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Research Note

Effect of Heme and Sulphydryl Reagents on Reactivity of the β-93 Sulphydryl Group of Bovine Apohemoglobin and Oxyhemoglobin

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In this paper, the pH-dependence of the second-order rate constant for the reaction of 5, 5'dithiobis (2-nitrobenzoic acid), DTNB with 2, 2'-dithiodipyridine, 2-DTP, with bovine apohemoglobin and oxyhemoglobin in the presence or absence of sodium n-dodecyl sulphate, SDS, has been studied.

The sulphydryl group of bovine apohemoglobin in native and denatured form reacts faster with DTNB and 2-DTP than that of bovine oxyhemoglobin, demonstrating that heme reduces the sulphydryl reactivity of hemoglobin. Generally, 2-DTP reacts faster with the sulphydryl group of denatured bovine apohemoglobin and oxyhemoglobin while DTNB reacts faster with native bovine apohemoglobin and oxyhemoglobin. This shows that unlike the native form where the hydrophobic moieties are buried within the molecule, in the denatured form the hydrophobic moieties are exposed which subsequently influences the reaction of 2-DTP. Quantitative analysis reveals that two ionizable groups are linked to the sulphydryl reaction in the native form, namely His HC3 (146) β and CysF9 (93) β while CysF9 (93) β is the modulating factor in the denatured form. pK_1 and pK_2 are assigned to HC3 (146) β and CysF9 (93) β, respectively.

INTRODUCTION

The sulphydryl reactivities of hemoglobins have been intensively studied [1-6], however, the effect of heme on the sulphydryl reactivity of apohemoglobin and oxyhemoglobin with 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 2, 2'-dithiodipyridine (2-DTP) in the presence or absence of sodium n-dodecyl sulphate (SDS) has not received considerable attention. Recently, the authors have reported the kinetics for the reaction of DTNB with denatured human hemoglobin A with multiple sulphydryl groups using SDS as a denaturant, where the reaction was biphasic at lower pH and triphasic at higher pH.

Bovine hemoglobin has two sulphydryl groups located on the β-chain at position F9 (93), therefore, there are no sulphydryl groups on the α-chain [7]. The CysF9 (93)β sulphydryl is invariant in virtually all hemoglobins. In this paper, comprehensive pH dependence studies have been conducted regarding the reaction of bovine apohemoglobin and oxyhemoglobin with DTNB and 2-DTP in the presence or absence of SDS. Furthermore, the pK_a values of the ionizable groups linked to the sulphydryl group are determined.

MATERIALS AND METHODS

Hemoglobin was prepared according to the normal laboratory procedures [8]. The acid-acetone method was carried out to obtain globin from hemoglobin. The HCl-acetone solution (6mM) was placed in a -20°C cooling bath, where the temperature was maintained using dry liquid air. Hemoglobin solution (2.5%) deionized by exhaustive dialysis was introduced drop by drop into the HCl-acetone solution. The mixture was vigorously stirred by a magnetic stirrer for 15 minutes. Globin precipitates; heme remains in the supernatant. Globin was obtained through centrifuge at -20°C for 20 minutes at 2000 r.p.m.. Then, the white precipitate was washed with freshly distilled acetone at -20°C, dissolved in cold distilled water and immediately lyophilized. It was stored in the cold room[9]. DTNB and 2-DTP were purchased from

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Sigma Chemical Company Ltd. and were used without any further treatment. SDS was purchased from BDH and used as supplied.

DTNB solution was prepared by dissolving 0.9907 g of DTNB in 0.05M phosphate buffer (pH = 7.6) in a 250 ml volumetric flask and the mixture was magnetically stirred for four days. The solution was then filtered and its concentration was determined through spectrophotometric measurement of the optical density at 412 nm after reacting it with an excess of mercaptoethanol. A molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ was assumed for 3-carboxylato 4-nitrothiophenolate [6]. A 0.01 M 2-DTP solution was prepared by dissolving 22 mg of 2-DTP in 20% ethanol in a 10 ml volumetric flask [1]. Its concentration was also determined spectrophotometrically at 281 nm in phosphate buffer (pH = 7.0, I = 0.2), using an absorptivity of 9.73 $mM^{-1}cm^{-1}$ for 2-thiopyridone [10].

DTNB KINETICS

The DTNB kinetic was studied on a Shimadzu computerized double beam UV-160 spectrophotometer at 27°C. Solutions of hemoglobin (10μM) were prepared in phosphate buffers (pH = 5.6 to 8.0) and borate buffers (pH > 8.0), each with total ionic strength of 0.05 M. The solutions were allowed to equilibrate at 27°C. A 1 ml aliquot of each solution was pipetted into a 1 × 1 cm cuvette which was subsequently placed in the cell compartment of the spectrophotometer. A few microliters of DTNB of known concentration was measured with a Finn pipette into a glass rod shaped in a shallow spoon rod. The rod was used to add the DTNB solution and to stir the DTNB-hemoglobin mixture. The absorbance of the mixture was monitored as a function of time at 412 nm. Each kinetic run was repeated twice under identical experimental conditions.

The same procedure was employed for the kinetics of the reaction of DTNB with bovine apohemoglobin at 27°C. The bovine apohemoglobin concentration was 5μM.

In the case of denaturation, each buffer solution contains 10 mM SDS which is the critical micelle concentration of SDS for proteins [11]. Hemoglobin solution [10 μM] was added to each buffer solution containing SDS. The hemoglobin solutions were allowed to equilibrate for an hour before employing the DTNB kinetic procedure. Apohemoglobin solutions [5μM] were prepared in buffer solutions containing SDS. Each kinetic run was repeated twice under identical experimental conditions. The concentration of DTNB in the cuvette ranged between 30 and 60 μM.

Apparent second order rate constants, k_{app}, were calculated using the second order rate equation. The molar absorption coefficients used in the calculations at 27°C have been reported before as a function of pH for 3-carboxylato 4-nitrothiophenolate [12], the product of the DTNB reaction.

2-DTP KINETICS

The change in absorbance was observed at 343 nm for the reaction of hemoglobin with 2-DTP in the presence or absence of SDS. The color produced is due to the formation of 2-thiopyridone. The same procedure was repeated for the reaction of 2-DTP with bovine apohemoglobin in the presence or absence of SDS. Each kinetic run was repeated twice under identical experimental conditions.

The apparent second order rate constants, k_{app}, were calculated using the second order rate equation. The molar absorption of 2-thiopyridone previously determined was used which is independent of pH and temperature [1]. The hemoglobin and apohemoglobin concentrations were 5 and 10 μM, respectively. The 2-DTP concentration ranged between 20 and 100 μM.

RESULTS AND DISCUSSION

Titration of bovine apohemoglobin and oxyhemoglobin with DTNB and 2-DTP in the presence or absence of SDS is limited to pH < 9, due to the fact that the rate of hydrolysis of disulphide bonds increases remarkably above pH 9 [13].

It is generally accepted that the reactions of sulphydryl groups of simple compounds and proteins are 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 any given sulphydryl reagent will depend on whether such a reagent is charged or not [1]. It has been suggested that the reactivity of the sulphydryl group depends on two factors, the conformation of the sulphydryl group and the electrostatic effects of the charged ionizable groups on the proteins as well as the pK of the sulphydryl group [1].

On the basis of these facts, the following expression can be tentatively considered for the apparent second order rate constant, k_{app}, for the reaction of the sulphydryl group with sulphydryl reagent [2]:

$$k_{app} = k_1 \frac{K_1}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]} \quad , \qquad (1)$$

where k_1 is the limiting apparent second-order rate constant at high pH for the sulphydryl group, which is linked to the ionization of imidazole with the ionization constant K_1 and k_2 is the limiting apparent second-order rate constant at high pH when the sulphydryl reactivity is linked to the ionization of cysteine with the ionization constant K_2. The first fractional term is the fraction of the neutral form of the histidine, while

the second fractional term is the fraction of the thiol anion form of the sulphydryl.

If the sulphydryl is the only modulating factor, Equation 1 may be expressed in a simpler form as:

$$k_{app} = k_2 \frac{K_2}{K_2 + [H^+]}. \quad (2)$$

Both sulphydryl reagents (DTNB and 2-DTP) exhibited satisfying second order kinetics with bovine apohemoglobin and oxyhemoglobin for all pH values studied at 27°C.

The pH-k_{app} profiles for the reaction of DTNB and 2-DTP with native bovine oxyhemoglobin at 27°C is shown in Figure 1. The reaction with the negatively charged DTNB is approximately three to five times faster than the uncharged reagent 2-DTP within the pH range of 5.6 to 7.8 and about two times faster than 2- DTP at pH > 7.8. The differences in rate between the two reagents show that positively charged groups are responsible for the increase in the reaction rate of DTNB.

The second-order rate constant shown in Figure 1 was compared with data presented in Table 1, within the pH range studied. Apohemoglobin is not soluble in buffer solutions at pH > 6.2. In spite of the shortcoming about solubility of apohemoglobin in buffer solutions, pH > 6.2, an interesting result is obtained within the pH range of 5.6 to 6.2.

High-resolution x-ray studies have revealed that the heme is inserted in a cleft between the E and F helices. The iron is linked covalently to the imidazole nitrogen of the proximal F8 histidine.

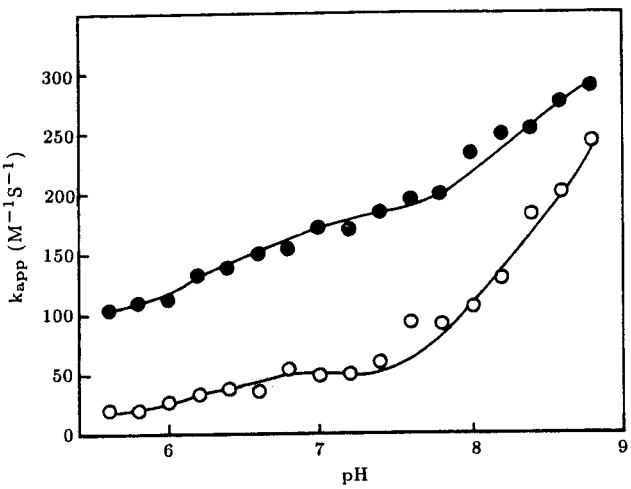

Figure 1. Reaction of sulphydryl reagents with CysF9 (93) β sulphydryl of bovine oxyhemoglobin, dependence of k_{app} on pH at 27°C. The standard error of each point is about 5%. The lines through the experimental points are best-fit lines calculated with Equation 1 (• = DTNB and o = 2-DTP).

Table 1. Reaction of DTNB and 2-DTP with bovine apohemoglobin, dependence of k_{app} on pH at 27°C. Each point is the mean of three determinations.

pH	DTNB $k_{app}(M^{-1}s^{-1})$	2-DTP $k_{app}(M^{-1}s^{-1})$
5.6	702 ± 13	528 ± 10
5.8	781 ± 13	625 ± 10
6.0	801 ± 15	666 ± 10
6.2	988 ± 10	749 ± 9

The heme is stabilized by a large number of interatomic contacts [14]. The removal from the bovine oxyhemoglobin by the acid-acetone method exposes the proximal and distal histidines to the solvent. The exposed histidines are: His E7 (58) α, His F8 (87) α, His E7 (63) β and His F8 (92) β. The rate of reaction for DTNB with apohemoglobin (Table 1) is approximately six to eight times faster than that of oxyhemoglobin (Figure 1), within the pH range studied. The observed differences in the reaction rate is due to free proximal and distal histidines which act as a catalyst. The reaction rate of 2-DTP with bovine apohemoglobin is approximately twenty times faster than that of oxyhemoglobin. This might be due to the increase in the number of non-polar amino acid residues initially linked to the heme that are exposed to the solvent. 2-DTP is relatively unaffected by the charges on the protein but is primarily influenced by changes in the pK of the thiol group or tertiary and quaternary structural changes in the R-state [1]. This result shows that 2-DTP is very reactive with sulphydryl group in a more hydrophobic environment while DTNB reacts faster in a hydrophilic environment. Reactivities of these reagents with denature apohemoglobin and oxyhemoglobin using SDS are also discussed in this paper.

Equations 1 and 2 were used to analyze the k_{app} versus pH profiles for the DTNB and 2-DTP using a non-linear parameter-optimization program [15,16]. The solid lines are theoretical best-fit lines obtained using Equation 1 for all the pH-k_{app} profiles, however Equation 2 showed a poor fit. It is clear that Equation 1 gives the best fit to the data in Figure 1. The fitting parameters are shown in Table 2. In the author's recent reports [2,3], pK_1 value is assigned to His HC3 (146) β and pK_2 value to CysF9 (93) β, therefore, the results are in good agreement with earlier pK_a values reported. The pK_1 5.6 or 6.0 is assigned to His HC (146) β and pK 8.6 is assigned to CysF9 (93) β for the two sulphydryl reagents.

Table 2. Reaction of DTNB and 2-DTP with bovine oxyhemoglobin. Best-fit parameters employed for fitting the data in Figure 1 with Equation 1.

	$k_1(M^{-1}s^{-1})$	$k_2(M^{-1}s^{-1})$	pK_1	pK_2
DTNB	173	178	5.6	8.5
2-DTP	48	321	6.0	8.6

Figures 2 and 3 show that the reaction rate of 2-DTP with denatured bovine apohemoglobin and oxyhemoglobin is approximately eight to eleven times faster than that of DTNB. These differences reflect the characteristic of the sulphydryl reagents. Earlier, it was stated that DTNB is charge sensitive unlike 2-DTP, therefore, 2-DTP will react faster with sulphydryl group in a more hydrophobic environment than that with DTNB. It should be noted that SDS is an anionic surfactant that denatures protein and interacts with positively charged groups of the protein [11]. In this case, SDS must have ruptured and broken the hydrogen bonds and salt bridges holding the α- and β-chains, and exposing the buried hydrophobic moieties to the solvent. Subsequently, the environment around the reactive sulphydryl center becomes hydrophobic, in which 2-DTP will be expected to react faster than DTNB.

The decrease observed for DTNB is expected because the imidazole groups that should have catalyzed its reaction have interacted with SDS. In addition, the structural transformation plays an important role in the sulphydryl reactivities of the sulphydryl group of the apohemoglobin and hemoglobin in the denatured form. In the denatured form, the structural transformation favours 2-DTP over DTNB because of their characteristics.

A theoretical assumption already made [17] could be invoked to account for the differences observed in the reaction rates for DTNB and 2-DTP, knowing fully well that positive charged groups have interacted with SDS. This assumption will be used to account for the trend in the pH range of 5.6 to 8.8 (Figures 2 and 3). The assumption is that the thiol group undergoes ionization in two stages in which one pK_a value is the modulating factor in the acidic medium while the other pK_a is the modulating factor in the alkaline medium. This assumption holds only for the reaction of 2-DTP with sulphydryl group of denatured form where there is a gradual monotonic increase in the reaction rates.

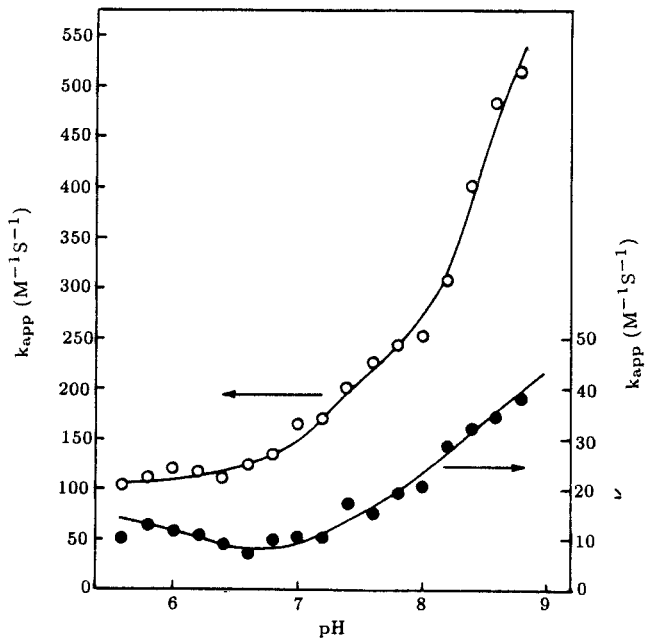

Figure 2. Reaction of sulphydryl reagents with CysF9 (93) β sulphydryl of bovine oxyhemoglobin in the presence of SDS dependence of k_{app} on pH at 27°C. The standard error of each point is about 5%. The lines through the experimental points are best-fit lines calculated with Equation 2 (• = DTNB and o = 2-DTP).

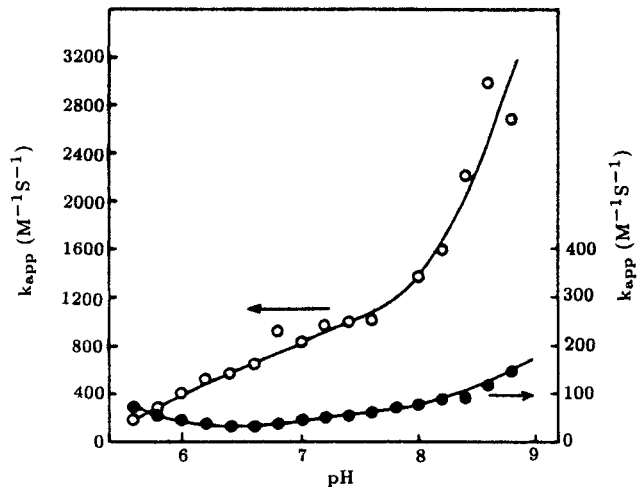

Figure 3. Reaction of sulphydryl reagents with CysF9 (93) β sulphydryl of bovine apohemoglobin in the presence of SDS, dependence of k_{app} on pH at 27°C. The standard error of each point is about 8%. The lines through the experimental points are best-fit lines calculated with Equation 2 (• = DTNB and o = 2-DTP).

The pH-k_{app} profiles for DTNB (Figures 2 and 3) show that there is a slight down-up transition. This down-up transition could be traced to the interaction of SDS with positive charged groups. It has been reported that the number of SDS-protein complexes formed increases from pH 5.6 to 7.0 and later decrease at pH > 7.0 [11]. This shows that the rate of reaction is inversely proportional to the number of SDS-protein complexes formed.

Equations 1 and 2 were used to analyze the k_{app} versus pH profiles for the DTNB and 2-DTP reaction (Figures 2 and 3). The solid lines are theoretical best-fit lines which were obtained using Equation 2 for all the pH-k_{app} profiles, however, Equation 1 showed a poor fit. The best-fitting parameters are reported in Table 3. The pK_2 values are in good agreement with pK_a value of thiol group already reported in the literature [2,3].

Figures 4 and 5 show close similarity with Figures 1 and 2 when they are compared with Table 1. In

Table 3. Reaction of sulphydryl reagents with denatured bovine apohemoglobin and hemoglobin. Best-fit parameters employed for fitting the data in Figures 2 and 3 with Equation 2.

	Hemoglobin		Apohemoglobin	
	DTNB	2-DTP	DTNB	2-DTP
$k_2(M^{-1}s^{-1})$	69	972	141	4540
pK_2	8.64	8.63	8.40	8.38

Figures 4 and 5, the reaction rate of DTNB with denatured apohemoglobin is approximately three to five times faster than with denatured hemoglobin, while the reaction of 2-DTP with denatured apohemoglobin is about two to five times faster than with denatured hemoglobin. There are four reasons which might be responsible for this general trend: (1) the characteristics of sulphydryl reagent with respect to charge sensitivity, (2) the hydrophobic environment of the reactive sulphydryl group, (3) the increase in the number of non-polar amino acid residues that are initially linked with heme and are now exposed to the solvent in denatured apohemoglobin and (4) the structural transformation of the denatured apohemoglobin and oxyhemoglobin.

Figures 6 and 7 show the comparisons of the pH-k_{app} profiles of native and denatured bovine oxyhemoglobin with DTNB and 2-DTP, respectively. In Figure 6, the reaction rate of DTNB with native oxyhemoglobin is ten times faster than that of denatured oxyhemoglobin. Figure 7 shows that 2-DTP reacts five times faster with denatured oxyhemoglobin than with native oxyhemoglobin. In this case, DTNB

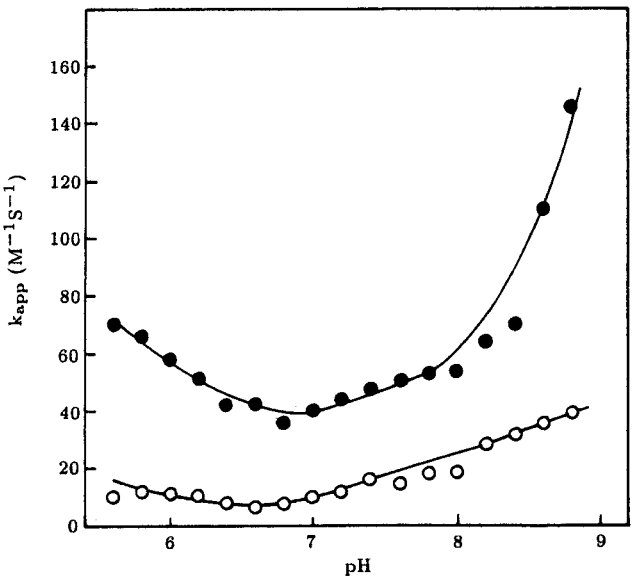

Figure 4. Reaction of DTNB with CysF9 (93) β sulphydryl of bovine apohemoglobin and oxyhemoglobin in the presence of SDS, dependence of k_{app} on pH at 27°C. The lines are fitted with Equation 2. Standard error is about 5% (• = apohemoglobin and o = oxyhemoglobin).

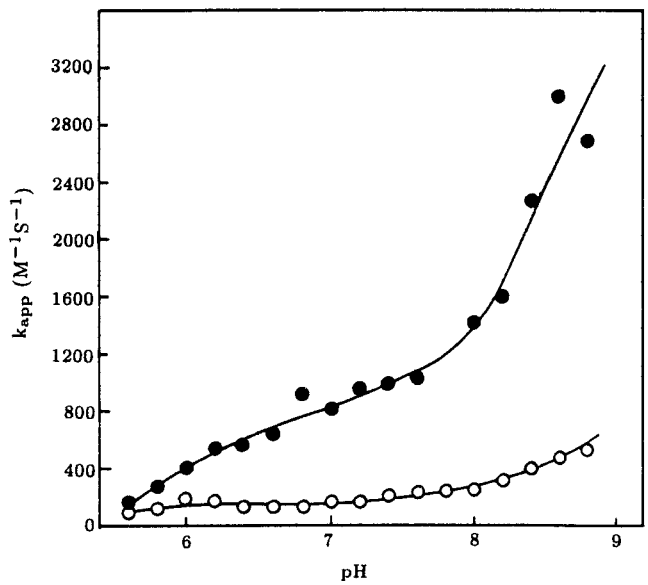

Figure 5. Reaction of 2-DTP with Cys F9 (93) β sulphydryl of bovine oxyhemoglobin and hemoglobin in the presence of SDS, dependence of k_{app} on pH at 27°C. The standard error of each point is about 8% (• = apohemoglobin and o = oxyhemoglobin).

is very reactive with sulphydryl group in a more hydrophilic environment while that of 2-DTP will react fast with sulphydryl group in a more hydrophobic environment. Generally, polar residues are exposed while non polar residues are buried in the native form. In the denatured form, non-polar and polar residues are exposed, therefore it can be concluded that 2-DTP is a better sulphydryl reagent for investigating the sulphydryl group of the denatured protein than DTNB.

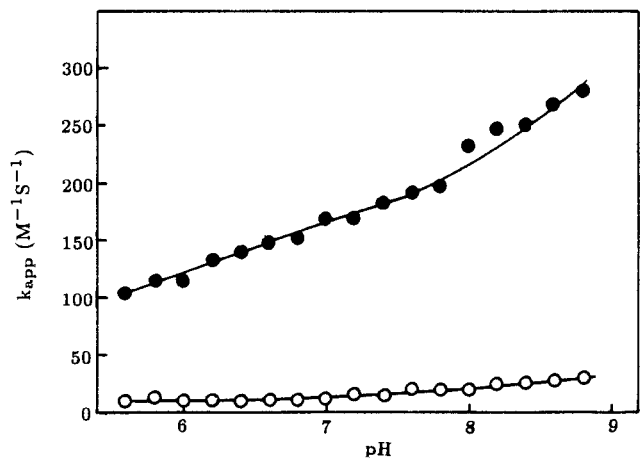

Figure 6. Reaction of DTNB with Cys F9 (93) β sulphydryl of native and denatured bovine oxyhemoglobin, dependence of k_{app}, on pH at 27°C. The standard error of each point is about 5% (• = native oxyhemoglobin and o = denatured oxyhemoglobin).

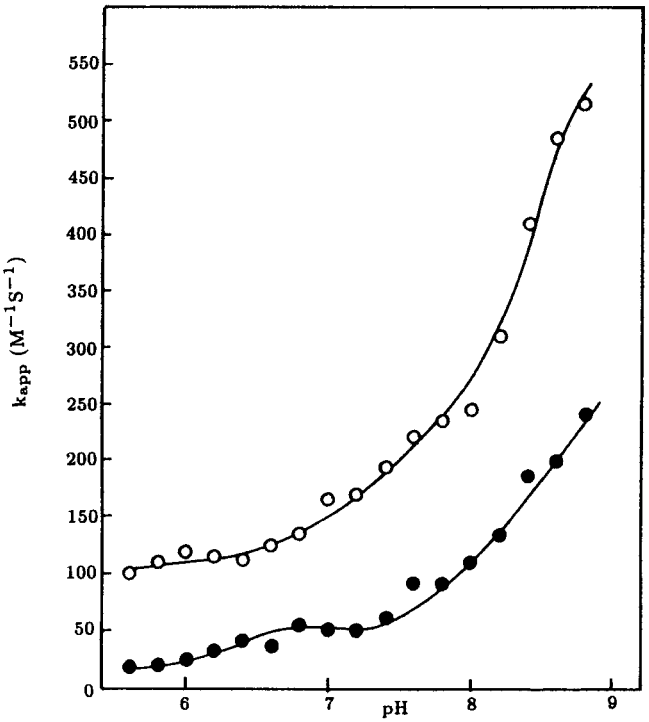

Figure 7. Reaction of 2-DTP with Cys F9 (93) β sulphydryl of native and denatured bovine oxyhemoglobin, dependence of k_{app} on pH at 27°C. The standard error of each point is about 5-8% (• = native oxyhemoglobin and o = denatured oxyhemoglobin).

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