# THE REACTION OF IMIDAZOLE

# WITH

# FERRIHEMOGLOBIN AND FERRIMYOGLOBIN

BY

ISMAT A. ABU-ISA

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## ABSTRACT

A thermodynamic study has been made of the equilibria which are set up in very dilute aqueous solutions containing the hemoproteins ferrihemoglobin or ferrimyoglobin with the monodentate ligand imidazole.

Although the reaction involves single bonding of the tertiary imidazole nitrogen to the sixth, and only, free coordination position of the hematin Fe(III) ion, a number of overlapping ionization equilibria occur, making the measured equilibrium constant a complicated function of the hydrogen ion concentration of the solution. From a mathematical analysis of the variation of $K_{meas.}$ with pH, the acid ionization constants have been evaluated and discussed. The enthalpy and entropy changes for the reaction were obtained from measurements of $K_{meas.}$ at three temperatures.

One very interesting result obtained was a "heme-linked" ionization of an acidic group in the hemoprotein-imidazole complex with pK $\sim$10.3. It is postulated that this group is the imidazole imino =NH group which in free imidazole is an extremely weak acid with pK = 14.5. The significance of this effect is discussed.

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# INTRODUCTION

The work described in this dissertation was undertaken solely from a physico-chemical point of view. Its direct aim was the elucidation of some of the finer chemical and structural features that characterize hemoproteins, and the comparison of the chemical behaviour of the iron atom in these special complexes with its behaviour in other inorganic and organic coordination compounds.

The reaction studied is fundamentally very simple. It involves the bonding of one monodentate ligand, imidazole, to Fe(III) at the centre of an enormous complex ion, the hemoprotein.

The choice of the ligand imidazole was deliberate. For, it is perhaps the strongest amine which exists largely as an unprotonated base in neutral solution, and hence is an especially effective catalyst of hydrolysis. Moreover, imidazole in the form of histidine is now known to be associated with the centre of enzymic activity in several proteins, and in the case of hemoglobin and myoglobin it forms the actual point of linkage between the protein and the heme iron. Imidazole therefore plays a central role in the transmission of charge and other effects from the protein to the heme iron in hemoproteins.

The hemoproteins are themselves very interesting molecules. Thus, ferrihemoglobin is the Fe(III) oxidized

form of the physiologically important blood pigment and oxygen carrier (ferro-) hemoglobin, and ferrimyoglobin is the Fe(III) oxidized form of the corresponding muscle pigment and oxygen store (ferro-) myoglobin. Both molecules are made up of a protein (globin) conjugated to an iron porphyrin (heme) disc. Myoglobin is the simplest known hemoprotein, with one globin and one heme, and a molecular weight about 18,000. Hemoglobin has four glbins and four hemes per molecule, with a molecular weight about 68,000. In both cases, the ferrihemoprotein is far more stable than the reduced ferro form, is easy to purify and crystallize, and is characterized by sharp absorption band which make accurate physico-chemical measurements relatively simple to carry out. In both cases, Fe is octahedrally bonded, four strong bonds being in the heme plane, the fifth also a strong bond to the protein, and the sixth being loosely attached to an $H_2O$ molecule (or to -OH in strongly alkaline solutions, in which case this sixth bond is also fairly strong). The stability of the iron-porphyrin four bonds as well as the iron-protein fifth bond makes one of the most characteristic features of hemoproteins when compared to coordination compounds in general. It enables the hemoproteins to form a wide variety of coordination complexes in a straightforward bimolecular reaction involving the replacement of the water molecule (or the OH group) at the sixth coordination position.

The chemistry and structure of these molecules is at

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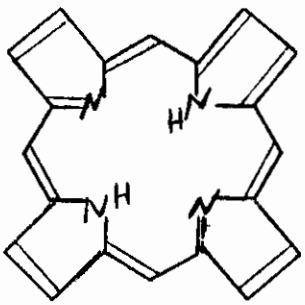
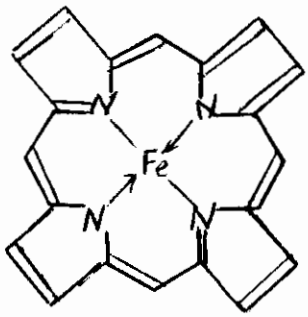
The chemistry and structure of these molecules is at

present known with a remarkable degree of detail. The structural analysis by Perutz, of hemoglobin, and Kendrew, of myoglobin, is discussed in the historical section below.

It is necessary to define further the terms "heme" and "globin". Hemes are metallo-porphyrins, the porphydrin ring being a cyclic tetra-pyrrole compound with four methene bridges and a system of eleven conjugated double bonds as shown below (left)




The number of possible substituents at the eight positions of the pyrrole rings is of course very large, but actually they are very much restricted in nature and in most of the porphyrins positions, 1, 3, 5 and 8 are occupied by methyl groups.

When a metallic ion is introduced into the porphyrin it assumes the central position of the great planar ring, forming coordinate bonds with the four nitrogens and displacing the two imino hydrogen atoms. This point is very important because the charge on the metallic ion is thereby reduced by 2.

Thus, if Fe(II) is introduced, the formal charge on the heme will be zero and the resulting compound is known as ferroheme, while if Fe(III) is introduced a net positive charge (+1) will be the formal charge of the heme and the compound will then be called ferriheme.

In both hemoglobin and myoglobin the porphyrin found is the isomer protoporphyrin IX, with the following substituents on the pyrrole ring: Methyl groups on positions 1, 3, 5 and 8; Vinyl groups on positions 2 and 4; Propionic acid groups on positions 6 and 7. The heme of both hemoglobin and myoglobin is represented above (right).

Globin is a large protein molecule formed of many different amino acids totalling over 150 residues. Though the general spacial structure of the myoglobin "globin" and the hemoglobin "globins" is similar, yet there are differences in their amino acid composition. Globins of the same hemoprotein but of different species also differ in the amino acid composition. Such differences are even found between fetal and adult hemoproteins of the same species. The following table shows part of the amino acid composition of the hemoproteins used in this work.

Further details about the composition and configuration of the amino acids in globin are given below in the section on structure.

Table I

| Amino Acid | Number of Residues per Molecule | | |
|---|---|---|---|
| | Horse Mb [1] | Whale Mb [2] | Horse Hb [3] |
| Glycine | 15 | 11 | 48 |
| Alanine | 15 | 17 | 54 |
| Valine | 7 | 8 | 50 |
| Leucine | 17 | 18 | 75 |
| Isoleucine | 9 | 9 | 0 |
| Serine | 5 | 6 | 35 |
| Aspartic acid | 10 | 8 | 51 |
| Glutamic acid | 19 | 19 | 36 |
| Histidine | 10 | 12 | 36 |
| Lysine | 18 | 19 | 38 |
| Phenylalanine | 7 | 6 | 33 |
| Other amino acid residues | .. | .. | .. |
| | 152 | 153 | 542 |

# HISTORICAL

## I.- Survey of the Problem

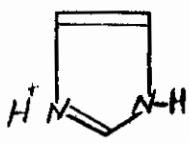
Although hemoglobin, as the red pigment of erythrocytes and oxygen carrier of the blood, had been known for a long time, it was not until Theorell[4] isolated and crystallized ferrimyoglobin from horse heart muscle in 1932, and Keilin and Hartree[5] devised a good method for purifying and crystallizing blood hemoglobin in 1936, that physico-chemical measurements became at all possible and meaningful.

The first study of a reaction between imidazole and a hemoprotein was reported by Russel and Pauling[6] in 1939. These authors carried out a magnetic titration on bovine ferrihemoglobin with imidazole, from which they calculate the (dissociation) equilibrium constant for the resulting complex. The titration was done at 25°C., at three pH values, but the precise experimental conditions, degree of purity of hemoglobin etc. were not recorded. Neither were the values of the measured equilibrium constant recorded directly. The authors stated that the variation of their (three) results with pH could be interpreted on the assumption that in the ferrihemoglobin-imidazole complex there is an acidic (heme-linked) group with $pK \sim 9.5$.

Cogwill and Clark[7] have also investigated the coordination of a large number of imidazole derivatives with ferrimesoporphyrin a heme compound not conjugated to protein. However, in this case the reactions are complicated by dimeri-

zation etc. and the paper contains no data relevent to the present work.

Scheler[8] has published a detailed study of the imidazole-ferrihemoglobin system. From spectral measurements he concluded that the reaction is reversible only in neutral solutions, and that the absorption spectrum of the complex is very markedly dependent on pH. On this basis, Scheler suggested that three types of complexes are formed depending on pH, in which imidazole has respectively the following structures:



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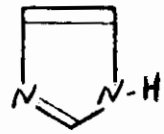

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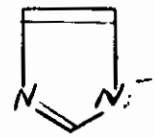


III

the third being what he called a "salt-like compound" which forms at pH 10-11, with 50% conversion of form II to III occurring at pH $\sim$ 10.4. It is interesting to note that this apparently guessed value is very close to the value which was obtained in the present work on the basis of precise measurements (as described later in the discussion). Furthermore Scheler's results at pH 8.0, ionic strength I = 0.02, and at five different temperatures, led to $\Delta H^{\circ} = -4.30$ Kcal/mole and $\Delta S^{\circ} = -3.40$ eu, for this reaction. The enthalpy change is of course itself a function of pH as will be shown in the discussion below.

II.- The Imidazole Hypothesis:

The idea has recently been gaining ground that the protein linkage to the heme in both hemoglobin and myoglobin is through imidazole (histidine). The spectroscopic similarity between denatured hemoglobin and hemochromes (in which it is known that the fifth and sixth coordination positions are occupied by nitrogen atoms of two nitrogenous bases) drew attention to the amino acid which could be responsible for the formation of hemochrome in denatured hemoglobin. It was suggested by Küster[9] as early as 1927 that imidazole as part of the amino acid histidine in globin was directly bonded to the iron atom in the heme, and this was assumed to be reasonable on the grounds that the histidine content of globin was large (cf., Table 1). However, Haurowitz[10] opposed this view because of the low affinity of histidine for heme, and Holden and Freeman[11] also opposed it on the basis of their work which showed that the ability of denatured ox globin to form hemochromes was diminished by treatment with $HNO_2$, a procedure which does not affect imidazole. The origin and early development of the imidazole hypothesis up to about 1945 is very well summarized by Lemberge and Legge[12].

In 1933, Conant[13] made the unproved suggestion that in hemoglobin the iron atom is not only linked to the protein through imidazole but is actually linked on both sides of the heme disc to the imidazole residues of histidines, one bond being very strong and the other weak. It is at

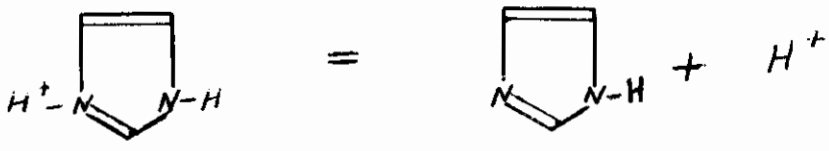
this weak link that oxygen and other groups can form bonds with the iron atom.

In 1937, Cohn et al[14] titrated horse carboxy-hemoglobin and showed that between pH 5 and 9 the results could be explained by postulating the presence of 13 histidine residues with apparent pK = 5.7 and 20 histidine residues with apparent pH = 7.5. The number of histidine residues indicated is identical with the value found analytically by Vickery[15], but little less than the number accepted at present (see Table 1).

The work of Wyman et al[3b,16,17,18] gave one of the most important lines of evidence in favour of the imidazole hypothesis. The work produced the following results:

1. Differential acid-base titrations of ferrohemoglobin and oxyhemoglobin indicated that between pH 4.5 and 6.1 oxyhemoglobin is a weaker acid that ferrohemoglobin, while between pH 6.1 and 9.0 it is a stronger acid. Outside this limit the acidity of the two proteins is the same.

2. The effect of temperature on the differential acid-base titration led to a value about 7 Kcal./mole for the enthalpy of ionization of the titrable acidic groups in hemoglobin which appear between pH 6 and 8. Both facts support the view that such an ionization belongs to the acid dissociation of imidazole, namely



for which pK = 7.1 and $\Delta H$ = 6900 cal./mole[19].

Wyman interpreted his results by assuming that the iron is situated between two imidazoles, and from his analysis he obtained the following pK values for the two heme-linked acidic groups which produced the above effect:

| | $pK_1$ | $pK_2$ |
|---|---|---|
| hemoglobin | 5.25 | 7.81 |
| oxyhemoglobin | 5.75 | 6.80 |

Based on Wyman's findings, Coryell and Pauling[20] gave an extremely interesting explanation for the change in acidity on the introduction of an oxygen molecule into hemoglobin. Their argument was based on changes in bond type and the stability of resonance hybrids in hemoglobin and oxyhemoglobin. They attributed one ionization to the proximal heme-linked group, and the other (and weaker) effect to the distal heme-linked group. Resonance effects account for the change in the acid strength of the proximal group, and steric factors for the change in the acid strength of the distal group. However, this interpretation does not fit other thermodynamic data on ferrihemoglobin reported by George and Hanania[21].

More recently Corwin and Reyes[22] showed that an imidazole-ferroprotoporphyrin complex is capable of combining with molecular oxygen in the ratio of 0.94 - 0.97 moles of $O_2$ to one mole of the complex. It is known of course, that reversible combination with $O_2$ is characteristic of myoglobin and hemoglobin. However, in this case, deoxygenation

of the imidazole-protoporphyrin complex requires vacuum and a temperature of 100°C. which is certainly not normal for hemoproteins.

Though the imidazole hypothesis can account for many of the hemoglobin and myoglobin reactions, Roughton[23], points out that it is unable to account for at least one important reaction, namely, the direct combination of $CO_2$ with oxylabile groups in hemoglobin. Only free amino groups have been found to form carbamino compounds according to the equation

$$RNH_2 + CO_2 \longrightarrow R\text{-}NH\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH$$

Imidazole does not form such compounds. Furthermore, Roughton criticized the lack of precision in previous experimental work, and the lack of rigour in identifying groups on the basis of indirectly measured pK and $\Delta H$ values.

Recently O'Hagan[24] has argued against the imidazole hypothesis in favour of the carboxyl side chain of the heme. But his work is not very clear.

Evidence for the presence of an $H_2O$ molecule attached to the sixth coordination position of the heme iron in hemoglobin was given by Haurowitz[3c] and by Keilin[25]. This fact seems now to be directly supported by the structural analysis of hemoglobin and myoglobin crystals which also makes it nearly certain that the iron is linked to the protein through histidine.

III.- Structure of Hemoproteins and Related Problems:

Because of the profound effect of neighbouring groups on the properties of the iron atom in hemoproteins, the value of a knowledge of their detailed geometrical structure has long been recognized. This knowledge has been generally sought along two distinct lines: indirect evidence from physico-chemical studies in solution relating to the position of the heme relative to the surface of the globin molecule etc., and direct structural analysis, by X-ray and optical methods, of the crystalline structure of these molecules. Both methods of approach have yielded a remarkable amount of useful information about myoglobin and hemoglobin.

The first major contribution was made by Pauling and his coworkers in a series of papers from 1935 to 1949. It has been known that in the reaction with molecular oxygen, the four $O_2$ molecules that attach to the four hemes in hemoglobin do not do so independently of each other. Pauling[26] suggested a mathematical treatment of this "heme-linked interaction" taking a simple picture of the four hemes of hemoglobin at the corners of a square. In a later model, Pauling[3d] used the crevice hypothesis where the hemes would be embedded within the hemoglobin molecule in such a way that a pushing apart of the molecule must occur in order that the ligand molecule may fit in. By this, the first ligand molecule, once it is in, will loosen the structure of hemoglobin making it easier for the second ligand molecule to fit in. The third molecule

will fit in with greater ease and so on.

Experimental evidence for the crevice hypothesis was sought in the reaction with alkyl isocyanides. St. George and Pauling[27] found no appreciable difference in the affinity of ferroheme for ethyl, isopropyl and tert-butyl isocyanides. However, under the same conditions, 35°C. and pH 6.8, the affinity of ferrohemoglobin for isocyanides decreased 3-fold and 200-fold respectively on going along the series. This suggested steric hindrance in support of the crevice idea. Another observation which seems to support a steric theory of heme-heme interaction is that the oxygen affinity of hemoglobin increases and heme-heme interaction becomes smaller with decreasing ionic strength of the solution[28,29]. The removal of salt loosens the hemoglobin structure, makes it easy for the ligand to attach itself, and decreases heme-heme interaction, if the later is mainly due to steric factors.

Keilin[25], however, argued strongly against a crevice structure in hemoglobin. His evidence rested mainly on the fact that hemoglobin combines with large molecules such as nitrobenzene and 4-methyl imidazole, which suggests that the heme iron is not embedded in a crevice but is readily accessible.

Similar conclusions were arrived at by Kendrew and Parish[30] who found the crystalline form and unit cell dimensions etc. of (sperm whale) ferrimyoglobin to be almost

identical with the corresponding properties of serveral imidazole complexes of ferrimyoglobin, and by George and Hanania[31] whose thermodynamic data on the ferrimyoglobin-cyanide reaction in dilute aqueous solution showed that the entropy change is in very great contrast with the corresponding data on ferricytochrome-c, a hemoprotein in which there is little doubt about a crevice configuration.

The above review shows that there have been many contradicting ideas concerning the structure of hemoproteins and its related problems. The final answer, at least on the structure of their crystals, came from the X-ray studies made by Kendrew and by Perutz and their coworkers[32-41] especially their recent work on the three dimensional fourier synthesis of myoglobin[40] and hemoglobin[41].

The results on myoglobin obtained from a 6Å and a 2Å resolution study show that the crystals are monoclinic, with two molecules in the unit cell, the molecule measuring 43x35x25 Å. The heme appears to be a disc surrounded by a complicated folding single rod of polypeptide in which the amino acids are arranged in the $\alpha$-helix chain configuration. On the proximal side of the heme, the iron atom seems to be bonded to histidine, as predicted; and on the distal side to an $H_2O$ molecule, again as predicted. Furthermore, the second histidine (imino) nitrogen appears to be hydrogen bonded to a carbonyl group in the polypeptide chain. Beyond the $H_2O$ molecule, on the distal side, is another

histidine (or glutamine) molecule possibly also hydrogn-bonded to the $H_2O$ molecule[42].

For hemoglobin, from the 5.5 Å resolution analysis, Perutz obtained the following facts:

1.- The crystals are half occupied by water of hydration, and the dimensions of the spheroidal molecule are 64x55x50 Å.

2.- Each hemoglobin molecule consists of four separate units identical in pairs. The nonidentical pairs are quite similar. The only difference between them being few gaps in one showing less electron density. Apart from the areas of low electron densities, myoglobin has configuration similar to that of the hemoglobin units.

3.- The heme groups in hemoglobin lie in four different pockets on the surface of the molecule. Each pocket is formed by the fold of a polypeptide chain which appears to make four points of contact. The iron atoms of the hemes lie at the corners of an irregular tetrahedron, with distances 33.4 and 36.0 Å between symetrically related pairs. The iron atoms of neighbouring pockets are 25 Å apart. The heme of myoglobin is also seen to lie in a pocket on the surface of the molecule.

4.- Horse hemoglobin has four cysteine residues, but only two sulfhydryl groups react with mercury in the native protein[43]. From the positions of the mercury atoms, it was inferred that each of the two active sulfhydryl groups is

about 13 Å away from one iron atom and 21 Å away from another. The positions of the unreactive sulfhydryl groups are still unknown.

5.- The heme groups as seen from above, are too far apart for steric heme-heme interaction. The latter must therefore be of some indirect type which is still unknown.

Rigg[29] showed that the bonding of the sulfhydryl groups reduces heme-heme interaction. These groups occupy key positions close to the heme linked histidines on one hand, and points of contact between two different subunits on the other. The possibility that they play a part in the transmition of charge and other effects in hemoglobin is logical.

Along with Perutz's and Kendrew's X-ray studies on the structure of hemoproteins, chemical studies were conducted for the identification of N-terminal amino acid residues. Various values for the N-terminal valyl residues have been reported in the literature but recent work done by Levitt and Rhinesmith[44], on horse hemoglobin, shows that there are 4 N-terminal residues per molecule; and that the $\alpha$ chains have the N-terminal sequence val. leuc., while the $\beta$ chains have the sequence val. glutamic. leuc.

In myoglobin, there is one terminal nitrogen, as expected from the fact that its protein consists of one polypeptide chain[45].

## THEORY

All available evidence supports the view that the reaction of ferrihemoglobin (or ferrimyoglobin) with imidazole involves single replacement of $H_2O$ at the sixth octahedral coordination position in the Fe(III) complex, so that

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

where $Fe\text{-}(H_2O)$ represents ferrihemoglobin (or ferrimyoglobin) without indicating the other five bonds or the effective charge of +1 on Fe; Im represents imidazole, and Fe-Im the resulting complex. The thermodynamic equilibrium constant is given in the usual way in terms of activities $a_i$:

$$K = \frac{a_{Fe\text{-}Im}}{a_{Fe\text{-}(H_2O)} \times a_{Im}} \qquad (2)$$

At a first glance, it might appear easy to obtain K from measurements involving concentrations (rather than activities). For, since the ligand is a neutral molecule, and since the ratio of the activity coefficient of the reactant to that of the complex ion is not expected to differ much from unity, it could be argued that K will not vary much with ionic strength especially as the measurements are being made in very dilute aqueous solutions ( $Fe\text{-}(H_2O)) \sim 5 \times 10^{-6}$, and $(Im) \sim 10^{-2}$ M). This may indeed be approximately true[8], but it cannot be regarded as accurate thermodynamic determination. At best it is quasi-thermodynamic.

The main difficulty arises from uncertainties about charges on the various species which are involved in the simultaneous (and partly overlapping) acid-base equilibria, as is shown below.

In view of the uncertainties, it was decided to make the bulk of the measurements at the constant ionic strength I = 0.20. Consequently equation 2 above can be rewritten in the form

$$K_{meas.} = \frac{(\text{Total Complex})}{(\text{Total Hemoprotein})(\text{Total Imidazole})} \qquad (3)$$

where brackets indicate molar concentrations, and $K_{meas.}$ is the measured equilibrium constant at a set of given conditions.

The dependence of $K_{meas.}$ on pH is fairly complicated, but in essence it can be described on the basis of three postulates:

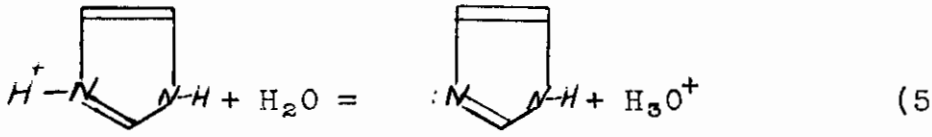
1.- The species Fe-OH, which is the conjugate base of the reactant Fe-($H_2O$) is unreactive. The concentration of Fe-($H_2O$) is clearly a function of the pH of the solution, since

$$Fe\text{-}(H_2O) + H_2O = Fe\text{-}OH + H_3O^+ \qquad (4)$$

for which[21,46] the ionization constant $K_{Fe} \sim 10^{-9}$. There is evidence, both theoretical and experimental[21], for the Fe-OH bond being strong, and hence for Fe-OH being non-reactive. The observed equilibrium constant for the reaction should therefore decrease as the pH increases, with an inflection point

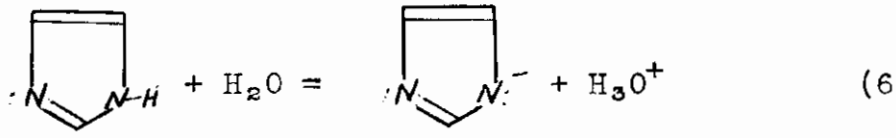
around pH 9.

2.- The ligand imidazole reacts as the conjugate base in which the tertiary nitrogen atom has a lone pair of electrons available for coordination. The ionization occurs in two stages:



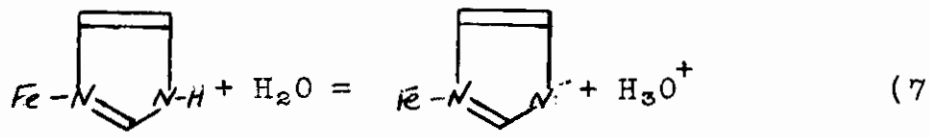
(5)

for which[19] $K_1 \sim 10^{-7}$, and



(6)

for which[47] $K_2 \sim 10^{-14}$. The latter ionization will naturally not be expected to affect the measured equilibrium constant, unles measurements could be extended beyond pH $\sim$13 or so. However, in the ferrihemoprotein-imidazole complex, conjugation of imidazole to the heme should stabilize the anion and thus increase the acid strength of the imino =NH group considerably. One may therefore write a third ionization



(7)

for which $K_3 > K_2$. In fact the main purpose of these studies is to determine the value of $K_3$ and the relevant thermodynamics of that ionization.

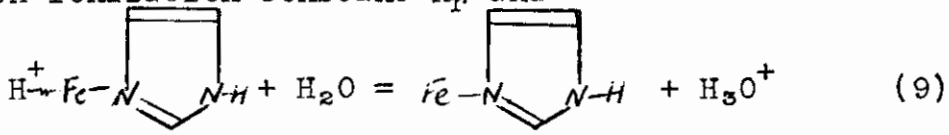
The effect of pH on the observed equilibrium constant will in this case be the reverse of the first effect; that is, as the pH of the s olution increases, the proportion of

imidazole in the form of its conjugate base increases, and the reaction with ferrihemoglobin (or ferrimyoglobin) is favoured.

3.- There is good evidence[48] also for the participation of at least one "heme-linked" ionization which probably occurs on the protein or a porphyrin side chain. The heme-linked effect occurs at pH $<$ 7. Since the group responsible for this ionization exists in the reactant (ferrihemoprotein) and in the product (the complex), two corresponding equilibria should be considered:

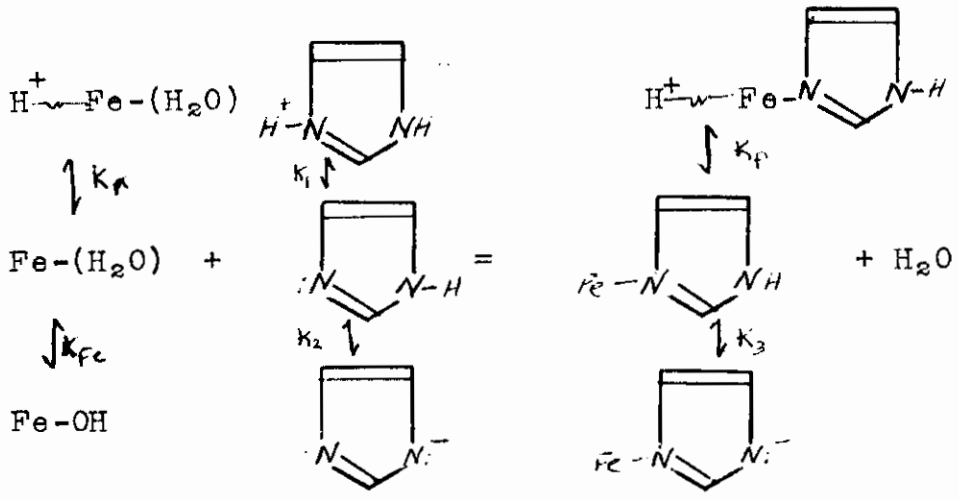
$$H^+\text{-}\sim\text{-}Fe\text{-}(H_2O) + H_2O = Fe\text{-}(H_2O) + H_3O^+ \qquad (8)$$

with ionization constant $K_r$ and

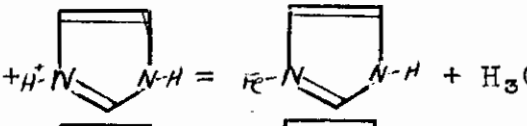


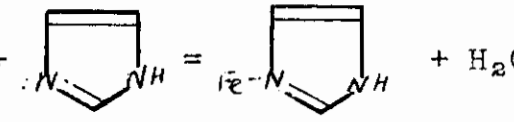
with ionization constant $K_p$. If $K_r$ and $K_p$ are identical, there could be no heme-linked effect, and this could mean that there is no interaction between the ionization of the acidic group in question and bonding of imidazole to the iron atom. The extent to which $K_p$ differs from $K_r$ measures the strength of the heme-linked effect. The results of the present work suggest a small but measurable effect between pH 6 and pH 7.

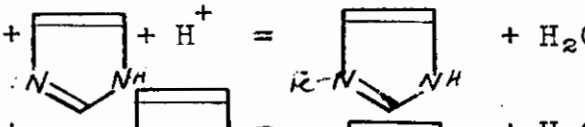
The following scheme now combines all above equilibria (note that the effective charge on the Fe ion is not indicated).

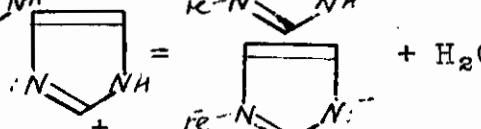


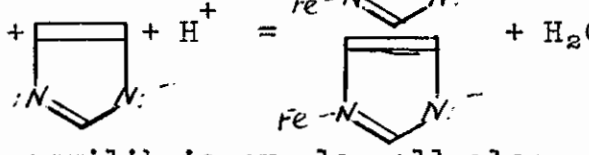
Another way of illustrating the dependence of this reaction on pH is to write the overall equilibrium which predominates at various pH values. Omitting for the moment the minor heme-linked effect, and remembering that the three imidazole ionizations have $K_1 \sim 10^{-7}$, $K_2 \sim 10^{-14}$ and $K_3 > K_2$ (say $10^{-11}$), whereas $K_{Fe} = 10^{-9}$ one obtains the following set of equations (again not showing the effective charge on Fe):

pH < 7  (10)

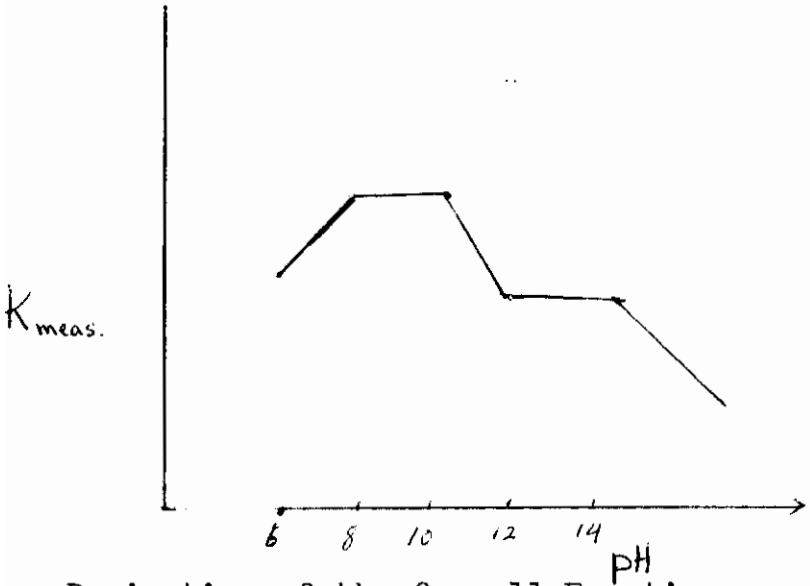
pH 7-9  (11)

pH 9-10.5  (12)

pH 10.5-14  (13)

pH > 14  (14)

Of course the equilibria overlap all along, but they suggest the following type of variation of $K_{meas.}$ with pH. This, in fact, is in accord with the experimental results.




Derivation of the Overall Equation:

The mathematical relation between the various parameters and the measured equilibrium constant for the reaction can be derived on the above basis.

First consider the ionization of the hemeprotein. The main one is given in equation 4, for which

$$K_{Fe} = \frac{(Fe\text{-}OH)\ h}{(Fe\text{-}(H_2O))} \qquad (15)$$

where h stands for the hydronium ion concentration, $(H_3O^+)$. Hence,

$$\frac{(Fe\text{-}(H_2O))}{(Fe\text{-}OH)} = \frac{h}{K_{Fe}}$$

and $$\frac{(Fe\text{-}(H_2O))}{(Fe\text{-}(H_2O)) + (Fe\text{-}OH)} = \frac{h}{K_{Fe} + h}$$

But $(Fe\text{-}(H_2O))$ is the concentration of reactive ferrihemoglobin

(or ferrimyoglobin) and $(Fe\text{-}(H_2O)) + (Fe\text{-}OH)$ is the total concentration of ferrihemoglobin (or ferrimyoglobin). Hence, the concentration of reactive ferrihemoglobin (or ferrimyoglobin) is,

$$(Fe\text{-}(H_2O)) = \frac{h}{K_{Fe} + h} \times (\text{total ferrihemoprotein}) \qquad (16)$$

likewise, the heme-linked ionization on the reactant is given by equation 7, for which

$$K_r = \frac{(Fe\text{-}(H_2O)) \times h}{(H^+\!\!\sim\!\!Fe\text{-}(H_2O))} \quad . \qquad (17)$$

Using the same steps as in the above derivation, it can be shown that,

$$(Fe\text{-}(H_2O)) = \frac{K_r}{K_r + h} \times (\text{total ferrihemoprotein}). \qquad (18)$$

Consequently, the combined effect is to make

$$(Fe\text{-}(H_2O)) = \frac{h}{K_{Fe} + h} \times \frac{K_r}{K_r + h} \times (\text{total ferrihemoprotein}). \qquad (19)$$

Next, consider the ionization of imidazole. Referring to equations 5 and 6 above, and using the symbols $H^+$-Im, Im and $Im^-$ for the species involved, it follows that

$$(Im) = \frac{h}{K_2 + h} \times \frac{K_1}{K_1 + h} \times (\text{total imidazole}). \qquad (20)$$

Finally, consider the product, the coordination complex. Referring to the above set of equilibria, it can similarly

be shown that

$$(Fe\text{-}Im) = \frac{h}{K_3 + h} \times \frac{K_p}{K_p + h} \times (\text{total complex}) \qquad (21)$$

We may now define the true equilibrium constant for the reaction between $Fe\text{-}(H_2O)$ and Im by

$$K = \frac{(Fe\text{-}Im)}{(Fe\text{-}(H_2O))(Im)} \qquad (22)$$

whereas $K_{meas.}$ is defined in equation 3 in terms of the total concentrations. It follows that the true equilibrium constant K is related to the measured value $K_{meas.}$ as shown here:

$$K = K_{meas.} \frac{\frac{h}{K_3 + h} \times \frac{K_p}{K_p + h}}{\frac{h}{K_{Fe} + h} \times \frac{K_r}{K_r + h} \times \frac{h}{K_2 + h} \times \frac{K_1}{K_1 + h}} \qquad (23)$$

At, say, 25° and I = 0.20, the value of K is constant, as are the values of $K_{Fe}$, $K_1$, $K_2$, $K_3$, and $K_p$. But $K_{meas.}$ is a complicated function of pH given by

$$K_{meas.} = K \times \frac{\frac{h}{K_{Fe} + h} \times \frac{K_r}{K_r + h} \times \frac{h}{K_2 + h} \times \frac{K_1}{K_1 + h}}{\frac{h}{K_3 + h} \times \frac{K_p}{K_p + h}} \qquad (24)$$

This equation can be simplified in two ways

1.- pH $<$ 7: under these conditions $h \gg K_3$, $K_2$, $K_{Fe}$ and hence the equation reduces to

$$K_{meas.} = K \times \frac{K_r}{K_p} \times \frac{K_p + h}{K_r + h} \times \frac{K_1}{K_1 + h} \qquad (25)$$

But there is no direct graphical method of obtaining the values of $K_r$ and $K_p$ from the experimental data.

2.- pH 9 - pH 12: under these conditions $h \ll K_r$, $K_p$, $K_1$ and $h \gg K_2$.

Hence the equation reduces to:

$$K_{meas.} = K \times \frac{\frac{h}{K_{Fe} + h}}{\frac{h}{K_3 + h}} = K \times \frac{K_3 + h}{K_{Fe} + h} \qquad (26)$$

therefore, $K_{meas.} (K_{Fe} + h) = KK_3 + K \times h$

In this case, it is easy to obtain the value of $K_3$ from a plot of $K_{meas.} (K_{Fe} + h)$ against h, that is against $(H_3O^+)$.

## EXPERIMENTAL PROCEDURE

The experimental procedure involves

a.- Preparation of stock solutions which include

i - Preparation of different buffers at different pH values and constant ionic strength.

ii - Preparation of ferrihemoglobin and ferrimyoglobin solutions of proper concentration.

iii - Preparation of imidazole solutions in buffer and of the required concentrations.

b.- It also involves mixing of these solutions in the right proportions, at a constant temperature, and following the extent of the reaction spectrophotometrically.

I.- Preparation of Stock Solutions:

i - Buffers: The correct ratio of the weak acid and its salt are dissolved together in a given volume of deionized and redistilled water, to give the required pH value. The amount of acid and salt added are calculated so as to give an ionic strength of 0.22 to the solution. Because of the low solubility of one or both constituents of some buffers, it was found that NaCl should be added to the solution to give it the relatively high ionic strength of 0.22.

The buffers used in this study consisted of:

| | | | | | |
|---|---|---|---|---|---|
| 1.- KHphthalate / NaOH | for | the | pH | range | 4.0 - 6.0 |
| 2.- $NaH_2PO_4.H_2O$ /NaOH | " | " | " | " | 5.8 - 8.0 |
| 3.- $H_3BO_3$ / NaOH | " | " | " | " | 8.0 - 10.2 |
| 4.- Glycine / NaOH | " | " | " | " | 10.0 - 11.0 |
| 5.- Anhyd. $NaHPO_4$ / NaOH | " | " | " | " | 11.0 - 12.0 |

In the last three sets of buffers, NaCl was used to bring up the ionic strength of the solution to 0.22.

All the chemicals used in the preparation of above buffers were analytically pure reagents and were not further purified before use.

ii - Preparation of ferrihemoglobin and ferrimyoglobin solutions: Horse oxy-hemoglobin, prepared and purified by Dr. George I.H. Hanania, was used for the preparation of ferrihemoglobin solutions. The oxy-hemoglobin was first oxidized with $K_3Fe(CN)_6$, and then exhaustively dialyzed in deionized water, to get rid of $K_3Fe(CN)_6$ and of all other tra ces of salts found originally in the oxy-hemoglobin. After dialysis the ferrihemoglobin was diluted with deionized and redistilled water to the proper concentration ($\sim 5 \times 10^{-6}$), and used.

Horse heart ferrimyoglobin was also prepared and purified by Dr. George I.H. Hanania. The ferrimyoglobin was simply diluted with the deionized and redistilled water and put for use.

Another type of horse heart ferrimyoglobin prepared and purified by the "Nutritional Biochemicals Corporation,

Cleveland, Ohio", was tried but failed to work. It's absorption spectrum was taken and the absorption band expected at 409.5 mμ for myoglobin was found shifted to 407 mμ. It probably contained some impurities. To purify it, it was recrystallized from ammonium sulfate solution and dialized. The resulting myoglobin was still impure and could not be used. The trial to purify it by electrophoresis also failed.

Whale ferrimyoglobin, purified and recrystallized, was kindly provided by Dr. M.F. Perutz, F.R.S., of Cambridge University, England. This myoglobin was dialized in deionized water; diluted with deionized and redistilled water and put for use.

All the above hemoprotein solutions were stored in a refrigerator freezer, because of their ease of denaturation if left for a long time at room temperature. Before use, the solutions were titrated to the pH of the buffer used in the specific run. If this is not done, the precise control of pH of a series of solutions becomes very difficult.

iii - Preparation of imidazole solutions: Analytically pure imidazole was purchased from "British Drug Houses Ltd." and was dried in a desiccator over calcium chloride. Standard solutions of the dried imidazole were prepared in buffers, after titrating them to the pH of the specific buffer used in each case. (For the same reason as above).

II.- Measurement of the Equilibrium Constant:

After the stock solutions were prepared for each run,

they were mixed together in the appropriate proportions. The mixing was made in a number of test tubes, usually around seven. The test tubes were cleaned, rinsed with deionized and redistilled water and dried before use. Except for the first test tube which was left as a blank, and the last one where excess imidazole was added, different volumes of standard imidazole solution were pipetted into the tubes. Buffer solution was then pipetted into the tubes, usually 9.0 ml of it into the first, and the required volume in the rest, so as to make the total volume of imidazole solution and buffer solution 9.0 ml in each case. The mixtures were then transferred to a thermostat and left there for around ten minutes to attain the constant temperature of the thermostat.

The temperature of the thermostat was controlled by a "Techam" tempunit (Techne, Cambridge, Ltd.). This tempunit keeps the temperature constant within $\pm$ 0.2°C. The temperature is measured with a sensitive thermometer which reads to within 0.1°C. From this thermostat water was circulated through the spectrophotometer and the pH meter, so as all measurements could be made at the same temperature.

After thermostating the solutions, 1.0 ml of ferrihemoglobin or ferrimyoglobin stock solution was added to each of the test tubes, and left for a couple of minutes to ensure equilibration. The reaction of ferrihemoglobin or ferrimyoglobin with imidazole is very fast, and does not need more than two minutes to equilibrate.

The absorbancies of the different solutions were then measured on a Zeiss Spectrophotometer (PMQ II). In the early part of the work a Beckman DU Spectrophotometer was used. The readings were taken at a wavelength of 405 mμ and 409.5 mμ which are the wavelength at which maximum absorbancy of ferrihemoglobin and ferrimyoglobin respectively occurs. In alkaline media (pH$>$ 9), the aborbancy of ferrimyoglobin at 409.5 mμ becomes very close to that of the complex, and measurements were therefore made at 417 mμ, where the maximum absorbancy of the complex (Fe-Im) occurs.

When the spectrophotometric readings were taken for all the solutions, the pH values of the solutions were measured individually. The measurements were taken for most of the runs on a pH meter (Radiometer, PH 4, type PHM4c, No. 39538). The pH meter used to be standardized before taking the measurements of each run by a standard buffer with slightly lower pH value, and rechecked with another buffer with slightly higher pH value than the one expected for the set of solutions. The pH values of the standard buffers at different temperatures as given by the National Bureau of Standards[49] are summarized below. These solutions were selected to give a maximum stability with respect to changes of concentration and slight contamination, and the error would not exceed $\pm$ 0.01 pH units.

| Buffer | Concentration | Temperature | | | |
|---|---|---|---|---|---|
| | | 0°C. | 10°C. | 25°C. | 38°C. |
| KHphthalate | 0.05M | 4.008 | 4.000 | 4.008 | 4.034 |
| $Na_2B_4O_7$ | 0.01M | 9.466 | 9.331 | 4.181 | 9.085 |
| $Na_2CO_3$ | 0.05M | 11.887 | 11.612 | 11.266 | 11.000 |

All above solutions will have an ionic strength of around 0.20, because a total volume of 9.0 ml was pipetted in each case from the buffer solution and the imidazole solution in buffer, and made up to 10.0 ml by the addition of 1.0 ml of water solution of ferrihemoglobin or ferrimyoglobin. Since the ionic strength of the buffer is 0.22, and neglecting the ionic strength contribution of the very dilute solutions of ferrihemoglobin or ferrimyoglobin and imidazole; the ionic strength of the resulting solution will be very nearly 0.20.

III.- Calculation of the Equilibrium Constant ($K_{meas.}$):

Method A:

In order to check the stoichiometry of the reaction, one set of data was analyzed in the following manner:

Assume that n moles of imidazole react with one mole of ferrihemoglobin to form one mole of complex $Fe\text{-}Im_n$.

$$Fe\text{-}(H_2O) + n\ Im \longleftrightarrow Fe\text{-}Im_n + H_2O \qquad (27)$$

$$K_{meas.} = \frac{(Fe\text{-}Im_n)}{(Fe\text{-}(H_2O))(Im)^n} \qquad (28)$$

hence $\log K_{meas.} = \log \frac{(Fe\text{-}Im_n)}{(Fe\text{-}(H_2O))} - n \log(Im)$

and $\log \frac{(Fe\text{-}Im_n)}{(Fe\text{-}(H_2O))} = \log K + n \log(Im)$ (29)

A plot of $\log \frac{(Fe\text{-}Im)}{(Fe\text{-}(H_2O))}$ versus log(Im) will have a slope of n which is the number of imidazole moles reacting with one mole of $Fe\text{-}(H_2O)$, and an intercept equal to $\log K_{meas.}$. The sample run below gives the analysis of one set of data and shows that $n = 1.00 \pm 0.02$, and $K_{meas.} = 21.4$ in good agreement with the value of $K_{meas.}$ obtained by the two other methods discussed below (see Table 2). The calculation of $\frac{(Fe\text{-}(H_2O))}{(Fe\text{-}Im)}$ is similar to the one discussed in method B below.

Method B:

The usual method of calculating $K_{meas.}$ was the following: Let $A_o$ stand for the absorbancy of pure ferrihemoglobin or ferrimyoglobin as measured from the solution of the first test tube where no imidazole is added. And $A_{100}$ stand for the absorbancy of the complex as measured from the last test tube where excess imidazole was added. Then the percent complex formed in any of the other solutions having absorbancy A will be:

$$\% \text{ complex} = \frac{A_o - A}{A_o - A_{100}} \times 100 \qquad (30)$$

and $$K_{meas.} = \frac{\% \text{ complex}}{(100 - \% \text{ complex})(Im)} \qquad (31)$$

hence calculating % complex from above relation (equation 30) and knowing the imidazole concentration, the equilibrium constant is easily calculated from equation 31.

Method C:

The defect of the above method is the difficulty of finding $A_{100}$, since excess imidazole tends to denature the hemoprotein. Hence, a second graphical procedure was sought and used. It follows from the above equation (30) that

$$\frac{(\text{total complex})}{(\text{total ferrihemoprotein})} = \frac{A_0 - A}{A_0 - A_{100}} \Big/ 1 - \frac{A_0 - A}{A_0 - A_{100}}$$

$$= \frac{A_0 - A}{A - A_{100}}$$

and therefor that

$$K_{meas.} = \frac{(\text{total complex})}{(\text{total ferrihemoprotein})(Im)} = \frac{A_0 - A}{(A - A_{100})(Im)} \qquad (32)$$

hence

$$\frac{A_0 - A}{(Im)} = KA - KA_{100}$$

and

$$A = \frac{A_0 - A}{(Im)} \times \frac{1}{K} + A_{100} \qquad (33)$$

Plotting A versus $\frac{A_0 - A}{(Im)}$, the slope of the straight line will be equal to $\frac{1}{K}$ and the intercept to $A_{100}$.

In the case of runs taken in alkaline medium and where the wavelength is taken at maximum absorbancy of the complex it can be shown easily, that the following mathematical relation exist in this case:

$$A = - \frac{A - A_o}{(Im)} \times \frac{1}{K} + A_{100} \qquad (34)$$

Taking the value of $A_{100}$ from the intercept, K can also be calculated by the first method. In fact the calculation of K in many of the runs was made by both methods and used to give the same result within very small differences.

IV.- Sample Run:

A sample run was chosen at random, and is discussed here as a representative of all other runs. All stock solutions for this run were prepared as stated above. The concentration of the imidazole stock solution was 0.500M. It happened that in this particular run, the pH chosen was 9.8 which is about the pH expected of imidazole aqueous solution. Hence no titration for the imidazole was needed. But the myoglobin was titrated to this pH before use. The temperature at which the run was made was 35.0 $\pm$ 0.2°C. The following data were recorded.

Whale Ferrimyoglobin and Im May 4, 1961

Stock Solutions

1. Whale ferriMb solution in base prepared on April 24, 1961
2. (Im) = 0.500M and pH 9.8 " " May 4, 1961
3. Buffer solution pH 9.8 and
   I = 0.22 " " April 11, 1961

Conditions

1. Temperature = 35 ± 0.2°C.
2. Average pH = 9.835 I = 0.20
3. A taken at $\lambda$ = 417 mμ.

| Solutions | $(Im)x10^2$ | pH | A |
|---|---|---|---|
| 1. 1.0 ml Mb + 0.0 ml Im + 9.0 ml buffer | 0.00 | 9.791 | 0.730 |
| 2. 1.0 ml Mb + 0.4 ml Im + 8.6 ml buffer | 2.00 | 9.810 | 0.789 |
| 3. 1.0 ml Mb + 0.6 ml Im + 8.4 ml buffer | 3.00 | 9.823 | 0.808 |
| 4. 1.0 ml Mb + 0.8 ml Im + 8.2 ml buffer | 4.00 | 9.834 | 0.820 |
| 5. 1.0 ml Mb + 1.0 ml Im + 8.0 ml buffer | 5.00 | 9.840 | 0.834 |
| 6. 1.0 ml Mb + 1.2 ml Im + 7.8 ml buffer | 6.00 | 9.845 | 0.842 |
| 7. 1.0 ml Mb + 1.4 ml Im + 7.6 ml buffer | 7.00 | 9.858 | 0.847 |
| blank / blank at the beginning | | | 0.000 |
| blank / blank at the end | | | 0.000 |

Table II

Data and Experimental Results for the Sample Run Described in the Text

$T = 35.0 \pm 0.2^{\circ}C$; $I = 0.20$; $\lambda = 417$ mμ; pH = 9.810-9.858

| $(Im) \times 10^2$ | $\log(Im) \times 10^2$ | A | $\frac{A-A_0}{(Im)}$ | % complex | $\log\frac{(Fe-Im)}{(Fe-(H_2O))}$ | $K_{meas.}$ |
|---|---|---|---|---|---|---|
| 0.00 | 0.000 | 0.730 | - | 00.0 | - | - |
| 2.00 | 0.301 | 0.789 | 2.95 | 29.5 | -0.378 | 20.9 |
| 3.00 | 0.477 | 0.808 | 2.60 | 39.5 | -0.185 | 21.3 |
| 4.00 | 0.602 | 0.820 | 2.25 | 45.0 | -0.087 | 20.4 |
| 5.00 | 0.699 | 0.834 | 2.08 | 52.0 | +0.035 | 21.7 |
| 6.00 | 0.778 | 0.842 | 1.87 | 56.0 | +0.105 | 21.2 |
| 7.00 | 0.845 | 0.847 | 1.67 | 58.5 | +0.149 | 20.1 |
| excess(graph) | - | 0.930 | - | 100 | - | - |

Following the procedure discussed above for calculating the measured equilibrium constant the following values were obtained.

Method A:

$$\log \frac{(Fe\text{-}Im)}{(Fe\text{-}(H_2O))} = \log K_{meas.} + n \log (Im)$$

From the plot shown in figure 1, of $\log \frac{(Fe\text{-}Im)}{(Fe\text{-}(H_2O))}$ versus $\log (Im) \times 10^2$, the value of n (from the slope) was found to be 1.0. This confirms the supposition that only one mole of imidazole reacts with one mole of ferrimyoglobin (or ferrihemoglobin). The value of the intercept which is equal to $\log K_{meas.} + 2n$ was found to be -0.667.

Hence $\log K_{meas.} = 1.333$

and $K_{meas.} = 21.5$

Method B:

From the relation $K = \frac{\% \text{ complex}}{100 - \% \text{ complex}} \times \frac{1}{(Im)}$ the values of $K_{meas.}$ were calculated as reported above (Table II). The average $K_{meas.}$ was found to be $K_{meas.} = 20.9 \pm 0.5$.

Method C:

Plotting A versus $\frac{A - A_o}{(Im)}$ (as shown in fig. 2) the value of $K_{meas.}$ was found to be $K_{meas.} = 20.8 \pm 0.4$.

The three values of $K_{meas.}$, as expected, are in quite good agreement.

In all above measurements and readings the errors introduced were very small. The readings were reproducable within $\pm$ 1%.

Fig. 1

(from data in Table II)

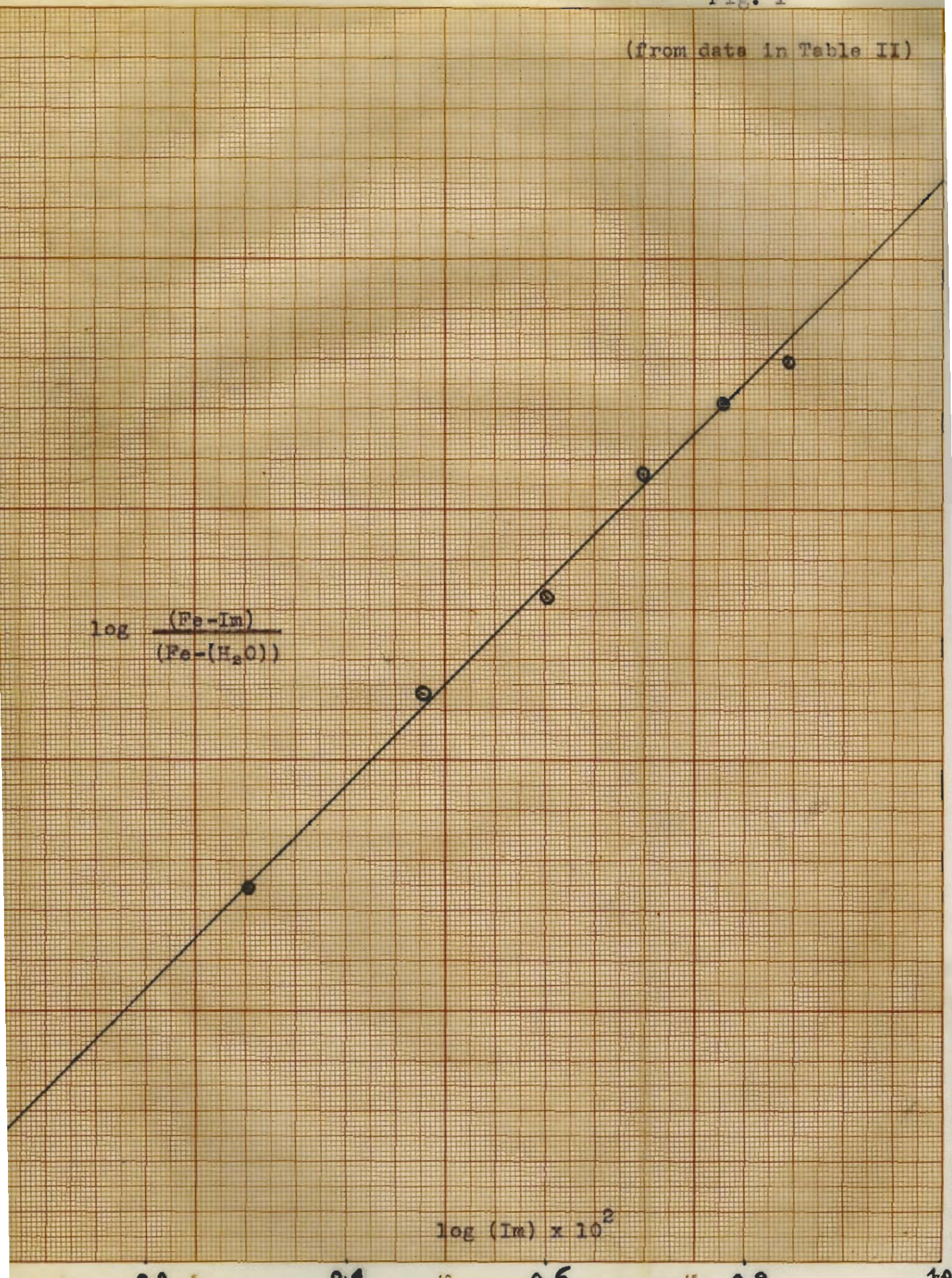
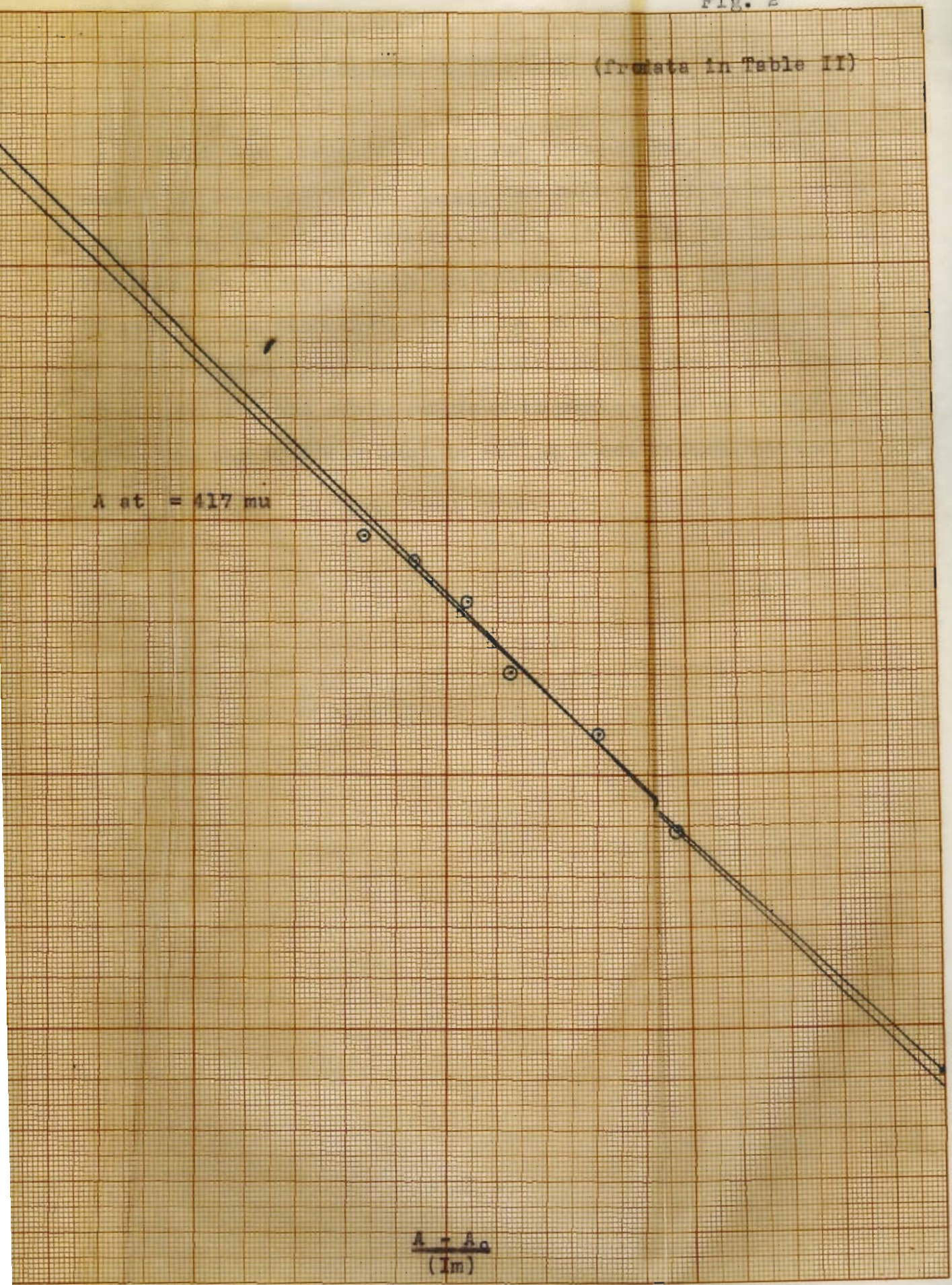


Fig. 2

(from data in Table II)



## RESULTS AND CALCULATIONS

Using the above procedure and precautions, a large number of measurements of $K_{meas.}$ was made on (1) Horse ferrihemoglobin, (2) Horse ferrimyoglobin, (3) Whale ferrimyoglobin. The data are summarized in the following tables (Table III, IV and V). In each case, the value of $K_{meas.}$ represents the arithmetic mean (with the mean deviation) of about six determinations, and the range (minimum to maximum) of pH values among the six mixtures used in making these determinations.

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Table III

Variation of $K_{meas.}$ with pH for the Reaction of Horse Ferri-Hemoglobin and Imidazole  I = 0.20

| Temperature °C. | pH | $K_{meas.}$ | Buffer used |
|---|---|---|---|
| 25°C. | 5.96 - 6.16 | 32.8 ± 5 | $NaH_2PO_4.H_2O$/NaOH |
| " | 6.02 - 6.19 | 26.4 ± 2 | " |
| " | 6.02 - 6.14 | 28.9 ± 2 | " |
| " | 6.10 - 6.26 | 59.5 ± 1 | " |
| " | 6.39 - 6.59 | 66.9 ± 2 | " |
| " | 6.49 - 6.69 | 116 ± 2 | " |
| " | 6.92 (av.) | 180 ± 4 | " |
| " | 7.00 - 7.10 | 195 ± 4 | " |
| " | 7.17 - 7.27 | 213 ± 2 | " |
| " | 7.41 - 7.51 | 245 ± 13 | " |
| " | 7.59 - 7.71 | 264 ± 3 | " |
| " | 8.00 - 8.06 | 326 ± 2 | $H_3BO_3$/NaOH |
| " | 8.08 - 8.20 | 292 ± 2 | " |
| " | 8.18 - 8.25 | 250 ± 10 | " |
| " | 8.46 - 8.52 | 230 ± 3 | " |
| 35°C. | 6.15 (av.) | 34.2 ± 1.5 | $NaH_2PO_4.H_2O$/NaOH |
| " | 6.24 - 6.35 | 46.2 ± 1.5 | " |
| " | 6.39 - 6.48 | 51.4 ± 3 | " |
| " | 6.76 - 6.92 | 91.8 ± 6 | " |
| " | 6.80 - 6.90 | 133 ± 3 | " |
| " | 6.98 - 7.08 | 152 ± 2 | " |

Table IV

Variation of $K_{meas.}$ with pH for the Reaction of Horse Ferri-myoglobin and Imidazole T = 25.0 ± 0.2°C. and I = 0.20

| pH | $K_{meas.}$ | Buffer used |
|---|---|---|
| 5.205 - 5.234 | 5.56 ± ? | KHphthalate/NaOH |
| 5.510 - 5.531 | 6.04 ± 1 | " |
| 5.535 - 5.579 | 7.13 ± 0.9 | " |
| 5.550 - 5.613 | 7.41 ± ? | " |
| 5.730 - 5.770 | 9.76 ± 1.4 | " |
| 5.775 - 5.788 | 13.3 ± 0.3 | " |
| 5.950 - 5.988 | 19.8 ± 1.5 | " |
| 7.340 - 7.450 | 140 ± 4 | $NaH_2PO_4.H_2O$/NaOH |
| 7.520 - 7.647 | 149 ± ? | " |
| 7.600 - 7.730 | 160 ± 3 | $H_3BO_3$/NaOH |
| 7.750 - 7.871 | 160 ± 5 | $NaH_2PO_4.H_2O$/NaOH |
| 8.020 - 8.074 | 184 ± 8 | $H_3BO_3$/NaOH |
| 8.170 - 8.187 | 118 ± 5 | " |

Table V

Variation of $K_{meas.}$ with pH for the Reaction of Whale Ferrimyoglobin and Imidazole  I = 0.20

| Temperature °C. | pH | $K_{meas.}$ | Buffer used |
|---|---|---|---|
| 20°C | 9.952 - 9.975 | 32.3 ± 0.6 | $H_3BO_3$/NaOH |
| " | 10.140 - 10.237 | 28.2 ± 0.5 | " |
| " | 10.411 (av.) | 20.8 ± 0.5 | " |
| " | 10.758 - 10.831 | 14.3 ± 0.1 | Glycine/NaOH |
| " | 11.155 - 11.254 | 10.5 ± 0.4 | " |
| 25°C | 8.095 - 8.120 | 113 ± 3.0 | $H_3BO_3$/NaOH |
| " | 9.635 - 9.660 | 29.0 ± 1.0 | " |
| " | 10.102 - 10.135 | 20.6 ± 0.5 | " |
| " | 10.230 (av.) | 19 - 24 | " |
| " | 10.300 (av.) | 18.6 ± 0.4 | Glycine/NaOH |
| " | 10.362 - 10.460 | 17.8 ± ? | $H_3BO_3$/NaOH |
| " | 10.375 - 10.440 | 14.7 ± ? | Glycine/NaOH |
| " | 10.578 - 10.605 | 14.6 ± ? | " |
| " | 11.040 - 11.081 | 5.5 ± ? | " |
| " | 11.989 - 12.011 | 4.4 - 4.9 | " |
| 35°C | 9.810 - 9.859 | 20.8 ± 0.4 | $H_3BO_3$/NaOH |
| " | 9.950 or 10.04 | 17.0 ± 1.4 | " |
| " | 10.054 - 10.075 | 15.3 ± 0.3 | " |
| " | 10.235 - 10.303 | 12.0 ± 1.6 | Glycine/NaOH |
| " | 10.432 - 10.515 | 11.4 ± 0.2 | " |

## Calculation of K for the Ferrihemoglobin-Imidazole Reaction

The data for horse ferrihemoglobin given in Table III covers the pH range 6.0 - 8.5. This makes the analysis difficult, for equation 25 cannot be directly plotted as equation 26.

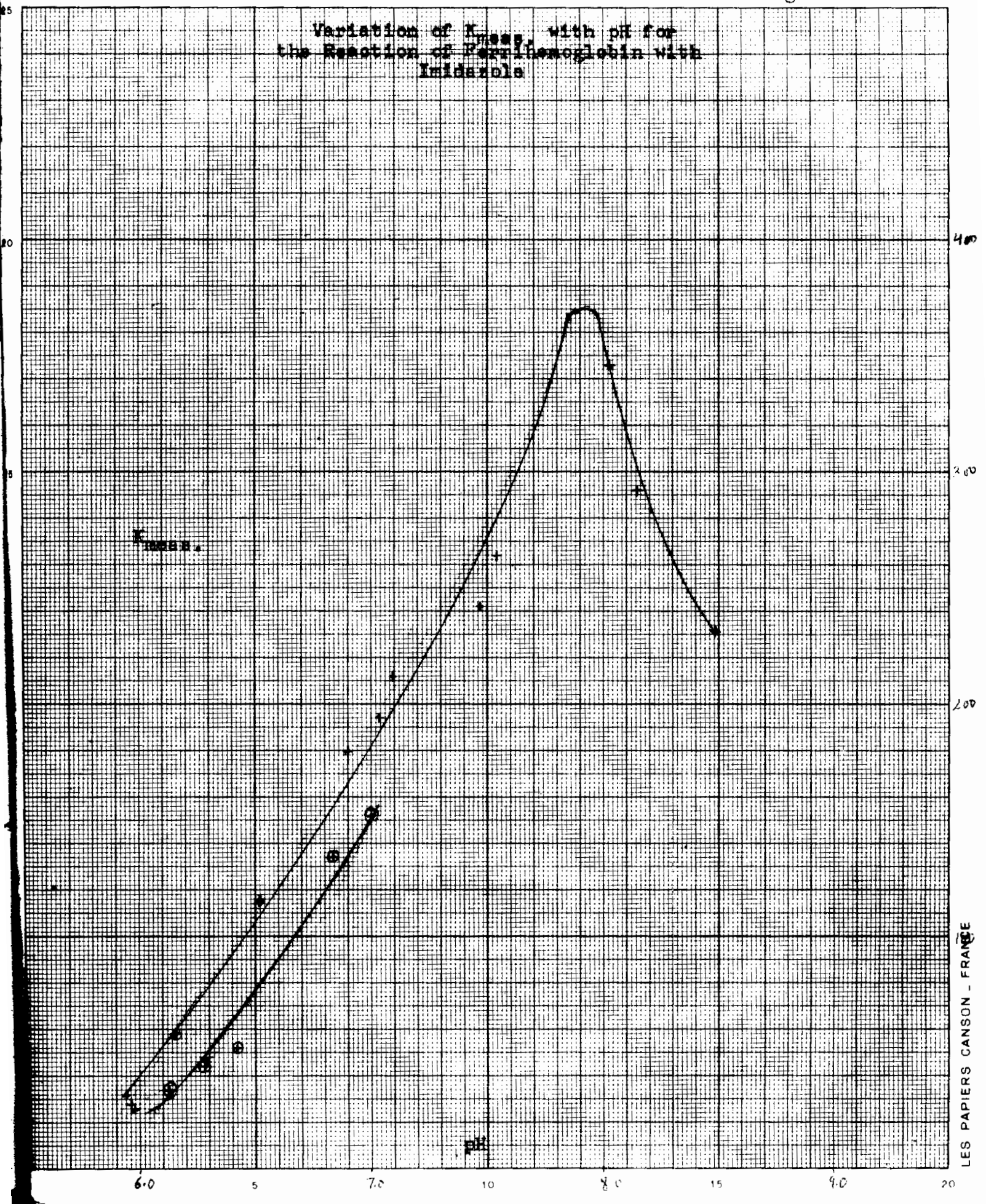
Nevertheless, by taking reasonable values for $K_r$, $K_p$ and $K_1$, we can calculate K from equation 25 for each of the given pH values. A preliminary calculation was carried out on the data covering pH 6 - 7 and using the values: $K_r \sim 10 \times 10^{-8}$; $K_p \sim 5 \times 10^{-8}$; $K_1 \sim 8 \times 10^{-8}$. The results showed that K, the true equilibrium constant for the ferrihemoglobin-imidazole reaction which corresponds to the ferrimyoglobin one below, is of the order of 400 at 25°C.

## Calculation of the Apparent Enthalpy for the Ferrihemoglobin-Imidazole Reaction

Although the results obtained on horse ferrihemoglobin are not sufficient for a good calculation of the enthalpy change, we can still use the data at 25° and 35°C. given in Table III and plotted in figure 3.

The approximate value around pH 6.5 was found to be $\Delta \overset{\circ}{H} = -5{,}300$ cal./mole.

Fig. 3


Variation of $K_{meas.}$ with pH for the Reaction of Ferrihemoglobin with Imidazole
$K_{meas.}$
pH
6.0
7.0
8.0
9.0
400
200
LES PAPIERS CANSON - FRANCE

Calculation of K and $K_3$ for Ferrimyoglobin

The value of the true equilibrium constant K, (which refers to equation 11 and is defined in equation 23) and of the heme-linked ionization constant $K_3$ (which refers to equation 7) can now be calculated by applying equation 26 to the data on Whale ferrimyoglobin given in Table V.

Figure 4 shows the (linear) relation between $K_{meas.}(K_{Fe}+h)$ and hydrogen ion concentration h at 35°C. Two lines are drawn to show the limits of the slope. The value of $K_{Fe}$ was assumed to be the same as that for horse myoglobin[21a], viz. $110\times10^{-11}$ at 20°C., $120\times10^{-11}$ at 25°C. and $140\times10^{-11}$ at 35°C. All points show the limits of variation in the parameters. The results are:

$$K = \text{Slope} = 141 \pm 15$$

$$K \times K_3 = \text{Intercept} = 1.12 \times 10^{-8}$$

therefore

$$K_3 = 8.01\ (\pm\ 0.70) \times 10^{-11}$$

$$pK_3\ (35°C.) = 10.10 \pm 0.04$$

Similar plots were made at 20°C. and at 25°C. using the data given in the same table. The results were as follows:

At 20°C., $K = 310 \pm 45$

$K_3 = 3.23\ (\pm\ 0.48) \times 10^{-11}$

$pK_3 = 10.49\ (\pm\ 0.06)$

At 25°C., $K = 255 \pm 45$

$K_3 = 4.15\ (\pm\ 0.70) \times 10^{-11}$

$pK_3 = 10.38\ (\pm\ 0.07)$

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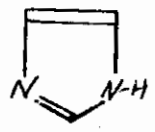
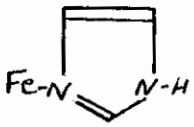
$pK_3 = 10.38 (\pm 0.07)$

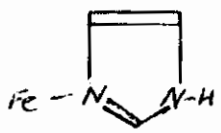
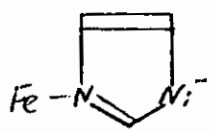
Calculation of $\Delta H^\circ$ and $\Delta S^\circ$ for K and $K_3$

The true equilibrium constant K, for which the values at 20, 25 and 35°C. were calculated above, refers to the reaction:

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

which has already been defined in equation 11, and which is the overall equilibrium predominating around pH 8. From these three values of K, the enthalpy change $\Delta H^\circ$ can be obtained in the usual way from the plot of pK vs. $1/T(^\circ K)$, as shown in figure 5.

Similarly, the values of $K_3$ at 20, 25 and 35°C., which were calculated above, refer to the heme-linked ionization on the complex

$$\text{  } + H_2O = \text{  } + H_3O^+$$

already defined in equation 7. For comparison with the above, the values of $pK_3$ have been plotted on figure 5 also. The results are as follows:

$$\Delta H^\circ = 2.303\ R\ \frac{d(pK)}{d(1/T)}$$

$$\Delta S^\circ = \frac{\Delta H^\circ}{T} - 2.303\ R\ (pK)$$

For K: $\Delta H^\circ = -\ 11{,}100\ (\pm\ 1{,}700)$ cal./mole

$\Delta S^\circ = -\ 26.4\ (\pm\ 9)$ e.u.

For $K_3$: $\Delta H^\circ = +\ 11{,}300\ (\pm\ 1{,}700)$ cal./mole

$\Delta S^\circ = -\ 9.6\ (\pm\ 4)$ e.u.

Calculation of the Apparent Enthalpy of Reaction

The data obtained in the present work are not extensive enough to permit the calculation of the apparent $\Delta H^\circ$ over a wide pH range. However, it is possible to do so for whale ferrimyoglobin in the pH range 9.8 - 10.6.

In figure 6, the experimental results taken from Table V show the variation of $K_{meas.}$ with pH at the three temperatures. Interpolating at constant pH, and plotting a series of log K vs. 1/T lines, we obtain the following approximate values:

at pH 9.8, $\Delta H^\circ = -\ 8{,}200$ cal./mole

" " 10.2, $\Delta H^\circ = -\ 7{,}500$ " "

" " 10.6, $\Delta H^\circ = -\ 6{,}200$ " "

these values are to be compared with the value $\Delta H^\circ = -11{,}100$ cal./mole calculated above for the true equilibrium constant, that is around pH 8. The progressive drop in the value of $\Delta H^\circ$ with increasing temperature will be commented on in the discussion below.

Fig. 4

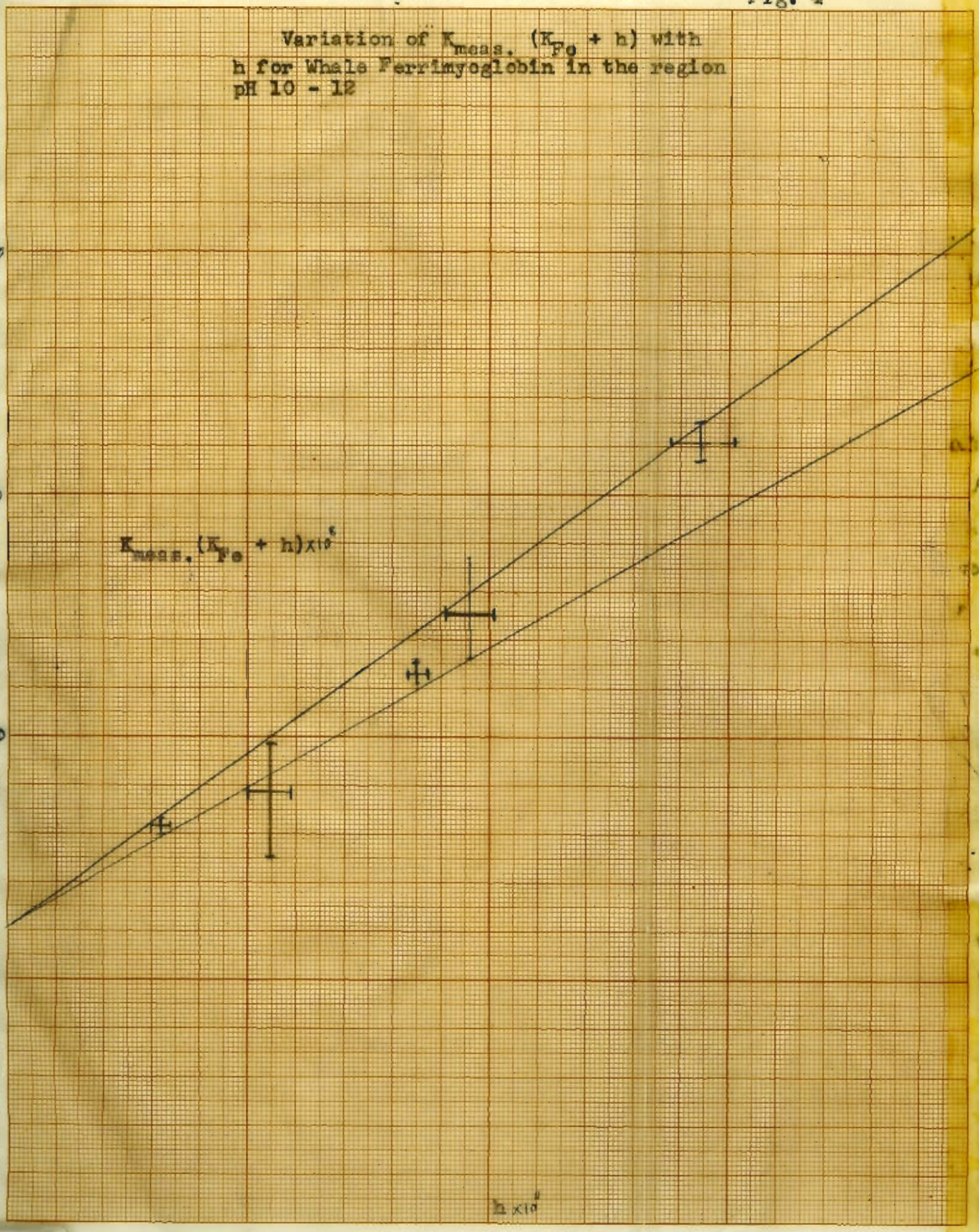

Variation of $K_{meas.}(K_{Fe} + h)$ with h for Whale Ferrimyoglobin in the region pH 10 - 12
$K_{meas.}(K_{Fe} + h) \times 10^5$
4.0
3.0
2.0
1.0
$h \times 10^4$
5
10
15
20

Fig. 5

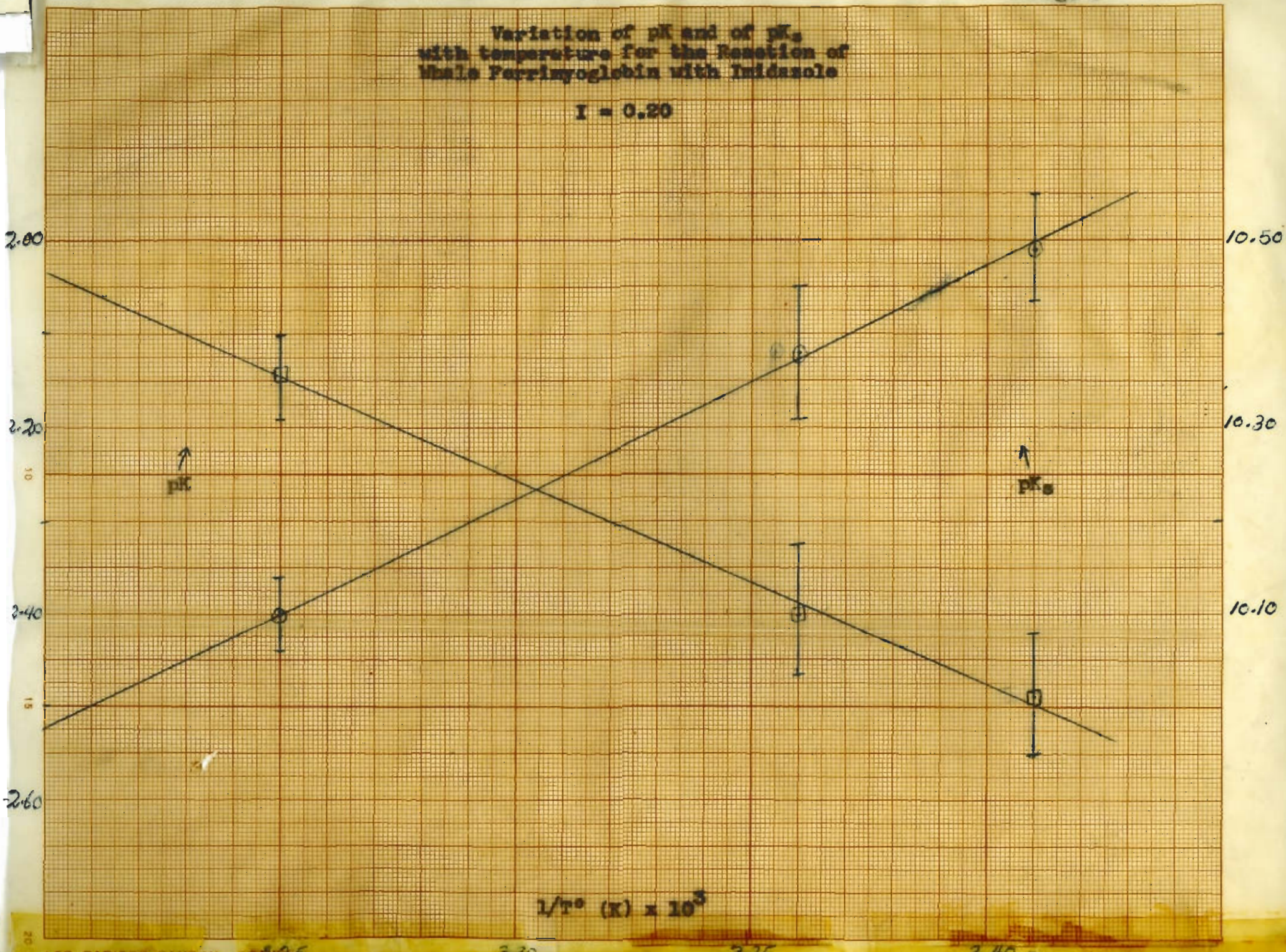
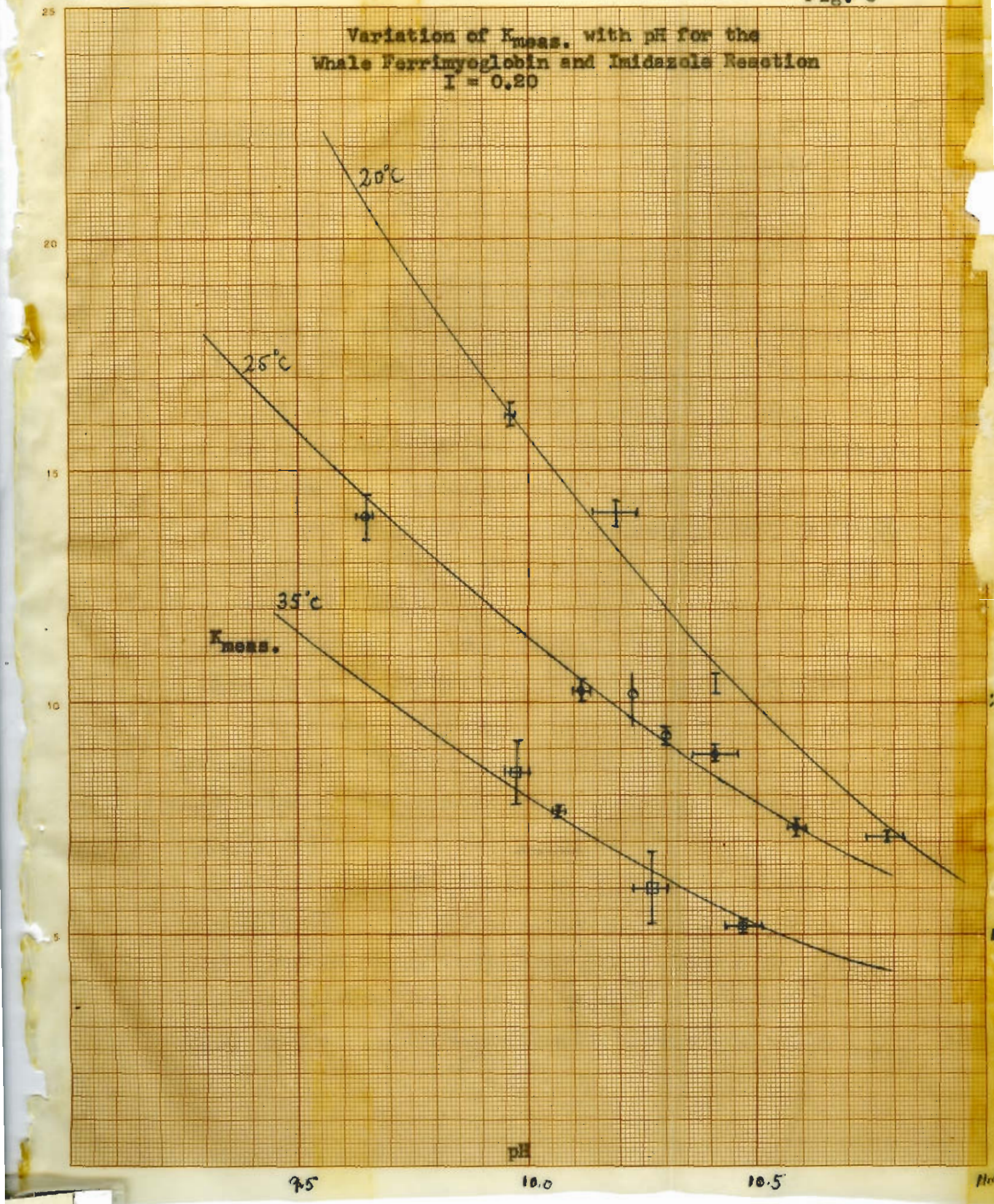

Variation of pK and of pK$_s$
with temperature for the Reaction of
Whale Ferrimyoglobin with Imidazole
I = 0.20
2.00
2.20
2.40
2.60
10.50
10.30
10.10
pK
pK$_s$
1/T° (K) x 10$^3$
3.30
3.35
3.40

Fig. 6


Variation of $K_{meas.}$ with pH for the
Whale Ferrimyoglobin and Imidazole Reaction
I = 0.20
20°C
25°C
35°C
$K_{meas.}$
25
20
15
10
5
pH
9.5
10.0
10.5

## DISCUSSION

Difficulties: In this type of work there are several inherent difficulties, both experimental and theoretical. Experimentally the following difficulties were met.

1 - Work was done on two species of myoglobin and one hemoglobin. It would have been better to concentrate on one species and obtain extensive data, but it was not possible to make a fresh preparation when the material available was consumed. Nevertheless it is interesting to be able to compare the results obtained on different types of hemoprotein.

2 - The structural sensitivity of the hemoprotein molecule (hemoglobin more so than myoglobin) made it difficult to work unless the conditions were very carefully chosen and reproducibility checked again and again at every stage. Otherwise, denaturation and subsequent fluculation of the protein produced very abnormal spectrophotometric results.

3 - In the early stages of the work, the instruments used and the methods of thermostating were inadequate, and most of the results obtained that way had to be discarded because of their low precision.

4 - As was seen above the analysis of the results is very sensitive to hydrogen ion concentration. Moreover since every run involved measurements on a series of buffered solutions which did not have identical pH (because of the change in the relative concentration of imidazole) every $K_{meas.}$

value obtained/had an error not only from the averaging of results but also from the variation of pH within the run. This can be seen in the way the figures were drawun, where the limits of error were indicated.

Theoretically also there is an inherent difficulty in this kind of work. For, not only does one has to find, by mathematical analysis of experimental data, several heme-linked ionization constants ($K_r$, $K_p$ and $K_3$) but it is necessary also to take into account the probability that each of these constants is itself a function of the parameter being investigated.

Variation among Species: Preliminary measurements which have been done on both myoglobins show that under exactly the same conditions, they give different thermodynamic equilibrium constants in their reaction with imidazole. This suggest, that the protein plays a role in the reaction though a secondary one. It also confirms what other workers in the field of hemoproteins and enzymes have observed in many other reactions (see for example reference 21b).

Heme-Linked Ionizations:

1 - Ionizations in the region pH 6 - 7. The variation of $K_{meas.}$ with pH in the region pH $<$ 7 could only be explained on the basis of an ionization of an acidic group linked to the heme in the ferrihemoprotein. George and Hanania[48] have already published an analysis of this effect showing that the observed effect is the result of a difference in the acid

strength of the group between its state in the reactant and the product of the reaction. That is, depending on the extent of interaction between bonding of the ligand to the iron and dissociation of the heme-linked acidic group, the effect observed may be negligibly small, or just perceptible if $|pK_r - pK_p| \sim 0.2$, or more pronounced as this difference becomes larger.

In the case of ferrihemoglobin reacting with imidazole, the above results showed that $|pK_r - pK_p| \sim 0.3$.

2 - Ionization in the pH range 10 - 11. In the case of whale ferrimyoglobin the above analysis of results showed that an ionization of a heme-lined acidic group occurred in the product (the complex), but not in the reactant. This is to be expected if it is an ionization connected with the ligand imidazole.

It is interesting to note in this connection that N-Methyl Imidazole, which forms a similar complex with ferri-hemoproteins but does not have H on the imino N of imidazole, does not show a heme-linked effect in this pH range[8]. This supports the view that the heme-linked ionization with $pK_3 \sim 10.3$ obtained above, is probably that of the imino group of imidazole as defined in equation 13.

If so, it becomes even more interesting to note that the corresponding ionization in free imidazole[47] has $pK_2 = 14.5$. Evidently this group, which is an extremely weak acid in imidazole becomes $10^4$ times stronger in the complex. This

could be accounted for on the basis of resonance with the iron in the heme which stabilizes the anion.

The only work of similar type that seems to have been done was reported by Hanania[50] and Irvine who found a $10^3$ times increase in the strength of an (-OH) oxime group upon coordination to Fe(II) in aqueous solutions.

The question now arizes as to why this ionization did not also appear in the reactant (ferrihemoprotein). For, if the imidazole hypothesis is true the iron atom is linked to an imidazole (histidine) on the protein side of the heme. One of three conclusions can be drawn:

i - There is no imidazole bonded to the iron on the protein side. (see the section on imidazole hypthesis above).

ii - There is an imidazole (histidine) bonded to iron, and the H of its imino group is hydrogen bonded to a carbonyl group in the polypeptide chain[40]. In this case it could be argued that the effect of hydrogen bonding, coordination to the metal, and the effect of histidine linkage, etc., have altered the acid strength so much that it became very weak ($pK > 12$) or very strong (pK 6 - 7). In the former case it could not be measured, and in the latter case it would be identical with the other heme-linked effect discussed above.

iii - Even if the imino group in imidazole (histidine) were around the pH region 10 - 11, it would still be possible not to detect it if $|pK_r - pK_p| < 0.2$.

Another line of evidence in this regard comes from an

examination of $\Delta H^\circ$ and $\Delta S^\circ$ values. The above results showed that, for the heme-linked ionization in this range (i.e. $pK_3$), $\Delta H^\circ = +11{,}300$ and $\Delta S^\circ = -9.6$ e.u. These thermodynamic data are not unreasonable for weak nitrogen bases and ammonium groups. But, one must take into account the effect of coordination to a metal on the thermodynamics of ionization of a heme-linked group. If this group is in fact the imino group of imidazole, with $pK_2 = 14.5$ shifting to $pK_3 = 10.3$ in the complex, then the $\Delta F^\circ$ has changed by about 5.7 Kcal./mole. This could arize either/a due to change in enthalpy or in entropy or both. The maximum contribution from enthalpy is obviously 5.7 Kcal./mole, which would make $\Delta H^\circ = 11{,}300 - 17{,}000$ cal./mole for the ionization of the imino group in imidazole. Unfortunately the heat of ionization of this group has not been determined, possibly because it is experimentally difficult to do so. However it is a crucial measurement from the point of view of this work.

Variation of $\Delta H^\circ$ for the Ferrimyoglobin-Imidazole Reaction with pH: It was shown above that the apparent enthalpy of reaction, obtained from $K_{meas.}$ values, varied with pH. This is a consequence of the fact that the overall equilibrium changes with pH as shown in equations 10 - 14.

Starting with the reaction around pH 8, for which the true equilibrium constant K leads to a value $\Delta H^\circ = -11{,}100$ cal./mole, and using $\Delta H^\circ = +6{,}900$ for the ionization of the tertiary nitrogen of imidazole, it follows that at pH 7

(equation 11) $\Delta H^{\circ}$ = - 4,200 cal./mole. This value could not be tested on myoglbin because no data were available, but the corresponding hemoglobin results (see figure 3, Table III) showed an enthalpy of around 5 Kcal./mole.

It is very strange that Scheler[8] reported $\Delta H^{\circ}$ = -4.3 Kcal./mole for the corresponding hemoglobin reaction at pH 8. Unfortunately he did not publish the details of his results, and so the discrepancy can not be explained.

Again using the value $\Delta H^{\circ}$ = - 11,100 cal./mole together with $\Delta H^{\circ}$ = + 5,700 cal./mole for the ionization of ferrimyoglobin, one gets $\Delta H^{\circ}$ = - 16,800 cal./mole for the reaction in equation 12. However this overlaps with the heme-linked ionization around pH 10.3, for which $\Delta H^{\circ}$ = + 11,300 cal./mole. The result is that at pH $>$ 11 the apparent $\Delta H^{\circ}$ for the reaction should approach the value - 5, 500 cal./mole. The above calculation in the section on results showed that, the apparent $\Delta H^{\circ}$ varied from - 8,200 at pH 9.8 to - 6,200 at pH 10.6. These values are in fairly good agreement with expectation. Naturally, this can be tested more precisely as more data become available.

Finally, it is very important and very interesting to note that no conclusions can be drawn about the nature of the heme-linked group, from the pK of its ionization, because the latter changes greatly in the free and in the coordinated form of the group. (Compare $pK_2$ = 14.5 to $pK_3$ = 10.3).

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