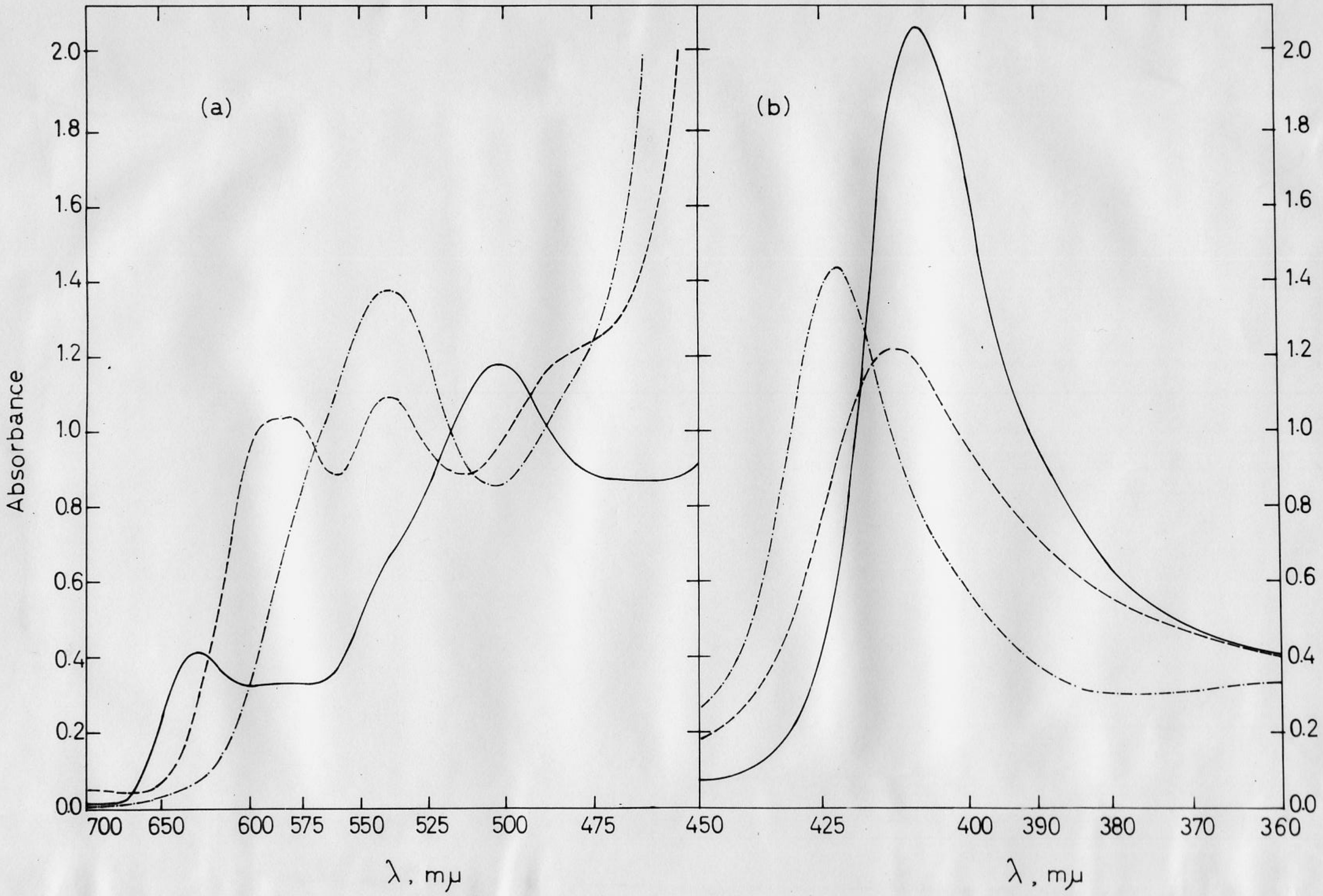


Figure 2 - Absorption spectra of acidic (—————) and alkaline (- - - - - -)
sperm whale ferrimyoglobin IV and cyanide complex (.-.-.-.-.-.-):
(a) visible region, concentration = 1.16×10^{-4}M.
(b) Soret region, concentration = 1.16×10^{-5}M.

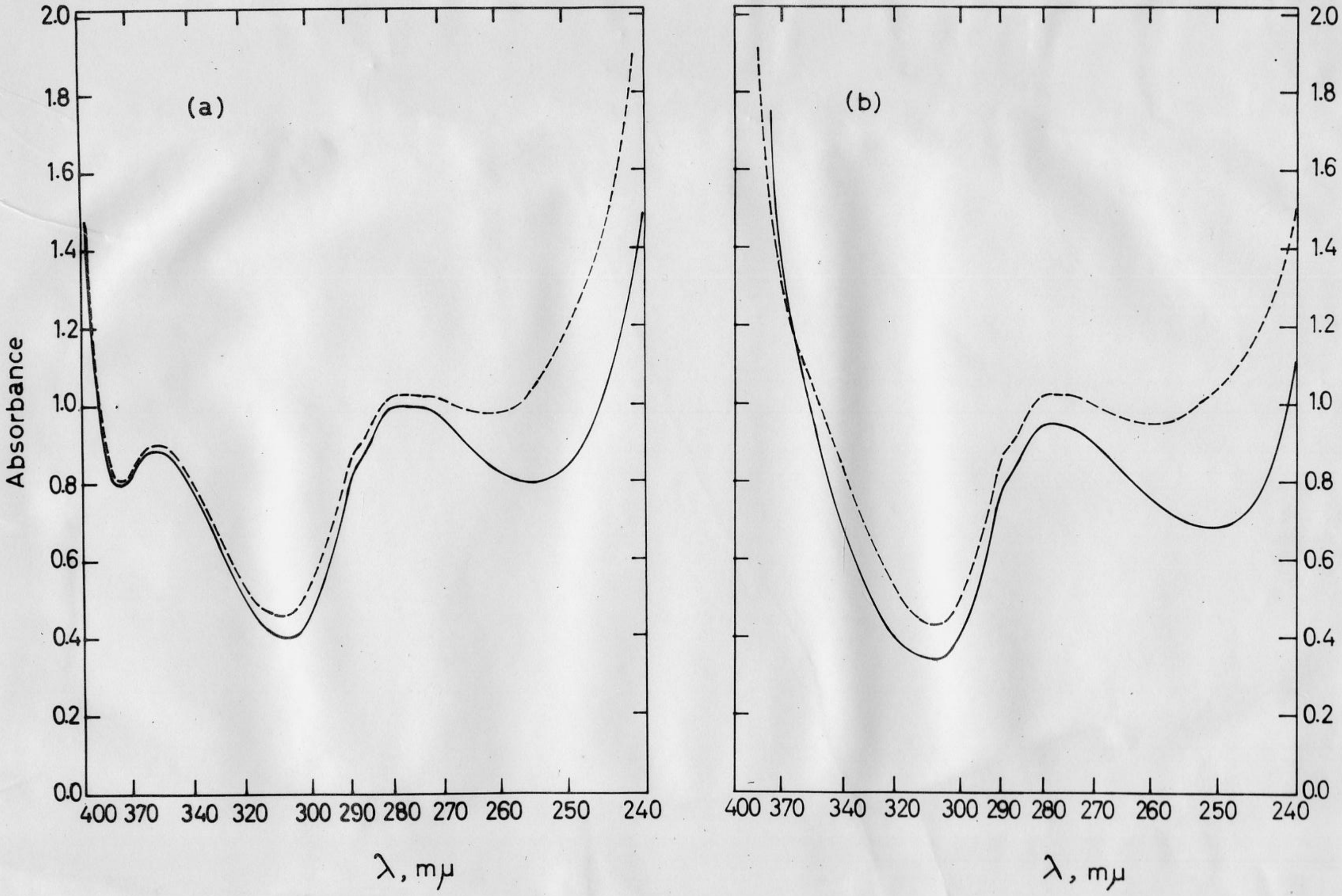


Figure 3b - Absorption spectra of 2.90 x 10^{-5}M solutions of Acidic (—————) and alkaline (- - - -) sperm whale ferrimyoglobin IV in the ultraviolet.

Figure 3a - Absorption spectra of 2.90 x 10^{-5}M solutions of the cyanide complex at pH 7 (————) and pH 11 (- - - - -) in the ultraviolet.

The first quantitative study of a cyanide reaction with myoglobin was the magnetic susceptibility study of Coryell, Stitt, and Pauling (1937) on horse ferrimyoglobin, at pH 4.77 and high ionic strength. The authors represented the reaction by the equation;

$$Fe^{+} + HCN = FeCN + H_3O^{+}$$

By analogy with the fluoride reaction, Havemann in 1944 gave the following mechanism of ferrihemoglobin with cyanide:

$$pH < 6.5 \quad Fe^{+}H_2O + HCN = FeCN + H_3O^{+}$$

$$pH > 6.5 \quad FeOH + HCN = FeCN + H_2O$$

but he did not consider the possibility of CN^{-} ion participating in the reaction.

Chance (1952) carried out a kinetic study of the reaction of cyanide with horse ferrimyoglobin and ferrihemoglobin over the range of pH 5 to 10, and observed the increase in rate with rising pH and its subsequent drop beyond pH 9. From this Chance concluded that HCN is the only reactant in the mechanism:

$$Fe^{+}H_2O + HCN \quad Fe^{+}CNH + H_2O$$

$$Fe\text{-}OH + HCN \quad Fe\text{-}CN + H_2O$$

From an extensive kinetic and equilibrium study of the cyanide reaction of horse ferrimyoglobin, Hanania (1953) concluded that both the HCN and CN^{-} species participate in the reaction, and that only CN^{-} is a ligand, and that the equilibrium is influenced not only by the ionization of H_2O and HCN, but also by another (heme-linked) ionization from an adjacent but unknown chemical group. On this basis, the mechanism would be :

$$Fe^{+}H_2O + HCN \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Fe\text{-}CN + H_3O^{+}$$

$$Fe^{+}H_2O + CN^{-} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Fe\text{-}CN + H_2O$$

with two corresponding reactions involving the conjugate base of the heme-linked group. The rate of reaction with CN^- was found to be 100 times faster than the rate of reaction with HCN.

The Problem of Heme-linked Effects

Thermodynamic study of ferrimyoglobin reactions leads to deeper understanding of interactions between the reactive site, the iron atom, and its protein environment. This is the problem of heme-linked effects.

Thus, it has been observed that the equilibrium constant for the oxygenation of ferromyoglobin, a reaction which does not explicitly involve H^+, still shows a pH dependence (Wyman, 1948). It has also been observed that,in the formation of the cyanide (and fluoride) complexes of ferrimyoglobin, this kind of pH variation is superimposed on the mass law effect of H^+ and the H^+-dependent concentrations of HCN and CN^- (Hanania, 1953). This pH dependence, 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

the nature of the ligand attached to the iron. In the chemical sense, it is assumed that the ionizing group is in the amino acid residues of the protein to which the heme is attached.

Historically, this problem started with the discovery (Bohr, 1904) that the oxygen equilibrium of blood was influenced by changes in the partial pressure of carbon dioxide. It was Henderson (1920) who 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.

German and Wyman (1937) made the first differential acid-base titration on horse ferrohemoglobin and oxyhemoglobin. They showed that in the physiological range oxyhemoglobin was a stronger acid than hemoglobin, and that at pH < 6, the situation was reversed. Wyman and Ingalls (1941) interpreted the above results, and similar data obtained by other workers, by assuming four identical sets of two oxygen-linked acidic groups in hemoglobin, one of which is rendered stronger, and the other weaker, upon oxygenation. Wyman (1939a) analyzed the then available enthalpy data and concluded that the enthalpy of ionization for a heme-linked group at pH < 4.5 is characteristic of carboxylic acids; between pH 6 and 8 it is characteristic of imidazolinium $\geqslant NH^+$ groups; and at pH > 9.5 it corresponds to that expected for $-NH_3^+$ groups such as ϵ-amino groups of lysine. From the pH variation of the enthalpy of the hemoglobin oxygenation reaction, Wyman (1939b) obtained $\Delta H = 6500$ cal/mole for the ionization of both oxygen-linked groups, and concluded that both are imidazolinium groups of histidine.

Furthermore, he attributed the opposite effects, which were exerted on the ionizations by the introduction of oxygen, 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 .

Coryell and Pauling (1940) gave a structural interpretation of the above effects in which it was assumed that the heme was between two imidazoles, closer to one (the proximal) than to the other (the distal). It is noteworthy that the recent crystal structure determination of sperm whale ferrimyoglobin (Kendrew,1963) has shown that the environment of the heme is in close agreement with the picture of proximal and distal histidines used in Pauling's interpretations.

However, several lines of investigation by other workers necessitated a wider interpretation of the heme-linked concept. For instance, measurements of the ionization constant for the iron-bound water molecule in ferrihemoglobin and ferrimyoglobin (George and Hanania, 1953 and 1957) have shown that the effective charge on the hematin iron becomes progressively more negative at higher pH. This effect is screened out by high salt concentrations, which suggests that it is an electrostatic effect due to non-specific interaction with charged groups in the protein.

Hanania and Irvine (1962a and b) have considered the role of the metal ion in their studies of the effect of coordination on ionization in selected chelates of iron and cobalt (Hanania and

Irvine, 1964), 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 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.

The variety of apparent heme-linked and other charge effects encountered is further illustrated in the recent work on ferrihemoglobin thermodynamics and on ferrimyoglobin kinetics. Thus, Beetlestone and Irvine (1964) 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, with only slight changes in pK. The authors concluded that the variations in ΔH and $T\Delta S$ reflect difference in electrostatic interactions originating from different charge configurations in each of the hemoglobins and found no need to invoke specific ionizations of heme-linked groups. Ansium, Beetlestone, and Irvine (1966) also found that the enthalpy of the reaction of azide with ferrihemoglobin varied with pH in a marked and complicated manner, with a maximum effect around the protein's isoionic point. Again, a general electrostatic factor was proposed.

The kinetics of the cyanide reaction of sperm whale ferrimyoglobin have recently been investigated with specific reference to the problem of heme-linked effects. From a detailed study in the pH range 5 to 7, Awad and Badro (1967) concluded that the protein undergoes a conformational change accompanied by 5 simultaneous ionizations with intrinsic pK = 5.66, in addition to an overall heme-linked charge effect. Van Ploeg and Alberty (1968) have analyzed the pH 5 to 10 profile of the same reaction and concluded that only one heme-linked ionizing group with pK = 6.0 accounts adequately for the whole variation of observed rate with pH. It should be noted, however, that this pH profile is not very detailed.

In the present work, we attempt a detailed thermodynamic investigation of the cyanide reaction with ferrimyoglobin to test the above ideas. Specifically, the work should answer the question of whether there are heme-linked effects in the reaction, and if so whether the effects are essentially chemical or electrostatic, or whether both effects concur.

T H E O R Y

The ligand-bonding reactions of hemoproteins may be divided into two general classes:

1. Ferrohemoproteins tend to combine strongly with neutral ligands like O_2, NO, CO. The reactions occur at the sixth (the only available) octahedral coordinating position on the iron(II) atom which carries a formal charge of zero. In the case of sperm whale ferromyoglobin, there appears to be no H_2O or other ligand group at the reactive site (Nobbs, Watson, and Kendrew, 1966). These reactions can nevertheless be represented by a 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 involve replacement of H_2O by ligand anion at the sixth coordination position. In this case, 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 of this second type and is concerned with the binding of the cyanide ligand to the hematin iron(III) atom of ferrimyoglobin:

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

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

$$K^{o} = a_{FeCN} / a_{Fe^{+}H_2O} \cdot a_{CN^{-}} \qquad (4)$$

At finite ionic strength I, the equilibrium constant K is defined in terms of the molar concentrations of the species at equilibrium:

$$K = (FeCN) / (Fe^{+}H_2O)\,(CN^{-}) \qquad (5)$$

The relation between K^{o} and K is :

$$K^{o} = K \cdot y_o / y_+ y_- \qquad (6)$$

where y_o, y_+, y_- are the molar activity coefficients of the complex, reactant (ferrimyoglobin), and ligand (cyanide) respectively. It may be assumed that, in the dilute solutions used, activity coefficients obey a Debye-Huckel relation:

$$-\log y_i = A.Z_i^2.\, f(I) \qquad (7)$$

where A = 0.510 at 25^{o}, Z_i is the charge, and the appropriate Debye-Huckel function may be taken as $f(I) = I^{1/2} / (1 + 6I^{1/2})$ on the basis of 18 Å for the closest distance of ionic approach to the iron atom from the electrolyte. Since $y_o \simeq 1$, it follows that at 25^{o}

$$\log K^{o} = \log K + 1.02\, f(I) \qquad (8)$$

K, in turn, is obtained from the experimentally determined K_{obs}. However, one expects K_{obs} to be influenced by pH in two ways:

Specific ionizations:(1) Ionization of the iron-bound water molecule (K_{Fe}). (2) Ionization of hydrocyanic acid (K_{HCN}).

Other effects: (1) Heme-linked ionizations.(2) Conformational charge effects.

The specific ionization effects arise because the reactant and the ligand are involved in acid-base equilibria and do not exist as single species throughout the experimental range of pH 6 to 10. The resulting equilibria may be represented as follows:

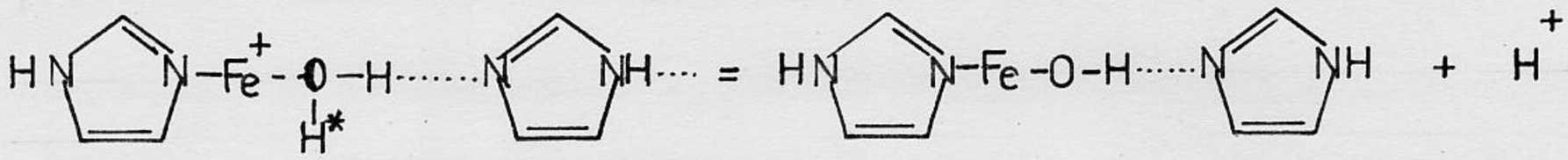
$$\begin{array}{ccccccc} & & \mathrm{HCN} & & & & \\ & & \Big\uparrow K_{HCN} & & & & \\ \mathrm{Fe^{\ddagger}H_2O} & + & \mathrm{CN^-} & = & \mathrm{Fe\text{-}CN} & + & \mathrm{H_2O} \qquad (9) \\ \Big\uparrow K_{Fe} & & & & & & \\ \mathrm{Fe\text{-}OH} & & & & & & \end{array}$$

Consequently, the experimentally determined equilibrium constant K_{obs}, which refers to equation 9, is a function of composite quantities and is defined in terms of the total molar concentrations of reactants and products:

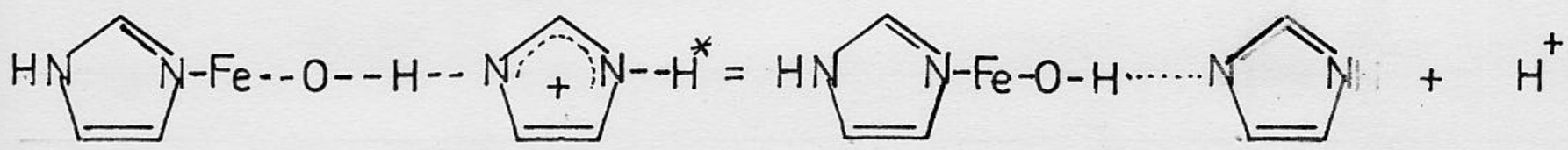
$$K_{obs} = \Sigma(\text{complex}) \,/\, \Sigma(\text{Mb}) \,.\, \Sigma(\text{cyanide}) \qquad (10)$$

(a) Effect on K_{obs} of the ionization of iron-bound H_2O

The acid ionization of the iron-bound water molecule at the sixth coordination position in ferrimyoglobin may be represented in one of two thermodynamically equivalent forms. On the one hand, it may be regarded as the ionization of a proton (with asterisk) from the iron-bound H_2O molecule which is itself hydrogen-bonded to the conjugate base of imidazolinium nitrogen of the E 7 distal histidine:



Alternatively, (Caughey, 1966), the same chemical equilibrium may be regarded as the ionization of a proton from an imidazolinium group on the E 7 distal histidine:



For present purposes this equilibrium may be simply represented as follows:

$$Fe^{+}H_2O \quad = \quad Fe\text{-}Oh \quad + \quad H^{+} \qquad (11)$$

for which one defines an ionization constant K_{Fe}:

$$K_{Fe} \quad = \quad h \cdot (Fe\text{-}OH) \,/\, (Fe^{+}H_2O) \qquad (12)$$

where $(Fe\text{-}OH)/(Fe^{+}H_2O)$ is the ratio of molar concentration of conjugate base to acid obtained spectrophotometrically, and h is the H^{+} ion activity obtained from pH measurements on the usual assumptions (Bates, 1964). K_{Fe} is of the order 10^{-9}.

Since $(Mb) = (Fe^{+}H_2O) + (Fe\text{-}OH)$, it follows from equation 12 that :

$$(Fe^{+}H_2O) \quad = \quad (Mb) \cdot h \,/\, (K_{Fe} + h) \qquad (13)$$

consequently by combing equations 5, 10, and 13, the single effect of the water ionization on the measured equilibrium K'_{obs} would be:

$$K'_{obs} \,/\, K \quad = \quad h \,/\, (K_{Fe} + h) \qquad (14)$$

where K'_{obs} refers to the observed formation constant of equation 9 assuming the ferrimyoglobin-cyanide complex and the ligand cyanide both exist as single species. This single effect is illustrated in Figure 4a for which the calculated values of the function $h/(K_{Fe} + h)$ are given in Table II which also includes relevant data on the ionization constants K_{Fe} and K_{HCN}. The theoretical curve shows that on this basis, the reactivity of ferrimyoglobin towards ligand should drop along a titration curve with inflexion point around pH 9.

(b) Effect on K_{obs} of ionization of HCN

Izatt et al (1962) have established thermodynamic parameters for the ionization of hydrocyanic acid in water:

$$HCN = H^+ + CN^- \qquad (15)$$

for which the ionization constant K_{HCN} is about 10^{-9}, and is defined by:

$$K_{HCN} = h \cdot (CN^-) / (HCN) \qquad (16)$$

the terms being used as above. It can be shown by argument similar to that above that

$$K''_{obs} / K = K_{HCN} / (K_{HCN} + h) \qquad (17)$$

where K''_{obs} is the formation constant on the basis that ferrimyoglobin and the complex exist as the single species. Equation 17 shows that the hydrocyanic acid ionization, unlike that of the water molecule, favors the reaction at higher pH, and that the variation of K''_{obs} with pH follows a titration curve with an inflexion point at about pH 9. Again, this single effect is illustrated in Figure 4b for which the calculated values are given in Table II.

Table II

Data for Theoretical Curves (I = 0.010 M)

pH	$h/(K_{Fe}+h)$ 25.0°	$K_{HCN}/(K_{HCN}+h)$ 25.0°	f(h) 25.0°	f(h) 15.0°	f(h) 35.0°
6.0	0.998	0.0006	0.0006	0.0004	0.001
6.5	0.996	0.002	0.0019	0.0011	0.004
7.0	0.987	0.007	0.007	0.003	0.011
7.5	0.959	0.021	0.020	0.011	0.033
8.0	0.881	0.064	0.056	0.032	0.085
8.5	0.701	0.178	0.125	0.078	0.164
8.6	0.650	0.214	0.139	0.090	0.175
8.7	0.597	0.255	0.152	0.103	0.180
8.8	0.539	0.302	0.163	0.115	0.1875
8.9	0.483	0.352	0.170	0.126	0.1870
9.0	0.426	0.406	0.173	0.135	0.180
9.1	0.365	0.468	0.171	0.141	0.169
9.2	0.319	0.520	0.166	0.148	0.157
9.3	0.270	0.578	0.156	0.144	0.142
9.5	0.190	0.684	0.130	0.132	0.109
9.7	0.129	0.774	0.100	0.111	0.079
10.0	0.069	0.872	0.060	0.071	0.044
10.5	0.023	0.955	0.022	0.03	0.016
11.0	0.007	0.986	0.007	0.009	0.005
11.5	0.002	0.996	0.002	0.003	0.0016
12.0	0.0007	0.998	0.0007	0.001	0.0005

Ionization Constants

(data used in calculating theoretical curves)

Temperature °C	I (M)	$10^{10}K_{Fe}$ (eq.12)	$10^{10}K_{HCN}$ (eq.16)
15.0	0.010	9.55	3.59
25.0	0.010	13.5	6.84
35.0	0.010	19.5	11.4
25.0	0.100	11.9	8.13

K_{Fe} data taken from Hanania (private communication).

K_{HCN} data taken from Izatt et al (1962).

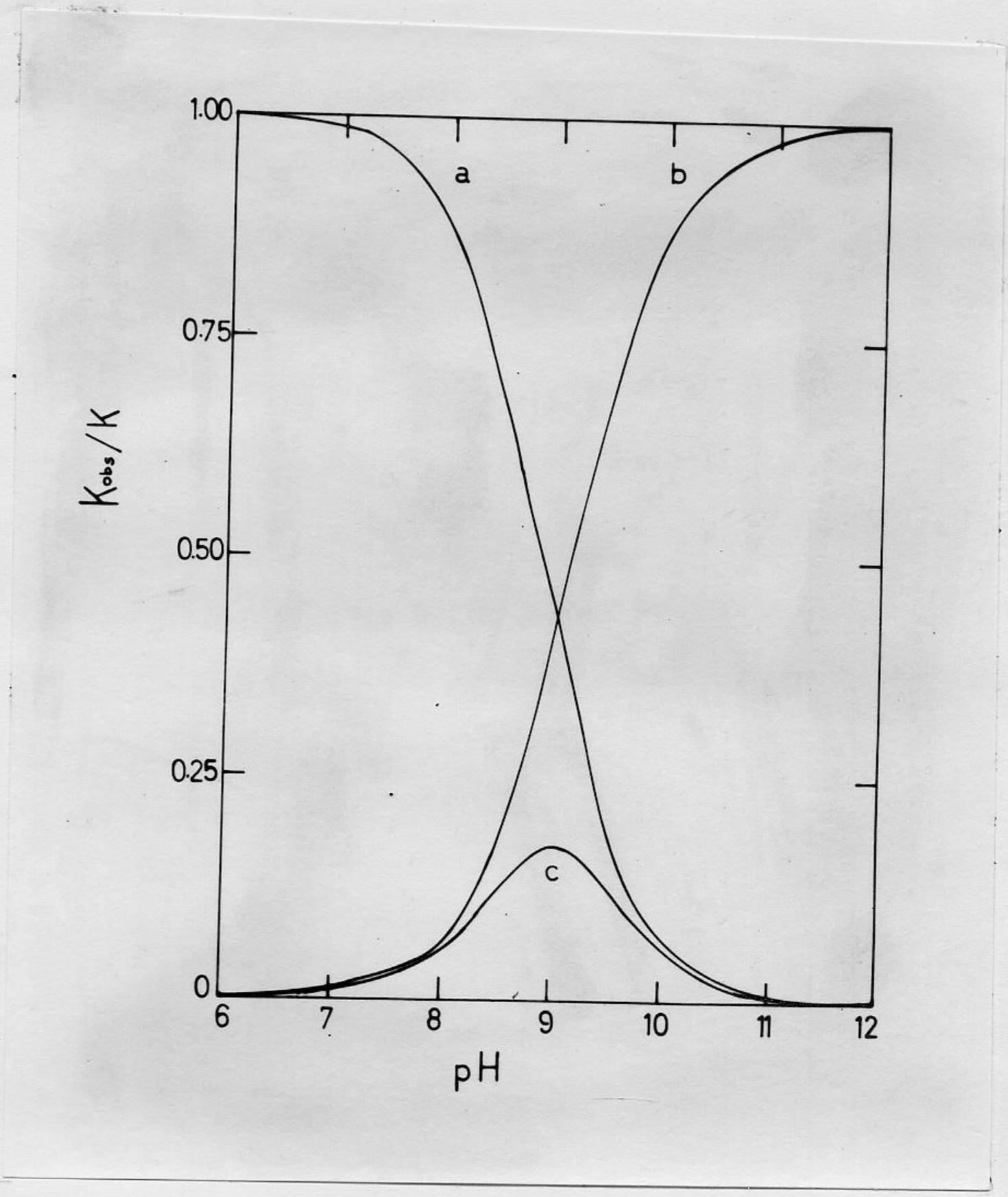


Figure 4 - Theoretical curves for effect of acid-base equilibria on the measured equilibrium constant K_{obs} for the reaction of sperm whale ferrimyoglobin with cyanide at 25.0°

(a) iron-bound water (equation 14)

(b) cyanide (equation 17)

(c) combined effect of curves a and b (equation 18).

(c) Effect on K_{obs} of both ionizations

The combined effect of the two ionizations, in reactant (K_{Fe}) and in ligand (K_{HCN}), is obtained in a similar manner and leads to the following relation:

$$K''_{obs} / K = \frac{h}{(K_{Fe} + h)} \cdot \frac{K_{HCN}}{(K_{HCN} + h)} = f(h) \qquad (18)$$

where K''_{obs} is the measured formation constant on the basis that both ferrimyoglobin and cyanide exist as single species, and K the equilibrium constant as defined for equation 5. The ratio K''_{obs} / K is also defined as f (h), the function which gives the pH dependence due to these two factors. The values of f (h) have been computed and are given in Table II at three temperatures (to be used also for plotting theoretical curves in the Results chapter).

Figure 4c illustrates this effect which because of the balancing of the two ionizations leads to a nearly symmetrical bell-shaped pH profile with its maximum around pH 9.

Thus on the basis of the two known ionizations, the equilibrium constant K which is defined in equation 5 for the equilibrium reaction (equation 3), is seen to be derived from the measured reaction equilibrium constant K_{obs} through the relation:

$$K = K_{obs} \cdot \frac{(K_{Fe} + h)}{h} \cdot \frac{(K_{HCN} + h)}{K_{HCN}} \qquad (19)$$

(d) Other pH effects

If the above treatment is inadequate to account fully for effect of pH on equilibrium constant, there are two further approaches to the problem:

1. One can invoke the operation of a heme-linked ionization either in reactant, or in complex, or in both. The effect of such an ionization can be computed in the same way as above and the new resulting functions are then tested against available experimental data (George and Hanania, 1955, and other papers).

2. Alternatively one can analyze data on the variation (if any) of K with pH, treating it as one would an acid-base titration curve, On this basis the calculations yield a value for the "intrinsic pK" and for the number of protons involved in the equilibrium. The effect could be attributed to a conformational transition in the protein. A recent application of this approach was made by Awad and Badro (1967) in their kinetic study of the cyanide reaction.

E X P E R I M E N T A L

I. Materials

(a) Myoglobin: All work reported in this study was done using sperm whale ferrimyoglobin IV, a microhomogeneous major component prepared and kindly donated by Dr. E. S. Awad. This component had been chromatographically fractionated from commercial sperm whale myoglobin which was purchased from Seravac Laboratories (Pty. Ltd., Colnbrook, England) as lyophilized and salt free sample (batch) prepared from skeletal muscle. The commercial product was shown by Awad (1968) to be chromatographically identical with the material used by Edmundson and Hirs (1962) in their studies of amino acid sequence, and by Kendrew and coworkers (1963) in their crystallographic work.

(b) Potassium Cyanide: AnalaR grade potassium cyanide was purchased from the British Drug Houses. It was titrated against standard silver nitrate solution before the start and after the end of measurements, and was found to be 99.0% pure. The sample was kept in a dessicator over calcium chloride and was used throughout without further purification.

(c) Water: Deionized redistilled carbon dioxide free water was used in the preparation of all the solutions and buffers. Tap water was passed through a two-bed ion-exchange (Deminerolit mack 4E, United Water Softners Ltd., London), redistilled from a commercial glass still (Laughborough Glass Co., England). Then it was boiled to remove carbon dioxide. In later stages of the work, steam boiled water was redistilled and then boiled to remove CO_2.

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

(d) Buffers: The pH range in the present study extended from 6 to 10. Three different sets of buffers were used to cover this range:

1. $NaH_2PO_4.2H_2O$ / NaOH for the pH range 6 - 8.
2. TRIS / HCl for the pH range 7 - 9.
3. H_3BO_3 / NaOH for the pH range 8 - 10.

Mixtures were made to contain, as far as possible, the same total molar concentration of buffer, with varying salt concentration to make constant total molar ionic strength I. For experiments at I= 0.010 M, the buffers were made to I = 0.011M to take into account the dilution effect of the ferrimyoglobin solution. The buffers for experiments at I = 0.100 M were made to I = 0.111 M.

The computation of ionic strength was based on the contribution of the following ions:

1. Phosphate buffer: Na^+, Cl^-, $H_2PO_4^-$, $HPO_4^=$.
2. TRIS buffer : Na^+, Cl^-, $(HOCH_2)_3NH_3^+$.
3. Borate buffer : Na^+, Cl^-, $H_2BO_3^-$.

Tables IIIa, b, c, d contain detailed numbers of quantities used in preparing the different buffers.

Table III a

Phosphate Buffer I = 0.011 M

Amounts of reagents used for making 250 ml. buffer.

Nominal pH of buffer	NaH_2PO_4 0.020M ml	NaOH 0.020M ml	NaCl 1.0mg/ml ml	Measured pH of reaction mixture
5.8	50.0	3.66	93	6.11
6.0	50.0	5.64	90	6.20
6.2	50.0	8.55	83	6.39
6.4	50.0	12.60	74	6.55
6.6	50.0	17.74	62	6.71
6.8	50.0	23.60	51	6.91
7.0	50.0	29.54	37	7.12
7.2	50.0	34.90	23	7.30
7.4	50.0	39.34	12	7.51
7.6	50.0	42.74	4	7.67
7.8	50.0	45.17	0	7.81
8.0	50.0	46.85	0	7.88

Table III b

TRIS Buffer I = 0.011 M

Amounts of Reagents used for making 250 ml buffer

pH	TRIS 0.050M ml	HCl 0.050M ml	NaCl 1.0mg/ml ml	Measured pH of reaction mixture
7.4	50.0	41.4	41	7.47
7.6	50.0	38.4	50	7.55
7.8	50.0	32.5	67	7.81
8.0	50.0	26.8	94	8.03
8.2	50.0	21.9	97	8.20
8.4	50.0	16.5	114	8.47
8.6	50.0	12.2	126	8.66
8.8	50.0	8.1	137	8.85
9.0	50.0	5.0	146	9.08

Table III c

Borate Buffer I = 0.011 M

Amounts of reagents used for making 250 ml Buffer

pH	H_3BO_3 0.050M ml	NaOH 0.050M ml	NaCl 1.0mg/ml ml	Measured pH of reaction mixture
8.0	50.0	4.00	150	8.13
8.2	50.0	5.90	144	8.28
8.4	50.0	8.55	137	8.48
8.6	50.0	12.00	127	8.66
8.8	50.0	16.40	114	8.85
9.0	50.0	21.40	99	9.03
9.2	50.0	26.70	85	9.21
9.4	50.0	32.00	68	9.39
9.6	50.0	36.85	53	9.65
9.8	50.0	40.85	42	9.74
10.0	50.0	43.90	33	9.90

Table III c

Borate Buffer I = 0.011 M

Amounts of reagents used for making 250 ml Buffer

pH	H_3BO_3 0.050M ml	NaOH 0.050M ml	NaCl 1.0mg/ml ml	Measured pH of reaction mixture
8.0	50.0	4.00	150	8.13
8.2	50.0	5.90	144	8.28
8.4	50.0	8.55	137	8.48
8.6	50.0	12.00	127	8.66
8.8	50.0	16.40	114	8.85
9.0	50.0	21.40	99	9.03
9.2	50.0	26.70	85	9.21
9.4	50.0	32.00	68	9.39
9.6	50.0	36.85	53	9.65
9.8	50.0	40.85	42	9.74
10.0	50.0	43.90	33	9.90

Table III d

Borate Buffer I = 0.111 M

Amount of reagents used for making 200 ml buffer

pH	H_3BO_3 0.75M ml	NaOH 0.75M ml	NaCl 10.0mg/ml ml	Measured pH of reaction mixture
7.8	50.0	2.00	1439	7.32
8.0	50.0	4.00	1123	7.70
8.2	50.0	5.90	1053	7.95
8.4	50.0	8.55	936	8.21
8.6	50.0	12.00	760	8.51
8.8	50.0	16.40	585	8.77
9.0	50.0	21.40	439	9.05

pH	H_3BO_3 0.50M ml	NaOH 0.50M ml	NaCl 10.0mg/ml ml	Measured pH of reaction mixture
9.2	50.0	26.70	526	9.23
9.4	50.0	32.00	439	9.46
9.6	50.0	36.85	222	9.70
9.8	50.0	40.80	117	9.93
10.0	50.0	43.90	000	10.24

II. Apparatus

The experimental work involved measurements of absorbancy and pH, at given temperature and ionic strength, of equilibrium mixtures of ferrimyoglobin and cyanide. For this purpose the following equipment and apparatus were used:

(a) Spectrophotometer: Absorbancy measurements were made in a Unicam SP.500 spectrophotometer (Unicam Instruments Ltd., Arbury works, Cambridge, England) fitted with a cell holder for 40.0 mm glass cells which was thermostated by water circulating from a constant temperature bath. This allowed control of the solutions' temperature to $\pm$ 0.05° or better.

(b) pH Meter: All pH measurements were made on a Radiometer pH meter, model PHM 4c (Radiometer Co., Copenhagen, Denmark). The instrument was fitted with a Faraday cage and a thermostated assembly which circulated water around both the glass and saturated calomel electrodes' compartments. A small polyethylene tube containing agar-saturated KCl was used to connect the two electrolytes, and the junction was renewed when necessary by slicing a bit off the end of the tube.

(c) Temperature Control: A thermostat (Wilkens-Andersons Lo-Temp Bath, Chicago, U.S.A.) was used for control of temperature from 14° to 35°C. Water from this thermostat was circulated around the pH measurement cell and the spectrophotometer's cells. A telethermometer thermistor probe (Yellow Springs Instrument Co., Ohio, U.S.A.) was used for measuring the temperature of the solutions in the spectrophotometric cells.

Temperatures were calibrated against a set of calibrated thermometers certified by the National Physical Laboratory, London, in 1961.

(d) Volumetric Apparatus: 10 ml volumetric flasks, Kimax or Pyrex grade A were used for preparing the different solutions.

1 ml volumetric pippetes, Pyrex grade A, were used for delivering the myoglobin solution. A 0.50 ml graduated pipet was used for pipeting the cyanide solution.

III. Procedure

(a) Preparation of Solutions: Three solutions were used in every experiment:

i) Ferrimyoglobin IV dissolved in deionized redistilled CO_2-free water was filtered, diluted with water to give a stock concentration 1.0×10^{-5}M.

ii) Potassium cyanide stock solution about 0.1 M was accurately prepared in very weak borate buffer pH 10 freshly every 2-3 days. Subsequent dilutions in stages were made with the same buffer daily.

iii) Buffers: A series of 0.011 M buffers with a difference between consecutive ones were prepared as described above, and were kept in glass stoppered bottles. Runs were made as soon as possible after the preparation of solutions.

(b) Equilibrium measurements:

1.00 ml of myoglobin solution was pipetted into a 10 ml glass stoppered volumetric flask with a 1 ml volumetric pipette. Few milliliters of buffer were added after which the required cyanide

solution amount was added with a 0.50 ml graduated pipette. The volumetric flask was then filled up to the mark with the buffer and was closed with the glass stopper. The solution was mixed and put in the water bath for attaining equilibrium at the pre-set temperature . The solution was left for at least one hour to achieve equilibrium. After that, the solution was mixed again and put immediately into a 40.0 mm spectrophotometric cell. The absorbance of this solution, which contains an equilibrium mixture of ferrimyoglobin and its cyanide complex and cyanide, was read at 410 nm. The blank, water against water, was also measured at the beginning and at the end of each run.

For every run at given temperature and pH, a series of equilibrium mixture was prepared containing the same total ferrimyoglobin concentration (usually around 1 x 10^{-6}M) and varying cyanide concentrations (between 0.50and 5.0 x 10^{-6}) chosen to yield 40 - 60% complex formation.

(c) pH Measurements:

The solution which was used for measuring absorbance was then transferred into the thermostated cell containing the glass electrode for pH measurement. All pH calibration and measurements were made following specifications of the U.S. National Bureau of Standards (Bates, 1964). The pH of standard buffers was measured before and after each run, and in case of slight change in the standard pH, the appropriate small corrections were introduced into the various pH readings. Such corrections varied between zero and ± 0.010 units normally.

IV. Calculation of K_{obs}

The equilibrium constant K_{obs} is obtained from measurements of absorbancy in the following way. Equation 10 is rewritten in the form

$$K_{obs} = (C) / (Mb) \cdot (CN) \qquad (20)$$

where the symbols refer to the total molar concentrations at equilibrium of complex, reactant and ligand, respectively.

The ratio (C) / (Mb) is obtained from the absorbancy of the solution. It can be shown that:

$$(C) / (Mb) = (A_o - A) / (A - A_\infty) \qquad (21)$$

where a A_o stands for the absorbancy of ferrimyoglobin solution without cyanide, A the absorbancy of a solution with the added cyanide, and A_∞ for the absorbancy of the solution with excess cyanide, representing 100% complex formation, all solutions containing the same total protein concentration.

The equilibrium concentration is of cyanide is $(CN)=(KCN)_o - (C)$; that is the total concentration of added KCN minus the quantity reacted. This calculation is based on the stoichiometry of equation 3, and takes into account the % complex formed at equilibrium as deduced from the absorbancies, namely $100(A_o - A) / (A_o - A_\infty)$.

A typical run is reported below for illustration.

V. Sample Rum

Stock Solutions:

Ferrimyoglobin IV : made in deionized redistilled CO_2-free water.

Potassium Cyanide : 0.0985 N in 0.001 M borate buffer of pH 10.2.

Working Solutions:

Ferrimyoglobin stock filtered and diluted to ~1 x 10^{-5} M. Concentration determined from absorbancy at 410 nm (ϵ = 157 x 10^3 $M^{-1}cm^{-1}$ for acidic ferrimyoglobin).

Potassium cyanide stock diluted with borate buffer pH 10.2 to 5.00 x 10^{-5}M concentration.

Conditions: T: 25.0 ± 0.05°C I = 0.010 M pH: 8.125

Procedure: 1.00 ml of ferrimyoglobin solution + cyanide solution as indicated below + 0.011 M borate buffer to make volume up to 10.0 ml.

KCN ml	A	A_o-A	A-A_∞	% C	10^6(CN)	(C)/(Mb)	$10^{-6}K_{obs}$
0.00	0.485						
0.25	0.394	0.091	0.164	36.1	0.325	0.562	1.73
0.30	0.380	0.105	0.150	41.6	0.405	0.707	1.74
0.35	0.370	0.115	0.140	45.5	0.497	0.828	1.67
0.40	0.355	0.130	0.125	51.4	0.574	1.06	1.85
0.45	0.340	0.145	0.110	57.2	0.650	1.32	2.03
Solid KCN	0.230						

R E S U L T S

Following the detailed procedure described above, the equilibrium constant for the ferrimyoglobin cyanide reaction was determined over the following ranges of conditions:

Ferrimyoglobin concentration:		from 0.8 to 1.2 x 10^{-6} M.
Cyanide concentration	:	from 0.50 to 5.0 x 10^{-6} M.
Ionic strength	:	I = 0.010 M and 0.10 M.
Temperature	:	15.0°, 25.0°, and 35.0°C.
pH	:	6 - 8 Phosphate buffer.
	:	7 - 9 TRIS buffer.
	:	8 -10 Borate buffer.

The value of K_{obs} (equation 10) for every set of conditions was obtained as the average of about 3 determinations for around 40 - 60 % reaction. The results are reported in Tables IV, V, VI, and are illustrated in Figures 5, 6, 7, 8, and 9. In every case, the corresponding value of the equilibrium constant K (equation 19) for the hypothetical pH-independent reaction was calculated using the parameters listed in Table II. In all tables of data, values of equilibrium constants K_{obs} and K are reported to two significant figures. Although the precision of individual measurements was much higher, general consideration of the various errors shows that the overall limits of uncertainty vary between 1 and 10 % of the magnitude of equilibrium constants depending on particular conditions.

Table IV a

Variation of Equilibrium Constant with pH

Phosphate buffer at 15.0°C and I = 0.010 M

pH	$10^{-6}K_{obs}$ (eq. 10)	$10^{-8}K$ (eq.19 & Table II)	log K
6.14	0.33	6.7	8.826
6.21	0.41	7.0	8.845
6.43	0.56	5.8	8.763
6.61	0.80	5.5	8.740
6.82	1.0	4.2	8.623
7.00	1.5	4.2	8.623
7.18	2.2	4.1	8.613
7.36	2.7	3.4	8.532
7.49	3.2	3.0	8.477
7.64	4.3	2.9	8.462
7.76	5.6	2.9	8.462
7.88	7.5	3.1	8.491

Table IV b

Variation of Equilibrium Constant with pH

Phosphate buffer at 25.0°C and I = 0.010 M

pH	10^{-6}Kobs (eq. 10)	10^{-8}K (eq.19 & Table II)	log K
6.10	0.24	2.8	8.447
6.21	0.29	2.6	8.415
6.39	0.33	2.9	8.462
6.55	0.50	2.1	8.322
6.71	0.69	2.0	8.301
6.91	1.0	1.8	8.255
7.12	1.4	1.6	8.204
7.30	2.0	1.5	8.176
7.51	2.4	1.2	8.079
7.67	3.0	1.0	8.000
7.81	3.6	0.93	7.968
7.88	3.8	0.85	7.929

Table IV c

Variation of Equilibrium Constant with pH

Phosphate buffer at 35.0°C and I = 0.010 M

pH	$10^{-6}K_{obs}$ (eq. 10)	$10^{-8}K$ (eq.19 & Table II)	log K
6.14	0.14	0.90	7.954
6.20	0.16	0.89	7.949
6.41	0.24	0.83	7.919
6.58	0.34	0.79	7.898
6.77	0.48	0.73	7.863
6.95	0.63	0.64	7.806
7.13	0.95	0.61	7.785
7.29	1.2	0.57	7.756
7.41	1.5	0.55	7.740
7.57	1.9	0.50	7.699
7.70	2.1	0.43	7.634
7.80	2.8	0.41	7.613

Table V

Variation of Equilibrium Constant with pH

TRIS buffer at 25.0°C and I = 0.010 M

pH	$10^{-6}K_{obs}$ (eq. 10)	$10^{-7}K$ (eq.19 & Table II)	log K
7.47	1.2	6.3	7.799
7.55	1.3	5.8	7.763
7.81	1.8	4.6	7.663
8.03	2.2	3.7	7.568
8.20	2.9	3.6	7.556
8.39	3.5	2.8	7.447
8.48	5.3	4.4	7.644
8.67	5.8	3.9	7.591
8.85	5.3	3.2	7.505
9.08	4.4	2.5	7.398
9.40	2.4	1.7	7.230

Table VI a

Variation of Equilibrium Constant with pH

Borate buffer at 15.0°C and I = 0.010 M

pH	$10^{-6}K_{obs}$ (eq. 10)	$10^{-7}K$ (eq.19 & Table II)	log K
8.12	3.9	9.8	7.991
8.30	3.4	5.9	7.771
8.54	3.1	3.7	7.568
8.75	3.7	3.4	7.532
8.93	3.0	2.4	7.380
9.10	3.2	2.3	7.354
9.28	2.4	1.7	7.220
9.49	1.8	1.4	7.130
9.66	1.8	1.6	7.190
9.82	1.0	1.0	7.017
9.98	0.75	0.98	6.991

Table VI b

Variation of Equilibrium Constant with pH

Borate buffer at 25.0°C and I = 0.010 M

pH	$10^{-6}K_{obs}$ (eq. 10)	$10^{-7}K$ (eq.19 & Table II)	log K
8.12	1.8	2.6	7.415
8.27	2.0	2.2	7.346
8.29	2.0	2.1	7.332
8.45	2.7	2.3	7.362
8.48	2.7	2.2	7.346
8.66	2.5	1.7	7.230
8.84	2.1	1.3	7.100
8.85	2.1	1.3	7.100
9.03	2.7	1.6	7.193
9.21	2.5	1.5	7.182
9.39	1.9	1.3	7.114
9.57	1.2	1.0	7.000
9.71	0.85	0.86	6.935
9.87	0.65	0.86	6.935

Table VI c

Variation of Equilibrium Constant with pH

Borate buffer at 35.0°C and I = 0.010 M

pH	$10^{-6}K_{obs}$ (eq. 10)	$10^{-7}K$ (eq.19 & Table II)	log K
7.94	1.3	1.7	7.230
8.10	1.5	1.5	7.176
8.33	1.6	1.2	7.065
8.52	2.0	1.2	7.079
8.73	2.0	1.1	7.033
8.92	1.5	0.81	6.909
9.09	1.2	0.70	6.845
9.29	1.0	0.70	6.845
9.48	0.75	0.67	6.826
9.63	0.60	0.68	6.829
9.78	0.45	0.66	6.816

Table VI d

Variation of Equilibrium Constant with pH

Borate buffer at 25.0°C and I = 0.10 M

pH	$10^{-6}K_{obs}$ (eq. 10)	$10^{-7}K$ (eq.19 & Table II)	log K
8.21	1.7	1.7	7.230
8.51	1.9	1.3	7.114
8.77	1.8	0.95	6.978
9.06	2.2	1.1	7.041
9.23	1.7	1.0	7.000
9.46	1.1	0.70	6.845
9.71	0.70	0.63	6.799
9.93	0.50	0.64	6.806
10.25	0.20	0.50	6.699

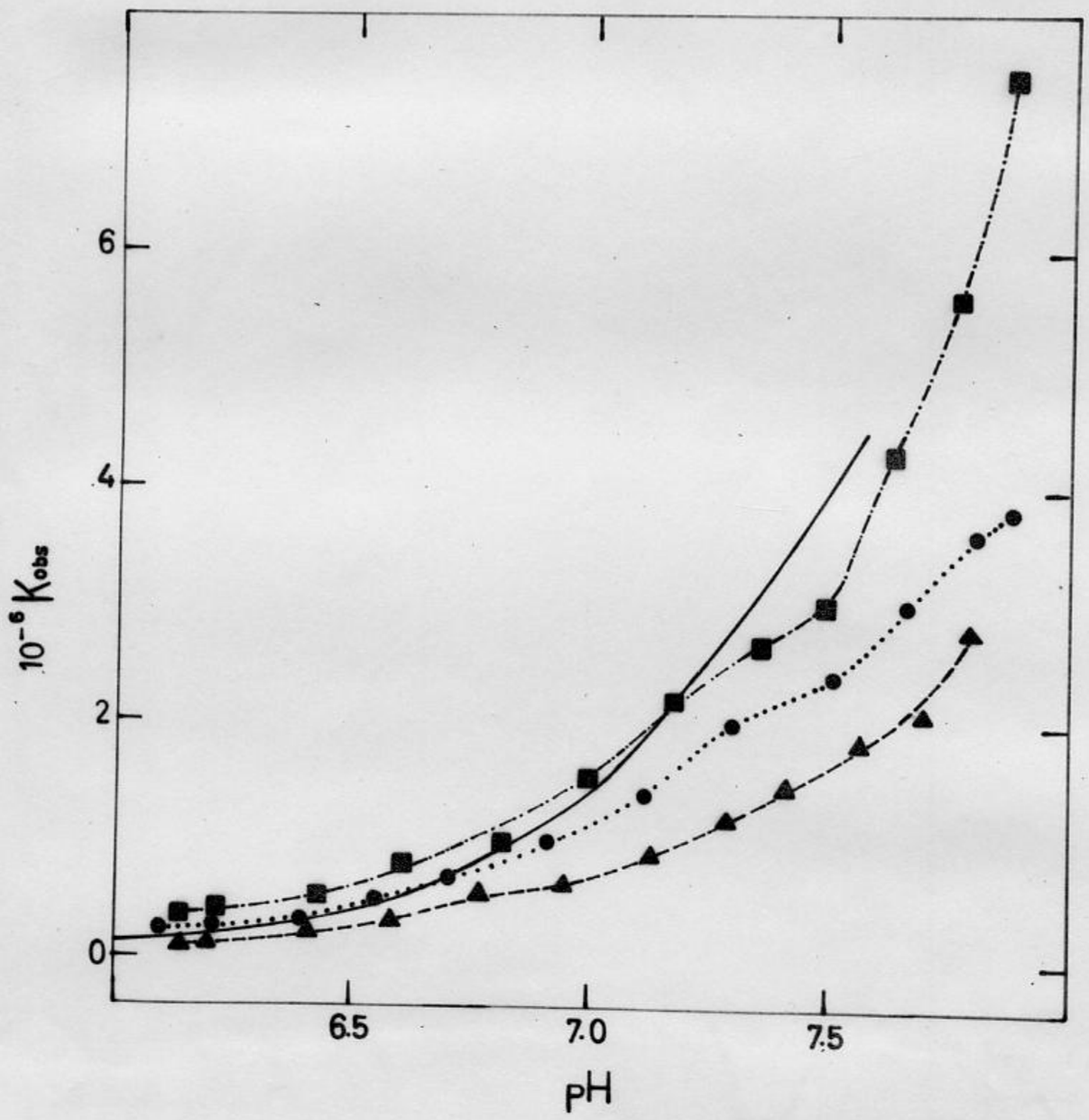


Figure 5 Variation of the measured equilibrium constant K_{obs} of the reaction of sperm whale ferrimyoglobin IV with cyanide in phosphate buffer at 15.0° (-.-.-.-.), 25.0° (.....) and 35.0° (- - - - -), all at I = 0.010 M. Solid line is theoretical curve at 25.0° and I = 0.010 (see Theory)

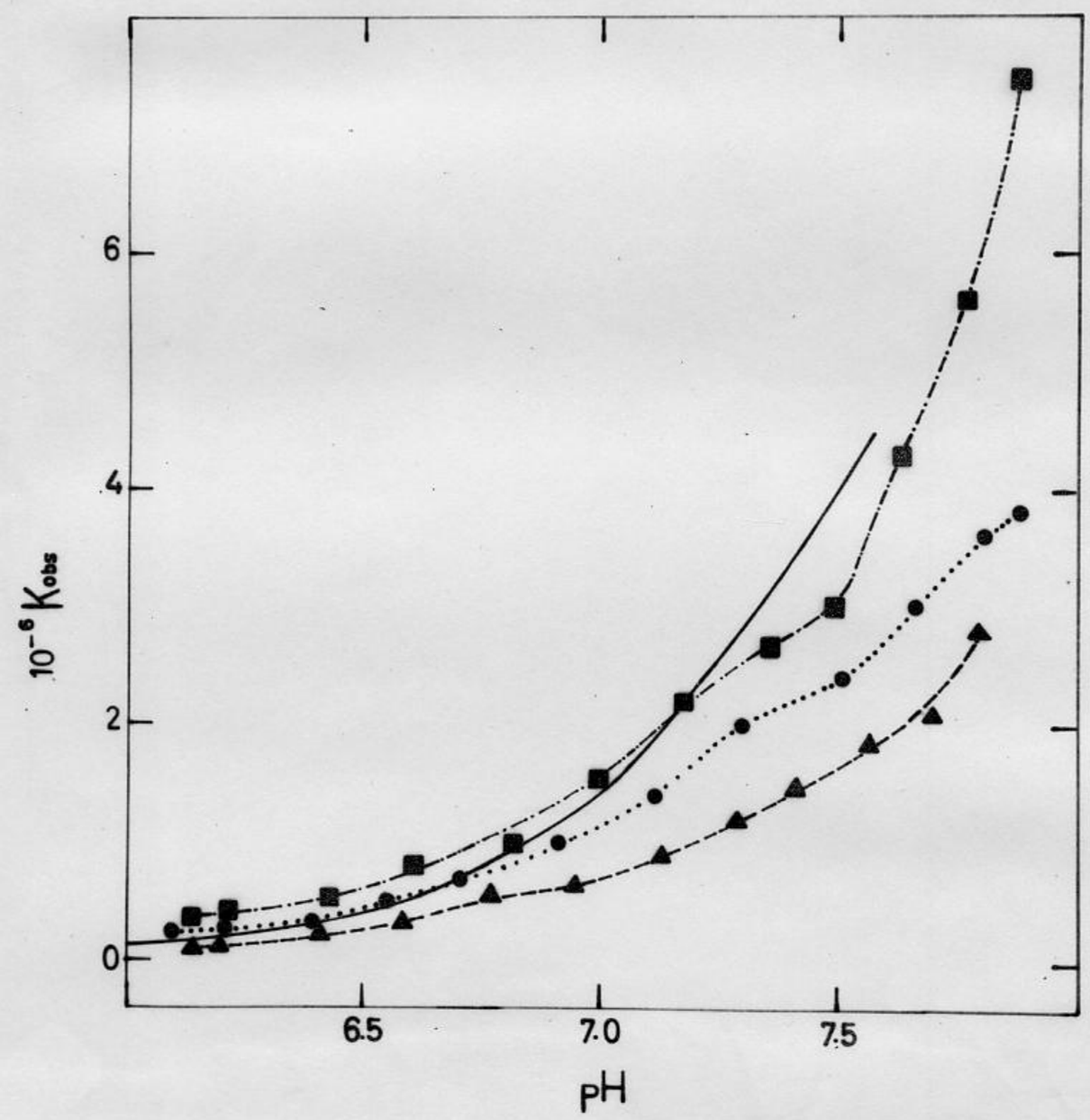


Figure 5 Variation of the measured equilibrium constant K_{obs} of the reaction of sperm whale ferrimyoglobin IV with cyanide in phosphate buffer at 15.0° (-.-.-.-.), 25.0° (.....) and 35.0° (- - - - -), all at I = 0.010 M. Solid line is theoretical curve at 25.0° and I = 0.010 (see Theory)

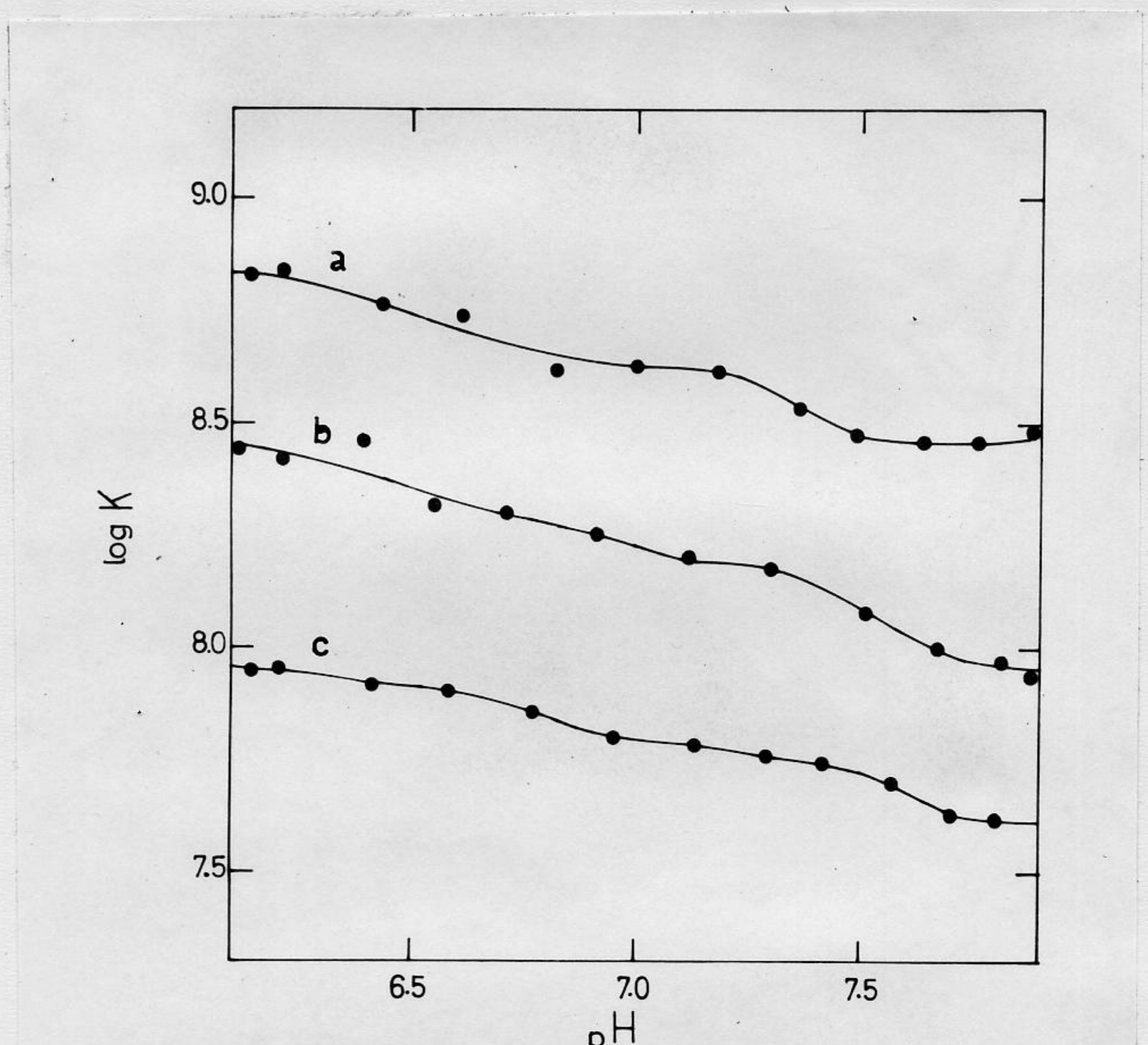


Figure 6 - Variation of log K the derived equilibrium constant (equation 19) with pH in phosphate buffer at I = 0.010 M: (a) 15.0° (b) 25.0° and (c) 35.0°.

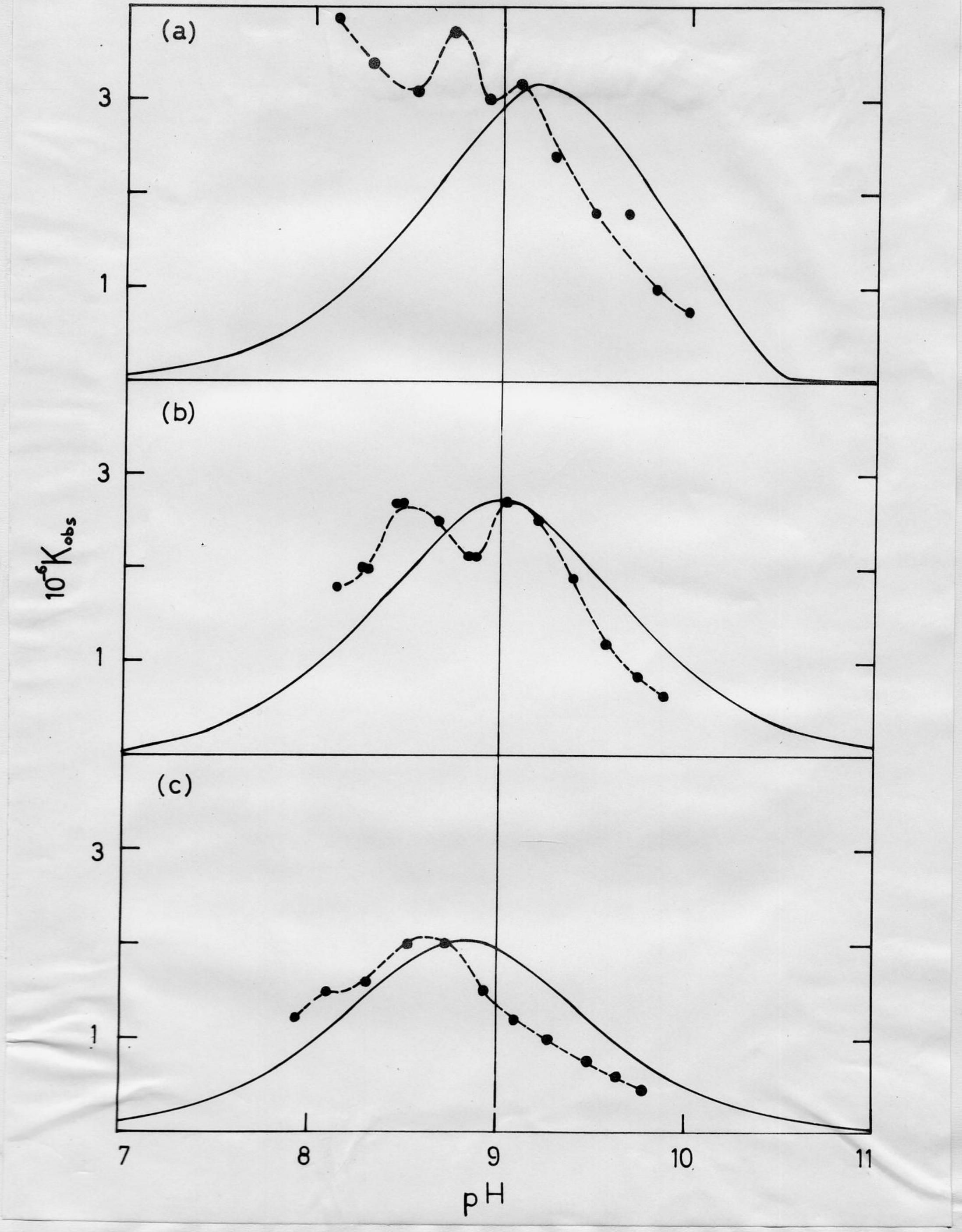


Figure 7 - Variation of the measured equilibrium constant K_{obs} in borate buffer at I = 0.010 M: (a) 15.0°. (b) 25.0° (c) 35.0°. Solid lines (————) are theoretical curves at the three temperatures (see Theory)

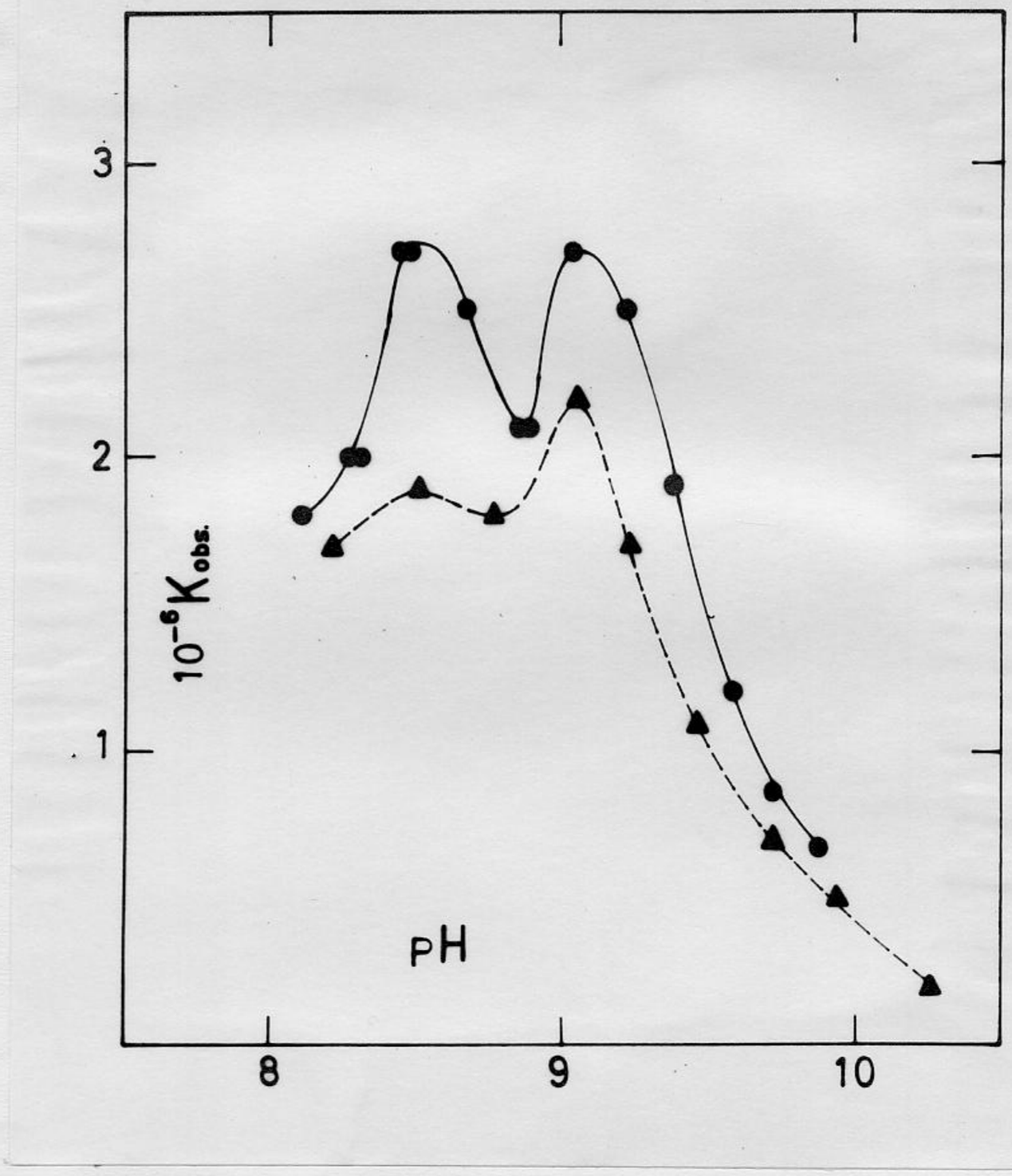


Figure 8 - Variation of measured equilibrium constant K_{obs} in borate buffer at 25.0°: I = 0.010 M (————); I = 0.100 M (- - - -).

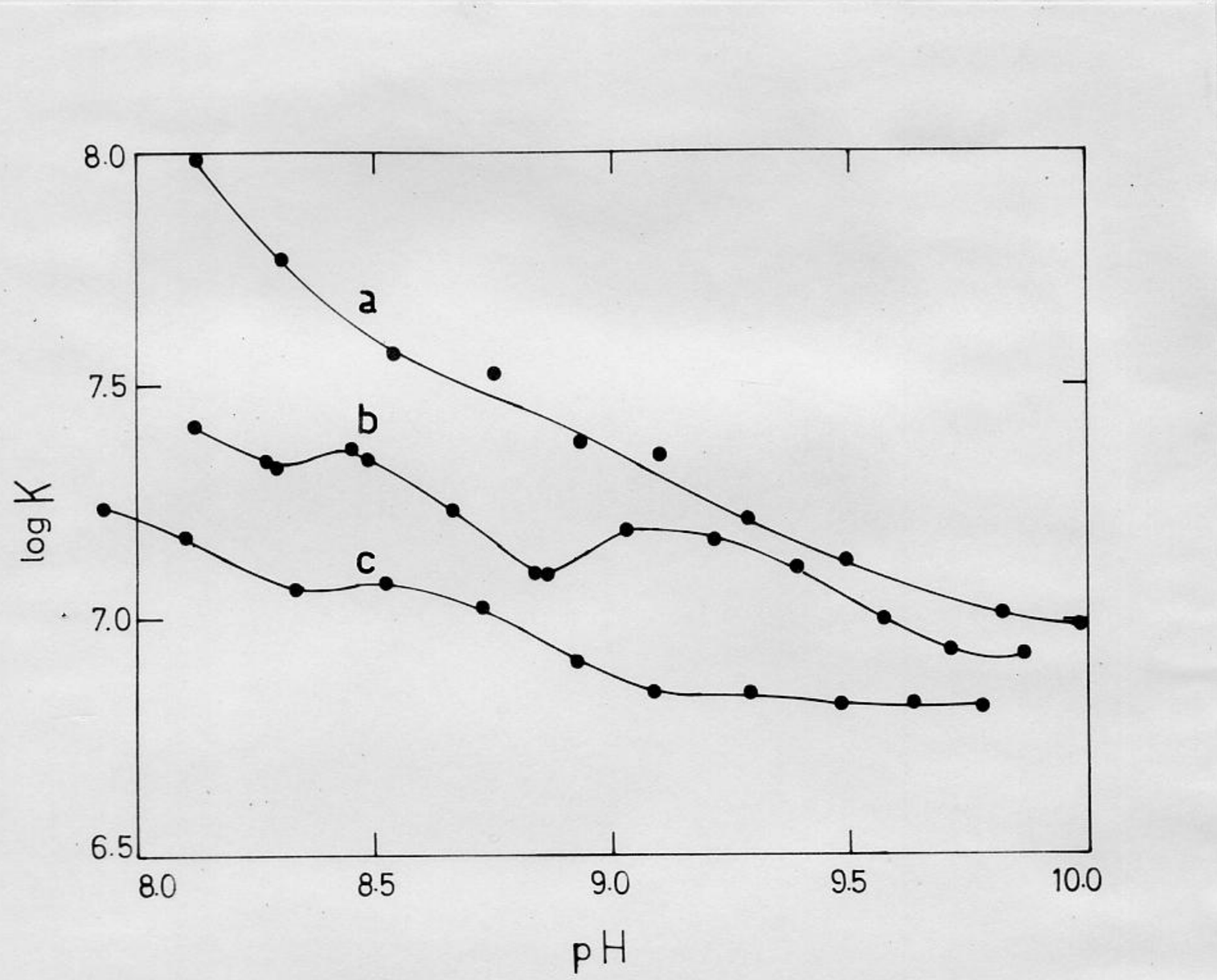


Figure 9 - Variation of log K the derived equilibrium constant (equation 19) with pH in borate buffer at I = 0.010 M: (a) 15.0° (b) 25.0° and (c) 35.0°

Tables IVa, b, and c include the results of measurements in phosphate buffers at I = 0.010 M at three temperatures : 15.0°, 25.0°, and 35.0°C. The pH variation of the measured equilibrium constant K_{obs} is shown in Figure 5, and the pH variation of log K, the derived equilibrium constant, is shown in Figure 6. Figure 5 also shows the theoretical variation of K_{obs} with pH at 25.0° and I = 0.010 M on the basis of the two known ionizations in reactant and ligand, that is using the function f(h) in equation 18 with an assumed constant $K = 2.0 \times 10^{8}$. This was taken in view of the fact that $10^{-8}K$ varies from 2.8 to 0.85 (Table IV) and there is no a priori reason for deciding which is the real constant.

Inspection of the data shows that whereas the equilibrium constant, as expected, increases with rising pH, this pH variation does not precisely fit the theoretical curve (see Figure 5), thereby suggesting the operation of other H^+ effects on the reaction.

It is noteworthy that the variation of log K with pH (Figure 6) resembles two successive weak acid-base titration curves with intrinsic pK 6.6 and 7.5 respectively. The effect is seen at all three temperatures. However, the data are neither extensive nor precise enough to warrant quantitative treatment of these apparent titrations.

Table V gives the corresponding results of measurements in Tris(hydroxymethyl)aminomethane / hydrochloric acid buffer at the same ionic strength I = 0.010 M and at one temperature 25.0°. Comparison of these results with the above phosphate results and also

with the borate results (following) definitely shows a marked specific salt effect of buffer on the equilibrium constant. Thus at pH 7.8, the measured K_{obs} in TRIS buffer is one half the corresponding value in phosphate buffer, and the results of the measured K_{obs} in TRIS are definitely higher than those measured in borate buffer. This complication limits thermodynamic discussion of results to comparison between data obtained in the same buffer medium.

The major part of the work was carried out in borate buffer covering the pH range 8 to 10 and containing the same total borate concentration.

The results in borate solutions at I = 0.010 M and 15.0°, 25.0°, and 35.0°C are given in Tables VIa, b, and c respectively. Table VId contains results in borate buffer at I = 0.100 M and 25.0°. Figure 7 shows the pH variation of the measured equilibrium constant K_{obs}. Figure 7 also shows the theoretical variation of K_{obs} with pH at 15.0°, 25.0°, and 35.0°, at I = 0.010 M, on the basis of the two known ionizations and using the function f(h) in equation 18 with an assumed constant $10^{-7}K$ = 2.16, 1.56, and 1.08 for the three temperatures respectively. Figure 8 illustrates the difference in pH profiles for the measured equilibrium constant at 25.0° between the two electrolyte conditions of ionic strength I = 0.010 M and 0.100 M. Figure 9 shows the variation of log K with pH in borate buffer at I = 0.010 M at the three temperatures.

Inspection of these results first shows the expected effect

of the two ionizations on the measured equilibrium constant. The shift in the peak of the theoretical curves with temperature is paralleled by a similar shift in the experimental curves for K_{obs} as a function of pH (Figure7).

However, it is apparent from the results that there are two additional effects, one occuring in the region of pH >9 and the other at pH < 9. The data at high pH show a greater dependence of K_{obs} on pH than that of the theoretical curve. A heme-linked ionization in the complex, not operative in the reactant, could be introduced to account for this discrepancy. On this basis, ferrimyoglobin-cyanide would act as a weak acid with pK ~10. But the data so far available do not extend to sufficiently high pH for quantitative treatment of this problem to be possible.

The other effect is more complicated. In the first place, the pH profile of K_{obs} (Figure 7) shows a maximum around pH 8.6 at all three temperatures. The effect, however, is seen to be considerably diminished at higher ionic strength (Figure 8), a fact which strongly suggests that electrostatic interactions are involved. When the data are converted into log K plots (Figure 9), the resulting variations are seen to go beyond the regular titration curves, especially when compared with the corresponding results for pH 6 to 8 (Figure 6).

In view of the complicated pH effects noted above, it is

not surprising to find that the corresponding enthalpy of reaction is also a complicated function of pH.

To obtain the value of ΔH for the hypothetically pH-independent reaction (equation 3), one can interpolate graphically from Figures 6 and 9 at any given pH the value of K (equation 19) at the three temperatures 15.0°, 25.0°, and 35.0°; the mean ΔH would then be obtained from the thermodynamic relation :

$$\Delta H = - 4.576 \left(d \log K / d \left(1/T \right) \right)_p$$

Reference to Figures 6 and 9 immidiately shows that the plots of log K versus 1/T deviate markedly from linearity specially so around pH 8.2 and also pH 9.2. These uncertainties may, on the one hand, reflect the sensitivity of borate buffers with respect to K_{Fe} at pH 8.2, and on the other hand, the sharp drop in K at 9.2. Because of the large uncertainties in ΔH that non-linearity causes, an alternative procedure was followed. At every pH, three ΔH values were computed from a pair of temperatures (15.0° and 25.0°, 25.0° and 35.0°, 15.0° and 35.0°) and these values were plotted to show the limits of uncertainty. This was done for the entire range of pH 6 - 10. The results are shown in Figure 10.

It is evident that the enthalpy of reaction ΔH for equation 3 is far from being pH-independent. In fact, one major transition is seen to occur from pH 8 to 9 with a corresponding decrease of at least 10 kcal/mole in the exothermicity of reaction. In addition, there

may be two other small perturbations, one around pH 7 and one around pH 9. The results do not cover the regions of pH <6 and pH >10 to ascertain whether a constant enthalpy occurs there.

In view of the above, it is clearly not possible to specify the thermodynamic parameters for the reaction. Instead, one can only give apparent quantities at 25.0° and I = 0.010 M, based on mean ΔH values. Thus taking averages from the ΔH/pH plot of Figure 10, one obtains values of $\Delta H_{average}$ as a function of pH (Table VII, illustrated in Figure 11). From this, and the K values in Tables IVb and VIb, the value of ΔG°, and hence ΔS are computed from the thermodynamic relations:

$$\Delta G^{\circ} = - RT\ln K$$

$$\Delta S = (\Delta H - \Delta G^{\circ}) / T$$

The results are given in Table VIII. Although the limits of uncertainty are wide, varying from $\pm$ 1 to 10% on K and from $\pm$3 to 50% on ΔH, the results are nevertheless striking. For, apart from the minor and somewhat uncertain perturbations at pH 7 and pH 9, the transition from a region with $\Delta H = -18$ to one with $\Delta H = -4$ kcal/mole is unmistakable. Likewise, the corresponding large change in the entropy of reaction from -26 to +20 e.u. is also definite.

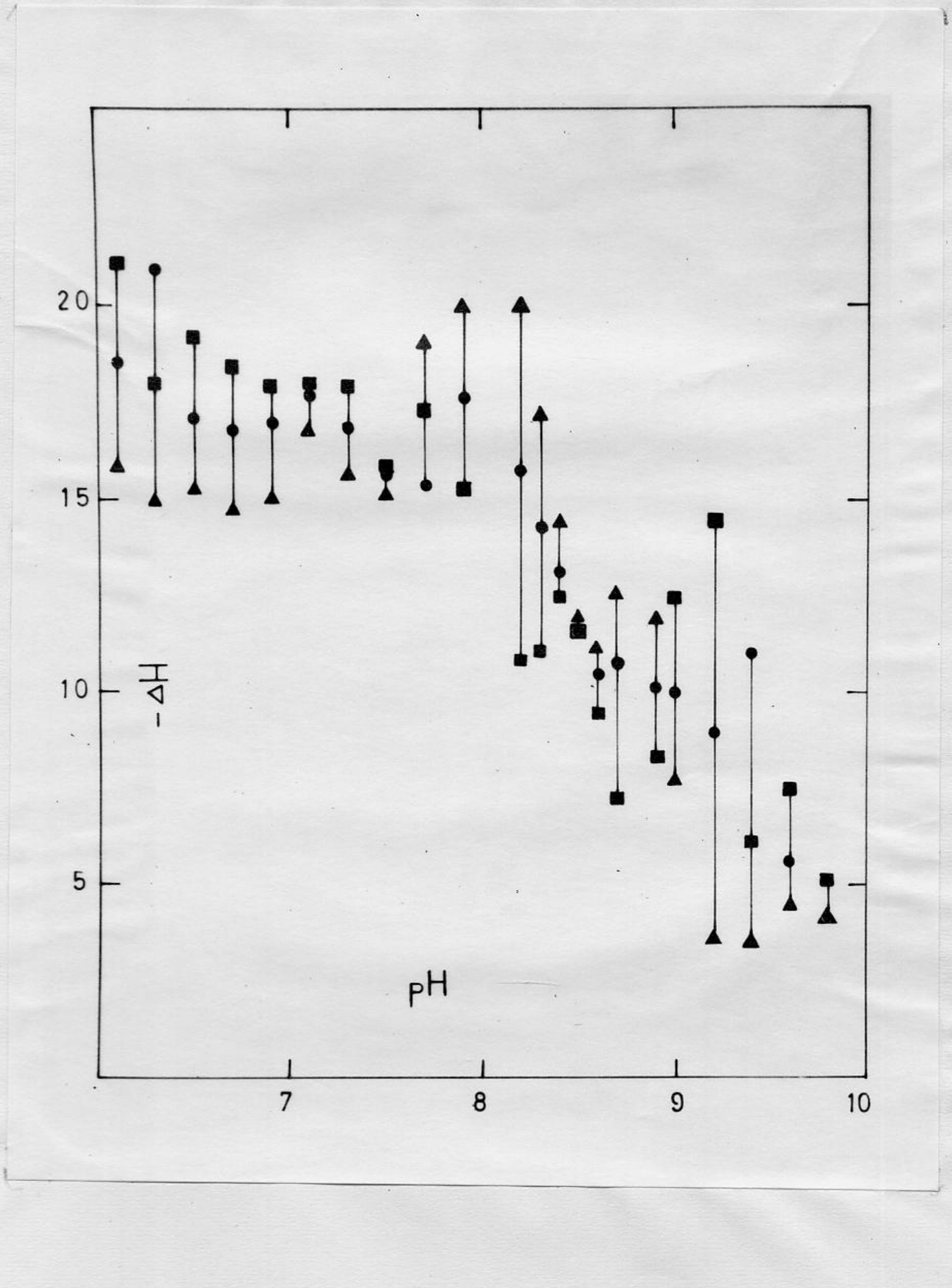


Figure 10 - Variation of ΔH with pH. ▲ ΔH calculated from 15.0° and 25.0° data. ■ ΔH calculated from 25.0° and 35.0°. ● Δ H calculated from 15.0° and 35.0° data.

Table VII

Variation of mean ΔH with pH

(Mean about 25°C, at I = 0.010 M)

Buffer	pH	$-\Delta H$ Kcal/mole	Buffer	pH	$-\Delta H$ Kcal/mole
Phosphate	6.1	18.5	Borate	8.2	17.8
"	6.3	18.0	"	8.3	14.9
"	6.5	17.5	"	8.4	13.0
"	6.7	16.7	"	8.5	11.4
"	6.9	17.6	"	8.6	10.2
"	7.1	17.9	"	8.7	10.5
"	7.3	17.0	"	8.9	10.2
"	7.5	15.8	"	9.0	8.9
"	7.7	17.5	"	9.2	6.9
"	7.9	18.4	"	9.4	9.0
			"	9.5	7.2
			"	9.6	6.4
			"	9.7	5.3
			"	9.8	4.1

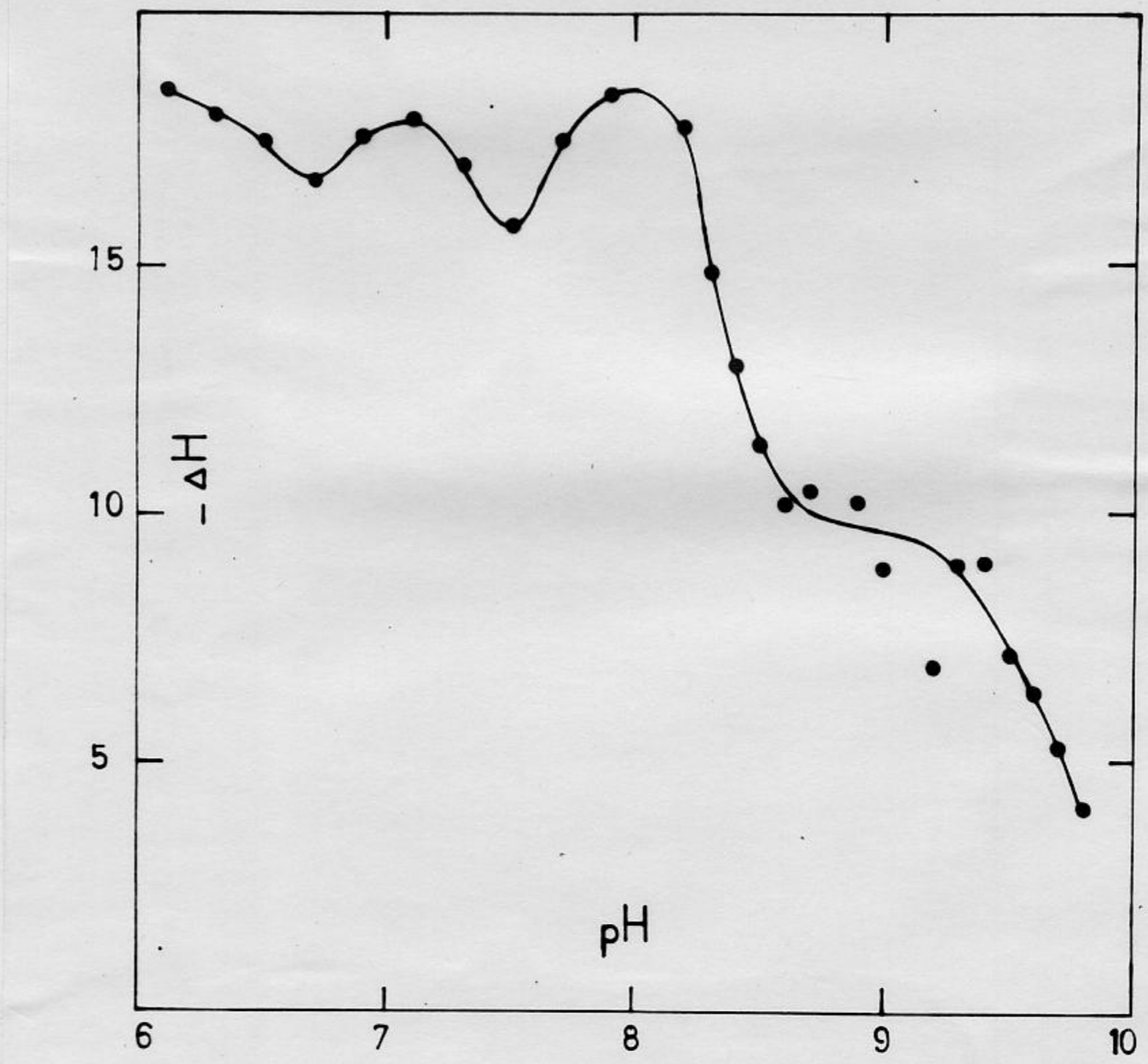


Figure 11 - Variation of $\Delta H_{average}$ with pH,
(Table VII, values averaged from ΔH values of Figure 10)

Table VIII

Ferrimyoglobin-Cyanide Reaction (eq.3)

Apparent Thermodynamic Quantities

$T = 25.0^{\circ}$, $I = 0.010$ M.

Data from Tables IV b, VI b, and VII.

Uncertainties : K (±1 - 10%), ΔH (±3 - 50%)

pH	$10^{-7}K$ (M^{-1})	$-\Delta G^{\circ}$ (kcal/mole)	$-\Delta H$ (kcal/mole	ΔS (e.u.)
6.1	28	11.5	18.5	-23
6.5	20	11.4	17.5	-20
6.9	18	11.3	17.6	-21
7.5	12	11.1	15.8	-16
7.9	8.5	10.9	18.4	-25
8.2	2.4	10.1	17.8	-26
8.5	2.2	10.0	11.4	-05
9.0	1.6	9.8	8.9	+03
9.5	1.0	9.6	7.2	+08
9.8	0.86	9.5	4.1	+20

D I S C U S S I O N

In the present work, an attempt was made to measure and evaluate thermodynamic parameters for the reaction between sperm whale ferrimyoglobin IV and cyanide ligand in the pH range 6 - 10. Although the reaction is relatively simple, it is complicated by two major factors: (1) the limitations imposed on the experimental conditions by the fact that the reaction is highly favored ($K \sim 10^8$); (2) various ionizations and electrostatic effects of the protein environment.

It is first necessary to determine limits of uncertainty for the results obtained. The measured equilibrium constant K_{obs} is of the order 10^6, and for measuring it spectrophotometrically at 410 nm, ferrimyoglobin concentrations used were between 0.8 and 1.2 x 10^{-6}M, with cyanide concentrations between 0.5 and 5.0 x 10^{-6}M. The equilibrium concentration of cyanide was in some cases, less than 2 x 10^{-7}M which made calculations subject to large errors. Also unspecific binding of CN^- to the protein may be significant. In fact, plots of log (complex/ferrimyoglobin) versus log (CN^-) from the relation

$$\log (C)/(Mb) = \log K_{obs} + n \log (CN^-) \qquad (22)$$

indicate that beyond 60% complex formation, n tends to deviate from 1, the value predicted from the availibility of only one site for the

reaction at the iron atom of the heme. To circumvent this difficulty, K_{obs} was averaged from results of runs within 40 - 60% reaction. For all these reasons, the values of K_{obs} are given to two significant figures, indicating limits of uncertainty which vary between + 1 and 10%, depending on conditions.

Another factor to consider in assessing the reliability of thermodynamic data is the role of buffer ion effects. In our case, the reaction was run in three buffer mixtures: phosphate pH 6 - 8, TRIS pH 7 - 9, and borate pH 8 - 10. The values obtained for the measured equilibrium constant K_{obs} in the three pH regions do not overlap, thus indicating the reality of specific salt effects. This observation confirms the view that the investigation of pH effects on equilibria should strictly be confined to systems in the same buffer and electrolyte environment. It is interesting to note, however, that no such discrepancy occured upon comparison of the enthalpy of reaction derived from data in two buffer systems, phosphate and borate. This suggests either that the two buffer systems have similar enthalpies of ion association with the protein or that a cancellation of other effects occurs.

Two specific acid-base equilibria are the major factors which determine the pH-dependence of K_{obs} in the investigated pH region. They are the ionizations of the iron-bound water molecule (equation 12) and the ionization of hydrocyanic acid (equation 16). It is fortunate that data for both equilibria are established (Table II); thus the equilibrium constant (equation 5) which is derived for the reaction

of equation 3 is obtained from the measured equilibrium constant K_{obs} and the known parameters for the two acid-base equilibria (equation 18).

From these results, at several temperatures, ΔH values are obtained. Inspection of Figure 10 shows that uncertainties in ΔH vary between 3 and 50%, the upper limit being limited to few points only (pH 8.2, 9.2, and 9.4). Apart from these, the average uncertainty would be ± 10 to 15%.

With these limits in mind, analysis of Table VIII shows that there is marked decrease in the enthalpy of the reaction ranging from about - 18 kcal/mole at pH 8.2 to about - 4.0 kcal/mole at pH 9.8. However,there is an opposite increase in the entropy of reaction which

	pH 8.2	pH 9.8	Change in thermodynamic parameter
ΔG^{o} kcal/mole	- 10.1	- 9.5	slight decrease in free energy
ΔH kcal/mole	- 17.8	- 4.1	very large decrease in exothermicity
ΔS e.u.	- 26	+ 20	very large increase in entropy

results from the fact that the free energy for the reaction is substantially constant with only a slight decrease in its value (0.6 kcal/mole). This is an indication that enthalpy and entropy effects tend to balance out.

The above results appear to confirm the observations of

Ansium, Beetlestone, and Irvine (1966) in their comparative study of the reaction of ferrihemoglobin A and C with azide, as well as the original work of Beetlestone and Irvine (1964) on the iron-bound water ionization in various hemoglobins. In their studies, these authors pointed out the curious balancing of enthalpy and entropy factors in hemoglobin reactions. Furthermore, the azide reaction showed maximum exothermicity near the isoionic point. On this basis, they interpreted this effect in terms of electrostatic interaction between the iron atom and the various charged groups on the protein surface. In the present work a similar phenomenon appears. Thus, the variations with pH of the enthalpy and entropy of the cyanide reaction are large but nearly equal and in opposite directions; and the maximum exothermicity of the reaction occurs around pH 8 which is close to the isoionic point of sperm whale ferrimyoglobin 8.07 (Sabri, 1968).

One interpretation of this effect would be the above electrostatic interaction idea. In an alternative approach, one could invoke a change in the degree of ionic hydration around the protein molecule which accompanies the ionization of acidic side groups (Table I). It may be assumed that cationic sites have stronger structuring effect on solvent molecules than anionic carboxylate side groups do, where the negative charge is presumably delocalized. On this basis, one would expect stronger hydration around pH 8 than that at pH 9. This effect is reflected in the large difference in the enthalpy of the reaction at both pH regions. Stronger structuring at pH 8 explains the more negative entropy values.

On the other hand at pH 9, the molecule has a net negative charge and the cationic NH_3^+ of lysine side chains are neutralized, which will result in less ordering and subsequent positive entropy effect.

The only other thermodynamic data available on ligand bonding reactions of sperm whale ferrimyoglobin are for imidazole (Baghdoyan and Hanania, 1965). In that work, which was carried out at the higher ionic strength I = 0.1 M, the enthalpy of reaction around pH 7 was found to be $\Delta H = -2.2 \pm 0.7$ kcal/mole, and no significant pH variation of the enthalpy was noted. However, in view of our observation that charge effects in the cyanide reaction are reduced considerably by increasing ionic strength from I = 0.010 M to I = 0.100 M, the possibility remains that a similar effect may appear in the imidazole reaction at lower ionic strengths. The fact that imidazole is a neutral ligand and a heterocyclic molecule makes it particularly interesting to find out whether or not a corresponding phenomenon would be discovered for the imidazole reaction at sufficiently low ionic strength.

Reference can be made to the equilibria study of the horse ferrimyoglobin-cyanide reaction at I = 0.1 M (Hanania, Ph.D. thesis,1953). Those early results showed that the enthalpy $\Delta H = -18$ kcal/mole for pH ~7 region. However, more complete data are not available for high pH range to enable determination of ΔH as a function of pH. Furthermore the results afor the two proteins are at different ionic strengths, hence no comparison can be done at this stage.

In view of the above uncertainties, it is clear that much more detailed work should be done on a number of comparable systems under carefully controlled conditions if one is to be able to decide on the thermodynamic significance of the data. In particular it seems that the following aspects of the reaction require further investigation:

1. Study of the sperm whale ferrimyoglobin-cyanide reaction at much higher ionic strength, where changes in hydration of the protein and concomitant conformational and general electrostatic effects could be partly or totally masked.

2. Extending the work to pH < 6 and pH > 10 aiming at arriving at constant values for the equilibrium constant K and the enthalpy of the reaction ΔH. In the particular case of the region pH > 10, the data should help verify the presence or absence of a specific ionization in the cyanide complex.

3. Study of the reaction of cyanide with a ferrimyoglobin having its isoionic point at a pH different from that of sperm whale ferrimyoglobin. (such as horse ferrimyoglobin pI = 7.44, modified acetylated sperm whale ferrimyoglobin pI = 6.36)(Sabri, 1968). Since the major effect appears in the vicinity of the isoionic point, this effect would then shift to a pH corresponding to the isoionic point of the protein concerned.

4. Corresponding study of the ferrimyoglobin reaction with another ligand whose pK_a is far from the pH region of the proteins isoionic point. In the case of cyanide, the acid-base equilibrium of the ligand operates in the region of the major effect, and though the derived

equilibrium constant was corrected for this ionization, it is still instructive to examine results where the two effects do not overlap to any significant degree.

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