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QUATENARY STRUCTURE OF METHEMOGLOBIN II.

PULSE RADIOLYSIS STUDY OF

THE BINDING OF OXYGEN TO THE VALENCE-HYBRID

PROGRESS REPORT

Mordechai Chevion*, Yael A. Ilan**, Amram Samuni***,
Tikva Navok* and Gidon Czapski**

Department of Cellular Biochemistry*, Physical Chemistry**
and Molecular Biology***,

The Hebrew University of Jerusalem, Jerusalem, Israel

Work carried out during the period:
December 1st 1978 to November 30th 1979

Prepared for the U.S. Energy Research & Development Administration
Under Contract No. EY-76-C-02-3221

DISCLAIMER

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Footnotes

1) A review summarizing the lines of evidence in favor of this model was recently published (Shulman, R.G., Hopfield, J.J. and Ogawa, S. (1975), Quarterly Rev. Biophys. 8, 325-421).

2) Abbreviation used:

DSS - 2,2-dimethyl-2-silapentane-5-sulfonate

IHP - inositol hexaphosphate.

3) The transition between the high-spin form of adult human methemoglobin and its low-spin forms was also studied by low-temperature EPR spectroscopy both in the absence and in the presence of IHP. The results show a pK = 8.2 for this transition in the absence of organic phosphates, while in the presence of IHP the pK is shifted to about 8.5 (M. Chevion and J. Peisach, unpublished results).

4) Y.A. Ilan and G. Czapski, unpublished results.

Summary

The pulse-radiolysis of solutions of adult human methemoglobin was used in order to reduce a single heme-iron within the protein tetramers. The valence-hybrids thus formed were reacted with oxygen. Kinetics of the reactions were studied. The effects of pH and inositol-hexaphosphate were examined.

The kinetics of the ligation of oxygen to stripped valence-hybrids showed a single-phase behaviour at the pH range 6.5-9. As the pH was lowered below 6.5 a second, slower phase became apparent. In the presence of IHP, above pH 8, the kinetics of oxygen binding was of a single-phase. As the pH was lowered a transition to a second, slower phase was noticed. Below pH 7 the slower phase was the only detectable one. The analysis of the relative contribution of the faster phase to the total reaction as a function of the pH showed a typical transition curve characterized by a pK = 7.5 and a Hill parameter n = 2.9.

On this basis it is concluded that human adult stripped methemoglobin resides in an R quarternary structure while the presence of IHP stabilizes the T structure at pH below 7.5. This transition between the quaternary structures of methemoglobin cannot be accounted for by the switch between the high-spin and the low-spin states of the ferric iron. This switch of spin state takes place at pH > 8.2.

The concept that the two-state model actually describes the hemoglobin tetramer has been a subject for many studies[1]. This model has been refined so that the protein is now visualized as residing in either of two sets of quaternary structures. In each set the structures are closely related.

Various markers have been used for the determination of the quaternary structure of a hemoglobin or its derivative. Besides the direct structure elucidation using X-ray diffraction studies (1-3), kinetics of ligand-association or ligand-dissociation have been used to determine the affinity state of the tetramer and to correlate it with its quaternary structure (4-6). For nitrosyl hemoglobin two spectroscopic markers were used. These are the NMR line which is 14 ppm downfield from DSS[2] in H_2O (7) and the negative peak at 285 nm in circular dichroism spectra (8), both of which are monitors of characteristics inherent in the globin moiety of the molecule. Recently, another marker of the heme characteristics has been proposed (9-12). According to this suggestion the low temperature EPR spectrum of nitrosyl derivatives of various human hemoglobin variants changes markedly when the tetramer switches its quaternary structure that is coupled to its affinity state.

In a recent study (13) we suggested that by using the pulse radiolysis technique the quaternary structure of methemoglobin can be characterized by following the kinetics of CO binding to its valence-hybrid. The rate constant for CO binding to the valence-hybrid is dependent upon the pH and the presence of organic phosphates (13,14). The change in this rate constant indicates a transition between affinity states (11) that is correlated with a switch between quaternary structures.

In this work we further explore the suggestion that the kinetics of ligand binding, indeed, can serve as a marker for the quaternary structure of the protein. Here, the affinity state of the radiolytically formed valence-hybrid is monitored by studying its oxygenation. Special attention is given to the relation between spin state and the quaternary structures.

Materials and Methods

Human adult hemoglobin (hemoglobin A) was freshly prepared from venous blood drawn in the presence of heparin. The cells were washed three times with isotonic saline and osmotically lysed for 1 h at 4° by adding 4 volumes of distilled water. The unlysed cells and debris were removed by centrifugation (10,000 x g, 15 min). The hemolysate containing approximately 1 g of protein was then passed through a Sephadex G-25 column (4 x 50 cm) which was equilibrated with 0.01 M phosphate buffer, pH 6.8, in order to remove organic phosphates. The red eluate was applied to a carboxymethylcellulose column (4 x 50 cm) equilibrated with the same buffer. Hemoglobin was eluted using a pH gradient of phosphate buffer (6.7 to 8.0). The major fraction was used for preparation of methemoglobin A.

A 4-fold molar excess (per heme) ferricyanide was added to the hemoglobin solution and allowed to react for 15 min. The solution was then applied to a Sephadex G-25 column (4 x 50 cm) and eluted with 0.01 M phosphate buffer, pH 6.8. The amount of residual oxyhemoglobin was determined optically. When it exceeded more than 3%, the protein was again treated with ferricyanide.

Inositol hexaphosphate (IHP) was purchased from Sigman Co. All other chemicals used were of the highest analytical grade available and were used without any further purification. Protein solutions buffered with 0.01 M phosphate were prepared in triply distilled water immediately before irradiation.

Organic buffering systems like Tris were avoided as they react with the radicals formed.

Methemoglobin concentration throughout the irradiation experiments was 20μM on tetramer basis. The protein solutions were deoxygenated by flushing with argon. Each sample was diluted with half the volume of an air-saturated buffer solution. The pH was varied between 6.2-8.9.

All solutions contained 0.1 M tert-butanol which scavenges all the OH radicals produced during the radiolysis. The organic radicals formed from the alcohol do not react with the hemoprotein in the time span of the reactions studied (15). Consequently, the e^-_{aq} is left as the sole reactive species.

The initial concentration of the hydrated electrons following the pulse was 5 μM but only one third of them reacted with the protein. The rest reacted with oxygen yielding $\dot{O}_2^-$ which decayed by self recombination. Under these experimental conditions the reduction of the methemoglobin resulted in a single-heme reduced methemoglobin tetramer (13, 15-18). The pulse radiolysis set-up has been previously described (17).

Results

O_2 binding to the IHP-free and IHP-bound valence-hybrids.

Methemoglobin solutions were pulse-irradiated in the presence of oxygen. The hydrated electrons, e^-_{aq}, thus formed, reduced (eq. 1) single subunits within the methemoglobin tetramers, $(MHb)_4$, producing valence-hybrids, $(MHb)_3Hb$ (13, 15-18).

(1) $(MHb)_4 + e^-_{aq} \rightarrow (MHb)_3Hb$

(2) $(MHb)_3Hb + O_2 \rightarrow (MHb)_3HbO_2$

The reaction of the valence-hybrid with oxygen (eq. 2) was spectrophotometrically followed at 435 nm. The change in transmittance accompanying the process was recorded on a fast scan oscilloscope and processed by an on-line computer.

At pH 7.5, in the absence of IHP, the reaction of oxygen binding was pseudo first-order with respect to oxygen. The observed rate constant k, was found to depend linearly on $[O_2]$, and the second-order rate constant calculated on a tetrameric basis was, $k_2 = 3.5 \times 10^7 M^{-1} sec^{-1}$.

In the presence of IHP ([IHP]/[tetramer] = 5), at the same pH, the reaction kinetics had no longer a simple first-order character. Instead, the reaction exhibited a clear biphasic behaviour.

Analysis of the kinetic curve. The experimentally observed points of such a typical reaction are presented in fig. 1, along with best fit lines. Let us define $\Delta A_r = A_r - A_M$ as the change in absorbance due to the reduction of a single heme in the tetramer. Thus, it is the difference in absorbance between the radiolytically formed, unligated valence-hybrid (A_r) and the unirradiated "parent" methemoglobin (A_M). $\Delta A_o = A_r - A_\infty$ is the change in absorbance associated with the ligation process, i.e. it is the difference in absorbance between the deoxy and the oxy forms of the valence-hybrid. Figure 1, curve a is a computer-best-fit using a single-phase first order rate expression (eq. 3).

$$(3) \quad \Delta A_t = \Delta A_o \exp[-kt]$$

Curve b is a computer best fit of the same data, using a two-phase first order rate expression (eq. 4).

$$(4) \quad \Delta A_t = \Delta A_o^f \exp[-k^f t] + \Delta A_o^s \exp[-k^s t]$$

Equation 4 accounts for two concomitant reactions characterized by the rate constants k^f and k^s for the faster and slower reacting species, respectively. The relative contributions of the faster phase to the reaction (θ^f) is evaluated from equation 5:

$$(5) \quad \theta^f = \Delta A_o^f / \Delta A_o$$

where ΔA_o^f is calculated from eq. 4 and ΔA_o is the change in absorption for the oxygenation reaction studied at the same pH in the absence of IHP.

$$(6) \quad \theta^s = 1 - \theta^f$$

All the reaction curves which did not conform to the single exponential rate expression (eq. 3) were analyzed as biphasic processes (eq. 4) and the rate constant for each phase, as well as the relative contribution of each phase to the total change, were calculated: The values evaluated for the two rate constants k^f and k^s together with the rate constant k determined in the IHP-free system are presented in fig. 2.

The biphasic character of the oxygenation of the valence-hybrid is consistent with a reaction of two different populations of the valence-hybrid, both of which are IHP-bound. The fast reacting species resembles that formed in the absence of IHP.

Effect of pH on the oxygenation reaction of the valence-hybrid. The oxygen binding reaction to the valence-hybrid (eq. 2), in the absence of IHP, was studied at the pH range 6-9, and the dependence of k on the pH is shown in fig. 2. Below pH 6.5 in the absence of IHP, the reaction was biphasic and two rate constants, k^f and k^s, were evaluated according to eq. 4 (fig. 2). The pH dependence of this reaction was examined in the presence of IHP as well.

At pH values where the analysis indicated two different contributions, evaluation of two rate constants and two relative contributions was carried out as explained above. In cases where one of the contributions was less than 10% of the total change and the quantitative determination of its extent was not possible, the reaction was treated as a simple first-order one. The values of ΔA_o^f, ΔA_o^s and ΔA_o measured at the various pH values are summarized in Table I. The effect of pH on the two rate constants, k^f and k^s, is illustrated in fig. 2 (although it does not show their relative weights). The results indicate that the rate constant of the fast reacting phase, k^f, gradually increases with pH, similarly to k, while k^s remains constant over the pH range studied.

When equation 3 has been arbitrarily fitted to each of the reaction curves, even those showing biphasic behaviour, a new set of apparent rate constants (k_{app}) has been derived. These rate constants determined over the same pH range are presented in fig. 3. The data thus treated resulted in a sigmoidal curve of k_{app} vs. pH (fig. 3). This curve resembles those, similarly obtained, for the binding reaction of CO to the valence-hybrid previously studied using flash-photolysis (19) and pulse-radiolysis techniques (13).

Relative contributions of the slow and fast reacting phases.

Using the data given in Table I, the relative contributions of the slow-reacting and fast-reacting valence-hybrids were calculated at the various pH values. Fig. 4 shows the relative contribution of the fast phase, θ^f, as a function of pH. The solid line is a best fit of the Hill equation $\theta^f = \{1 + 10^{n(pK-pH)}\}^{-1}$ with n = 2.9 and pK = 7.5.

DISCUSSION

The R ⇄ T transition

In the presence of IHP, the pH-dependence of the rate constant of oxygen ligation to the valence-hybrid showed a sigmoidal behaviour that consists of three parts: at low pH (pH ≤ 7) at intermediate pH (7 < pH < 8), and at high pH (pH ≥ 8) (fig. 4). At any given pH, in either of both extreme regions (when the pH was either below 7 or above 8) the analysis of each of the kinetic curves of the oxygenation revealed that the reaction is a simple, single-phase one. At any intermediate pH, on the other hand, each of the kinetic curves was shown to result from varying levels of relative contributions from two reactions. One contribution corresponds to the reaction of oxygen with the valence-hybrid at pH < 7, and the second corresponds to the rate constant at pH > 8. Thus, it is suggested that the sigmoidal curve (fig. 4) represents a transition between two states of the valence-hybrid with different reactivities towards oxygen. By analogy to the case of CO-binding (13), this transition reflects a change in the affinity state of the valence-hybrid. As no conformational changes could be detected between the moment of initial reduction of the heme and the completion of the ligation reaction it is further suggested that fig. 4, in fact, demonstrates the transition between the two affinity states of the "parent" methemoglobin. This transition in affinity states, associated with the R ⇄ T switch in quaternary structure, is characterized by a pK = 7.5 and a Hill coefficient n = 2.9.

The binding constant of methemoglobin to IHP decreases with increasing pH (20,21). At pH > 8, IHP weakly binds to the protein. Thus, one could have suggested that the sigmoidal curve in fig. 4 actually reflects the binding of IHP to methemoglobin and the transition demonstrates the switch

between IHP-bound and IHP-free protein rather than a change in affinity state. This possibility can be ruled-out considering the following results: The same kinetic constants as well as the same relative contributions were evaluated when the experiments were compared. In one, a slight excess of IHP was added at pH 7.5, while in others up to 13-fold excess in the concentration of IHP was used.

In the absence of IHP and above pH 6.5 the kinetics of oxygen binding to the valence-hybrid obeyed a single-phase expression. At pH 6.5 a slower phase became apparent. As the pH was further lowered the biphasic character became more prominent and the relative contribution of the slow phase increased and reached ~20% at pH 6. Both rate constants, those of the fast and of the slow reacting species, are equal to the corresponding ones measured in the presence of organic phosphates. Thus, it seems that there is an equilibrium between the two quaternary structures of the methemoglobin even in the absence of IHP. As the pH is lowered, the T quaternary structure becomes more favourable. This conclusion is similar to the findings with nitrosyl derivatives of various human hemoglobins variants that showed the analogous effect of protons and organic phosphates on the quaternary structure of the hemoglobin tetramer (9-12).

Comparison between the ligation of oxygen and of carbon-monoxide to the valence-hybrid.

We have shown that the study of the kinetics of ligation of the valence-hybrids by the pulse radiolysis technique can be used to monitor the quaternary structure of the methemoglobin tetramer. The same behaviour is expected when either oxygen or carbon-monoxide is used. The analysis and the results shown in this work with oxygen are more accurate and more significant as compared to those which were calculated for the reaction with CO (13). Due to the fact

that at the intermediary pH region contributions from 3 different reactions with 3 different rate constants were involved, the analysis for the reaction with CO was less accurate. Each of the kinetic curves was analyzed as if it is composed of only two reactions while the third component could not be taken into account and, therefore, ignored (13). It is impossible to apply the analysis introduced in this study to the data earlier published, with CO. Instead, if one applies the former analysis to the results in this present study a different transition curve is evaluated (fig. 3). This new sigmoidal curve is characterized by pK = 8 and n = 2.1. These parameters as well as the assymetric shape of the curve are very similar to those published earlier (13). In conclusion, there is no contradiction between the two studies; the difference in the values of the pK and n are due to a different, more meaningful and more accurate analysis introduced in this study.

The R $\rightleftarrows$ T transition and the switch in spin states.

Methemoglobin can exist in either of two different spin states. The aquomet form which is a high spin compound (20), and the hydroxymet form which is a low spin one (22). The transition between the two spin states takes place at pH above 8 in the absence of organic phosphates (22, 23) and is shifted to a higher pH in the presence of IHP^3. It was suggested by Perutz (3) that the transition between the two quaternary structures in hemoglobin is coupled to the switch of spin state. This suggestion takes into account the larger diameter of the high-spin compound; the iron in this case, cannot fit into the matrix of the porphyrin ring and, thus, it exerts tension of the molecule that favours the T quaternary structure. It is clearly demonstrated here, that these two transitions of quaternary structure and spin states do not coincide, but rather there is a full pH unit difference between them.

Inequivalence between α and β subunits

In a previous study (13) we had found that when the valence-hybrid resides in a low-affinity state, the kinetics of CO-binding exhibited a prominent biphasic character. This finding was in accordance with an earlier report by Gray and Gibson (24). In the present communication, the reaction of the valence-hybrid with oxygen, rather than with carbon-monoxide, was studied and this biphasicity could not be noticed.

In a recent article (25) a biphasic behaviour in the ligation of oxygen to the valence-hybrid was shown even in the absence of IHP. It was claimed (25) that this biphasicity is an expression of a latent chain-inequivalence toward oxygen. It was suggested (25) that the electron does not react indiscriminately with α and β chains during the reduction of methemoglobin (eq. 1) but rather that there is a preference for its reaction with one specific chain. This suggestion (25) does not agree with two earlier reports (15,17). Also, when the reduction of the methemoglobin is carried out in the presence of an excess of ferricyanide, and the oxidation of the just-formed valence-hybrid is followed spectrophotometrically - no biphasicity could be detected[4]. This suggestion (25) by itself cannot provide an explanation for the fact (13, 18) that the latent biphasicity is expressed only under certain conditions, (with CO, rather than with O_2, and only in the presence of IHP) and not always. Thus, it seems that there is indeed an inequivalence between the α and the β subunits (independently of whether their formation is in equal or unequal amounts). Furthermore, the expression of this chain inequivalence is induced by the presence of organic phosphates.

CONCLUSIONS

1. In the presence of IHP, the reaction of oxygen binding to the valence-hybrid is a single phase at the regions where pH $\leq$ 7 or pH $\geq$ 8 and a biphasic one at 8 > pH > 7. In the absence of IHP biphasicity becomes apparent as the pH is lowered to 6.5.

2. The analysis of the kinetics of the oxygenation reaction enables the resolution of two contributions; a slow reacting and a fast reacting valence-hybrid.

3. The pH-dependence of the relative contribution of the reacting species is a sigmoidal curve that represents a transition between quaternary structures. The T $\rightleftarrows$ R transition is characterized by a pK = 7.5 and a Hill coefficient n = 2.9.

4. The T $\rightleftarrows$ R transition and the switch in spin state of the ferric iron do not coincide. A full pH unit separates between the two phenomena.

5. No chain inequivalence could be noticed in the ligation of the valence-hybrid with oxygen even in the presence of IHP. In this respect the ligation of CO is different. There, a pronounced biphasicity is clearly seen.

Acknowledgment

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Table I.

Values of ΔA (435 nm) resulting from the oxygenation reactions of the fast reacting and slow reacting valence-hybrids.

pH	ΔA_o	ΔA_o^f	ΔA_o^s
7.0	0.09		0.05
7.35	0.087	0.011	0.035
7.5	0.095	0.044	0.023
7.7	0.085	0.057	0.02
7.85	0.075	0.067	0.012
8.1	0.075	0.075	
8.9	0.05	0.055	

$\Delta A_o = A_r - A_\infty$, is the absorbance difference measured in the absence of IHP. ΔA_o^f and ΔA_o^s are the absorbance differences contributed by the fast-reacting and slow-reacting phases of the oxygen binding reaction in the presence of IHP. These values were evaluated through curve fitting as explained in the text.

Legend to Figures

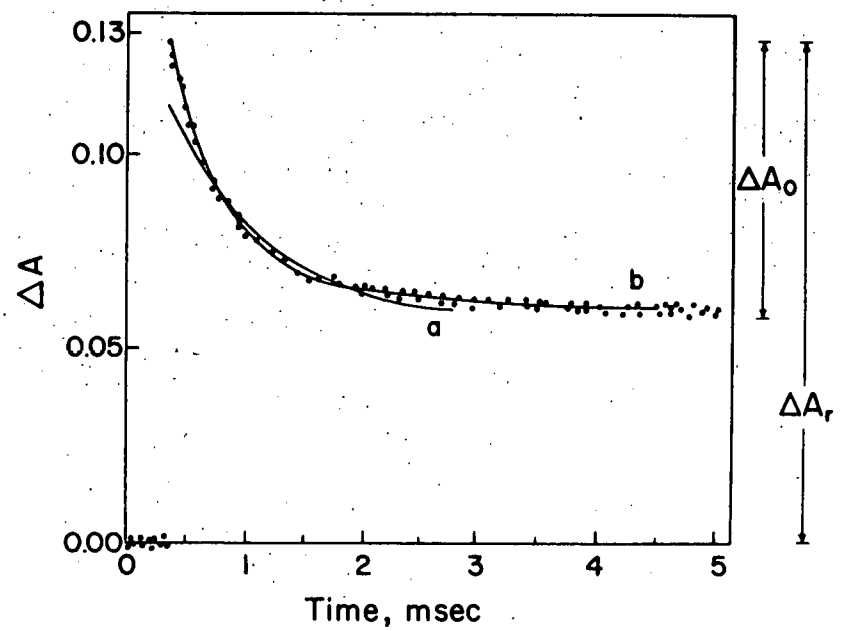
Figure 1: The change of absorbance with time for oxygen binding to the valence-hybrid in the presence of IHP. Curve a is a computer best fit using a simple first-order kinetic expression (eq. 3). Curve b is a best fit of the same data points using a biphasic kinetic expression (eq. 4).

λ = 435 nm; [metHb] = 20μM tetramer; $[e^-_{aq}]$ = 5μM; $[O_2]$ = 83μM; [phosphate] = 0.02M, pH 7.5.

Figure 2: pH dependence of the pseudo first-order rate constants for the binding of oxygen to the valence-hybrid. In the absence of IHP: k(O,●); in the presence of IHP: k^f(Δ), k^s(▲).

Figure 3: pH dependence of k_{app} for the reaction of oxygen with the valence hybrid in the presence of IHP. All experimental conditions are as in Fig. 2. The apparent rate constants k_{app} have been evaluated from the reaction curves using single first order rate equations.

Figure 4: pH dependence of the relative contribution (θ^f) of the fast-reacting valence-hybrid to the overall reaction. ($\theta^f = \Delta A^f_o/\Delta A_o$). The values of θ^f have been calculated from the experimental data shown in Table I. The solid line is a best fit of the Hill equation $\theta^f = \frac{1}{1+10^{n(pK-pH)}}$ to the experimental values of θ^f.


0.13
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ΔA
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Time, msec
a
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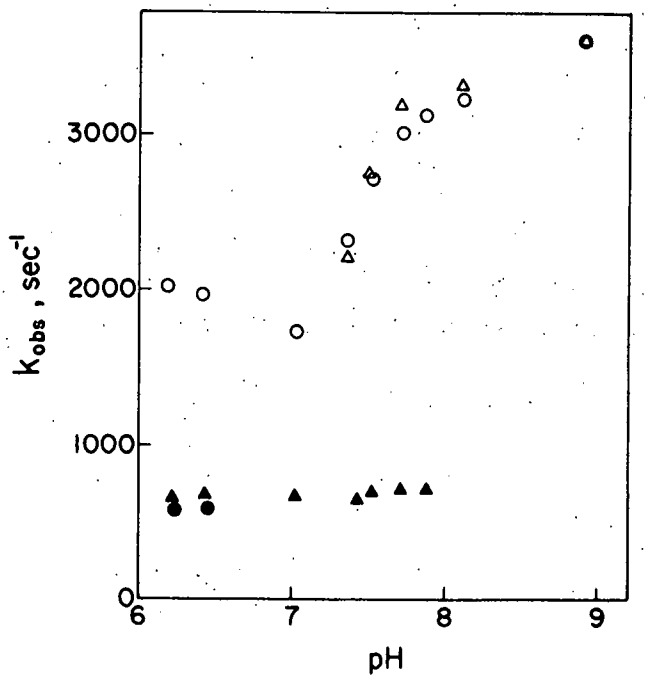
Fig. 1

Fig. 2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2000
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k_obs, sec⁻¹
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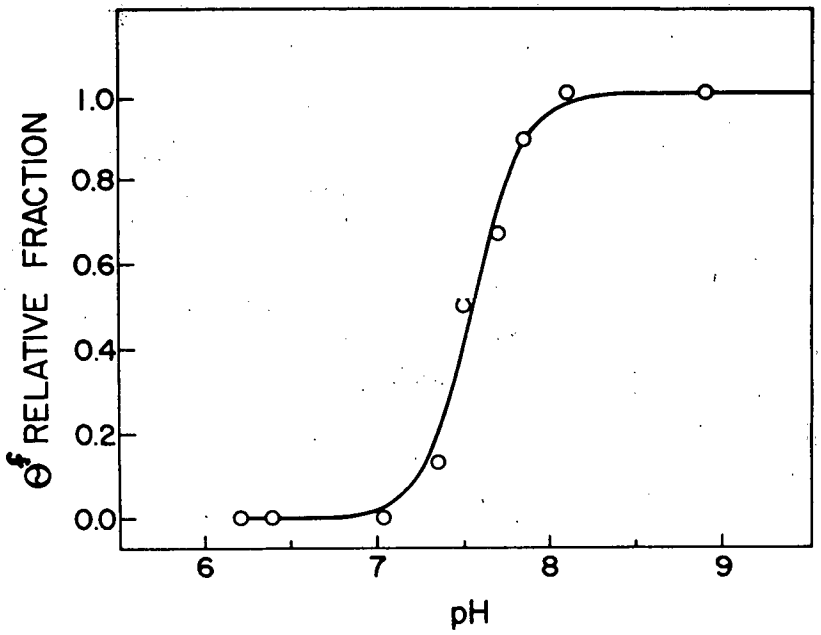
pH


θ^f RELATIVE FRACTION
1.0
0.8
0.6
0.4
0.2
0.0
6
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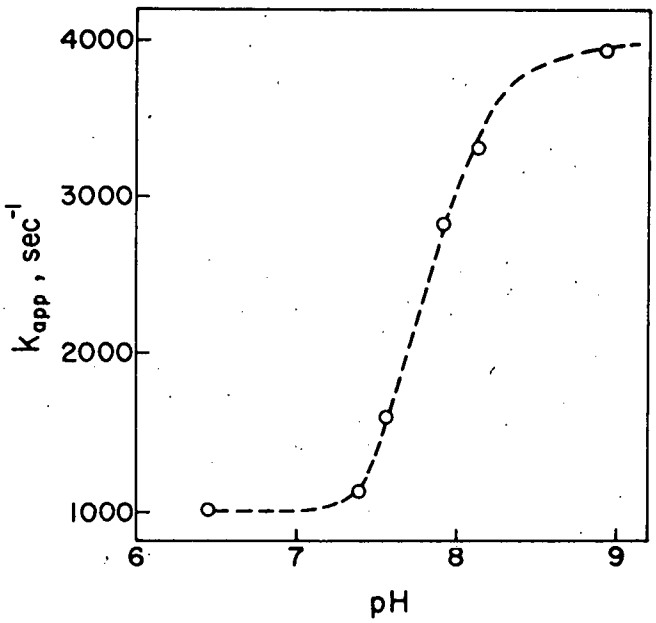
8
9
pH

Fig. 3


k_{app}, sec^{-1}
4000
3000
2000
1000
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8
9
pH

Fig. 4