Equilibrium and Kinetic Studies of the Reaction of Aquomet Derivative of Pigeon Haemoglobin with 5,5[1] Dithiobis (2-Nitrobenzoic Acid).

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ABSTRACT

The sulphydryl groups Cysf9(93)β and Cysβ5(23)β present in the aquomet derivative of pigeon haemoglobin were investigated for their equilibrium and kinetic parameters by monitoring their reaction with 5,5¹- dithiobis (2-nitrobenzoic acid) (DTNB) with the aid of a UV-visible spectrophotometer. At specific pH (5.7-9.0) the absorbances of mixtures of varying DTNB concentrations (0.015mM-0.34mM) and a fixed concentration of aquomet haemoglobin (50mM haem) thermostated at 25ºC was measured at a wavelength of 412nm. Data was analyzed with the aid of a computer program written on Micromaths scientist software. Equilibrium constant Ke_q varied between 17.3 and 0.02 for both sulphydryl groups reflecting a decrease by almost three orders of magnitude between pH 5.7 and 9.0. The apparent second order reverse rate constant (k_r) for the reaction of the sulphydryl groups with DTNB was calculated to be 26.29 to 63,516 $dm^3mol^{-1}sec^{-1}$ and 4.31 to 1358.203$dm^3mol^{-1}sec^{-1}$ between pH 5.7-9.0 for Cysf9(93)β and Cysβ5(23)β, respectively.

Elucidation of the apparent second order reverse rate constant (k_r) which has not been feasible from kinetic experiments was made possible from this study. A quantitative assessment of the pH-dependence profile shows that k_r increases by almost three orders of magnitude for Cysf9(93)β and two orders of magnitude for Cysβ5(23)β between the pH range studied indicating higher reactivity for Cysf9(93)β. This information is vital for a comprehensive understanding of the kinetics and reactivity of the sulphydryl groups in pigeon haemoglobin (aquomethaemoglobin).

(Keywords: aquomethaemoglobin, pigeon, DTNB, equilibrium constants, *Cysf9(93)β, Cysβ5(23)β*)

INTRODUCTION

Haemoglobin is a respiratory pigment present in the erythrocytes of most vertebrates. It transports oxygen from the lungs to other tissues of the body. Haemoglobin has the distinct characteristics of reversibly binding oxygen without alteration of the ferrous state of the iron (White 1997). *In vitro* studies have shown that haemoglobin can be a broad monoxygenase catalyst, exhibiting the property of a monoxygenase enzyme. Thus catalysis by haemoglobin display typical Michaelis- Menton kinetics, depending on the native protein (Mieyal and Starke 1994).

The side chain in cysteine residue of haemoglobin has a sulphydryl group (SH). Reactive oxygen species can directly affect the conformation and activity of sulphydryl containing proteins by oxidation of their thiol moiety (Barcroft 1925). Due to the reactivity of the sulphydryl groups, they are most studied extensively of all the amino acid residues. (Antonini and Brunori 1971, Taketa et al. 1980, Okonjo et al. 1995, 1996, and Okonjo and Nwozo 1997).

The number of sulphydryl groups has been determined by spectroscopic titration with sensitive reagents and this has been found to be generally less than the total number of the cysteines in the molecule as determined from the amino acid sequence because some thiols(sulphydry groups) are masked at a subunit interface or hidden in a hydrophobic region not accessible to reagents. The number of titratable sulphydryl groups in haemoglobin depends on the thiol reagent used (Okonjo et al., 1979). Mercurial reagents titrate all the free thiol groups in haemoglobin, whereas non mercurial reagents titrate only those thiols which can form the thiolate

ion. For example, the major chicken haemoglobin has ten sulphydryl groups (Kleinschmidt and Sgourous, 1987) of which eight are titratable with P-Chloromercuri(11)benzoate (PCMB) in the intact haemoglobin. Only four of these are titratable with 5,5[1]- dithiobis (2-nitrobenzoic acid, (DTNB) a non-mercurial reagent (Okonjo and Nwozo, 1997 and Antonini and Brunori, 1971).

Ten sulphydryl groups are present in pigeon haemoglobin, four of these are titratable with DTNB, while eight are titratable with P-MB. The locations are at [a]Cysf9(93)β, [a]Cysβ5(23)β, (sulphydryl groups titratable with all sulphydryl reagents), [b]CysH4(126)β, [b]CysH13(130)α (sulphydryl groups titratable with only organic mercurials) and [c]CysG11(104)α (masked sulphydryl groups).

Different animal haemoglobins have different numbers of sulphydryl groups, with the exception of tadpole haemoglobin; all haemoglobins investigated so far contain cysteines (Jocelyn 1972). A similar work into the equilibrium study of the oxy-haemoglobin of pigeon with DTNB has been previously reported (Akpoveta and Osakwe, 2008). The results enabled the determination of the apparent second order reverse rate constant (kr) which has not been experimentally feasible from kinetic studies. This finding prompted the present research into the equilibrium determination of the reaction of aquomet derivative of pigeon haemoglobin with DTNB so as to ascertain if the elucidation of Kr will also be possible. The mechanism of reactions which is by thiol-disulphide exchange is shown in Figure 1.

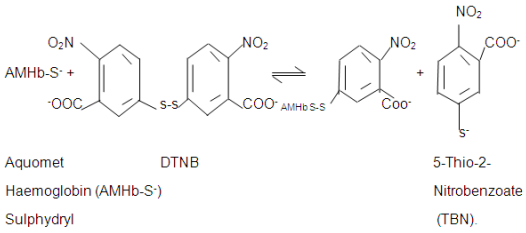

Figure 1: Mechanism of Thio-Disulphide Exchange.

EXPERIMENTAL METHOD

Preparation of Reagents

Standard Buffer Solution (SBS): SBS of pH 9.22 ± 0.02 and pH 4.00 ± 0.02 were prepared by dissolving one tablet of each with distilled water in 100ml flask and making up to the mark.

Phosphate Buffers: 0.4M NaOH and 0.4M NaH_2PO_4 were prepared by dissolving 16g and 62.4g of each respectively in a one liter standard volumetric flask and making up to the mark with distilled water. The phosphate buffer solutions of ionic strength 0.05M at specific pH were prepared by mixing specified amounts of 0.4M NaOH, 0.4M NaH_2PO_4 and NaCl crystals and then making up to the mark with distilled water in a one liter volumetric flask. The pH of each buffer solution was checked on a radiometer type PHM 85 precision pH meter which had been earlier standardized with standard buffer solutions of pH 4.00 ± 0.02 and 9.22 ± 0.02.

Borate Buffer Solutions (0.05M, pH 8.0-9.0): 0.3M NaOH and 0.3M boric acid were prepared by dissolving 12g and 18.55g of their pellets respectively with distilled water and making up to one liter volumetric flask. Borate buffer solutions of specific pH were prepared by mixing specified amounts of the stock 0.3M NaOH, 0.3M boric acid and NaCl with distilled water in a one liter volumetric flask after which the pH of each buffer was confirmed on a pH meter.

5,5[1] dithiobis (2-nitrobenzoic Acid) (DTNB): A 50mM solution of DTNB was prepared by dissolving 0.4954g of DTNB reagent in 95% ethanol and making up to 25ml with it. 25ml of 0.2M NaH_2PO_4 was prepared by dissolving 0.78005g of the salt (f.w=156.01, $NaH_2PO_4.2H_2O$) in distilled water and making it up to 25ml. 50ml Na_2HPO_4 was also prepared by dissolving 1.4196g of the anhydrous salt (f.w=141.96) in distilled water and making it up. 50ml Na_2HPO_4 was then titrated with 1.5ml NaH_2PO_4 to pH 7.989. 17ml of 0.2M phosphate buffer (pH 7.989) was added to the 25ml 50mM D.T.N.B solution to give a solution of pH 6.58. The concentration of the solution was 29.07mM.

Other Reagents: Acid Citrate Dextrose Anticoagulant (ACD), saline solution, and dialyzing solution were prepared using standard laboratory procedures (Beetlestone, 1975).

Preparation of Dintzis Column: Dintzis column which is used for deionizing haemoglobin was prepared by packing a fixed bed of different resins (Figure 2). The packed resins were hydrogen form resin, acetate form resin and Ammonium form resin (Dintzis, 1952).

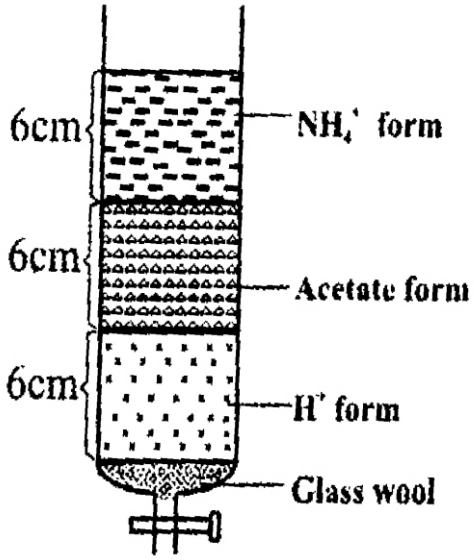

Figure 2: Dintzis Column.

Preparation of Aquomet-Haemoglobin: Pigeon birds were slaughtered and their blood drained into a beaker containing 80cm^3 of acid citrate dextrose anticoagulant. Standard haemoglobin laboratory procedures were then followed for the preparation of oxyhaemoglobin (Beetlestone, 1975). 0.33g of potassium ferricyanide was dissolved in 1ml distilled water to give approximately 1M solution. Two fold excess of the freshly prepared potassium ferricyanide was added to a known concentration of oxyhaemoglobin. The volume of 1M $K_3fe(CN)_6$ required was calculated using the equation:

$$v = 2MV$$

where,

v = Volume of 1M $K_3fe(CN)_6$
M= Concentration of oxyhaemoglobin.
V = Volume of oxyhaemoglobin

The dark brown oxidation product, aquomet-haemoglobin was then deionized by passing it through the Dintzis column to remove organic phosphate and excess potassium ferricyanide.

Determination of Aquomet-Haemoglobin Concentration: 0.1ml of aquomet-haemoglobin was added to 10ml of distilled water. A few crystals of recrystallized potassium cyanide were then added. The absorbance of the resulting solution was taken at 540nm using a Zeiss MH Q11 spectrophotometer. The concentration was determined in moles per haem using the equation:

$$C = \frac{A_{540}}{\in \iota} \times \frac{V+v}{v}$$

where C = Concentration, A = Absorbance
$\in$ = Molar extinction coefficient
ι = Pathlenght
V = Volume of distilled water
v = Volume of aquomet-haemoglobin

The molar extinction was taken to be 1.09 x 10^4 M^{-1} (Austin and Drabkin 1935-36 and Drabkin and Austin, 1935-36).

Determination of Equilibrium Constant (Ke_q) for the Reaction of DTNB with the Sulphydryl Groups of Aquomet-Haemoglobin

10ml of 50µM(50µM in reactive sulphydryl groups) aquomet-haemoglobin solution in buffer of known pH, ionic strength 0.05M were measured into eighteen clean dry test tubes each. The volume and concentration of 29.07mM DTNB stock solution were varied between 5.0µl-120µl, 0.015mM-0.35mM and added to the different test tubes, stirred and left to equilibrate between 5 and 6 hours at 25ºc in a thermostated water bath.

The absorbance of each solution was read at 412nm on a Zeiss M4 Q11 UV – visible spectrophotometer. The concentration of 5-thio-2-nitrobenzoate (TNB) which is the product of the DTNB reaction was calculated from the change in absorbance, taken a molar absorption coefficient of 14000M^{-1} cm^{-1} for TNB. The equilibrium constant (Ke_q) for each test tubes were calculated using a program specially designed on the computer (Micromaths scientist software), and the average value of Ke_q for each pH was taken.

The pH of the resulting mixtures was taken at the end of each equilibration on the thermostat using a radiometer type PHM 85 precision pH meter. The procedure was repeated for all the phosphate ($5.6 \leq pH \leq 8.0$) and borate buffers ($8.0 \leq pH \leq 9.0$) with ionic strength of 0.05M. The negative logarithm to base ten of the equilibrium constants were calculated and plotted against their corresponding pH values.

RESULTS AND DISCUSSION

DTNB reacts only with the thiolate anion form of the sulphydryl group (Okonjo et al., 1995, Okonjo and Nwozo, 1971, Okonjo and Okia, 1993, and Okonjo and Adejoro, 1993). Its reaction is given as:

$$\text{AMHbSH} + \text{DTNB} \overset{K_{SH}}{\longleftrightarrow} \text{H}^+ + \text{AMHbS}^- + \text{DTNB} \overset{K_{eq}}{\longleftrightarrow}$$

$$\text{H}^+ + \text{AMHbS.TNB} + \text{TNB} \overset{K_{TNB}}{\longleftrightarrow} \text{AMHbS.TNB} + \text{TNBH}.$$

In the equation above, K_{SH} is the dissociation constant of the suphydryl group. K_{eq} is the equilibrium constant to be determined and K_{TNB} is the dissociation constant of $TNBH^+$. The equilibrium constant for the ionization of the sulphydryl group, K_{SH} is given by:

$$K_{SH} = \frac{[H^+][AMHbS][DTNB]_s}{[AMHbSH][DTNB]_s} = \frac{[H^+][AMHbS]_s}{[AMHbSH]_s} \quad \textbf{(1)}$$

$$K_{eq} = \frac{[H^+][AMHbS.TNB][TNB]}{[H^+](AMHbS^-]_s\,[DTNB]_s} = \frac{[AMHbS.TNB][TNB]}{AMHbS]_s\,[DTNB]_s} \quad \textbf{(2)}$$

$$K_{TNB} = \frac{[H^+][AMHbS.TNB][TNB]}{(AMHbS.TNB]\,[TNBH^+]} = \frac{[H^+][TNB]}{[TNBH^+]} \quad \textbf{(3)}$$

The subscript S denotes the unreacted species.
It has been shown that K_{eq} is given by the expression (Adebayo, 2005):

$$Keq = \frac{[TNB]^2\left\{1+\frac{[H^+]}{K_{TNB}}\right\}}{\left\{\frac{[AMHb]_t\text{-}TNB\left\{1+\frac{[H^+]}{K_{TNB}}\right\}}{1+\frac{[H^+]}{K_{SH}}}\right\}[DTNB]_t - [TNB]\left\{1+\frac{[H^+]}{K_{TNB}}\right\}}$$

$$= \frac{[TNB]^2\left\{1+\frac{[H^+]}{K_{TNB}}\right\}\left\{1+\frac{[H^+]}{K_{SH}}\right\}}{\left\{[AMHb]_t - [TNB]\left\{1+\frac{[H^+]}{K_{TNB}}\right\}\right\}\left\{[DTNB]_t - [TNB]\left\{1+\frac{[H^+]}{K_{TNB}}\right\}\right\}} \quad \textbf{(4)}$$

A knowledge of the total aquomet-haemoglobin concentration, total DTNB concentration and concentration of TNB formed at equilibrium will allow for the determination of the equilibrium constant (Keq) for the DTNB reaction from the equation above as long as the ionization constant of $TNBH^+$, K_{TNB} and K_{SH} are also known (Adebayo, 2005). pK_{SH} of the CysF9[93]β sulphydryl group ranges between 8 and 8.6 (Okonjo et al., 1996, Okonjo et al., 1995., and Okonjo and Okia, 1993).

A value of 8.3 was adopted for the purpose of the work. PK_{TNB} has been previously determined by Nwosu (2004). The value was 5.267 at 25^0C. The TNB concentration can be determined from the absorbance of each solution at 412nm using an extinction coefficient of 14,000.

Effect of pH on the Equilibrium Constant for the Reaction of Pigeon Aqomet-haemoglobin with DTNB

The effect of pH on the equilibrium constant for the reaction is shown on Table 1 and Figure 3, respectively. Figure 3 shows a plot of $-\log_{10}K_{eq}$ as a function of pH.

Table 1: Dependence of Negative Logarithm to base ten of the Equilibrium Constant, $-\log_{10}K_{eq}$ on pH for the Reaction of 5,5[1]-Dithiobis (Nitrobenzoic Acid) (DTNB) with Pigeon Aquomet-Haemoglobin at 25ºC.

pH	K_{eq}	$-\log_{10}K_{eq}$
5.75	17.319± 0.73	-1.24
5.85	13.110± 0.463	-1.12
6.10	6.43± 0.27	-0.81
6.29	3.81± 0.17	-0.58
6.46	1.912± 0.071	-0.28
6.60	1.306± 0.088	-0.12
6.81	0.786± 0.106	-0.10
7.04	0.442± 0.0211	0.36
7.16	0.324± 0.0211	0.49
7.38	0.21± 0.012	0.67
7.60	0.15±0.0074	0.82
7.79	0.088±0.0161	1.05
8.01	0.077±0.0026	1.11
8.22	0.06±0.0097	1.23
8.43	0.04±0.0022	1.36
8.60	0.032±0.0015	1.50
8.80	0.030±0.0011	1.51
9.99	0.028±0.0018	1.55

Conditions: *Phosphate buffers pH 5.6-7.9 and borate buffers 8.0-9.0 (ionic strength 0.05M, added salt, NaCl), stock DTNB concentration, 0.02907M, haemoglobin concentration, 50µM in reactive sulphydryl groups (50µM haem), volume of haemoglobin used, 10ml. Molar extinction of 1.4 x 10^4 M^1cm^1 was assumed for the 5-thio-2-nitrobenzoate (TNB) formed, pK_{SH} = 8.3, pK_{TNB} = 5.267.*

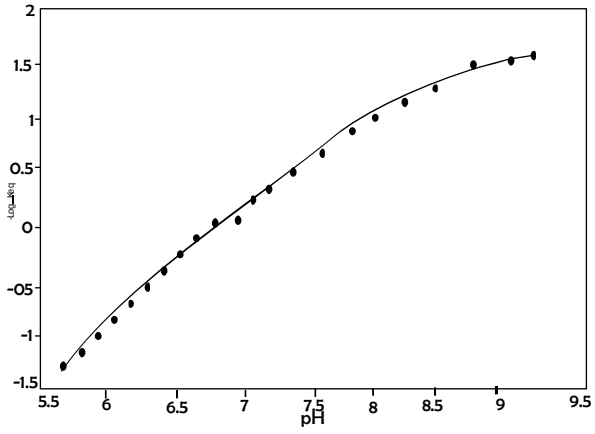

Figure 3: Dependence of Negative Logarithm to base ten of the Equilibrium Constant, $-\log_{10}K_{eq}$ on pH for the Reaction of 5,5[1]-Dithiobis (Nitrobenzoic Acid) (DTNB) with Pigeon Aquomet-Haemoglobin at 25ºC.

Conditions: *Phosphate buffers pH 5.6-7.9 and borate buffers 8.0-9.0 (ionic strength 0.05M, added salt, NaCl), haemoglobin concentration, 50µM in reactive sulphydryl groups, stock DTNB concentration. 0.02907 M in 0.2M phosphate buffer, pH 6.5; volume of stock DTNB used 5-120uL; 25ºC.*

K_{eq} varies strongly between pH 5.7 and 9 to about three orders of magnitude as seen in Table1. This variation was also observed when compared with the results obtained for the reaction of the oxy-haemoglobin of pigeon with DTNB (Akpoveta and Osakwe, 2008).

Dependence of the Apparent Second Order Reverse Rate Constant on pH

Only four sulphydryl groups are reactive towards DTNB, they are Cysf9(93)β and CysB5(23)β found on the two beta chains of the aquomet-haemoglobin structure, respectively. The apparent second order forward rate constant (k_f) for this reaction as a function of pH has been previously determined (Nwozo, 1999).

Since the apparent second order reverse rate constant cannot be determined experimentally, it is possible to calculate K_r if k_f and K_{eq} are known since $k_r=k_f/K_{eq}(dm^3mol^{-1}sec^{-1})$. k_r was calculated for the two different reacting sulfhydryl groups Cysf9(93)β and CysB5(23)β and their results presented in Tables 2 and 3.

kr increases by almost two orders of magnitude between pH 5.7 and 9.0 for the reaction of both sulphydryl groups. Their pH dependence profile was also found to be simple as evident in Figures 3 and 4.

Kr values obtained for the reaction of CysB5(23)β as compared to that for Cysf9(93)β shows that there was at least a six fold decrease in the reactivity of CysB5(23)β at pH 5.7 to over 40 fold decrease in the reactivity at pH 9.0. This indicates that the reactivity of CysB5(23)β appears to be slower than that of Cysf9(93)β. As such CysB5(23)β corresponds to the slow phase while Cysf9(93)β corresponds to the fast phase. A similar trend was also observed in the results obtained from the study of the reaction of the oxy-haemoglobin of pigeon with DTNB (Akpoveta and Osakwe, 2008) showing strong correlation and agreements in both studies.

Table 2: Dependence of the Apparent Second Order Forward Rate Constant (k_f), Equilibrium Constant (Keq) and Apparent Second Order Reverse Rate Constant (k_r) on pH for the Reaction of 5,5[1]-Dithiobis (Nitrobenzoic Acid)(DTNB) with CysF9(93)β of Pigeon Aquomet-Haemoglobin.

pH	k_f($dm^3mol^{-1}sec^{-1}$)	K_{eq}	$k_r=k_f/K_{eq}$ ($dm^3mol^{-1}sec^{-1}$)
5.6	455.4	17.32	26.3
5.8	455.4	13.11	13.1
6.0	599.4	6.43	93.2
6.2	707.9	3.81	185.8
6.4	692.4	1.912	362.2
6.6	580.1	1.306	444.2
7.0	952.4	0.442	2154.8
7.2	990.2	0.324	3056.2
7.4	992.1	0.12	4724.4
7.6	1159	0.15	7726.7
7.8	1311.3	0.088	14901.5
8.0	1477	0.077	19181.8
8.2	1577.7	0.06	26294.5
8.4	1436	0.04	35900
8.6	1430.5	0.032	45412.7
8.8	1600	0.031	51779.9
9.0	1703.5	0.027	63516

Table 3: Dependence of the Apparent Second Order Forward Rate Constant (k_f), Equilibrium Constant (Keq) and Apparent Second Order Reverse Rate Constant (k_r) on pH for the Reaction of 5,5[1]-Dithiobis (Nitrobenzoic Acid)(DTNB) with CysB5(23)β of Pigeon Aquomet-Haemoglobin.

pH	k_f($dm^3mol^{-1}sec^{-1}$)	K_{eq}	$k_r=k_f/K_{eq}$($dm^3mol^{-1}sec^{-1}$)
5.6	74.72	17.32	4.31
5.8	---	13.11	---
6	59.03	6.43	9.18
6.2	58.14	3.81	15.26
6.4	67.2	1.91	35.15
6.6	57.22	1.31	43.81
6.8	53.15	0.79	67.62
7	44.5	0.44	100.68
7.2	36.51	0.32	112.69
7.4	33.1	0.21	157.6
7.6	37.53	0.15	250.2
7.8	30.35	0.09	344.83
8	18.45	0.08	239.61
8.2	16.48	0.06	274.67
8.4	28.84	0.04	720.93
8.6	33.34	0.032	1058.32
8.8	33.35	0.031	1079.29
9	36.43	0.027	1358.20

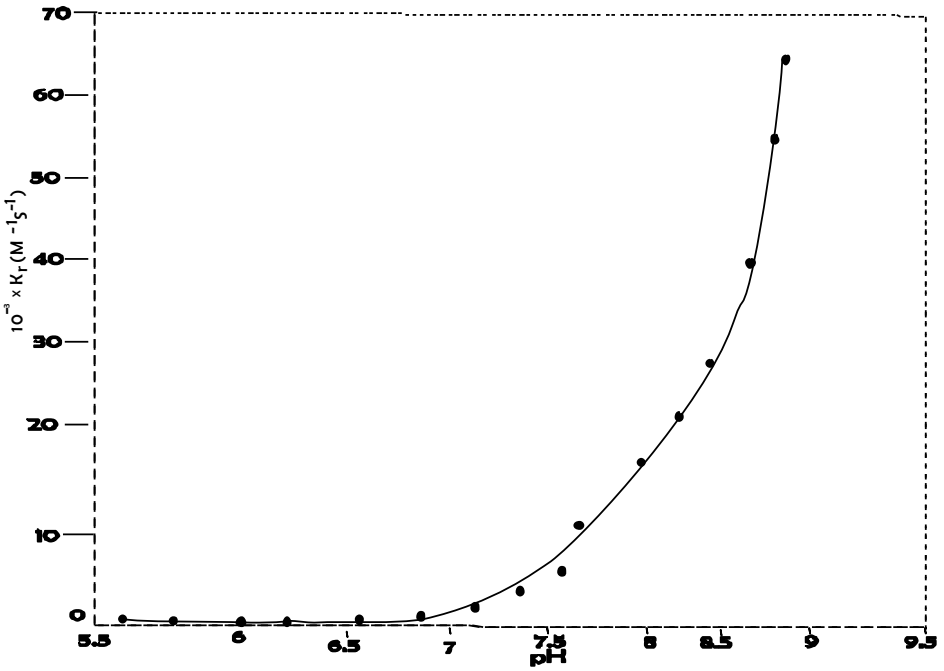

Figure 4: Dependence of K_r, the Apparent Reverse Second Order Rate Constant, on pH for the reaction CysF9 (93)β Sulphydryl Group of Pigeon Aguomet-haemoglobin with DTNB.

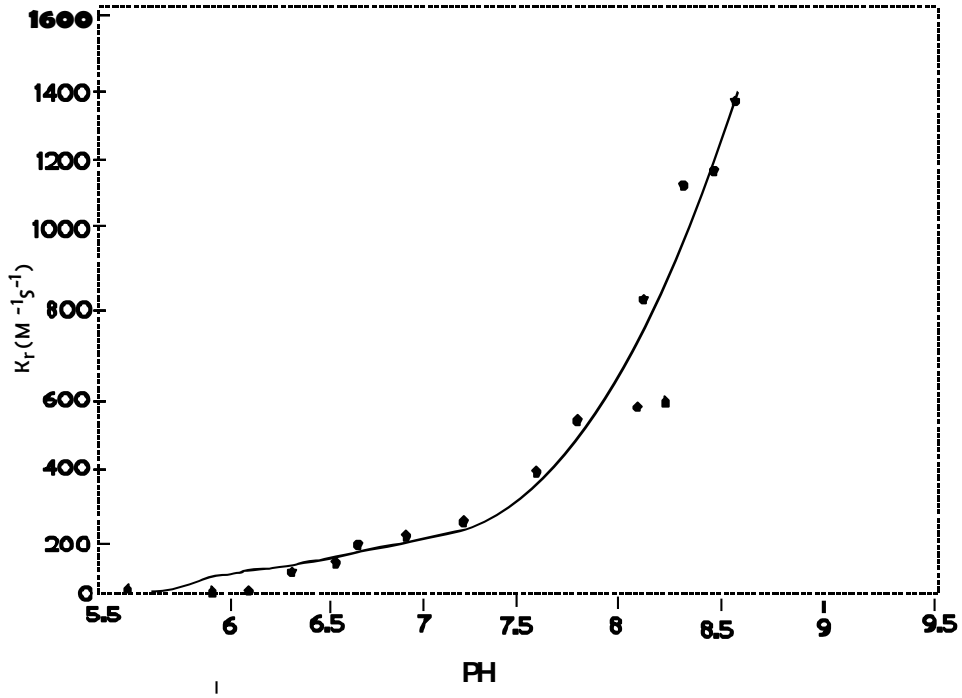

Figure 5: Dependence of K_r, the Apparent Reverse Second Order Rate Constant, on pH for the reaction CysB5(23)β Sulphydryl Group of Pigeon Aguomet-haemoglobin with DTNB.

CONCLUSION

Elucidation of the apparent second order reverse rate constant (k_r) which has not been feasible from kinetic experiments was made possible from this study. The results of equilibrium constant (K_{eq}) from this work and the apparent second order forward rate constant (k_f) from previous study enabled this breakthrough. A knowledge of the equilibrium constant (K_{eq}) also has the great benefit of enabling the determination of the apparent second order reverse rate constant (kr), without necessarily carrying out a concentration dependence of Kobs, the pseudo- first order rate constant (Pladziewicz et al., 1986). It could also be inferred that Cysf9(93)β showed higher reactivity than CysB5(23)β since higher k_r and k_f values were obtained over its entire pH range.

The findings of this work and those previously reported by Akpoveta and Osakwe (2008) indicates that Cysf9(93)β corresponds to the fast phase and shows more reactivity towards D.T.N.B as compared with CysB5(23)β.

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