ACID-BASE EQUILIBRIA

IN

FERRIMYOGLOBIN

BY

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ABSTRACT

The present work is a thermodynamic study of the ionization of ferrimyoglobin (of sperm whale) in dilute aqueous solution. The ionization is generally considered as involving a water molecule bonded at the sixth coordinating position of the iron(III) atom:

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

Using precision spectrophotometric methods, the iron content of this hemoprotein was determined, and on this basis the standard absorption spectra were mapped out for the acid and its conjugate base from 200 to 2500 mμ.

The ionization constant for the above equilibrium was measured within the pH range 8 to 10, at ionic strengths ranging from 0.20 to less than 0.001M, and at 25.0° and 34.0°C. Experiments were also performed at several temperatures between 15.0 and 34.0°C., all at constant I = 0.020M, for the calculation of the enthalpy of the ionization.

The effect of ionic strength on pK is marked and is reversed at $I < 0.010M$. Linear extrapolation from this region to zero ionic strength leads to the following results:

$$pK^{0} \ (25.0^{\circ}C.) = 8.99 \pm 0.025$$

$$\Delta H^{0} \ (\text{mean over } 15\text{-}34^{\circ}C.) = 7.2 \pm 0.6 \text{ Kcal/mole}$$

$$\Delta S^{0} \ (25.0^{\circ}C.) = -17.0 \pm 2.1 \text{ cal/mole/deg}$$

The limiting slopes of the plots of pK versus f(I) lead to the conclusion that the effective charge on the heme iron is -1, and not +1 which is the formal charge of the iron(III) atom in ferrimyoglobin. The source of this negative charge is discussed in relation to the question of secondary ionization and interaction effects in the molecule.

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LIST OF SYMBOLS

$Fe^{+}(H_2O)$, also A	Ferrimyoglobin, conjugate acid.
Fe-OH, also B	Ferrimyoglobin, conjugate base.
a	Activity.
( )	Concentration.
f	Activity coefficient.
I	Ionic strength (mole/liter).
f(I)	$\sqrt{I}/(1 + \sqrt{I})$.
Z_i	Effective charge of an ion i.
Z_A	Effective (ionic strength) charge on acidic ferrimyoglobin.
Z_B	Effective (ionic strength) charge on alkaline ferrimyoglobin.
λ	Wavelength.
A(x mμ)	Absorbancy at wavelength x mμ.
Å	Angstroms.
"Tris"	Tris-(hydroxymethyl)-aminomethane.
$>$ or $<$	Greater or smaller.
N.B.S.	National Bureau of Standards.

INTRODUCTION

From a physiological point of view, the iron-protein complex, myoglobin is important as the muscle pigment responsible for oxygen storage. This function is performed by the molecule in its iron(II) form, ferromyoglobin. However, as the substance is much more stable in its iron(III) form, ferrimyoglobin, it is the latter which has been the subject of much recent physico-chemical study. This interest in ferrimyoglobin is justified by the fact that the molecule possesses certain remarkable properties.

As a coordination compound, ferrimyoglobin possesses very useful properties. For, it is a metal complex in which the iron(III) atom is octahedrally bonded at six positions, five of which are firmly occupied by nitrogen atoms, the sixth position having a loosely attached water molecule. The contrast with inorganic Fe^{3+}_{aq} is evident. Inorganic cations can undergo a series of hydrolysis or ligand bonding reactions the interpretation of which is extremely difficult. The hemoprotein, however, presents the case of a complex ion with one single bond available for reaction, and with very sharp absorption spectra to aid precision spectrophotometric techniques. Moreover, its reactions are, in general, relatively slow and clean.

As an iron-protein complex(a hemoprotein), myoglobin is the simplest molecule known, and yet it possesses most of the characteristics of large protein molecules. From this point of view, the molecule serves as an ideal model system, since through the study of secondary interaction effects between the iron atom and its environment, a more

detailed understanding of the specificity of reactions involving the more complicated hemoproteins can be arrived at.

Ferrimyoglobin was first crystallized by Theorell[1] in 1932. Since then, the molecule has been studied by a rapidly increasing number of investigators. Kinetic and equilibrium studies of ligand bonding reactions of horse ferrimyoglobin[2] have been carried out to parallel corresponding work on ferrihemoglobin (which is obtained from blood). More recently, sperm whale ferrimyoglobin has been studied extensively; the crystal structure has been determined in detail by X-ray analysis, the substance has been fractionated by column chromatography into five components the amino acid composition of each of which has been determined, and the amino acid sequence in the protein is also being determined (see Structure).

In view of the fact that this recent work on sperm whale ferrimyoglobin is yielding extremely valuable information about the structure of solid crystals, it is particularly appropriate to obtain thermodynamic data for this hemoprotein in aqueous solution. It would be very interesting to find out the extent to which information derived from structural work on the solid crystal confirms the corresponding results obtained from physico-chemical studies of the same substance in aqueous solution.

In aqueous solution, the reactions of ferrimyoglobin involve the iron-bound water molecule, and consequently involve acid-base equilibria. The measurement of ionization constants for these equilibria is therefore fundamental to any thermodynamic study of reactions.

Although ionization studies on horse ferrimyoglobin have been studied (see Review of Previous Work), no corresponding work on sperm whale ferrimyoglobin has been published.

The present work is concerned with the fundamental study of the molecule in dilute aqueous solution, involving the determination of iron content, standard absorption spectra, and the measurement of ionization constants.

HISTORICAL

I. Review of Previous Work

Although it was only recently that the evidence for the bonding of a water molecule to the iron atom in hemoglobin and in myoglobin became generally accepted*, it has for a long time been known that a reversible acid-base equilibrium, with a distinct colour change, occurs between two forms of ferrihemoglobin. This equilibrium has been investigated by several workers in the hemoprotein field.

Gamgee (1868) was the first to observe the reversible colour change[3]. However, this fact remained unnoticed until 1935 when Austin and Drabkin[4] measured spectrophotometrically the ionization constant of ferrihemoglobin from canine blood, and showed that:

a) The ionization was single; that is, it involved one hydrogen ion per hematin iron atom.

b) The measured pK' (corresponding to pK of equation 4 in the present work) was 8.120 $\pm$ 0.010 at I = 0.10M (at an unspecified temperature).

In their paper, Austin and Drabkin also stated that, at $I < 0.15M$, the variation of pK' with $\sqrt{I}$ is linear with a slope of about -0.6; or

$$\Delta pK' = -0.6 \quad \Delta\sqrt{I}$$

It should be noted that Austin and Drabkin's results cannot be as accurate as appears above. For, if their published spectra are used in calculating pK values at the various ionic strengths reported in their

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* The evidence is summarized under "Structure of Myoglobin" below.

paper, the dependence of pK' on $\sqrt{I}$ will be seen to be scattered over a wide range. This is illustrated in Figure 1, where pK' values were calculated from Austin and Drabkin's data using equation 4. It can be seen in Figure 1 that, taking all their data into account, the mean value of pK' at I = 0.10M is 8.17 ± 0.10 (maximum deviation), and not 8.120 ± 0.010 as reported. Furthermore, the extrapolated value of pK' at zero ionic strength pK^0 = 8.37 ± 0.09 (at that unspecified temperature).

Figure 1 also shows the two experimental results of Coryell Stitt and Pauling[5] who, in 1937, titrated bovine ferrihemoglobin magnetically and reported the following pK values for the same ionization at 24°C.

I (M)	0.20	1.3
pK	8.15 ± 0.02	8.56

From these values they concluded that the linear slope of pK versus $\sqrt{I}$ was about +0.6, a value which they mistook to be the same as the slope -0.6 found by Austin and Drabkin. In fact, linear extrapolation from these two points would lead to pK^0 = 7.89, as shown in Figure 1. But such an extrapolation is, of course, of no thermodynamic significance.

Coryell et al thus thought that they had verified Austin and Drabkin's results by another method, but obviously the two sets of results are neither identical nor contradictory. In fact, they are complementary, the curve in Figure 1 passing through a minimum between the two sets of data and having slopes of nearly equal value but opposite signs.

Bowen[6], in 1947, was the first to record absorption curves and molar extinction coefficients for myoglobin (obtained from horse heart) and

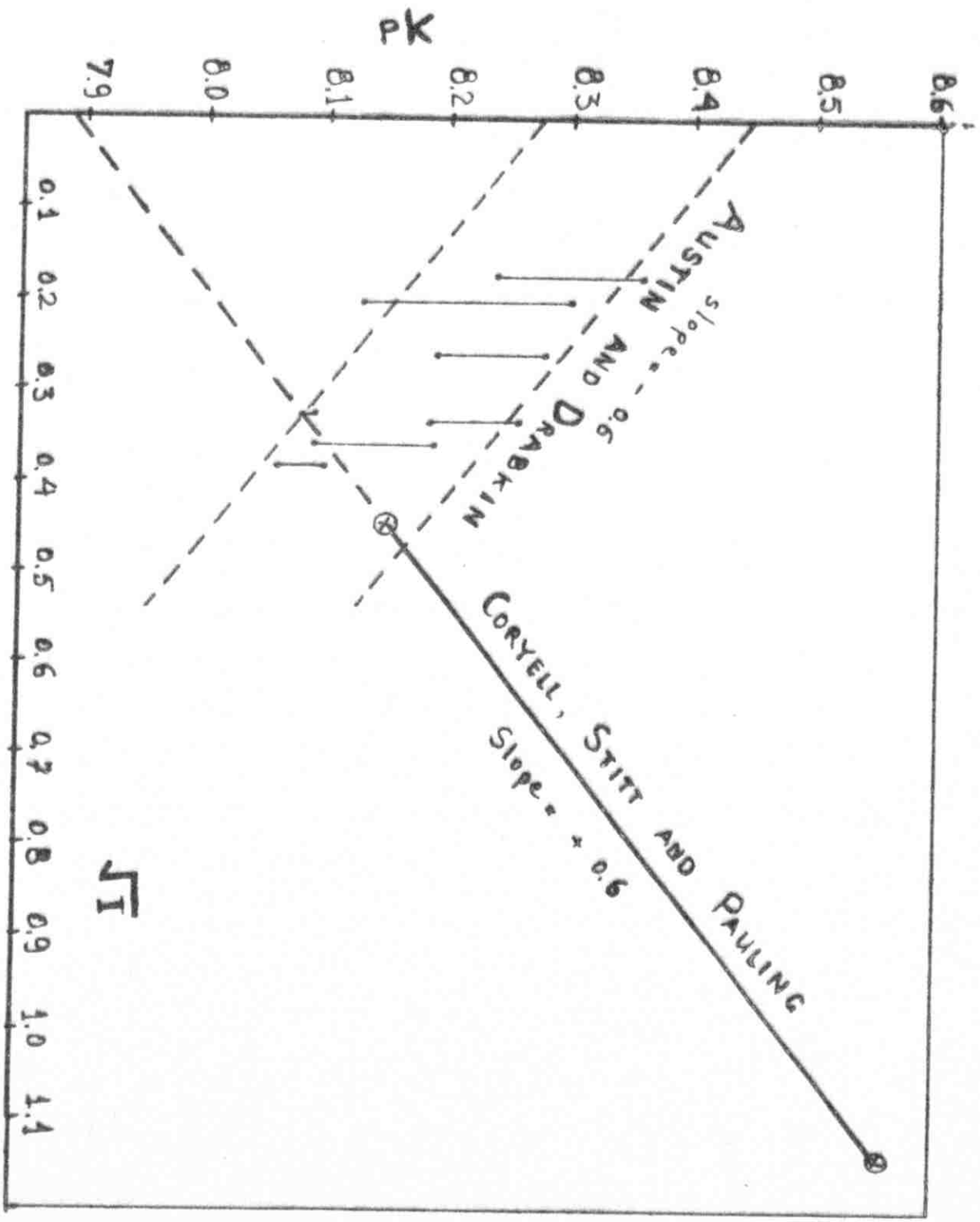

Figure 1 - Variation of pK with Ionic Strength (Data of A.&D., and C.S.&P.)

several of its derivatives at wavelengths from 450 to 1000 mμ and at different pH values. He stressed the importance of the influence of pH in spectrophotometric studies, but did not attempt to calculate the ionization constant deciding that, owing to effects of pH, it was difficult to duplicate measurements. No quantitative results were published by him.

Theorell and Ehrenberg[7] in 1951, however, did not encounter such difficulty. They carried out experiments on (unpurified) horse ferrimyoglobin and reported the following:

a) The variation of absorption spectrum with pH could be readily reproduced, at different pH values in the wavelength range of 450 to 650 mμ. Three isobestic points were identified.

b) Ferrimyoglobin has a well-defined pH-stability range. Rapid destruction occurs at pH values less than 4.60 or more than 11.75. Therefore all experiments should be carried on between these two limits.

c) The transition from neutral (the conjugate acid) to alkaline ferrimyoglobin follows a single equivalent dissociation curve.

d) The influence of ionic strength upon pK, measured spectrophotometrically, was as follows:

I (M)	0.02	0.1	0.20	2.0
pK	8.92	8.95	9.01	9.17

(temperature unspecified; data plotted in Figure 2)

They concluded that their results could best be fitted by the expression:

$$pK = 8.84 + 0.56 \sqrt{I} / (1 + \sqrt{I})$$

where $pK^o = 8.84$ at $I = 0$. The corresponding pK^o value from a magnetic

titration carried out by the authors was 8.77 ± 0.04.

Like Coryell, Stitt and Pauling, these workers did not extend their measurements to sufficiently low ionic strengths, and consequently they reported a positive limiting slope in their equation. Here again extrapolation to pK^0 gives the wrong value.

It was George and Hanania[8,9] who first studied this problem in detail and published reliable thermodynamic data on the ionization of horse heart ferrimyoglobin. Their relevant work appeared in two papers (1952 and 1957), and their results may be summarized as follows:

a) They confirmed previous work which showed that the ionization is single and reversible.

b) They extended the measurement of pK over a wide range of I from 0.7 down to 0.001M and so they were able to demonstrate the reversal of the salt effect, that is the change in slope of pK versus a function of I at $I < 0.01M$ (Figure 2).

c) They showed that pK can be extrapolated against a number of functions of I, ($\sqrt{I}$; $\sqrt{I} / (1 + \sqrt{I})$; $\sqrt{I} / (1 + 2\sqrt{I})$), all equally probable. Consequently, pK^0 values have uncertainty of ± 0.025.

d) Thermodynamic data for this ionization were given (see Table V).

e) From the limiting linear slope of pK versus $\sqrt{I} / (1 + \sqrt{I})$, and on the basis of a Debye-Hückel equation, they calculated an effective charge of -2 on the acid, and consequently -3 on the conjugate base. That is, although the formal charge in ferrimyoglobin is +1, sufficient negative charge is transmitted to the iron atom at low ionic strengths to give it a net effective negative charge. Furthermore, they demonstrated that the effective charge varies with pH (see Discussion).

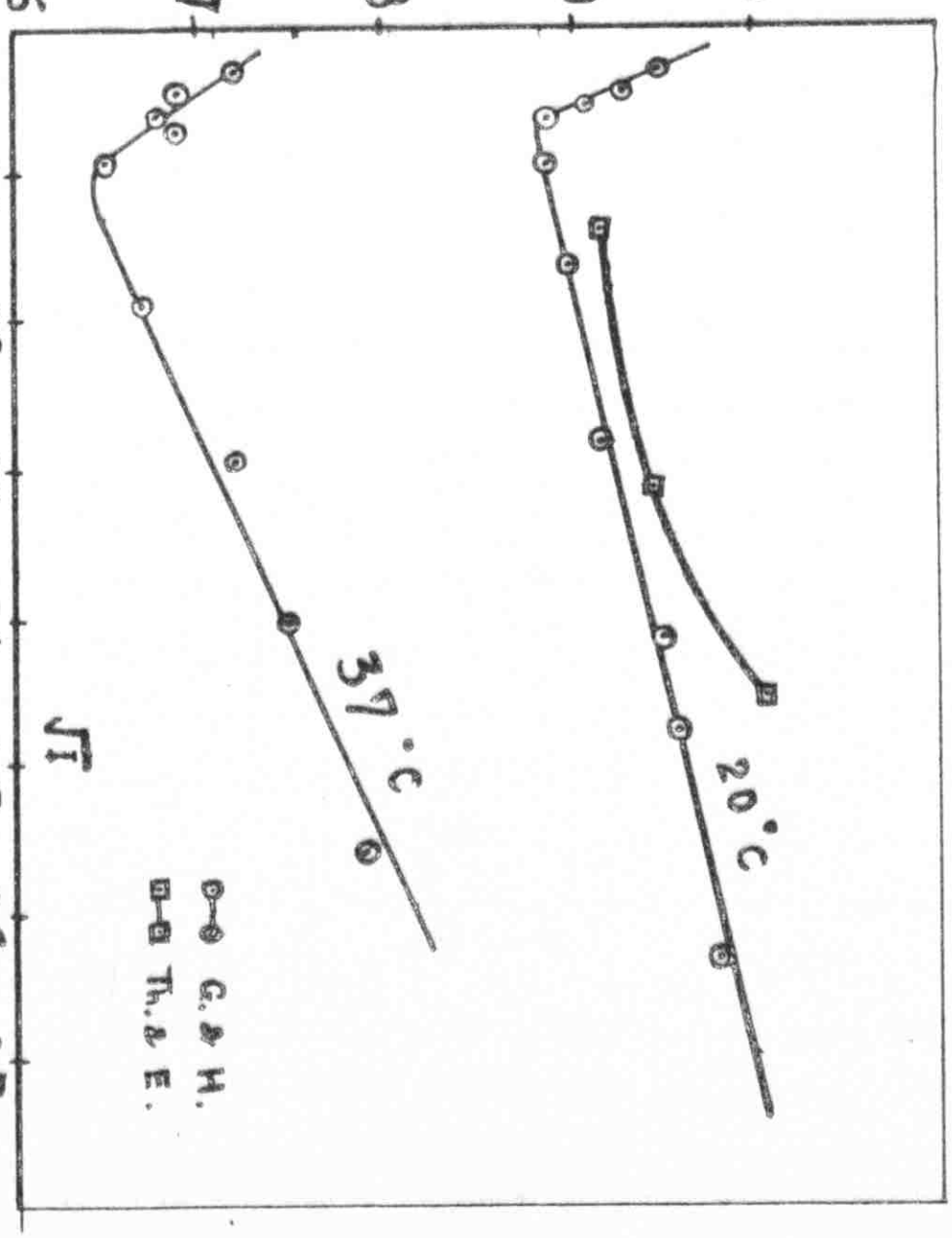

Figure 2 - Variation of pK with Ionic Strength (Data of Th.&E., and G.&H.)

II. The Structure of Ferrimyoglobin

Myoglobin, and to a lesser extent hemoglobin, are the only proteins for which the crystal structure has been determined. The work on myoglobin has been carried out by Kendrew[10,11] and coworkers (1955 - 1963) and the corresponding work on hemoglobin by Perutz[12] and coworkers, both at Cambridge, England.

The three-dimentional X-ray crystal analysis of sperm whale ferrimyoglobin has been done at three resolutions of progressively increasing refinements: 6 Å, 2 Å, and lately at 1.4 Å (1963).

On the basis of Kendrew's findings we may now describe the structure of ferrimyoglobin in the following section:

The Molecule

Myoglobin crystals are monoclinic, the structure of the molecule (Figure 3) being compact with hardly any volume of internal empty space. Very little water is held inside the molecule. The molecular weight is about 18,000.

Myoglobin is a conjugated protein consisting of a globular protein (globin) conjugated to a single heme disc (iron porphyrin). Thus it has one equivalent Fe per mole.

The heme disc is situated nearly perpendicularly to the surface of the molecule and buried within, the hydrophilic end of the heme (the end containing the polar propionic acid groups) being at the surface.

The Protein

Globin is a large protein molecule, a single polypeptide chain consisting of a sequence of about 150 to 155 amino acids. The amino acid composition of myoglobin varies for different species. In fact, such

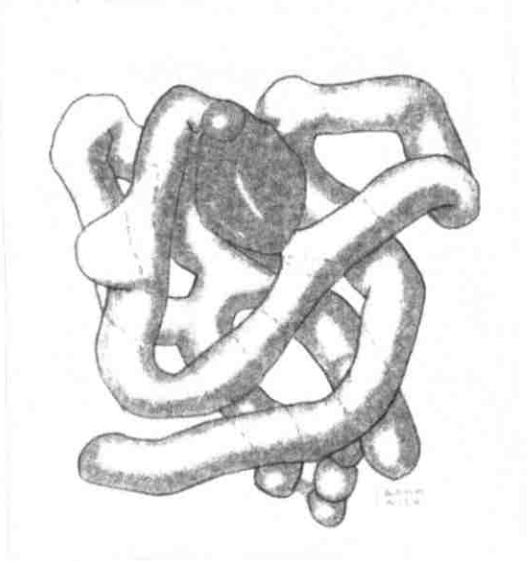

Figure 3 - Model of the myoglobin molecule, derived from the 6Å Fourier Synthesis[11].

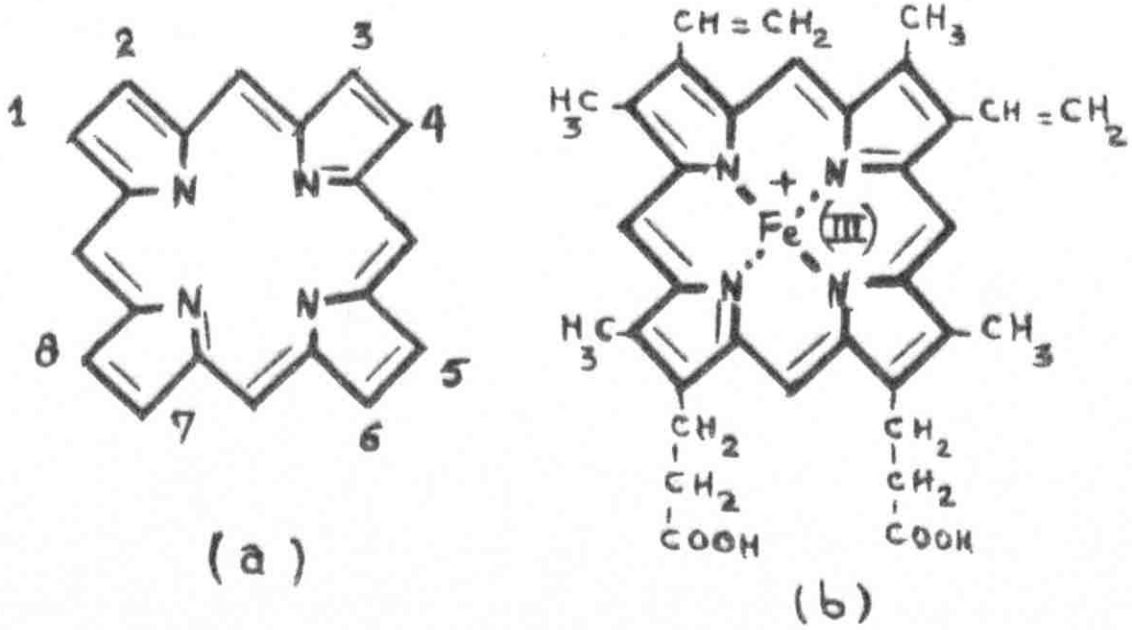

Figure 4 a. Porphyrin ring

4 b. Iron-Protoporphyrin IX (heme)

differences are found even among fetal and adult hemoproteins of the same species.

These amino acids form a right-handed α-helix chain. They are folded in a complex and unsymmetrical manner to form a flattened roughly triangular prism with dimentions about 45 by 35 by 25 $\mathring{A}$.

Almost all the side chains containing polar groups are on the surface, having their polar ends on the outside. The interior of the molecule is thus almost entirely made up of nonpolar residues, closely packed and in van der Waals contact with their nearest neighbours.

Edmundson and Hirs[13] used chromatographic methods to fractionate sperm whale myoglobin. They achieved resolution into five hemoprotein components which were subjected to quantitative amino acid analysis, by tryptic digestion and end group analysis. (Table I). They give the molecular weight of myoglobin, on basis of detailed analysis of components IV & V, including contribution of the heme and the terminal water, as 17,816.

Their results supplement and in general confirm those obtained by X-ray analysis.

The Heme

Hemes are metalloporphyrins; the porphyrin ring is a planar cyclic tetrapyrrole with four methene bridges and a system of eleven conjugated double bonds as shown below (Figure 4a). In ferrimyoglobin the porphyrin is the isomer protoporphyrin IX, (Figure 4b).

When a metallic ion is introduced into the porphyrin, it assumes the central position of the planar ring, forming coordinate bonds with the four nitrogen atoms and displacing the two imino hydrogen atoms, thereby

Table I

Comparison of Amino-Acid Composition of the Chromatographic Components of Sperm Whale Ferrimyoglobin (Edmundson and Hirs[13])

Amino-Acid	Number of Residues (to nearest integer)				
	Component I	II	III	IV+V	V
Aspartic acid	9	8	8	8	8
Glutamic acid	18	19	19	19	19
Glycine	11	11	11	11	11
Alanine	15	17	17	17	17
Valine	8	8	8	8	8
Leucine	16	18	18	18	18
Isoleucine	8	9	9	9	9
Serine	7	6	6	6	6
Threonine	5	5	5	5	5
Methionine	2	2	2	2	2
Proline	5	4	4	4	4
Phenylalanine	6	6	6	6	6
Tyrosine	4	3	3	3	3
Histidine	10	12	12	12	12
Lysine	17	19	19	19	19
Arginine	4	4	4	4	4
Tryptophane	1	2	2	2	2
Amide Ammonia	-	-	-	(7)	(7)
Total	146	153	153	153	153

reducing the charge of the metallic ion by two. Thus in ferrimyoglobin, where the central ion is Fe(III), the net positive charge will be +1 and will represent the formal charge of the heme.

The iron atom is octahedrally bonded, four strong bonds being in the plane of the heme. The fifth, also a strong bond, is coordinated to the protein through a ring nitrogen atom of a histidine residue, the so-called proximal hemelinked histidine. At the sixth coordination position a water molecule is loosely bonded (or -OH in strongly alkaline solutions in which case this sixth bond is also fairly strong). It is believed that this water molecule is hydrogen-bonded to another histidine (called the distal),

Two facts should be noted here:

a) It has not been possible to make direct X-ray analysis of the free heme on account of the instability and strong tendency toward polymerization of the molecule. Hence structural evidence has been derived indirectly from work on analogues of hemes such as the phthalocyanines.

b) Kendrew has recently reported that the iron atom lies more than 1/4 angstrom out of the plane of the group. This, however, is of no direct relevence to the present work.

The Iron-Bound H_2O

It seems to be generally accepted at present that a water molecule is loosely bonded at the sixth coordination position on the iron atom in myoglobin. The evidence for this is circumstantial, and may be summarized as follows:

a) A large number of aquo-ions and simple coordination compounds are known in which the bonding of H_2O, or at least of $-OH^-$, is unequivocal.

b) In general, it is found that the changes in absorption spectra and paramagnetic susceptibility which accompany the reactions of ferrihemoglobin and ferrimyoglobin with F^-, CN^-, or other ligands, are similar to the changes which accompany the ionization. These ligands are known to form direct bonds with the iron atom, and it therefore seems reasonable to conclude that the ionizing group is also iron-bonded.

c) The dehydration (drying) of hemoglobin is known to lead to spectral changes which are characteristic of hemochromogens (where the iron is known to be completely bonded to nitrogen atoms). Haurowitz[14] proved that the change is reversible, and that oxyhemoglobin, where O_2 occupies the sixth bonding position, does not undergo this change. Keilin and Hartree[15] also showed that ferrihemoglobin undergoes this spectral change but that the cyanide, fluoride and azide derivatives (where the ligands are known to be iron-bonded) do not form hemochromogens.

d) The recent crystal structure analysis of Kendrew and Perutz is consistent with the evidence for H_2O bonding. This, however, refers to the solid crystal and not to aqueous solution.

George and Lyster[16] have discussed the evidence for and against a two-bond crevice structure in hemoglobin. In such a state the sixth bond of the iron would be attached to another group on the protein, though not necessarily a nitrogen. Cameron[17] has shown that hemoglobin M, an abnormal human hemoglobin has a two-bond crevice structure, but this is an exceptional abnormality.

It remains true that the cumulative evidence for iron-bound H_2O in aqueous solutions is fairly good.

THEORY

On the above basis, we may represent the acid-base equilibrium which is investigated in the present work as the ionization of the water molecule bound at the sixth coordination position of iron. Thus

$$\underset{\text{(brown)}}{Fe^{+}(H_2O)} \quad = \quad \underset{\text{(red)}}{Fe\text{-}OH} \; + \; H^{+} \qquad (1)$$

where $Fe^{+}(H_2O)$ stands for a molecule of the conjugate acid in ferrimyoglobin with a formal charge of +1, and Fe-OH the conjugate base with its formal charge zero. The other five bonds of the iron atom are not shown.

For convenience, equation 1 may be rewritten in the form:

$$A \; = \; B \; + \; H^{+} \qquad (1a)$$

The thermodynamic ionization constant is defined in terms of activities:

$$K^{o} \; = \; \frac{a_B \cdot a_{H^+}}{a_A} \qquad (2)$$

and hence:

$$pK^{o} = \log \frac{a_A}{a_B} - \log \; a_{H^+} \qquad (3)$$

In this work, an observed ionization constant was obtained using the ratio of molar concentrations of conjugate acid and base (measured spectrophotometrically) and pH of the solution (measured relative to primary standards on a pH-meter). That is, the experimental pK at finite ionic strengths is given by:

$$pK = pH \; + \; Log \; \frac{(A)}{(B)} \qquad (4)$$

In order to compare these two equations, equation 3 should first be rewritten in the form:

$$pK^{o} = \log \frac{(A)}{(B)} + \log \frac{f_A}{f_B} - \log a_{H^+} \qquad (5)$$

where the activities of the conjugate acid and base have been replaced by molar concentrations and their corresponding activity coefficients f_A and f_B.

On combining equations 4 and 5 we now obtain:

$$pK = pK^{o} - \log \frac{f_A}{f_B} + pH + \log a_{H^+} \qquad (6)$$

a relation which shows how an experimental pK value depends on the composition of the solution (ionic strength). The term (pH + log a_{H^+}) would cancel out if pH = - log a_{H^+}. We should now discuss this point in further detail.

It is known that pH may be defined in several ways. However, following primary standards set by the U.S. National Bureau of Standards, it may be assumed that the difference between log a_{H^+} and measured pH under the experimental conditions of the present work is not greater than the limits of experimental uncertainty. In particular, the following points may be noted:

a) Both the standard buffers used, and the test solutions measured, were fairly dilute aqueous solutions. Concequently, the liquid junction potential between the saturated calomel electrode and the standard buffer could not have been significantly different from that between the calomel electrode and the test solution. Thus[18a] liquid junction potential = -0.2 mv. (3.5N KCl versus 0.1N NaCl); and +0.6 mv. (3.5N KCl versus 0.1N KCl), which corresponds to an uncertainty of ± 0.007 in pH.

b) All measurements were made on solutions within the pH range 6-10, that is where the concentration of both H^+ and OH^- is extremely small.

This further limits the values of liquid junction potential and thereby reduces errors.

c) The normal pH standard employed was a 0.01M solution of Borax[18b] with pH = 9.18 ± 0.01 at 25.0°C, and test solutions were in the range pH 8-10. Within this small range of pH difference between test and standard solutions, the assumption of hydrogen-electrode behaviour would be expected to hold fairly accurately.

Since the N.B.S. standard[18c] for pH measurement gives the nearest approximation to log a_{H^+}, and in view of the above discussion, it seems reasonable to assume that the present experimental work is consistent with the assumption: pH + log a_{H^+} = 0 to within the experimental error ± 0.01.

Consequently, for our purposes, equation 6 simplifies to:

$$pK = pK^0 - \log \frac{f_A}{f_B} \qquad (7)$$

Effect of Ionic Strength:

Equation 7 above shows that the measured pK should depend on the total molar ionic strength I of the medium, and that its value will approach pK^0 as $I \longrightarrow 0$. This dependence of pK on the I may be assumed to follow a Debye-Hückel type of relationship at sufficiently low ionic strengths. Using the simplified equation, at 25°C

$$- \log f_i = \frac{0.50\, Z_i^2 \sqrt{I}}{1 + \sqrt{I}} \qquad (8)$$

and substituting into equation 7 yields:

$$pK = pK^0 + 0.50\,(Z_A^2 - Z_B^2)\ \frac{\sqrt{I}}{1 + \sqrt{I}} \qquad (9)$$

where Z_A is the charge in the acid A, namely $Fe^+(H_2O)$, and Z_B that on

its conjugate base B. It is evident that $Z_A = Z_B + 1$.

Using the formal charges $Z_A = +1$ and $Z_B = 0$, equation 9 gives

$$pK = pK^o + 0.50 \frac{\sqrt{I}}{1 + \sqrt{I}} \qquad (10)$$

which leads to the conclusion that the theoretical limiting slope of a plot of pK experimental values against the above function of ionic strength is +0.50.

On the other hand, the observed limiting slope may itself be used in conjunction with equation 9 to calculate the effective charge Z_A, and hence Z_B. As mentioned in section "Historical" above, the limiting slope is actually negative not positive. Therefore, if equation 9 is applied to the data, the effective charge turns out to be negative although iron (III) carries a formal charge of +1 (see Discussion).

Effect of Temperature

Assuming no significant changes in heat capacities over the range of temperature investigated (15 - 35°C.), the Van't Hoff equation gives the dependence of pK on temperature and yields the average value of the enthalpy of ionization over the temperature range covered:

$$\Delta H^o = RT^2 \frac{d\ln K^o}{dT} = 2.303R \frac{dpK^o}{d(1/T)} \qquad (11)$$

Strictly, ΔH^o is obtained from the thermodynamic pK^o values. However, it may also be assumed that at very low ionic strengths $\Delta H \simeq \Delta H^o$.

The value of the standard free energy change in the ionization equilibrium is:

$$\Delta F^o = -RT \ln K^o = 2.303\ RTpK^o \qquad (12)$$

and the corresponding standard entropy change is given by:

$$\Delta S^{o} = \frac{\Delta H^{o} - \Delta F^{o}}{T} \qquad (13)$$

Thus the enthalpy and entropy of ionization may be calculate from pK data.

It should be noted here, that the above simplified derivations assume a single ionization equilibrium. However, experiment shows that pK tends to increase with increasing pH especially at pH $>$ 9 and that the effect becomes more pronounced at lower ionic strengths. This observation suggests that a number of linked ionization effects also appear in the molecule. This phenomenon is considered in the Discussion Section below.

EXPERIMENTAL

I. Ferrimyoglobin

Although ferrimyoglobin from horse heart muscle had previously been investigated[2] no corresponding work on whale ferrimyoglobin has been reported in the literature. It was therefore necessary to establish for this newly isolated myoglobin its purity, iron content, standard absorption spectra, and the thermodynamics of its ionization in dilute aqueous solution.

Sperm whale ferrimyoglobin was obtained from two sources. The first series of experiments was made on a sample which was received as an ammonium sulfate filter cake of crystalline ferrimyoglobin*. It was stored in a deep freeze. The sample was used mainly for the measurement of pK at 25°C.

The data for iron content, spectra and ionization constants at 34.0°C. were obtained using lyophilized sperm whale ferrimyoglobin purchased in salt-free form from Seravac Laboratories**, marked batch I. This was stored in a refrigerator and was used without chromatographic separation into components***. Its iron content was determined by a phenanthroline method and the results were used in establishing extinction coefficients for the standard absorption spectra of the acid and its conjugate base.

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* Kindly provided by Dr. J. Kendrew, Laboratory for Molecular Biology, University of Cambridge, England.

** Seravac Laboratories Pty. Ltd., Colnbrook, England.

*** Edmundson and Hirs (see ref. 13)

To test whether the ferrimyoglobin obtained from the two different sources was the same, and consequently whether results obtained from the two were comparable, determinations of pK under identical conditions were independently carried out on both samples. The results were the same, within the limits of experimental error.

A. Determination of iron in myoglobin as tris-(o-phenanthroline)-iron(II) complex:

The percentage of iron in the ferrimyoglobin samples which were used in the present studies was determined according to the procedure of Dr. B. Cameron*[17]. Experimental details of the determination are as follows:

The sample to be assayed (if solid about 5 mg, if liquid up to 0.2 ml) is transferred to a 10 ml volumetric flask. To this are added 0.1 ml of 70% $HClO_4$ and 0.1 ml of 30% H_2O_2, and the solution is digested in a hot water bath for half an hour. After cooling, the sample is diluted with a little water, and 0.1 ml. of 10% aqueous $NH_2OH \cdot HCl$ is then added. The mixture is left to stand for ten minutes, after which 1.0 ml of 0.5% o-phenanthroline in 50% ethanol is added, followed immediately by 1.0 ml of pyridine. All reagents and solutions used in this determination should be repeatedly redistilled or recrystallized until completely iron-free. Glassware should be thoroughly cleansed and kept in H_2SO_4 - HNO_3 mixture.

The order of adding reagents is very important in obtaining an immediate color development of the complex, and one which is stable (for several days).

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* This procedure is based on a modification (A.D. Adler, unpublished results) of the o-phenanthroline method for the determination of Fe in biological materials (G. Barkan and B.S. Walker, J. Biol. Chem., 138, 37-42 (1940); D.L. Drabkin, J. Biol. Chem., 140, 380-396 (1941)).

The absorbancy of the resulting complex is read against water at λ = 509 mμ, which is the absorption peak of the red complex ion*.

Measurements on Solid

Three solid samples of ferrimyoglobin were taken directly from the bottle as received (Seravac Batch I) and were accurately weighed**, (by difference). These were treated according to the above procedure. Simultaneously, three blank determinations were also made, omitting ferrimyoglobin but treating them exactly the same way as the samples.

Calculations were based on the standard extinction coefficient of 11.0×10^3 M^{-1} cm^{-1} for the tris-(o-phenanthroline)-iron(II) complex as determined on the same stock by Dr. B. Cameron[17]. This extinction coefficient was averaged from determinations by two different complexing agents: Mohr's salt: $Fe(NH_4)_2(SO_4)_2.6H_2O$; and Oesper's salt: $FeC_2H_4(NH_3)_2(SO_4)_2.4H_2O$.

The following data and results were obtained:

Blanks	A(509 mμ)
1	0.008
2	0.008
3	0.008

Mean A(509 mμ) for blank = 0.008.

Samples	Weight(mg.± 0.2%)	A(509 mμ) Corrected for blank	%Fe
1	5.514	0.304_5	0.280
2	7.346	0.408	0.282
3	6.806	0.374	0.279

Average % Fe in ferrimyoglobin (on non-dry weight basis)=0.280±0.001.

---

* These measurements were made on a Zeiss PMQ II spectrophotometer.

** A Cahn Electrobalance was used, with range from 1 μg. to 100 mg with a precision up to ± 0.1 μg.

B. Determination of the extinction coefficient for the ferri-myoglobin cyanide complex:

The cyanide complex of ferrimyoglobin is a convenient standard by which concentrations of ferrimyoglobin solutions can easily be measured. It was therefore decided to determine accurately the molar extinction coefficient of ferrimyoglobin-cyanide complex on the basis of the above iron determination. This standard would then be used in obtaining extinction coefficients for the absorption spectra (Section C below).

About 5 mg of ferrimyoglobin, or if liquid up to 0.1 ml sample, is transferred to a 10 ml volumetric flask. In the case of the solid sample some water is added to dissolve the crystals. To ensure that the myoglobin is present completely in its iron(III) state, a tiny crystal of $K_3Fe(CN)_6$ is added to oxidize any possible trace of ferro- or oxy-myoglobin. Some solid KCN is then added to form the cyanide complex which is known to be stable and has large favorable formation constant. The red colour of the complex appears almost immediately. As a further precaution the mixture is always made up to 10 ml with a buffer solution of pH about 7. The object of buffering the mixture is to prevent possible decomposition or side reactions which are also known to occur at low pH.

The absorbancy of the resulting solution is read against water, at λ = 540 mμ.

Measurement on Solid

Three solid samples of the same stock ferrimyoglobin that was used above were weighed accurately on the Cahn Electrobalance, and the cyanide complex was formed according to the above procedure.

The concentrations of the ferrimyoglobin samples were calculated

based on the percentage of iron determined above. The data and results obtained were the following:

Spectrophotometer blank (water versus water) A = 0.003

Sample	Weight(mg ± 0.2%)	Cyanide complex A(540 mμ) corrected for blank	$10^{-3} \epsilon (M^{-1}cm^{-1})$
1	5.806	0.311	10.6_8
2	6.503	0.346	10.6_1
3	7.314	0.386	10.5_3

Therefore average value of ϵ from measurements on solid samples = $(10.6_1 \pm 0.06) \times 10^3\ M^{-1}\ cm^{-1}$.

Note: This value is to be averaged with the result from a corresponding determination using ferrimyoglobin solution. (Given in the following section).

Measurement on Solution

The above determinations were carried out on solid ferrimyoglobin. However, it was also necessary to test whether the solid is completely soluble upon dissolution in water and whether all the iron is hematin iron.

A dialyzed concentrated solution of the same stock ferrimyoglobin was prepared, filtered, and the two determinations described above were repeated.

a) Iron determination:

Three 0.20 ml samples of this stock solution and three blanks were treated in accordance to the above mentioned procedure for the iron determination. The following data were obtained:

Blanks	A(509 mμ) versus water
1	0.009
2	0.010
3	0.008

Mean blank A(509 mμ) = 0.009

Samples	A(509 mμ) versus water corrected for blank
1	0.315
2	0.317
3	0.317

Mean A(509 mμ) = 0.316_5

b) Extinction of ferrimyoglobin-cyanide complex:

Three 0.20 ml samples of the same stock solution of ferrimyoglobin were treated according to the directions of the cyanide determination. The following results were obtained:

Spectrophotometric blank (water versus water) A (540 mμ) = 0.002

Samples	A(540 mμ) versus water corrected for blank
1	0.309
2	0.310
3	0.310

Mean A(540 mμ) = 0.309_7

Since ϵ = 11.0 x 10^3 M^{-1} cm^{-1} for the phenanthroline complex, it follows that

$$\frac{11.0 \times 10^3}{\epsilon_{CN}} = \frac{\text{A(phenanthroline complex)}}{\text{A(ferrimyoglobin-cyanide complex)}}$$

which gives ϵ of the cyanide complex = 10.7_7 x 10^3

Averaging all values (3 values on solid and 3 averaged values on solution):

For ferrimyoglobin-cyanide, ϵ (540 mμ) = (10.7±0.07) x 10^3 M^{-1} cm^{-1}.

Conclusions

The above experiments lead to the following observations on the properties of the ferrimyoglobin samples which were used in the present study.

a) The solid ferrimyoglobin in stock was analyzed on different occasions and was found, to within 1% error, to contain 0.280% Fe in every case. This fact leads to the conclusion that solid ferrimyoglobin is equilibrated against moisture of the air, and that it is stable.

In order to use iron content for an estimation of molecular weight of myoglobin it would be necesaary to take the water content of the crystals also into account. Dr. B. Cameron[17] has carried out the iron determination on the sample stock of ferrimyoglobin after drying it at 105°C to constant dry weight. His results give an average iron content of 0.306%. On this basis, the molecular weight of myoglobin would be 18,250 (see Edmundson and Hirs[13] value of 17,812 obtained for amino acid analysis of components IV and V, Table I).

b) Similar iron determinations on batch II (a new preparation of ferrimyoglobin purchased from the same Company - Seravac) gave the same results as batch I, within 1%. It therefore appears that the commercial preparation is reproducible.

It is necessary to note, however, that batch II was not further used for other experimental work in the present study.

c) Comparison of results obtained above on solid sample (section A) with those on the solution (section B), shows that unless there are fortuitous effects which balance out,

i) There is no significant amount of insoluble (denatured)

material which would be lost upon dissolving the solid.

ii) There is no significant amount of non-heme iron which would otherwise appear as iron-phenanthroline complex but not as ferrimyoglobin-cyanide complex.

d) In view of the foregoing discussion, the average value $\epsilon = 10.7 \times 10^3\ M^{-1}\ cm^{-1}$ has been taken as the standard extinction coefficient for the ferrimyoglobin-cyanide complex. Consequently the absorption spectra for acid and alkali forms of the ferrimyoglobin (section C) have been plotted based on this value. Similarly, all determinations of the concentration of ferrimyoglobin solutions were based on this value as standard.

C. Absorption spectra of ferrimyoglobin - conjugate acid and base:

The absorption spectra of both acid and alkali forms of ferrimyoglobin were mapped out very carefully from 200 to 2500 mμ on the Zeiss PMQ II spectrophotometer. About 300 points were taken for each spectrum. The details are given below:

a) Solid ferrimyoglobin was dissolved in water, dialyzed in ice-cold water, then filtered to make a concentrated stock solution about 5×10^{-4} M.

b) For the acid, the stock ferrimyoglobin was diluted with NaH_2PO_4-NaOH buffer, I $\sim$ 0.02M, to about pH 6.

For the base, the stock ferrimyoglobin was diluted with Na_2HPO_4-NaOH buffer, I $\sim$ 0.05M, to about pH 11.5. However, this stock was not sufficiently concentrated for the infrared spectrum of alkali ferrimyoglobin, in which case a more concentrated stock was prepared (7×10^{-4}M) and its pH raised to 11 with dilute alkali.

The concentration in every case was adjusted to give optimal absorbancy readings against water.

c) The absorbancy of the buffer used in each case was also read against water over the entire range of wavelength. These values, comprising the spectrophotometric blank, were subtracted from the corresponding absorbancy readings of ferrimyoglobin at each wavelength, for the acid as well as the base.

d) Concentrations of the ferrimyoglobin were determined on the stock, after dilution, by the cyanide method discussed above, taking the extinction coefficient for ferrimyoglobin-cyanide as 10.7×10^3 M^{-1} cm^{-1}.

The standard absorption spectra are shown in Figure 5 (a,b,c) (near UV, visible, and near IR), and the main band characteristics are summarized in Table IIa and IIb.

The comparison of these spectra with those for horse ferrimyoglobin is referred to in the Discussion section.

Table IIa

Acidic Ferrimyoglobin

Characteristics of Absorption Spectra

λ (mμ)	10^{-3} ϵ (M^{-1} cm^{-1}) maxima	minima	shoulder
2500-2000			~ 0.6
1660		0.022	
1510	0.409		
1370		0.025	
1040	0.893		
715		0.150	
635	3.55		
605		2.71	
595-585	2.76		
578		2.71	
505	9.47		
460		7.01	
409.5	156.7		
311		11.6	
290			~ 26.7
280	31.2		
252		23.0	
below 225	∞		

Table IIb

Basic Ferrimyoglobin

Characteristics of Absorption Spectra

λ (mμ)	$10^{-3} \epsilon$ (M^{-1} cm^{-1}) maxima	minima	shoulder
2500-2000			~ 0.190
1700			~ 0.015
1660		-0.006	
1520	0.085		
1440			~ 0.067
1365		0.003	
1070- 980			0.104-0.142
825	0.754		
740			~ 0.554
675		0.421	
595			8.66
583	8.82		
565		7.51	
542	9.24		
515		7.11	
414	97.2		
358			33.5
313- 310		14.0	
290			28.2
281	32.1		
264		30.0	
below 225	∞		

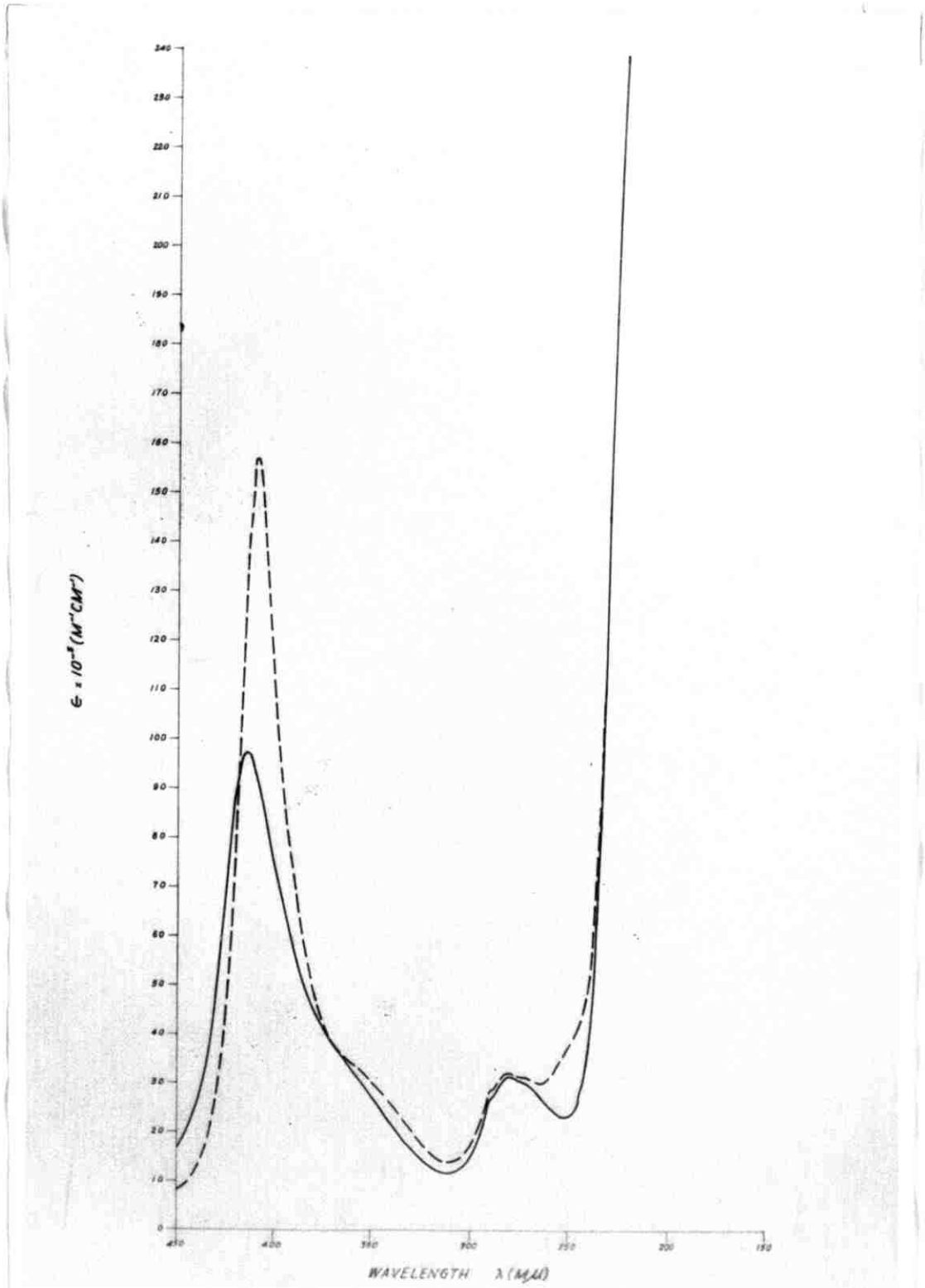

Figure 5a - Absorption Spectra for Acidic and Alkali forms of Ferrimyoglobin

Near U.V.

----- Acidic ; ——— Alkali .

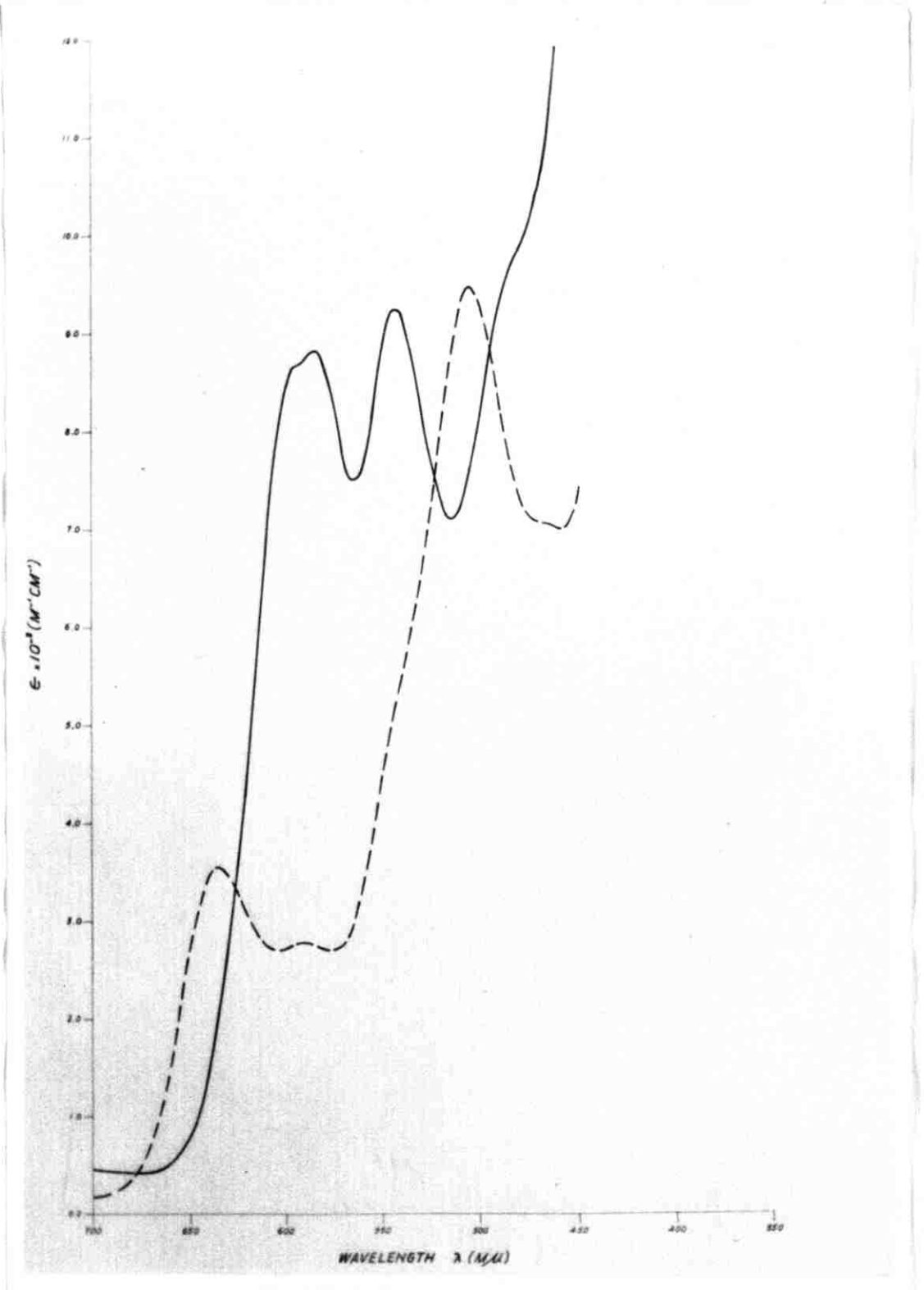

Figure 5b - Absorption Spectra for Acidic and Alkali forms of Ferrimyoglobin

Visible

----- Acidic ; ——— Alkali .

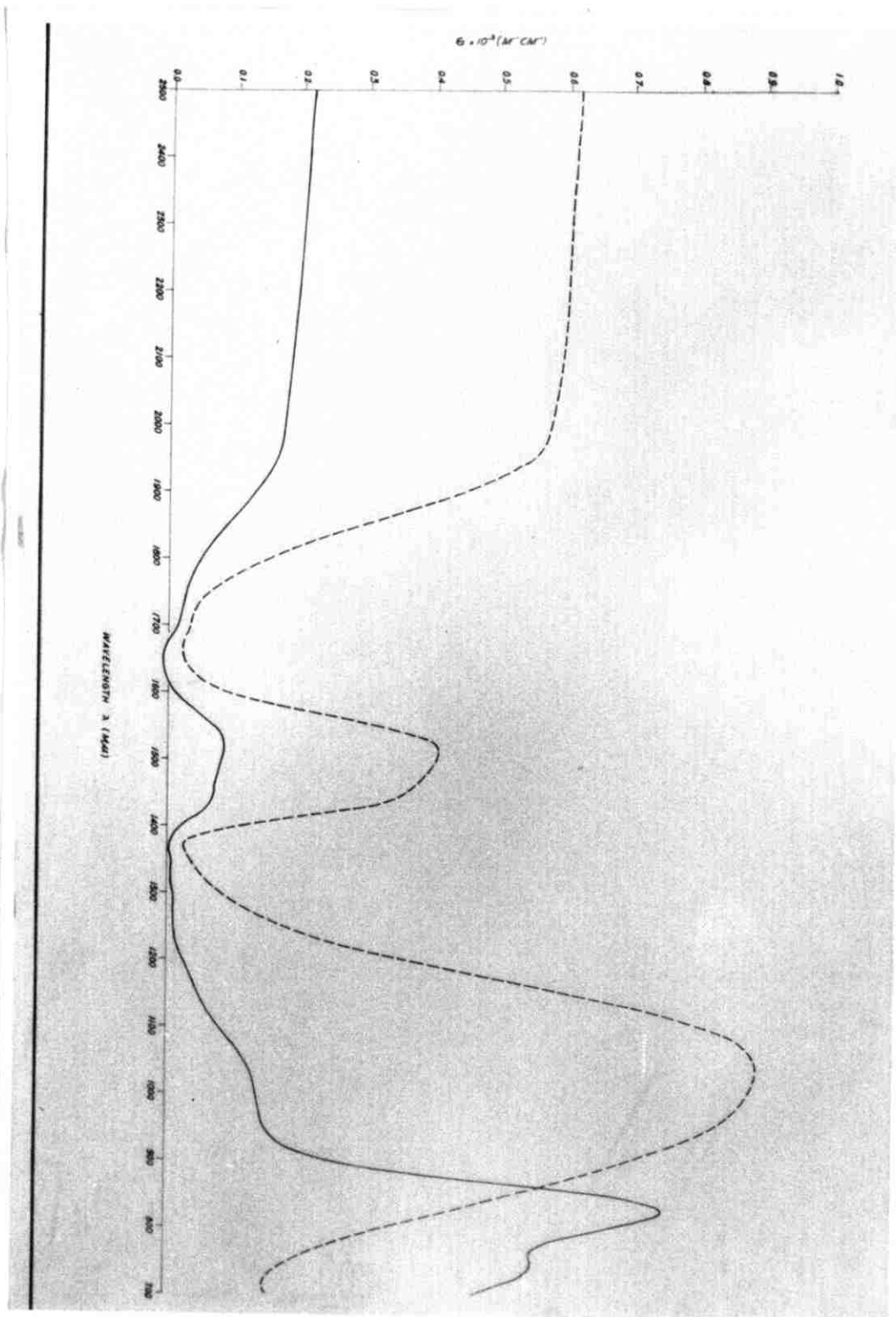

Figure 5c - Absorption Spectra for Acidic and Alkali forms of Ferrimyoglobin

Near I.R.

----- Acidic ; _____ Alkali .

II. Measurement of Ionization Constant

The ionization constant of ferrimyoglobin, referring to the equilibrium in equation 1, was measured spectrophotometrically over a range of pH, I and T. A series of buffered solutions was prepared for every experiment, and pK was calculated from equation 4. Experimental details are described in this section.

A. Solutions:

1) Buffers: Experiments were usually made on buffered solutions within the pH range 8 to 10. For this pH range a series of borate buffers was found most suitable.

The buffers were prepared by mixing initially calculated amounts of boric acid and sodium hydroxide. Since buffers at higher pH require bigger proportions of NaOH to H_3BO_3, it follows that the resulting ionic strengths of the various solutions would not be constant but would increase with increasing pH. Hence, in order to prepare a series of such buffers at constant total ionic strength, it was necessary to add progressively smaller amounts of the neutral salt, NaCl, to the series of buffers prepared between pH 8 and 10. Molar ionic strengths were computed on the basis of Na^+, $H_2BO_3^-$, and Na^+, Cl^- concentrations. The volume was made up by adding de-ionized, distilled and boild out (CO_2-free) water.

Thus it was possible to prepare a series of about ten buffers at the same ionic strength, covering the pH range of 8 to 10 with a difference of about 0.2 pH units between successive ones.

Buffers were prepared at ionic strengths 0.22, 0.11, 0.055, 0.022, 0.011 and 0.0055M, and these were further diluted as necessary.

Two other buffered solutions were prepared. A $NaH_2PO_4.H_2O$-NaOH

buffer at pH 6.0 - 6.2 was the reference for 100% $Fe^{+}(H_2O)$. A glycine - NaOH at pH about 11 was used as reference for Fe-OH.

All chemicals were of analytical grade and were used for making the above buffer mixtures without further purification.

2) Solution mixtures: Solutions for measurement were prepared in initially washed and dried test tubes. 1.0 ml of the stock ferrimyoglobin (dissolved in de-ionized distilled water, and dialysed overnight) was pipetted into each one of these test tubes, to which 9.0 ml of buffer was added, a different one from the set to each, thus forming a series of 10.0 ml solutions of ferrimyoglobin of same total concentration (about 5×10^{-6}M) and ionic strength (0.20, 0.10, 0.050, 0.020, 0.010 and 0.0050M), but different pH values.

At intermediate and lower ionic strengths, solutions were prepared using the same stock buffers. The procedure mentioned above was followed, except that less than 9.0 ml of the buffer was used, water being added to make the volume up to 10.0 ml. The water used was de-ionized distilled and boiled out.

The reference acid and base solutions were prepared similarly, care being taken to have the acid solution at the same ionic strength as the set. The reason for taking this precaution was that the absorbancy of acidic ferrimyoglobin varies with ionic strengths (see section D).

For the very low ionic strengths, 20.0 ml of solution were prepared by doubling the amounts necessary. This was done deliberately to render possible the measurement of absorbancy and pH simultaneously, and to minimize changes of pH and ionic strength caused by the absorption of carbon dioxide from the atmosphere especially at very low I and high pH.

After making up the solutions, the test tubes were covered with wax paper, shaken and transferred to the thermostat at the desired temperature. (Most experiments were performed at 25.0° and 34.0°C).

B. Apparatus and procedure:

The solutions were thermostated in a bath with automatically controlled cooler and heater*, temperature being controlled to ± 0.1° or better. From the thermostat, water was circulated through the spectrophotometer cell compartment and the solution-vessel of the pH-meter, so that all the measurements could be made at the same constant temperature. (Control within ± 0.2°C. or better).

The temperature of the solution in the cuvette of the spectrophotometer was taken as the temperature of the whole run. It was measured with a "tele-thermometer"**, which reads to within 0.1°C. This thermometer was calibrated against a standard thermometer.***

Spectrophotometric measurements

The test tubes were left in the water bath for about ten minutes so that the solution would attain the temperature of the bath. Then they were taken out one at a time, their contents mixed, and the absorbancies of the solutions were measured on a spectrophotometer**** at a wavelength of 410 mμ, using distilled water as the blank.

A pair of matched quarts cuvettes of 1.0 cm light path were used after being left in dilute nitric acid (6N) for about ten minutes and

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* Lo-Temp Bath; Wilkens-Anderson Co., Chicago, U.S.A.

** Yellow Springs Instrument Co. Inc., Ohio, U.S.A.

*** Calibrated by the National Physical Laboratory, England, and certified in 1961.

**** Unicam SP 500 quartz spectrophotometer. 200-1000 mμ range, Cambridge, England.

rinsing well with distilled water. These cells were always rinsed with alconox solution and kept in very dilute (0.1N) nitric acid.

Absorbancy readings were taken at 410 mμ because, at this wavelength, the conjugate acid has a sharp peak and because there is a large difference in the absorbancies of the acid and base forms. Care was taken to have the ferrimyoglobin concentration such that the optical scale would be accurate and the differences of absorbancies would be a maximum among consecutive solutions. Optimal conditions were found to be at $A \simeq 0.8$ for $Fe^{+}(H_2O)$ so that actual readings on the solution were in the range $A = 0.4$ to 0.6.

Measurement of pH

All pH measurements were taken on a pH meter* which read to within 0.002 pH units. A reference calomel electrode (K400) and a glass electrode (Radiometer, Type G 200B) were used. The instrument was first standardized at the desired temperature with Borax buffer. (see below). Then the pH of each one of the solutions was measured immediately after the absorption was read.

In the case of very dilute buffers at the lowest ionic strengths, where CO_2 effects became significant, it was necessary to measure pH simultaneously with the measurement of absorbancy; this was done with the assistance of another person.

After taking all readings, the instrument was again checked with the standard buffer. Usually there was good agreement, the maximum

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* pH meter 4, type PHM 4c, Radiometer Co., Copenhagen, Denmark.

discrepancy being about 0.05 pH unit. This difference was corrected for in the calculations.

Standard buffers used for the standardization of the pH-meter were:

Buffer		Standard pH at t^0 C				
		15	20	25	30	35
Borax	0.01M	9.27	9.22	9.18	9.14	9.10_5
KHphthalate	0.05M	4.00	4.00	4.01	4.01	4.02
KH_2PO_4	0.025M		6.88	6.86	6.85	
Na_2HPO_4	0.025M					

The values given above are by the National Bureau of Standards[18d].

C. Sample Run

A typical run is reproduced here in detail to give an idea about the procedure followed. This run does not illustrate the phenomenon of pK values progressively increasing with rise in pH (see Discussion).

Date of experiment: March 26, 1963

Stock solutions:

1. Ferrimyoglobin solution in water (approximately 5×10^{-5}M)
2. H_3BO_3 - NaOH buffers (0.15M) of pH 8.0 8.2 8.4 8.6 8.8 9.0 9.2 9.4 9.6 9.8 all adjusted with NaCl to total I = 0.22M
3. Glycine - NaOH buffer of pH~11
4. Phosphate buffer pH~6.2, I = 0.22M

Dilutions were made with de-ionized, distilled boiled out water.

Conditions:

1. T = 34.0 ± 0.2°C.
2. I = 0.020M
3. Absorbancies taken at λ = 410 mμ.

Calculation of pK:

For each of the nine test solutions, pK was calculated using equation 4. The ratio of acid to base forms is obtained from the relation

$$\frac{\text{(Alkaline ferrimyoglobin)}}{\text{(Acidic ferrimyoglobin)}} = \frac{A_{acid} - A_{mixture}}{A_{mixture} - A_{alkali}}$$

where A is the absorbancy. In this run, A_{acid} and A_{alkali} were taken to represent 100% of each form. The data and calculation are shown in Table III.

Table III

Sample Run

Test Solutions	pH	A(410mμ)	log A/B	pK (calc.)
2.0 ml Mb + 2.0 ml buffer pH 6.2 + 16.0 ml H_2O *	~6	0.445		
2.0 ml Mb + 9.0 ml buffer pH 11.1 + 9.0 ml H_2O**	~11	0.258		
1. 2.0 ml Mb + 2.0 ml buffer pH 8.0 + 16.0 ml H_2O	8.010	0.415	+0.719	8.729
2. 2.0 ml Mb + 2.0 ml buffer pH 8.4 + 16.0 ml H_2O	8.410	0.388	+0.358	8.765
3. 2.0 ml Mb + 2.0 ml buffer pH 8.6 + 16.0 ml H_2O	8.590	0.372	+0.194	8.780
4. 2.0 ml Mb + 2.0 ml buffer pH 8.8 + 16.0 ml H_2O	8.780	0.349	-0.023	8.755
5. 2.0 ml Mb + 2.0 ml buffer pH 9.0 + 16.0 ml H_2O	8.960	0.331	-0.194	8.766
6. 2.0 ml Mb + 2.0 ml buffer pH 9.2 + 16.0 ml H_2O	9.140	0.316	-0.347	8.793
7. 2.0 ml Mb + 2.0 ml buffer pH 9.4 + 16.0 ml H_2O	9.315	0.300	-0.538	8.777
8. 2.0 ml Mb + 2.0 ml buffer pH 9.6 + 16.0 ml H_2O	9.485	0.289	-0.702	8.780

Mean pK = 8.77 ± 0.01

Water versus water at the beginning A(410 mμ) = 0.000

Water versus water at the end A(410 mμ) = 0.000

pH standard at the beginning = 9.105

pH standard at the end = 9.100

* Reference acid

** Reference base

D. Investigation of Specific Effects

1) Buffers: The results of pK measurements reported in this thesis were all obtained on solutions containing borate buffers where the total borate concentration was 0.067M at I = 0.10M, and proportionately less at lower ionic strengths.

The possibility of specific buffer effects appearing was investigated by carrying out two parallel runs, one with a series of borate buffers and the other with tris-(hydroxymethyl)-aminomethane buffers, both runs at 25.0°C. and I = 0.10M. The experiment was performed as described above for the sample run, and a pK value was calculated from every experimental point within the range of pH 8 - 10. The results are plotted as pK versus pH in Figure 6.

It is evident from the figure that borate buffers give the most consistent set of results. Thus all 10 points, including the two different borate concentrations and covering 14.5% to 90.8% Fe-OH formation, give the mean pK = 8.96 ± 0.02.

On the other hand, there is a distinct specific effect due to "tris" buffer. Even for the case where "tris" concentration is the same as that of borate (0.067M) there is a difference of 0.05 - 0.02 between the two sets of results, the three "tris" points giving a mean pK = 8.92 ± 0.015. The discrepancy is far greater in the case of the more concentrated "tris" buffer, and the results vary much with pH.

Of course this experiment does not prove that specific effects are entirely absent in solutions containing borate buffers. In any case the solutions contained varying concentrations of sodium chloride which were needed to make up a constant total ionic strength, and it is conceivable

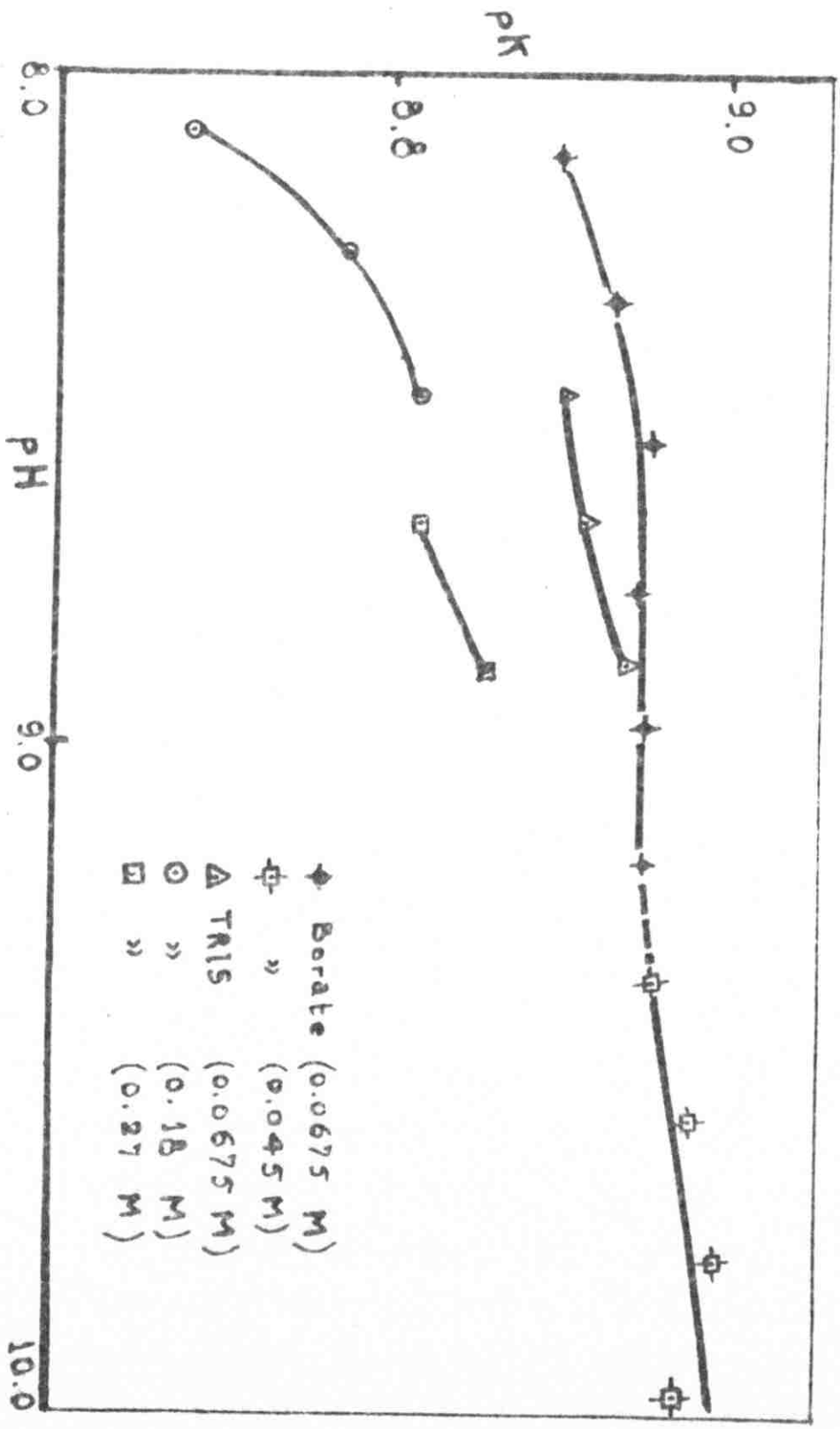

Figure 6 - Investigation of Specific Buffer Effects

T = 25.0°C. I = 0.10M

that the combined effect of buffer and salt is a constant error in these measurements. Nevertheless, the degree of internal agreement in the case of borate buffers was considered sufficient for present purposes.

2) Effect of Ionic Strength on Absorption Spectrum of $Fe(H_2O)$: A number of preliminary observations in the early stages of the work seemed to indicate that the absorbancy of 100% $Fe(H_2O)^+$ at its Soret peak (410 mμ) varies a little with ionic strength. This phenomenon was investigated as follows:

The wavelength range λ = 405 to 415 mμ was carefully mapped out for $Fe(H_2O)^+$ at four ionic strengths. The absorbancies were corrected to 100% acid using the measured pH of the solution, the approximate pK under the same conditions (as given in Table IV) and the estimated ratio of absorbancies of acid to base. The curves are plotted in Figure 7 and the results are summarized below:

Curve	a	b	c	d
I (M)	0.0050	0.010	0.050	0.10
A(410 mμ)	0.672	0.674	0.677_5	0.680_5

Thus there is an increase in absorbancy of about $1^1/_4\%$ paralleling the increase in I from 0.0050 to 0.10M. Although this is hardly beyond the limits of experimental error it seems to be a definite trend which is internally consistent and uniform.

This effect was also examined by comparing the ratio of absorbancies of the acidic to alkaline forms of ferrimyoglobin, (A)/(B), for every experiment performed. The ratio seems to vary between 1.70 - 1.75 for

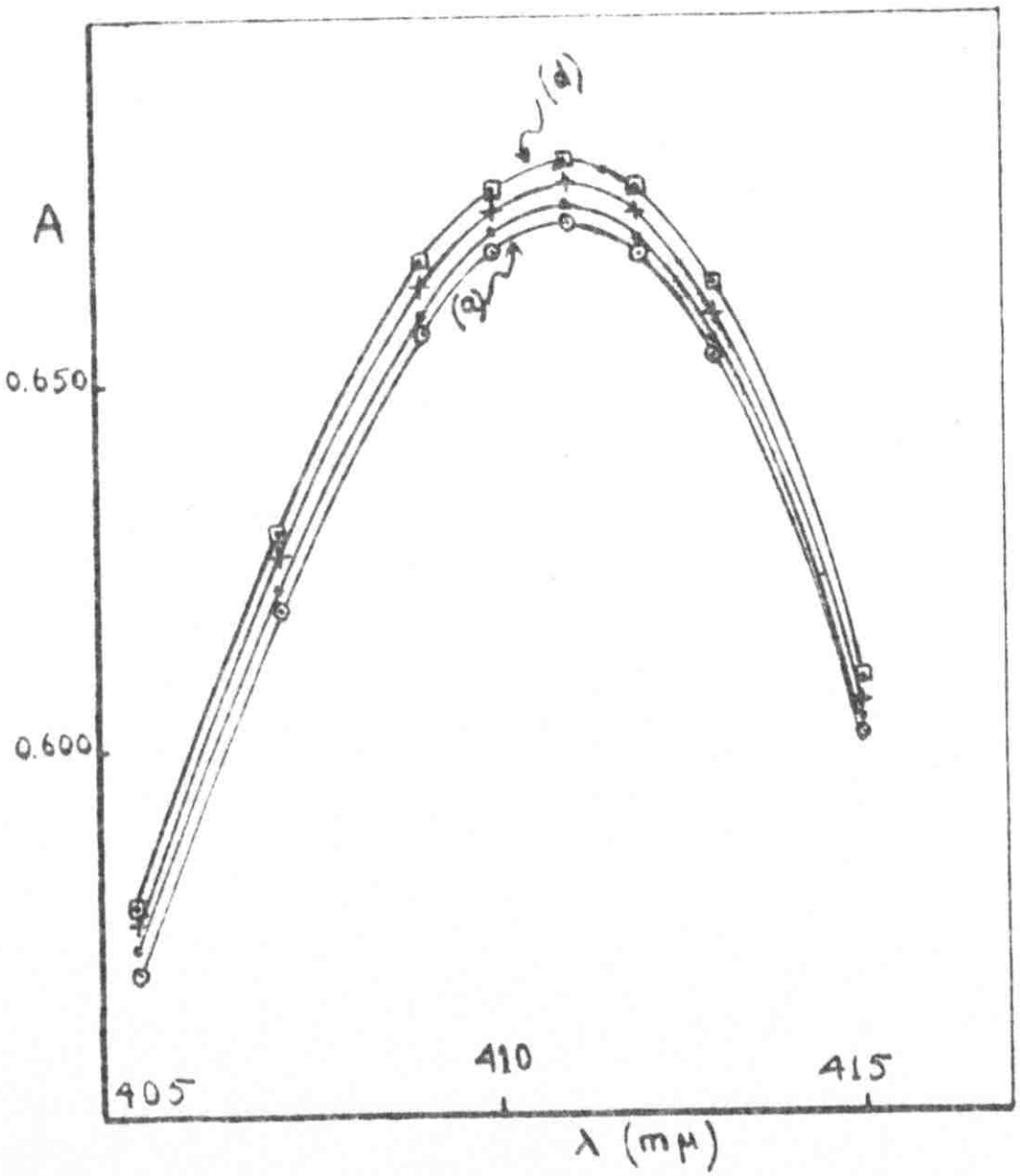

Figure 7 - Soret Absorption Band of $Fe^{+}(H_2O)$

T = 25.0°C. (I = 0.0050(a); 0.010 (b);
0.050 (c); 0.10 (d)).

cases where $I \leq 0.02$, and 1.75 - 1.80 where $I \sim 0.10M$, representing an increase of about 3 ± 3%. Now, if it is assumed that the absorbancy of 100% Fe-OH at 410 mμ does not vary with ionic strength, it follows that the above increase in the ratio (A)/(B) is due to the effect of ionic strength on the absorbancy of 100% $Fe^{+}(H_2O)$. This would be in accord with the above conclusion.

3) Other Effects: A number of other experiments were performed to test the possibility of binding of Cl^- ions and the binding of H^+(heme-linked ionization) in the pH range 6 to 7. Small but consistent effects were detected in both cases though only ~1% changes were observed in the absorbancy of $Fe^{+}(H_2O)$. However, the results are far from conclusive since the effects of H^+, Cl^-, buffer or acid anions, as well as total I, could not be tested independently, since they are related to each other in such a way that when one varies some of the other variables also change accordingly.

In any case, these second-order effects do not alter the precision with which pK is determined (which is calculated from measurements around pH 9).

RESULTS

The procedure described in the previous chapter was followed in determining pK within the pH range 8 - 10, at ionic strengths from 0.20 to 0.001M, and at temperatures from 15.3 to 34.0°C. (Most experiments were done at 25.0 and 34.0°C.).

Each pK value reported is the average for a run which normally has 8 to 10 solutions, and pK is given as the arithmetic mean ± mean deviation. In the case of solutions where $I < 0.02M$, the variation of pK with pH (see Discussion) became large, consequently pK was limited to the mean of values obtained within the range 30 - 60% alkaline form (from about four solutions only).

Table IVa gives a summary of the results at 25.0 and 34.0°C. at several values of I, and Table IVb gives the corresponding pK values at I = 0.02M at five different temperatures. The data are also plotted in Figures 8 and 9.

Figure 8 shows clearly the reversal of the salt effect, the slope of pK versus $\sqrt{I}/(1+\sqrt{I})$ changing from positive to negative around I = 0.01M, as ionic strength decreases.

Upon linear extrapolation of the experimental pK values at low ionic strength to I = 0, thermodynamic pK^0 values are obtained. The slope of the extrapolation line can also be measured. The results are given below:

T(°C.)	25.0	34.0
pK^0	8.99 ± 0.025	8.82 ± 0.025
Limiting Slope	-1.4	-1.6

From the two pK^o values, the enthalpy of ionization may be calculated using equation 11

$$\Delta H^o = 2.303\ R\ \frac{\Delta(pK^o)}{\Delta(1/T)} = 7.8 \text{ Kcal/mole.}$$

Δ H at I = 0.02M may also be calculated from the data in Table IVb and Figure 9, and assuming that $\Delta H^o \sim \Delta H$

$$\Delta H^o = 2.303\ R\ \frac{d(pK)}{d(1/T)} = 6.6 \text{ Kcal/mole.}$$

Hence, average $\Delta H^o = 7.2 \pm 0.6$ Kcal/mole.

At 25°C., using equations 12 and 13

$$\Delta F^o = -R\ T\ \ln K^o = 12.26 \pm 0.03 \text{ Kcal/mole.}$$

Therefore $\Delta S^o = (\Delta H^o - \Delta F^o)/T = -17.0 \pm 2.1$ cal/mole/deg.

The effective charge Z_A may also be calculated using equation 9. Taking the average limiting slope as -1.5, it follows that

$$\text{Slope} = \frac{d(pK)}{d\left\{\sqrt{I}/(1+\sqrt{I})\right\}} = 0.5\left\{Z_A^2 - (Z_A - 1)^2\right\} = -1.5$$

and so, $Z_A = -1$

$$Z_B = Z_A - 1 = -2$$

Table IVa

Variation of pK with Ionic Strength at 25.0 and 34.0°C.

I (M)	$\sqrt{I}/(1+\sqrt{I})$	pK at 25.0°C.	pK at 34.0°C.
0.20	0.308	9.08 ± 0.03	-
0.20	0.308	9.00 ± 0.02	-
0.20	0.308	9.00 ± 0.03	-
0.10	0.240	8.95 ± 0.03	8.87 ± 0.01
0.10	0.240	8.96 ± 0.02	-
0.050	0.183	8.94 ± 0.025	8.80 ± 0.01
0.020	0.124	8.91 ± 0.02	8.77 ± 0.01
0.010	0.091	8.88 ± 0.02	8.75 ± 0.02
0.0077	0.081	8.90 ± 0.02	-
0.0050	0.066	8.91 ± 0.01	8.75 ± 0.01
0.0041	0.060	8.91 ± 0.025	-
0.0033	0.055	8.94 ± 0.01	-
0.0028	0.050	-	8.75 ± 0.025
0.0025	0.048	8.93 ± 0.02	8.74 ± 0.01
0.0020	0.042	-	8.76 ± 0.01
0.0017	0.040	8.93 ± 0.02	-
0.0011	0.032	8.95 ± 0.03	8.76 ± 0.01
0.0011	0.032	-	8.78 ± 0.02
0.00085	0.028	8.95 ± 0.03	-
0.00085	0.028	8.94 ± 0.02	-

Table IVb

Variation of pK with Temperature at I=0.02M

T(^{o}C)	10^{3}(1/T) (^{o}K)	pK
15.3	3.47	9.07 ± 0.01
20.5	3.40_{5}	8.99 ± 0.01
25.0	3.35_{5}	8.91 ± 0.02
30.0	3.30	8.86 ± 0.03
34.0	3.26	8.77 ± 0.01

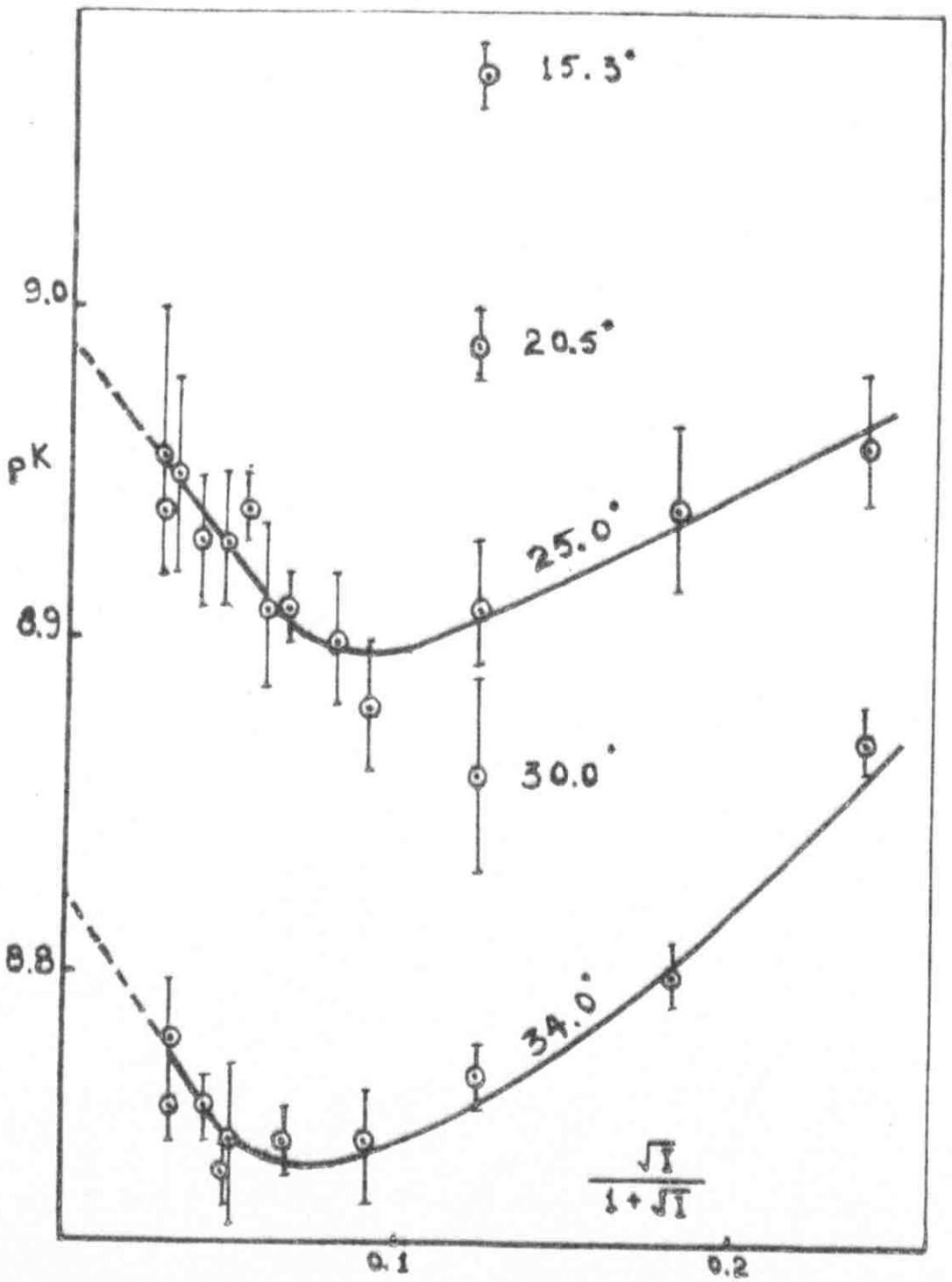

Figure 8 - Variation of pK for Sperm Whale Ferrimyoglobin with Ionic strength and Temperature.

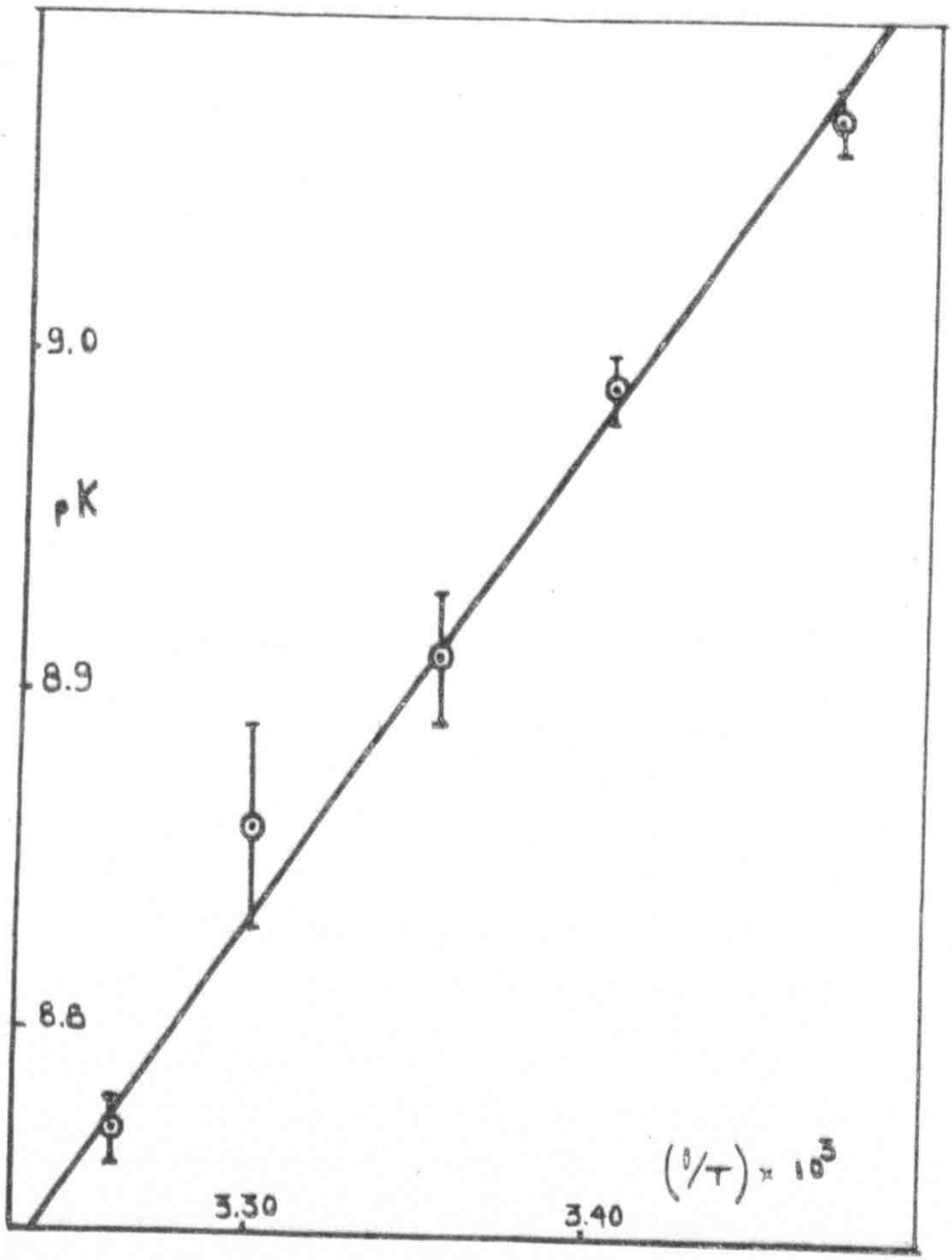

Figure 9 - Variation of pK with Temperature, Sperm Whale Ferrimyoglobin, I = 0.02M.

DISCUSSION

In the preceding chapters, an attempt has been made to obtain certain fundamental data on sperm whale ferrimyoglobin in dilute aqueous solution. This information should form the basis of further work on this substance and should complement the detailed structural information which X-ray crystallographic studies are yielding for the solid.

The main feature of the ionization of acidic ferrimyoglobin is that it refers to the water molecule bonded to iron. The indirect evidence for this was discussed in the early part of the thesis. It is now of interest to ask why the thermodynamic constants obtained, namely $pK^0 = 8.99$; $\Delta H^0 = 7.2$ and $\Delta S^0 = -17$ at 25^0C (see Results), are far from similar to the corresponding values for the ionization of a water molecule, namely $pK_w = 14.0$; $\Delta H^0 = 13.5$ and $\Delta S^0 = -18.7$ at 25^0C.

The difference is explained mainly by the fact that the ionizing water molecule in this study is not independent of its hemoprotein environment as is the case of a free water molecule, but it is coordinated to a large complicated molecule through a metal, and this is expected to affect the ionization considerably (Hanania and Irvine[19]). A comparison of enthalpy and entropy values shows that the major difference between the case of free water and that of the water in ferrimyoglobin is reflected, in enthalpy (decreased endothermicity) and not in entropy. Hence, the effect of coordination to the metal is one of bonding rather than a change in the structure of the molecule itself.

Another main feature of the above results is that the ionization is influenced very markedly by neutral salts. The addition of sodium

chloride enhances the degree of ionization up to ionic strengths of about 0.01M, but further addition of the salt represses the ionization. Thus for the graph of pK versus $\sqrt{I}/1+\sqrt{I}$) (Figure 8), the slope is positive at $I > 0.01$ and negative at $I < 0.01$M. This reversal of the salt effect may be examined in terms of a Debye-Hückel extended equation.

Assuming that a Debye-Hückel limiting law is applicable to the region of dilute solutions, and using the average limiting slope of -1.5, the effective charge on acidic ferrimyoglobin was calculated to be -1 (see Results) and not +1, in this low ionic strength region.

Since the slope, and consequently the effective charge, vary with ionic strength, it must be due to some electrostatic rather than resonance effects. Moreover, as this effect of negative charge diminishes with very slight increase of neutral salt concentration and disappears completely at $I > 0.01$M, it follows that the (electrostatic) interaction between the negative charges and the iron atom is very weak and easy to mask. This conclusion is of significance in structural considerations and in the interpretation of second-order effects.

One of these secondary effects is the variation of pK with pH at very low I, observed in the present work. As pointed out in the chapter on Results, pK was found to increase with increasing pH and the effect became progressively greater at lower ionic strengths. In fact, this effect became so pronounced at $I < 0.02$M, that only values of pK obtained from solutions where ferrimyoglobin was 30 - 60% in alkaline form could be meaningfully used to calculate pK. The extent of this effect may be seen in the following set of results obtained at 25°C. and I = 0.0041M.

pH	8.30	8.51	8.68	8.89	9.06	9.24	9.40	9.54	9.72
pK	8.86	8.86	8.90	8.93	8.93	8.98	9.02	9.07	9.11

George and Hanania[9] have studied this effect in more detail for the corresponding ionization in horse ferrimyoglobin. Having observed the dependence of pK on pH at low I, the authors interpolated values of pK at constant pH and at varying I values, and obtained pK as a function of I. From this they concluded that the effective charge on iron in ferrimyoglobin changes from about +1 to nearly -4 as the alkalinity of the solution increases from pH $<$ 8 to pH $>$ 9.8, as can be seen in the following table:

pH	pK^0	limiting slope	effective charge on Fe-(H_2O)
7.8	8.81	+0.5	+1
8.6 to 9.3	9.07	-2.5	-2
9.8	9.25	-4.5	-4

To account for this negative charge transmitted to the iron atom, the immediate environment of the iron (heme as well as protein) should be taken into consideration. The porphyrin ring has two propionic acid side chains at positions 6 and 7 (see Figure 4a and b). The protein has one terminal carboxylic acid group, while in the long peptide chain there are some amino acids that have side groups (like -COOH or $-NH_3^+$). Thus, amino acids like aspartic acid and glutamic acid impart a negative charge while amino acids like lysine lose their positive charge upon ionization thereby releasing the effect of some negative charge. Hence, although most of the acidic

groups in the molecule are probably located at considerable distances from the heme iron, they are nevertheless able to influence the effective charge on the iron, and consequently the ionization of the water molecule which is coordinated to it. This observation indicates that there are electrostatic inductive effects in the molecule.

It would be interesting at this stage to compare the results obtained in the present study on sperm whale ferrimyoglobin with corresponding data on other hemoproteins. A summary of available data[20] is given in Table V .

Table V

Comparison Of Thermodynamic and Charge Properties of different Hemoproteins

Hemoprotein	$pK^o(25^oC)$	ΔH^o(Kcal/mole)	ΔS^o(e.u.)	Z	I.E.P.	I(critical)
Ferrimyoglobin, sperm whale muscle	8.99±0.025	7.2 ± 0.6	-17.0±2.1	-1		0.01
Ferrimyoglobin, horse heart	8.97±0.02	5.75±0.67	-21.6±2.4	-2	7.0	0.01
Ferrihemoglobin, horse blood	8.81±0.02	3.91±0.49	-27.2±1.8	-2	7.0	0.2-0.3
Ferrihemoglobin, chironomous blood	8.19	3.8	-25	-4		
Ferrihemoglobin, Soybean Nodules (slow component)	8.49_2±0.02	2.15±0.75	-31.5±2.6	-1	4.4	0.05

Inspection of the data in Table V shows that sperm whale and horse ferrimyoglobins are very similar in their thermodynamics of ionization and in the salt effect. There is, however, a difference which is probably quite significant. At $I < 0.01M$, the effective charge on $Fe(H_2O)$ in whale is -1.

while that in horse is -2. Since, as mentioned above, the charge is effected by interactions with the heme and probably the protein as well, this observation suggests that there is a difference in the environment of the heme of these two species.

An inspection of visible absorption spectra of whale and horse ferrimyoglobins also shows a small difference which might be significant. In general the spectra are quite similar (for horse spectra refer to George and Hanania[8]), with extinction coefficients of whale ferrimyoglobin about 3% higher than that of horse. However, at 583-5 mμ and 595 mμ there is a double band in alkaline horse ferrimyoglobin, whereas in whale the 583 mμ is present but the 595 one is a shoulder about 2% lower in extinction coefficient. This difference is very small but nevertheless it is interesting. For, it is known that absorption at this wavelength is related to the Fe-OH bond character (being maximum for a completely ionic bond, and minimum for a completely covalent bond). Consequently, any differences in absorption at this 595 mμ band might reflect differences in bonding between the two cases. This is in line with the above conclusion about structural difference.

In conclusion, it may be useful to outline some of the unsolved problems relating to the present study and to indicate possible lines of future work in this area. There are three major questions to be answered:

a) Do the chromatographically pure components of ferrimyoglobin (see Structure) show any differences? It is known[13] that only component I, which forms 3% of the mixture, is significantly different in amino acid composition from the other four components, and yet all components must be different in charge distribution since they are separated on ion-exchange

columns. It is necessary to investigate the possible differences in thermodynamic and charge properties among these components.

b) To what extent do second-order interactions and ionizations influence the data obtained on pK?

The present work has shown that absorbancy of $Fe^{+}(H_2O)$ varies slightly with ionic strength. No corresponding inspection at the alkaline end was made but it may prove useful to do so. In anycase, it remains to be shown what this effect is due to. It may involve binding of salt or buffer ions; it may be a heme-linked ionization; and it may also involve a combination of factors.

The direct spectrophotometric measurement of a heme-linked ionization should be tried, since it is known indirectly from a number of studies[2] that such an ionization appears, around pH 6, and that it is not masked by neutral salts. Preliminary experiments have shown that this is possible to do, although the change in absorbancy is less than 1%. Thus, to obtain unequivocal results, it will be necessary to work with very concentrated solutions of myoglobin and with the proper precautions.

The binding of chloride ions may also be tested for in the acid region by measuring changes in absorbancy of ferrimyoglobin at constant pH and I with varying concentrations of Cl^{-}. Here again, preliminary experiments have shown that a slight effect occurs, but this could not be attributed solely to Cl^{-} since it was not possible to isolate one variable (Cl^{-} concentration) from all the other parameters (other ion concentrations).

c) The effect of temperature on the 595 mμ absorption of alkaline ferrimyoglobin should give an idea about the equilibrium between the ionic

and covalent forms of the Fe-OH bond[21]. In view of the theoretical interest in this problem, it would be particularly useful to obtain further information on this matter and to compare the results with those of horse ferrimyoglobin.

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