EFFECT OF ELECTROSTATICALLY LINKED IONIZABLE GROUPS ON SULPHYDRYL REACTIVITIES OF HEMOGLOBINS

BY

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ABSTRACT

The kinetics of the reactions of two sulphydryl reagents with the CysF9(93)β sulphydryl group of various human and animal hemoglobin derivatives have been studied as a function of pH at 20°C, at an ionic strength of 0.05 M. A quantitative analysis of the data was possible only for the 2,2'-dithiobispyridine data. For the 5,5'-dithiobis[2-nitrobenzoic acid] data, the equation relating the apparent second order rate constant to the model parameters was complex and therefore not useful for quantitative analysis. However, interesting results were observed for the 5,5'-dithiobis [2-nitrobenzoic acid]reaction. As a general conclusion, it is suggested that tne reactivity of the CysF9(93)β sulphydryl depends on two factors: the conformation of the sulphydryl group and the electrostatic effects of the charged ionizable groups on the protein.

In order to determine the nature of the charged groups affecting sulphydryl reactivity, the reaction of hemoglobin with another sulphydryl reagent, was

studied by potentiometric difference titration as a function of pH at 20°C and ionic strength 0.05 M. Differences were observed for various derivatives for the parameter Δh^+ the number of protons released per sulphydryl group reacted. These differences reflect differences in tertiary structure between the derivatives studied rather than differences in quaternary structure.

DEDICATION

This thesis is dedicated to

JESUS CHRIST

The only true Saviour

ACKNOWLEDGEMENT

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CERTIFICATION

I certify that the work reported in this thesis was carried out under my supervision by Christopher Olumuyiwa Aboluwoye in the Department of Chemistry, University of Ibadan.

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CHAPTER 1

INTRODUCTION

Living organisms require sulphur, which is needed in different forms by different organisms. Bacteria use it as the free element; plants use it as sulphate or sulphide and higher animals use it as Cysteine, Cystine and Methionine residues of proteins (1).

It is important to understand the physiological significance of the sulphydryl groups in living organelles. Thiols in plants have been reported to play a vital role in the primary steps of photo-synthesis (2). In the eye, the retina and the lens are the two chief tissues containing thiol groups. In the lens, protein thiols constitute about 75% of the total thiols present. Crystallins, the trans-parent lens thiol protein, is the most abundant (3). The opacity of the lens is increased, and normal vision is reduced, by precipitation of crystallins (1). Molecules in the retina containing thiol groups include glutathione and the visual pigment rhodopsin. Rhodopsin has four thiol groups per molecule in the

dark. During illumination the bound carotenoid component (11-Cis retinal) isomerises and dissociates off. The thiol content of the residual protein (Opsin) increases to eight per molecule (1).

Rhodopsin ——————> Opsin + trans-retinal

4SH 8SH

Binding of the aldehyde group of the retinal rhodopsin involves one thiol group directly (4-6). These thiol groups are probably present in rhodopsin but are not titratable until there is a change in conformation during conversion to opsin which exposes them.

Free sulphydryl groups play a critical role in the catalytic mechanism of a wide variety of enzymes. Modification of such sulphydryl groups inactivates such enzymes. Free sulphydryl groups can be modified by direct oxidation, or by reaction with an alkylating or disulphide reagent. Modification of free sulphydryl groups in cells can result in a complex sequence of events culminating in cell death. Whilst such toxic cell damage often leads to necrosis, where cell death is the result of the failure of endogenous systems to

compensate, it is also possible that programmed cell death (apoptosis) is initiated - the result of an active process (7). Many compounds that are toxic through the formation of reactive intermediates affect cellular levels of reduced glutathione (GSH). Whilst attention was initially concentrated on the role of adduct formation with consequent depletion of cellular GSH content, it is now recognised that oxidation of GSH to its disulphide, GSSG, is also an important mechanism in compromising host defence. Oxidation may be caused directly by oxidizing species such as the reactive metabolite of paracetamol, N-acetyl-P-benzoquinoneimine, menadione and t-butyl-hydroperoxide (7). It has been established that modification of the free sulphydryl groups by these toxic compounds results in blebbing (potentially lethal changes) which can be reversed or prevented by the addition of dithiothreitol, a direct-acting thiol reductant. Thus, there is good evidence that modification of protein thiol groups can result in cell death (7).

The intracellular Ca^{2+} concentration is rigorously maintained at around 0.1μM, against an extracellular concentration of more than 1μM, as Ca^{2+} plays a key role as a second messenger in the regulation of many cell activities (7). The Ca^{2+} gradient is maintained by a number of ATP-dependent enzymes, the most important of which appear to be located in the plasma membrane (Ca^{2+} - transporting ATP_{ases}) and in the endoplasmic reticulum. Ca^{2+}-transporting ATPases are sulphydryl containing enzymes which can be inhibited by thiol-group modification with compounds such as paracetamol, menadione and t-butylhydroperoxide. This results in Ca^{2+} homoestasis. All of these Ca^{2+}-regulating enzymes are inhibited by cytotoxins and their activity can largely be restored by dithiothreitol (7).

Plasmodium falciparum trophozite is a malaria causative organism. It has a sulphydryl containing enzyme known as Cysteine proteinase (8). Cysteine causes the degradation of hemoglobin during the erythrocyte stage or the cleavage of cell-surface proteins during the merozoite stage in the life cycle of plasmodium (8). The activity of cysteine proteinase

can be inhibited by the two peptide inhibitors (1) Leupeptine and (2) L-transepoxy-succinyl-leucyl-amido-(4-quanidino)-butane (E-64) (9). They inhibit the proteolysis of globin and also the degradation of hemoglobin by Cysteine proteinase (9). Specific inhibitors of this enzyme might provide new means of antimalarial chemotherapy (9).

A: SULPHYDRYL REACTIVITIES OF NON-PROTEINS

The sulphydryl groups in non-proteins are very reactive unless masked, as is the case in some proteins. Their reactivity is due to the thiolate anion (RS^-) which is over five hundred times more nucleophilic than the corresponding alkoxy, RO^-, analogue (10). The most abundant naturally occuring non-protein thiol is glutathione (11). Non-protein thiol groups mainly react by substitution, addition, elimination, and oxidation mechanisms. They also form stable chelation complexes with many metals, especially heavy metals like mercury, silver, copper, iron and arsenic. Mercury forms the most stable complexes with sulphydryl groups over the entire pH range. At low pH, Hg^{2+} is the most specific of all the thiol combining reagents

and it is for this reason that mercurials have been extensively used for assaying sulphydryl groups in proteins (12), although other reagents are now available which are more convenient to use (13,14).

The reaction of methyl methanethiosulphonate with the thiol groups of glutathione, cysteine, 2-mercaptoethanol, 3-mercaptopropionic acid, 2-mercaptoethylamine, and 5-mercapto-2-nitrobenzoic acid have been studied by potentiometric difference titration as a function of pH (15). This method has revealed the ionization behaviour of thiol groups in simple compounds which has helped to elucidate the ionizations of groups that are thermodynamically linked to the thiol group (15).

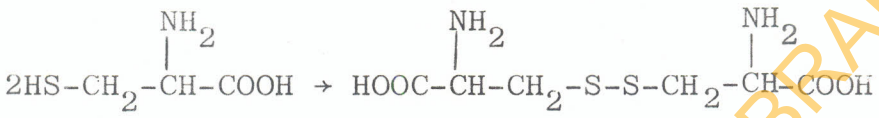
The kinetics of the reaction of 2-mercaptoethanol with2,2'-dipyridyl disulphide in its various states of ionization have been studied at 20^{o}C and at a single pH (16,17). The result obtained from this study has shown that the 2,2'-dipyridyldisulphide di-cation reacts faster than the 2,2'-dipyridyldisulphide monocation which in turn reacts faster than the neutral 2,2'-dipyridyldisulphide. This confirms the effect of charges on the ionization behaviour of the thiol groups (16,17).

The kinetics of the reaction of the negatively charged, 5,5'-dithiobis[2-nitrobenzoic acid] and uncharged 2,2'-dithiobispyridine with the thiol group of glutathione have been determined as a function of pH (18). The result obtained from this study shows that glutathione reacts at the same rate with both reagents over the pH range studied. This indicates that, for a simple thiol compound, the charge on an attacking reagent is unimportant, provided any charged group on the thiol compound is not near the thiol group (18).

B: SULPHYDRYL REACTIVITIES OF SOME PROTEINS.

Thiol groups do undergo autoxidation in the presence of catalysts such as free metals (19) like Hg, Pt, Au and traces of metals (1) like metal complexes or selenium compounds such as selerite. The factors which influence the rate of oxidation include temperature, pH and buffer medium, type of catalyst, oxygen tension and the nature and concentration of the thiol to be oxidized (19).

Free cysteine is stable either as the crystalline hydrochloride or in acid solution. In neutral solution, it autoxidizes to cystine



L - Cysteine L - Cystine.

Cysteine and Cystine residues account for the SH and SS groups in proteins (19).

A considerable amount of work has been done on the pH dependence of the reactions of protein thiol groups. The Cysteine-25 in papain, the thiol group in bovine serum albumin, and the thiol groups of Calf-thymus histone react with a number of sulphydryl reagents (15,20-23). The results obtained in such studies have been used to characterize the ionization behaviour of the thiol groups in such proteins (15,20-23).

The kinetics of the reaction of the thiol group of the plant enzyme papain with iodoacetamide, chloroacetamide, and fluoroacetamide give sigmoid pH profiles (23). The kinetics of the reaction of the thiol group of thiosubtilisin(a bacterial enzyme) with mercaptoacetate and haloacetamide have been studied

as a function of pH. The kinetics are pH dependent and are affected by charged groups in the micro-environment of the thiol groups (23). Iodoacetamide was reported to be more reactive than other halo-acetamides (24).

The kinetics of the reaction of the sulphydryl group of Calf-thymus histone F3 with 5,5'-dithiobis [2-nitrobenzoic acid] has also been studied as a function of pH. The reaction followed second order kinetics and the apparent second-order rate constant obtained for the histone was surprisingly 4,000 times higher than that of glutathione (22). This study was extended to investigate electrostatic and confor-mational effects on the reaction. This was done in order to provide an insight into the mechanism of the reaction, to determine how the tertiary structure and charges of the protein give rise to the observed super reactivity. It was found that charged groups play an important role in the reactivity of the thiol group (25).

The kinetics of the reactions of the active-centre thiol groups of papain and ficin with the

two-protonic-state reactivity probes 2,2'dipyridyl disulphide, n-propyl-2-pyridyldisulphide and 4-(N-amino ethyl 2' -pyridyl disulphide) 7-nitrobenzo-2-oxa-1,3-diazole were studied over a wide range of pH (26-31). The results indicate that the differences between the reactivities of ficin and papain towards the cationic forms of the alkyl 2-pyridyl disulphide probes may be due to a cationic site in ficin without an analogue in papain (26-30), and the striking difference in the shapes of the pH-rate profiles for the reactions of the two enzymes with 4-(N-aminoethyl 2' 2'-pyridyl disulphide) 7-nitrobenzo-2-oxa-1,3-diazole suggests differences in the mobilities or dispositions of the active-centre histidine imidazole groups with respect to relevant hydrophobic binding areas (28). The evidence from reactivity-probe studies that the papain catalytic mechanism involves substantial repositioning of the active-centre imidazole group during the catalytic act does not apply to ficin. It would be expected that the pKa of the carboxy group in ficin should be higher than it is if ficin contains an aspartic acid residue analogous to aspartic acid -

158 in papain. This is however not so because of the absence of an aspartic acid - 158 analogue in ficin.

C: SULPHYDRYL GROUPS OF HEMOGLOBIN.

(i) NATURE OF SULPHYDRYL GROUPS IN HEMOGLOBIN

The number of thiol groups in various hemoglobins has been determined by titration. There are no disulphide groups except in frog. Dog hemoglobin has four sulphydryl groups at positions G18(111)α and F9(93)β, all of which are titrable with mercurials (32). Only two of these, however, react with non-mercurial sulphydryl reagents (32). The number of thiol groups and their positions are given in Table 1 for some animal hemoglobins (33,34).

In human hemoglobin, the two sulphydryl groups which are titrable are at position F9(93)β while the others are located at G14(112)β and G11(104)α, respectively. Though unreactive in normal hemoglobin, the thiol group at G14(112)β is much more reactive than that at F9(93)β if the protecting α-chains are removed (35). Horse and rabbit hemoglobins have no

Table 1: The number of thiol groups and their positions in hemoglobin species.

	Thiol groups per molecule		
Hemoglobin	Titrable	Total	Location
Human Adult	2	6	CysF9(93)β CysG14(112)β CysG11(104)α
Human Fetal	2	4	CysF9(93)γ CysG11(104)α
Dog	2	8	CysF9(93)β CysG14(112)β CysG11(104)α CysG11(111)α
Horse	2	4	CysF9(93)β CysG11(104)α
Ox	2	2	CysF9(93)β
Rabbit	2	4	CysF9(93)β CysG11(104)α
Monkey	2	6	CysF9(93)β CysG14(112)β CysG11(104)α
Echidna	2	4	CysF9(93)β CysG11(104)α

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Horse	2	4	CysF9(93)β CysG11(104)α
Ox	2	2	CysF9(93)β
Rabbit	2	4	CysF9(93)β CysG11(104)α
Monkey	2	6	CysF9(93)β CysG14(112)β CysG11(104)α
Echidna	2	4	CysF9(93)β CysG11(104)α

sulphydryl group at position G14(112)β unlike the human hemoglobins (33,34).

The transformation of deoxyhemoglobin kempsey in its R-state to T-state in the presence of inositol hexakiphasphate (IHP) has been studied at a single pH (36). The result has shown that R → T transition of deoxyhemoglobin kempsey is also accompanied by a marked lowering of the sulphydryl reactivity (36). It is due to the change in quaternary structure produced by IHP and not the changes in tertiary structure that alone accompany deoxygenation in the absence of IHP which causes the drop in sulphydryl reactivity. In addition, the reaction of p-hydroxymercuric benzoate (PMB) with sulphydryl group of oxyhemoglobin and deoxyhemoglobin in the absence of IHP differ widely, by up to 80-fold. This is due to the conformational changes of the hemoglobin derivatives. The reaction of PMB with β^{93} sulphydryl group of aquomethemoglobin and azidomethemoglobin in the presence of IHP have been studied at a single pH. The result indicates that the reaction rates are similar for the two forms; therefore, to the extent that reactivity

of β^{93} - SH reflects conformation, there is no difference in the presence of IHP for the high spin and low spin forms (37).

The orientations of the reactive Cys F9(93)β thiol group in hemoglobin derivatives like Oxy -, carbonmonoxy-, aquomet-, and deoxyhemoglobin differ (38). The position of the CysF9(93)β thiol group influences its reactivity. The reactivity of CysF9(93)β differs in carbonmonoxy - and aquomethemoglobin. The second order rate constant of the reaction with p-mercuribenzoate is twice as large, and that with 2,2'-dithiobispyridine four times as large in aquomethemoglobin as in carbonmonoxy-hemoglobin (39). These differences arise from the change in equilibrium between the mainly external position of the sulphydryl group in aquomethemoglobin and its mainly buried position in carbonmonoxy-hemoglobin (39). The CysF9(93)β thiol group in aquomethemoglobin is in equilibrium between two conformations: one with the side chain pointing into a pocket enclosed by parts of helices F, G, and H, and the other with the side chain pointing into the solvent. The difference fourier map shows that in

carbonmonoxyhemoglobin the side chain always has the first of these conformations, accounting for the decreased reactivity of this sulphydryl group in carbonmonoxyhemoglobin compared to that in aquo-methemoglobin (39).

In deoxyhemoglobin, the side chain always has the external conformation, whilst the pocket between helices F, G and H is occupied by the side chain of Try HC2(145)β, whose OH^- group is hydrogen-bonded to the carbonyl group of Val FG5(98)β. The reactivity of the thiol group is low in deoxyhemoglobin because access to it is hindered by the carboxy-terminal residue His HC3(146)β which is held firmly in position by salt-bridges in deoxyhemoglobin but is probably free-moving in carbonmonoxyhemoglobin (38). In the recent study (40), it has been shown that the sulphydryl group of deoxyhemoglobin exists in two conformational forms in constrast to the previous finding (40).

The Cys F9(93)β thiol group of oxyhemoglobin alternates between a major and a minor site, in conjunction with Tyr HC2(145)(41). In the major conformation, both share the internal pocket between helices F and H. The Cys F9(93)β thiol group spends

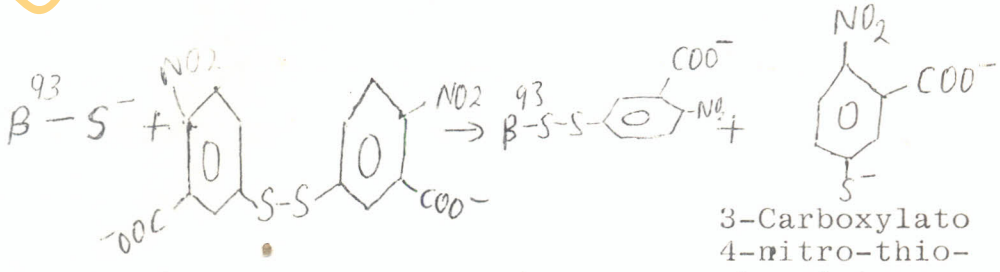
only 30 percent of the time in the minor conformation in an area that is exposed to the solvent (41).

(ii) REACTION OF HEMOGLOBIN SULPHYDRYL GROUPS WITH VARIOUS REAGENTS.

Thiolate anions reactivity with many sulphydryl reagents like p-hydroxymericuribenzoate, iodoacetate, and iodoacetamide (32,42), 2,2'-dithiobispyridine, 5,5'-dithiobis [2-nitrobenzoic acid](13,22), N-ethylmaleimide (43) and fluoropyruvate (44) have been widely used to study the reactivities of thiol groups spectrophotometrically.

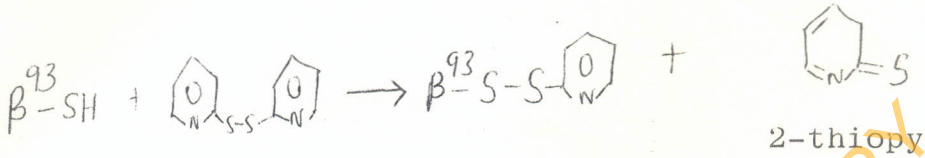
Treatment of hemoglobin with an excess of certain disulphide compounds leads to reaction with the Cys F9(93)β thiol group of the protein, due to the formation of a mixed disulphide (33). The reaction of hemoglobin with disulphide compounds may be represented as follows:

(a) With 5,5'-dithiobis(2-nitrobenzoic acid)



3-Carboxylato 4-nitro-thiophenolate.

(b) With 2,2'-dithiobispyridine.



2-thiopyridone.

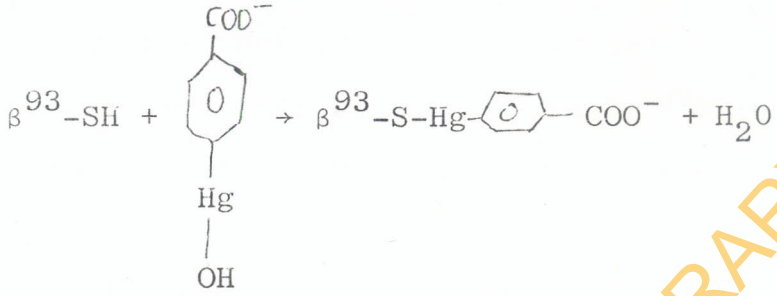
(c) With methyl methanethiosulphonate

$$\beta^{93}\text{-SH} + CH_3\text{-}\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}\text{-S-}CH_3 \rightarrow \beta^{93}\text{-S-S-}CH_3 + CH_3SO_2^- + H^+$$

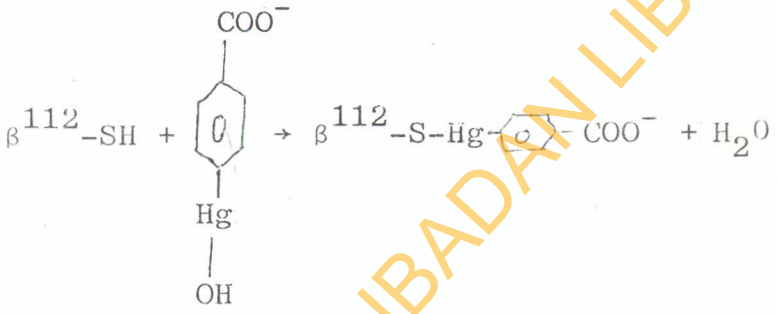
While non-mercurial reagents are sensitive only to the Cys F9(93)β thiol group, p-hydroxymercuribenzoate reacts with both the Cys F9(93)β and CysG14(112)β thiol groups (33). P-hydroxymercuribenzoate reacts more rapidly with CysF9(93)β at neutral pH than with Cys G14(112)β. The difference in reactivity between these groups is such that it can now be stated that the Cys G14(112)β in hemoglobin is masked while Cys F9(93)β is free.

Modification of the Cys G14(112)β residue affects the ability of the β-chains to aggregate with each other (33). The reaction of hemoglobin with mercurial reagent may be represented as follows:

(a) With p-hydroxymercuribenzoate [PMB]



(b)



(iii) EFFECTS OF SULPHYDRYL GROUP MODIFICATION

Blockage of the thiol groups of hemoglobin by treatment with N-ethylmaleimide, iodoacetate, mercurials or other sulphydryl reagents causes the following changes:

(a) The affinity for oxygen is increased and the co-operative interaction between the subunits - which is normally responsible for the sigmoid shape of the oxygen saturation curve - is decreased.

(b) The acid Bohr effect is increased while the alkaline Bohr effect is reduced (40,45).

(c) After treatment with mercurials, the protein dissociates slowly, first to αβ-dimers, and then to monomers.

(d) Mercurial treated hemoglobin has reduced solubility and tends to precipitate spontaneously from solution (46).

(iv) pH-DEPENDENCE STUDIES OF THE REACTIVITIES OF THE THIOL GROUPS OF HEMOGLOBIN

Work has been done on the kinetics of reaction of hemoglobin with sulphydryl reagents at a single pH (32,47). These kinetic studies have shown that the velocities of reaction of iodoacetamide, iodoacetic acid, N-ethylmaleimide, and 5,5'-dithiobis(2-nitro-benzoic acid) with the sulphydryl groups of human oxy-, carbon- monoxy- and deoxyhemoglobin (47). The rates of reaction of the liganded hemoglobins were shown to be faster (4 to 32 times) than those of deoxyhemoglobin. However, the velocity of reaction of oxyhemoglobin was consistently greater (1.5 to 1.7) than that of carbonmonoxyhemoglobin at pH 7.15 (32).

The kinetics of reaction of the Cys F9(93)β thiol groups of various human oxyhemoglobins (having mutations involving charged groups) with the negatively charged 5,5'-dithiobis(2-nitrobenzoic acid) and the uncharged 2,2'-dithiobispyridine have been studied as a function of pH at 5°C (18). In normal human oxyhemoglobin A, the second-order rate constants with 5,5'-dithiobis(2-nitrobenzoic acid) as reactant are approximately three to four times higher than with the uncharged 2,2'-dithiobispyridine, reaching a maximum difference at pH 7.3 (18). The difference in behaviour between the two reagents reflects the presence of one or more positively charged groups near the CysF9(93)β thiol group. This reactivity profile is likely due to one or more histidine residue (18). In the pH 9 region the thiolate ion becomes the common modulator of reactivity. The uncharged reagent, 2,2'-dithiobispyridine, is relatively unaffected by these charges, and it is primarily influenced by changes in the pK of the thiol group or by tertiary and quaternary structural changes (18). In this study (18), the aim was to test the effect of charged groups, introduced

by mutations, on sulphydryl reactivity. In these mutant hemoglobins - Osler Try HC2(145)β → Asp, Wood His FG4(97)β → Leu, Malmo His FG4(97)β → Gln, Yakima Asp G1(99)β → His, Kempsey Asp G1(99)β → Asn, Andrew-Minneapolis Lys H22(144)β → Asn, Chesapeake Arg FG4(143)α → Leu, Fetal His H21(143)β → Ser and Lys H22(144)β → Arg, and S-hemoglobin Glu A3(6)β → Val - residue substitutions at the $\alpha_1\beta_2$ interface which increase oxygen affinity alter the reactivity of the CysF9(93)β thiol group toward an uncharged reagent such as 2,2'-dithiobispyridine. These studies indicate that the sulphydryl reactivities of various hemoglobin derivatives; but they do not provide any quantitative analysis to account for this fact.

(d) AIM OF THE PRESENT STUDY

A series of studies has been reported that negatively charged ligands such as azide, cyanide, fluoride, formate and thiocyanate ions bind to aquomethemoglobin (48-57). Aquomethemoglobin can also bind some neutral ligands like methylamine, imidazole, and hydrogensulphide (48). The binding of most ligands

to aquomethemoglobin is kinetically complex, due to differences in the reactivities of the α- and β-chains, each chain reacting with most ligands at its characteristic rate (49). The situation is further complicated by the fact that these reactions are subject to the electrostatic effects of ionizable groups on the protein moiety (54,55). The number and nature of such "heme-linked" ionizable groups have been determined in some ferric heme-proteins from the pH dependence of the kinetics of ligand binding (50-57). Analyses of the data have revealed that there are three sets of "heme-linked" ionizable groups that influence ligand binding to aquomethemoglobin. They are: (1) a set of carboxylic acid groups; (2) a set of histidine and terminal amino acid groups; and (3) the acid-alkaline aquomethemoglobin transition (56,57).

The limitation of this kind of study (50-57) is that it is only applicable to the determination of the nature and number of sets of ionizable groups in ferric but not in ferrous heme proteins, since the latter react only with uncharged ligands.

It is generally accepted that the reaction of sulphydryl groups in proteins is via nucleophilic attack, on a sulphydryl reagent, by the thiolate anion (20,32). This should provide a means for the determination of the pKs for the ionizations of charged ionizable groups in ferrous heme-proteins containing sulphydryl groups, since the negatively charged thiolate anion should be electrostatically linked to the ionizable groups in these proteins.

Specifically, the present study has the following aims:

(i) To determine the ionization constants of Cys F9(93)β sulphydryl groups of derivatives of various hemoglobin species by studying the pH dependence for the reaction with uncharged sulphydryl reagents.

(ii) To investigate the nature of ionizable groups of hemoglobin that are electrostatically linked to the Cys F9(93)β sulphydryl group - by investigating the pH dependence of the reactivity with a charged sulphydryl reagent.

(iii) To investigate the effect of species differences, and differences in tertiary structure, on sulphydryl reactivity so as to provide a rational explanation for differences in sulphydryl reactivities among various hemoglobin species.

CHAPTER 2

A: PREPARATION OF MATERIALS

(i) PREPARATION OF PRECISION BUFFER SOLUTIONS.

(a) TYPE S1500: This solution was prepared by dissolving 3.402 g potassium dihydrogen phosphate and 4.450 g di-sodium hydrogen phosphate dihydrate crystals in distilled water and making up to (58) the mark in a one-litre volumetric flask.

(b) TYPE S1510: This solution (58) was prepared by dissolving 1.816 g potassium dihydrogen phosphate and 9.501 g di-sodium hydrogen phosphate dihydrate crystals in distilled water and making up to the mark in a one-litre volumetric flask.

All the chemicals used were reagent grade products of British Drug Houses.

The precision buffers were used for standardizing the pH meter at a given temperature using the pH versus temperature conversion Table (58).

TABLE 2

The pH versus Temperature conversion table.

$t^{o}C$	pH value for Type S1500 buffer	pH value for Type S1510 buffer
0	6.984	7.531
5	6.951	7.497
10	6.923	7.469
15	6.900	7.445
20	6.881	7.426
25	6.865	7.410
30	6.853	7.397
35	6.844	7.386
37	6.841	7.383
38	6.840	7.381
40	6.838	7.377
45	6.834	7.370
50	6.833	7.363
55	6.834	7.370
60	6.836	7.370
70	6.845	7.370
80	6.859	-
90	6.877	-
95	6.886	-

(ii) PREPARATION OF PHOSPHATE BUFFERS, IONIC STRENGTH 0.05 M (pH 5.6-8.0)

Phosphate buffers were prepared with reagent grade chemicals from British Drug Houses. A stock solution of 0.4 M sodium hydroxide was prepared by dissolving 16.0 g sodium hydroxide pellets in carbon-dioxide free distilled water in a one-litre volumetric flask. The solution was made up to the mark with distilled water. Sodium dihydrogen phosphate (0.4 M) prepared by dissolving 62.4 g of sodium dihydrogen phosphate dihydrate crystals in 1 litre of solution; 1 M sodium chloride solution was prepared by dissolving 58.44 g of sodium chloride crystals in 1 litre of solution.

Phosphate buffers as specific pH values were prepared by mixing appropriate amounts of 0.4 M sodium hydroxide and 0.4 M sodium dihydrogen phosphate (59). The ionic strength of each buffer solution was made up to 0.5 M by the addition of a specific amount of NaCl (59). The solution was made up to the mark with distilled water and the pH of each buffer solution was checked on a Radiometer pH meter Type PHM 4d with

a Radiometer GK2401C glass electrode.

(iii) PREPARATION OF BORATE BUFFERS, IONIC STRENGTH 0.05 M (pH 8.0-9.0)

A stock solution of 0.3 M sodium hydroxide was prepared by dissolving 12 g of sodium hydroxide pellets in distilled water and making up to the mark in a one-litre volumetric flask. A stock solution of 0.3 M boric acid was prepared by dissolving 18.55 g of boric acid crystals in distilled water and making up to the mark in a one-litre volumetric flask. Borate buffers at specific pH values were prepared by mixing the specified amounts of 0.3 M sodium bydroxide stock solution and 0.3 M boric acid stock solution in a one-litre volumetric flask (59). The ionic strength of each buffer solution was made up to 0.05 M by the addition of a specified amount of sodium chloride stock solution into the volumetric flask. The solution was made up to the mark with distilled water. The pH of each buffer solution was checked on a Radiometer pH meter Type PHM 4d with a Radiometer GK2401C glass electrode.

(iv) PREPARATION OF CARBON MONOXIDE GAS

Carbon monoxide gas was prepared by reacting concentrated sulphuric acid (VI) acid with sodium methanoate.

$$HCOONa_{(s)} + H_2SO_{4(\ell)} \rightarrow NaHSO_{4(s)} + H_2O_{(\ell)} + CO_{(g)}$$

The carbon monoxide gas evolved was passed through two wash bottles containing distilled water. Carbon monoxide is not soluble in distilled water and so the distilled water merely removes the acid vapour that may have issued along with the gas. The carbon monoxide was stored in a gas trap bottle for use (60).

(v) PREPARATION OF DEOXYGENATING SOLUTION

A solution of chromic sulphate was prepared by dissolving 500 g chromic sulphate $Cr_2(SO_4)_3$ in 5 litres of 1 M tetraoxosulphate (VI) acid. Zinc amalgam was prepared as follows: 7 g mercuric nitrate dihydrate ($Hg(NO_3)_2.2H_2O$) was dissolved in one-litre of 1 M nitric acid solution. Zinc metal (500 g) was added to the mercuric nitrate solution and allowed to stand for twenty minutes. The resulting zinc amalgam was washed many times with distilled

water. It was poured into two bubble bottles. Sufficient chromic sulphate solution was added to the zinc amalgam and the mixture was allowed to stand for six days (61).

(vi) PREPARA TION OF CONSTANT BOILING HYDROCHLORIC ACID

Hydrochloric acid (36% V/V, 200 ml) was placed in a one-litre volumetric flask containing a small amount of distilled water. A hygrometer was used to determine the specific gravity of the HCl solution. Small amounts of distilled water were added until the hygrometer indicated a specific gravity of 1.1. At this specific gravity, 152 ml of distilled water had been added.

A simple distillation process was carried out with a thermometer to monitor. A condenser was acctached to the side tube, giving an all glass set-up. Dilute HCl (specific gravity 1.1, 300 ml) was placed in the flask and distilled at a rate of approximately 4 ml (per minute). The distillage was collected in a small pyrex flask and was poured into a measuring cylinder. When about 160 ml of the distillate had been collected,

a further 60 ml was collected at a constant temperature of 110^{O}C. The receiver was removed and stoppered. The distillate collected at 110^{O}C is the constant boiling HCl.

To prepare 0.01 M HCl from the stock, a glass stoppered flask was cleaned and dried. This was weighed with a sensitive Metler balance. Without touching the flask with fingers directly, exactly 18.02 g of constant boiling HCl was weighed out. This was washed into a one-litre volumetric flask with distilled water. The flask was stoppered and the solution mixed thoroughly (61).

(vii) PREPARATION OF ACD ANTICOAGULANT

An acid-citrate-dextrose (ACD) anticoagulant solution was prepared by dissolving a mixture of tri-sodium citrate dihydrate (5.10 g), Citric acid monohydrate (1.6 g) and anhydrous dextrose (2.4 g) distilled water in a 200 ml volumetric flask. The solution was thereafter made up to the mark with distilled water (59).

(viii) PREPARATION OF DRABKINS' SOLUTION

A mixture of potassium ferricyanide (0.05 g), potassium cyanide (0.125 g) and sodium hydrogen carbonate (2.5 g) was dissolved in distilled water in a 250 ml volumetric flask. The solution was up to the mark with distilled water and was kept in a dark bottle to protect it from light (59).

(ix) BLOOD SAMPLES

(a) Normal human blood containing hemoglobin A was obtained from the Blood Banks at the University College Hospital, Ibadan and also Oyo State Hospital Blood Bank, Total Garden, Ibadan.

(b) Sickle cell blood was obtained from the Haematology Clinic, University College Hospital, Ibadan.

(c) Dog and Horse blood were obtained from the Veterinary Teaching Hospital, Department of Veterinary Medicine, University of Ibadan, Ibadan.

(d) Rabbit blood was obtained from the Rabbitary Section, Department of Animal Science, University of Ibadan, Ibadan and Dr. G. Kolawole's Rabbitary Depot.

(x) PREPARATION OF OXYHEMOGLOBIN

Hemoglobins were prepared by a standard laboratory procedure (62). The blood samples were centrifuged for 20 minutes at 5°C at 18,000 r.p.m. Using a IEC HT centrifuge. The red cells were washed three times with saline solution (4.75 g NaCl/500 ml). After each washing, the resulting mixture was centrifuged at 8,000 r.p.m. for 15 minutes. Then the cells were lysed by shaking vigorously with an equal volume of ice-cold distilled water. The mixture was centrifuged at 12,000 r.p.m. for 20 minutes. Hemoglobin was then decanted from the beneath the cake of cell debris. After making it 5% (W/V) in sodium chloride, it was kept at 5°C for 20 minutes during which non-heme proteins precipitated. At the maximum speed of the centrifuge, 18,000 r.p.m., the hemoglobin solution was further centrifuged for 20 minutes. The hemoglobin solution was then dialysed. Each dialysis solution was brought to pH 6.5-7.5 with phosphate buffer (each dialysing solution consists of 0.4 M Na_2HPO_4 (5 ml); 0.4 M NaH_2PO_4(10 ml) and NaCl(0.29 g)).

Three changes of dialysis solutions were made at intervals of three hours.

To free oxyhemoglobin from small ions and organic phosphate, it was passed slowly through a Sephadex G-25 column previously equilibrated with distilled water.

Oxyhemoglobin concentrations were determined by taking the absorbance, at 540 nm, of 0.1 ml of oxyhemoglobin mixed with 10 ml of Drabkins' solution. Using a millimolar molar absorptivity of 109 for the cyanomethemoglobin complex, the hemoglobin concentration was determined in moles per heme (59,62). Hemoglobin was stored at 5^{o}C and used within four days of preparation.

(xi) PREPARATION OF CARBONMONOXYHEMOGLOBIN.

Carbon monoxide gas bubbled through oxyhemoglobin for about 20 minutes to convert it to carbonmonoxyhemoglobin. The carbonmonoxyhemoglobin concentration was determined by taking the optical density, at 537.5 nm, of 0.1 ml of carbonmonoxyhemoglobin mixed with 10 ml of distilled water. The concentration was determined in moles per heme assuming a millimolar molar

absorptivity of 140 (60).

(xii) PREPARATION OF AQUOMETHEMOGLOBIN.

With a two-fold excess of potassium ferricyanide solution oxyhemoglobin of known concentration was oxidised to aquomethemoglobin. Excess ferricyanide was removed by gel filtration on a Sephadex G-25 column previously equilibrated with distilled water. The concentration of aquomethemoglobin was determined by measuring the optical density at 540 nm of a mixture of 0.1 ml aquomethemoglobin and 10 ml of Drabkins' solution. The molar absorptivity (per heme) at 540 nm was assumed to be 10.9×10^3. Aquomethemoglobin samples were used within three days of preparation (62).

(xiii) PREPARATION OF AZIDOMETHEMOGLOBIN

A known concentration of sodium azide stock solution was prepared with distilled water using freshly recrystallized sodium azide (British Drug Houses) which had previously been left to dry for several days in a dessicator.

The observed equilibrium constant, K_{obs}, for azide binding to aquomethemoglobins A and S had been determined as a function of pH at 20°C with ionic strength of 0.05 (63). On the basis of the observed equilibrium constant, a calculated amount of sodium azide solution was added to a known concentration of aquomethemoglobin solution at a given pH to give 99% reaction. This increased the pH and the solution was titrated back to the starting pH with acid of known molarity. The mixture was allowed to equilibrate for two hours in a thermostated bath at 20°C.

The amount of azide suitable to give 99% reaction was calculated using the equation below (45).

$$[N_3^-]_T = \frac{99}{K_{obs}} + 0.99C$$

where $[N_3^-]_T$ = Total azide concentration

C = Total quomethemoglobin concentration

K_{obs} = Observed equilibrium constant at a given pH.

The amount of azide required to give 99% reaction for horse, dog and rabbit aquomethemoglobins was similarly calculated from a knowledge of their equilibrium constants at 20°C and as a function of pH.

(xiv) PREPARATION OF DEOXYHEMOGLOBIN

To free oxyhemoglobin from small ions and organic phosphates, the hemoglobin was passed slowly through a Sephadex G-25 column previously equilibrated with distilled water. The oxyhemoglobin concentration was determined as described above.

Oxyhemoglobin was placed in a jacketted (thermostated) titration vessel on a magnetic stirrer and was stirred continuously. Nitrogen gas was passed for 3 hours through two bubble bottles containing chromic sulphate and zinc amalgam deoxygenating solutions and through 0.4 M NaOH solution and then distilled water, into the titration vessel. The deoxygenated solution was transferred from the jacketted (thermostated) vessel into the free air jacketted (thermostated) titration vessel using a sigmamotor instrument.

(xv) PREPARATION OF STOCK SOLUTIONS OF 5,5'-DITHIOBIS [2-NITROBENZOIC ACID] [DTNB]

DTNB was purchased from Sigma Chemical Company and was used without further treatment. A known weight of DTNB was dissolved in phosphate buffer pH 7.6 of ionic strength 0.05 in a 500 ml volumetric flask, and the mixture was magnetically stirred for four days. The solution was then filtered and its concentration was determined spectrometrically by measuring the optical density at 412 nm after reacting it with excess mercaptoethanol. A molar absorptivity of 13,600 M^{-1} cm^{-1} was assumed for 3-carboxylato 4-nitro-thiophenolate (32).

(xvi) PREPARATION OF STOCK SOLUTIONS OF 2,2'-DITHIOBIS-PYRIDINE (DTP)

DTP was purchased from Sigma Chemical Company and was used without further treatment. A known weight of DTP was dissolved in 95% ethanol to make a 0.045 M solution in a 100 ml volumetric flask (18). Its concentration was also determined spectrophotometrically at 281 nm in phosphate buffer (pH 7.0 I = 0.2), using a molar absorptivity of 9.73 mM^{-1} cm^{-1} for 2-thiopyridone (14).

(xvii) PREPARATION OF 0.01 M STOCK SOLUTION OF METHYL METHANETHIOSULPHONATE (MMTS).

Methyl methanethiosulphonate was purchased from Sigma Chemical Company and was used without further treatment. MMTS is a very poisonous compound; it causes lung cancer if inhaled. A known volume of MMTS (Specific gravity 1.23) was pipetted into a 0.05 M NaCl solution using an Agla syringe with a surgical needle attached.

B: EXPERIMENTAL PROCEDURES

(a) (i) CYANIDE KINETICS

The kinetics were studied under pseudo-first-order conditions with a Zeiss PMQ II spectrophotometer. The cell compartment was thermostated with a Lauda Table Cryostat Model TUK 30D. Heme solution (2 μM) of aquomethemoglobin were prepared in phosphate buffers [pH 5.6-8.0] or borate buffers [pH 8.0-9.0], each of ionic strength 0.05. These solutions were allowed to temperature equilibrate in the Lauda thermostat at 20°C. A 10 ml aliquot of aquomethemoglobin was pipetted into a 2x2 cm spectrophotometric cell. After allowing for temperature equilibration, the transmittance of the solution in the spectrophotometer was recorded.

For the kinetic runs, potassium cyanide solutions were prepared fresh each day. A few microliters of 0.01 M cyanide solution to give a desired concentration was measured with a Finn pipette and transferred to a glass rod [one end of which was shaped in the form of a shallow spoon] which also served as the stirrer. The transmittance of the mixture was recorded at given intervals of time at 405 nm. Reactions were followed for at least 1.5 half-lives before a few crystals of potassium cyanide were added to determine the transmittance at the completion of the reaction. Each kinetic run was repeated twice under identical experimental conditions. This procedure was repeated at a single KCN concentration across the pH range on a single day.

Linear plots were analyzed by linear least squares regression. Mean values of the experimental parameters were determined with the weighted mean equation

$$\bar{X}_w = \frac{\sum_{i=1}^{N} \frac{X_i}{S_i^2}}{\sum_{i=1}^{N} \frac{1}{S_i^2}} \qquad \ldots (1)$$

where S_i is the standard deviation of parameter X_i and N is the number of values of X_i.

(ii) 5,5'-DITHIOBIS(2-NITROBENZOIC ACID) KINETICS

The reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with hemoglobin derivatives was monitored at 412 nm on a Zeiss PMQ II spectrophotometer. The cell compartment was thermostated with a Lauda Table Cryostat Model TUK30D. Heme solution (10 μM) of each hemoglobin derivative were prepared in phsophate buffers [pH 5.6-8.0] or borate buffers [pH 8.0-9.0], each of ionic strength 0.05. These solutions were allowed to temperature equilibrate in the Lauda thermostat at 20°C. A 10 cm^2 aliquot of hemoglobin was pipetted into a 2x2 cm spectrophotometric cell. The reaction was started by adding 5,5'-dithiobis (2-nitrobenzoic acid) solution to the sample cell with a Finn pipette. Stirring of the mixture was ensured with a magnetic stirrer fitted to the base of the cell holder.

The transmittance of the solution, T, was followed at 412 nm. The yellowish colour produced is due to the formation of 3-carboxylato-4-nitro-thiophenolate (TNB).

Using appropriate extinction coefficients at various pH values (22), the absorbance values were employed to calculate the concentration of TNB, x, produced at time, t. Values of the apparent second-order rate constant, $k_{(app)}$, were calculated with the second-order rate equation

$$k_{(app)} = \frac{1}{(a-b)} \cdot \frac{1}{t} \ln \frac{b(a-x)}{a(b-x)} \quad \ldots (2)$$

In this equation, a and b are the initial concontrations of DTNB and hemoglobin respectively. The hemoglobin concentration of all experimental solutions was 10 μM heme [5 μM in reactive sulphydryl group]. The DTNB concentration ranged between 20 μM and 100 μM. The pH values of experimental solutions were determined on a Radiometer GK2401C combined electrode. Each kinetic run was repeated twice under identical experimental conditions.

(iii) 2,2'-DITHIOBISPYRIDINE KINETICS

The reaction of 2,2'-dithiobispyridine with hemoglobin derivatives was monitored at 343 nm on a Zeiss PMQ II Spectrophotometer. The cell compartment was thermostated with a Lauda Table Cryostat Model

TUK 30D. Solutions of each derivative (10 μM Heme) of a hemoglobin species were prepared in phosphate buffers [pH 5.6-8.0] or borate buffers [pH 8.0-9.0], each of ionic strength 0.05. These solutions were allowed to equilibrate in a thermostat at 20°C. A 10 cm^3 aliquot of hemoglobin was pipetted into 2x2 cm spectrophotometric cell. The reaction was started by adding 2,2'-dithiobispyridine to the sample cell with a Finn pipette. Stirring of the mixture was ensured with a magnetic stirrer fitted to the base of the cell holder. The change in transmittance, T, was followed at 343 nm. The colour (deep brown) produced is due to the formation of 2-thiopyridone [TP]. The absorptivity of TP previously determined (18) was used. It was independent of pH and temperature (18). Transmittance readings were converted to absorbance using this equation

$$A = \log\frac{100}{T} \qquad \ldots (3)$$

where

A = absorbance

T = transmittance.

The concentrations of TP, x, produced at time, t, were calculated from the transmittance readings. Values of the apparent second-order rate constant, k_{app}, were calculated with the second-order rate equation, (equation 2). The hemoglobin concentration of all experimental solutions was 10 μM heme (5 μM in reactive sulphydryl group). The 2,2'-dithiobispyridine concentration ranged between 50 μM and 200 μM. The pH of experimental solutions were determined on a Radiometer PHM 4d pH meter equipped with a Radiometer GK2401C combined electrode. Each kinetic run was repeated twice under identifical experimental conditions.

(iv) KINETICS OF HYDROLYTIC CLEAVAGE OF MODIFIED HEMOGLOBIN

Mixed disulphide hemoglobins are very stable at pH 8.0 but are cleaved with increasing rates as the pH increases (64). Since some of the kinetic experiments reported in this thesis were done at pH > 8.0 hydrolytic cleavage was monitored after 5,5-dithiobis(2-nitrobenzoic acid) was added to hemoglobin and the mixture allowed to react for about 2 hours. To remove excess (DTNB) aliquots were passed through a Sephadex-G25 column previously equilibrated with distilled water.

The rate of TNB cleavage from the hemoglobin - TNB complex was followed at 412 nm at pH 9.2. This hydrolysis method was also applied to hemoglobin - TP complex. The rate of TP hydrolysis from hemoglobin - TP complex was monitored at 343 nm at pH 9.2.

(b) POTENTIOMETRIC DIFFERENCE TITRATION: PROTON RELEASE ON MMTS BINDING TO HEMOGLOBIN.

Measurements of pH were made on a Radiometer PHM 4d pH meter standardized at the appropriate temperature (20^{o}C) with two precision buffer solutions types S1500 and S1510, pH 6.88 and 7.43, respectively (58).

The reaction of MMTS with the CysF9(93)β sulphydryl group of hemoglobin was monitored potentiometrically at 20^{o}C, as a function of pH, in the absence of buffer ions between pH 5.6 and 9.6. Hemoglobin was passed into a jacketted (thermostated) titration vessel, at the neck of which a Radiometer GK2401C combined electrode had been sealed. In order to eliminate CO_2 completely from the solution, nitrogen gas was passed through bubble bottles containing 0.4 M NaOH and distilled water and then over the hemoglobin solution which was stirred

magnetically. In the case of oxyhemoglobin, "Carbosorb" non-deliquescent, self-indicating soda lime granules (about 10-16 mesh) were used to eliminate CO_2. After about 30 minutes, the solution in the titration vessel was brought to the desired pH by adding small quantities of CO_2-free 0.01 M hydrochloric acid or 0.01 M sodium hydroxide solution. The hemoglobin concentration was determined spectrophotometrically and was about 0.4 mM in heme. To give 100% sulphydryl reaction, a known volume equivalent to 0.2 mM MMTS was added to the titration vessel at a given pH. On addition of MMTS, the pH of the solution decreased and was brought back to the original pH with a known quantity of 0.1 M or 0.01 M carbonate-free NaOH in an Agla syringe. This procedure was repeated at various pH values with fresh samples of hemoglobin. Each potentiometric titration was repeated once or twice under identical experimental conditions.

The proton release per thiol group reacted was calculated using the equation

$$\frac{\Delta h^{+}}{SH} = \frac{\text{Molarity of NaOH x volume of NaOH used}}{\text{Molarity of thiol group in hemoglobin x total volume of aliquot}} \quad \ldots (4)$$

CHAPTER 3

RESULTS

A: DEPENDENCE OF THE KINETICS OF CYANIDE BINDING TO AQUOMETHEMOGLOBIN ON pH.

The kinetics of cyanide binding to horse and rabbit aquomethemoglobins were studied as a function of pH at ionic strength 0.05. This study was undertaken to determine the pK values of the "heme-linked" ionizable groups of these aquomethemoglobins and is an extension of similar work reported earlier (56) on human A and S, pigeon and guinea pig aquomethemoglobins.

The kinetics of cyanide binding of three aquomethemoglobin species have been reported to be monophasic: the α- and β- chains were found to react at the same rate (9,56). The reaction was monitored under pseudo first order conditions. This was accomplished by reacting aquomethemoglobin with at least a tenfold excess of potassium cyanide in this study. An essential isolation of each of two reacting species can be made by adjusting their concentrations so that one of them, which is present in considerable excess, is effectively maintained at constant concentration.

For instance, for the reaction,

$$A + B \rightarrow AB \qquad \ldots (5)$$

the rate equation is

$$\frac{-d[A]}{dt} = \frac{-d[B]}{dt} = k[A][B] \qquad \ldots (6)$$

where k is the second order rate constant. If $[A]_o$ and $[B]_o$ are the initial concentrations and $[A]_o$ is much greater than $[B]_o$, that is, $[A]_o \geqslant 10[B]_o$, the rate equation becomes

$$\frac{-d[A]}{dt} = \frac{-d[B]}{dt} = k'[B] \qquad \ldots (7)$$

where $k' = k[A]_o$ is the pseudo first order rate constant.

For the above reaction, the rate equation may also be written as:

$$\frac{-d[a-x]}{dt} = \frac{-da}{dt} + \frac{dx}{dt}$$

where a is the initial concentration of the reacting species, and x is the amount reacted at time, t. Since a is a constant, $\frac{da}{dt} = 0$.

Therefore,

$$\frac{dx}{dt} = k'[b-x] \qquad \ldots (8)$$

On integration one obtains

$$\ln b - \ln[b-x] = k't \qquad \ldots (9)$$

In this work the kinetics have been studied spectrophotometrically under conditions where the Beer-Lambert law applies. From the Beer-Lambert law, the absorbance of a solution is related to the concentration of the absorbing species by the equation

$$E = \varepsilon c l$$

where E is the absorbance, ε is the molar absorptivity c is the concentration of absorbing species, and 1 is the path length.

Let E_o be the absorbance at zero time; E_∞ the absorbance at the compeltion of the reaction, and E_t the absorbance at time t after the initiation of the reaction. The following proportionalities hold:

$\frac{x}{b}$, the fraction reacted at time, t, is proportional to

$$\frac{E_o - E_t}{E_o - E_\infty} ;$$

$\frac{b-x}{b}$, the fraction remaining at time t is proportional to

$$\frac{E_t - E_\infty}{E_o - E_\infty}$$

Therefore, equation 9 can be expressed in terms of absorbance as:

$$\ln[E_o - E_\infty] - \ln[E_t - E_\infty] = k't \qquad \ldots (10)$$

Since the α and β subunits of aquomethemoglobin react with cyanide ion at the same characteristic rate (49, 56), the following stoichiometric equation describes the homogeneous reaction:

$$Hb^{+} + CN^{-} \underset{k_{-1(app)}}{\overset{k_{1(app)}}{\rightleftharpoons}} HbCN \qquad \ldots (11)$$

In this equation, $k_{1(app)}$ is the apparent second order combination rate constant and $k_{-1}(app)$ is the apparent first order dissociation rate constant.

Under pseudo first-order conditions, the observed rate constant, k_{obs}, is related to the apparent rate constants by the equation

$$k_{obs} = k_{1(app)}[CN^{-}] + k_{-1}(app) \qquad \ldots(12)$$

Figure 1 shows, for rabbit aquomethemoglobin, typical plots of $-\ln[E_t - E_\infty]$ against time (c.f. Equation 10) at various fixed cyanide concentrations at a given pH. The plots are monophasic; similar plots were obtained for horse aquomethemoglobin (not shown). These monophasic plots strongly indicate that the α- and β- subunits are kinetically equivalent with respect to cyanide binding, as has been reported for other aquomethemoglobins (56).

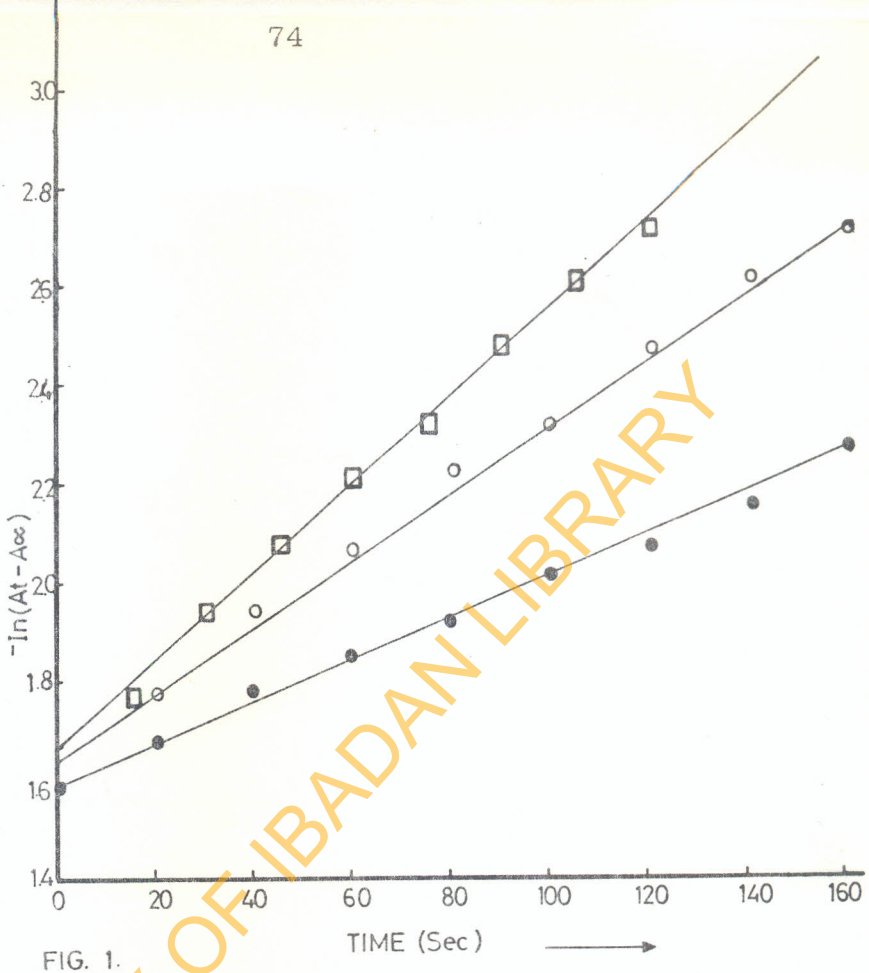


FIG. 1.

Pseudo first-order rate plots for the binding of cyanide ion to rabbit aquomethemoglobin. Conditions: Phosphate buffer, pH = 6.2; ionic strength = 0.05 M; 20°C [MetHb] = 2 μM heme. The KCN concentrations are 30 μM [filled circles], 60 μM (open circles) and 80 μM (squares) See Appendix 1 Table 6.

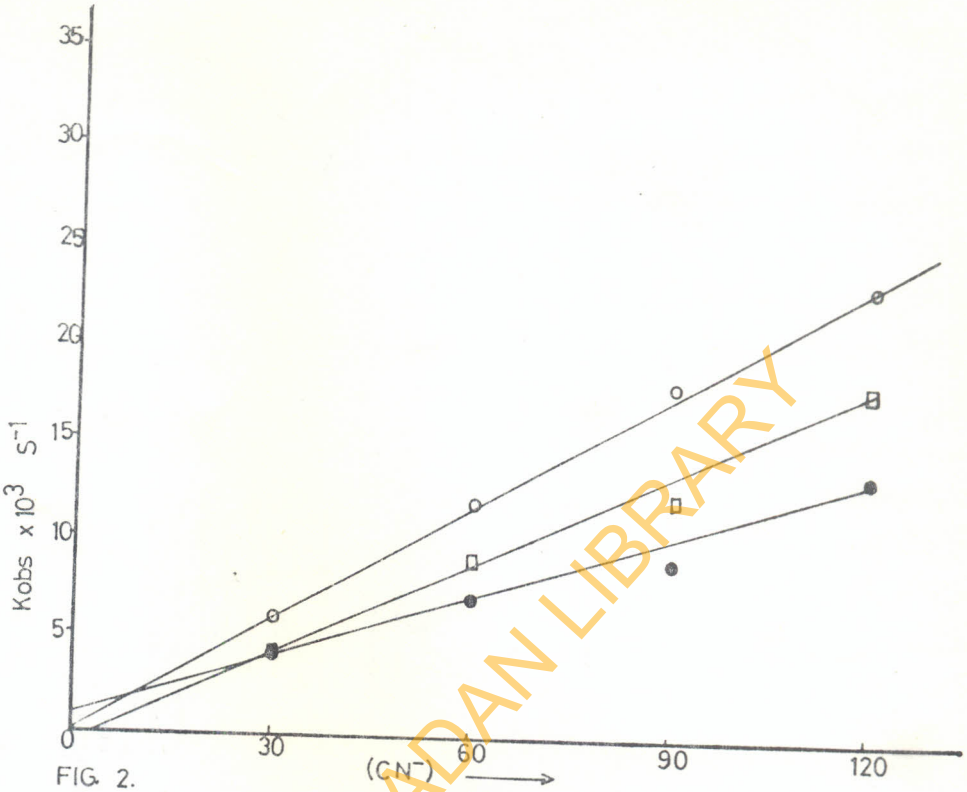


FIG. 2.

Dependence of observed rate constant, k_{obs}, on cyanide concentration at 20°C for rabbit aquomethemoglobin pH = 6.2 [filled circles], pH = 7.0 [open circles] and pH = 8.6 [Squares] See Appendix 1 Table 8.

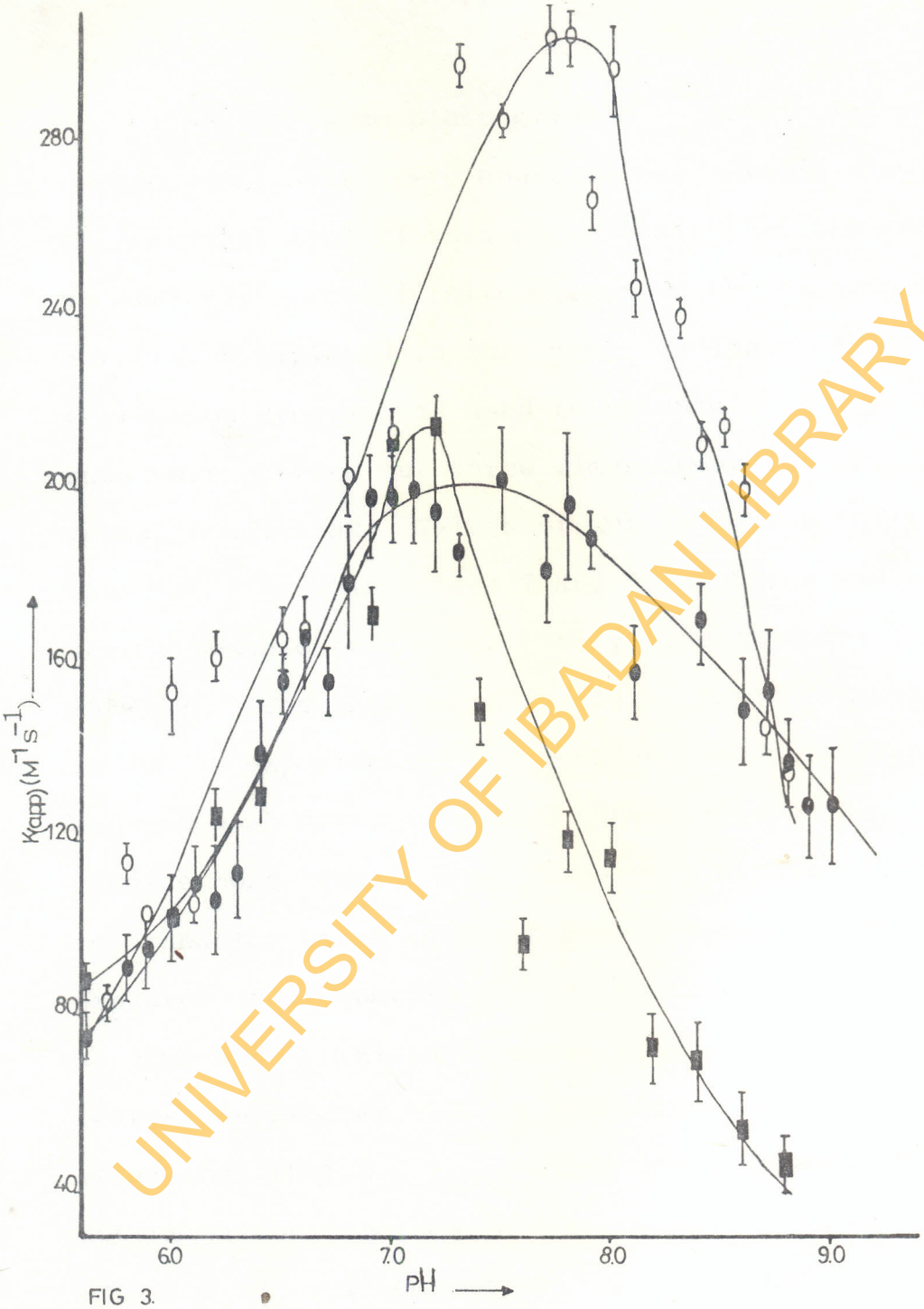


FIG 3.

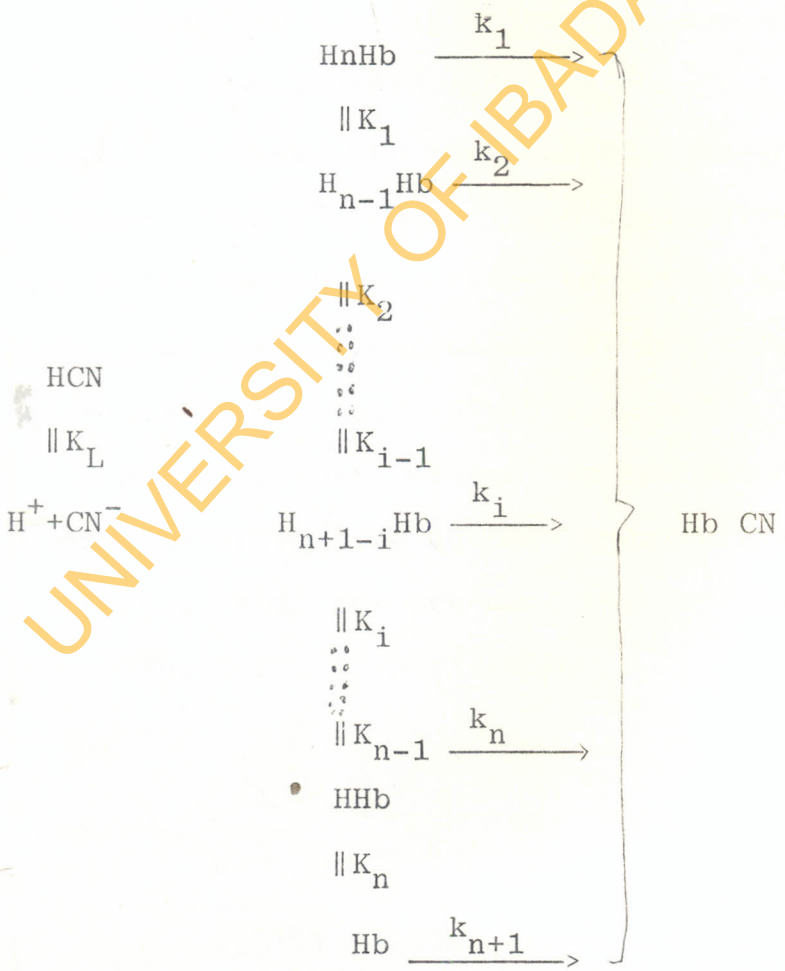
Dependence of the apparent second order rate constant for cyanide binding to aquomethemoglobin, k_{app}, on pH at 20°C I = 0.05. These are theoretical lines obtained from the computer. Human A [filled squares], Horse [open circles], and Rabbit [filled circles]. See Appendix I Tables 9 and 10 for horse and rabbit aquomethemoglobin respectively.

The slopes of these plots give k_{obs}, the observed pseudo first-order rate constant for cyanide binding. The straight line plots also indicate that the reaction is strictly pseudo first-order under the experimental conditions employed in this work. A linear least squares regression program was used to calculate k_{obs}. Kinetic runs were carried out three times under the same experimental conditions. Values of mean k_{obs} for individual runs are listed in Tables 7 and 8 for horse and rabbit aquomethemoglobins, respectively [See Appendix 1 pages 157-159]. The intercepts in Fig. 1 are not equal but it is due to experimental error which does not affect our experimental data. Figure 2 shows plots of k_{obs} versus cyanide concentrations at fixed pH. Similar plots were obtained for horse aquomethemoglobin (not shown). As expected from equation 12, the plots are linear, Values of $k_1(app)$ were obtained from the slopes by least squares regression program. It is known from the results of Job et al (54) that $K_{-1(app)}$ values are very small and their determination from the intercepts of plots according to equation 12 would be subject to a great deal of uncertainty. No attempt was therefore made to evaluate $k_{-1(app)}$. Figure 3 shows plots of $k_{1(app)}$ against pH for horse and rabbit aquomethemoglobins. The plot for human A aquomethemoglobin (56) is included for comparison. Tables

9 and 10 (see Appendix 1 Pages 161-162) list the values of $k_{1(app)}$ for horse and rabbit aquomethemoglobin, respectively at various pH values.

The pH profiles for horse and rabbit aquomethemoglobins are similar to those of other aquomethemoglobins (56). Such pH dependence data have been satisfactorily accounted for with Scheme I(56).

SCHEME I



In the above scheme, n ionizable groups are linked electrostatically to the heme-protein Hb. HnHb, $H_{(n-1)}Hb$,...,$H_{(n+1-i)}Hb$,...,HHb and Hb are protein species having n, (n-1),...,(n+1-i),... 1 and 0 protons bound, respectively to the electrostatically heme-protein linked ionizable groups. K_L is the ionisation constant of hydrogen cyanide. The k_i (i = 1,2,3,... n+1) are the second-order rate constants for the reaction of cyanide ion binding to various aquomethemoglobin species. The kinetic model of Scheme I assumes that the protolytic steps are much faster than the cyanide ion binding steps (56, 66). This assumption has been justified (56) by reference to direct kinetic studies of reactions involving protons (67-70). Such reactions are diffusion-controlled (67-70).

Assuming that, of the two species HCN and CN^-, CN^- binds exclusively to aquomethemoglobin (54), it can be shown (56) that for Scheme I the expression for $k_{1(app)}$ (See equation 12) is given by

$$k_{1(app)} = \frac{K_L}{K_L+[H^+]} \cdot \frac{k_1[H^+]^n + \sum_{i=1}^{n+1} k_i[H^+]^{n+1-i} \prod_{j=1}^{i-1} K_j}{[H^+]^n + \sum_{i=1}^{n+1}[H^+]^{n+1-i} \prod_{j=1}^{i-1} K_j} \quad \ldots (13)$$

Using a computer program developed for this purpose (71),

equation 13 was employed to fit the $k_{1(app)}$ versus pH profiles by parameter optimization (Figure 3). The best fits to the horse and rabbit aquomethemoglobin data in Figure 3 were obtained with $n = 3$, in agreement with previously published data (56). The lines in Figure 3 are theoretical lines calculated from equation 13, and the fitting parameters are shown in Table 3. Details of the fitting procedure have been given elsewhere (56, 57).

The results of the computer fit (Table 3) and those obtained previously (56) strongly indicate that three sets of "heme-linked" ionizable groups influence the binding of cyanide ion, and of other anions, to aquomethemoglobin derivatives of animal species. They are: (1) a set of carboxylic acid groups; (2) a set of histidine and terminal amino acid groups; and (3) the acid-alkaline aquomethemoglobin transition (56,57).

The limitation of the kind of study described above is that it is only applicable to the determination of the nature and number of sets of ionizable groups in ferric heme proteins (50-57) but not in oxy hemeproteins, since the latter react only with uncharged ligands. The rest of the work reported in this thesis is aimed at determining the nature of these ionizable groups in ferrous hemoglobin.

TABLE 3

FITTINGS PARAMETERS USED TO CALCULATE THE THEORETICAL LINES FIGURE 3 ACCORDING TO EQUATION 13 (c.f. REFERENCE 56).

Parameter	n = 3		
	MetHb A*	MetHb Horse	MetHb Rabbit
$10^{-6}k_1M^{-1}S^{-1}$	0.6	1.10	0.4
$10^{-4}k_2M^{-1}S^{-1}$	29.1	7.20	5.2
$10^{-3}k_3M^{-1}S^{-1}$	0.7	1.60	1.4
$k_4M^{-1}S^{-1}$	0.0	0.00	0.0
pK_1	5.22	5.33	5.60
pK_2	6.23	7.05	6.77
pK_3	8.00	8.22	8.25
pK_L	9.30	9.29	9.12

* = Data from Ref. 56.

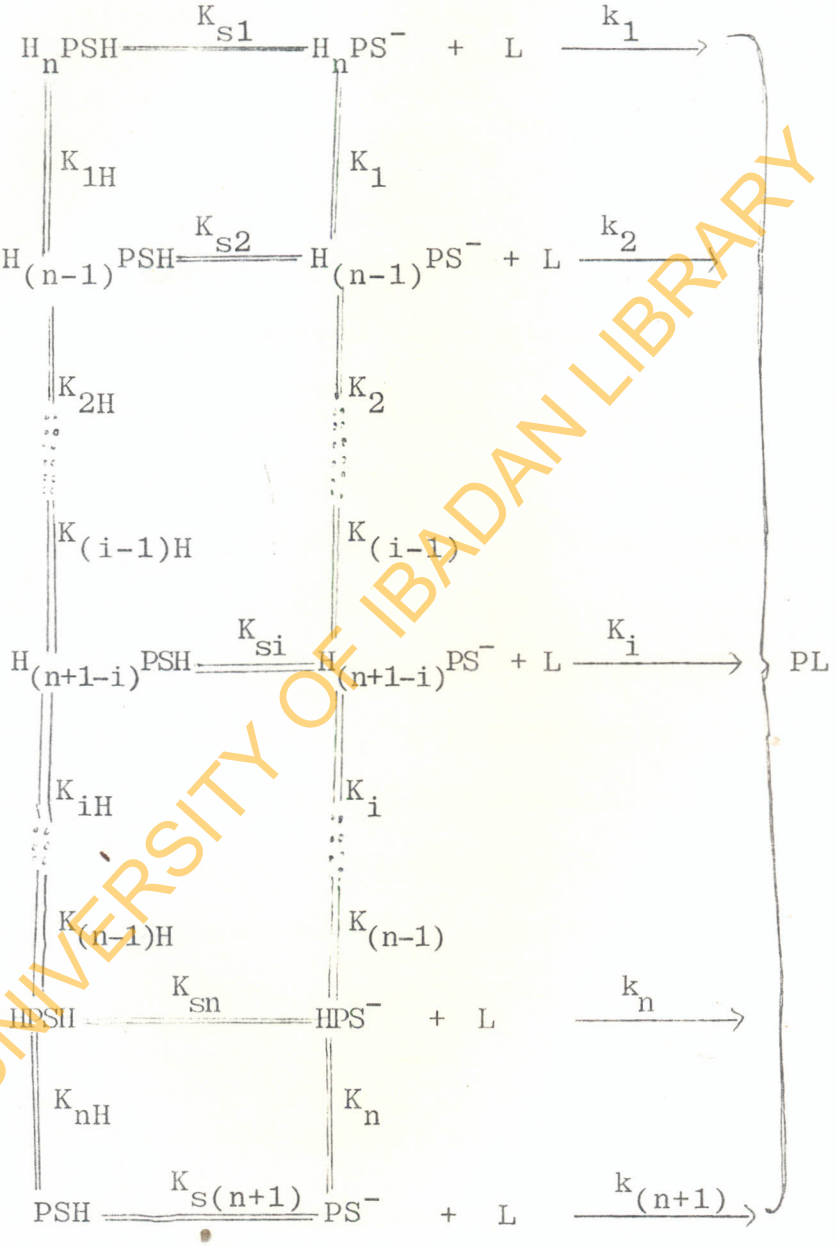
B: pH DEPENDENCE OF THE KINETICS OF REACTION OF THE β^{93} SULPHYDRYL GROUPS OF HEMOGLOBIN.

It is generally accepted that the reactions of sulphydryl groups of simple compounds and in proteins is via nucleophilic attack by the thiolate anion. Since the sulphydryl group in any given protein is surrounded by charged groups, the reaction profile exhibited for reaction with any given sulphydryl reagent will depend on whether such reagent is charged or not (74-76)

(i) Kinetics of reaction of 5,5'-dithiobis(2-nitrobenzoic acid) with hemoglobin.

5,5'-dithiobis (2-nitrobenzoic acid)(DTNB) is a charged sulphydryl reagent. Therefore the pH profile of the kinetics of its reaction with ferrous hemoglobin should be similar to that for cyanide ion binding to aquomethemoglobin. Therefore to account for the pH dependence of the DTNB reaction, a Scheme (Scheme II) sikilar to Scheme I was employed.

SCHEME II



In the above scheme, n ionizable groups are linked electrostatically to the thiol groups of a protein P. H_nPSH, $H_{(n-1)}PSH$, ..., $H_{(n+1-i)}PSH$,....,HPSH and PSH are protein species having n, (n-1), ..., (n+1-i),...,1 and O protons bound, respectively, to the electrostatically thiol linked ionizable groups. Each of these species has a protonated thiol group.

H_nPS^-,..., $H_{(n+1-i)}PS^-$,...,HPS^- and PS^- are the corresponding thiolate anion forms, respectively. The k_i ($i = 1,2,3,...,n+1$) are the second-order rate constants for the reaction of a sulphydryl reagent L with the thiolate thiolate anion forms of the various species. The K_{Si} are the protolytic dissociation constants of the thiol group in species i. K_{iH} (K_i) are the protolytic dissociation constants of the electrostatically thiol-linked ionizable groups for the neutral thiol (thiolate anion) forms. The species in the above scheme may be represented by the general formula:

$H_{(n+1-i)}PSH$ for the neutral thiol forms and

$H_{(n+1-i)}PS^-$ for the thiolate anion forms ($i = 1,2,3,....,n+1$). It is assumed that the protolytic steps of the scheme are much faster than the thiol reaction steps (56)

i.e., binding of L to the thiolate anion species. In terms of the parameters of the above scheme and on the basis of the above assumption, the apparent second-order rate constant of the reaction in Scheme II is given by:

$$k_{(app)} = \frac{k_{n+1} + \sum_{i=1}^{n} k_i [H^+]^{n+1-i} [\prod_{i=1}^{n} K_i]^{-1}}{1 + \sum_{i=1}^{n} [H^+]^{n+1-i} [\prod_{j=i}^{n} K_j]^{-1} + \frac{[H^+]}{K_{s(n+1)}} \sum_{i=1}^{n} [H^+]^{n+2-i} [\prod_{j=1}^{n} K_{j+H}]^{-1}} \quad \ldots (15)$$

Equation 15 is similar to Equation 13 employed to account for the pH dependence of cyanide binding to aquomethemoglobin (56). A comparison of Equation 15 with equation 13 shows, however, that the former contains many more unknown parameters than the latter. These additional parameters are the K_{jH} (j = 1,2,...,n) and $K_{s(n+1)}$. For n = 3 Equation 13 contains 8 unknown parameters. Equation 15, on the other hand, contains 14 unknown parameters for n = 3. The formula for the $k_{(app)}$ obtained from the scheme proposed to analyze the DTNB data contained several parameters, that could not be separated, therefore, making the model unidentifiable. The DTNB data presented in this thesis are therefore discussed only qualitatively.

The pH dependences of the DTNB reaction with human oxy-, carbonmonoxy-, azidomet- and aquomet- hemoglobins are presented in Figures 4-7. In each figure the profiles for normal and sickle cell hemoglobin are compared. Each point is the mean of at least three determinations. In each case $k_{(app)}$ first increases with pH and attains a maximum value around pH 7. Thereafter $k_{(app)}$ decreases, reaches a minimum around pH 7.6 and then increases once again with pH. This behaviour is different from the kinetic pH profile for cyanide binding to aquomethemoglobin (compare Figures 4-7 with Figure 3 above the Figures 3 and 4 of Ref. 56). The increase of $k_{(app)}$ at high pH may be attributed to increased ionization of the β^{93} sulphydryl group with increasing pH. Examination of Equation 15 shows that the third term in the denominator,

$$\frac{[H^+]}{K_{s(n+1)}} \sum_{i=1}^{n} [H^+]^{n+2-i} \left[\prod_{j=1}^{n} K_{jH}\right],$$

decreases with increasing pH. The magnitude of this term will vary most sensitively around pH > $pK_{s(n+1)}$. Hence around pH>$pK_{s(n+1)}$ $k_{(app)}$ increases. This will hold only if all the other terms do not change at the pH under consideration.

The profiles of normal and sickle cell hemoglobin

may not be compared for each derivative. Figure 4 shows that for most of the pH range 5.6 to 9.0 oxyhemoglobin A reacts faster than oxyhemoglobin S. The same result is demonstrated dramatically in the carbonmonoxy derivatives (Figure 5) where at pH 7, normal hemoglobin reacts about seven times faster than sickle cell hemoglobin.

In the azidomethemoglobin derivatives, however, both hemoglobins appear to react at the same rate throughout the pH range 5.6 to 9.0 (Figure 6). Figure 7 shows an equally dramatic difference for the aquomethemoglobin derivatives of the two hemoglobins.

The differences observed between normal and sickle cell hemoglobin (Figures 4,5,7) are surprising and are the reverse of what would have been expected on electrostatic grounds. Since it is known that sickle cell hemoglobin has a higher positive net charge than normal hemoglobin (82) it would be expected to react faster with the negatively charged DTNB. That this is not the case indicates that factors other than the net charge are more significant, as has been pointed out previously (56,57). These factors will be discussed later together with the finding (Figure 6) that the azidomethemoglobin derivatives react at the same rate.

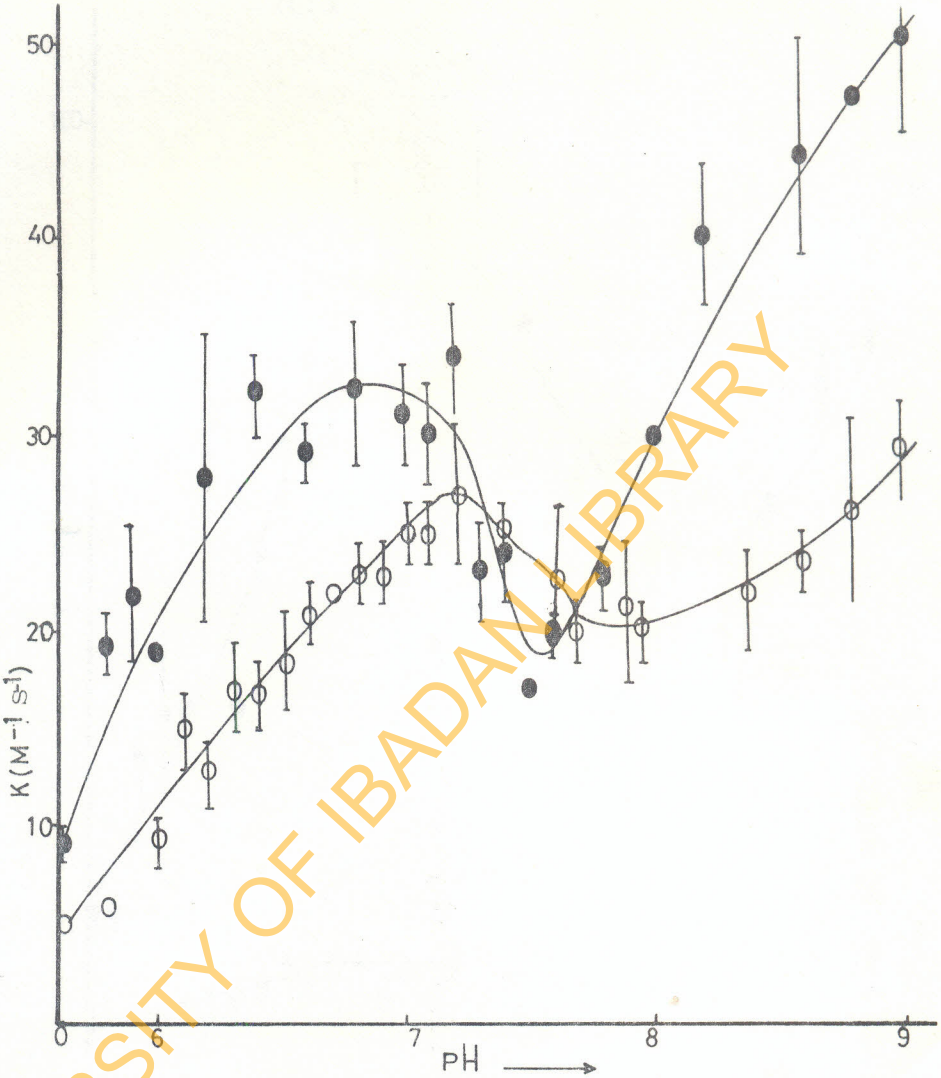


FIG 4.

Dependence of the apparent second order rate constant k_{app}, on pH for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of oxyhemoglobins at 20°C I = 0.05. Human A (filled circles) Seen Table 12 and Human S (open circle) see Table 16 in Appendix II.

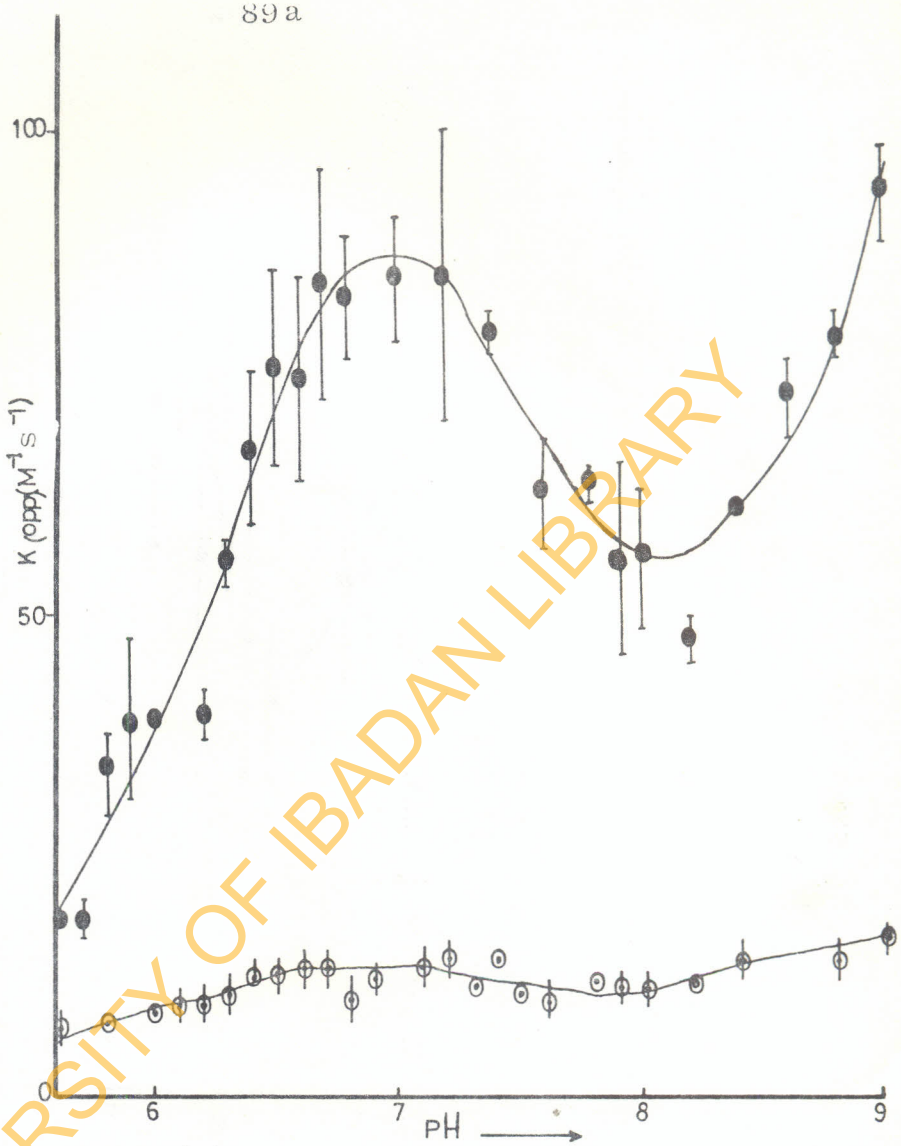


FIG 5.(a)

Dependence of the apparent second order rate constant k_{app}, for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of carbonmonoxyhemoglobins at 20°C I = 0.05. Human A [filled circles) see Table 13 and Human S (open circles) see Table 17 in Appendix II.

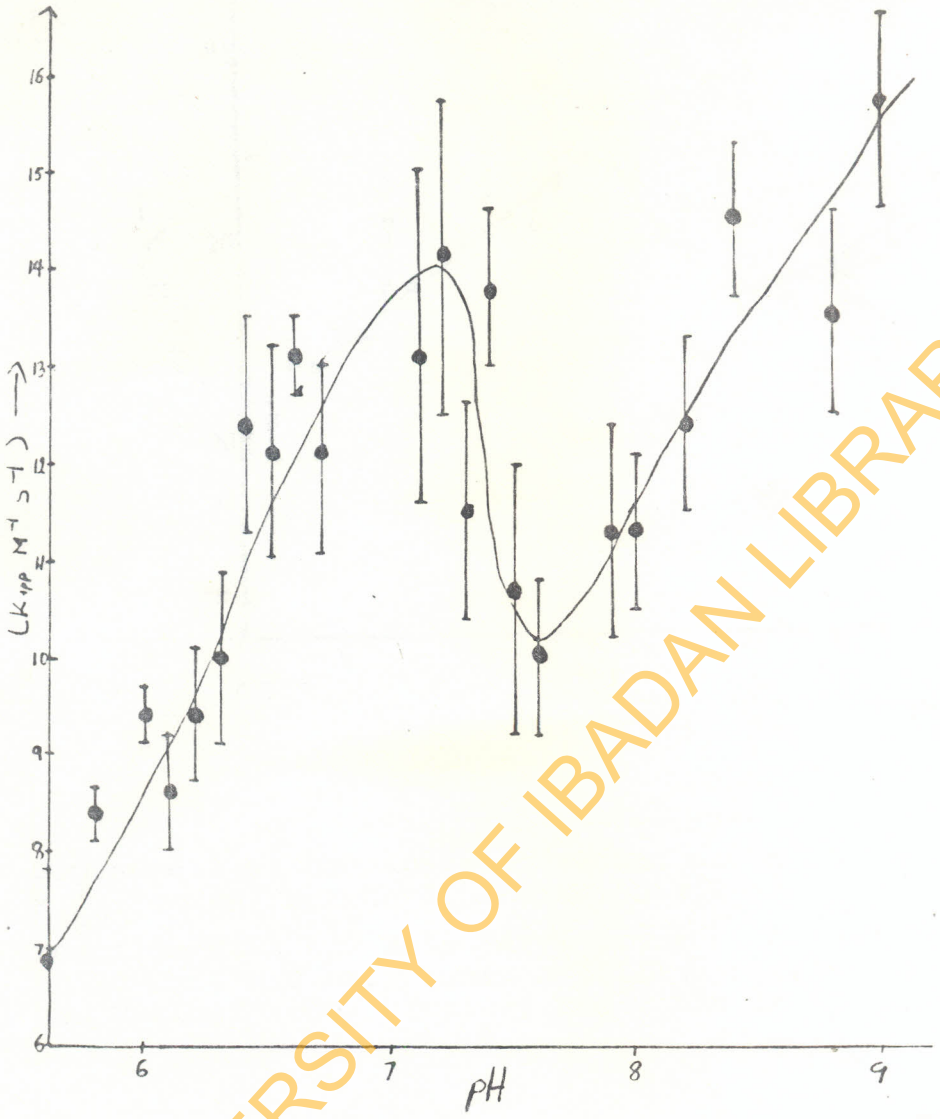


Fig. 5b: Dependence of the apparent second order rate constant, k_{app} on pH for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of carbonmonoxyhemoglobin S at 20°C I = 0.05.

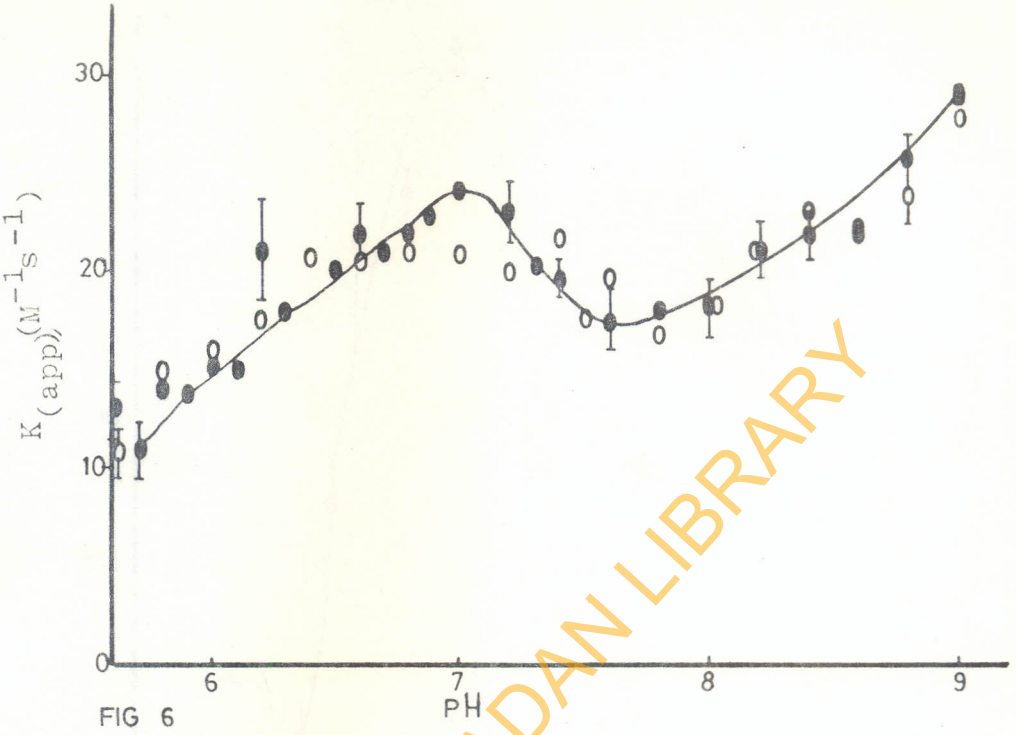


FIG 6

Dependence of the apparent second order rate constant, k_{app}, on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with β^{93} sulphydryl group of azidomethemoglobins at 20°C I = 0.05. Human A (filled circles) see Table 14 and Human S (open circles) see Table 18 in Appendix II.

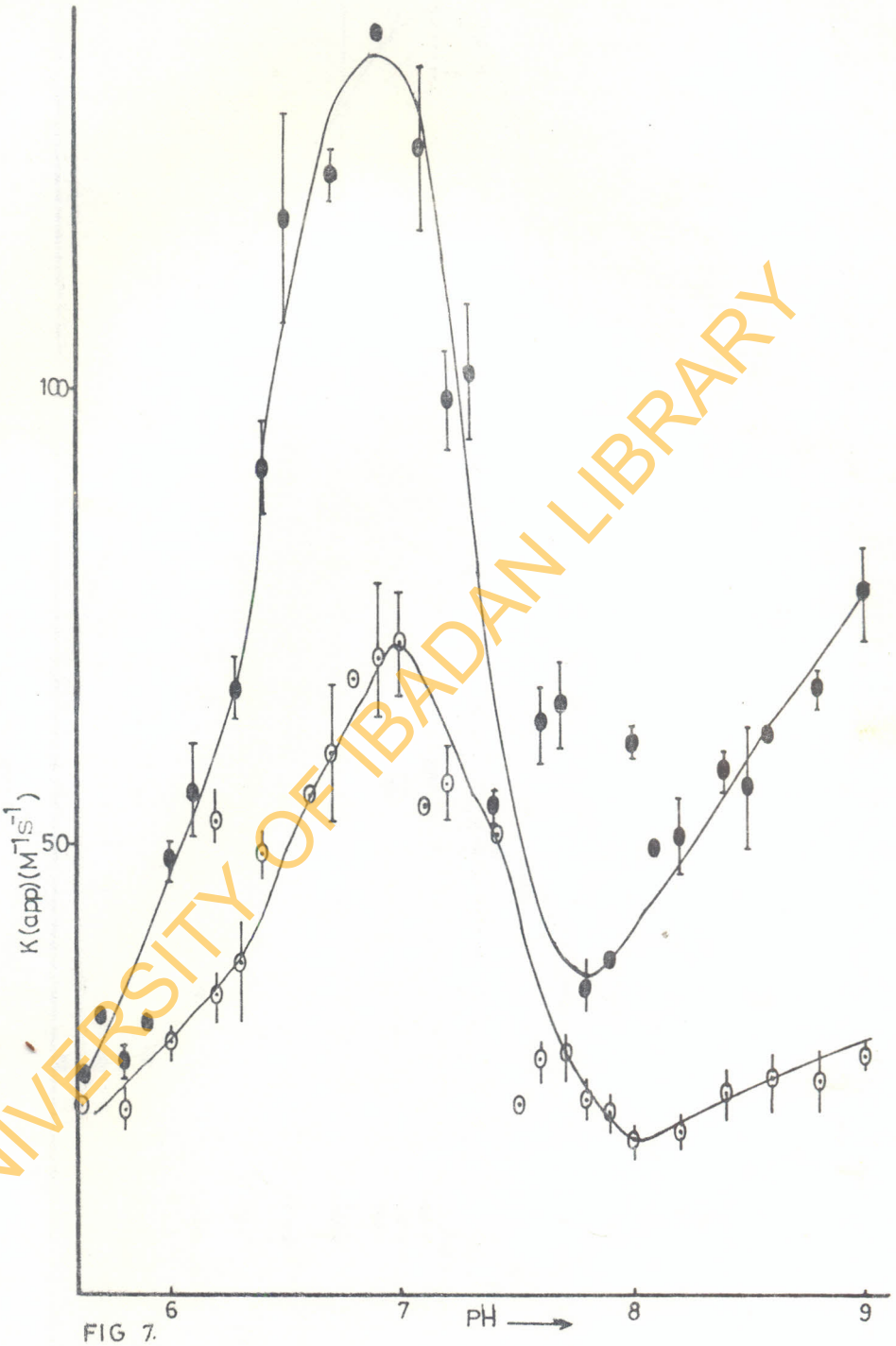


FIG 7.

Dependence of the apparent second order rate constant k_{app}, on pH for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of aquomethemoglobins at 20°C I = 0.05. Human A (filled circles) see Table 15 and Human S (open circles) see Table 18 in Appendix II.

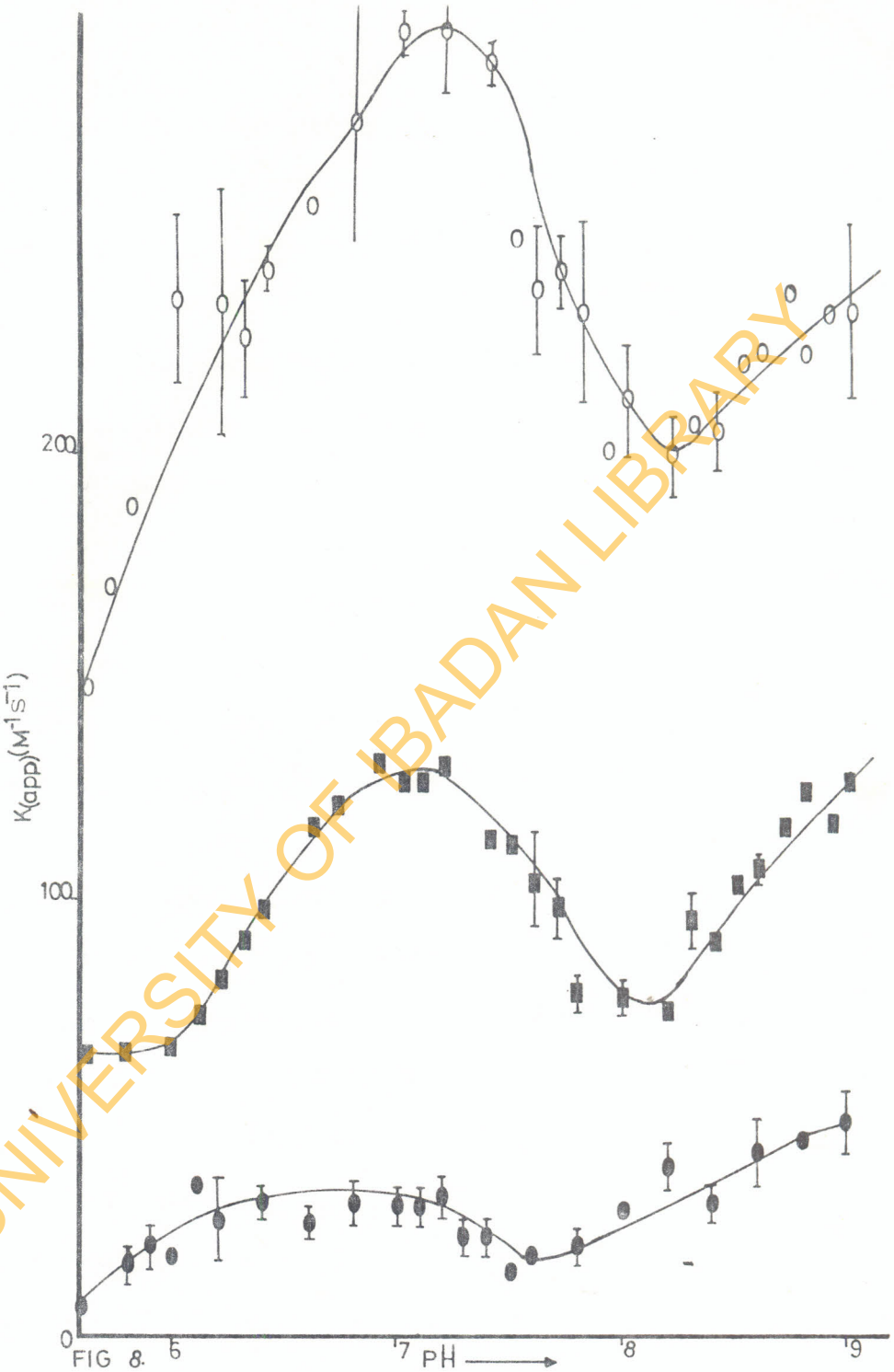


FIG 8.

Dependence of the apparent second order rate constant, k_{app}, on pH for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of oxyhemoglobins at 20°C I = 0.05. Human A (filled circles) ,see Table 12, Horse (filled squares) see Table 20 and Rabbit (open circles) see Table 24 in Appendix II.

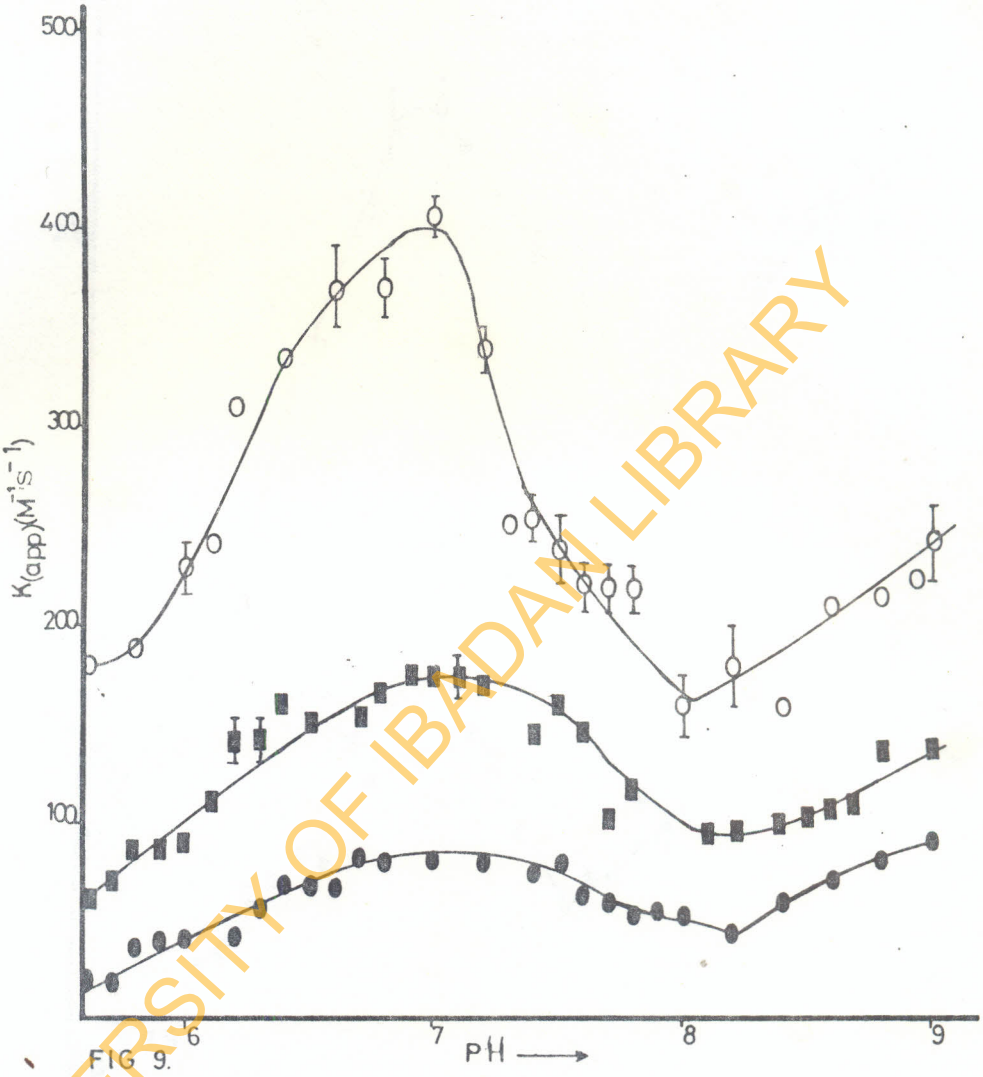


FIG 9.

Dependence of the apparent second order rate constant, k_{app} on pH for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of carbonmonoxyhemoglobins at 20°C I = 0.05. Human A (filled circles), see Table 13, Horse (filled squares) see Table 21 and Rabbit (open circles) see Table 25 in Appendix II.

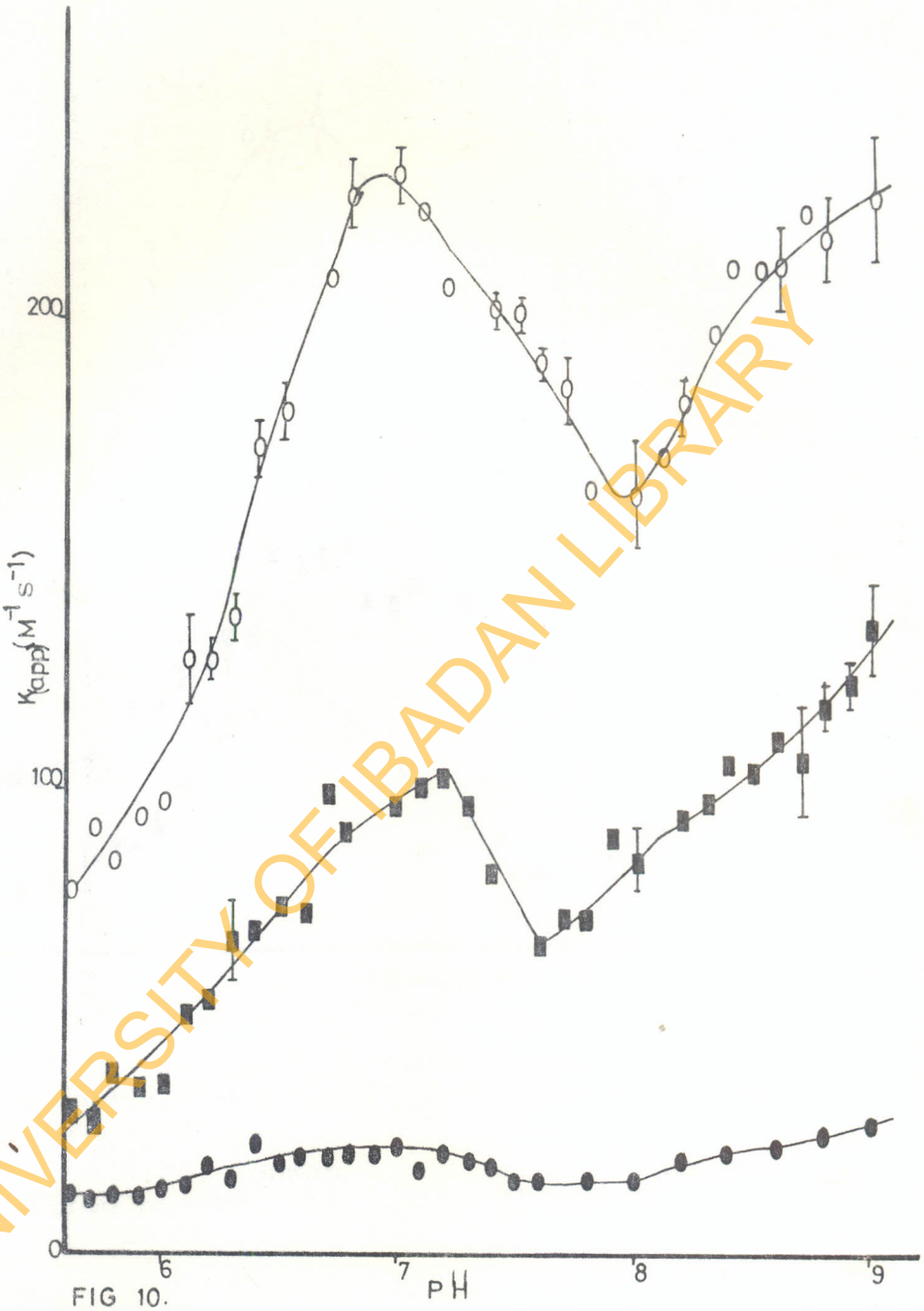


FIG 10.

Dependence of the apparent second order rate constant, k_{app} on pH for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of azidomethemoglobins at 20°C I = 0.05. Human A (filled circles) see Table 14, Horse (filled squares (see Table 22 and Rabbit (open circles) see Table 26 in Appendix II

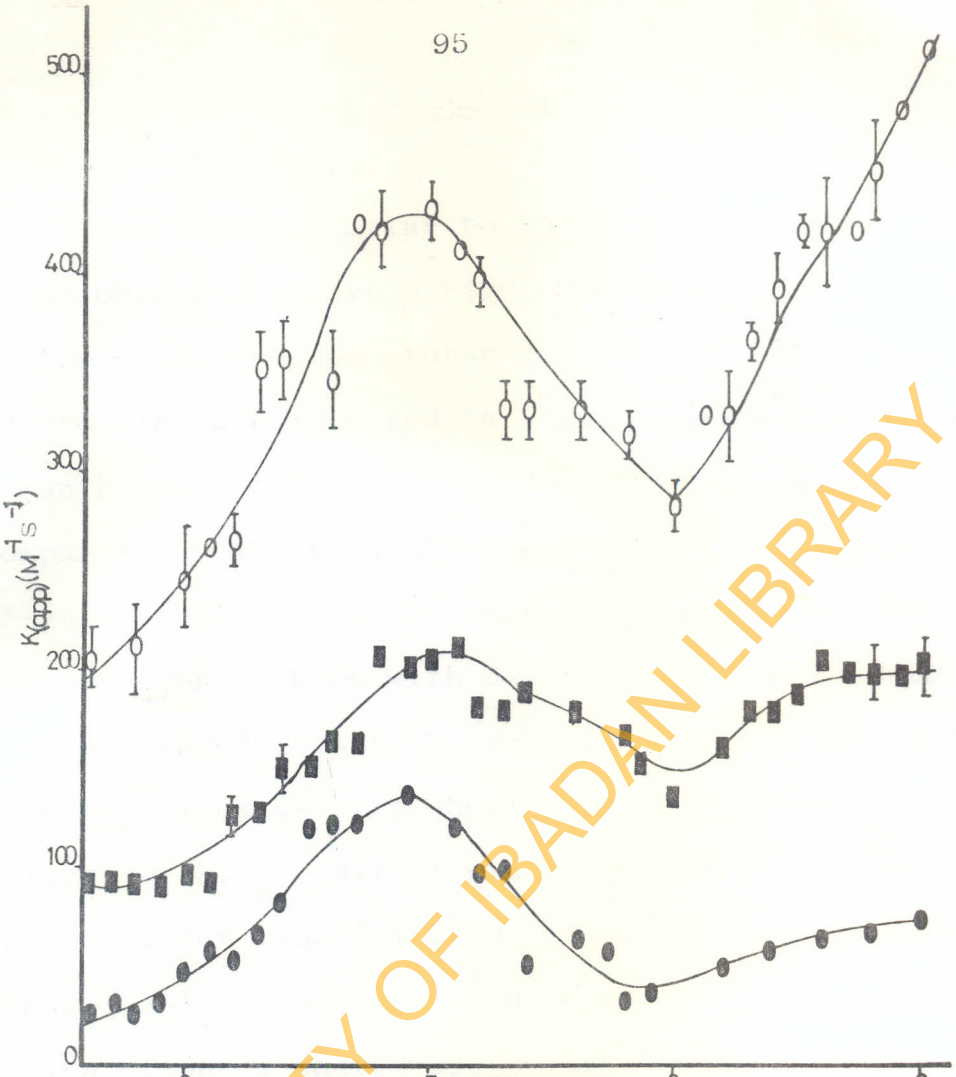


FIG 11.

Dependence of the apparent second order rate constant, k_{app} on pH for the 5,5'-dithiobis(2-nitrobenzoic acid) reaction with β^{93} sulphydryl group of aquomethemoglobins at 20°C, I = 0.05. Human A (filled circles) see Table 15, Horse (filled squares) see Table 23 and Rabbit (open circles) see Table 27 in Appendix II.

Experiments similar to those done on human hemoglobin derivatives (Figures 4-7) were carried out on two other species: horse and rabbit. The $k_{(app)}$ versus pH profiles are reported in Figures 8-11. The data for human hemoglobin A are included in each figure for comparison. The similar shapes of the pH profiles (Figures 4-11) allow a general conclusion to be made about the reaction of DTNB with derivatives of all hemoglobin species, namely, that the underlying mechanism is the same in all cases. It should be noted, however, that values for $k_{(app)}$ differ widely for different hemoglobin species under identifical experimental conditions (See Figures 8-11). A comparison of the $k_{(app)}$ values in the region of pH 7, where $k_{(app)}$ assumes its maximum value, show that rabbit hemoglobin reacts at between 4- and 10- fold faster than hemoglobin A and about 2-fold faster than horse hemoglobin. Also, for each hemoglobin species, the most reactive derivative is the aquomethemoglobin, followed by the carbonmonoxy- and oxyhemoglobin derivatives. The azidomethemoglobin derivatives are the least reactive.

(ii) Kinetics of reaction of 2,2'-dithiobispyridine with hemoglobin

In principle, it should be possible to employ Equation 15 to determine the parameters of Scheme II for the

reaction of a charged sulphydryl reagent, such as DTNB, with hemoglobin. However, as pointed out above, this aim was defeated by the unidentifiability of the kinetic model So it was not possible to determine the ionization constant of the β^{93} sulphydryl group, one of the parameters of Scheme II. To get around this difficulty an uncharged sulphydryl reagent, 2,2'-dithiobispyridine (2-DTP) was selected. On account of its uncharged nature in the experimental pH range of 5.6 to 9.0, 2-DTP, unlike DTNB would not be subject to the electrostatic effects of ionizable groups on the protein during its interaction with the hemoglobin sulphydryl group. Its rate of reaction will depend solely on the state of ionization of the sulphydryl group.

The $k_{(app)}$ versus pH profiles for the reaction of 2-DTP with derivatives of various hemoglobin species are shown in Figures 12-20. As can be seen from these figures, $k_{(app)}$ increases monotonously with pH. This is in sharp contrast with the profiles obtained for the DTNB reaction (Figures 4-11). The 2-DTP profiles are also much simpler, and each resembles a simple titration curve. This result indicates that the underlying mechanism is also much simpler.

The immediate conclusion that can be arrived at by comparing the profiles, for the DTNB reaction (Figures 4-11) with those for the 2-DTP reaction (Figures 12-20) is that the electrostatic effects of the protein ionizable groups present in the DTNB reaction are absent or negligible in the 2-DTP reaction.

Although the 2-DTP profiles (Figures 12-20) resemble simple titration curves for the sulphydryl group of hemoglobin, the picture is a little bit more complicated than this. An examination of the X-ray structure of hemoglobin in the region of the β^{93} sulphydryl group (38,41,87) shows that this sulphydryl group can take up two alternative conformations: an external and an internal conformation. Both conformations are in dynamic equilibrium (40). In the external conformation, the sulphydryl is exposed to water, a medium with a dielectric constant of 80; in the internal conformation the sulphydryl is in a hydrophobic pocket in the protein. A value of 4 has been suggested for the dielectric constant inside a protein, (72,73). It is therefore to be expected that the pK for the ionization of the sulphydryl will differ in the two conformations.

If the sulphydryl pK differs in the two conformations

the apparent second order rate constant for the reaction with 2-DTP is given by

$$k_{(app)} = k_{ext}.F \cdot \frac{K_{ext}}{K_{ext}+[H^+]} + k_{int}(1-F)\frac{K_{int}}{K_{int}+[H^+]} \quad \ldots (16)$$

The derivation of equation 16 is shown in Appendix VI page 220. In Equation 16, k_{ext} is the plateau value at high pH of the apparent second-order rate constant for reaction in the external conformation; k_{int} is the corresponding value for the internal conformation; f is the fractional population of the external conformation; K_{ext} is the ionization constant of the sulphydryl group in its external conformation; and K_{int} is the corresponding value for the internal conformation. The fractional terms in Equation 16 take account of the fact that only the thiolate anion is the reactive species. Equation 16 is valid only if the transition between the sulphydryl conformations is fast compared to the reaction with 2-DTP. This condition is fulfilled because the rate of the sulphydryl conformational transition 5×10^4 at 11^oC (40) is several orders of magnitude faster than the rate of the 2-DTP reaction.

If the β^{93} sulphydryl group exists in only one conformation, external or internal, the $k_{(app)}$ versus pH profile should resemble a simple titration curve for the

ionization of the sulphydryl group. In this case Equation 16 assumes the simpler forms

$$k_{(app)} = k_{ext} \frac{K_{ext}}{K_{ext} + [H^+]} \quad \ldots (17a)$$

or

$$k_{(app)} = k_{int} \frac{K_{int}}{K_{int} + [H^+]} \quad \ldots (17b)$$

All the $k_{(app)}$ versus pH profiles for the 2-DTP reaction (Figures 12-20) were analyzed with Equations 16 and 17 using a parameter optimization program. In fitting data with Equation 16, k_{ext} F and $k_{int}(1-F)$ were each treated as a single parameter. The validity of either Equation 16 or 17 for a given hemoglobin derivative was judged by comparing the relative goodness-of-fit of each equation to the experimental data. Examples of such comparisions are given in Figures 15 and 16 for aquomethemoglobins A and S, respectively. The full lines in these figures are the fits with Equation 16 while the broken lines are the fits with Equation 17. It is clear that Equation 16 gives the better fit to the data. Similar results were obtained for the aquomethemoglobin derivatives

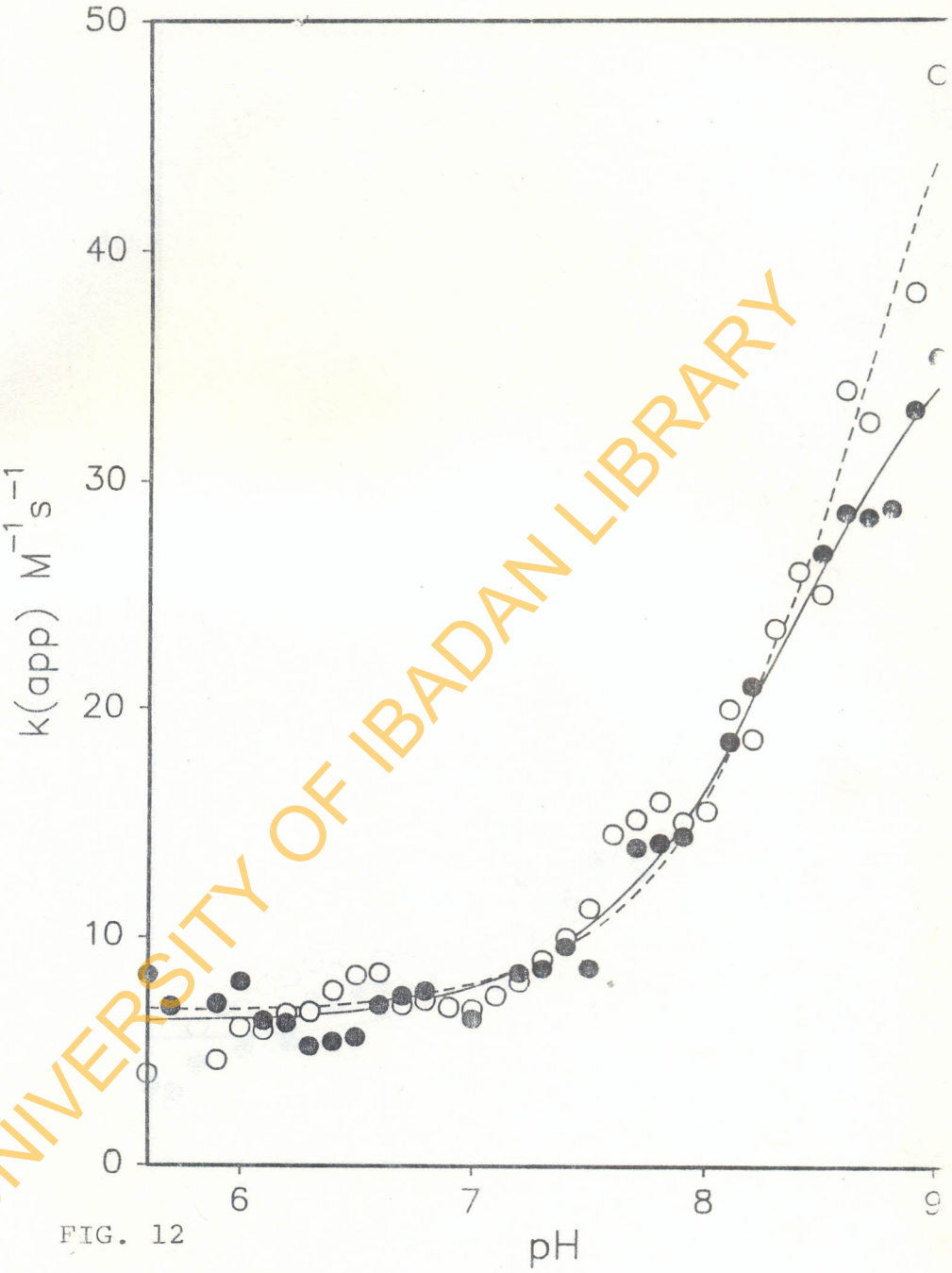


FIG. 12

Dependence of the apparent second-order rate constant, k_{app}, on pH for the 2,2'-dithiobispyridine reaction with β^{93} sulphydryl group of oxyhemoglobins at 20°C I = 0.05. Human A (filled circles with full line) see Table 29 and Human S (open circles with broken lines) see Table 33 in Appendix III. Standard error $\leq M^{-1}s^{-1}$ fit with Equation 17b.

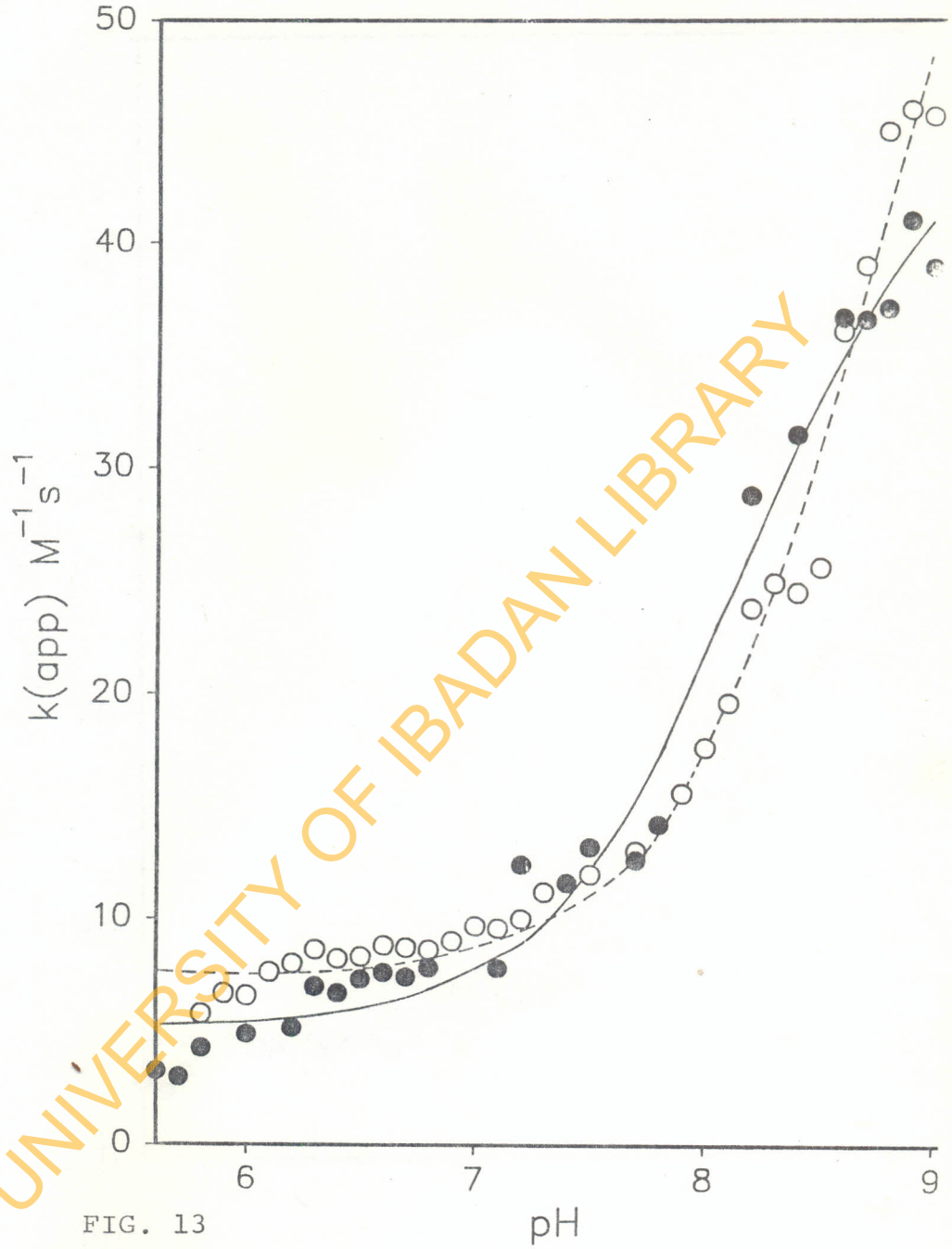


FIG. 13

Dependence of the apparent second-order rate constant, k_{app} on pH for the 2,2'dithiobispyridine reaction with β^{93} sulphydryl group of carbonmonoxyhemoglobins at 20°C I = 0.05. Human A (filled circles with full lines) see Table 30 and Human S (open circles with broken lines) see Table 34 in Appendix III. Standard error $\leq 1\ M^{-1}s^{-1}$ fit with equation 17b.

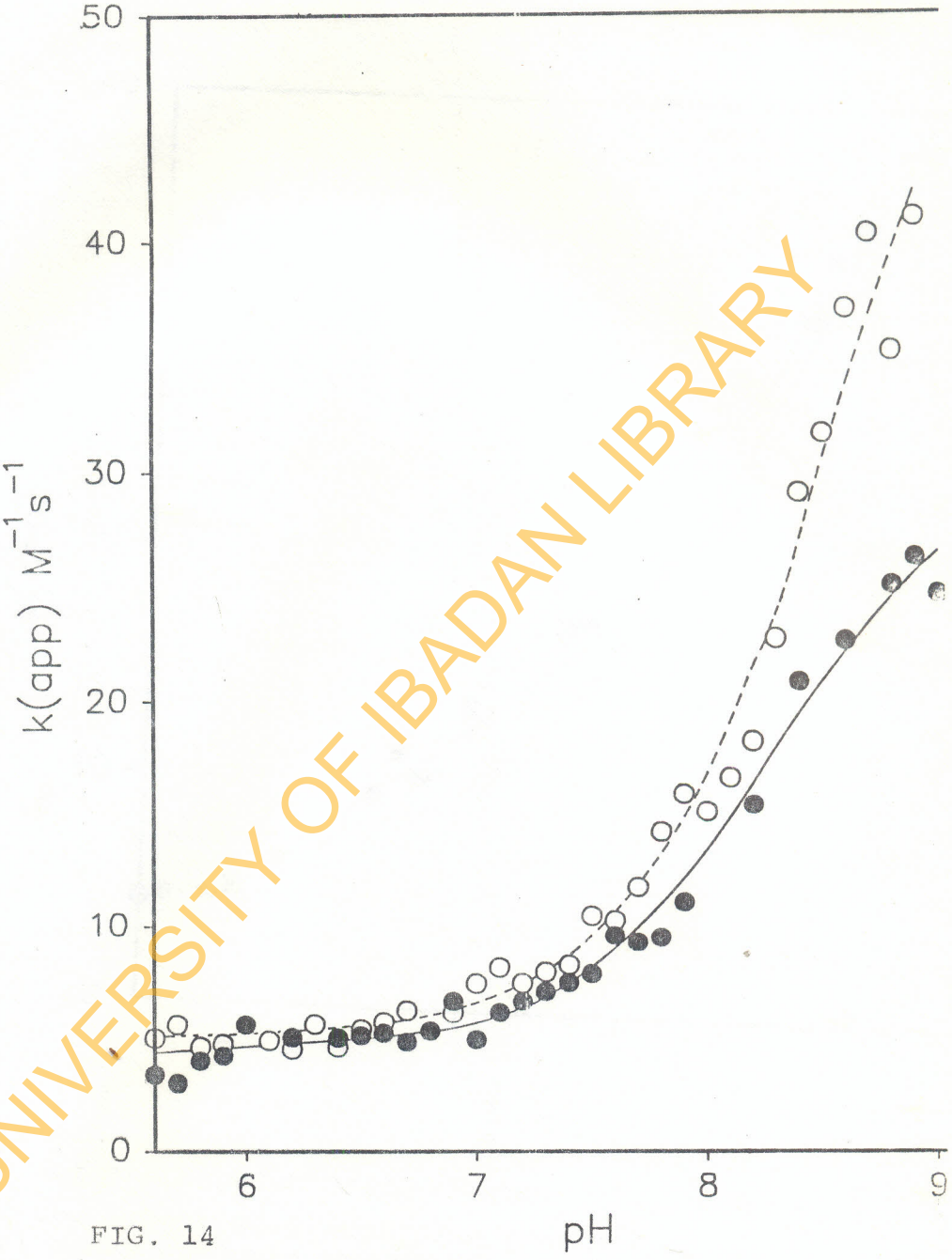


FIG. 14

Dependence of the apparent second-order rate constant, k_{app}, on pH for the 2,2'-dithiobispyridine reaction with β^{93} sulphydryl group of azidomethemoglobins at 20°C I = 0.05. Human A (filled circles with full lines) see Table 31 and Human S (open circles with broken lines) see Table 35 in Appendix III. Standard error ≤ 1 $M^{-1}s^{-1}$. Fit with equation 17b.

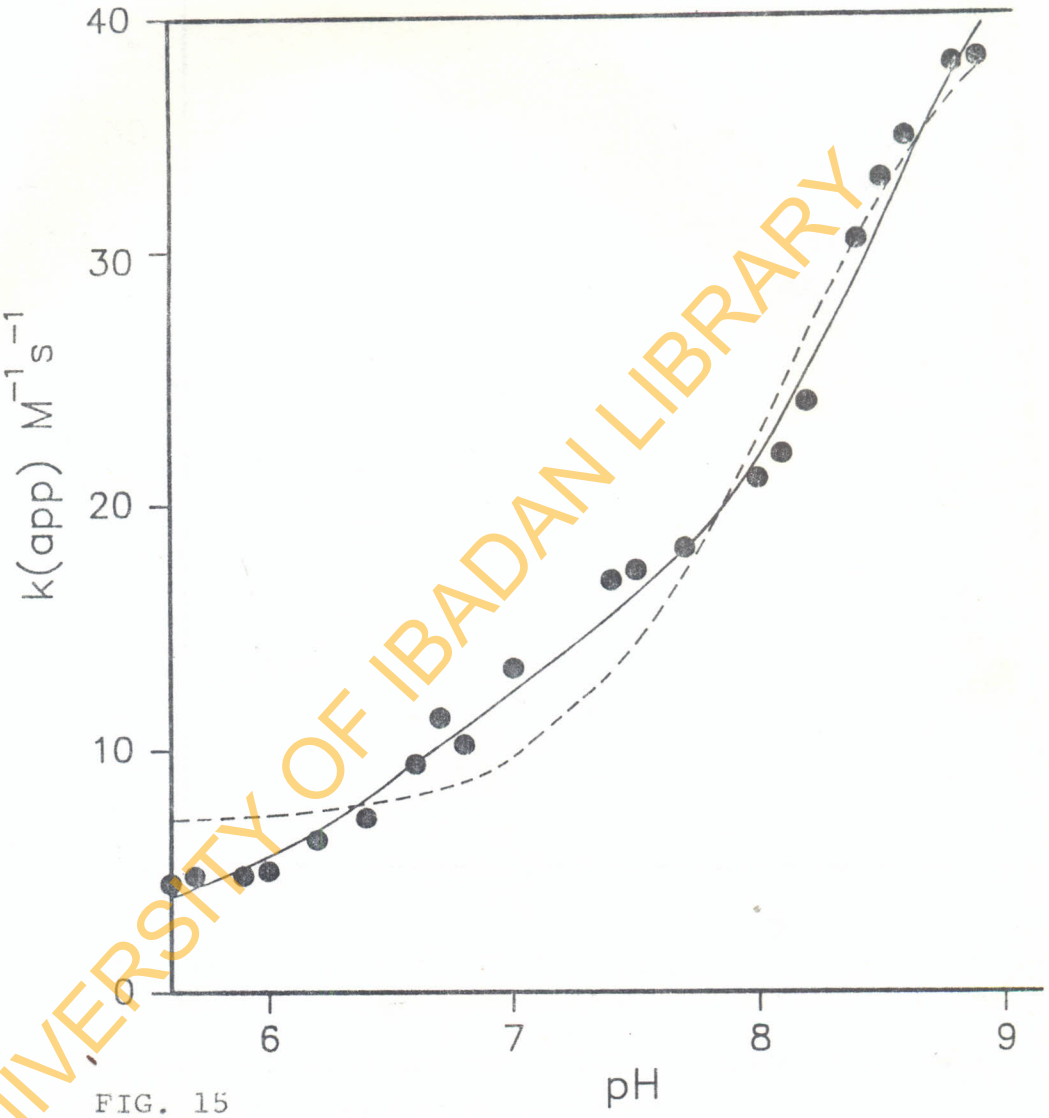


FIG. 15

Dependence of the apparent second-order rate constant, k_{app}, on pH for the 2,2-dithiobispyridine reaction with β^{93} sulphydryl group of human aquomethemoglobin A at 20°C I = 0.05. Broken lines fitted with equation 17b while full line fitted with equation 16.

Standard error $\leq 1\ M^{-1}s^{-1}$. Table 32 in Appendix III.

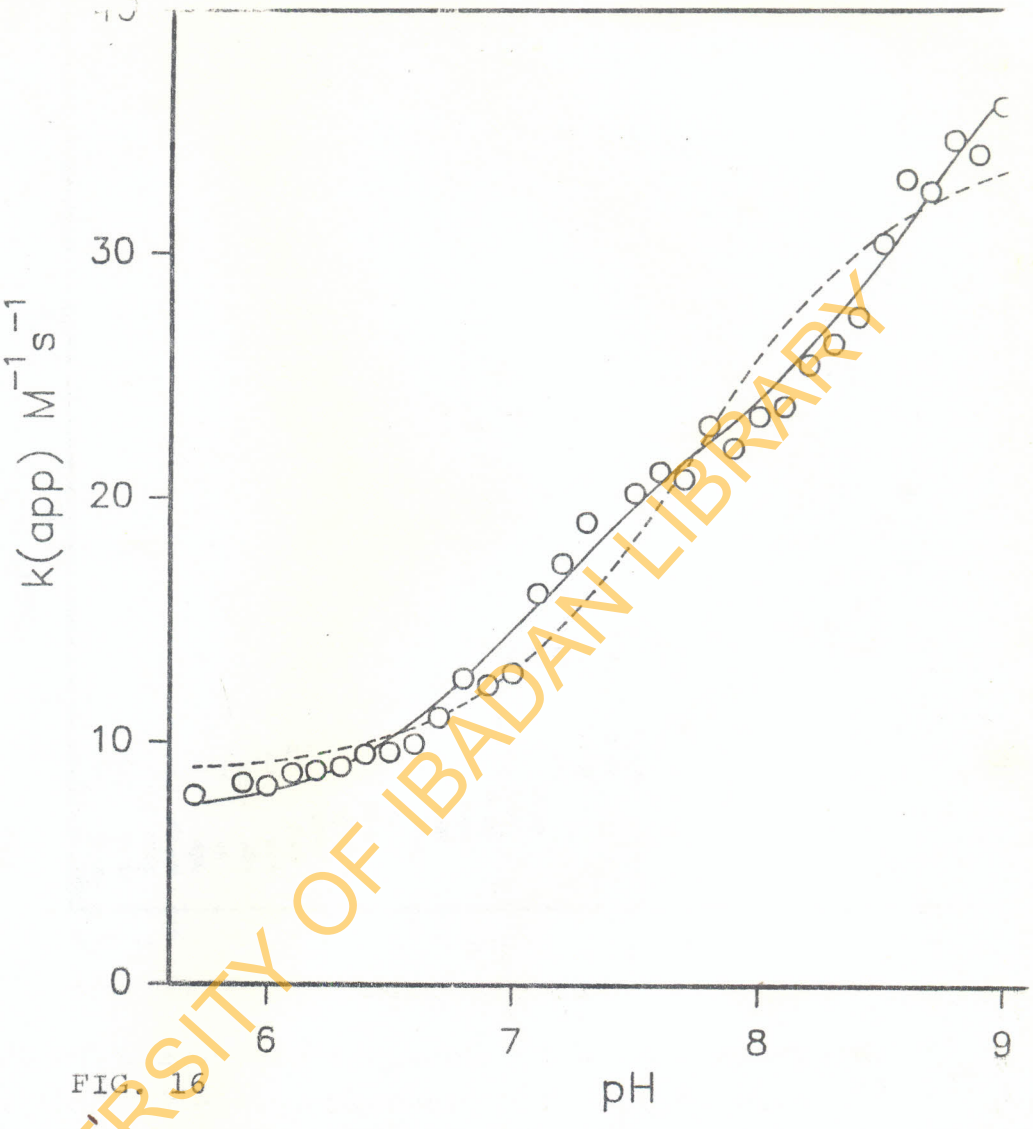


FIG. 16

Dependence of the apparent second-order rate constant, k_{app}, on pH for the 2,2'-dithiobispyridine reaction with β^{93} sulphydryl group of human aquomethemoglobin S at 20°C I = 0.05. Broken lines fitted with equation 17b while full lines fitted with equation 16.

Standard error $\leq 1\ M^{-1}s^{-1}$. Table 36 in Appendix III.

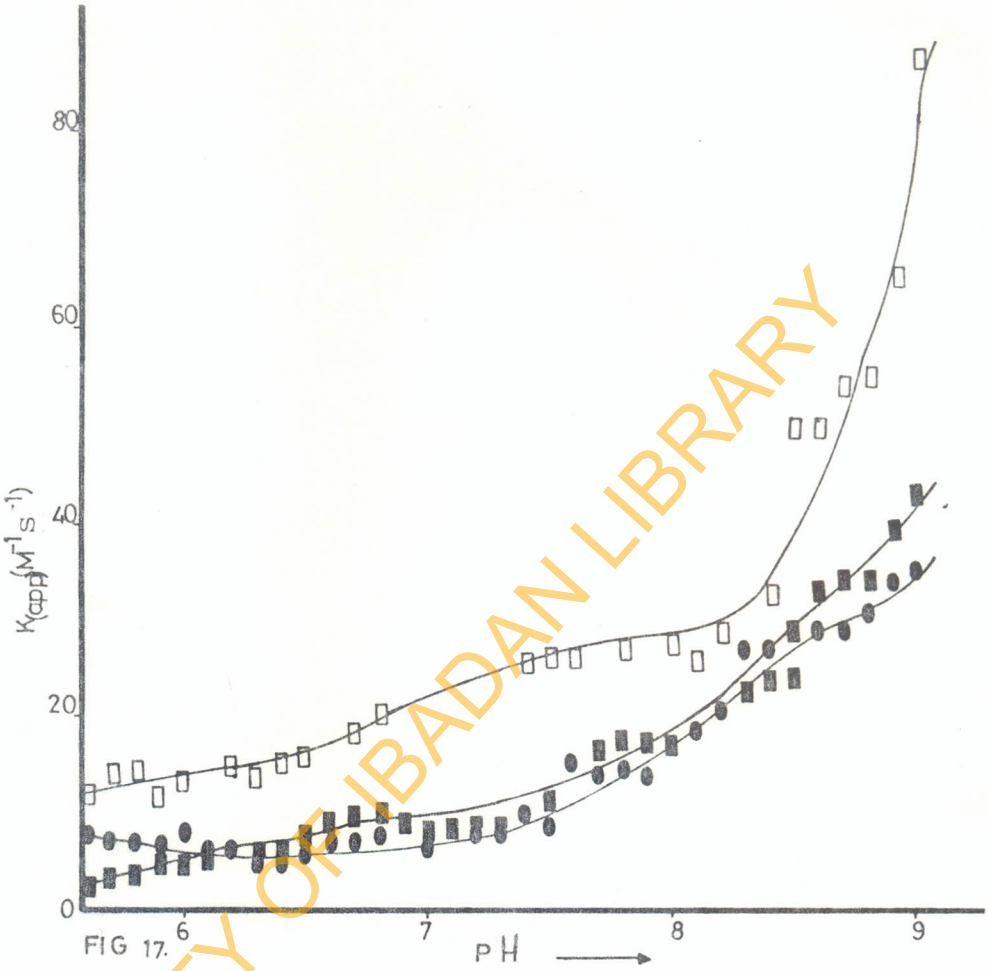


FIG 17.

Dependence of the apparent second-order rate constant, k_{app} on pH for the 2,2'-dithiobispyridine reaction with β^{93} sulphydryl group of oxyhemoglobins at 20°C I = 0.05. Human A (filled circles) see Table 29, Dog (filled squares) see Table 37, and Rabbit (open squares) see Table 41. Standard error $\leq 1.0\ M^{-1}s^{-1}$, fitted with equations 16 and 17b.

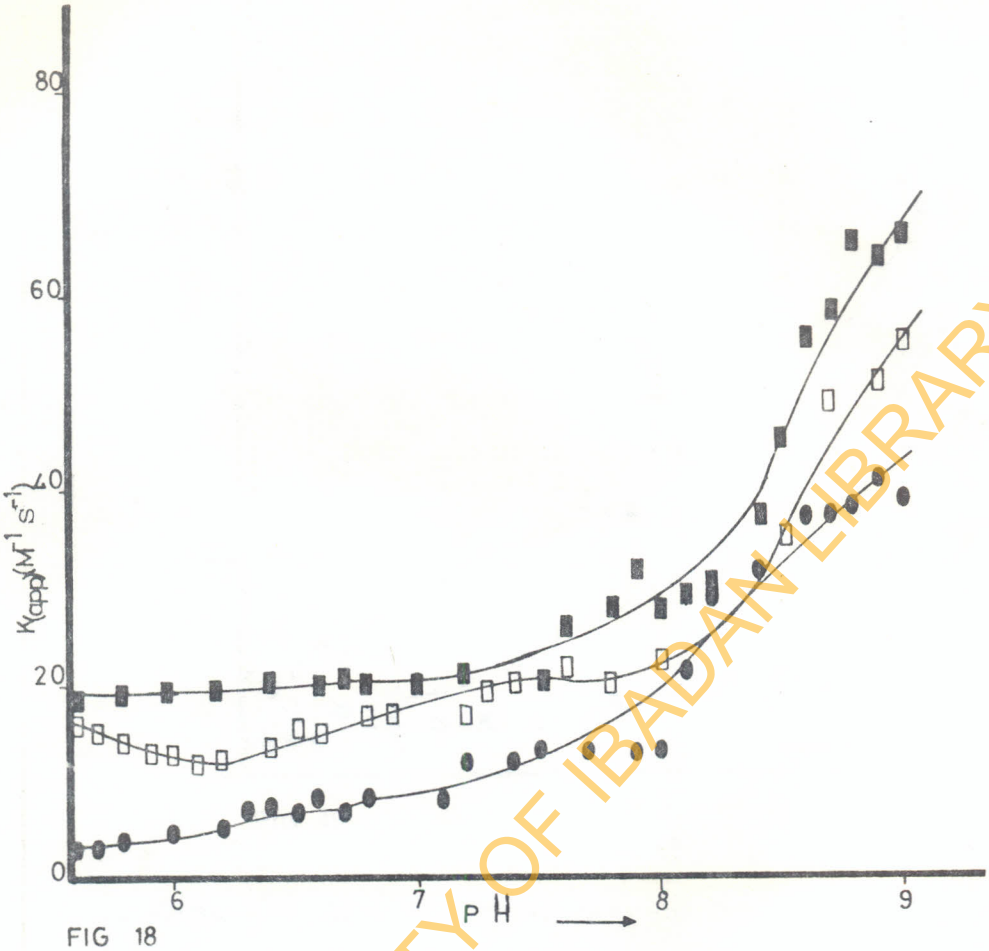


FIG 18

Dependence of the apparent second-order rate constant, k_{app} on pH for the 2,2'-dithiobispyridine reaction with β^{93} sulphydryl group of carbonmonoxyhemoglobins at 20°C. I = 0.05 Human A (filled circles) see Table 30, Dog (filled squares) see Table 38, and Rabbit (open squares) see Table 42 in Appendix II.

Standard error $\leq 1.0\ M^{-1}s^{-1}$. Best fit with equation 17b.

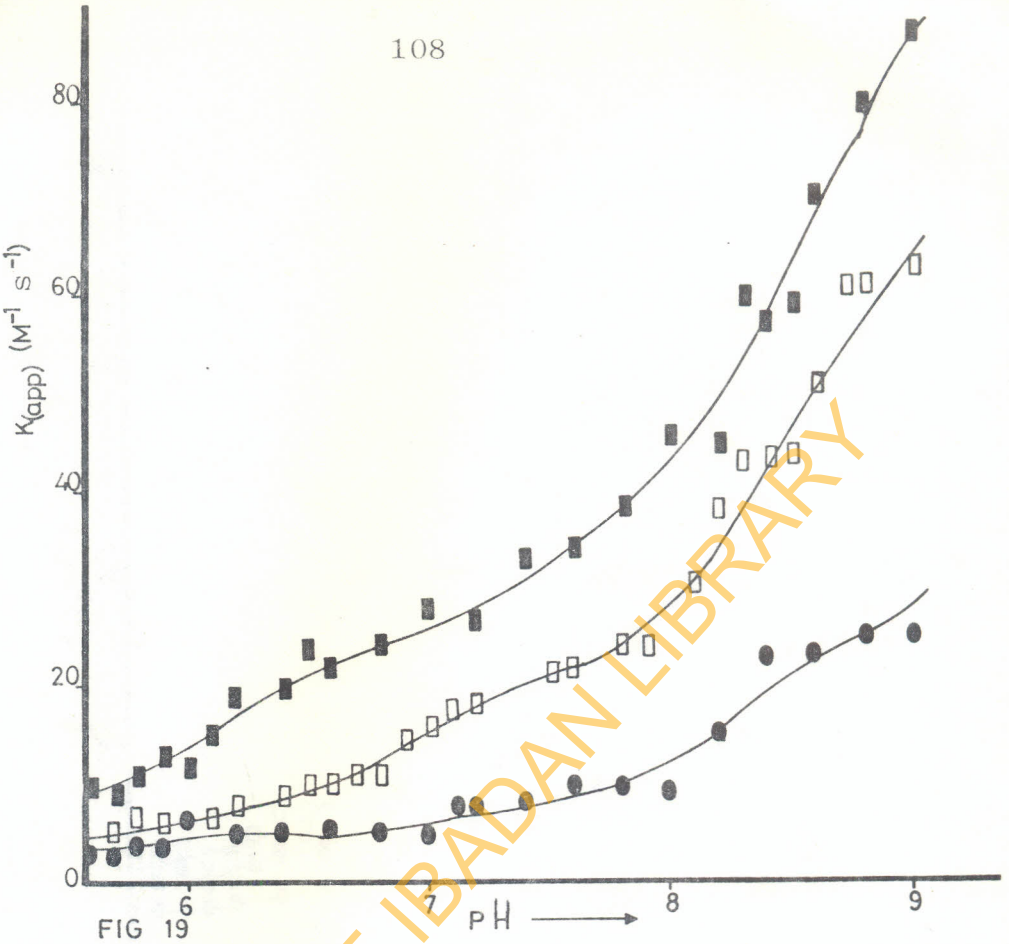


FIG 19

Dependence of the apparent second-order rate constant, k_{app}, on pH for the 2,2'-dithiobispyridine reaction with β^{93} sulphydryl droup of azidomethemoglobins at 20°C. I = 0.05. Human A (filled circles) see Table 31, Dog (filled squares) see Table 39 and Rabbit (open squares) see Table 43 in Appendix III.

Standard error $\leq$ 1.0 $M^{-1}s^{-1}$. Best fit with equations 16 and 17b.

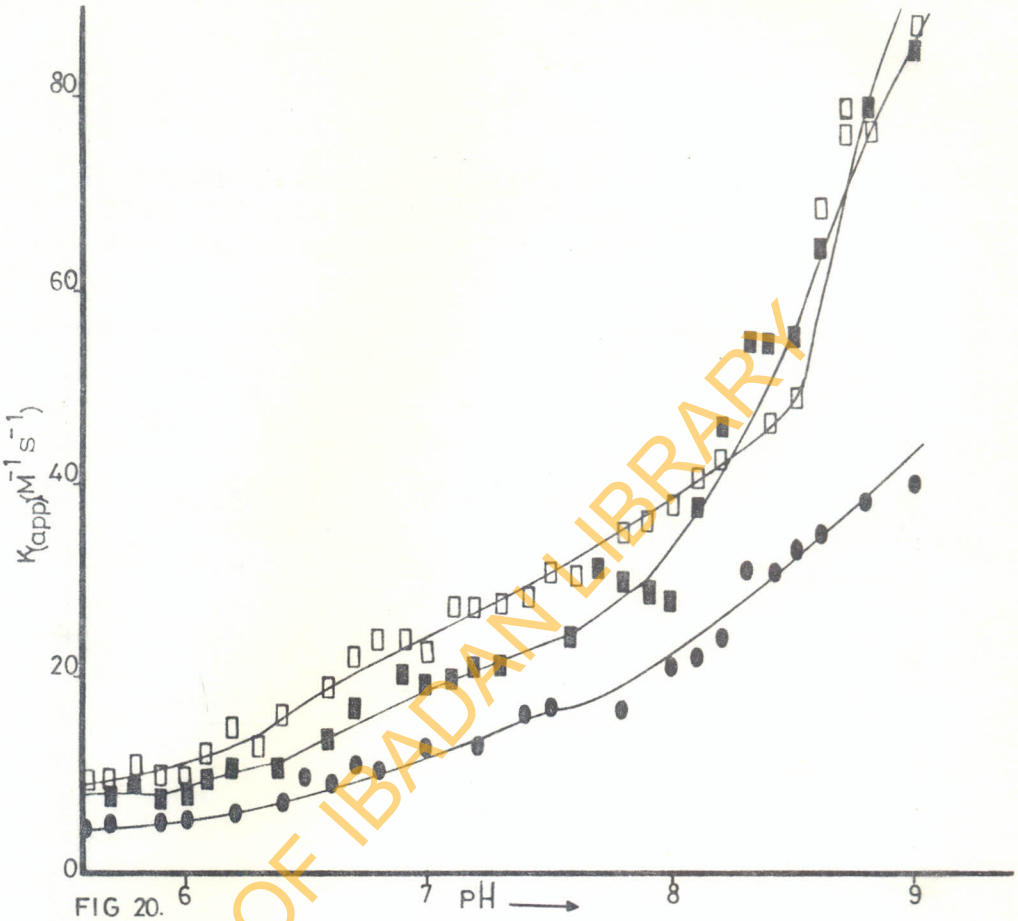


FIG 20.

Dependence of the apparent second-order rate constant, k_{app} on pH for the 2,2'-dithiobispyridine reaction with β^{93} sulphydrul group of aquomethemoglobins at 20°C I = 0.05. Human A (filled circles) see Table 32, Dog (filled squares) see Table 40 and Rabbit (open squares) see Table 44 in Appendix III.

Standard error $\leq$ 1.0 $M^{-1}s^{-1}$. Best fit with equation 16.

TABLE 4

REACTION OF AQUOMETHEMOGLOBINS WITH 2,2'-DITHIOBIS-PYRIDINE. BEST-FIT PARAMETERS EMPLOYED FOR FITTING IN FIGURES WITH EQUATION 16 OF THE TEXT

Aquomethemoglobin	k_{ext} F $[M^{-1}s^{-1}]$	$k_{int}(1-F)$	pK_{ext}	pK_{int}
A	11.8	36.3	6.51	8.54
S	15.9	22.8	7.05	8.80
Dog	16.67	98.86	5.80	8.54
Rabbit	26.8	118.2	6.13	8.94

Table 5

Reaction of Hemoglobin derivatives with 2,2'-dithiobis-pyridine best-fit parameters employed for fitting in figures with equations 16 and 17b of the text.

Oxyhemo-globin	k_{ext} F	$k_{int}(1-F)$	pK_{ext}	pK_{int}
A	-	33.9	-	8.35
S	-	53.7	-	8.65
Dog	8.62	54.07	5.82	8.76
Rabbit	20.31	114.3	5.64	9.10
Carbonmonoxy-hemoglobin				
A	-	41.1	-	8.18
S	-	62.3	-	8.72
Dog	-	94.21	-	8.86
Rabbit	-	71.48	-	8.86
Azidomethe-moglobin				
A	-	26.4	-	8.29
S	-	53.4	-	8.54
Dog	27.29	85.46	5.91	8.62
Rabbit	14.39	67.75	6.11	8.53

of the other hemoglobin studied in this work (Figure 20). The best-fit parameters are reported in Table 4. The validity of Equation 16 for the aquomethemoglobin data indicates that the β^{93} sulphydryl group is in a state of dynamic equilibrium between two different conformations. This is in line with the X-ray data (38, 41, 87).

The $k_{(app)}$ versus pH profiles of the carbonmonoxy derivatives of all species (Figure 18) were best fitted with Equation 17. The fitting parameters are reported in Table 5. It should be noted that the pK_s of the sulphydryl group calculated from these fits are similar to pK_{int} values reported for the aquomethemoglobin derivative in Table 4 and the widely different from the pK_{ext} values in Table 4.

Thus Equation 17b, rather than Equation 17a, is valid for the carbonmonoxy derivatives. Although Equation 17a and 17b are formally equivalent, the results for carbonmonoxyhemoglobin show that it is important to distinguish between them because the approximate pK_{int} value is known.

In contrast to the straight forward results obtained with the aquomet and carbonmonoxy derivatives, the picture is more complicated for the oxy and azidomet derivatives.

From Table 5 it is seen that for rabbit and dog hemoglobins the best fits were provided by Equation 16. By contrast Equation 17b gave the best fits for human hemoglobins. One is led to conclude that the oxy and azidomet derivatives of human hemoglobins have their sulphydryl groups in the internal conformation, whereas those of rabbit and dog are in dynamic equilibrium between the internal and external conformations. Thus the oxy and azidomet derivatives of rabbit and dog behave like the aquomet derivatives (compare Tables 4 and 5).

Examination of Tables 4 and 5 allows some conclusions to be drawn about the nature of the β^{93} sulphydryl group. A comparison of pK_{ext} values for all derivatives of the hemoglobins studied in this work shows that they are all similar, ranging between 5.6 and 7.0, with a mean value of 6.12±0.16. Similarly, values for pK_{int} are similar in magnitude, ranging between 8.2 and 9.1, with a mean value of 8.64±0.06. The finding that the pK of the β^{93} sulphydryl group differs by 2.5 pK units in its internal and external conformations has implications for hemoglobin function, at least at first sight. These will be discussed later in the thesis.

C: POTENTIOMETRIC DIFFERENCE TITRATION OF THE β^{93} SULPHYDRYL GROUP OF HEMOGLOBIN WITH METHYL METHANETHIOSULPHONATE.

Methyl methanethiosulphonate (MMTS) was introduced in 1975 for use as a sulphydryl reagent (88,89), and has been employed to characterize the ionization behaviour of the active-site thiol group in papain (21), in bovine serum albumin (15) and in other proteins (90-93). Like 2-DTP MMTS is a neutral reagent carrying no charge. It is therefore also free of the electrostatic effects of nearby ionizable groups when it reacts with a sulphydryl group in a protein. The major advantage of MMTS is that it blocks a thiol sulphur atom with a small neutral non-hydrogen bonding methylthio group and thereby produces a minimal perturbation in the structure of the protein (20). The major disadvantage of this reagent, however, is that, in contrast to DTNB and 2-DTP, no colour change is produced when MMTS interacts with a sulphydryl group.

The reaction of MMTS with a sulphydryl group is via nucleophilic attack by the thiolate anion. This reaction may be depicted as follows:

$$CH_3S(=O)_2SCH_3 + RS^- + H^+ \underset{\text{RSH}}{\rightarrow} RSSCH_3 + CH_3SO_2^- + H^+ \qquad ...(18)$$

The reaction of MMTS with a sulphydryl group therefore gives rise to the release of protons and can therefore be monitored potentiometrically, provided buffer ions and CO_2 are excluded from the reaction medium.

The amount of protons produced in the above reactions, Δh^+, varies with pH for a given protein (15,21). As written formally above, Equation 18 seems to imply that the protons produced come entirely from the ionization of the sulphydryl group. This is however not the case. In its anionic form, RS^-, the sulphydryl group in a protein would be electrostatically linked to charged, ionizable groups in the protein. When the charge on thiol anion is neutralized by reaction with MMTS the electrostatically linked ionizable groups on the protein have their pK_s changed. This gives rise to a release of protons. Therefore determination of the proton release as a function of pH should, in principle, give information on those ionizable groups that are electrostatically linked with the sulphydryl group in a protein. The initial attempt to obtain information on the nature and number of these groups by reaction with DTNB had been unsuccessful because of a scheme (Scheme II) and an equation (Equation 15) that contained too many unidentifiable parameters.

For a protein ionizable group i with ionization constant K_i which, for simplicity, is assumed to be independent of other ionizable groups in the protein, the fractional population of the ionized state is

$$\frac{K_i}{K_i+[H^+]} = \frac{10^{-pK_i}}{10^{-pK_i}+10^{-pH}} ;$$

and the fractional population of the unionized state is

$$\frac{[H^+]}{[H^+]+K_i} = \frac{10^{-pH}}{10^{-pH}+10^{-pK_i}} .$$

So, for this ionizable group i the fractional change in its proton content upon ionization at a given pH is

$$\frac{10^{-pH}}{10^{-pH}+10^{-pK_i}} - \frac{10^{-pK_i}}{10^{-pK_i}+10^{-pH}}$$

When the sulphydryl group in a protein is methylthiolated (Equation 18), the negative charge on the thiolate anion is lost. Consequently, ionizable groups that are electrostatically linked to the thiolate anion lose protons to the reaction medium. For n such ionizable groups the proton release Δh^+ is given by

$$\Delta h^{+} = \sum_{i=1}^{n} \left(\frac{10^{-pH}}{10^{-pH} + 10^{-pK_i}} - \frac{10^{-pK_i}}{10^{-pK_i} + 10^{-pH}} \right) \quad \ldots(19)$$

for each sulphydryl group methylthiolated. The above equation has not taken the ionization of the sulphydryl group itself into account. For each thiol anion that is methylthiolated one neutral thiol group ionizes. This gives rise to one more fractional term

$$\frac{10^{-pH}}{10^{-pH} + 10^{-pK_i}}$$

in the above equation, without the corresponding term

$$\frac{10^{-pK_i}}{(10^{-pK_i} + 10^{-pH}}$$

Since the ionized form of the thiol is converted completely to the methylthiol form. Taking the sulphydryl group ionization into consideration, the above equation (Equation 19 takes the form

$$\Delta h^{+} = \sum_{i=1}^{n+1} \frac{10^{-pH}}{10^{-pH} + 10^{-pK_i}} - \sum_{i=1}^{n} \frac{10^{-pK_i}}{10^{-pH} + 10^{-pK_i}} \quad \ldots(20)$$

The above equation has been derived on the assumption that ionizable groups in a protein are independent of each

other. In reality this is not the case.

In order to use Equation 20 for data analysis, however, the simplified method of Simms (81) is used. The pH dependence of the proton content of a polyprotic substance containing n ionizable groups is analyzed as if it were a mixture of n hypothetical groups which independently ionize with dissociation constants $G_i (i = 1,2,3,\ldots,n)$. The constants G_i are of course different from K_i and are merely constants obtained from analyses of the data, not real ionization constants. Appropriate relationships can be derived to relate G_i and K_i (80). We therefore write

$$\Delta h^+ = \sum_{i=1}^{n+1} \frac{10^{-pH}}{10^{-pH} + 10^{-pG_i}} - \sum_{i=1}^{n} \frac{10^{-pG_i}}{10^{-pH} + 10^{-pG_i}} \quad \ldots (21)$$

Figures 21-26 show plots of Δh^+ versus pH for various hemoglobin derivatives. The general shape of the curves is the same for each hemoglobin derivative, irrespective of the hemoglobin species, and resembles the kinetic pH profile for DTNB binding (Figures 4-11). Computational difficulties have so far made it impossible to analyze these data quantitatively. Consequently the

following discussion of the data will be merely qualitative.

The data for the deoxy, carbonmonoxy and oxy derivative of hemoglobin A are compared in Figure 21. Similar comparisons are made for horse and rabbit in Figures 23 and 25, respectively. In Figure 21 values of Δh^+ are highest for deoxyhemoglobin and lowest for oxyhemoglobin. In fact the values for deoxyhemoglobin and carbonmonoxyhemoglobin are closer to each other through most of the pH range than those of oxyhemoglobin and carbonmonoxyhemoglobin

Both oxy- and carbonmonoxyhemoglobin have the R quaternary structure, whereas deoxyhemoglobin has the T quaternary structure (78). The result in Figure 21 thus indicates that Δh^+ does not arise from a change in quaternary structure of the protein upon methylthiolation. The quaternary structure change of hemoglobin is related to the Bohr effect. Below pH 6 the binding of ligand to deoxyhemoglobin gives rise to an uptake of protons from solution (the acid Bohr effect); above pH 6 ligand binding causes protons to be ejected into solution (the alkaline Bohr effect) (40). In Figures 21 and 23 the difference in Δh^+ between deoxyhemoglobin and oxyhemoglobin has the correct sign for the acid Bohr effect but the wrong sign

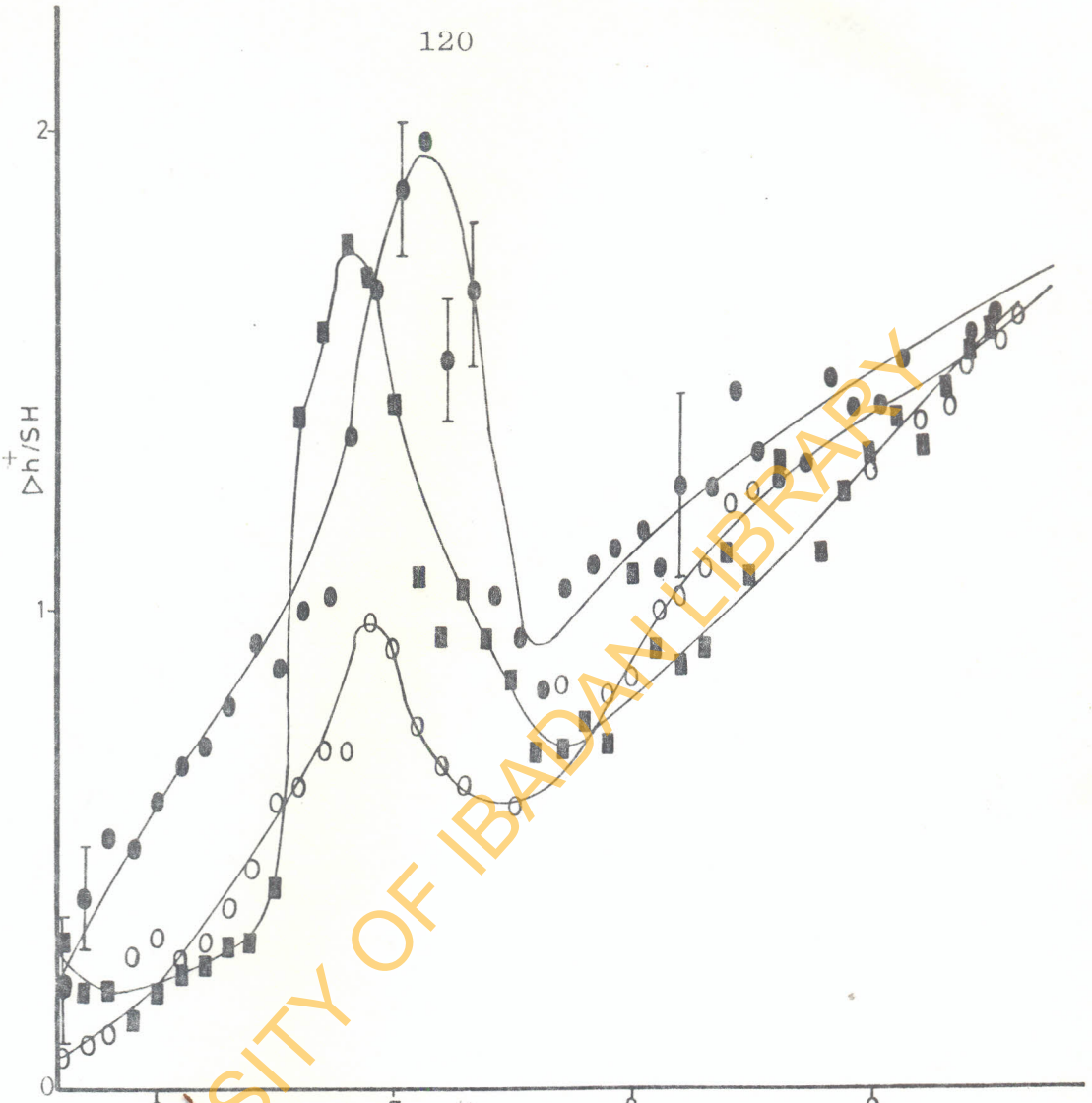


FIG 21.

Proton release, Δh^+, per β^{93} sulphydryl group as a function of pH for the methyl methanethiosulphonate reaction with human hemoglobin A derivatives at 20°C I = 0.05. Oxyhemoglobin (open circles) see Table 46, Carbonmonoxy-hemoglobin (filled squares) see Table 47, and deoxyhemoglobin (filled circles) see Table 48 in Appendix IV. Standard error lies between 0.01 and 0.05.

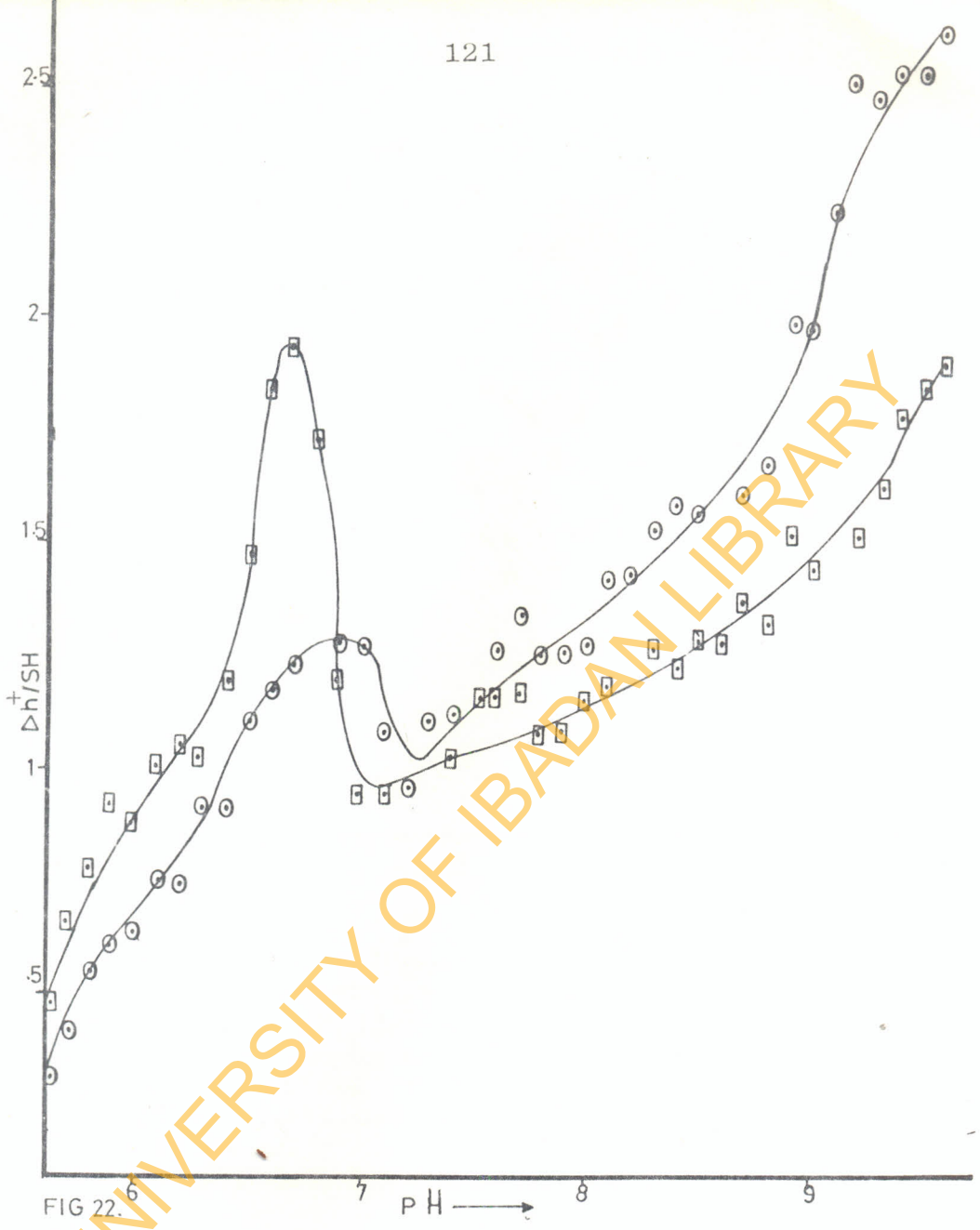


FIG 22.

Proton release, Δh^+, per β^{93} sulphydryl group as a function of pH for the methyl methanethiosulphonate reaction with human hemoglobin A derivatives at 20°C I = 0.05. Azidomethemoglobin (squares with dots) see Table 49 and aquomethemoglobin (open circles with dots) see Table 50 in Appendix IV.

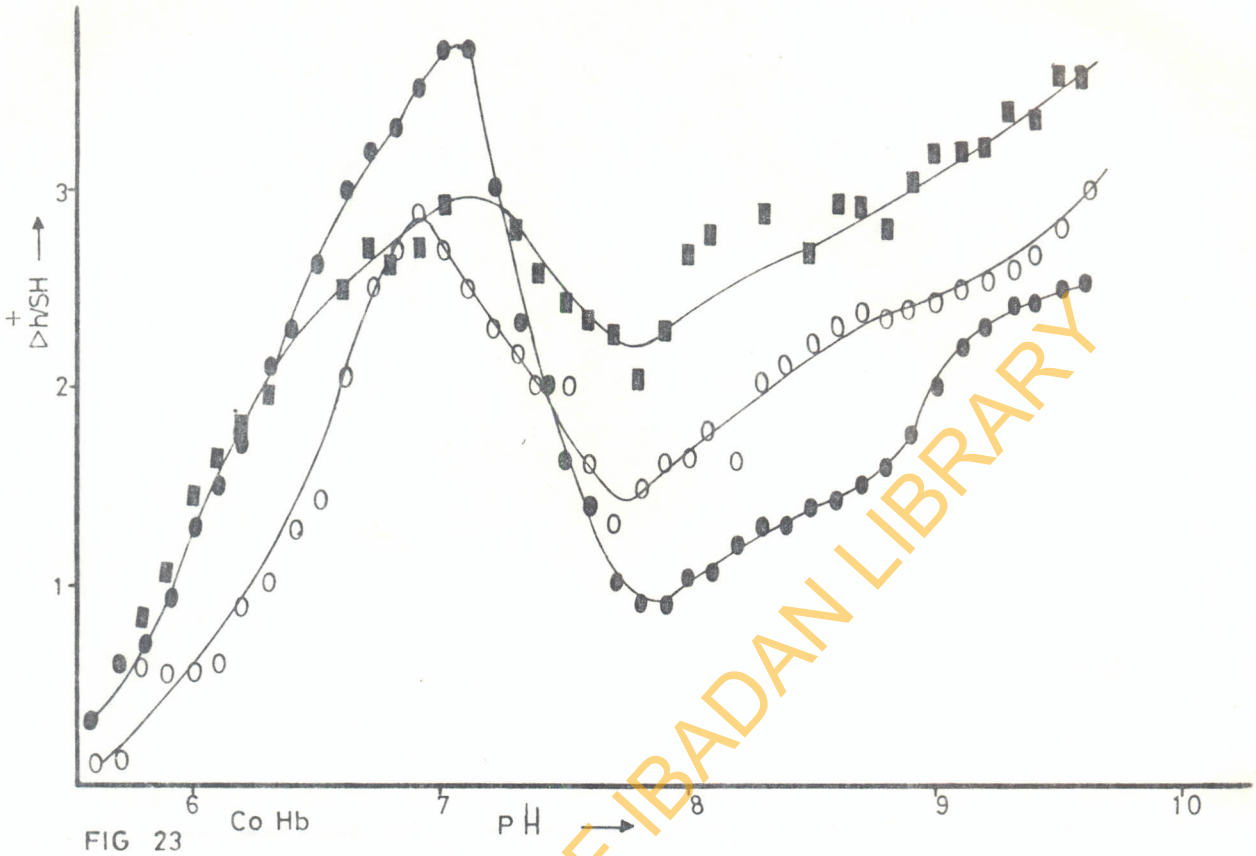


FIG 23

Proton release, Δh^+, per β^{93} sulphydryl group as a function of pH for the methyl methanethiosulphonate reaction with horse hemoglobin derivatives at 20°C I = 0.05. Oxyhemoglobin (open circles) see Table 51, carbonmonoxy-hemoglobin (filled squares) see Table 52, and deoxyhemoglobin (filled circles) see Table 53 in Appendix IV. Standard error lies between 0.01 and 0.07.

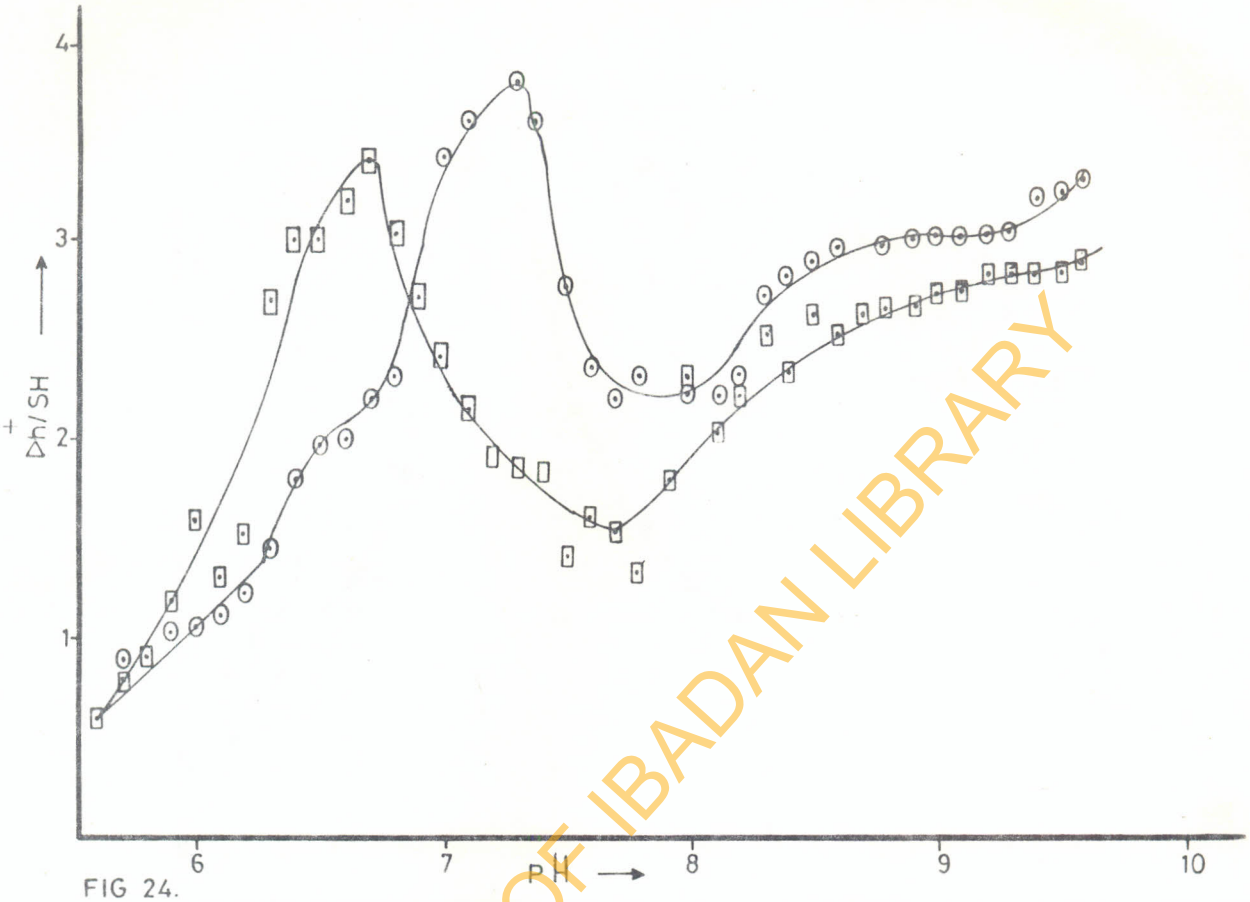


FIG 24.

Proton release, Δh^+, per β^{93} sulphydryl group as a function of pH for the methyl methanethiosulphonate reaction with horse hemoglobin derivatives at 20°C I = 0.05. azidomethemoglobin (squares with dots) see Table 54 and aquomethemoglobin (open circles with dots) see Table 55 in Appendix IV. Standard error lines between 0.01 and 0.07.

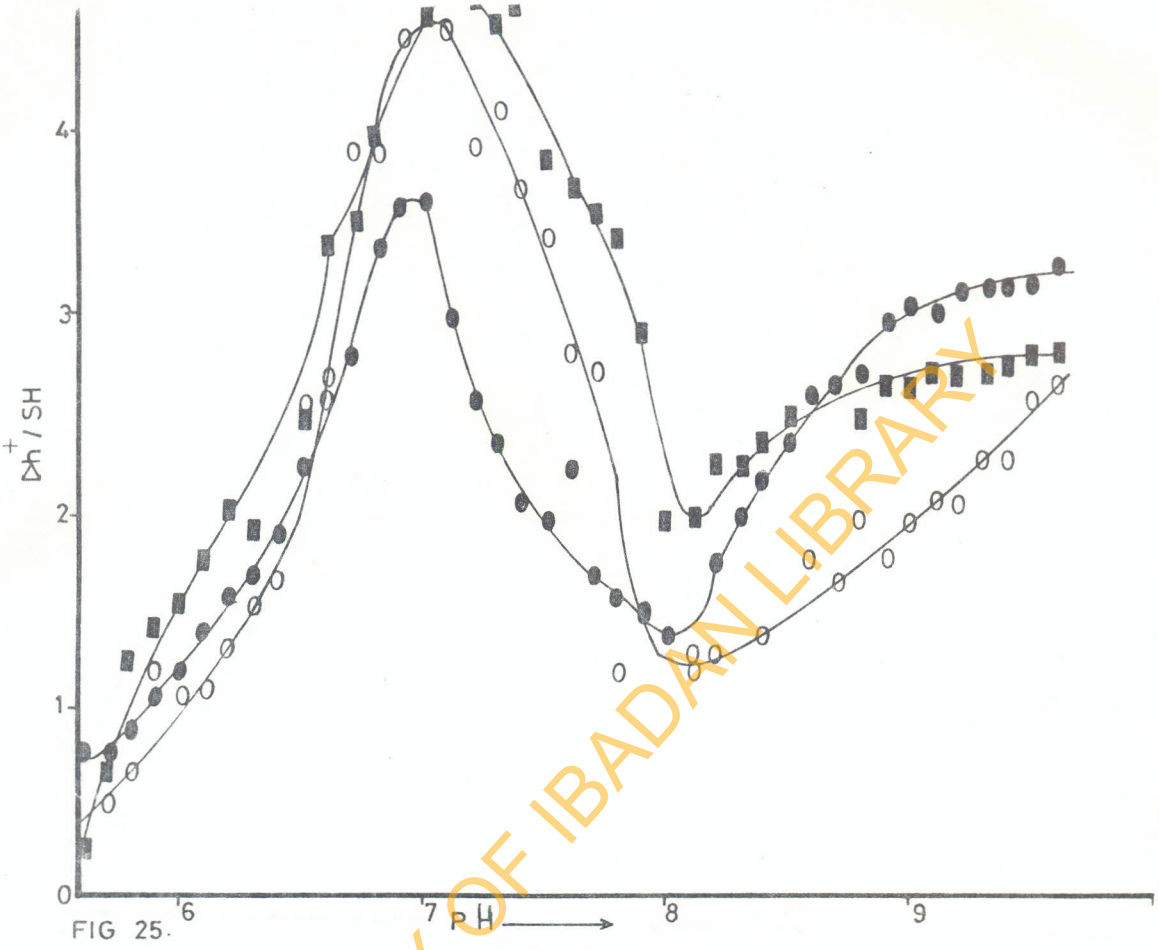


FIG 25.

Proton release, Δh^+ per β^{93} sulphydryl group as a function of pH for the methyl methanethiosulphonate reaction with rabbit hemoglobin derivatives at 20°C I = 0.05. oxyhemoglobin (open circles) see Table 56, carbonmonoxyhemoglobin (filled squares) see Table 57, and deoxyhemoglobin (filled circles) see Table 58 in appendix IV. Standard error lies between 0.01 and 0.07.

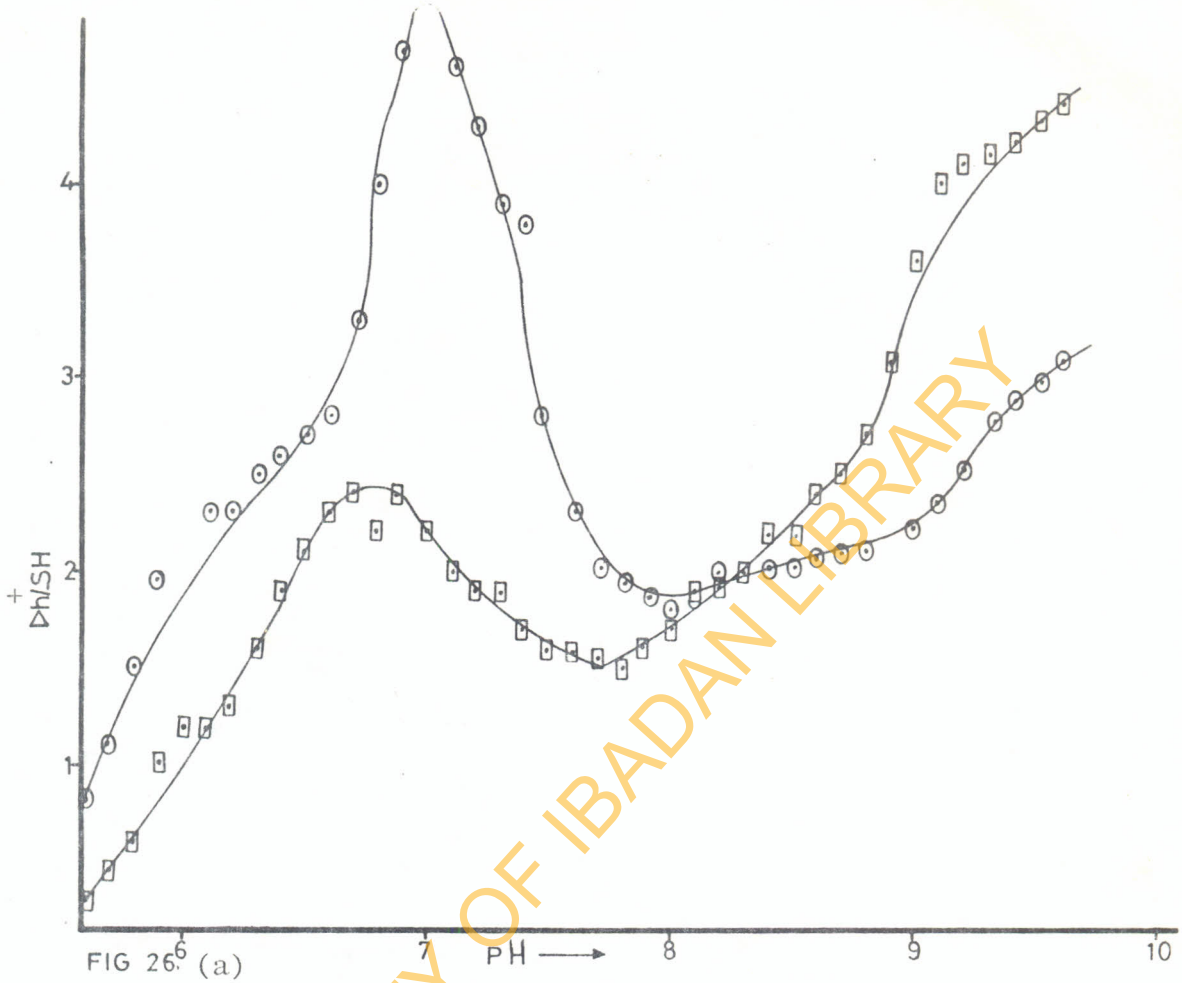


FIG 26 (a)

Proton release, Δh^+, per β^{93} sulphydryl group as a function of pH for the methyl methanethiosulphonate reaction with rabbit hemoglobin derivatives at 20°C I = 0.05. azidomethemoglobin (squares with dots) see Table 59 and aquomethemoglobin (open circles with dots) see Table 60 in Appendix IV. Standard error lies between 0.01 and 0.07

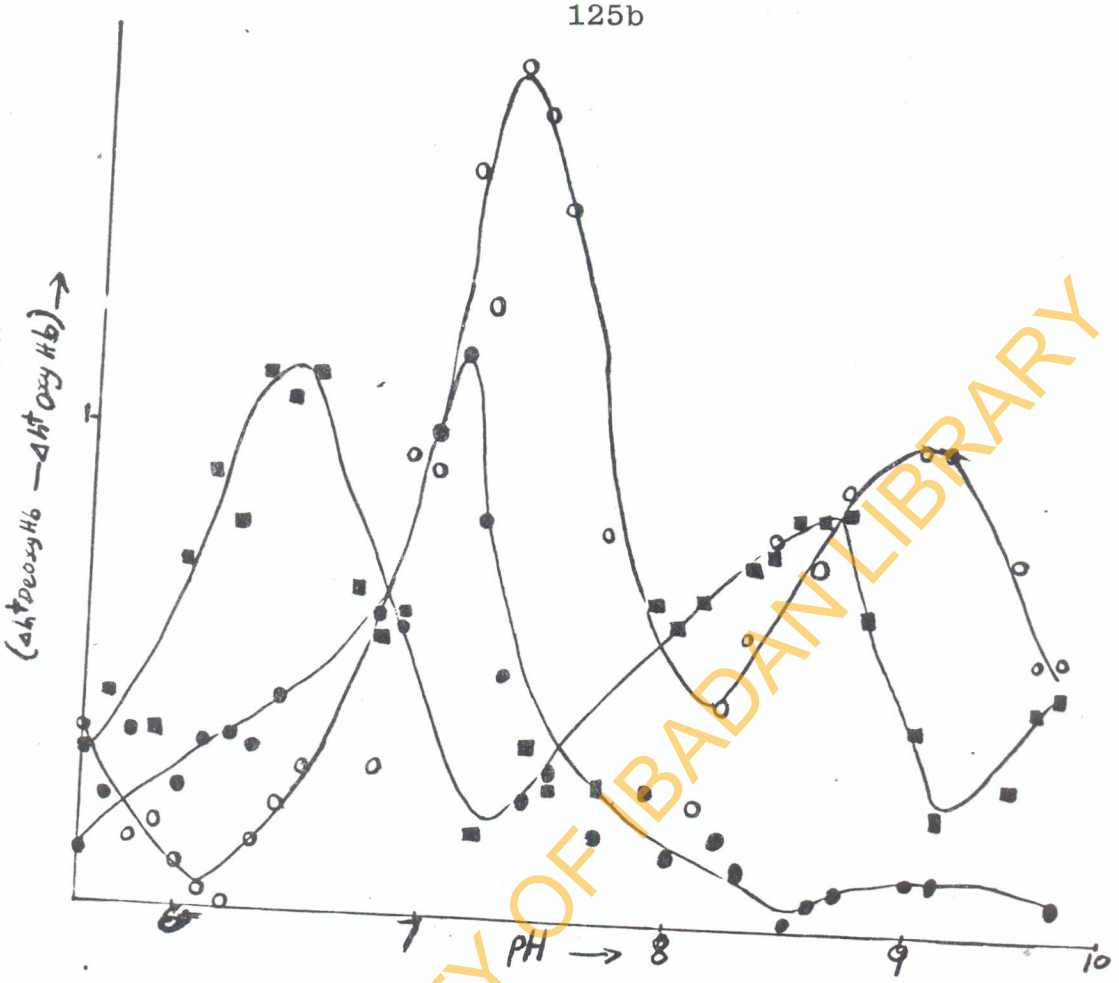


Fig. 26b: (Δh^+ DeoxyHb - Δh^+ OxyHb) per sulphydryl group as a function of pH for the methyl methanethiosulphonate reaction with hemoglobin species.
Human HbA (filled circles), Horse (filled squares) and Rabbit (open circles). See Table 61 Appendix IV.

for the alkaline Bohr effect. In Figure 25 the sign of the Δh^+ difference is correct for both forms of the Bohr effect for the transition deoxyhemoglobin to oxyhemoglobin, but not for deoxyhemoglobin to carbonmonoxyhemoglobin. Plots of Δh^+ (deoxy)- Δh^+ (oxy) versus pH for the data in Figure 26(b) do not resemble the Bohr effect. Thus one can rule out quaternary structure differences as the basis for differences in Δh^+.

In Figures 22, 24 and 26 the Δh^+ versus pH profiles of aquomethemoglobin and azidomethemoglobin are compared. For each hemoglobin the Δh^+ values at low pH are higher for the aquomet derivative than for the azidomet derivative. This situation is reversed at higher pH, and Δh^+ for the azidomet becomes higher than for the aquomet derivative. These results may be explained as follows. It has been demonstrated that when aquomethemoglobin reacts with azide there is an uptake of protons from solution (63). A quantitative analysis of this phenomenon has shown that the uptake of protons is due to ionizable groups on aquomethemoglobin whose pK_s rise on formation of the azidomet derivative (57). Such increases in pK arise because the potential field sensed by these ionizable groups becomes more negative when the positive charges on the iron atoms

are neutralized (57). Thus the same ionizable groups with low pK_s in aquomethemoglobin have relatively higher pK_s in the azidomet derivative.

Consequently such ionizable groups give up their protons at low pH upon methylthiolation of aquomethemoglobin but do not in azidomethemoglobin. At more alkaline pH these groups give up their protons more readily in azidomethemoglobin, and Δh^+ is relatively higher at alkaline pH compared to that of aquomethemoglobin whose groups have already lost their protons at lower pH values.

CHAPTER 4

DISCUSSION

[A] KINETICS OF CYANIDE ION BINDING

The kinetics of cyanide ion binding to aquomethemoglobin have been studied before as a function of pH (56). Two conclusions were drawn from this study:

(i) that the $k_{(app)}$ pH profiles for different aquomethemoglobins cannot be explained on the basis of net charge;

(ii) that contributions are made to $k_{(app)}$, at any given pH, by various hemoglobin species (see Scheme I) in such a manner that both the rate constant K_i and the relative population of each species must be taken into account, not merely the rate constant only (56).

This second point is important when making comparisons between Figure 3 and Table 3, which reports the parameters employed to fit the theoretical lines in Figure 3.

It is obvious in Figure 3 that $k_{(app)}$ for horse is much higher than for the other hemoglobins. On the surface one would therefore expect k_i values for horse to be higher than for the other two species. Consideration of Equation 13 shows, however, that in calculating the

contribution of each species i in Scheme I to $k_{(app)}$, each k_i is multiplied by a factor reflecting the relative population of species i. These relative populations are highly sensitive to pH (See Figure 5 of Ref. 56) and are therefore important determinants of the value of $k_{(app)}$. So the parameters reported in Table 3 are satisfactory and are in general agreement with those reported for other hemoglobin (56). In any case, the major purpose of undertaking the cyanide binding study was to provide initial estimates for a computer analysis of the DTNB reaction. Unfortunately, this latter analysis proved impossible to carry out because the equation derived for it (Equation 13) contained unidentifiable parameters.

[B] pH DEPENDENCE OF THE KINETICS OF REACTION OF THE β^{93} SULPHYDRYL GROUP OF HEMOGLOBIN.

(i) Kinetics of 5,5'-dithiobis[2-Nitrobenzoic acid] reaction

Despite the setback of not being analyzed quantitatively, the data on the DTNB reaction (Figures 4-11) are interesting in qualitative terms.

In Figures 4-7 human hemoglobins A and S are compared. In every case, except the azidomet derivatives (Figure 6),

hemoglobin A reacts faster than hemoglobin S. This result is rather surprising because it is known that hemoglobin S has a higher net positive charge than hemoglobin A, since Glu A3(6)β in the latter appears as a valine in hemoglobin S (82-84). Consequently, hemoglobin S, with its higher positive charge , would be expected to react faster with a negatively charged molecule like DTNB. That this is not the case indicates that factors more important than net charge determine the reactivities of the β^{93} sulphydryl group.

Considerable attention has been paid to the influence of quaternary structure on the reactivity of the β^{93} sulphydryl group (36, 39, 95, 96), with little consideration being given to effects arising from differences in tertiary structure (47, 80). All the hemoglobin derivatives studied with DTNB (Figures 4-11) have the R quaternary structure. This allows for differences in their reactivities to be attributed to differences in tertiary structure (80), in a straight forward manner. For example, the fact that aquomethemoglobin reacts faster than carbonmonoxyhemoglobin (compare Figures 5 and 7) may be reconciled with the findings from X-ray difference Fourier analyses which show that in aquomethemoglobin the β^{93} sulphydryl group is more

exposed to the solvent than it is in carbonmonoxyhemoglobin (87). Moreover, it is now established that the β^{93} sulphydryl group of hemoglobin exists in a dynamic equilibrium between two tertiary conformational states (40, 41). It is likely that the position of this dynamic equilibrium will be sensitive to species differences and will also differ for different hemoglobin derivatives. These considerations allow for the assignment of the differences observed in Figures 4,5,7-11 to differences in the position of the dynamic equilibrium of the sulphydryl group in various species and derivatives. The finding (Figure 6) that the reactivities of the azidomet derivatives of hemoglobins A and S are equal throughout the pH range studied indicates that in these derivatives the β^{93} sulphydryl group is equally exposed to the solvent. In the previous study (18), it was observed that there is no difference in the $k_{(app)}$ obtained for oxyhemoglobins A and S contrary to our own findings. The differences between our results and that of Hallaway may be due to differences in the ionic strength since our results were obtained at 0.05 M while Hallaways results were obtained at an ionic strength of 0.2 M.

Bettlestone et al (85) confirmed that the free energies and enthalpies of ionisation for three different aquomethemoglobins A, S and C are different at low ionic strength ($I \leqslant 0.05$) but are the same at higher ionic strength ($I \geqslant 1.00$). Confirming the effect of ionic strength on hemoglobin reaction, Bettlestone et al. (86) have shown that the pK values of aquomethemoglobins A and C when azide ion binds differ at low ionic strength but are similar at higher ionic strength.

(ii) Kinetics of 2,2'-dithiobispyridine reaction.

As far as data analysis is concerned, the 2-DTP data (Figures 12-20) have turned out to be the most fruitful of all the data reported in this thesis. In all cases it was not possible to undertake experiments above pH 9, on account of complications arising from an increased rate of hydrolysis of disulphide bonds at higher pH (64) and also for DTNB data. Figures 27 and 28 show the hydrolytic cleavage for the disulphide bonds of hemoglobin using DTNB and 2-DTP, respectively. The reaction of 2-DTP with hemoglobin A derivatives was carried out (See Appendix V Table 63) at higher pH values.

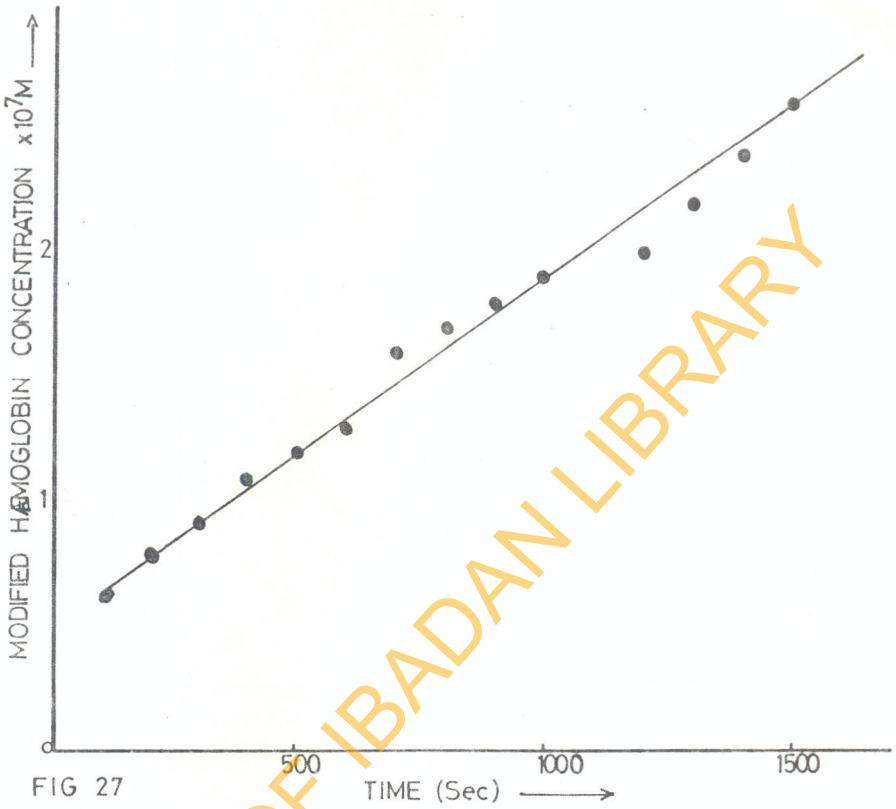


FIG 27

Hydrolytic cleavage of modified hemoglobin A using 5,5'-dithiobis(2-nitrobenzoic acid) at pH 9.2 at 20°C I = 0.05. Using a linear least square program $k_{obs} = 5 \times 10^{-9}$ M s^{-1} (See Appendix V Table 61).

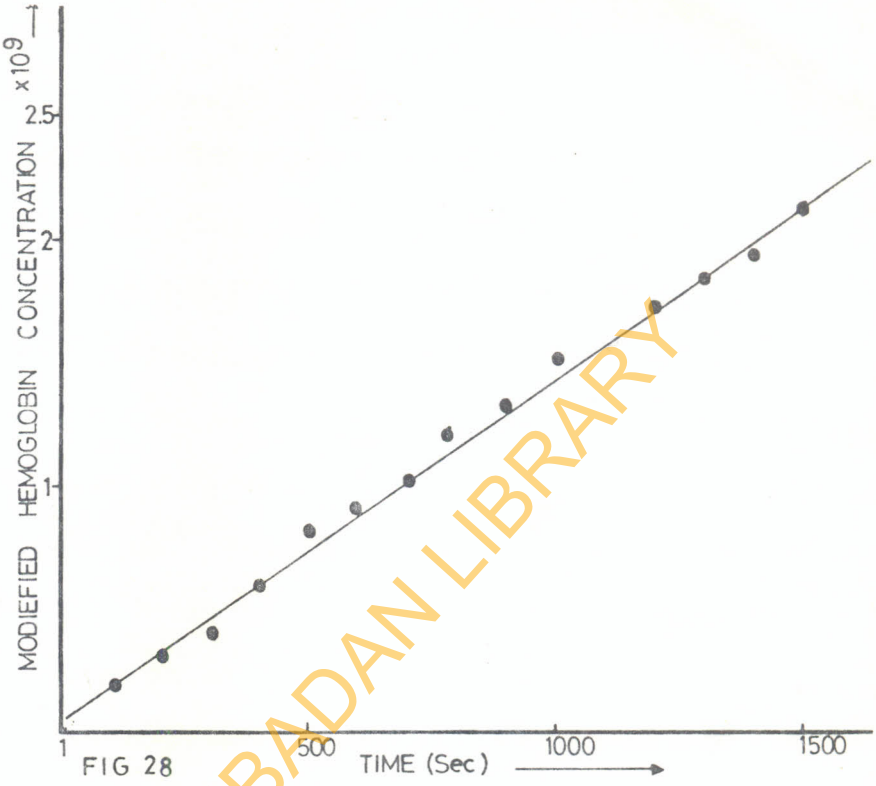


FIG 28

Hydrolytic cleavage of modified hemoglobin A using 2,2'dithiobispyridine at pH 9.2 at 20°C I = 0.05. Using a linear least square program $k_{obs} = 1.4 \times 10^{-11}$ M s^{-1} (See Appendix V Table 62).

The $k_{(app)}$ values are very low compared with $k_{(app)}$ values within the pH range of 5.6 to 9.0. It is believed that as soon as the 2-DTP reacts with hemoglobin, hydrolytic cleavage sets in at higher pH (64) (See Appendix V Table 63). As a result it was not experimentally possible to get the curves to "level off" as would be expected at higher pH. This defect has however been taken care of to a large extent by the use of computer analyses. The results of these analyses (Tables 4 and 5) indicate strongly that the pK of the β^{93} sulphydryl group depends on its conformation, its pK in its internal conformation differing from that of its external conformation by 2.5 pK units. As can be seen in Tables 4 and 5, pK_{ext} values for different hemoglobin species and derivatives lie within a narrow range. Similar remarks are valid for pK_{int}. The pK_{ext} for hemoglobin species does not in hemoglobin species affect the sulphydryl reactivities.

Deoxyhemoglobin crystals are normally associated with the β^{93} sulphydryl group in the external conformation; similarly oxyhemoglobin is normally associated with the internal conformation of this sulphydryl (38,87). Assuming from Tables 4 and 5 that the β^{93} sulphydryl group of deoxyhemoglobin has a pK about 2.5 pK units lower than that of oxyhemoglobin, conversion of deoxyhemoglobin

to oxyhemoglobin should give rise to a contribution of a reverse Bohr effect by the sulphydryl group. Calculation shows that at pH 7.5 this should amount to about $-0.4\ H^+$ per mole heme for hemoglobin A . The experimentally determined Bohr effect at this pH is $+0.5\ H^+$ per mole heme (78). Does it mean that this value would have been $+0.9\ H^+$ per mole heme but for the reverse Bohr effect/contribution of the sulphydryl group? What contribution, if any does the β^{93} sulphydryl group make to the alkaline Bohr effect? These questions are important in the light of reports of the possible existence of reverse Bohr groups, especially in hemoglobins in which Lys E F6 (82)β is replaced by neutral or negatively charged groups (79, 97).

The value $-0.4\ H^+$ per mole heme at pH 7.5 calculated above for the reverse Bohr contribution of the β^{93} sulphydryl group of hemoglobin A is based on two assumptions (i) that its conformation in deoxyhemoglobin crystals is fully external, and (ii) that its conformation in oxyhemoglobin is fully internal.

However, data from temperature-jump studies have shown that in deoxyhemoglobin in solution the sulphydryl group is in a rapid dynamic equilibrium between the external

and internal conformations (40). Moreover, it has been demonstrated from X-ray studies at $-2^{o}C$ that in oxyhemoglobin the sulphydryl group is 30% in the external conformation (41).

This finding is also supported by temperature - jump data on carbonmonoxyhemoglobin (40). Calculations from the temperature - jump data give the fractional populations of the external sulphydryl conformation as 12.9% and 6% in deoxyhemoglobin and oxyhemoglobin, respectively, rather than the 100% and 0% previously assumed. The value -0.4 H^{+} per mole heme at pH 7.5 calculated for the reverse Bohr contribution of the β^{93} sulphydryl groups must therefore be multiplied by the factor (12.9-6)/(100-0). This results in a value of -0.03 H^{+} per mole heme as the actual reverse Bohr contribution of the β^{93} sulphydryl, a value that is within the error of Bohr effect determinations. Thus, inspite of a difference of 2.5 pK units in the pH of the sulphydryl group in the internal and external conformations this group makes no significant contribution to the Bohr effect. The use of the data obtained at $-2^{o}C$ to explain our present results at $20^{o}C$ can be best be assumed approximate. This is because we have not lost sight of the temperature difference and its consequential effects.

Although a direct physiological role for the β^{93} sulphydryl group of hemoglobin is ruled out by the above considerations, an indirect role for this group is

indicated by the finding that the magnitudes of the acid and alkaline Bohr effects are changed upon chemical modification of this group (45, 75, 77). A structural explanation for the effect of sulphydryl modification on the alkaline Bohr effect was provided recently (40).

[C] POTENTIOMETRIC DIFFERENCE TITRATION OF METHYL METHANETHIOSULPHONATE REACTION.

Despite the setback of not being analyzed quantitatively, the data on the MMTS reaction (Figures 21-26) show some interesting results in qualitative terms.

In Figure 21, the data for the deoxy, carbonmonoxy and oxy derivatives of hemoglobin A are compared. Similar comparisons are made for horse and rabbit in Figures 23 and 25. Oxyhemoglobin and carbonmonoxyhemoglobin have the R quaternary structure, whereas deoxyhemoglobin has the T quarternary structure (78). The result in Figure 21 thus indicates that Δh^+ does not arise from a change in quaternary structure of the protein upon methylthiolation. The difference in Δh^+ per mole heme is due to the differences in the tertiary structures of hemoglobin derivatives (80).

The quaternary structure is related to Bohr effect (40). The plots of Δh^+ (deoxy) - Δh^+ (oxy) versus pH for the data in Figures 26(b) (page 125b) do not

resemble the Bohr effect. Thus one can conclude that the differences in Δh^+ per mole heme is not due to the differences in the quaternary structure but due to the differences in the tertiary structure (80).

In Figures 22, 24 and 26 the Δh^+ versus pH profiles of aquomethemoglobin and azidomethemoglobin are compared. It has been established that when azide ion binds to aquomethemoglobin protons are taken up (57, 63). At low pH, Δh^+ values for aquomet derivative are higher than for the azidomet derivative whereas at high pH the reverse is the case. It can be concluded that the variation is due to the same ionizable groups with low pK_s in aquomethemoglobin that have relatively higher pK_s in the azidomethemoglobin. In addition, the variation in Δh^+ versus pH for aquomet derivative and azidomet derivative (Figures 22, 24 and 26) is likely due to the position of β^{93} sulphydryl group of hemoglobin which exists in a dynamic equilibrium between two tertiary conformational states (40,41). It is believed that the position of this dynamic equilibrium will be sensitive to species differences and will also differ for different hemoglobin derivatives.

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APPENDIX I

TABLE 6

Typical Results of the Kinetics of the Binding of Cyanide ion to Rabbit Methemoglobin at 20°C I = 0.05

pH = 6.2					pH = 6.2		
Time (Sec)	$k_{obs} = 4.09 \times 10^{3} s^{-1}$, $[CN^-] = 30\mu M$		$k_{obs} = 6.94 \times 10^{3} s^{-1}$ $[CN^-] = 60\mu M$		Time (Sec)	$k_{obs} = 9.27 \times 10^{3}$ $[CN^-] = 90\mu M$	
	$D_t - D_\infty$	$-\ln(D_t - D_\infty)$	$D_t - D_\infty$	$-\ln(D_t - D_\infty)$		$D_t - D_\infty$	$-\ln(D_t - D_\infty)$
0	0.205	1.585	0.205	1.585	0	0.205	1.585
20	0.188	1.672	0.169	1.775	15	0.169	1.775
40	0.169	1.775	0.155	1.936	30	0.143	1.935
60	0.157	1.855	0.125	2.076	45	0.125	2.076
80	0.146	1.925	0.110	2.207	60	0.110	2.207
100	0.134	2.008	0.098	2.320	75	0.098	2.320
120	0.125	2.082	0.085	2.469	90	0.084	2.468
140	0.116	2.153	0.074	2.602	105	0.074	2.602
160	0.105	2.256	0.066	2.723	120	0.066	2.723

The k_{obs} values were obtained using linear least squares regression program.

TABLE 7

Dependence of the Mean Pseudo-First Order Rate Constant on pH at various Cyanide Concentrations at 20°C I = 0.05M for Horse aquomethemoglobin = 2 μM heme

pH	$[CN^-]$ = 30 μM $k_{obs} \times 10^3$	$[CN^-]$=60 μM $k_{obs} \times 10^3$	$[CN^-]$=90 μM $k_{obs} \times 10^3$	$[CN^-]$=120 μM $k_{obs} \times 10^3$
5.6	2.24	4.15	9.20	-
5.7	3.06	3.77	8.74	10.35
5.8	2.95	4.80	9.86	12.40
5.9	3.22	4.16	9.22	11.98
6.0	3.27	5.57	-	13.62
6.1	3.94	4.31	11.76	13.26
6.2	3.86	6.34	-	13.28
6.3	-	5.37	14.11	17.62
6.4	4.44	6.88	-	17.05
6.5	-	6.39	15.71	20.53
6.6	5.49	9.14	-	22.22
6.7	5.41	9.31	-	23.29
6.9	7.01	-	24.54	27.13
7.0	7.32	11.62	-	26.07
7.1	2.84	11.59	23.75	31.64
7.2	2.83	11.17	26.45	30.35
7.3	-	12.20	24.44	33.05
7.4	-	9.60	26.39	31.69

TABLE 7 (contd.)

pH	$[CN^-]$ = 30 μM $k_{obs}x10^3$	$[CN^-]$=60 μM $k_{obs}x10^3$	$[CN^-]$=90 μM $k_{obs}x10^3$	$[CN^-]$=120 μM $k_{obs}x10^3$
7.5	-	13.00	23.80	38.59
7.6	-	13.00	29.36	36.67
7.7	-	12.80	26.18	34.19
7.8	7.71	13.83	24.94	30.60
7.9	7.41	13.59	22.70	30.60
8.0	7.50	12.52	26.12	33.83
8.1	4.00	12.41	23.64	29.10
8.2	3.87	9.46	18.25	-
8.3	4.30	9.44	16.83	22.83
8.4	4.29	7.33	15.42	23.17
8.5	1.88	7.04	14.26	29.14
8.6	1.85	6.48	13.16	-
8.7	2.92	6.06	11.90	14.70
8.8	2.93	4.35	10.22	14.80
8.9	2.43	4.00	10.18	-
9.0	2.39	5.10	9.12	12.18

At fixed pH and Cyanide concentration, the k_{obs} value is mean of three k_{obs} values.

TABLE 8

Dependence of the Mean Pseudo-First Order Rate Constant on pH and $[CN^-]$ at 20°C for [Rabbit Met Hb] = 2μM Heme.

pH	$[CN^-]$=30 μM $k_{obs} \times 10^3$	$[CN^-]$=60 μM $k_{obs} \times 10^3$	$[CN^-]$=90 μM $k_{obs} \times 10^3$	$[CN^-]$=120 μM $k_{obs} \times 10^3$
5.6	-	4.46	5.88	8.86
5.7	4.45	-	5.78	9.47
5.8	5.20	5.50	7.34	10.99
5.9	5.30	-	7.86	11.62
6.0	5.20	6.43	8.51	12.86
6.1	5.41	6.98	9.51	13.69
6.2	4.09	6.94	9.27	13.96
6.3	4.29	7.19	9.63	14.69
6.4	3.09	8.02	10.92	16.16
6.5	3.24	7.62	12.31	17.56
6.6	3.50	9.35	14.18	18.74
6.7	4.03	10.16	14.50	19.51
6.8	5.39	10.88	15.03	21.93
6.9	5.60	11.27	15.62	24.00
7.0	5.76	11.69	18.39	23.29
7.1	6.05	12.52	15.83	25.16
7.2	6.04	12.03	16.45	24.03
7.3	6.17	11.91	17.29	22.39
7.4	6.55	12.89	15.60	26.28

TABLE 8 (contd.)

pH	$[CN^-]$=30 μM $k_{obs} \times 10^3 s^{-1}$	$[CN^-]$=60 μM $k_{obs} \times 10^3 s^{-1}$	$[CN^-]$=90 μM $k_{obs} \times 10^3 s^{-1}$	$[CN^-]$=120 μM $k_{obs} \times 10^3 s^{-1}$
7.5	7.01	12.43	18.20	25.47
7.6	6.86	13.61	14.67	27.25
7.7	7.61	13.89	16.25	25.04
7.8	6.18	12.23	16.54	24.43
7.9	6.76	12.33	18.48	23.57
8.0	6.97	14.35	18.56	27.61
8.1	7.72	13.47	17.25	29.44
8.2	6.46	12.89	17.54	25.92
8.3	7.69	9.96	13.90	26.90
8.4	5.17	10.34	13.83	21.19
8.5	5.58	8.81	12.37	14.53
8.6	4.59	9.08	12.60	18.44
8.7	4.94	7.99	12.71	18.90
8.8	3.49	6.93	9.27	15.36
8.9	3.50	8.55	9.54	14.42
9.0	3.76	7.38	9.70	14.86

TABLE 9

Apparent Second order rate constant for Cyanide binding to Horse Methemoglobin at 20°C I = 0.05

pH	$k_{(app)}$ $[M^{-1}s^{-1}]$	pH	$k_{(app)}$ $[M^{-1}s^{-1}]$
5.6	75.57±6.00	7.8	305.30±5.00
5.7	83.07±3.00	7.9	265.90±5.33
5.8	111.60±2.30	8.0	295.60±4.33
5.9	104.50±3.00	8.1	245.20±3.00
6.0	152.70±6.00	8.3	239.70±3.33
6.1	107.40±3.00	8.4	210.00±5.00
6.2	162.70±3.34	8.5	215.80±3.45
6.5	167.50±5.00	8.6	140.90±3.70
6.6	166.70±3.40	8.7	137.70±3.77
6.8	204.90±7.00	8.8	
7.0	213.00±3.00		
7.1	328.50±3.40		
7.3	326.10±4.00		
7.5	284.70±3.00		
7.7	309.40±7.00		

Each $k_{(app)}$ is mean of three $k_{(app)}$ values at a fixed pH linear least squares regressionprogram was used to calculate the $k_{(app)}$.

TABLE 10

Apparent second order rate constant for Cyanide binding to Rabbit Methemoglobin at 20°C I = 0.05

pH	$K_{(app)}$ $[M^{-1}s^{-1}]$	pH	$K_{(app)}$ $[M^{-1}s^{-1}]$
5.6	73.33±5.00	7.2	194.60±13.11
5.8	91.50±6.00	7.3	185.33±3.47
5.9	94.17±7.23	7.5	203.50±10.00
6.0	107.20±8.00	7.7	182.70±10.00
6.1	111.80±7.50	7.8	196.90±15.00
6.2	106.50±11.00	7.9	188.60±4.62
6.3	112.10±10.00	8.0	220.40±10.02
6.4	140.40±10.00	8.1	158.80±8.96
6.5	158.80±4.62	8.2	210.10±16.00
6.6	168.50±7.03	8.3	203.50±5.00
6.7	155.80±6.44	8.4	171.80±7.00
6.8	179.20±12.00	8.6	150.20±10.00
6.9	198.50±8.13	8.7	155.30±11.00
7.0	197.60±8.20	8.8	128.30±8.00
7.1	202.40±10.33	8.9	118.00±10.00
		9.0	118.70±11.00

At fixed pH, the $k_{(app)}$ was calculated using the linear least squares regression program.

APPENDIX II

TABLE 11

Typical Results of the Kinetics of the Reactivity of 5,5'-Dithiobis[2-Nitrobenzoic Acid][DTNB] to the β^{93} Sulphydryl group of carbonmonoxyhemoglobin s at 20°C
I = 0.05 [CoHbSH] = 5 μM [DTNB] = 100 μM pH = 5.6

Time	Transmittance 1	k_1	Transmittance 2	k_2	Transmittance 3	k_3
40	99.0	8.76	99.1	7.86	99.0	8.76
60	98.8	7.04	98.9	6.44	98.4	9.48
80	98.5	6.65	98.7	5.73	98.2	8.04
100	98.0	7.18	98.4	5.69	98.0	7.18
120	97.7	6.93	98.1	5.67	97.8	6.61
140	97.5	6.49	97.7	5.94	97.6	6.21
160	97.3	6.16	97.5	5.68	97.4	5.92
180	97.1	5.91	9.20	6.13	97.0	6.13

$k_1^* = 6.89 \pm 0.85$ $k_2^* = 6.14 \pm 0.75$ $k_3^* = 7.74 \pm 0.75$

$k_{(app)}^{**} = 6.92 \pm 0.75$

* = Average of the second order rate constants.

** = mean of the averaged second order rate constants

Second-order rate equation 2 was used for the calculation of $k_{(app)}$.

TABLE 12

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with human oxyhemoglobin A at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	8.75±0.87	7.5	17.03±0.37
5.8	19.81±1.25	7.6	20.39±0.61
5.9	21.93±3.01	7.8	22.88±1.12
6.0	19.06±0.09	8.0	30.36±0.25
6.1	35.07±2.15	8.1	36.74±2.02
6.2	27.94±7.25	8.2	40.82±2.74
6.4	31.97±1.36	8.4	31.39±2.36
6.6	28.89±1.03	8.6	44.38 4.72
6.8	32.20±2.83	8.8	47.09 0.54
7.0	30.63±1.95	9.0	49.79 5.00
7.1	30.02±1.88		
7.2	33.54±1.71		
7.3	23.26±1.88		
7.4	24.03±1.89		

At fixed pH, k_{app} is mean of three k_{app} values

TABLE 13

Dependence of Apparent Second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with human carbonmonoxyhemoglobin A at 20°C I=0.05.

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	17.81±0.56	7.2	84.90±14.50
5.7	17.67±0.55	7.4	77.83±0.51
5.8	34.17±2.03	7.5	80.03±12.01
5.9	38.72±7.25	7.6	61.78±4.80
6.0	38.78±0.19	7.8	63.19±0.50
6.2	39.18±1.72	7.9	55.33±8.00
6.3	55.45±1.13	8.0	55.59±13.00
6.4	67.01±6.55	8.2	47.32±1.82
6.5	75.00±14.00	8.4	59.59±0.06
6.6	73.40±9.00	8.6	72.41±3.08
6.7	83.85±22.00	8.8	77.84±1.02
6.8	82.12±5.49	9.0	93.93±3.86
7.0	84.46±5.00		

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 14

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with human azidomethemoglobin A at 20°C I = 0.05.

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	13.65±1.00	7.1	19.55±0.02
5.7	10.94±1.33	7.2	23.21±1.33
5.8	13.73±0.33	7.3	20.22±0.02
5.9	13.29±0.02	7.4	20.16±0.67
6.0	14.98±0.04	7.5	17.71±0.05
6.1	15.16±0.01	7.6	16.68±1.00
6.2	20.77±2.00	7.8	17.54±0.67
6.3	17.62±0.33	8.0	17.52±0.02
6.4	25.65±1.33	8.0	17.93±0.67
6.5	19.92±0.01	8.2	21.01±1.00
6.6	22.17±1.33	8.4	21.65±0.67
6.7	20.72±0.05	8.6	21.96±0.33
6.8	22.41±0.02	8.8	25.79±1.33
6.9	22.91±0.03	9.0	29.22±0.33
7.0	23.69±0.02		

At fixed pH, $k_{(app)}$ is mean of three k_{app} values.

TABLE 15

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with human aquomethemoglobin A at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	24.20±0.45	7.6	63.57±2.79
5.7	31.92±0.11	7.7	65.07±4.08
5.8	26.90±1.29	7.8	34.78±2.13
5.9	29.56±0.20	7.9	38.19±0.87
6.0	48.72±1.56	8.2	50.95±3.00
6.1	56.35±4.61	8.4	58.62±1.39
6.2	52.60±1.89	8.6	60.59±1.08
6.3	67.01±1.73	8.8	66.50±0.60
6.4	82.63±2.80	9.0	77.60±4.09
6.5	119.21±2.38		
6.6	121.71±3.40		
6.7	122.78±6.00		
6.9	138.76±8.22		
7.1	120.71±11.00		
7.2	98.26±4.00		
7.3	100.96±6.62		
7.4	54.01±1.27		

At fixed pH, $k_{(app)}$ is a mean of three $k_{(app)}$ values.

TABLE 16

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with human oxyhemoglobin S at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	5.02±0.16	7.1	25.32±1.46
5.8	5.69±0.48	7.2	27.83±2.65
6.0	9.73±0.65	7.4	25.50±2.39
6.1	15.37±1.41	7.6	22.75±3.41
6.2	13.01±1.08	7.7	19.81±0.85
6.3	16.90±1.67	7.9	20.60±3.30
6.4	17.00±1.22	8.0	19.90±0.80
6.5	18.59±1.96	8.4	21.85±2.20
6.6	20.60±0.72	8.6	22.71±1.10
6.7	21.79±2.01	8.8	25.99±4.50
6.8	23.02±0.21	9.0	28.58±1.83
6.9	23.47±0.81		
7.0	25.43±1.16		

At fixed pH, $k_{(app)}$ is mean of three $k_{(app)}$ values.

TABLE 17

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with human carbonmonoxyhemoglobin S at 20°C I=0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	6.93±0.75	7.3	11.49±1.28
5.8	8.43±0.21	7.4	13.75±0.70
6.0	9.39±0.23	7.5	10.67±1.43
6.1	8.56±0.50	7.6	10.05±0.80
6.2	9.38±0.59	7.8	12.33±0.63
6.3	10.02±0.75	7.9	11.37±1.00
6.4	12.37±1.00	8.0	11.38±1.00
6.5	12.12±0.95	8.2	12.44±0.67
6.6	13.11±0.32	8.4	14.50±0.79
6.7	12.14±0.88	8.8	13.50±0.93
6.8	10.08±1.62	9.0	15.68±1.06
6.9	11.67±1.45		
7.1	13.06±1.72		
7.2	14.14±1.84		

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 18

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with human azidomethemoglobin S at 20°C I = 0.05

pH	k_{app} [M^{-1} s^{-1}]
5.6	11.94±1.00
5.8	15.62±0.02
6.0	15.71±0.01
6.2	17.96±0.04
6.4	21.57±0.03
6.6	20.93±0.02
6.8	21.71±0.03
7.0	20.56±0.67
7.2	20.22±0.67
7.4	22.29±0.03
7.6	19.95±0.04
7.8	17.02±0.67
8.0	18.67±0.30
8.2	20.94±1.00
8.4	23.11±0.02
8.6	22.44±0.33
8.8	23.86±1.00
9.0	28.29±0.33

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 19

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with human aquomethemoglobin S at 20°C I = 0.05

pH	k_{app} [$M^{-1} s^{-1}$]	pH	k_{app} [$M^{-1} s^{-1}$]
5.6	21.42±1.61	7.4	54.51±2.98
5.8	20.76±0.75	7.5	21.11±0.72
6.0	28.22±1.12	7.6	26.24±1.25
6.2	32.71±1.85	7.7	26.30±1.98
6.3	36.09±5.50	7.8	21.06±1.11
6.4	49.26±2.02	7.9	20.37±0.70
6.5	39.22±4.53	8.0	16.90±1.05
6.6	55.31±0.03	8.2	18.33±1.34
6.7	59.02±7.85	8.4	21.83±1.80
6.8	68.34±1.00	8.6	22.71±2.52
6.9	70.33±7.83	8.8	22.95±3.30
7.0	71.68±4.00	9.0	25.42±3.24
7.2	55.78±3.33		

At fixed pH, $k_{(app)}$ is mean of three k_{app} values.

TABLE 20

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with Horse Oxyhemoglobin at 20°C I = 0.05.

pH	k_{app} [$M^{-1} s^{-1}$]	pH	k_{app} [$M^{-1} s^{-1}$]
5.6	65.68±1.50	7.5	116.62±0.02
5.8	66.80±0.02	7.6	105.63±9.25
6.0	67.63±0.40	7.7	99.85±5.00
6.1	74.18±0.03	7.8	80.02±2.49
6.2	86.24±0.50	8.0	78.34±2.50
6.3	92.91±0.30	8.2	76.13±1.00
6.4	98.90±10.00	8.3	95.94±5.11
6.5	123.31±5.00	8.4	91.94±0.30
6.6	116.15±0.23	8.5	102.41±1.67
6.7	122.35±0.23	8.6	107.31±2.00
6.9	129.98±0.50	8.7	117.82±0.05
7.0	126.62±2.53	8.8	124.95±0.03
7.1	125.53±0.02	8.9	116.49±0.33
7.2	129.12±0.67	9.0	127.08±0.30
7.4	115.22±3.00		

At fixed pH, $k_{(app)}$ is mean of three k_{app} values.

TABLE 21

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with Horse carbonmonoxyhemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	60.97±1.17	7.2	168.66±1.50
5.7	69.64±3.33	7.4	146.73±4.02
5.8	85.33±3.00	7.5	167.23±6.38
5.9	84.49±2.33	7.6	148.29±2.59
6.0	89.67±4.48	7.7	101.17±3.67
6.1	123.02±13.33	7.8	121.02±0.27
6.2	141.07±5.00	8.1	99.31±7.89
6.3	143.17±5.55	8.2	97.12±9.33
6.4	160.51±4.44	8.4	104.68±3.00
6.5	149.37±2.00	8.5	106.21±4.33
6.6	186.41±2.50	8.6	123.33±8.30
6.7	153.40±1.00	8.7	124.72±0.35
6.8	164.82±7.00	8.8	138.32±10.0
6.9	174.83±8.3	9.0	141.01±4.11
7.0	168.91±0.32		
7.1	174.65±5.00		

At fixed pH, k_{app} is mean of three k_{app} values

TABLE 22

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-dithiobis[2-nitrobenzoic acid] reaction with Horse Azidomethemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	35.29±0.20	7.6	68.62±1.40
5.7	31.57±0.03	7.7	73.87±3.80
5.8	40.25±0.04	7.8	73.00±4.67
5.9	37.53±0.03	7.9	90.36±2.00
6.0	38.76±0.31	8.0	85.70±5.33
6.1	53.87±0.65	8.2	94.58±5.00
6.2	55.37±0.47	8.3	95.66±0.04
6.3	68.13±7.37	8.4	104.09±0.02
6.4	69.98±0.01	8.5	101.59±0.50
6.5	74.35±0.03	8.6	109.99±0.01
6.6	73.22±2.16	8.7	105.85±10.0
6.7	100.29±0.01	8.8	118.81±3.27
6.8	91.22±0.62	8.9	123.68±3.00
7.0	98.38±1.17	9.0	135.55±7.91
7.1	100.52±1.33		
7.2	103.40±3.29		
7.3	97.35±0.05		
7.4	82.85±0.09		

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 23

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with Horse Aquomethemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	91.05±0.67	7.3	180.35±0.30
5.7	91.00±1.11	7.4	190.52±6.51
5.9	90.09±1.67	7.6	187.47±1.85
6.0	100.68±0.14	7.8	165.25±5.97
6.1	99.27±0.33	7.9	150.43±0.33
6.2	125.17±7.33	8.0	136.06±0.30
6.3	126.26±2.00	8.2	160.00±0.35
6.4	153.74±6.00	8.3	181.10±0.40
6.5	150.47±0.12	8.4	183.84±5.67
6.6	162.90±2.70	8.5	193.00±2.00
6.7	166.72±0.60	8.7	205.19±7.89
6.8	204.95±1.67	8.8	198.80±10.00
6.9	202.23±1.50	8.9	194.58±0.50
7.0	207.78±4.67	9.0	198.80±10.00
7.1	213.13±2.00		
7.2	182.99±1.33		

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 24

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with Rabbit Oxyhemoglobin at 20°C I = 0.05

pH	$k_{app}[M^{-1}s^{-1}]$	pH	$k_{app}[M^{-1}s^{-1}]$
5.6	146.71±0.87	7.9	200.87±0.13
5.7	170.20±0.03	8.0	212.58±9.19
5.8	188.01±0.01	8.2	200.41±6.69
6.0	234.59±15.50	8.3	206.05±0.23
6.2	231.56±27.00	8.4	206.98±6.00
6.3	225.51±10.00	8.5	219.86±0.50
6.4	241.48±2.00	8.6	223.43±1.00
6.6	253.71±0.58	8.7	234.88±0.50
6.8	274.63±25.00	8.8	221.75±1.00
7.0	294.28±1.67	8.9	230.88±0.50
7.2	294.67±10.8	9.0	230.62±17.00
7.4	287.04±3.04		
7.5	247.36±0.03		
7.6	236.15±12.00		
7.7	240.49±6.44		
7.8	231.25±20.00		

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 25

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with Rabbit carbonmonoxyhemoglobin at 20°C I=0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	179.54±0.50	7.7	226.72±12.70
5.8	194.31±2.78	7.8	220.55±7.50
6.0	230.03±7.27	8.0	164.42±12.50
6.1	241.11±0.03	8.2	180.95±16.7
6.2	313.23±7.47	8.4	164.06±0.01
6.4	338.55±8.02	8.6	212.34±2.50
6.6	369.14±14.70	8.8	214.76±1.50
6.8	370.61±11.00	8.9	225.38±0.02
7.0	409.46±3.49	9.0	247.50±12.00
7.2	343.36±6.38		
7.3	256.04±0.50		
7.4	260.11±7.00		
7.5	244.60±12.3		
7.6	223.47±6.82		

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 26

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with Rabbit azidomethemoglobin at 20°C I=0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	79.29±0.41	7.6	190.05±1.01
5.7	91.92±0.50	7.7	185.74±4.52
5.8	84.07±1.48	7.8	163.46±1.4
5.9	94.85±1.00	8.0	163.66±9.04
6.0	97.52±0.91	8.1	169.99±0.08
6.1	128.89±7.41	8.2	182.88±3.33
6.2	128.80±2.45	8.3	196.81±0.67
6.3	136.11±3.33	8.4	209.95±0.02
6.4	173.54±3.96	8.5	208.57±1.00
6.5	179.97±2.67	8.6	208.35±7.05
6.7	208.77±0.02	8.7	221.66±0.33
6.8	225.28±5.17	8.8	216.34±7.89
7.0	230.81±4.39	9.0	225.36±11.85
7.1	222.37±1.33		
7.2	207.47±0.33		
7.4	202.66±0.05		
7.5	200.89±1.33		

At fixed pH, k_{app} is mean of three k_{app} values.

TABLE 27

Dependence of apparent second order rate constant [k_{app}] on pH for the 5,5'-Dithiobis[2-nitrobenzoic acid] reaction with Rabbit aquomethemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	205.92±11.10	8.0	283.36±7.98
5.8	214.04±17.20	8.0	294.32±4.35
6.0	246.49±19.20	8.1	333.07±2.03
6.1	262.36±0.02	8.2	331.83±14.10
6.2	268.44±5.93	8.3	367.64±2.79
6.3	352.38±15.60	8.4	392.27±13.50
6.4	355.54±14.20	8.5	424.43±3.00
6.6	345.08±22.50	8.6	427.94±24.4
6.7	426.22±2.62	8.7	420.43±3.00
6.8	419.06±14.10	8.8	450.55±25.00
7.0	433.09±9.36	8.9	488.67±0.67
7.1	411.04±3.33	9.0	514.92±1.47
7.2	397.39±6.05		
7.3	330.72±12.5		
7.4	330.22±10.2		
7.6	332.94±8.73		
7.8	318.20±5.14		

At fixed pH, k_{app} is mean of three k_{app} values.

APPENDIX III

TABLE 28

Typical results of the kinetics of the reactions of 2,2'-dithiobispyridine [DTP] with the β^{93} sulphydryl group of carbonmonoxyhemoglobin A at 20°C I = 0.05

[COHbSH] = 5 μM [DTP] = 100 μM pH = 5.6

Time	Trans-mittance 1	k_1	Trans-mittance 2	k_2	Trans-mittance 3	k_3
60	99.5	4.87	99.4	5.87	99.5	4.87
120	99.2	3.94	99.2	3.94	99.3	3.44
180	99.0	3.31	98.9	3.65	99.0	3.33
240	98.8	3.00	98.7	3.26	98.6	3.53
300	98.6	2.89	98.4	3.25	98.5	3.03
360	98.3	2.88	98.2	3.24	98.2	3.07
420	98.0	2.95	98.0	2.95	98.0	2.95
480	97.8	2.86	97.6	3.14	97.6	3.14
540	97.6	2.79	97.4	3.05	97.4	3.05

$k_1^* = 3.27 \pm 0.23$ $k^* = 3.42 \pm 0.31$ $k_3^* = 3.38 \pm 0.20$

$k_{(app)}^{**} = 3.36 \pm 0.05$

* = Average of the second order rate constants.
** = Average of the averaged second order rate constants.
Second-order rate equation 2 was used for the calculation.

TABLE 29

Dependence of apparent second order rate constant, $k_{(app)}$, on pH for the 2,2'-dithiobispyridine reaction with human oxyhemoglobin A at 20°C I = 0.05

pH	$k_{(app)}$ [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	8.31±0.05	7.4	9.52±0.19
5.7	6.89±0.23	7.5	8.59±0.04
5.9	7.00±0.16	7.6	15.02±0.07
6.0	8.01±0.25	7.7	14.10±0.17
6.1	6.40±0.06	7.8	14.25±0.11
6.2	6.31±0.20	7.9	14.47±0.15
6.3	5.35±0.02	8.1	18.70±0.54
6.4	5.46±0.05	8.2	21.00±0.03
6.5	5.81±0.10	8.3	26.64±0.12
6.6	7.00±0.02	8.5	26.88±0.15
6.7	7.33±0.02	8.6	28.70±0.05
6.8	7.70±0.11	8.7	28.46±0.10
7.0	6.44±0.15	8.8	28.93±1.10
7.2	8.31±0.20	8.9	33.15±0.11
7.3	8.46±0.14	9.0	35.36±0.25

Each k_{app}, is an averaged of three k_{app} values.

TABLE 30

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with human carbonmoboxyhemoglobin A at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	3.36±0.05	7.4	11.61±0.10
5.7	3.13±0.05	7.5	13.18±0.15
5.8	4.45±0.29	7.6	11.16±0.04
6.0	4.78±0.03	7.7	12.70±0.04
6.2	5.19±0.18	7.9	12.88±0.05
6.3	7.06±0.26	8.0	13.11±0.07
6.4	6.81±0.56	8.1	22.01±0.05
6.5	7.44±0.03	8.2	29.01±0.19
6.6	7.61±0.27	8.4	31.63±0.01
6.7	7.40±0.02	8.6	36.75±0.33
6.8	7.75±0.20	8.7	36.62±0.20
7.1	7.83±0.67	8.8	37.14±0.10
7.2	12.33±0.11	8.9	40.98±0.38
		9.0	38.83±0.50

Each k_{app} is the mean of three k_{app} values

TABLE 31

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with Human azidomethemoglobin A at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	3.40±0.10	7.2	6.62±0.08
5.7	3.20±0.10	7.4	7.53±0.23
5.8	4.20±0.08	7.6	9.51±0.07
5.9	4.48±0.02	7.8	9.48±0.03
6.0	5.61±0.03	8.0	9.20±0.04
6.2	5.04±0.15	8.2	15.42±0.09
6.4	4.89±0.52	8.4	23.25±0.10
6.6	5.35±0.56	8.6	23.34±0.05
6.8	5.32±0.30	8.8	24.96±0.29
7.0	4.94±0.20	9.0	24.60±0.20
7.1	6.62±0.08		

Each k_{app} is the mean of three k_{app} values.

TABLE 32

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with Human aquomethemoglobin A 20°C I = 0.05

pH	k_{app} [$M^{-1} s^{-1}$]	pH	k_{app} [$M^{-1} s^{-1}$]
5.6	4.52±0.17	7.4	26.80±0.02
5.7	4.89±0.20	7.5	17.28±0.26
5.9	4.88±0.03	7.8	16.52±0.20
6.0	5.00±0.03	8.0	21.05±0.27
6.2	6.28±0.13	8.1	22.12±0.01
6.4	7.32±0.43	8.2	24.13±0.11
6.5	10.33±0.67	8.3	31.18±0.03
6.6	9.55±0.25	8.4	30.91±0.21
6.7	11.43±0.30	8.5	33.40±2.25
6.8	10.33±0.24	8.8	38.03±0.19
7.0	13.36±0.02	8.9	34.17±0.09
7.2	13.27±0.16	9.0	39.92±0.20

Each k_{app} is the mean of three k_{app} values.

TABLE 33

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with human oxyhemoglobin S at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	3.85±0.13	7.4	9.83±0.32
5.9	4.52±0.36	7.5	11.20±0.16
6.0	5.87±2.53	7.6	14.79±0.10
6.1	5.82±0.16	7.7	15.21±0.21
6.2	6.44±0.32	7.8	15.87±0.24
6.3	6.68±0.20	7.9	15.13±0.34
6.4	7.56±2.33	8.0	15.55±0.97
6.5	8.25±0.13	8.1	19.97±0.45
6.6	8.44±0.42	8.2	18.57±0.88
6.7	7.23±0.26	8.3	23.68±0.43
6.8	7.29±0.20	8.4	25.94±0.64
6.9	6.97±0.46	8.5	25.01±0.52
7.1	7.27±0.22	8.6	33.86±0.52
7.2	7.97±0.10	8.7	32.64±1.03
7.3	8.89±0.11	8.8	31.19±1.48

Each k_{app} is the mean of three k_{app} values.

TABLE 34

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with human carbonmonoxyhemoglobin S at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	2.03±0.03	7.4	11.68±0.36
5.7	5.76±0.71	7.5	11.95±0.15
5.9	6.85±0.34	7.6	11.44±0.10
6.0	6.82±0.28	7.7	12.96±0.04
6.1	7.61±0.25	7.8	14.24±0.20
6.2	8.14±0.33	7.9	15.61±0.19
6.3	8.65±0.29	8.0	17.61±0.16
6.4	8.25±0.77	8.1	19.59±0.11
6.5	8.34±0.45	8.2	23.78±0.13
6.6	8.90±0.45	8.3	24.89±0.03
6.7	8.76±0.27	8.4	24.58±0.29
6.8	8.65±0.28	8.5	25.58±0.24
6.9	8.99±0.08	8.6	36.76±0.08
7.0	9.58±0.24	8.7	39.04±0.10
7.1	9.57±0.24	8.8	45.01±0.28
7.2	9.96±0.36	8.9	45.97±0.21
7.3	11.17±0.02	9.0	45.77±0.24

Each k_{app} is the mean of three k_{app} values.

TABLE 35

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with human azidomethemoglobin S at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	5.17±0.23	7.4	8.33±0.37
5.8	4.46±0.04	7.5	10.51±0.01
5.9	4.68±0.02	7.6	10.18±0.15
6.1	4.91±0.10	7.7	11.78±0.11
6.2	4.50±0.05	7.8	14.35±0.16
6.3	5.67±0.13	7.9	15.85±0.08
6.4	4.61±0.03	8.0	15.22±0.05
6.5	5.43±0.03	8.1	16.57±0.13
6.6	5.55±0.09	8.2	18.26±0.13
6.7	6.12±0.03	8.3	22.66±0.09
6.9	6.53±0.11	8.4	29.09±0.29
7.0	7.36±0.20	8.5	31.60±0.15
7.2	7.38±0.15	8.6	37.05±0.16
7.3	7.81±0.10	8.7	40.30±0.89
		8.9	41.01±0.34

Each k_{app} is the mean of three k_{app} values.

TABLE 36

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with human aquomethemoglobin S at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.7	7.81±0.10	7.5	20.21±0.02
5.9	9.40±0.10	7.6	21.25±0.07
6.0	9.10±0.10	7.7	20.88±0.03
6.1	8.50±0.11	7.8	23.01±0.20
6.3	9.09±0.10	7.9	22.20±0.06
6.4	9.51±0.04	8.0	23.37±0.11
6.5	9.59±0.17	8.1	23.89±0.17
6.6	9.82±0.34	8.2	25.52±0.33
6.7	11.32±0.15	8.3	26.33±0.07
6.8	12.57±0.30	8.4	27.39±0.14
6.9	12.33±0.08	8.5	30.38±0.27
7.0	12.82±0.09	8.6	32.92±0.20
7.1	16.09±0.10	8.7	32.53±0.14
7.2	17.34±0.09	8.8	34.59±0.18
7.3	19.10±0.34	8.9	33.96±0.03
7.4	22.27±0.31	9.0	35.94±1.67

Each k_{app} is the mean of three k_{app} values.

TABLE 37

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with dog oxyhemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1} s^{-1}$]	pH	k_{app} [$M^{-1} s^{-1}$]
5.6	3.62±0.09	7.4	10.01±0.03
5.7	3.68±0.04	7.5	10.61±0.08
5.8	4.19±0.05	7.6	14.77±0.08
5.9	4.56±0.05	7.7	16.03±0.07
6.0	5.32±0.09	7.8	16.84±0.04
6.1	5.35±0.04	7.9	16.95±1.00
6.2	6.02±0.67	8.0	17.15±0.02
6.3	6.48±0.03	8.1	18.94±0.02
6.4	6.30±1.08	8.2	20.06±0.01
6.5	6.87±0.04	8.3	22.97±0.07
6.6	8.91±0.02	8.4	23.65±0.33
6.7	8.67±0.03	8.5	23.81±0.06
6.8	8.63±0.12	8.6	32.78±0.05
7.0	7.89±0.03	8.7	33.64±0.03
7.1	7.93±0.02	8.8	34.43±0.33
7.2	9.15±0.13	8.9	39.32±1.07
7.3	9.24±0.10	9.0	42.68±0.40

Each k_{app} is the mean of three k_{app} values.

TABLE 38

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with dog carbonmonoxyhemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	18.69±0.33	7.8	28.44±0.30
5.8	19.20±0.06	7.9	31.78±0.30
6.0	19.04±0.06	8.0	27.73±0.30
6.2	19.84±0.03	8.2	29.35±0.07
6.4	19.13±0.34	8.3	35.98±0.50
6.6	19.78±0.09	8.4	38.43±0.35
6.7	21.73±0.84	8.5	45.24±0.60
6.8	18.19±0.08	8.6	55.11±0.04
7.0	19.87±0.30	8.7	57.69±0.02
7.2	20.61±0.20	8.8	65.39±0.67
7.5	20.18±0.23	8.9	64.14±0.33
7.6	25.72±0.05	9.0	66.43±0.33

Each k_{app} is the mean of three k_{app} values.

TABLE 39

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with dog azidomethemoglobin at 20°C I = 0.50.

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	9.74±0.03	7.2	28.17±0.04
5.7	9.30±0.14	7.4	33.45±0.18
5.8	10.62±0.22	7.6	33.56±0.02
5.9	12.69±0.30	7.8	38.07±0.33
6.0	12.17±0.40	8.0	45.22±0.35
6.1	14.58±0.03	8.2	45.17±0.30
6.2	19.16±0.12	8.3	59.72±0.33
6.4	19.84±0.30	8.4	57.26±0.28
6.5	23.52±0.02	8.5	59.22±0.20
6.6	21.59±0.27	8.6	69.61±0.04
6.8	35.84±0.08	8.8	79.34±0.34
7.0	27.85±0.34	9.0	87.01±0.27
7.2	28.17±0.04		

Each k_{app} is the mean of three k_{app} values.

TABLE 40

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reactions with dog aquomethemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	5.52±0.15	7.2	20.57±0.59
5.7	7.55±0.10	7.3	20.53±0.03
5.8	8.97±1.00	7.6	23.67±0.04
5.9	7.80±0.30	7.7	31.48±0.34
6.0	8.45±0.23	7.8	29.62±0.33
6.1	9.55±0.20	7.9	29.14±0.07
6.2	10.51±0.09	8.0	28.31±0.20
6.3	13.03±0.02	8.1	38.19±0.04
6.4	11.19±0.30	8.2	46.92±0.33
6.6	14.01±2.00	8.3	54.98±0.03
6.7	17.33±0.30	8.4	54.30±0.07
6.9	20.53±0.03	8.5	55.37±2.00
7.0	19.09±0.09	8.6	64.52±1.00
7.1	19.66±0.02	8.8	78.38±0.67
		9.0	84.49±0.35

Each k_{app} is the mean of three k_{app} values.

TABLE 41

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with Rabbit oxyhemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	11.58±0.05	7.4	24.67±0.30
5.7	13.39±0.44	7.5	26.38±0.20
5.8	13.98±1.10	7.6	26.35±0.10
5.9	12.00±0.15	7.8	27.02±0.06
6.0	13.44±1.00	8.0	27.79±0.15
6.2	15.45±0.18	8.1	26.13±0.20
6.3	14.00±0.28	8.2	29.52±0.09
6.4	15.39±0.02	8.4	33.53±0.29
6.5	15.67±0.43	8.5	50.84±0.03
6.6	15.67±0.21	8.6	50.23±0.75
6.7	18.45±0.24	8.7	54.41±0.30
6.8	20.42±0.90	8.8	54.82±0.43
7.0	20.58±0.98	8.9	64.94±0.50
7.1	24.07±0.30	9.0	83.45±1.43
7.3	21.55±0.13		

Ecah k_{app} is the mean of three k_{app} values.

TABLE 42

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with Rabbit carbonmonoxyhemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	15.81±0.25	7.3	21.32±0.05
5.7	14.69±0.11	7.4	18.27±0.30
5.8	14.48±0.48	7.5	20.07±0.03
5.9	13.89±0.30	7.6	22.15±0.19
6.0	13.12±0.55	7.7	22.33±0.10
6.1	12.02±0.30	7.8	20.28±0.19
6.2	11.77±0.22	8.0	22.91±0.29
6.4	14.71±0.04	8.2	23.63±0.41
6.5	15.44±0.20	8.4	31.27±0.28
6.6	15.20±0.20	8.5	35.21±0.18
6.8	16.58±0.30	8.6	55.77±0.50
6.9	16.57±0.23	8.7	49.20±1.45
7.0	16.28±0.20	8.9	51.75±1.03
7.2	16.59±0.50	9.0	55.45±0.90

Each k_{app} is the mean of three k_{app} values.

TABLE 43

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with Rabbit azidomethemoglobin at 20°C I = 0.05.

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	4.50±0.30	7.2	18.04±0.39
5.7	4.70±0.30	7.5	20.71±0.03
5.8	6.51±0.09	7.6	21.70±1.44
5.9	6.19±0.03	7.8	23.56±0.26
6.0	6.22±0.04	7.9	24.10±0.67
6.1	7.32±1.06	8.1	31.09±0.27
6.2	8.39±0.20	8.2	37.98±0.23
6.4	9.12±0.29	8.3	43.48±0.36
6.5	9.94±0.96	8.4	42.94±0.09
6.6	10.00±0.20	8.5	44.24±0.08
6.7	10.65±0.83	8.6	50.51±0.04
6.8	10.91±0.04	8.7	61.90±0.14
6.9	14.86±0.26	8.8	61.16±0.15
7.0	16.03±0.38	9.0	62.56±0.78
7.1	16.79±0.18		

Each k_{app} is the mean of three k_{app} values.

TABLE 44

Dependence of apparent second order rate constant [k_{app}] on pH for the 2,2'-dithiobispyridine reaction with Rabbit aquomethemoglobin at 20°C I = 0.05

pH	k_{app} [$M^{-1}s^{-1}$]	pH	k_{app} [$M^{-1}s^{-1}$]
5.6	7.88±0.02	7.3	28.33±0.29
5.7	9.04±0.11	7.4	28.38±0.09
5.8	11.45±0.32	7.5	31.29±0.20
5.9	9.38±0.19	7.6	31.73±0.06
6.0	10.10±0.30	7.8	34.95±0.22
6.1	12.05±0.21	8.0	38.55±0.07
6.2	15.48±0.24	8.1	40.00±0.22
6.3	14.24±0.23	8.2	41.89±0.21
6.4	16.49±0.21	8.4	45.90±0.20
6.6	18.54±0.19	8.5	49.87±0.78
6.7	22.68±0.20	8.6	68.44±0.16
6.8	23.95±0.36	8.7	78.15±0.16
6.9	24.27±0.18	8.8	76.59±0.32
7.0	23.12±0.08	9.0	86.90±0.11
7.1	27.40±0.03		
7.2	26.87±0.08		

Each k_{app} is the mean of three k_{app} values.

APPENDIX IV

TABLE 45

Typical results of proton releast (Δh^+) per β^{93} Thiol group as a function of pH for methyl methanethiosulphonate reacting with Rabbit azidomethemoglobin at 20°C I = 0.05M

pH	Conc x 10^4 [HbSH]	Volume of the aliquot after the reaction	Volume of 0.01 M MMTS Used	Volume of 0.095 M NaOH Used	$\Delta h^+/SH$
5.6	1.78	23ml	0.5ml	0.003	0.069
5.6	1.78	23	0.5	0.002	0.046
5.7	1.78	23	0.5	0.004	0.092
5.7	1.78	23	0.5	0.004	0.092
5.8	1.78	23	0.5	0.005	0.115
5.8	1.78	23	0.5	0.006	0.138
5.9	1.78	22	0.5	0.008	0.194
5.9	1.78	22	0.5	0.009	0.218
6.0	1.78	22	0.5	0.013	0.315
6.0	1.78	22	0.5	0.012	0.291
6.1	1.78	21	0.5	0.010	0.254
6.1	1.78	21	0.5	0.009	0.229
6.2	1.78	21	0.5	0.012	0.305
6.2	1.78	21	0.5	0.010	0.254
6.3	1.78	22	0.5	0.013	0.315
6.3	1.78	22	0.5	0.014	0.340
6.4	1.78	22	0.5	0.015	0.364
6.4	1.78	22	0.5	0.016	0.388

TABLE 45 (contd.)

pH	Conc x 10^4 [HbSH]	Volume of the aliquot after the reaction	Volume of 0.01 M MMTS Used	Volume of 0.095 M NaOH Used	$\Delta h^+/SH$
6.5	1.78	22	0.5	0.017	0.412
6.5	1.78	22	0.5	0.018	0.437
6.6	1.78	22	0.5	0.019	0.461
6.6	1.78	22	0.5	0.019	0.461
6.7	1.78	22	0.5	0.020	0.485
6.7	1.78	22	0.5	0.020	0.485
6.8	1.78	22	0.5	0.018	0.437
6.8	1.78	22	0.5	0.019	0.461
6.9	1.78	22	0.5	0.020	0.485
6.9	1.78	22	0.5	0.021	0.509
7.0	1.78	22	0.5	0.019	0.461
7.0	1.78	22	0.5	0.018	0.437
7.1	1.78	22	0.5	0.017	0.412
7.1	1.78	22	0.5	0.016	0.388
7.2	1.78	22	0.5	0.016	0.388
7.2	1.78	22	0.5	0.015	0.364
7.3	1.78	22	0.5	0.015	0.364
7.3	1.78	22	0.5	0.014	0.340
7.4	1.78	22	0.5	0.014	0.340
7.4	1.78	22	0.5	0.013	0.315

TABLE 45 (contd.)

pH	Conc x 10^4 [HbSH]	Volume of the aliquot after the reaction	Volume of 0:01 M MMTS Used	Volume of 0.095 M NaOH Used	$\Delta h^+/SH$
7.5	1.78	22	0.5	0.0135	0.328
7.5	1.78	22	0.5	0.013	0.315
7.6	1.78	23	0.5	0.014	0.340
7.6	1.78	21	0.5	0.013	0.330
77	1.78	22	0.5	0.013	0.315
7.7	1.78	22	0.5	0.0125	0.303
7.8	1.78	21	0.5	0.012	0.305
7.8	1.78	21	0.5	0.011	0.280
7.9	1.78	22	0.5	0.013	0.315
7.9	1.78	22	0.5	0.014	0.340
8.0	1.78	22	0.5	0.015	0.364
8.0	1.78	22	0.5	0.014	0.340
8.1	1.78	22	0.5	0.015	0.364
8.1	1.78	22	0.5	0.0155	0.376
8.2	1.78	22	0.5	0.016	0.388
8.2	1.78	22	0.5	0.0162	0.392
8.3	1.78	22	0.5	0.017	0.412
8.3	1.78	22	0.5	0.0168	0.408
8.4	1.78	22	0.5	0.018	0.437
8.4	1.78	22	0.5	0.0175	0.435
8.5	1.78	22	0.5	0.017	0.412
8.5	1.78	22	0.5	0.018	0.439

TABLE 45 (contd.)

pH	Conc.x 10^4 [HbSH]	Volume of the aliquot after the reaction	Volume of 0.01 M MMTS Used	Volume of 0.095 M NaOH Used	$\Delta h^+/SH$
8.6	1.78	22	0.5	0.019	0.461
8.6	1.78	22	0.5	0.020	0.485
8.7	1.78	22	0.5	0.0205	0.497
8.7	1.78	22	0.5	0.021	0.509
8.8	1.78	22	0.5	0.022	0.534
8.8	1.78	22	0.5	0.023	0.558
8.9	1.78	22	0.5	0.025	0.606
8.9	1.78	22	0.5	0.026	0.631
9.0	1.78	22	0.5	0.030	0.728
9.0	1.78	22	0.5	0.032	0.776
9.1	1.78	22	0.5	0.032	0.776
9.1	1.78	22	0.05	0.033	0.801
9.2	1.78	22	0.5	0.034	0.825
9.2	1.78	22	0.5	0.033	0.801
9.3	1.78	22	0.5	0.035	0.849
9.3	1.78	22	0.5	0.036	0.873
9.4	1.78	22	0.5	0.037	0.898
9.4	1.78	22	0.5	0.038	0.922
9.5	1.78	22	0.5	0.039	0.946
9.5	1.78	22	0.5	0.0385	0.934
9.6	1.78	22	0.5	0.038	0.922
9.6	1.78	22	0.5	0.0385	0.934

TABLE 46

Proton release ($\Delta\overset{+}{h}$) per β^{93} Thiol group as a function of pH for methyl methanethiosulphonate reacting with oxyhemoglobin A. [O_2HbA] = 10 μM Heme: 0.01 M MMTS at 20°C I = 0.05.

pH	$\Delta\overset{+}{h}$/SH	pH	$\Delta\overset{+}{h}$/SH
5.6	0.068±0.020	7.2	0.667±0.06
5.7	0.095±0.003	7.3	0.650±0.08
5.8	0.117±0.020	7.4	0.744±0.03
5.9	0.291±0.110	7.5	0.582±0.11
6.0	0.318±0.13	7.7	0.840±0.01
6.1	0.282±0.06	7.9	0.810±0.01
6.2	0.308±0.04	8.0	0.855±0.01
6.3	0.396±0.12	8.1	1.003±0.13
6.4	0.459±0.06	8.2	1.013±0.01
6.5	0.590±0.05	8.3	1.080±0.01
6.6	0.633±0.05	8.4	1.125±0.01
6.7	0.710±0.12	8.5	1.1475±0.02
6.8	0.700±0.06	8.6	1.152±0.01
6.9	0.932±0.02	8.7	1.170±0.00
7.0	0.931±0.12	8.8	1.0505±0.00
7.1	0.771±0.06	8.9	1.0365±0.01
		9.0	1.269±0.00
		9.2	1.375±0.00
		9.3	1.422±0.00
		9.4	1.515±0.00
		9.5	1.572±0.00
		9.6	1.603±0.00

TABLE 47

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanethiosulphonate reacting with carbonmonoxyhemoglobin A [COHbA] = 10μM : 0.01 M MMTS at at 20°C I = 0.05.

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.282±0.01	7.5	0.854±0.01
5.7	0.180±0.02	7.6	0.710±0.09
5.8	0.200±0.01	7.7	0.700±0.06
5.9	0.152±0.047	7.8	0.77± 0.17
6.0	0.201±0.038	7.9	0.724±0.01
6.1	0.250±0.050	8.0	1.075±0.10
6.2	0.263±0.037	8.1	0.92±0.02
6.3	0.300±0.01	8.2	0.882±0.08
6.4	0.300±0.02	8.3	0.926±0.07
6.5	0.424±0.10	8.4	1.124±0.01
6.6	1.40±0.01	8.5	1.076±0.07
6.7	1.593±0.11	8.6	1.311±0.15
6.8	1.767±0.12	8.8	1.115±0.07
6.9	1.687±0.20	8.9	1.246±0.07
7.0	1.43±0.03	9.0	1.32±0.08
7.1	1.07±0.07	9.1	1.448±0.01
7.2	0.90±0.01	9.2	1.340±0.02
7.3	1.05±0.01	9.3	1.488±0.01
7.4	0.94±0.02	9.4	1.58±0.02
		9.5	1.64 ±0.02
		9.6	1.60±0.02

TABLE 48

Proton release (Δh^+) per β^{93} sulphydryl group as a function of pH for methyl methanethiosulphonate reacting with Deoxygenated Hemoglobin A [4×10^{-4}M] + 0.01 M MMTS at 20°C I = 0.05

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.175±0.13	7.7	1.0.17±0.00
5.7	0.328±0.10	7.8	1.005±0.03
5.8	0.447±0.05	7.9	1.087±0.04
5.9	0.423±0.08	8.0	1.013±0.01
6.0	0.565±0.03	8.1	1.038±0.00
6.1	0.637±0.02	8.2	1.211±0.18
6.2	0.676±0.00	8.3	1.207±0.04
6.3	0.760±0.00	8.4	1.417±0.04
6.4	0.899±0.00	8.5	1.140±0.00
6.5	0.826±0.01	8.6	1.216±0.10
6.7	0.968±0.07	8.7	1.264±0.01
6.8	1.322±0.02	8.8	1.443±0.08
6.9	1.534±0.09	8.9	1.384±0.14
7.0	1.844±0.13	9.01	1.380±0.13
7.1	1.936±0.06	9.1	1.486±0.00
7.2	1.484±0.12	9.3	1.205±0.06
7.3	1.160±0.21	9.4	1.531±0.16
7.4	0.999±0.17	9.5	1.573±0.15
7.5	0.890±0.02	9.6	1.665±0.00
7.6	0.797±0.00		

TABLE 49

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanethiosulphonate reacting with Azidomet Hemoglobin A [4×10^{-4} M] + 0.01 M MMTS at 20°C I = 0.05

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.304 ±0.05	7.7	1.347 ±0.01
5.7	0.421 ±0.02	7.8	1.214 ±0.01
5.8	0.551 ±0.02	7.9	1.214 ±0.01
5.9	0.632 ±0.01	8.0	1.276 ±0.04
6.0	0.643 ±0.02	8.1	1.410 ±0.03
6.1	0.757 ±0.03	8.2	1.457 ±0.04
6.2	0.750 ±0.01	8.3	1.519 ±0.04
6.3	0.922 ±0.03	8.4	1.590 ±0.02
6.4	0.911 ±0.01	8.5	1.578 ±0.01
6.5	1.102 ±0.01	8.6	1.478 ±0.01
6.6	1.180 ±0.06	8.7	1.605 ±0.01
6.7	1.224 ±0.05	8.8	1.670 ±0.01
6.9	1.281 ±0.01	8.9	1.897 ±0.06
7.0	1.284 ±0.05	9.0	1.981 ±0.05
7.1	1.083 ±0.05	9.1	2.224 ±0.35
7.2	0.950 ±0.08	9.2	2.220 ±0.03
7.3	1.135 ±0.08	9.3	2.506 ±0.03
7.4	1.150 ±0.06	9.4	2.492 ±0.03
7.5	1.308 ±0.01	9.5	2.528 ±0.01
7.6	1.217 ±0.01	9.6	2.622 ±0.02

TABLE 50

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanethiosulphonate reacting with aquomethemoglobin A [4×10^{-4} M] + 0.01 M MMTS at 20°C I = 0.05.

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.440±0.028	7.7	1.175±0.082
5.7	0.667±0.064	7.8	1.080±0.010
5.8	0.787±0.023	7.9	1.076±0.003
5.9	0.938±0.056	8.0	1.158±0.055
6.0	0.878±0.072	8.1	1.193±0.027
6.1	1.007±0.018	8.3	1.265±0.053
6.2	1.061±0.098	8.4	1.224±0.033
6.3	1.034±0.059	8.5	1.288±0.028
6.4	1.198±0.010	8.6	1.288±0.002
6.5	1.487±0.031	8.7	1.355±0.067
6.6	1.837±0.103	8.8	1.311±0.064
6.7	1.929±0.145	8.9	1.514±0.117
6.8	1.723±0.098	9.0	1.451±0.054
6.9	1.169±0.066	9.2	1.512±0.030
7.0	0.948±0.063	9.3	1.624±0.007
7.1	0.952±0.014	9.4	1.765±0.060
7.4	1.029±0.002	9.5	1.828±0.003
7.5	1.155±0.128	9.6	1.889±0.015
7.6	1.169±0.005		

TABLE 51

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanethiosulphonate reacting with oxyhemoglobin (Horse) OxyHb [4×10^{-4}M]+0.01 M MMTS at 20°C, I = 0.05.

pH	Δh^+/SH	pH	Δh^+/SH
5.6	0.072±0.03	7.7	1.350±0.00
5.7	0.184±0.002	7.8	1.543±0.013
5.8	0.608±0.07	7.9	1.625±0.00
5.9	0.593±0.05	8.0	1.658±0.033
6.0	0.580±0.07	8.1	1.773±0.013
6.1	0.595±0.07	8.2	1.623±0.27
6.2	0.948±0.14	8.3	2.018±0.013
6.3	1.035±0.00	8.4	2.098±0.07
6.4	1.285±0.07	8.5	2.213±0.02
6.5	1.490±0.00	8.6	2.315±0.015
6.6	2.098±0.07	8.7	2.403±0.033
6.7	2.503±0.07	8.8	2.320±0.00
6.8	2.705±0.00	8.9	2.390±0.02
6.9	2.908±0.07	9.0	2.435±0.00
7.0	2.705±0.00	9.1	2.470±0.04
7.1	2.500±0.07	9.2	2.570±0.00
7.2	2.355±0.04	9.3	2.653±0.013
7.3	2.165±0.00	9.4	2.705±0.00
7.4	2.048±0.02	9.5	2.846±0.00
7.5	2.003±0.06	9.6	3.043±0.07
7.6	1.558±0.07		

TABLE 52

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methomethanethiosulphonate reacting with carbonmonoxyhemoglobin (horse) CoHb Horse [4×10^{-4}M]+ 0.01 M MMTS at 20°C, I = 0.05

pH	Δh^+/SH	pH	Δh^+/SH
5.6	0.320±0.06	7.7	2.315±0.02
5.7	0.593±0.05	7.8	2.075±0.10
5.8	0.879±0.07	7.9	2.368±0.07
5.9	1.151±0.07	8.0	2.773±0.07
6.0	1.489±0.14	8.1	2.905±0.07
6.1	1.692±0.07	8.2	2.840±0.00
6.2	1.828±0.07	8.3	2.903±0.06
6.3	2.03 ±0.00	8.4	3.015±0.31
6.4	2.37±0.07	8.5	2.705±0.07
6.5	2.64±0.07	8.6	2.958±0.18
6.6	2.505±0.07	8.7	2.965±0.00
6.7	2.710±0.00	8.8	2.843±0.02
6.8	2.605±0.04	8.9	3.120±0.07
6.9	2.775±0.07	9.0	3.213±0.11
7.0	2.965±0.07	9.1	3.230±0.00
7.2	3.048±0.07	9.2	3.215±0.00
7.3	2.840±0.00	9.3	3.398±0.06
7.4	2.640±0.07	9.4	3.338±0.12
7.5	2.450±0.02	9.5	3.585±0.00
7.6	2.368±0.07	9.6	3.646±0.06

TABLE 53

Proton release ($\Delta\overset{+}{h}$) per β^{93} thiol group as a function of pH for methyl methanethiosulphate reacting with deoxyhemoglobin, Horse Deoxy Hb Horse [0.4 mM] +0.10 M MMTS at 20°C I = 0.05.

pH	$\Delta\overset{+}{h}$/SH	pH	$\Delta\overset{+}{h}$/SH
5.6	0.391±0.065	7.7	1.071±0.029
5.7	0.625±0.10	7.8	0.920±0.02
5.8	0.667±0.05	7.9	0.950±0.01
5.9	0.950±0.05	8.0	1.026±0.01
6.0	1.302±0.05	8.1	1.080±0.02
6.1	1.511±0.05	8.2	1.170±0.03
6.2	1.750±0.05	8.3	1.260±0.02
6.3	2.142±0.06	8.4	1.320±0.02
6.4	2.344±0.05	8.5	1.364±0.04
6.5	2.604±0.10	8.6	1.456±0.016
6.6	3.001±0.08	8.7	1.540±0.02
6.7	3.172±0.02	8.8	1.660±0.02
6.8	3.281±0.05	8.9	1.778±0.02
6.9	3.542±0.10	9.0	2.012±0.09
7.0	3.667±0.02	9.1	2.220±0.02
7.1	3.648±0.05	9.2	2.300±0.02
7.2	3.059±0.34	9.3	2.330±0.01
7.3	2.344±0.05	9.4	2.395±0.035
7.4	2.050±0.05	9.5	2.366±0.03
7.5	1.686±0.09	9.6	2.530±0.01
7.6	1.406±0.05		

TABLE 54

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanethiosulphate reacting with azido-methemoglobin Horse. [N_3 MetHb] = 0.4 mM, 0.01 M MMTS 20°C, I = 0.05.

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.604±0.06	7.7	1.504±0.012
5.7	0.791±0.02	7.8	1.263±0.06
5.8	0.901±0.02	7.9	1.865±0.06
5.9	1.209±0.11	8.0	2.286±0.12
6.0	1.593±0.06	8.1	2.002±0.06
6.1	1.318±0.00	8.2	2.183±0.00
6.2	1.538±0.11	8.3	2.516±0.033
6.3	2.577±0.05	8.4	2.305±0.00
6.4	3.032±0.00	8.5	2.625±0.011
6.5	3.083±0.05	8.6	2.463±0.037
6.6	3.184±0.15	8.7	2.615±0.02
6.7	3.487±0.05	8.8	2.624±0.034
6.8	3.008±0.00	8.9	2.580±0.05
6.9	2.707±0.06	9.0	2.769±0.07
7.0	2.406±0.00	9.1	2.720±0.018
7.1	2.106±0.06	9.2	2.790±0.066
7.2	1.865±0.06	9.3	2.768±0.022
7.3	1.624±0.06	9.4	2.911±0.05
7.4	1.820±0.24	9.5	2.801±0.05
7.5	1.395±0.061	9.6	2.933±0.033
7.6	1.588±0.024		

TABLE 55

Proton release ($\Delta\bar{h}^{+}$) per β^{93} thiol group as a function of pH for methyl methanethiosulphate reacting with aquomethemoglobin Horse. [MetHb Horse] = 0.4 mM, 0.01 M MMTS I = 0.05.

PH	$\Delta\bar{h}^{+}$/SH	pH	$\Delta\bar{h}^{+}$/SH
5.6	0.655±0.06	7.7	2.182±0.12
5.7	0.893±0.06	7.8	2.297±0.10
5.8	0.964±0.01	7.9	1,263±0.12
5.9	1.012±0.06	8.0	2.214±0.00
6.0	1.048±0.02	8.1	2.167±0.10
6.1	1.107±0.04	8.2	2.292±0.00
6.2	1.226±0.04	8.3	2.699±0.06
6.3	1.489±0.06	8.4	2.848±0.02
6.4	1.846±0.36	8.5	2.871±0.02
6.5	1.976±0.00	8.6	2.940±0.02
6.6	2.048±0.02	8.8	2.912±0.03
6.7	2.179±0.04	8.9	2.984±0.02
6.8	2.286±0.02	9.0	3.008±0.02
6.9	2.774±0.20	9.1	2.948±0.06
7.0	3.445±0.00	9.2	2.998±0.01
7.1	3.571±0.01	9.3	2.929±0.06
7.3	3.848±0.006	9.4	3.159±0.06
7.4	3.618±0.17	9.5	3.193±0.02
7.5	2.756±0.12	9.6	3.251±0.01
7.6	2.355±0.06		

TABLE 56

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanethiosulphonate reacting to oxyhemoglobin (Rabbit). $[O_2Hb]$ = 0.4 mM; 0.01 M MMTS at 20°C, I = 0.05

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.392±0.02	7.7	2.553±0.15
5.7	0.528±0.02	7.8	1.265±0.09
5.8	0.746±0.19	7.9	1.475±0.060
5.9	1.225±0.02	8.0	1.268±0.09
6.1	1.108±0.04	8.2	1.293±0.06
6.2	1.343±0.06	8.4	1.430±0.03
6.3	1.597±0.06	8.5	1.450±0.02
6.4	1.727±0.19	8.6	1.828±0.38
6.5	2.556±0.00	8.7	1.735±0.00
6.6	2 685±0.13	8.8	2.003±0.13
6.7	3.861±0.28	8.9	1.803±0.07
6.8	3.865±0.13	9.0	2.000±0.00
6.9	4.536±0.13	9.1	2.068±0.07
7.0	4.568±0.07	9.2	2.120±0.070
7.1	4.603±0.07	9.3	2.363±0.11
7.2	3.963±0.13	9.4	2.415±0.02
7.3	4.135±0.14	9.5	2.668±0.133
7.4	3.735±0.00	9.6	2.735±0.07
7.5	3.468±0.13		
7.6	2.685±0.02		

TABLE 57

Proton release (Δh^+) per β^{93} thiolgroup as a function of pH for methyl methanethiosulphonate reacting with carbonxyhemoglobin (Rabbit) CoHb Rabbit [$4x10^{-4}$M]+ [MMTS] = [0.01 M] at 20°C I = 0.05.

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.581±0.15	7.7	3.558±0.07
5.7	0.778±0.05	7.8	3.453±0.07
5.8	1.273±0.04	7.9	2.908±0.07
5.9	1.453±0.00	8.0	2.030±0.00
6.0	1.563±0.04	8.1	2.098±0.07
6.1	1.818±0.07	8.2	2.325±0.00
6.2	2.060±0.12	8.3	2.398±0.07
6.3	1.873±0.07	8.4	2.325±0.15
6.5	2.566±0.07	8.5	2.543±0.07
6.6	3.398±0.07	8.6	2.615±0.00
6.7	3.536±0.07	8.7	2.688±0.07
6.8	3.952±0.07	8.8	2.47 ±0.15
6.9	3.605±0.00	8.9	2.688±0.07
7.0	4.575±0.08	9.0	2.659±0.02
7.1	4.785±0.07	9.1	2.760±0.00
7.2	4.785±0.07	9.2	2.690±0.03
7.3	4.575±0.08	9.3	2.660±0.00
7.4	4.723±0.36	9.4	2.760±0.00
7.5	3.850±0.08	9.5	2.818±0.03
7.6	3.703±0.07	9.6	2.843±0.03

TABLE 58

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanesulphonate reacting with Deoxyhemoglobin Rabbit. DeoxyHb Rabbit [0.4μM] + 0.01 M MMTS at 20°C , I = 0.05.

pH	$\Delta h^+/SH$	pH	$\Delta h^+/SH$
5.6	0.759±0.063	7.7	1.740±0.12
5.7	0.758±0.062	7.8	1.566±0.06
5.8	0.891±0.08	7.9	1.508±0.00
5.9	1.056±0.012	8.0	1.439±0.05
6.0	1.194±0.082	8.1	1.303±0.14
6.1	1.390±0.002	8.2	1.779±0.11
6.2	1.581±0.09	8.3	2.046±0.09
6.3	1.742±0.074	8.4	2.242±0.11
6.4	2.252±0.097	8.5	2.378±0.06
6.5	2.252±0.097	8.6	2.588±0.035
6.6	2.589±0.13	8.7	2.646±0.024
6.7	2.829±0.37	8.8	2.657±0.012
6.8	3.363±0.05	8.9	2.843±0.05
6.9	3.575±0.05	9.0	3.029±0.012
7.0	3.620±0.06	9.1	3.086±0.00
7.1	3.057±0.023	9.2	3.121±0.012
7.2	2.693±0.024	9.3	3.156±0.00
7.3	2.366±0.06	9.4	3.191±0.012
7.4	2.062±0.12	9.5	3.237±0.012
7.5	2.001±0.18	9.6	3.318±0.023
7.6	2.262±0.06		

TABLE 59

Proton release ($\Delta\overset{+}{h}$) per β^{93} thiol group as a function of pH for methyl methanethiosulphonate reacting with azidmethemoglobin Rabbit. AzidometHb Rabbit [0.4 mM] 0.01 M MMTS at 20°C, I = 0.05

pH	$\Delta\overset{+}{h}$/SH	pH	$\Delta\overset{+}{h}$/SH
5.6	0.290±0.06	7.7	1.553±0.03
5.7	0.464±0.00	7.8	1.462±0.06
5.8	0.638±0.06	7.9	1.638±0.06
5.9	1.031±0.06	8.0	1.723±0.03
6.0	1.189±0.03	8.1	1.856±0.04
6.1	1.208±0.06	8.2	1.953±0.01
6.2	1.271±0.00	8.3	2.050±0.01
6.3	1.638±0.06	8.4	2.159±0.02
6.4	1.880±0.06	8.5	2.123±0.06
6.5	2.123±0.06	8.6	2.366±0.06
6.6	2.305±0.00	8.7	2.523±0.02
6.7	2.426±0.00	8.8	2.730±0.06
6.8	2.244±0.06	8.9	3.093±0.06
6.9	2.487±0.06	9.0	3.639±0.00
7.0	2.244±0.06	9.1	3.943±0.06
7.1	2.002±0.06	9.2	4.015±0.01
7.2	1.880±0.06	9.3	4.149±0.03
7.3	1.771±0.07	9.4	4.258±0.01
7.4	1.638±0.06	9.5	4.343±0.03
7.5	1.614±0.04	96	4.403±0.04
7.6	1.575±0.05		

TABLE 60

Proton release (Δh^+) per β^{93} thiol group as a function of pH for methyl methanethiosulphonate reacting with aquomethemoglobin Rabbit. MetHb [0.4 mM] +0.01 M MMTS at 20°C I = 0.05.

pH	Δh^+/SH	pH	Δh^+/SH
5.6	0.782±0.06	7.7	1.985±0.06
5.7	1.083±0.12	7.8	1.865±0.06
5.8	1.504±0.06	7.9	1.685±0.12
5.9	1.925±0.12	8.0	1.624±0.06
6.0	2.406±0.00	8.1	1.805±0.00
6.1	2.250±0.23	8.2	1.889±0.04
6.2	2.286±0.12	8.3	1.985±0.06
6.3	2.467±0.06	8.4	2.094±0.03
6.4	2.647±0.00	8.5	1.985±0.04
6.5	2.743±0.02	8.6	2.106±0.06
6.6	2.828±0.06	8.7	2.274±0.13
6.7	3.309±0.18	8.8	2.346±0.06
6.8	4.031±0.06	9.0	2.467±0.06
6.9	4.753±0.06	9.1	2.641±0.02
7.0	4.993±0.05	9.2	2.814±0.06
7.1	4.572±0.00	9.3	2.848±0.03
7.2	4.271±0.06	9.4	2.906±0.01
7.3	3.91±0.06	9.5	2.983±0.01
7.4	3.670±0.06	9.6	3.009±0.01
7.5	2.823±018		
7.6	2.286±0.12		

TABLE 61

(Δh^+ DeoxyHb - Δh^+ OxyHb) per thiol group as a function of pH for methyl methanethiosulphonate reacting with hemoglobin species at 20°C I = 0.05.

pH	Human A	Horse	Rabbit
5.6	0.107	0.319	0.367
5.7	0.233	0.441	0.230
5.8	0.360	-	0.145
5.9	-	0.370	0.169*
6.0	0.247	0.722	0.100*
6.1	0.355	0.916	0.054
6.2	0.368	0.802	0.016*
6.3	0.364	1.107	0.145
6.4	0.440	1.059	0.232
6.5	-	1.114	0.304*
6.7	-	0.669	-
6.8	0.622	0.576	0.305*
6.9	0.602	0.635	0.960*
7.0	1.005	-	0.948*
7.1	1.165	-	1.546*
7.2	0.817	0.179	1.270*
7.3	0.510	-	1.759*
7.4	0.255	0.317*	1.673*
7.5	0.312	0.309*	1.468*
7.7	0.177	0.279*	0.813*
7.9	0.277	0.675*	0.033

TABLE 61 (contd.)

pH	Human A	Horse	Rabbit
8.0	0.158	0.632*	0.171
8.1	0.035	0.693	0.250
8.2	0.198	-	0.486
8.3	0.127	0.758*	0.600
8.4	-	0.778*	0.812
8.5	0.008	0.849*	0.928
8.6	0.064	0.859*	0.760
8.7	0.094	0.863*	0.911
8.8	0.333	0.660*	-
9.0	0.111	0.423	1.029
9.1	0.111	0.250	1.018
9.4	-	0.310	0.776
9.5	-	0.474	0.569
9.6	0.062	0.510	0.583

* indicates that Δh^+ oxyHb is greater than that of Δh^+DeoxyHb.

APPENDIX V

TABLE 62

Typical Results of Hydrolytic Cleavage of modified Hemoglobin at 20°C , I = 0.05 pH 9.2
Using 5,5'-dithiobis[2-nitrobenzoic acid]

Time(sec)	modified hemoglobin concentration x 10^{7}M
100	0.6
200	0.8
300	0.9
400	1.1
500	1.2
600	1.3
700	1.6
800	1.7
900	1.8
1000	1.9
1200	2.0
1300	2.2
1400	2.4
1500	2.6

Using a linear least square program
$k_{obs} = 5 \times 10^{-9}$ M s^{-1}

TABLE 63

Typical Results of Hydrolytic cleavage of modified Hemoglobin at 20°C, I = 0.05, pH = 9.2 Using 2,2'-dithiobis pyridine

Time (sec)	modified hemoglobin concentration x 10^{9} M
100	0.2
200	0.3
300	0.4
400	0.6
500	0.8
600	0.9
700	1.0
800	1.2
900	1.3
1000	1.5
1200	1.7
1300	1.8
1400	1.9
1500	2.1

Using a linear least square program

$k_{obs} = 1.4 \times 10^{-11} M\ s^{-1}$.

TABLE 64

Dependence of the apparent second-order rate constant, k_{app}, on pH for the reaction 2,2'-dithiobispyridine with human hemoglobin A derivatives at 20°C I = 0.05

pH	O_2HbA $k[M^{-1}s^{-1}]$	CoHbA $k[M^{-1}s^{-1}]$	N_3MetHbA $k[M^{-1}s^{-1}]$	MetHbA $k[M^{-1}s^{-1}]$
9.2	14.47±0.10	15.53±0.08	10.35±0.06	15.75±2.76
9.4	15.35±0.08	16.44±0.02	11.74±0.08	18.91±0.25
9.6	16.84±0.04	16.93±0.02	13.90±0.04	22.62±0.30
9.8	17.10±0.10	19.75±0.10	16.28±0.04	24.11±0.04
10.0	18.44±0.08	20.81±0.07	17.48±0.02	32.20±0.11

APPENDIX VI

DERIVATION

$$k_{(app)} = k_{ext}.F.\frac{K_{ext}}{K_{ext}+[H^+]} + k_{int}(1-F).\frac{K_{int}}{K_{int}+[H^+]}$$

The CysF9(93)β sulphydryl group of hemoglobin exists in two conformational states at equilibrium:

sulphydryl external $\rightleftharpoons$ sulphydryl internal ...(1)

The rate constant, k_{ext}, for the reaction of 2,2'-dithiobispyridine in the external conformational state differs from that in the internal conformational state, k_{int}. Let the fractional population of the external conformational state be F; that of the thiol group in the internal conformational state is therefore (1-F).

Assuming that the sulphydryl group is fully ionized, the apparent second order rate constant is the weighted sum of k_{ext} and k_{int}.

i.e. $k_{app} = k_{ext}.F + k_{int}(1-F)$ ... (2)

However, the sulphydryl group is in fact not fully ionized in the pH range under study. Only the thiolate anion is reactive. The fraction of the sulphydryl in the anionic form may be derived as follows. The equilibrium between the neutral and thiolate anion forms is

$$SH \overset{K}{\rightleftharpoons} S^- + H^+ \qquad \ldots (3)$$

where K is the ionization constant.

$$K = \frac{[S^-][H^+]}{[SH]} \qquad \ldots (4)$$

$$\frac{[H^+]}{K} = \frac{[SH]}{[S^-]} \qquad \ldots (5)$$

$$\frac{[H^+]}{K} + 1 = \frac{[SH]}{[S^-]} + 1 \qquad \ldots (6)$$

$$\frac{[H^+] + K}{K} = \frac{[SH] + [S^-]}{[S^-]} \qquad \ldots (7)$$

Reciprocalize equation (7), it becomes

$$\frac{K}{[H^+] + K} = \frac{[S^-]}{[SH]+[S^-]} \qquad \ldots (8)$$

Since the sulphydryl group exists in two conformational states, equation (2) can be modified by multiplying the rate constant in the external and the internal conformational states by $\frac{K_{ext}}{K_{ext} + [H^+]}$ and $\frac{K_{int}}{K_{int}+[H^+]}$, respectively.

Equation (2) can be rewritten as

$$k_{(app)} = k_{ext}\, F. \frac{K_{ext}}{K_{ext}+[H^+]} + k_{int}(1-F) \frac{K_{int}}{K_{int}+[H^+]} .$$