KINETICS OF THE REACTION

OF

SPERM WHALE FERRIMYOGLOBIN

WITH CYANIDE

BY

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ABSTRACT

The pH dependence of sperm whale ferrimyoglobin reaction with cyanide in the range pH 5-7, in tris-maleate-potassium chloride buffers of ionic strength 0.15 M was investigated at two temperatures 25.0°C and 15.6°C. The pH dependence of the observed second order reaction with cyanide was found to obey the empirical equation k_F = constant . $(H)^{-\frac{1}{2}}$. It was established that a shoulder exists in the pH-profile around pH 5.7. The pH-profile was interpreted in terms of a mechanism involving two forms of acid ferrimyoglobin. Where the shoulder represents transition between the two myoglobin species. Analysis of the transition region led to the conclusion that a simultaneous 4-proton transfer accompanies the conversion of one of the myoglobin species to the other. The pK_{int} of the ionizing groups involved at 25.0°C was 5.695, ΔH^0 = 2.28 kcalmole^{-1} and ΔS^0 = -1.84 calmole^{-1}deg^{-1}, suggesting hydrogen-bonded carboxyls.

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A	Absorbancy (cm^{-1})
ϵ	Molar absorption ($M^{-1}cm^{-1}$)
(I)	Ionic strength (M)
(T-M)	Tris-maleate buffer
$k_F, k_1, k_1', k_2, k_2', k_A, k_B$	Second order kinetic constants ($M^{-1}sec^{-1}$)
$K_a, K_{tr}, K_{int}, K_{CN}$	Equilibrium constants
Mb	Myoglobin molecule
H or (H^+)	Hydrogen ion concentration
χ	Specific conductance ($ohm^{-1}cm^{-1}$)

CHAPTER I

INTRODUCTION

Myoglobin from horse heart was first crystallized by Theorell in 1932[24], and has been since then isolated from a variety of other species. Myoglobin is 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 the iron(III) form, ferrimyoglobin, it is the latter which has been subject to many physico-chemical investigations.

Chemically, myoglobin is a hemoprotein containing the iron protoporphyrin IX complex in conjugation with the globin moiety. It has a molecular weight of about 18000[11]. Ferrimyoglobin is the first protein whose detailed three-dimensional X-ray crystal structure has been determined with resolutions of 2.0 and recently 1.4 Å [20,21]. Myoglobin crystals are monoclinic. The molecule is compact and very little water is held in it[21]. The protein, a single polypeptide chain of 155 amino-acid residues of known sequence[12], forms to a large extent a right-handed α-helix, folding in a complex manner to form a flattened roughly triangular prism with dimensions about 45 by 35 by 25 Å[21]. Myoglobin varies, although slightly, with the genetic species from which it is derived[4,5,10]. Recent work on protein fractionation using ion-

exchange chromatography[1,12,20], and starch gel electropheresis[23] showed that myoglobin is microheterogeneous.

The study of the physico-chemical properties of myoglobin is important due to the physiological role of myoglobin as well as to its close similarity to hemoglobin[9]. Thus the study of secondary interactions between the iron atom and its globin environment can also serve as a model for the understanding of the more complicated hemoprotein reactions, especially those of hemoglobin.

As a coordination compound, ferrimyoglobin presents the case of a complex ion with a single bond available for reaction with ligands such as H_2O, OH^-, CN^-, F^-, N_3^-, SCN^-, OCN^-, $HCOO^-$. Furthermore, these reactions are measurable, thus allowing the calculation of exact thermodynamic and kinetic constants.

The observation that the addition of a little cyanide to an aqueous solution of myoglobin leads to an abrupt change in the absorbancy was explained by Vles[26] as the formation of a cyanohematin. Holden[17] suggested that this reaction is irreversible. Pauling and coworkers[8,21], and Theorell and coworkers[25] concluded from magneto-chemical studies that the myoglobin cyanide bond is a covalent one (in contrast with the ionic bond between fluoride and myoglobin). It was left to Bennett and Ingram[3] to assign the d^2sp^3, a symmetrical octahedral configuration to the complex. It should be noted that the complex involves bonding of the carbon atom of cyanide[15] to the iron atom of the heme, and that hydrocyanic acid does not bind at all[7].

The equilibrium nature of the reaction of myoglobin with cyanide as well as the molecularity of this reaction in buffered and unbuffered solutions has been investigated[15,18]. Hanania[15] studied the reaction of equine myoglobin with cyanide in the pH range 5-12. He based the analysis of his data on a four-reaction mechanism assuming a heme-linked ionizing group:

$$PFe^{+}H_2O + CN^{-} \underset{k_{-1}}{\overset{K_1}{\rightleftharpoons}} PFeCN + H_2O$$

$$PFe^{+}H_2O + HCN \underset{k_{-1}}{\overset{k_1'}{\rightleftharpoons}} PFeCN + H_3O^{+}$$

$$HPFe^{+}H_2O + CN^{-} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} HPFeCN + H_2O$$

$$HPFe^{+}H_2O + HCN \underset{k_{-2}}{\overset{k_2'}{\rightleftharpoons}} HPFeCN + H_3O^{+}$$

where HP fefers to the protonated heme-linked ionizing group, and thus P refers to the conjugate base of this group on the globin moiety. The values of the intrinsic constants pertaining to this reaction mechanism according to Hanania are listed below:

$k_1 = 41$ $\qquad$ $k_1' = 110$

$k_2 = 150 \times 10^3$ $\qquad$ $k_2' = 3.7 \times 10^3$

However, due to the lack of experimental data on the temperature dependence of the forward rate, no activation energies were computed. Scheler and coworkers[6] have carried out work on the reaction of equine myoglobin (component I) with cyanide, but their work does not include a pH profile, nor an inveatigation of temperature dependence.

A detailed study of cytochrome C cyanide formation has been carried out by George and Tsou[14] who assumed the following mechanism:

$$\text{cyt C} + \text{HCN} \xrightarrow{k_{HCN}} \text{cyt C-CN} + H^+$$

$$\text{cyt C} + CN^- \xrightarrow{k_{CN}} \text{cyt C-CN}$$

on the basis of which they calculated the following kinetic constants:

		ΔE^* kcalmole^{-1}	ΔH^* kcalmole^{-1}	ΔS^* caldeg^{-1}mole^{-1}
k_{HCN}	0.0543	18.4 ± 0.4	17.8 ± 0.4	-5.9 ± 1.4
k_{CN}	15.2	17.0 ± 0.5	16.4 ± 0.5	0.5 ± 1.7

It is the aim of this work to study the reaction of sperm whale myoglobin with cyanide in the so-called "heme-linked" pH region in detail, in order to derive more exact thermodynamic and kinetic data and possibly throw light on the mechanism of the reaction.

CHAPTER II

EXPERIMENTAL

A. Materials

Myoglobin. Sperm whale skeletal muscle ferrimyoglobin, salt-free, lyophilized, Batch I, purchased from Seravac Laboratory (Moneyrow Green, Holyport, Maidenhead, Berks., England) was used without further purification. Work in this laboratory[1a] showed that the sample contained 5 per cent hemoglobin, the iron content was 0.307 per cent on dry weight basis, and the absorbancy ratio $A_{410m\mu}/A_{280m\mu}$ was 5.18.

Myoglobin stock solutions approximately 5×10^{-3} M were prepared immediately before use.

Potassium cyanide. B.D.H. (England) "Laboratory Reagent" potassium cyanide was titrated with silver nitrate to determine its purity and was labelled 96 per cent KCN. Only freshly prepared solutions of potassium cyanide were used in the kinetic work.

Sodium hydroxide. Merck (Darmstadt, Germany) "Pro-Analysi" sodium hydroxide pellets were used to prepare a saturated solution (approximately 19 N). This was filtered through glass wool in order to remove carbonate and used to make standard solutions as needed.

Tris,(2-amino-2-hydroxymethyl-propane-1:3-diol). B.D.H. (England) "Laboratory Reagent" Tris was recrystallized from ethanol.

All other chemicals were of reagent grade and were used without further treatment.

All solutions were made using distilled deionized water whose specific conductance was better than 10^{-6} $ohm^{-1}cm^{-1}$.

B. Methods

Preparation of buffer solutions. A stock solution 1.00 M in Tris and 1.00 M in maleic acid was prepared. Of this solution 5 ml were transferred to a 100 ml volumetric flask, a calculated volume of 0.2 N sodium hydroxide was added to give the desired final pH, and the volume was brought up to 100 ml with water. The pH of the resulting buffer solution was measured and solid potassium chloride was added in order to bring the ionic strength to 0.15 M, the amount of potassium chloride having been calculated according to equation (10) below on the basis of the measured pH. The pH of the final buffer solution containing potassium chloride was measured and found to be identical with the first pH measurement. The effect of temperature on the Tris-maleate-potassium chloride buffer system was found to be negligible.

The amounts of potassium chloride added to make up the ionic strength to 0.15 M was calculated according to the following

considerations. Let T represent Tris and TH^+ the conjugate acid of Tris, and if MH_2, MH^-, $M^=$ represent respectively maleic acid, acid maleate and maleate, then we can write the following equilibria:

$$MH_2 \xrightleftharpoons{K_1} MH^- + H^+$$

$$MH^- \xrightleftharpoons{K_2} M^= + H^+$$

$$TH^+ \xrightleftharpoons{K_3} T + H^+$$

where the values[16] of these ionization constants are:

$$K_1 = 1.42 \times 10^{-2}$$

$$K_2 = 8.57 \times 10^{-7}$$

$$K_3 = 8.30 \times 10^{-8}$$

At pH 5 all the maleic acid should be in the ionized form.

Electroneutrality requires that:

$$(MH^-) + (M^=) = (T^+) + (Na^+) \qquad (1)$$

When no sodium hydroxide is added to the mixture we may write:

$$2C^o_= + C^o_- = C^o_+$$

where $C^o_= \equiv (M^=)_{NaOH = 0}$

$C^o_- \equiv (MH^-)_{NaOH = 0}$

$C^o_+ \equiv (TH^+)_{NaOH = 0}$

when X meq of NaOH are added we have X meg of (Na^+) and X meg of (OH^-). The amount of X (OH^-) will distribute itself, according to the respective equilibria between acid maleate and Tris conjugate acid. Thus we may call X_M and X_T the fractions of X(OH^-) used up by acid maleate and the conjugate acid of Tris respectively. This leads to:

$$(C^{o}_{-} - X_M) + 2(C^{o}_{=} + X_M) = (C^{o}_{+} - X_T) + (X_M + X_T) \quad (2)$$

Then

$$I = \frac{C^{o}_{-} + C^{o}_{+}}{2} + 2\,(C^{o}_{=} + 2\,X_M) \quad (3)$$

Since (MH_2) $\quad C = C - (M^{=})$ (4)

and

$$K_2 = \frac{(H^{+}) \times (M^{=})}{(MH^{-})} \quad (5)$$

it follows that

$$C_{=} = (M^{=}) = \frac{C\,K_2}{(H^{+}) + K_2} \quad (6)$$

substituting $X_M = C_{=} - C^{o}_{=}$

we obtain

$$I = \frac{C^{o}_{-} + C^{o}_{+}}{2} + 2\,C^{o}_{=} + 2\,C_{=} - 2\,C^{o}_{=} \quad (7)$$

$$= \frac{C^{o}_{-} + C^{o}_{+}}{2} + \frac{2C\,K_2}{(H^{+}) + K_2} \quad (8)$$

Where pH $>$ 5 we may write:

$$\frac{C^{o}_{+} + C^{o}_{-}}{2} = C = 0.05\ \underline{M} \quad (9)$$

Thus

$$I = 0.05 + \frac{0.10\,K_2}{(H^{+}) + K_2} \quad (10)$$

The conductivity of the buffer solutions before the addition of potassium chloride was measured (Conductivity Bridge Model RC, 16B2, Industrial Instrument Inc. Ltd., Cedar Groove, N.J.). The cell constant was determined using 0.01 M potassium chloride as standard, whose specific conductance at 25°C was taken as 0.001322 $ohm^{-1}cm^{-1}$.[16]

A plot of the ionic strength calculated by equation (10) against the corresponding measured specific conductance was linear (see Fig. 1), showing that equation (10) is valid. After the addition of potassium chloride all buffer solutions had the same conductivity.

Spectra of ferrimyoglobin and ferrimyoglobin cyanide. The absorption spectra of ferrimyoglobin and ferrimyoglobin cyanide, were measured at ionic strength 0.15 M in Tris-maleate-potassium chloride buffer in the spectral regions 404 - 426 mμ (Table I, Fig. 2a) and 535 - 565 mμ (Table II, Fig. 2b). The value 103, 400 at 408 mμ of the maximum difference between the spectra of acid myoglobin and myoglobin cyanide was used throughout this work. Similarly the value of 55,600 at 555 mμ was used in kinetic runs where the myoglobin solution was more concentrated.

No effect on the absorbancy of myoglobin cyanide at 423 mμ due to pH or to temperature was detected within experimental error as shown in Table III.

pH measurement. All pH measurements were made with an accuracy of ± 0.002 pH unit, (Radiometer, pH-meter-4, type RH.M4C). The instrument was standardized with phosphate and with phthalate buffers before and after the pH measurements. The standardization readings before and after were in good agreement. The buffers used for the

Table I

Spectra of Myoglobin and Myoglobin Cyanide at near U.V.

Conditions: (Tris-Maleate) = 0.05 M
I = 0.15 M
pH = 5.50
T = 25.0° ± 0.2°C

λ	$\epsilon_{MbH_2O} \times 10^{-3}$	$\epsilon_{MbCN} \times 10^{-3}$	$\Delta\epsilon \times 10^{-3}$
404	151.1	54.42	96.68
405	157.0	56.22	100.7
406	161.4	58.69	102.7
407	164.2	60.71	103.4
408	166.0	63.41	102.5
409	165.7	65.21	100.5
410	163.9	69.03	94.87
411	160.1	72.63	87.47
412	157.5	76.01	76.49
419	78.03	103.9	-25.87
420	67.91	106.1	-38.19
421	61.16	107.5	-46.34
422	53.29	108.8	-55.51
423	47.00	109.5	-62.50
424	40.48	108.6	-68.12
425	35.30	106.8	-71.50
426	31.26	104.3	-73.04

Table II

Spectra of Myoglobin and Myoglobin Cyanide in the Visible Region

Conditions: (Tris-Maleate) = 0.05 M
I = 0.15 M
pH = 5.50
T = 25.0° ± 0.2°C

λ	$\epsilon_{MbH_2O} \times 10^2$	$\epsilon_{MbCN} \times 10^2$	$\Delta\epsilon \times 10^2$
535	63.5	105	41.5
540	59.4	107	47.6
545	54.5	106	51.5
550	48.9	104	55.3
555	42.8	98.4	55.6
560	38.5	92.4	53.9
565	35.6	86.2	50.6

Table III

Effect of pH and Temperature on the Absorbancy of Ferrimyoglobin Cyanide at 423 mu.

Conditions: (Tris-Maleate) = 0.05 M
I = 0.15 M

pH	$\epsilon^{423}_{25} \times 10^{-3}$	$\epsilon^{423}_{15} \times 10^{-3}$
5.0	109.4	109.6
5.5	109.6	109.6
6.0	109.6	109.4
6.5	109.3	109.5
7.0	109.6	109.4
7.5	109.5	109.5

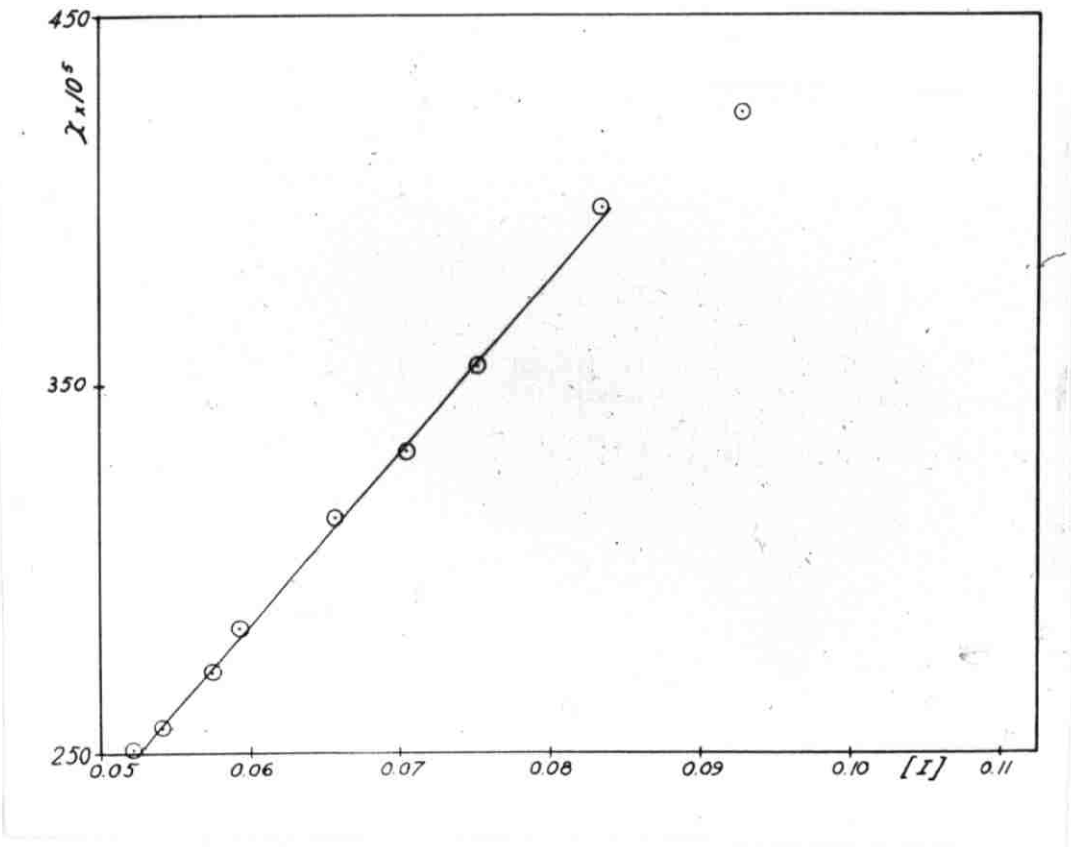


Fig. 1. Specific conductance χ in ohm^{-1} cm^{-1} versus the calculated ionic strength, according to equation (10), of tris maleate buffer solutions in the range pH 5 - 7 at 25°C.

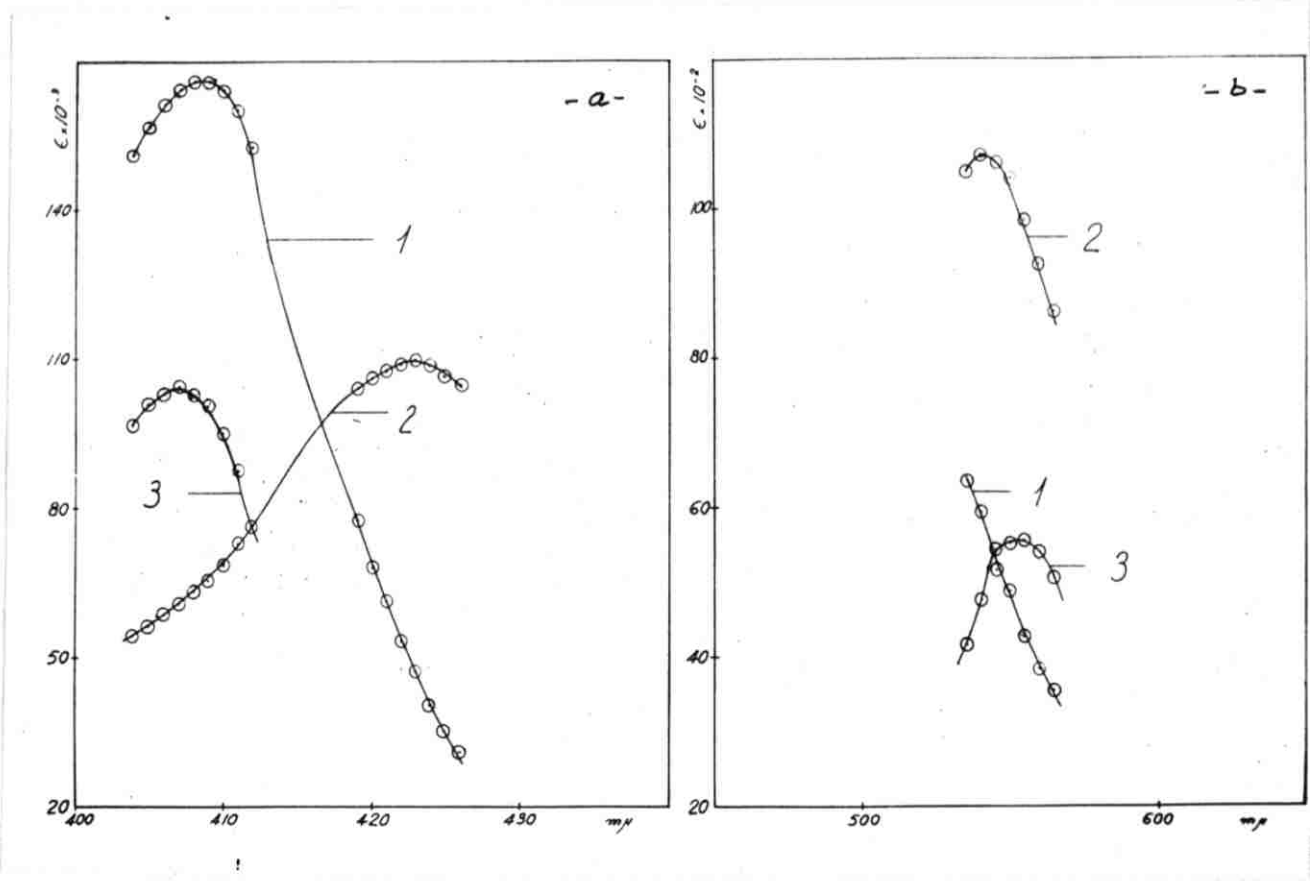


Fig. 2a. Absorption spectra of the Soret bands for (1) MbH_2O, (2) MbCN, (3) their difference.

b. Absorption spectra in the visible range for (1) MbH_2O, (2) MbCN, (3) their difference.

standardization of the pH-meter were the following N.B.S. standards[2]:

	15°C	25°C
Borax 0.01 M	9.26	9.18
KH phthalate 0.05 M	4.00	4.01
K_2HPO_4 0.025 M NaH_2PO_4 0.025 M	6.89	6.86

Temperature control and measurement. The reactants were brought to temperature equilibrium in a thermostated water bath, whose temperature was controlled within ± 0.2°C. The temperature in the reaction cell was measured by means of a thermo-couple "Tele-Thermometer" (Yellow-Springs Instrument Co. Inc., Ohio, U.S.A.) whose scale was standardized by thermometers calibrated by the National Physical Laboratory, England.

Measurement of the forward rate constant. The forward reaction of cyanide with ferrimyoglobin was followed spectrophotometrically (Zeiss Spectrophotometer, Model PMQII, Germany) and the change of absorbancy was automatically recorded (Sargent Recorder Model M.R., E. H. Sargent and Co., Scientific Laboratory Instrument, U.S.A.).

For kinetic runs at 25°C, 30 ml of ferrimyoglobin in Tris-maleate-potassium chloride buffer were transferred to the spectrophotometer cell, which was not thermostated. The recorder was thus

started, thus recording the absorbancy of acid ferrimyoglobin. Using a simple plunger , 10.0 ul of 0.100 M potassium cyanide were added to the reactants of the spectrophotometer cell, thus starting the reaction. At the end of the reaction the temperature was measured with the thermo-couple. This reading did not vary from 25°C more than ± 0.3°C.

Kinetic runs at 15.6°C were carried out in a jacketed-cell, through which thermostated water at 15.0°C was circulated. The 3.0 ml of ferrimyoglobin were left in the cell, and once the temperature reached 15.6°C, as measured with the thermo-couple, 10.0 ul of 0.100 M potassium cyanide were added.

The progress curves of the reaction of cyanide with acid ferrimyoglobin was obtained as a track of absorbancy versus time on the recorder chart, Fig. 3.

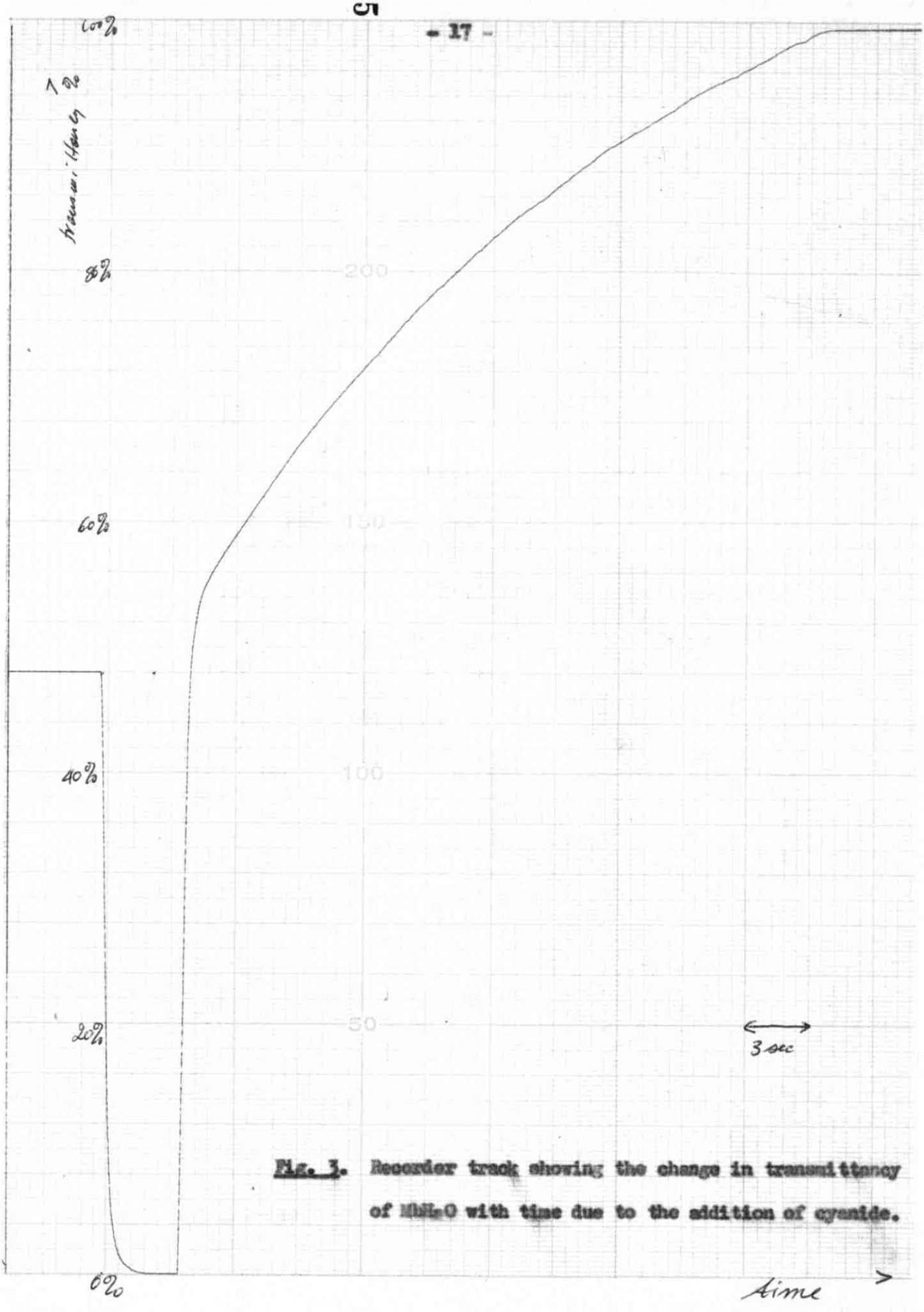


Fig. 3. Recorder track showing the change in transmittancy of MnL_2O with time due to the addition of cyanide.

CHAPTER III

RESULTS

Calculation of the observed first order rate constant (k_{obs}). In all runs a plot of log $(A - A_{\infty})$ versus time (t) gave a straight line showing good 1st order kinetics up to about 50 per cent reaction Fig. 4. The first order rate constant is then given by

$$k_{obs} = -2.303 \times (\text{slope}).$$

The effect of myoglobin concentration on k_{obs} was investigated, table IV, fig. 4. It was found that variation of the myoglobin concentration does not affect the rate constant, showing that k_{obs} is a genuine first order constant.

Calculation of the second order rate constant (k_F). Measurement of the forward rate constant with successive excess amounts of cyanide at the same myoglobin concentration, showed that k_{obs} is directly proportional to the stoichiometric cyanide concentration table V, Fig. 5. The observed second order constant k_F was therefore calculated from the relation

$$k_F = \frac{k_{obs}}{(CN)_{stoi}} .$$

The value of $(CN)_{stoi}$ throughout this work was 3.19×10^{-4} M.

Effect of pH and temperature on k_F. The pH profiles over the range pH

Table IV

Effect of Myoglobin Concentration on k_{obs}.

General conditions: (I) = 0.15 M
(T-M) = 0.05 M
T = 25.0 ± 0.2°C
(CN^-) = 9.57×10^{-4} M
pH = 5.06

$(Mb) \times 10^6$	$k_{obs} \times 10^3$ sec^{-1}	k_F $M^{-1} sec^{-1}$	λ mμ
3.72	68.1 ± 0.8	71.4 ± 0.9	408
12.70	70.7 ± 1.7	74.0 ± 1.7	-
17.44	69.0 ± 0.1	72.1 ± 0.1	-
23.45	71.3 ± 1.4	74.5 ± 1.5	-
23.68	68.2 ± 0.6	71.4 ± 0.6	555
58.94	69.5 ± 1.3	72.6 ± 1.3	-
127.3	69.9 ± 1.1	73.0 ± 1.2	-

$10^3 \times k_{obs}$ = (69.5±1.0) k_F = 72.7 ± 1.3

Table V

Effect of (CN) on k_{obs}.

General conditions:

(Mb) = 17.02×10^{-6} M

λ = 408 mμ

(I) = 0.15 M

(T-M) = 0.05 M

pH = 5.28

T = 25.0 ± 0.2°C

$(CN^-) \times 10^4$	$k_{obs} \times 10^3$ sec^{-1}	k_F M^{-1} sec^{-1}
2.88	24.2 ± 1.2	84.1 ± 4.1
4.90	39.7 ± 1.9	91.3 ± 4.0
6.72	57.6 ± 1.1	85.7 ± 1.5
9.60	80.0 ± 1.4	83.3 ± 1.5
14.40	118.0 ± 4.0	81.6 ± 3.1
24.00	200.0 ± 5.0	83.0 ± 2.4

k_F = 83.1 ± 2.8

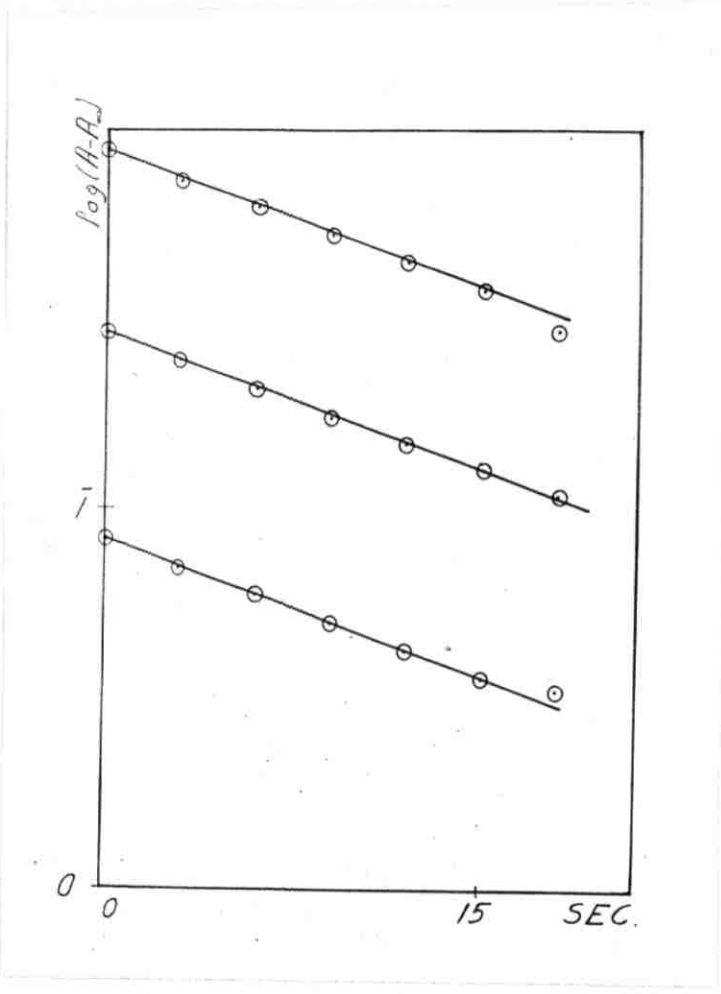


Fig. 4. General conditions: pH = 5.06
T = 25.0°C
(I) = 0.15 M
(T-M) = 0.05 M
(CN) = 9.57 x 10^{-4}

A plot of log (A - A_∞) versus (t) showing the first order kinetics over about 50 per cent reaction. Plots of log (A - A_∞) versus (t) where the initial (Mb) are

(Mb) = 3.72 x 10^{-6} M
(Mb) = 12.70 x 10^{-6} M
(Mb) = 17.44 x 10^{-6} M

showing the independence of k_{obs} on (Mb).

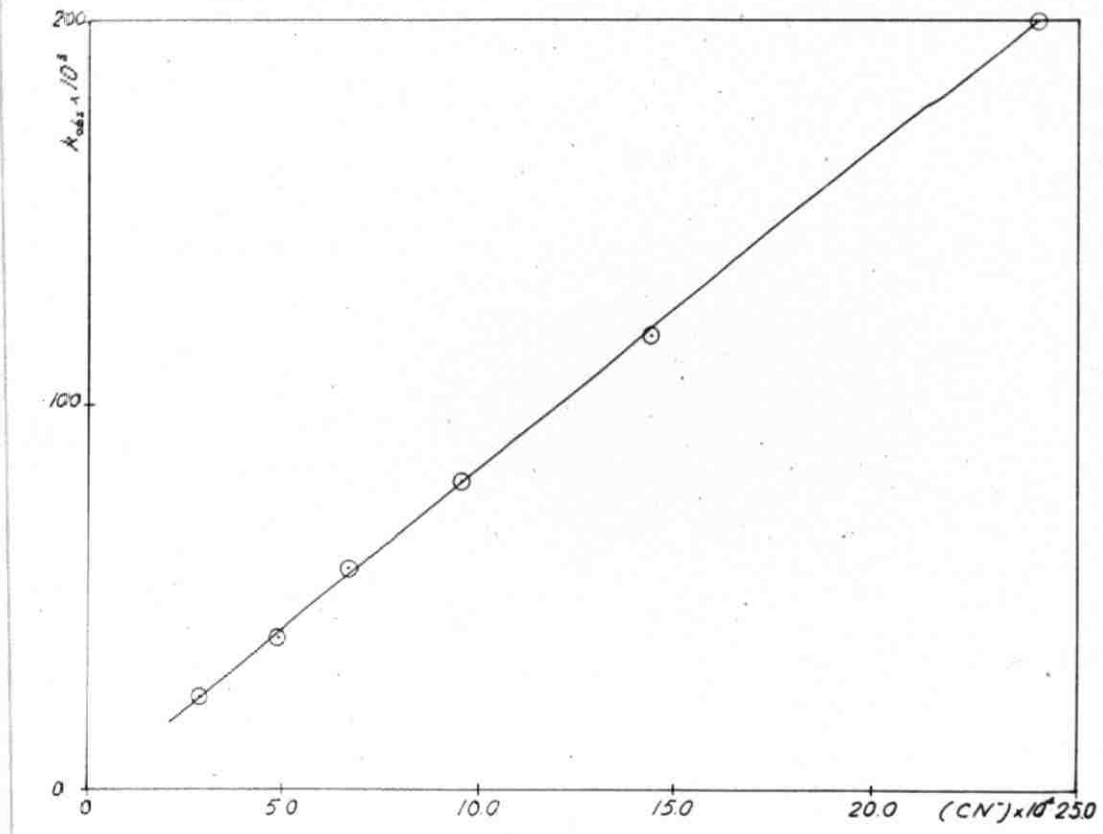


Fig. 5. General conditions: pH = 5.28
T = 25.0°C
I = 0.15 M
(T-M) = 0.05 M

Linear dependence of k_{obs} on the initial excess cyanide concentration.

5.2 - 7.0 at 25.0°C and at 15.6°C are shown in Fig. 6. The values plotted are given in tables VI , VII , where each k_F value represents the average of triplicate runs whose mean deviations are also given in tables VI , VII .

Analysis of the forward rate kinetic data. The calculation of kinetic constants from the pH profiles according to Hanania's four-reaction scheme[15] involves the arbitrary assignment of a value to either Ka or k_1 (as defined in the reaction scheme p. 3). Such a procedure leads to large uncertainties in the calculation of the remaining kinetic constants. Thus simplified reaction schemes were assumed in order to analyze the available data unambiguiously.

The values of the ionization constant of hydrocyanic acid , K_{CN}, used in the following calculations are:

	25.0°C	15.6°C	ΔH^o kcalmole^{-1}	ΔS^o caldeg^{-1}mole^{-1}
K_{CN}	6.16×10^{-10}	3.39×10^{-10}	10.4 ± 0.2	-3.9

according to the recent work of Izaat et al.[19].

A. A mechanism involving two reactions is assumed. It is assumed that hydrocyanic acid does not react with myoglobin and that a "heme-linked" ionizing group exists.

Symbolically:

$$HMnFe^{+}H_2O + CN^{-} \xrightarrow{k_2} HMbFeCN + H_2O$$

$$MbFe^{+}H_2O + CN^{-} \xrightarrow{k_2'} MbFeCN + H_2O$$

$$HMbFe^{+}H_2O \rightleftharpoons MbFe^{+}H_2O + H^{+} \quad (K_a)$$

Table VI

Experimental Kinetic Data at 25.0°C.

General Conditions:

$(T\text{-}M) = 0.05\ \underline{M}$

$(I) = 0.15\ \underline{M}$

$(CN^-) = 3.19 \times 10^{-4}\ \underline{M}$

$(Mb) \approx 5 \times 10^{-6}\ \underline{M}$

pH	$k_F M^{-1} sec^{-1}$	$\pm \delta$	pH	$k_F M^{-1} sec^{-1}$	$\pm \delta$
		Set I			
5.31	86.1	1.0	6.13	158.3	1.9
5.42	90.5	7.0	6.20	167.0	1.9
5.61	104.2	1.0	6.29	185.0	8.8
5.70	110.3	5.6	6.38	239.5	20
5.78	117.5	8.6	6.48	256.5	10
5.85	122.3	7.2	6.69	284.0	5.3
5.92	126.2	3.8	6.83	335	5.0
6.00	133.0	3.4	6.98	406	6.6
6.07	142.3	2.2			
		Set II			
6.20	173.3	3.2	6.57	260	1.2
6.29	195.5	10.0	6.69	310	3.2
6.38	205	7.2	6.83	357	6.9
6.48	234	3.2	6.98	400	6.9

Table VI cont'd

pH	$k_F M^{-1} sec^{-1}$	± δ	pH	$k_F M^{-1} sec^{-1}$	± δ
		Set III			
6.27	179.5	2.5	6.49	221.0	3.1
6.39	204.5	2.5	6.60	262.0	5.6
		Set IV			
5.31	87.6	2.2	5.85	122.5	5.3
5.42	96.0	1.9	5.92	126.8	5.3
5.52	106.5	3.1	6.01	141.0	2.1
5.61	113.2	2.8	6.07	153.0	2.8
5.70	113.2	1.9	6.14	159.8	1.0
5.78	114.0	2.8			
		Set V			
6.22	179.2	2.0	6.49	223	3.2
6.30	189.7	5.3	6.60	260	2.2
6.40	206	3.2	6.74	316	2.2
		Set VI			
5.22	82.7	3.5	5.63	118	1.6
5.38	102.7	9.1	5.68	117.4	5.3
5.46	102.9	2.2	5.73	112.7	5.3

Table VI cont'd

Set VI cont'd.

pH	$k_F M^{-1} sec^{-1}$	$\pm \delta$	pH	$k_F M^{-1} sec^{-1}$	$\pm \delta$
5.49	103.6	1.0	5.83	127.8	2.2
5.56	112.2	3.8	6.07	149	11.0
5.61	115.8	2.2			

Set VII

5.22	83.2	1.0	6.07	149	1.2
5.38	92.0	1.0	6.10	165	1.0
5.40	99.2	1.0	6.18	178	1.0
5.49	101.2	1.0	6.29	202.7	1.0
5.56	103.5	3.5	6.41	228	4.4
5.61	110.0	1.0	6.52	268.5	3.2
5.63	111.7	1.0	6.66	306	1.0
5.68	113.2	10.0	6.83	359	8.5
5.73	111.0	1.0	6.98	408	2.8
5.83	127.0	1.0			

Table VII

Experimental Kinetic Data at 15.6°C.

General Conditions:

$(T\text{-}M) = 0.05\ \underline{M}$

$(I) = 0.15\ \underline{M}$

$(CN^-) = 3.19 \times 10^{-4}\ \underline{M}$

$(Mb) \approx 5 \times 10^{-6}\ \underline{M}$

pH	$k_F M^{-1} sec^{-1}$	$\pm \delta$
5.22	43.2	2.2
5.38	52.6	1.2
5.46	55.1	1.0
5.49	53.6	1.0
5.56	57.4	1.0
5.61	58.3	1.6
5.63	58.6	1.0
5.68	61.6	1.0
5.73	62.4	1.0
5.83	63.6	1.0
6.07	72.6	1.2
6.10	8.08	1.0
6.18	85.5	1.0
6.29	95.9	2.8
6.41	104	4.4
6.52	119	2.2
6.66	146	2.2
6.83	184	1.6
6.98	228	3.5

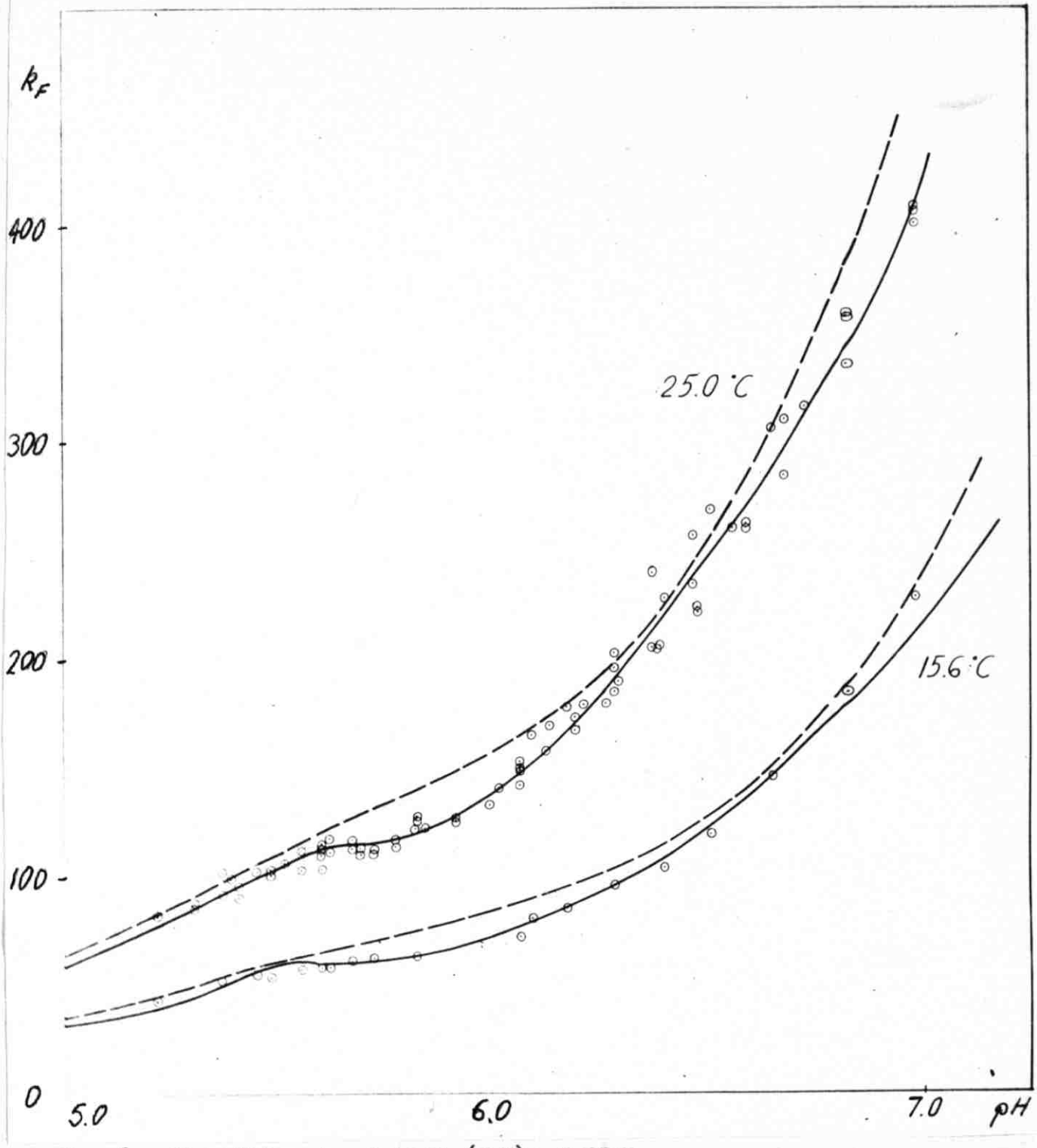


Fig. 6. pH-profiles, I = 0.15 M, (T-M) = 0.05 M.
Dashed line representing the theoretical curve of mechanism A.
Full line representing the theoretical curve of mechanism B.

The forward rate constant will be given by

$$k_F = k_2 \left(\frac{K_{CN}}{K_{CN} + H} \right) \left(\frac{H}{K_a + H} \right) + k_2' \left(\frac{K_{CN}}{K_{CN}+H} \right) \left(\frac{K_a}{K_a + H} \right) \quad (1)$$

in the range pH 5-7, $K_{CN} \ll H$.

Hence

$$k_F - k_2 = k_2' K_{CN} - H (k_F - k_2). \quad (2)$$

With pH approaching 7, $H/K_a \ll 1$,

hence

$$k_F = k_2 - K_{CN} K_a H k_F. \quad (3)$$

Plotting k_F against Hk_F according to equation (3) gives a straight line, whose intercept = k_2 and slope = $-K_{CN}K_a$. Inserting the value of k_2 in equation (2) and plotting $(k_F - k_2)$ versus $H(k_F - k_2)$ gives a straight line whose intercept = $k_2' K_{CN}$ (Fig. 7).

Thus the evaluation of all constants is made possible unambiguously.

The value of the intrinsic constants for this reaction system were found to be:

			kcalmole^{-1}		caldeg^{-1}mole^{-1}
	15.6°C	25.0°C	ΔH^0	ΔF^0	ΔS^0
k_2	78	147	-	-	-
k_2'	48,700	53,500	-	-	-
pK_a	5.11	5.13	-0.94	6.96	-28.7

The above values were used to plot a theoretical curve (dashed line in Fig. 6). It is seen that this curve does not fit the

experimental points in the region of the shoulder of the pH-profile.

The reaction scheme:

$$MbFe^{+}H_2O + HCN \xrightarrow{k_1'} MbFeCN + H_3O^{+}$$

$$MbFe^{+}H_2O + CN^{-} \xrightarrow{k_1} MbFeCN + H_2O$$

which neglects a "heme-linked" ionization did not lead to meaningful kinetic constants.

B. It is proposed that there exists an equilibrium of transition of acid myoglobin A, Mb_A, to acid myoglobin B, Mb_B, which can be represented as:

$$Mb_A \xrightleftharpoons{K_{tr}} Mb_B + nH^{+}.$$

The equilibrium involves a <u>simultaneous</u> release of n protons from the globin moiety of Mb_A to form the conjugate base Mb_B. For simplicity we shall assume that the prototropic groups are identical and non-interacting. Both of these myoglobin species react with cyanide as follows:

$$Mb_AH_2O + CN^{-} \xrightarrow{k_A} Mb_ACN + H_2O$$

$$Mb_BH_2O + CN^{-} \xrightarrow{k_B} Mb_BCN + H_2O$$

$$Mb_AH_2O \xrightleftharpoons{K_{tr}} Mb_BH_2O + nH^{+}.$$

In the range of pH 5-7, $K_{CN} \ll H$, hence for the region approaching pH 5,

$$k_F = k_A \frac{K_{CN}}{(H^+)}, \qquad (1)$$

and for the region approaching pH 7,

$$k_F = k_B \frac{K_{CN}}{(H^+)}. \qquad (2)$$

A plot of log k_F versus pH should give a straight line whose slope is unity. However, when the experimental data was plotted (Fig. 8a), the slope was found to be 0.5. This means that the observed second order forward rate constant, k_F, is empirically given by the relation:

$$k_F = \text{constant}.(H)^{-\frac{1}{2}}. \qquad (3)$$

Since it is difficult to escape the assumption that k_F involves the ionization of hydrocyanic acid, the kinetic interpretation of the empirical relation, equation (3), must include the term K_{CN}/H. The kinetic equation corresponding to equation (3) may be then written:

$$k_F = k_A \frac{K_{CN}}{H} q (H)^{\frac{1}{2}} \qquad (4)$$

(with a similar equation corresponding to k_B), which reduces to:

$$k_F = \frac{k_A q K_{CN}}{(H)^{\frac{1}{2}}}. \qquad (5)$$

No explanation in mechanistic terms can be offered at this point for the dependence of k_F on $(H)^{-\frac{1}{2}}$. Furthermore, it is not possible to separate k_A (or k_B) from q, but the product, $k_A q$ (or $k_B q$) can be calculated from the logarithmic plot (Fig. 8a), the values of which are listed in table VIII, together with the corresponding activation constants. The heat of activation, ΔH^*, and the entropy of activation, ΔS^*, include contributions due to the kinetic constant k_A

Table VIII

Kinetic and Thermodynamic Constants of Myoglobin Reaction with Cyanide

			ΔF^*	ΔH^*	ΔS^*
	15.6°C	25.0°C	kcalmole^{-1}	kcalmole^{-1}	calmole^{-1}deg^{-1}
$k_A q$	3.02×10^8	3.16×10^8	-5.06	0.92	2.01
$k_B q$	2.04×10^8	2.16×10^8	-4.96	1.15	2.05

			ΔF^0	ΔH^0	ΔS^0
	15.6°C	25.0°C	kcalmole^{-1}	kcalmole^{-1}	calmole^{-1}deg^{-1}
K_{int}	1.80×10^{-6}	2.02×10^{-6}	7.75	2.28	-1.84

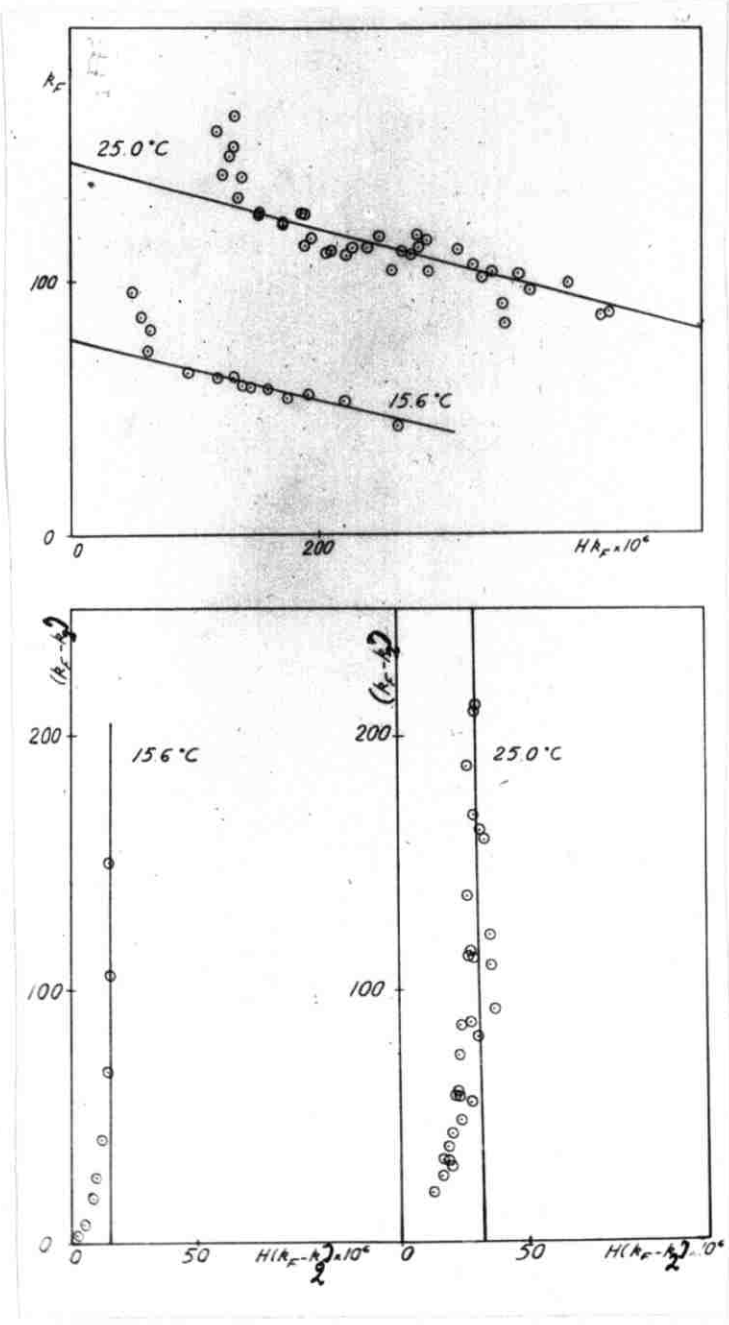


Fig. 7. Plots of equations 2 and 3,

(I) = 0.15 M, (T-M) = 0.05 M.

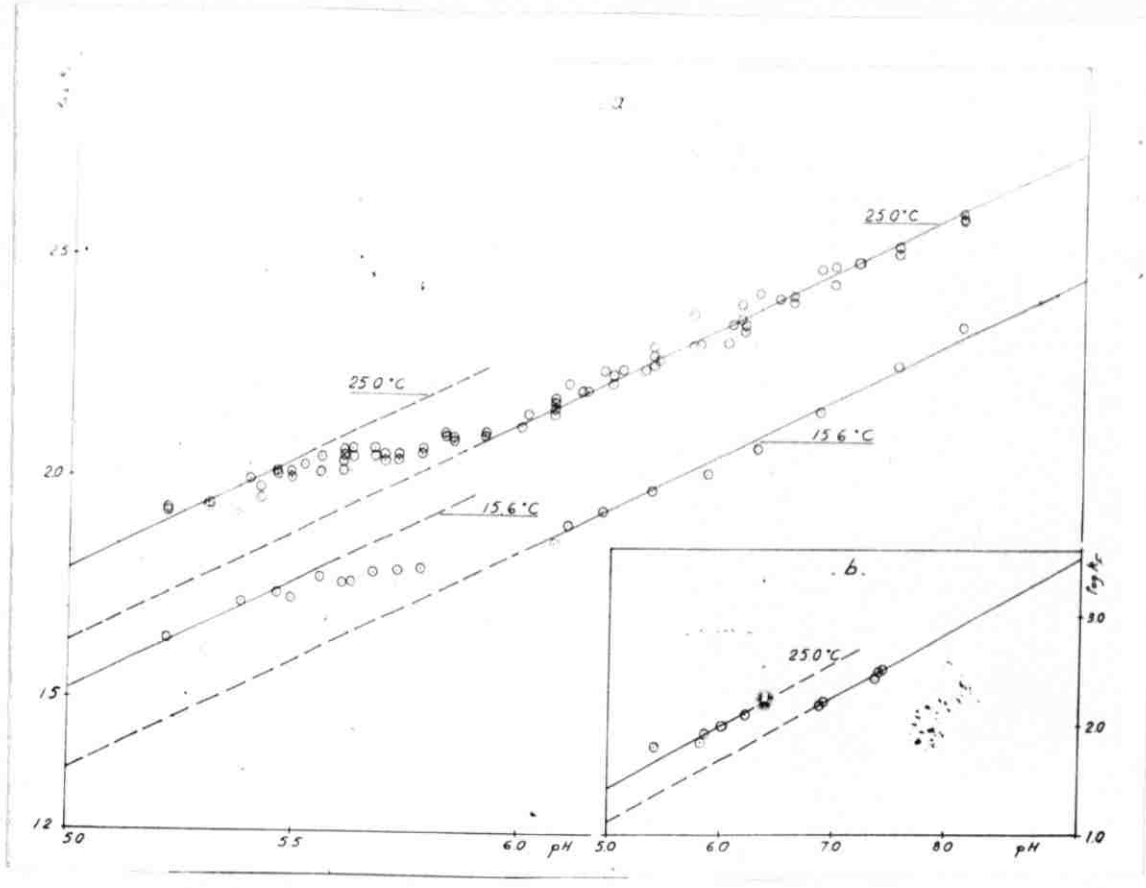


Fig. 8a. Logarithmic plot of equation (5),
(I)= 0.15 M, (T-M) = 0.05 M.

b. Logarithmic plot of equation (5), using Hanania's data, phosphate buffer, (I) = 0.10 M at 25°C.

(or k_B) as defined in the proposed reaction scheme above, as well as due to the unknown constant q, which, itself, may be a product of several true kinetic constants.

We shall now proceed to evaluate the number of protons, n, released in the transition from Mb_A to Mb_B. If we define α to be the fraction of myoglobin Mb_B, thus the fraction of Mb_A is $(1-\alpha)$. From the equilibrium

$$Mb_A \xrightleftharpoons{K_{tr}} Mb_B + nH^+ \tag{6}$$

where

$$K_{tr} = \frac{(Mb_B)\ (H)^n}{(Mb_A)} .$$

it follows that

$$npH = pK_{tr} + \log \frac{(Mb_B)}{(Mb_A)} = pK_{tr} + \log \frac{\alpha}{1-\alpha} \tag{7}$$

and $$n \frac{\partial pH}{\partial \alpha} = \frac{0.434}{\alpha(1-\alpha)} .$$

When $\alpha = 0.5$,

$$n = 1.74 \left(\frac{\partial \alpha}{\partial pH}\right)_{\alpha = 0.5.} \tag{8}$$

In the region of transition from Mb_A to Mb_B, the extent of the transition at any given pH is reflected kinetically in the value of k_F. Quantitatively the relation is

$$\log k_F = \alpha \log k_B + (1-\alpha) \log k_A,$$

hence

$$\alpha = \frac{\log k_F - \log k_B}{\log k_A - \log k_B} . \tag{9}$$

Since α is the fraction of myoglobin in the form Mb_B, which is subject to

the equilibrium equation (6). A plot of α, as defined by equation (9) versus pH should give a specific S-shaped titration curve of the type involving the simultaneous transfer of n protons. Such is found to be the case: for both temperature, 25.0°C and 15.6°C, the value of n = 4, as calculated by equation (8), gave good agreement with experimental points, as seen in Fig. 9, where the solid line represents the theoretical curve, assuming n = 4.

Presuming the above consideration, the value of the intrinsic pK of the prototropic group, involved in the equilibrium equation (6), can be evaluated:

Consider the two equilibria:

$$AH_n \overset{K_{tr}}{\rightleftharpoons} A + nH^+ \qquad (10)$$

$$GH \overset{K_{int}}{\rightleftharpoons} G + H^+ \qquad (11)$$

When equation (10) represents the ionization of the myoglobin molecule, associated with the transition of Mb_A to Mb_B; and equation (11) represents the ionization of each of the prototropic groups involved in this transition. It follows that

$$\frac{(Mb_B)}{(Mb_A)} = \frac{(G)}{(GH)} = \frac{\alpha}{1-\alpha} = W.$$

Hence

$$K_{tr} = W.(H)^n$$

and

$$K_{int} = W.(H)$$

Which, when written in the logarithmic form, give,

$$pK_{tr} = n\,pH + pW \qquad (12)$$

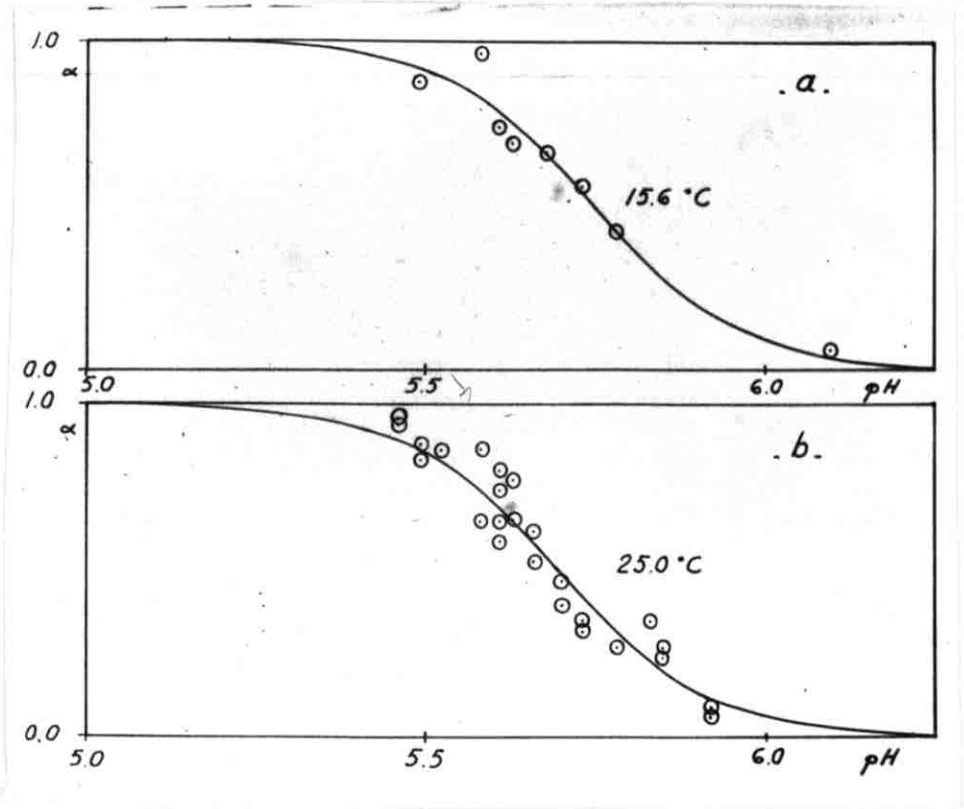


Fig. 9. A plot of α, as defined by equation (9), against pH. The solid line represents the theoretical curve assuming n = 4.

$$pK_{int} = pH + pW \qquad (13)$$

When

$$\alpha = 0.5, \quad pW = 0,$$

hence

$$pH = 1/n\ pK_{tr} = pK_{int}. \qquad (14)$$

Furthermore, we may note that it follows from equation (14) that

$$\frac{\partial pW}{\partial pH} = -n.$$

Using equation (14) we obtain from Fig. 9,

at 25.0°C $pK_{int} = 5.695$

and at 15.6°C $pK_2 = 5.745$

It is worth noting that the evaluation of n and pK_{int} according to the above considerations is valid whatever kinetic interpretation is assigned to the dependence of k_F on $(H)^{-\frac{1}{2}}$.

Using the above data, a theoretical curve was plotted (full line in Fig. 6), which is seen to fit the experimental points well over the whole pH-profile.

CHAPTER IV

DISCUSSION

The assumption of Pauling that there exists a "Proximal" histidine residue whose imidazole nitrogen atom is directly coordinated to the iron atom of the heme and in addition there exists a second "Distal" histidine residue, which is linked to the heme group by a hydrogen bond via the water molecule, has influenced the interpretation of a number of secondary effects in hemoglobin and myoglobin reactions, especially after the confirmation of Pauling's postulate by X-ray crystallography. This phenomenon has been termed the "heme-linked" effect.

In order to interpret the shoulder in the region of pH 6, Hanania[15] incorporated the concept of a "heme-linked" ionization in his kinetic analysis. Thus he regarded the pH-profile in the range of about pH 5-8 as composed of two sections: a first section, including the shoulder up to about pH 6, subject to a mechanism which involves a single heme-linked ionizing group (presumably the imidazole group of the proximal histidine residue) and a second section beyond about pH 6, whose mechanism only involves the cyanide ionization. In chemical terms this implies that the ionization of this group, causes a change through direct interaction, of

the electron density on the iron atom, thereby altering the thermodynamic and kinetic parameters related to the reaction of acid myoglobin with cyanide.

In contrast to the heme-linked effect hypothesis, the kinetic results of the present work, indicate the following mechanism, which does not invoke a heme-linked ionizing group. The logarithmic plot Fig. 8a leads to the conclusion that the reaction of myoglobin with cyanide follows the same mechanism over the entire range of pH 5-7. This view is based on the linearity of the logarithmic plot over large sections of the pH range investigated at both temperatures. Furthermore, it seems hardly coincidental that the slope in all the linear regions was found to be 0.5. Although at this point it is not possible to formulate a reaction scheme which will account for the empirical relation:

$$k_F = \text{constant} \cdot (H^+)^{-\frac{1}{2}},$$

in terms of intrinsic kinetic constants, it is clear that such a mechanism should be more complicated than the reaction scheme:

$$Mb_A H_2O + CN^- \xrightarrow{k_A} Mb_A CN + H_2O$$

$$Mb_B H_2O + CN^- \xrightarrow{k_B} Mb_B CN + H_2O.$$

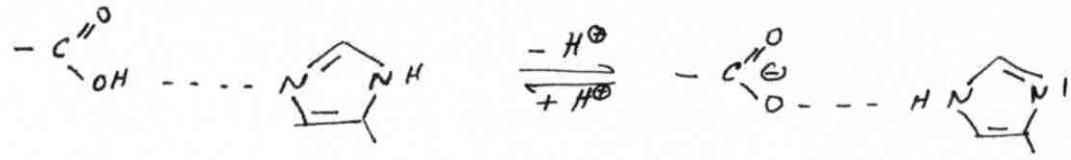
Whatever this complete reaction mechanism might be, it must be the same throughout the range pH 5-7. The discontinuity in the logarithmic plot, which corresponds to the shoulder in the pH-profile may be explained by the hypothesis that a 4-proton transfer equilibrium

exists between two acid myoglobin species, which are both present in the region of transition. The ionization of these 4 prototropic groups is responsible for a difference in the charge between Mb_A and Mb_B of 4 units. This charge difference on the globin moieties is transmitted to the iron atom of the heme group, as a long range smeared charge electrostatic effect, in accordance with Kirkwood's theory of electrostatic interactions in protein molecules. It is clear that this view predicts that k_Aq should be larger than k_Bq.

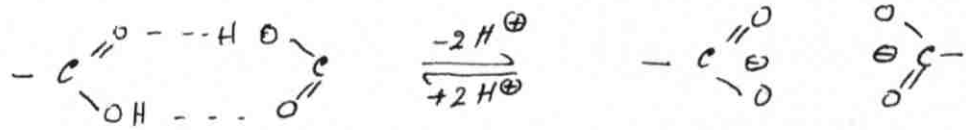
It is relevant to note that recently Beetlestone and Irvine[2a] have explained quantitatively the species variation of the pK of the equilibrium between acid hemoglobin and basic hemoglobin on the basis of Kirkwood's theory.

The values of $pK_{int} = 5.6$ and $\Delta H^0 \approx 2$ kcalmole^{-1} compare closely to the pK and ΔH^0 of a carboxyl group; the higher pK and ΔH^0 values observed can be accounted for by assuming H-bonding to a Lewis base of the type COOH ..... B. The group B might possibly be identified as a hydroxyl of serine or tyrosine, a carboxyl or carboxylate of glutamic or aspartic acid, or an imidazole of histidine. Of these possibilities the most plausible are the following two systems:

I



II



The present work does not allow the distinction between system I and II. However, a study of the optical rotation may decide this question, for system I does not involve any configurational changes, while system II, should involve a configurational change due to the electrostatic repulsion generated by the appearance of carboxylate groups.

It is of interest that when Hanania's data on the forward rate of the reaction of <u>equine</u> myoglobin with cyanide is analyzed according to equation (2), the same type of plot was obtained as in the present work, on sperm whale myoglobin (Fig. 8b). The calculated constants for equine myoglobin were found to be:

$\frac{K_{int}}{5 \times 10^{-7}}$	$\frac{k_A q \times 10^{-8}}{1.41}$	$\frac{k_B q \times 10^{-8}}{0.645}$

under the following experimental conditions: phosphate buffer, ionic

strength 0.10 M, at 25.0°C. It is seen that the order of magnitude of the values for equine myoglobin is comparable with sperm whale myoglobin. However, no absolute comparison can be made, since these values were obtained under different experimental conditions of ionic strength and buffer species. Chromatography of sperm whale and equine myoglobin on Sephadex CM - C50[1a] showed that sperm whale myoglobin is much more positively charged than equine. This is consistent with the lower values of $k_A q$ and $k_B q$ calculated for equine myoglobin.

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