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THE KINETICS OF HEMOGLOBIN DENATURATION IN ALKALI

by

Douglas R. Wilson

B.Sc.(Hons.) Simon Fraser University, 1975

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

April 1980

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The Kinetics of Hemoglobin Denaturation in Alkali

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ABSTRACT

The effects of chemical modifications and specific heme ligands on the kinetics of hemoglobin denaturation in alkali were investigated by both precipitation and spectral assay methods. For unmodified oxyhemoglobin both a fast and a slow reaction were observed. A model for the denaturation of oxyhemoglobin is proposed in which two reaction sequences compete for the native protein. The two pathways differ in that oxidation of ferrous heme follows denaturation in the first and precedes denaturation in the second. In the first (faster) reaction, the formation of native oxyhemoglobin monomers is the rate limiting step. Subsequent unfolding leads to the partial reduction of molecular oxygen with production of superoxide anion or hydrogen peroxide. These convert some of the native oxyhemoglobin in the alkaline reaction mixture to methemoglobin which, in turn, denatures rapidly by the second reaction sequence. The second pathway is not limited by formation of monomers and is characterized by the production of a hemichrome intermediate which is modified further in a slow reaction.

Human hemoglobin A was purified by ion exchange chromatography. Denaturations were carried out at pH 11.7 and 25.0°C in 0.025 M sodium phosphate, 0.100 M NaCl and were measured by both the absorbance decrease at 576 nm and the loss of protein due to precipitation after neutralization to pH 6.8 in the same buffer.

Denaturation of oxyhemoglobin by the first pathway (fast reaction) had a first-order rate constant of $0.42 \pm 0.02\ min^{-1}$. Full titration of the interfacial sulfhydryl groups with $HgCl_2$ was found to reduce this rate 23 fold while similar treatment with the larger mercurial p-mercuribenzoate was found to increase it 4 fold. This sensitivity of denaturation to modifications of the $\alpha_1\beta_1$ interface supports the rate limiting role of monomerization. Deoxyhemoglobin denatured only 7% faster by the first pathway than did oxyhemoglobin which suggests that they share the same rate limiting step and demonstrates that autoxidation is not a prerequisite for denaturation by the first pathway.

Methemoglobin was prepared by careful oxidation with $K_3Fe(CN)_6$. Alkaline denaturation occurred before the first measurement at 0.5 min. The rate limiting step for this derivative cannot be monomerization as for oxyhemoglobin since methemoglobin has a similar $\alpha_1\beta_1$ interface structure and stability to oxyhemoglobin and yet had a much higher denaturation rate. A hemichrome intermediate is formed by this reaction, which reacted further with a first-order rate constant of $0.014 \pm 0.002\ min^{-1}$. A similar intermediate observed with oxyhemoglobin reacted at the same rate. This indicates that some oxyhemoglobin is oxidized to methemoglobin before denaturation (the second pathway in the proposed scheme). For both

oxyhemoglobin and methemoglobin, the intermediate was soluble in the neutral buffer but not in neutral buffer containing 24% (w/v) $(NH_4)_2SO_4$. Also in both cases, the intermediate was rendered insoluble in neutral buffer by titration with 2 equivalents of $HgCl_2$ per tetramer prior to the start of the denaturation. Thus the intermediate appears to be identical whether oxyhemoglobin or methemoglobin is the starting material. As expected from the reaction scheme a lower amount of the intermediate was observed for oxyhemoglobin.

Oxidation of denatured hemoglobin during the fast reaction was clearly evident from the changes in the visible spectrum that parallel denaturation. Since oxygen is the only oxidant present, reduction to superoxide or peroxide should occur at the same time. The formation of these reactive species during oxyhemoglobin denaturation was indicated by concurrent sulfhydryl oxidation. A reduction in the titre of free sulfhydryl groups and production of disulfide-linked multimers were observed. Superoxide or peroxide should not be produced during methemoglobin denaturation and, as expected, sulfhydryl oxidation did not occur. These agents also cannot be present during the denaturation of deoxyhemoglobin because of the absence of oxygen.

DEDICATION

To Mom and Dad.

ACKNOWLEDGEMENTS

I am indebted to my senior supervisor, Dr. A.H. Burr, for his advice and unfailing positive attitude throughout the course of this work. His assistance with the preparation of this manuscript is especially appreciated. My thanks are due to the members of my committee, Dr. M.J. Smith and Dr. R.J. Cushley, for their suggestions and encouragement.

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INTRODUCTION

Studies of hemoglobin stability at alkaline pH date back to 1866 when Von Korber demonstrated that hemoglobin from the blood of newborn children is less susceptible to alkali than that of adults (Brinkman, et al, 1935). Today it is well known that the hemoglobin of human newborns is predominantly hemoglobin F, the immediate developmental precursor to adult hemoglobin or hemoglobin A The difference in alkaline stability between adult hemoglobin and hemoglobin F is the basis of a number of clinical procedures for measuring the level of foetal hemoglobin in human blood (Singer, et al, 1951; Jonxis, et al, 1956). Although this difference has been well known since the 19th century, a structural basis for it was not postulated until recently (Perutz, 1974).

Protein Denaturation

The function of globular proteins relies on the intactness of a specific ordered conformation which is commonly called the "native state". The persistence of the native state depends on the weak interactions between key functional groups distributed throughout the protein. The degree of interaction is dependent on the chemical and physical environment. Protein denaturation is a major shift of protein structure away from the native state

due to changes in these weak interactions. Minor changes of the native state such as the aggregation of protein molecules into a crystal are not considered denaturation.

The denaturation of globular proteins is usually accompanied by a decrease in their water solubility. Since non-polar amino acid residues within the interior of the protein molecule are frequently exposed by denaturation, the stabilizing interactions between protein and aqueous solvent is usually reduced and predictably aggregation of protein molecules is enhanced. The degree of precipitation of denatured protein can depend on many factors such as the concentration of the protein, the denaturation method and the physical state of the solvent (temperature, pH and ionic strength). Although precipitation is often used to measure denaturation, the tenuous relationship between the two phenomena should always be considered.

Most detailed investigations of the effects of pH extremes on protein denaturation have been confined to acid pH values. Undesirable irreversible changes are more prevalent at alkaline pH, of which the oxidation of cysteine sulfhydryl groups with formation of covalent disulfide linkages is the most widely discussed (Tanford, 1968). This type of covalent modification is a major problem in investigations where reversibility is to be investigated since it impairs refolding. Although disulfide

formation has not been demonstrated in past studies of the alkaline denaturation of hemoglobin, it is known to occur with thermal denaturation of oxyhemoglobin at physiological pH (Winterbourn, et al, 1974). Since sulfhydryl reactivities generally increase at alkaline pH (Means, et al, 1971) disulfide formation is very likely to be prevalent in the alkaline denaturation of oxyhemoglobin also, provided a suitable oxidant is present.

The denaturing effect of high temperature and extreme pH are not clearly separable because protein stability at high temperature is generally pH dependent. Denaturation of a protein at a particular acid or alkaline pH is often severely reduced or effectively terminated by lowering the temperature and could be regarded as having both pH and thermal denaturation components. Since the ionic state of the protein may differ substantially between an extreme pH and the most stable pH, the thermal denaturation mechanism may also differ. However information acquired from investigations of the thermal stability of a protein is often relevant to studies of protein denaturation at acid or alkaline pH.

The instability of globular proteins at extreme pH is often attributed to ionization of critical amino acid residues within the hydrophobic interior of the molecule such as histidines at acid pH and cysteines and tyrosines at alkaline pH. Amino acid

residues which are charged in the native structure of the protein at normal pH are typically confined to the protein surface and should contribute little to destabilization at pH extremes. The available detailed information on the structure of hemoglobin is thus a major asset in deciding the role of specific amino acid residues in the denaturation process. Because of this structural information, hemoglobin is an attractive experimental system for studies on protein denaturation.

Hemoglobin Structure

The 3-dimensional structure of hemoglobin was determined by the X-ray crystallographic studies of M.F. Perutz and collaborators (Perutz, et al, 1968). Mammalian hemoglobins are basically tetramers, constructed of two pairs of polypeptide chains, termed α and β, arranged tetrahedrally around a single two-fold symmetry axis. One molecule of heme, ferroprotoporphyrin IX, is inserted into a hydrophobic pocket of each chain. The heme component of hemoglobin structure is solely responsible for its characteristic visible spectrum.

The α and β chains consist of 141 and 146 amino acid residues respectively arranged into 8 α-helical segments which are normally labelled from the N terminus as segments A through H according to the convention of Watson and Kendrew

(Watson, et al, 1961). The non-helical segments are labelled AB through GH depending on the helical segments they separate or are labelled NA or HC if they occur at the N or C terminus respectively. The individual amino acids within each segment are numbered in the N to C direction such that each amino acid is specified by its position in the tertiary structure of the subunit. This system allows functionally relevant comparisons between the amino acid sequences of different hemoglobins and myoglobins.

Both chains have two relatively hydrophobic sites on their surfaces which bind the subunits together into tetramers. Subunit contacts are almost entirely between unlike subunits such that two types of subunit interfaces occur. The strongest interface, termed the $\alpha_1\beta_1$ interface, involves hydrophobic contacts between 34 amino acid residues belonging to segments B,G and H. The remaining interface, the $\alpha_1\beta_2$ interface, involves 19 residues from segments C,F and G in hydrophobic interactions.

The heme resides in a hydrophobic pocket created by segments C through G making approximately 90 van der Waals contacts with the polypeptide. The heme iron is covalently attatched to a histidine residue, called the proximal histidine, at position F8 and is normally displaced slightly out of the plane of the heme

towards this ligand. Molecular oxygen binds to the opposite side of the heme iron in oxyhemoglobin such that, including the four ligand positions occupied by the porphyrin, the iron is six-coordinate. In deoxyhemoglobin the oxygen binding site remains vacant such that the heme iron is only five-coordinate. Movement of the iron into the plane of the heme with the binding of oxygen is believed to be the trigger event for the cooperative heme-heme interaction which is characteristic of hemoglobin function.

A remarkable feature of the in vivo function of hemoglobin is the ability of the constituent ferrous heme to combine reversibly with O_2 without oxidation to the ferric state. Free ferrous heme in aqueous solution is rapidly oxidized by O_2 (Falk, 1964). The 3-D structure of the heme pocket must constrain the interaction between heme and O_2 such that oxidation does not occur. In denaturation, the integrity of the heme pocket will likely be lost. Oxidation of the heme iron is thus a predictable as well as observable consequence of the denaturation of oxyhemoglobin.

As well as exposing the heme to oxidation, denaturation of most hemoglobin derivatives is accompanied by changes in the ligand state of the heme. In general, both the fifth and sixth coordinate positions of the heme iron become occupied by

histidine imidazoles of the polypeptide chains. Heme compounds of this sort, in which both ligand positions are occupied by nitrogenous bases, are called "hemochromes" if the iron is in the ferrous state and "hemichromes" if the iron has been oxidized. Characteristic changes in the visible spectrum which accompany the formation of these derivatives are often used to monitor the denaturation reaction.

Denaturation Mechanisms

Denaturation of hemoglobin at extreme pH might be regarded as a series of conformational adjustments triggered by the ionization of particular amino acids in the polypeptide sequence. Because of the dependence of the native structure on the interaction of many amino acid residues, denaturation is predictably a cooperative phenomenon such that the rates obtained by the majority of these adjustments are governed by relatively few rate determining steps.

Dissociation of the native hemoglobin tetramers into $\alpha_1\beta_1$ dimers occurs readily, is reversible, and precedes alkaline denaturation of mammalian hemoglobins. At pH values ranging from 9.5 to 11.0, this dissociation has been demonstrated by both sedimentation velocity (Edelstein, et al, 1970) and light scattering (Flamig, et al, 1977) methods. Carboxyhemoglobin

(CO-hemoglobin) is found to dissociate completely at pH 11.0 (Hasserodt, et al, 1959). Because the rate constants obtained for dissociation into dimers (Flamig, et al, 1977) are much larger than those obtained for alkaline denaturation (Perutz, 1974), native hemoglobin must exist as $\alpha_1\beta_1$ dimers in the alkaline reaction mixture.

Compared to other globular proteins, human oxyhemoglobin is relatively stable at alkaline pH, requiring pH values greater than 11.0 to produce a significant denaturation rate at room temperature. If denaturation is triggered by ionization of specific critical amino acid residues then the required pH implicates only cysteine and tyrosine. Replacement of these residues by structurally similar species which do not ionize would be expected to confer greater alkali stability to the protein. The identity of the residues which are critical to the denaturation mechanism might then be obtained by comparing the alkaline denaturation kinetics of hemoglobin variants, hemoglobins from other species and chemically modified hemoglobins.

The rate of alkaline denaturation varies greatly between hemoglobins of different species. Human adult CO-hemoglobin denatures in 0.1N NaOH at 20°C with a half time of 20 sec while bovine hemoglobin is stable under the same conditions

(Haurowitz, et al, 1954). By correlating the differences in alkaline susceptibility of these and other hemoglobins with the presence or absence of 2 cysteines within the $\alpha_1\beta_1$ subunit interface at positions G11α and G14β and a tyrosine between segments A and H at position H8β, Perutz has deduced a reaction scheme which explains some of the basic features of the denaturation kinetics. In Perutz's model cleavage of the $\alpha_1\beta_1$ interface is the rate limiting step and the denaturation rate is equal to the difference between the rates of dissociation and reassociation. The ionization and subsequent hydration of the 2 interfacial cysteine residues should impair normal reassociation of unstable native monomers into stable dimers, and thus should increase the net rate of monomer formation. In the absence of the two interfacial cysteines, as in the case of bovine hemoglobin, subunit reassociation should not be impaired and thus the denaturation rate should be lower as is observed.

Perutz also suggests that dissociation of the native $\alpha_1\beta_1$ dimers into monomers is followed by the uncoiling of the individual subunits from the C-terminus. The tyrosine at position H8β is depicted as having a minor role in this process by enhancing the unfolding of the subunit. This proposal is supported by the alkali stability of the β subunits of hemoglobin Rainier, a hemoglobin variant in which another tyrosine, the penultimate C-terminal residue of the β subunit,

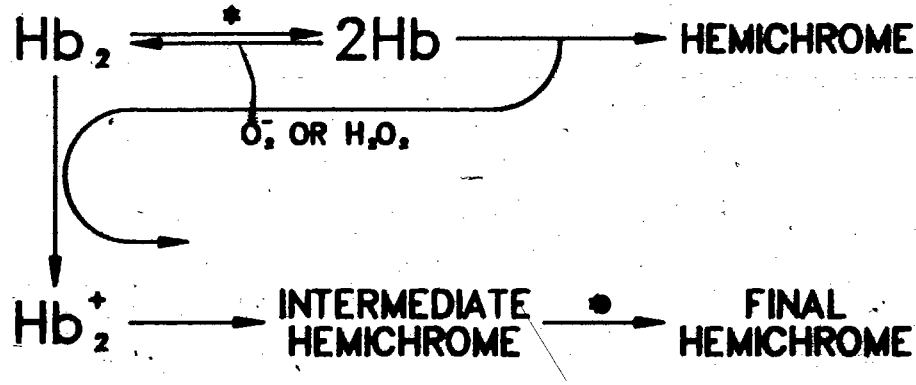
is replaced by cysteine. The alkali stability is thought to arise from a disulfide bridge between this cysteine and that normally at position F9β such that helices F and H are linked directly, preventing the movement necessary for the uncoiling of the latter helix. Since the $\alpha_1\beta_1$ interface links helices G and H, an intact interface would also be expected to prevent uncoiling from the C-terminus, thus explaining the stability of the intact $\alpha_1\beta_1$ dimers with respect to monomers.

Although Perutz's reaction scheme was intended for denaturation of hemoglobin in alkali, the differences between this scheme and one proposed for thermal denaturation of oxyhemoglobin at physiological pH values (Rachmilewitz, et al, 1971) merits consideration. In the latter scheme the subunit state of the initial reactants is not defined, but unlike Perutz's scheme, autoxidation of oxyhemoglobin to less stable methemoglobin is presented as an obligatory step prior to denaturation. Methemoglobin denatures much more readily than oxyhemoglobin at all pH values (Rieder, 1970).

A major consequence of the conversion of oxyhemoglobin to methemoglobin is a large reduction in the affinity of globin for heme (Bunn, et al, 1966). Because of this, some authors have suggested that a displacement or expulsion of the heme is responsible for the instability of methemoglobin relative to ferrous iron derivatives such as oxyhemoglobin (Rieder, 1970).

Complete expulsion of heme has been proposed as an initial step in the denaturation of certain unstable genetic variants of hemoglobin characterized by amino acid substitutions in the vicinity of the β chain heme (Jacob, et al, 1970). A major advantage of reaction schemes based on displacement of the heme is that they account for the stabilizing effect of cyanide. This ligand impairs both methemoglobin denaturation (Winterbourn, et al, 1974) and heme exchange (Bunn, et al, 1966).

To account for my results I have adopted a reaction scheme in which denaturation of oxyhemoglobin occurs in two parallel reactions; one initiated by monomerization and the other by oxidation to methemoglobin:



The denaturation of oxyhemoglobin monomers is assumed to be accompanied by the production of partially reduced forms of molecular oxygen such as superoxide anion and hydrogen peroxide. These oxidants, then convert some of the unreacted native

oxyhemoglobin to methemoglobin which denatures rapidly by a separate mechanism in which the rate determining step precedes formation of monomers. This step may be the expulsion of heme from one or both of the dimer subunits as observed for the acid denaturation of methemoglobin (Polet, et al, 1969).

I utilized a kinetic approach to explore the effect of heme ligands, chemical modifications and heme oxidation on the alkaline denaturation of hemoglobin. The reaction scheme diagrammed above accounts for all of the results I have obtained.

METHODS AND MATERIALS

Preparation of Hemoglobin Samples

Two levels of purification are commonly used in studies of human hemoglobin. Stroma-free hemolysates, in which 90% of the protein is a single hemoglobin component, can be prepared in a few hours from adult human blood. Although this level of purification is adequate for many experiments, more homogeneous samples are required to obtain reliable denaturation kinetics. The remaining protein impurities, which consist of non-heme proteins and two minor hemoglobin components, namely hemoglobin A2 and foetal hemoglobin, can be removed by ion exchange chromatography using DEAE-Sephadex A-50. Fully purified samples, prepared by the latter method, were used in all experiments where kinetic parameters were derived.

I. Preparation of Hemolysates

Hemolysates were prepared by a modified version of the method of Olson (Olson, 1976). Blood was drawn into a 20 cc Becton-Dickinson Vacutainer (lavender stopper) containing 0.17 ml of 15% K_3EDTA as an anticoagulent. The blood was then washed into a 50 ml polycarbonate centrifuge tube with 0.9% NaCl and diluted with the same to about 40 ml. The red blood cells were pelleted by centrifugation at 600xg and 4°C for 10 to 15 min in a swinging bucket rotor. The supernatant containing both

plasma protein and suspended white blood cells was then discarded completing the first cycle of the wash procedure. The cells were washed 3 more times in this manner and then lysed by mixing them with 3 volumes of glass distilled water and stirring slowly at 4°C for 1 hour.

The stromal impurities were removed by first adjusting the hemolysate to 3% NaCl by adding a suitable volume of 30% (w/v) NaCl and then, after 10 min of stirring, centrifuging the solution at 35,000xg and 4°C for 1 hour. The supernatant was collected with a Pasteur pipette taking care to avoid the viscous material on top of the pellet. Usually about 30 ml of stroma-free hemolysate is obtained at this point.

Before using the hemolysate it was established in a defined buffer system by gel filtration at 4°C. A 2.2x90 cm column of Sephadex G-25 (medium), previously equilibrated with 50 mM sodium phosphate, 0.10 M NaCl at pH 7.5 was used. A ratio of 13 volumes of gel to 1 volume of sample assured complete removal of 2,3-diphosphoglycerate, an organic phosphate which is normally found associated with hemoglobin (Berman et al, 1971). The sample was finally transferred to unbuffered 0.10 M NaCl by a second gel filtration step. Dilution was minimized in both gel filtration steps by discarding the leading and trailing edges of the hemoglobin bands. The purified hemolysates obtained were typically 3-5 g/dl in hemoglobin.

II. Preparation of Fully Purified Hemoglobin A

Further purification was accomplished by the ion-exchange chromatography method of Williams and Tsay (1973). The ion-exchange buffers were prepared as follows: Buffer A (50.0 mM Tris-HCl, pH 7.60 at 25°C) by diluting 231 ml of 1.000 N HCl and 36.6 g of Tris base to 6.00 l with distilled water; Buffer B (50.0 mM Tris-HCl, pH 8.40 at 25°C) by diluting 17.2 ml of 1.000 N HCl and 6.06 g Tris base to 1.000 l with distilled water; Buffer C (50.0 mM Tris-HCl, 0.200 M NaCl, pH 7) by diluting 46.6 ml of 1.000 N HCl, 6.06 g Tris base and 11.69 g NaCl to 1.000 l with distilled water. The Tris base was purchased from Swartz/Mann ("Ultra Pure" grade), the HCl (certified 1.000 N) and the NaCl (certified A.C.S.) were acquired from Fisher Scientific Company. Glass distilled water was used throughout and all buffers were equilibrated to 4 °C before use.

A 3.1x70 cm column of DEAE-Sephadex A-50 was equilibrated overnight in a 4°C cold room with 1-2 column volumes of buffer A. The ion exchanger was then overlayered with 1 cm of Sephadex G-25, also equilibrated with buffer A, to stabilize the bed surface and facilitate sample application. A peristaltic pump maintained the flow rate at 40 ml/hr.

The samples were prepared by the method described above for hemolysate except that the final gel filtration steps were replaced by a single gel filtration in which the hemoglobin is transferred to buffer A. The sample, containing about 1 g of hemoglobin, was then developed on the ion-exchange column with buffer A. A flow rate of 40 ml/hour was maintained for about 36 hours when hemoglobin A, which migrates between the non-adherent hemoglobin A2 fraction and the almost stationary foetal hemoglobin, began to elute from the bottom of the column.

A small, 2.6x8 cm column of DEAE-Sephadex A-50 in the same 4°C cold room was used to collect and concentrate the hemoglobin sample. It was initially equilibrated with an equal volume mixture of buffers A and B. When hemoglobin A reached the bottom of the first column, the outflow tubing was rearranged so that the column eluant and buffer B are pumped with equal flow rates into a Y-connector where they mix and continue into the collection column (Williams et al, 1973). When all of the desired hemoglobin has been collected in this fashion it is removed from the collection column by elution with buffer C. The resulting sample, usually 5-7% hemoglobin A, was then transferred to unbuffered 0.10 M NaCl by a final gel filtration step.

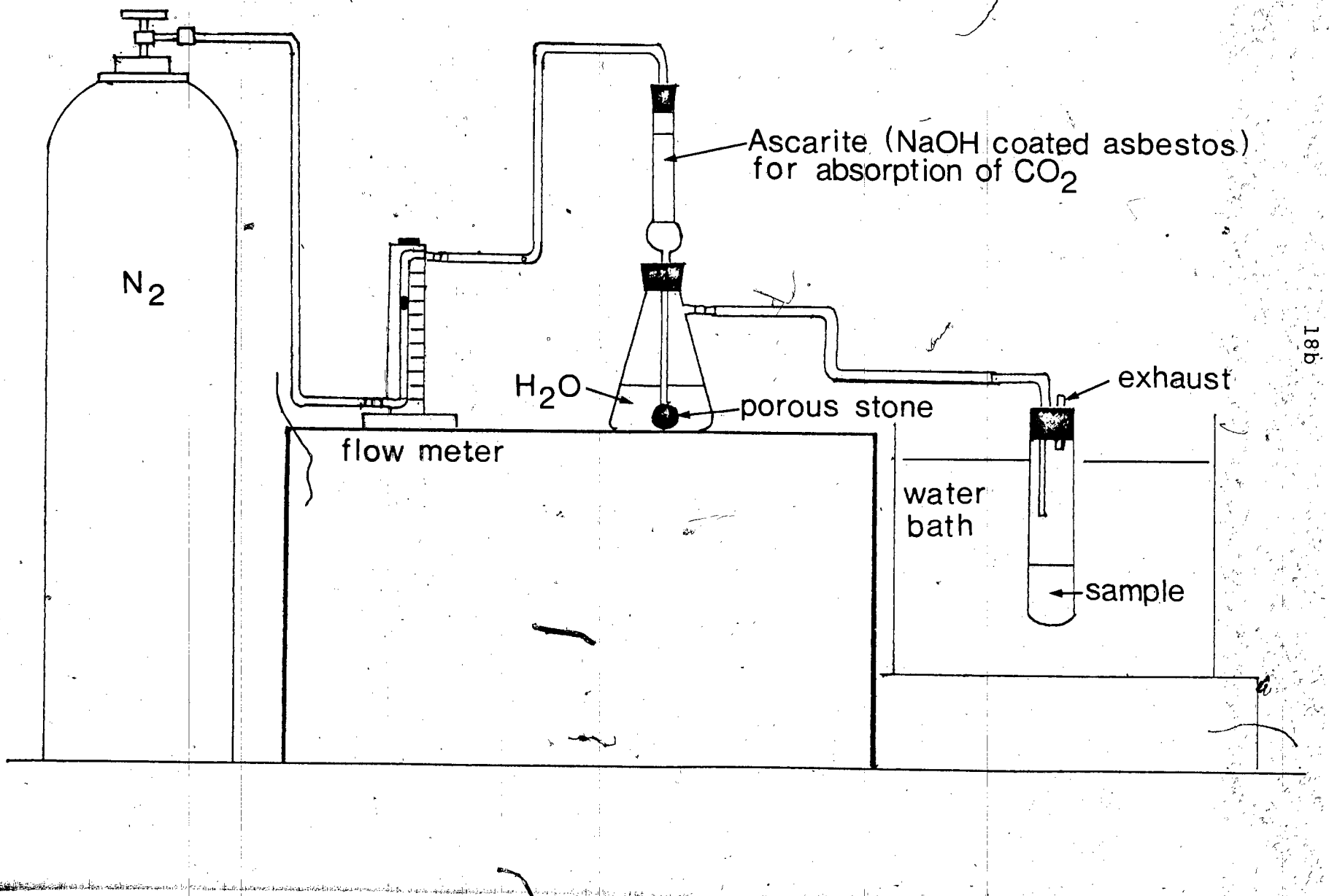
III. Preparation of Hemoglobin Derivatives

Deoxyhemoglobin samples were prepared immediately prior to use. First a 10 to 25 ml sample of oxyhemoglobin in 0.10 M NaCl was placed in a 30x200 mm test tube immersed in a thermostated, 25.0°C water bath. The sample was then exposed to a steady stream of CO_2-free, moist nitrogen (Union Carbide, technical grade) for at least 1 hour using the arrangement of apparatus depicted in Fig. 1. Just prior to starting a denaturation reaction by addition of nitrogen equilibrated alkaline buffer, $Na_2S_2O_4$ (sodium dithionite) was added to the sample in amounts not exceeding 0.2 mg/ml. A nitrogen atmosphere was maintained for the duration of the experiment.

Carbon-monoxide liganded hemoglobin was prepared and utilized with essentially the same method used with deoxyhemoglobin. Technical grade carbon monoxide (Union Carbide) was used instead of nitrogen and $Na_2S_2O_4$ was not used. Except when removing aliquots from the reaction mixture the exhaust port in the apparatus (Fig. 1) was connected to tygon tubing used to channel the outflowing CO into a fume hood.

Methemoglobin (ferric hemoglobin) samples were prepared by oxidation of the heme iron with potassium ferricyanide. Sufficient 0.20 M $K_3Fe(CN)_6$ was added to a measured volume

Figure 1. - Arrangement of apparatus used to maintain deoxyhemoglobin under an atmosphere of N_2 gas. The reaction mixture was sampled through the exhaust port using a 1 ml dispensing pipette. A special pipette tip was constructed from a 2 ml plastic syringe which was adapted to reach the bottom of the reaction vessel with a teflon catheter.


Ascarite (NaOH coated asbestos)
for absorption of CO_2
N_2
H_2O
porous stone
exhaust
flow meter
water
bath
sample

of oxyhemoglobin to achieve a 2-fold stoichiometric excess of the oxidant over the heme content of the sample. The sample was buffered with a pH of 9 or lower to ensure a reasonable oxidation rate (the rate decreases with increasing pH (Mansouri et al, 1973)). Tris-HCl buffered samples obtained directly from the collection column were normally used. After allowing 30 minutes for full oxidation the ferrocyanide and excess ferricyanide were removed by filtration through 12 to 15 sample volumes of Sephadex G-25 (fine) equilibrated with 0.10 M NaCl.

Mercurated hemoglobins were prepared just prior to use by diluting a concentrated aliquot of oxyhemoglobin (2 to 5 mM heme) to 0.4 mM heme with 0.10 M NaCl containing the appropriate amount of mercurial. By diluting the mercurial as much as possible prior to addition of hemoglobin, the undesirable effects of localized high concentrations of mercurial during sample mixing are minimized. When titrating the buried sulfhydryls, the mercurated samples were allowed to react for 2 hours when $HgCl_2$ was employed and 5 hours with p-hydroxymercury benzoate (PMB). The exposed sulfhydryls were considered to be mercurated immediately after mixing. $HgCl_2$-treated samples were found to gradually develop a precipitate and were therefore used within 4 hours of preparation.

Measurement of Hemoglobin Concentration

The hemoglobin concentration of both hemolysates and fully purified samples was determined by the method of Wootton and Blevin (1964) in which all of the hemoglobin is converted to cyanide liganded methemoglobin. The assay employs a solution which was prepared just prior to use by diluting 0.10 ml of 1.00 M NaH_2PO_4, 0.20 ml of 0.500 M KCN and 0.30 ml of 0.20 M $K_3Fe(CN)_6$ to 100 ml with distilled water. 7.5 ml aliquots of this solution are added to 25 ml volumetric flasks. Carefully measured amounts (usually less than 2 ml) of the hemoglobin sample (not CO-hemoglobin) are added to each of the flasks and the mixture is allowed to react for 15 minutes. 2.5 ml of 0.10 M sodium tetraborate is then added and the mixture is diluted to the mark with the first solution. The absorbance of the solution is determined at 540 nm and converted to the heme concentration of the sample using the extinction coefficient recomended by Van Assendelft and Zilstra (1975) of 11.0 mM^{-1} cm^{-1}.

Measurement of Methemoglobin in Oxyhemoglobin Samples

The amount of methemoglobin in oxyhemoglobin samples was determined by a modification of the method of Evelyn and Malloy (1938). This method assumes that the decrease in the absorbance

at 630 nm due to the addition of cyanide is solely due to changes in the absorbance characteristics of methemoglobin. It also assumes that the presence of ferricyanide and ferrocyanide in methemoglobin solutions does not effect their absorbance at 630 nm.

Four solutions were employed in this assay: Solution 1 (0.10 M Na phosphate, pH 6.5) was prepared by diluting 10.0 ml of 1.00 M NaH_2PO_4, 4.00 ml of 1.00 M NaOH to 100.0 ml with distilled water. Solution 2 (0.10 M Na phosphate, 1.00 mM $K_3Fe(CN)_6$, pH 6.5) was prepared identically to solution 1 except for the inclusion of 0.500 ml of 0.200 M $K_3Fe(CN)_6$. Solution 3 (0.10 M Na phosphate, 40 mM KCN, pH 6.5) was prepared by diluting 10.0 ml of 1.00 M NaH_2PO_4 and 8.00 ml of 0.500 M KCN to 100.0 ml. Solution 4 (0.10 M Na phosphate, 40 mM KCN, 1.00 mM $K_3Fe(CN)_6$, pH 6.5) was prepared identically to solution 3 except for the inclusion of 0.5 ml of 0.200 M $K_3Fe(CN)_6$. The solutions containing KCN were prepared fresh before use to minimize loss of cyanide as HCN fumes.

The methemoglobin content of an oxyhemoglobin solution is measured by mixing 2.00 ml of 0.400 mM(heme) hemoglobin dissolved in unbuffered 0.100 M NaCl with 2.00 ml aliquots of

each of the four solutions. The percentage methemoglobin is determined after allowing 30 minutes for reaction from the formula

$$\%Met = 100(A_1 - A_3)/(A_2 - A_4)$$

where A_1, A_2, A_3 and A_4 represent the 630 nm absorbances of the aliquots treated with solutions 1, 2, 3 and 4 respectively. With this method freshly prepared hemoglobin samples were always found to be less than 2% methemoglobin.

The Alkaline Denaturation Kinetics

Two buffer solutions were used routinely to assess the stability of hemoglobin samples at alkaline pH. An alkaline buffer (50.0 mM Na_3PO_4, 0.100 M NaCl, pH 12.0), prepared by diluting 100.0 ml of 1.000 N NaOH, 50.0 ml of 1.000 M NaH_2PO_4 and 5.84 g of NaCl to 1.0000 l with distilled water was used to initiate denaturation and a neutral buffer (25.0 mM Na phosphate, 0.100 M NaCl, pH 6.4), prepared by diluting 25.0 ml of 1.000 M NaH_2PO_4, 8.00 ml of 1.000 N NaOH and 5.84 g NaCl to 1.0000 l with distilled water was used to restore aliquots of the reaction mixture to near neutral pH.

A fixed set of denaturation conditions were used in the majority of experiments. 10.0 ml of 0.10 mM hemoglobin in 0.10 M NaCl was first placed in a 30x200 mm test tube. Both this test tube and a second test tube containing a larger aliquot of alkaline buffer were immersed in a 25.0°C, thermostated water bath and allowed to reach thermal equilibrium. The denaturation reaction was then initiated by abruptly adding 10.0 ml of the equilibrated alkaline buffer to the hemoglobin solution. The resulting reaction mixture, with a pH of 11.7, was maintained at 25.0°C for the duration of the denaturation. The extent of denaturation was then assessed at selected time intervals using one of the following assay procedures.

Because of the potential for variation in the denaturation kinetics due to different batches of hemoglobin or alkaline buffer, control reactions with air-equilibrated hemoglobin were used. These reactions were normally performed at the same time as the experimental reaction(s) so that the kinetic data obtained for a particular hemoglobin derivative could always be compared with that for an appropriate oxyhemoglobin control.

Solubility Assay Methods

The extent of denaturation at a particular time was determined by procedures based on decrease in solubility. The reaction was first terminated by addition of a 1.00 ml aliquot of the

reaction mixture to 4.00 ml of the neutral buffer. After allowing 2 to 4 hours for the denatured protein to aggregate it was removed from solution by centrifugation at 35,000xg for 30 minutes. The amount of protein remaining dissolved in the supernatant was then determined by measuring the absorbance at 275 nm. In the case of deoxyhemoglobin the supernatants were thoroughly oxygenated before measurement to remove residual dithionite which absorbs significantly at this wavelength.

The absorbance that would have been obtained if no denaturation had occurred was estimated as the absorbance of a mixture of 4.00 ml of neutralizing buffer, 0.500 ml of the alkaline buffer and 0.500 ml of the initial, unbuffered hemoglobin solution. The absorbance values obtained for the entire reaction were then expressed as fractions of the initial absorbance. In order to obtain precise estimates of the different kinetic parameters it is important to obtain data through the entire span of the reaction. The reaction was considered complete when the observation time exceeded 10 times the half-time of the slowest component.

Two solubility procedures were used which differ in the composition of the neutral buffer. In the "low salt assay", the neutral buffer was 25.0 mM Na phosphate, 0.100 M NaCl (pH 6.4). The "high salt assay" used an identical buffer except for the inclusion of 300 g/l of reagent grade $(NH_4)_2SO_4$. The two methods differ in that the absorption spectrum of the protein

which remains soluble with the high salt method is that of pure native hemoglobin whereas that with the low salt method indicates that a substantial level of hemichrome is present.

The Direct Absorbance Assay Method

An alternative method measures the changes in the visible spectrum (Fig. 2) that occur as hemoglobin is denatured. The extent of denaturation was assessed by measuring the absorbance decrease at 576 nm. The cuvette was maintained at 25.0°C by circulating water from a thermostated bath through a water-jacketed cuvette holder. The fraction of native undenatured protein present at a particular time was calculated using the equation

$$F = (A_t - A_f)/(A_i - A_f)$$

where A_t represents the absorbance at time t, A_f represents the final absorbance value and A_i represents the initial absorbance.

Sulfhydryl Oxidation

The process of sulfhydryl oxidation was followed by periodically removing aliquots and measuring the concentration of free cysteine sulfhydryl groups during exposure to alkali. The unreacted sulfhydryls were measured by the absorbance increase

at 250 nm due to their reaction with PMB. Two neutral buffers were used in this procedure, prepared by simple additions to the neutral buffer used in the low salt assay procedure. Buffer D included sodium dodecyl sulfate (SDS) at a concentration of 2.5% (w/v) and buffer E included, in addition, 0.15 mM PMB.

1.00 ml aliquots of the reaction mixture were neutralized with 4.00 ml of either buffer D or E at selected time intervals. The absorbances of the neutralized aliquots were then measured at 250 nm after allowing 2 or more hours to ensure equilibrium. By alternating between the 2 buffers, 2 absorbance versus time profiles were generated. The difference between the two absorbance profiles represents the absorbance of PMB plus the absorbance increase due to reaction of PMB with sulfhydryl groups. The absorbance of a hemoglobin-free reference solution (4.00 ml of buffer E, 0.500 ml of 0.100 M NaCl and 0.500 ml of alkaline buffer) was subtracted. The remaining absorbance increment was converted to sulfhydryls per hemoglobin tetramer using the molar extinction coefficient given by Boyer (1954) of 7.6×10^{-4} M^{-1} cm^{-1}.

The mercural in buffer E should prevent further disulfide formation in the SDS-solubilized protein. Some of the aliquots neutralized with this buffer were used to demonstrate the presence of disulfides using the SDS gel electrophoresis

procedure of Weber and Osborn (1975). Aliquots ranging from 0.050 to 0.20 ml of the neutralized aliquots were applied directly to 10% polyacrylamide 5.5 x 100 mm tube gels. 2-mercaptoethanol was added to a few of the gel samples. High molecular weight bands which were converted to lower molecular weight bands by 2-mercaptoethanol treatment were probably disulfide-linked multimers.

Analysis of the Reaction Kinetics

The denaturation data were fitted by APL programs to first or second-order models using an algorithm based on the non-linear least-squares procedure of Marquardt (1963). This iterative procedure converges rapidly on the minimum sum of squares by approximating the method of steepest descent when the minimum is distant and converting to a direct analytical method based on linearization of the fit equation when the minimum is near. In this manner Marquardt's procedure uses each of its component methods when they are most effective.

All of the models used in the curve fitting procedures were polynomials contructed from 3 types of terms; constant terms (i.e. B_0), first-order terms (i.e. $B_1\exp(-k_1t)$ and second order terms (i.e. $B_1/(1+k_1t)$). The second order terms are only appropriate for second-order reactions in which the ratio between the concentrations of the 2 molecular species involved

in the rate determining step remains constant. Terms were added to the fit model only if the resulting decrease in the fit sum of squares was assessed to be sufficient by a partial F-test (Bevington, 1969).

In addition to the fit itself, the APL programs provided relavent statistics on the quality of the fit as well as plots of the data and the fitted curve. Among the statistics were the standard deviation of the fit (SD) defined by

$$SD = \sqrt{\frac{\sum_{i=1}^{N}(YO_i - YE_i)^2}{N - P - 1}}$$

and the coefficient of determination of the fit (R^2) defined by

$$R^2 = 1 - \frac{\sum_{i=1}^{N}(YO_i - YE_i)^2}{\sum_{i=1}^{N}(YO_i - YM)^2}$$

where YO_i is the y-value obtained for the i^{th} data point, YE_i is that predicted by the fit model and YM is the mean y-value of the data. N is the number of data points and P is the number of parameters in the fit model. Note that R^2 represents the fraction of the overall variance of the data about YM that is accounted for by the fit model.

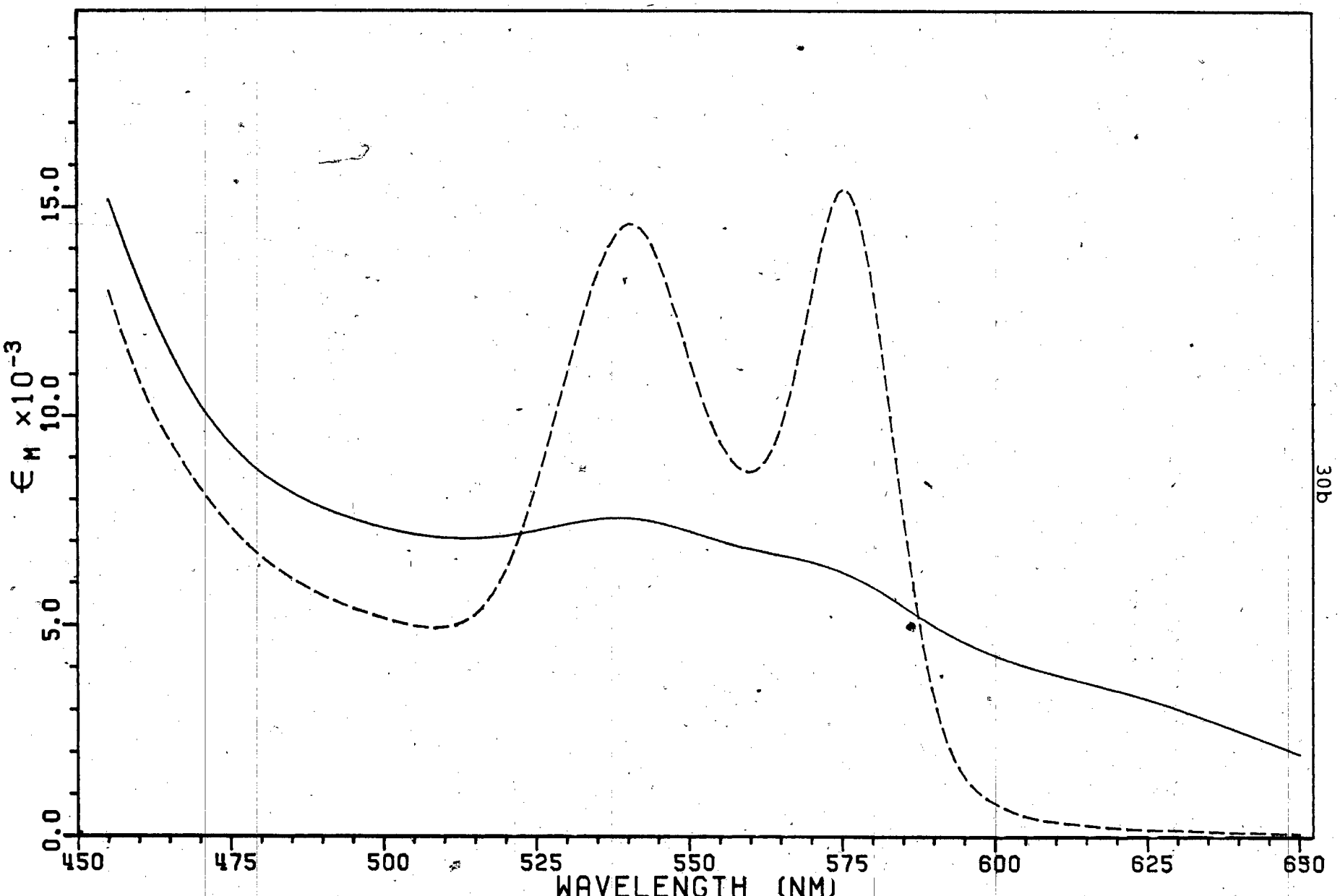
RESULTS

Oxyhemoglobin

The denaturation of oxyhemoglobin by alkali is accompanied by a reduction in absorbance at the oxyhemoglobin absorption maxima of 540 and 576 nm along with corresponding increases at visible wavelengths above 590 nm and below 520 nm (Fig. 2). This overall flattening of the absorption spectrum indicates conversion of oxyhemoglobin to hemichromes such that the direct absorbance procedure measures the kinetics of hemichrome formation. When the direct absorbance procedure is applied to oxyhemoglobin denaturation, the data are fit by a single-component first-order model with a half time of about 2 min (Fig. 3, Table 1).

The denaturation kinetics obtained with the high salt assay procedure appear identical to those obtained with the direct absorbance method (Fig. 3, Table 1). Thus the formation of alkaline hemichrome demonstrated by the direct absorbance procedure clearly corresponds with the loss of protein solubility demonstrated by the high salt method. The absorption spectra of the final supernatants obtained with this method were indistinguishable from that of oxyhemoglobin indicating that only the native oxyhemoglobin fraction of the reaction mixture remains soluble with this procedure.

Figure 2. - Absorption spectrum of the hemichromes resulting from exposing oxyhemoglobin to alkali for 20 min. (solid line). The absorption spectrum of native oxyhemoglobin (broken line) is supplied for comparison. Both spectra were obtained using concentrations of 0.10 mM heme. The native hemoglobin sample was unbuffered in 0.100 M NaCl with a measured pH of 7.6. The hemichrome in this spectrum and all subsequent spectra was dissolved in 25.0 mM Na_3PO_4, 0.10 M NaCl at pH 11.7. The values of the molar extinction coefficients in this spectrum and all subsequent spectra pertain to the heme concentrations.


$\epsilon_M \times 10^{-3}$
15.0
10.0
5.0
0.0
450
475
500
525
550
575
600
625
650
WAVELENGTH (NM)

30b

Figure 3. - Comparison of the denaturation kinetics of oxyhemoglobin as determined with the low salt procedure (+), the high salt procedure (x) and direct absorbance monitoring (*). The "remaining fraction" is either the fraction of the initial 275 nm absorbance that remained in solution after neutralization (low salt or high salt methods) or the remaining fraction of the total change in 576 nm absorbance (direct absorbance method). Models based on first order kinetics are applied in all three cases. The particular models and values of the fitted parameters are presented in Table 2. Denaturation was considered complete in 20 min with the direct absorbance procedure.

REMAINING FRACTION
0.0
0.2
0.4
0.6
0.8
TIME (MINUTES)
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0

Table 1 - Parameters and statistics for least squares fits as assessed with the low salt, high salt and direct absorbance assay procedures. First order models were used in all cases. The k_n values are the first-order rate constants in units of min^{-1}. The B_n values are a measure of the fraction of the overall change accounted for by each term of the fitted model. The statistics abbreviated "SD", "R^2" and "df" respectively represent the standard deviation, coefficient of determination and degrees of freedom for the fits. The errors in the parameters are standard deviations derived the error matrix for the particular fit.

APPLIED MODEL (ASSAY METHOD)	LINEAR PARAMETERS	NON-LINEAR PARAMETERS	FIT STATISTICS
$B_0 + B_1\exp(-k_1t) + B_2\exp(-k_2t)$ (LOW SALT)	$B_0 = 0.2255 \pm 0.0047$ $B_1 = 0.915 \pm 0.027$ $B_2 = 0.1199 \pm 0.0074$	$k_1 = 0.393 \pm 0.017$ ($T_1 = 1.76$ min) $k_2 = 0.0139 \pm 0.0025$ ($T_2 = 49.9$ min)	SD = 0.00846 $R^2 = 0.99837$ df = 17
$B_1\exp(-k_1t)$ (HIGH SALT)	$B_1 = 0.949 \pm 0.012$	$k_1 = 0.3399 \pm 0.0065$ ($T_1 = 2.039$ min)	SD = 0.0101 $R^2 = 0.99862$ df = 10
$B_1\exp(-k_1t)$ (DIRECT ABSORBANCE)	$B_1 = 0.9166 \pm 0.0022$	$k_1 = 0.3614 \pm 0.0012$ ($T_1 = 1.918$ min)	SD = 0.00200 $R^2 = 0.9999160$ df = 14

The kinetics observed with the low salt assay method are more complex (Figs 3 and 4). Precipitation occurs after a time lag of about 1 min with 2 clearly distinct rate components. A reproducible final level of soluble protein is observed for which the concentration was unaffected by changing the initial reactant concentration. The fastest and most prominent of the 2 components is kinetically equivalent to the single component obtained with the other 2 assays and there is very good agreement between the the kinetic parameters (B_1 and k_1) obtained with all 3 methods (Table 1). The less prominent slow component, representing the disappearance of an intermediate, is only observed with the low salt assay method. The precipitated protein could not be redissolved in the precipitation buffer. Table 2 contains parameters and statistics for least-squares fits to the low salt results based on first or second order kinetics. Partial F-test shows either of the 2 component models to be significantly better than the 1 component models at the 0.01 probability level.

In addition to the major changes in the visible spectrum of the reaction mixture due to conversion of native oxyhemoglobin to hemichrome, much slower changes occur due to some further alteration of the initial denaturation product. These appear as a further decrease in the 540 nm absorbance with corresponding increases at 480 nm and 610 nm (Fig. 5). It is of interest if

Figure 4. - Alkaline denaturation of oxyhemoglobin by the low-salt method. The fraction of the 275 nm absorbance remaining in solution after low-salt neutralization is plotted versus the neutralization time (points). A model consisting of a constant term and two exponential terms is fit to the data (solid line). The exponential terms (Table 1) are depicted separately by the broken lines. Only data for exposures greater than 1 min were used for the fit.

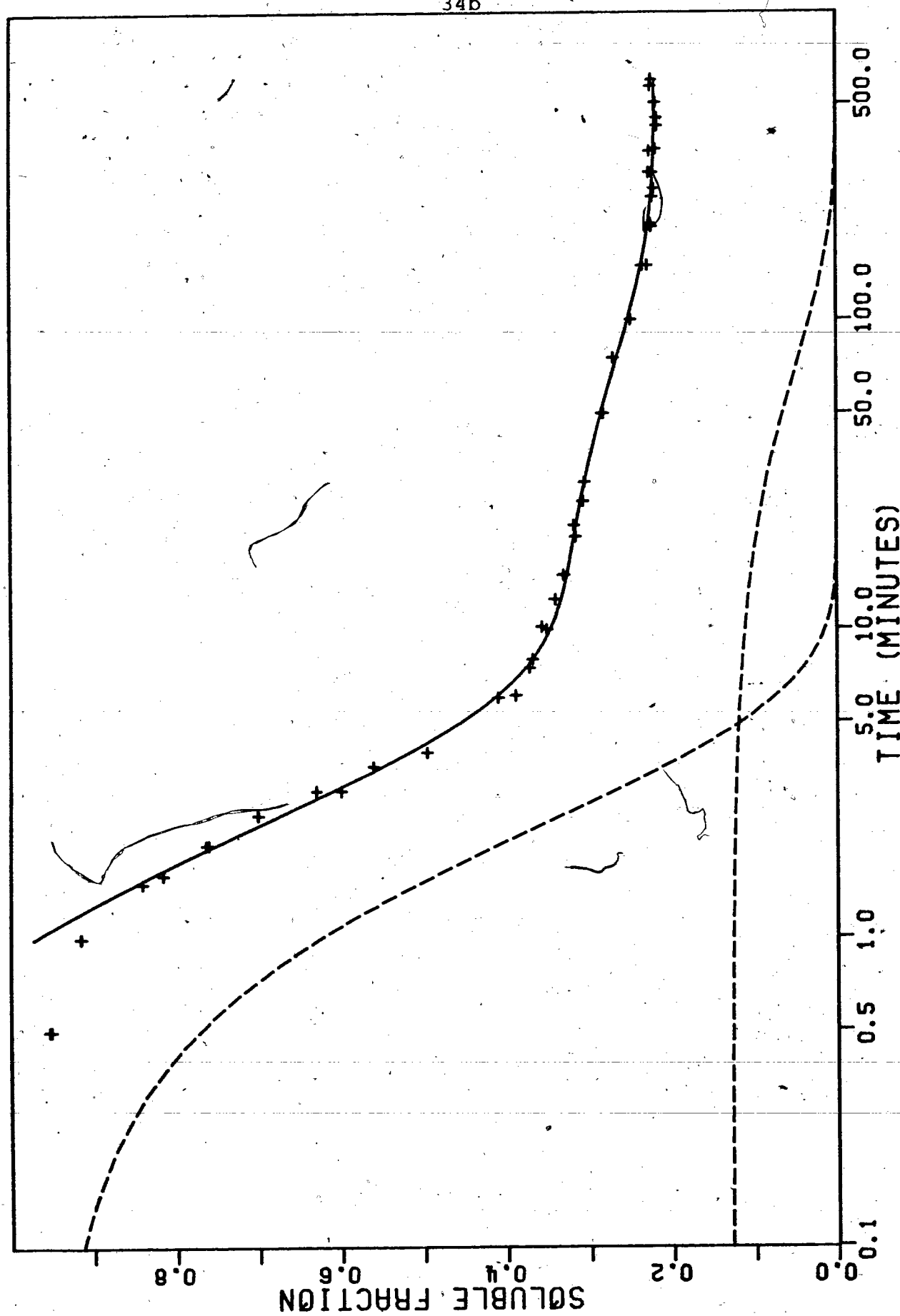
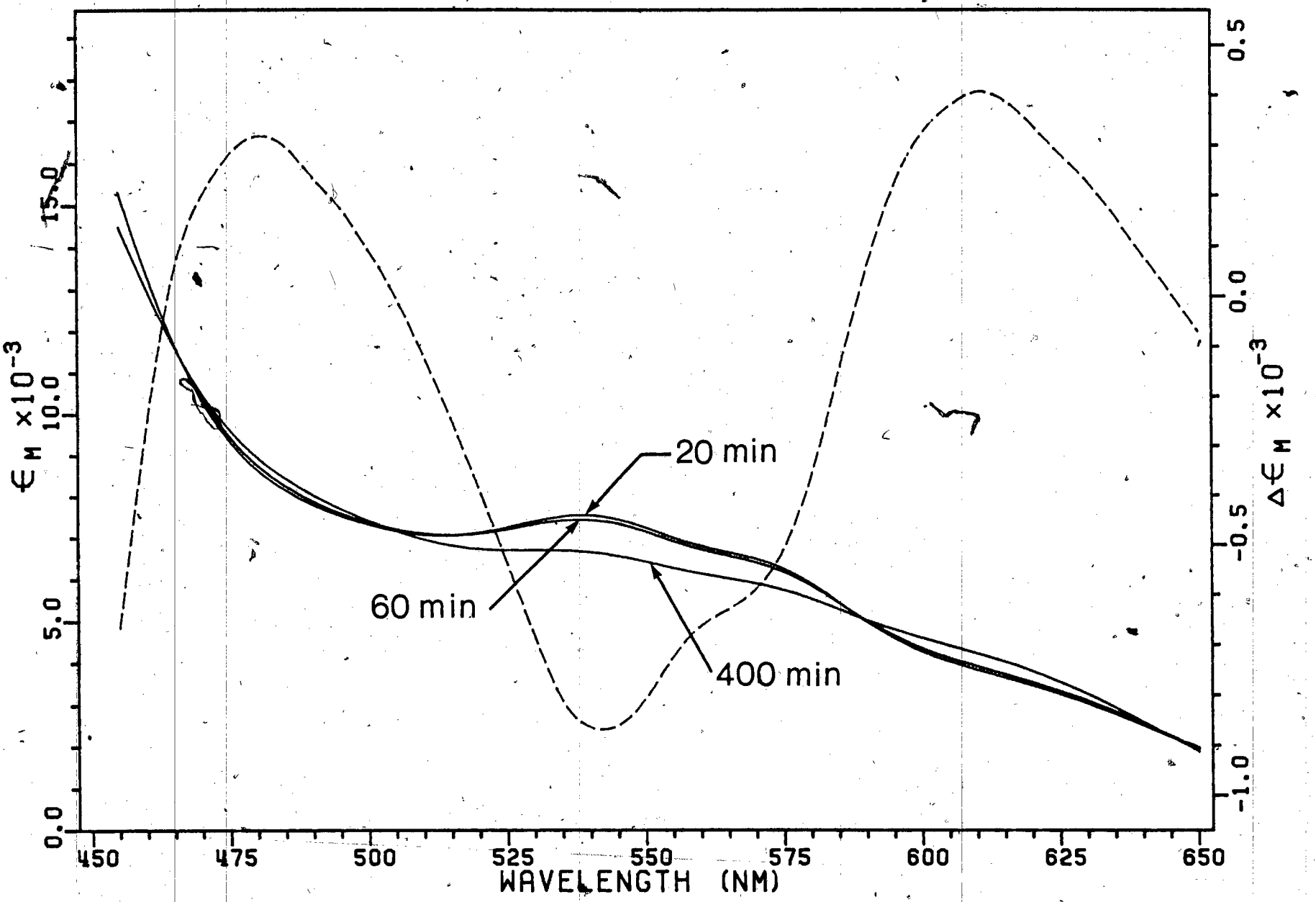

SOLUBLE FRACTION
0.0
0.2
0.4
0.6
0.8
TIME (MINUTES)
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0

Table 2 - Comparison of models applied to the kinetics of alkaline denaturation of oxyhemoglobin as measured with the low salt assay method. The parameters were estimated by non-linear least squares fits to the data plotted in Fig. 4. Data for reaction times less than 1 min was not used. The linear parameters are unitless and the non-linear parameters are in units of min^{-1}. The "T_n" values refer to reaction half times. The third fit model was used to generate the curves depicted in Fig. 4. Errors and statistics are explained in Table 1.

APPLIED MODEL	LINEAR PARAMETERS	NON-LINEAR PARAMETERS	FIT STATISTICS
$B_0 + B_1\exp(-k_1 t)$	$B_0 = 0.2526 \pm 0.0071$ $B_1 = 0.846 \pm 0.048$	$k_1 = 0.266 \pm 0.022$ ($T_1 = 2.61$ min)	SD = 0.0350 $R^2 = 0.9674$ = 37
$B_0 + B_1/(1+k_1 t)$	$B_0 = 0.2272 \pm 0.0046$ $B_1 = 1.96 \pm 0.30$	$k_1 = 1.40 \pm 0.29$ ($T_1 = 0.713$ min)	SD = 0.0202 $R^2 = 0.9892$ df = 37
$B_0 + B_1\exp(-k_1 t) + B_2\exp(-k_2 t)$	$B_0 = 0.2202 \pm 0.0030$ $B_1 = 0.953 \pm 0.025$ $B_2 = 0.1300 \pm 0.0065$	$k_1 = 0.425 \pm 0.016$ ($T_1 = 1.63$ min) $k_2 = 0.0144 \pm 0.0020$ ($T_2 = 48.0$ min)	SD = 0.00947 $R^2 = 0.99773$ df = 35
$B_0 + B_1\exp(-k_1 t) + B_2/(1+k_2 t)$	$B_0 = 0.2023 \pm 0.0059$ $B_1 = 0.949 \pm 0.028$ $B_2 = 0.162 \pm 0.010$	$k_1 = 0.438 \pm 0.021$ ($T_1 = 1.58$ min) $k_2 = 0.0218 \pm 0.0062$ ($T_2 = 45.9$ min)	SD = 0.0103 $R^2 = 0.99730$ df = 35

Figure 5. - Absorption spectrum of the oxyhemoglobin reaction mixture after 20, 60 and 400 min. The difference spectrum obtained by subtracting the absorbance at t=20 min from that at t=400 min is indicated by the broken line. The right-hand ordinate pertains to the difference spectrum.

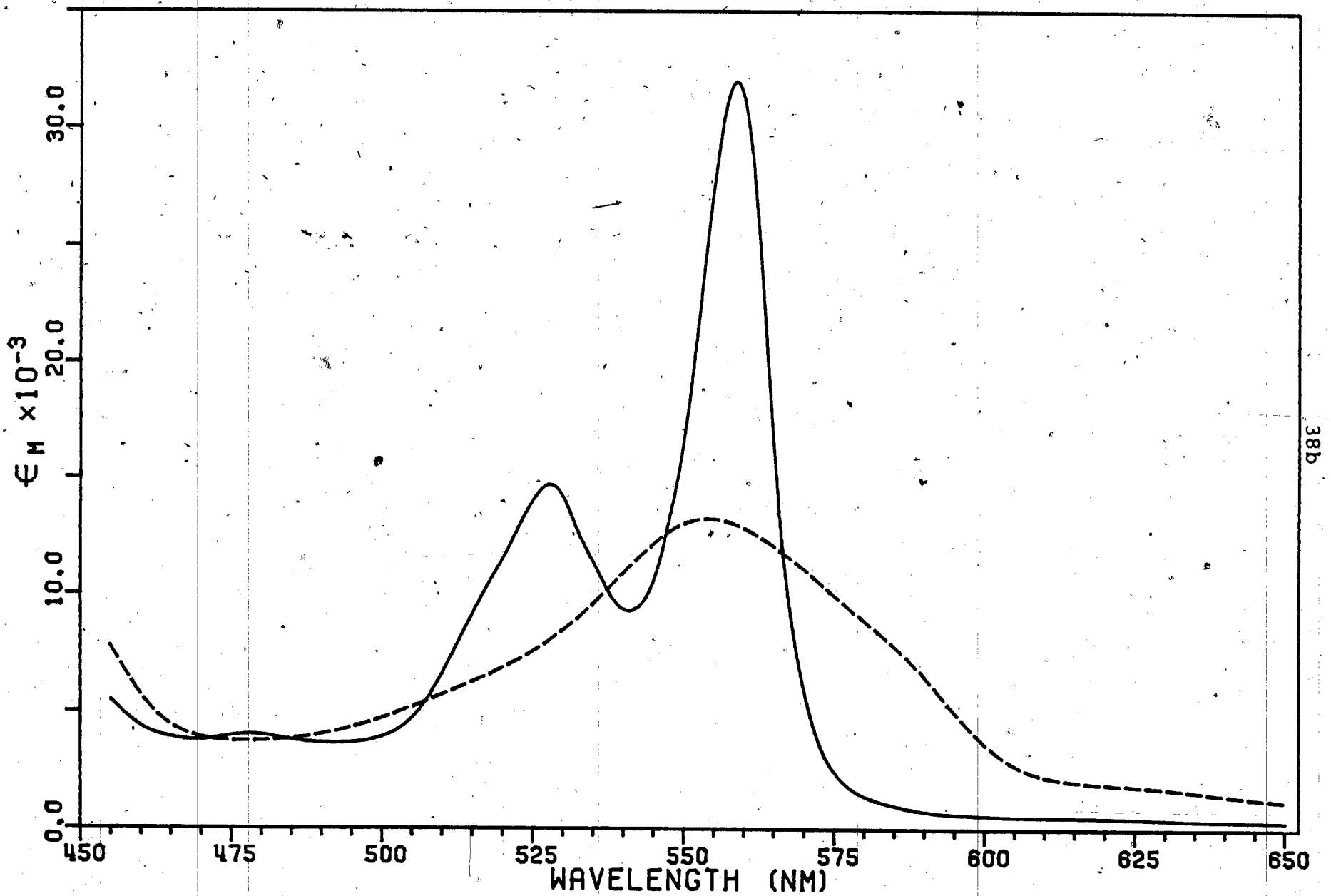

15.0
10.0
5.0
0.0
$\epsilon_M \times 10^{-3}$
0.5
0.0
-0.5
-1.0
$\Delta\epsilon_M \times 10^{-3}$
20 min
60 min
400 min
450
475
500
525
550
575
600
625
650
WAVELENGTH (NM)

the time course for these slow spectral changes is the same as that for the disappearance of the intermediate observed with the low salt assay. Using the rate constant for the latter reaction (k_2 of Table 2), one can predict that the hemichrome spectrum for 60 min of exposure to alkali should be positioned about half way between the 20 min and 400 min spectra if the two processes were identical. Since this is clearly not true, the slow change in the hemichrome spectrum and the slow component of the low salt assay must represent different reactions.

Deoxyhemoglobin

The denaturation of deoxyhemoglobin has characteristics that distinguish it from that of oxyhemoglobin. Firstly, the heme iron must remain in the reduced or ferrous state due to the absence of oxidants such as oxygen and the presence of the reducing agent dithionite. The visible absorption spectrum of native deoxyhemoglobin is converted to a spectrum with sharp absorption bands at 527 nm and 558 nm which are characteristic of hemochromes, the ferrous iron equivalent of hemichromes (Fig. 6). No further spectral changes are observed after 15 min of exposure to alkali, the time required for full conversion to hemochrome.

Figure 6. - Absorption spectrum of the hemochromes resulting from exposure of deoxyhemoglobin to alkali (solid line). The spectrum of native deoxyhemoglobin is included for comparison (broken line). The native deoxyhemoglobin was dissolved in unbuffered 0.100 M NaCl with a pH measured at 7.4. Concentrations were 0.050 mM heme for the hemochromes and 0.10 mM heme for the native protein.


30.0
20.0
10.0
0.0
$\epsilon_M \times 10^{-3}$
450
475
500
525
550
575
600
625
650
WAVELENGTH (NM)

38b

The reaction profile observed with the low salt assay is similar to that obtained with oxyhemoglobin (Fig. 7), in that some protein remains soluble. However, only one rate component was observed and the final level of soluble protein was considerably higher. The data fit a first-order model far better than a comparable second-order model (Table 3). The first-order rate constant is comparable to that obtained for oxyhemoglobin under identical conditions (Table 3).

In contrast to what was observed with oxyhemoglobin, the supernatants obtained with the low salt assay had visible spectra which were indistinguishable from that of native oxyhemoglobin (the neutralizing buffer contained O_2 and the samples were exposed to air after neutralization). Since full conversion to hemochromes occurs in less than 15 min of exposure to alkali (Fig. 6), the native oxyhemoglobin in these supernatants must have arisen by renaturation. When aliquots of the reaction mixture were neutralized with oxygen-free buffer and maintained at 4°C without O_2 for 16 hours, full renaturation to deoxyhemoglobin was obtained. The renaturation kinetics were not investigated.

Methemoglobin

Methemoglobin was much less stable than oxyhemoglobin to alkaline conditions. At least 1 fast reaction occurs within 30 sec of the start of the reaction with a product that is 20%

Figure 7. - Comparison of the denaturation kinetics of deoxyhemoglobin (+) and oxyhemoglobin (x) by the low salt method. The solid lines represent least-squares fits to the data based on first order kinetics and were generated using the first-order models and fit parameters presented in Table 3.

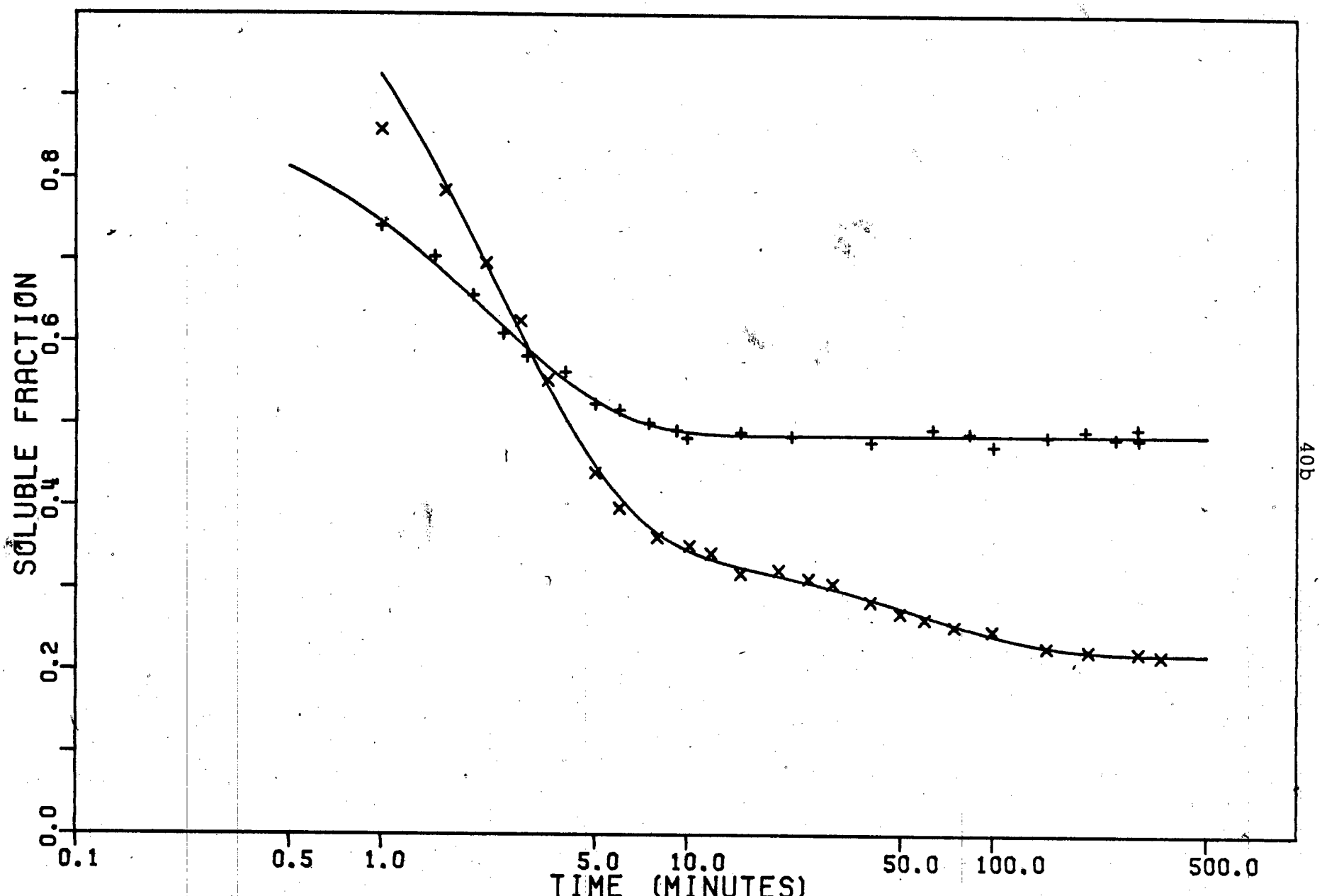

SOLUBLE FRACTION
0.0
0.2
0.4
0.6
0.8
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0
TIME (MINUTES)

Table 3 - Parameters and statistics for least squares fits to the alkaline denaturation kinetics of methemoglobin and deoxyhemoglobin. Results from control experiments with oxyhemoglobin are included for comparison. Single component first and second order models were applied to the methemoglobin and deoxyhemoglobin data while 2 component first order models were applied to the oxyhemoglobin controls. Much of the difference between the oxyhemoglobin controls is due to the use of different batches of alkaline buffer. The k_n values are in units of min^{-1}. Errors and statistics are explained in Table 1.

HEMOGLOBIN DERIVATIVE	APPLIED MODEL	LINEAR PARAMETERS	NON-LINEAR PARAMETERS	df	FIT STATISTICS
METHEMOGLOBIN	$B_0 + B_1\exp(-k_1 t)$	$B_0 = 0.281 \pm 0.010$ $B_1 = 0.532 \pm 0.016$	$k_1 = 0.0144 \pm 0.0012$ ($T_1 = 48.1$ min)	13	SD = 0.0232 $R^2 = 0.9892$
METHEMOGLOBIN	$B_0 + B_1/(1+k_1 t)$	$B_0 = 0.194 \pm 0.011$ $B_1 = 0.640 \pm 0.012$	$k_1 = 0.0184 \pm 0.0016$ ($T_1 = 54.3$ min)	13	SD = 0.0150 $R^2 = 0.99550$
OXYHEMOGLOBIN	$B_0 + B_1\exp(-k_1 t)$ $+ B_2\exp(-k_1 t)$	$B_0 = 0.2255 \pm 0.0047$ $B_1 = 0.915 \pm 0.027$ $B_2 = 0.1199 \pm 0.0074$	$k_1 = 0.393 \pm 0.017$ ($T_1 = 1.76$ min) $k_2 = 0.0139 \pm 0.0025$ ($T_2 = 49.9$ min)	17	SD = 0.00846 $R^2 = 0.99837$
DEOXYHEMOGLOBIN	$B_0 + B_1\exp(-k_1 t)$	$B_0 = 0.486 \pm 0.002$ $B_1 = 0.410 \pm 0.015$	$k_1 = 0.452 \pm 0.022$ ($T_1 = 1.53$ min)	19	SD = 0.00711 $R^2 = 0.99244$
DEOXYHEMOGLOBIN	$B_0 + B_1/(1+k_1 t)$	$B_0 = 0.475 \pm 0.005$ $B_1 = 1.5 \pm 1.0$	$k_1 = 4.2 \pm 3.4$ ($T_1 = 0.24$ min)	19	SD = 0.0160 $R^2 = 0.9619$
OXYHEMOGLOBIN	$B_0 + B_1\exp(-k_1 t)$ $+ B_2\exp(-k_1 t)$	$B_0 = 0.2206 \pm 0.0047$ $B_1 = 0.874 \pm$ $B_2 = 0.1346 \pm 0.0075$	$k_1 = 0.423 \pm 0.019$ ($T_1 = 1.64$ min) $k_2 = 0.0177 \pm 0.0025$ ($T_2 = 39.2$ min)	17	SD = 0.00772 $R^2 = 0.99811$

precipitated by the low salt buffer and completely precipitated by the high salt buffer. A slow reaction follows within the experimental time frame (Fig. 8). The single kinetic component represents a reaction of an intermediate which has lost the native structure since, like the intermediate observed for oxyhemoglobin, it is insoluble in high salt buffer.

A slight shift in the visible spectrum of the reaction mixture occurs during the transformation indicated by the low salt assay (Fig. 9). The spectra are typical of hemichromes. The observed changes are smaller than those observed with oxyhemoglobin (Fig. 5) and the difference spectrum obtained by subtracting the 2 extreme spectra is also different from that obtained with oxyhemoglobin. The positions of the 3 spectra are consistent with the kinetics of the slow reaction observed with the low salt assay and are not consistent with the kinetics of the slow changes in the hemichromes derived from oxyhemoglobin.

Carboxyhemoglobin

The effects of carbon monoxide on hemoglobin denaturation is seen in Fig. 10. A single rate component is observed with a much larger half time than the fast component obtained with identically treated oxyhemoglobin (Table 4). A colour change indicative of hemichrome formation was observed in the reaction

Figure 8. - Comparison of the alkaline denaturation kinetics of methemoglobin (+) and oxyhemoglobin (x) with the low salt assay. The solid lines represent least-squares fits based on the first-order models presented in Table 3.

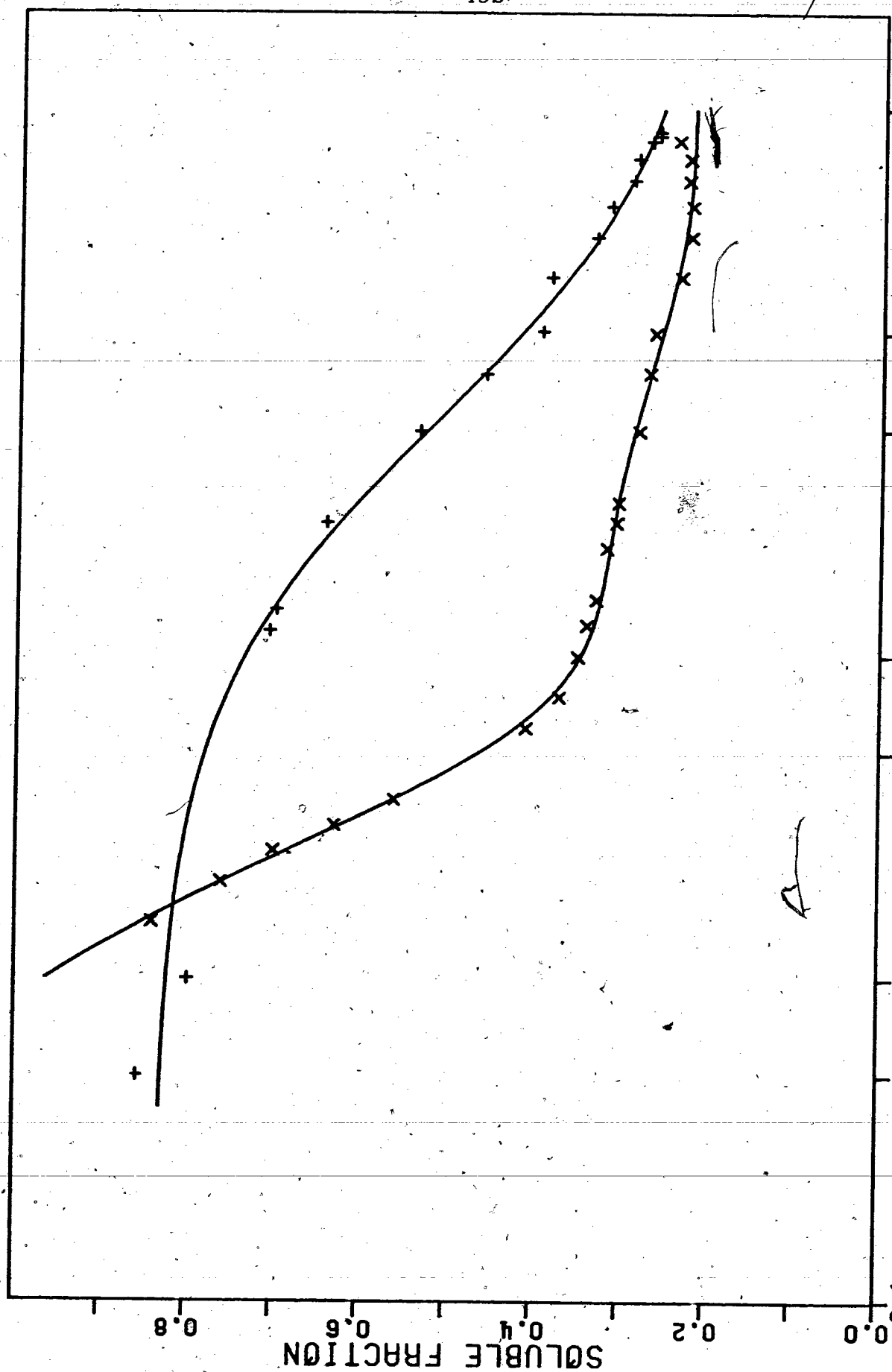
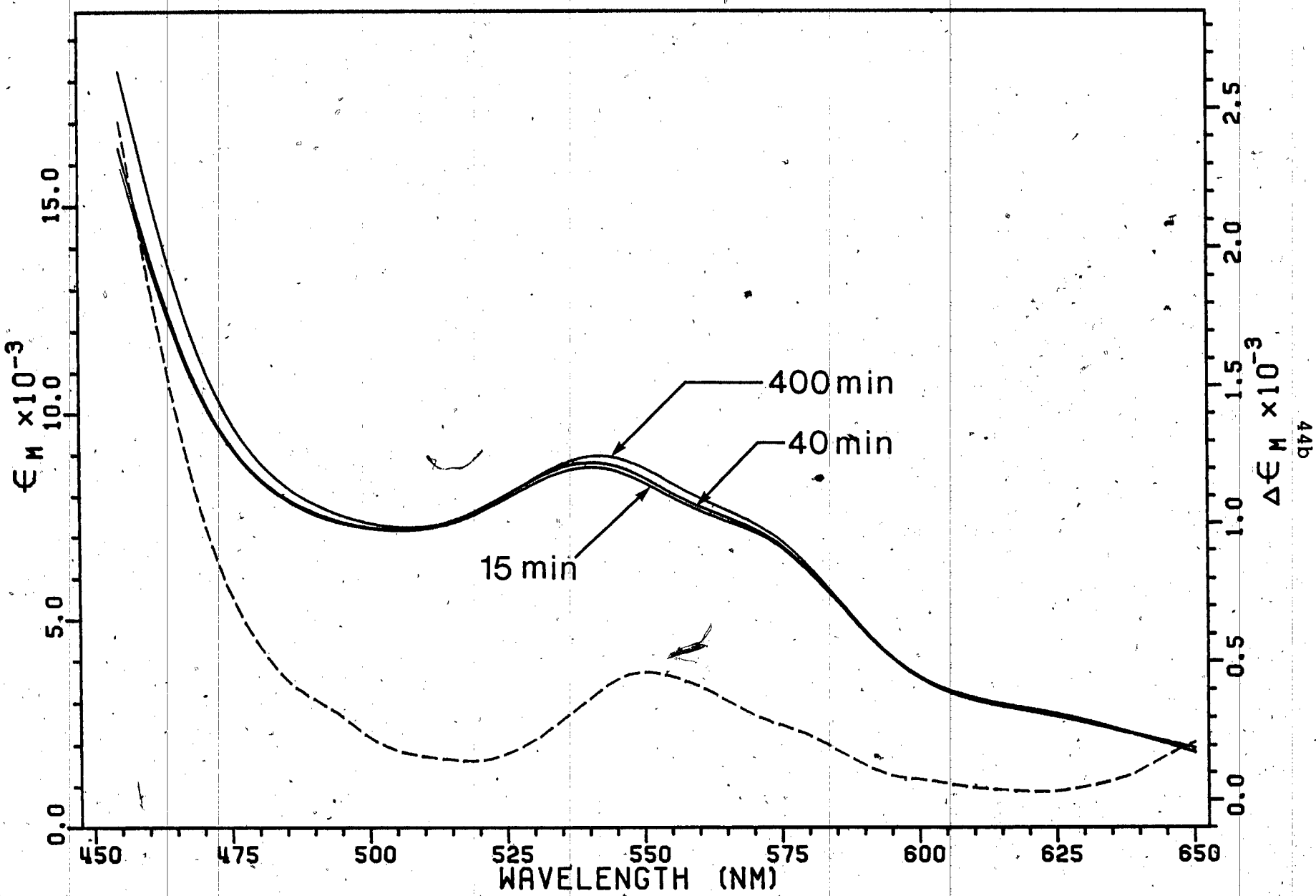

SOLUBLE FRACTION
0.0
0.2
0.4
0.6
0.8
TIME (MINUTES)
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0

Figure 9. - Absorption spectra of the reaction mixture derived from the alkaline denaturation of methemoglobin after 15, 40 and 400 min (solid lines). The difference spectrum obtained by subtracting the spectrum for t=15 min from that at t=400 min is also included (broken line). The right-hand axis applies to the difference spectrum.


400 min
40 min
15 min
$\epsilon_M \times 10^{-3}$
$\Delta\epsilon_M \times 10^{-3}$
WAVELENGTH (NM)
0.0
5.0
10.0
15.0
0.0
0.5
1.0
1.5
2.0
2.5
450
475
500
525
550
575
600
625
650

44b

Figure 10.- The denaturation kinetics of CO equilibrated (+) and air equilibrated (x) hemolysate as assessed with the low salt assay method. Denaturation was performed in 31.3 mM Na_3PO_4, 0.10 M NaCl at a pH of 11.8 and temperature of 25°C. Least squares fits based on first order components are represented by the solid lines. Values of the kinetic parameters are presented in Table 4.

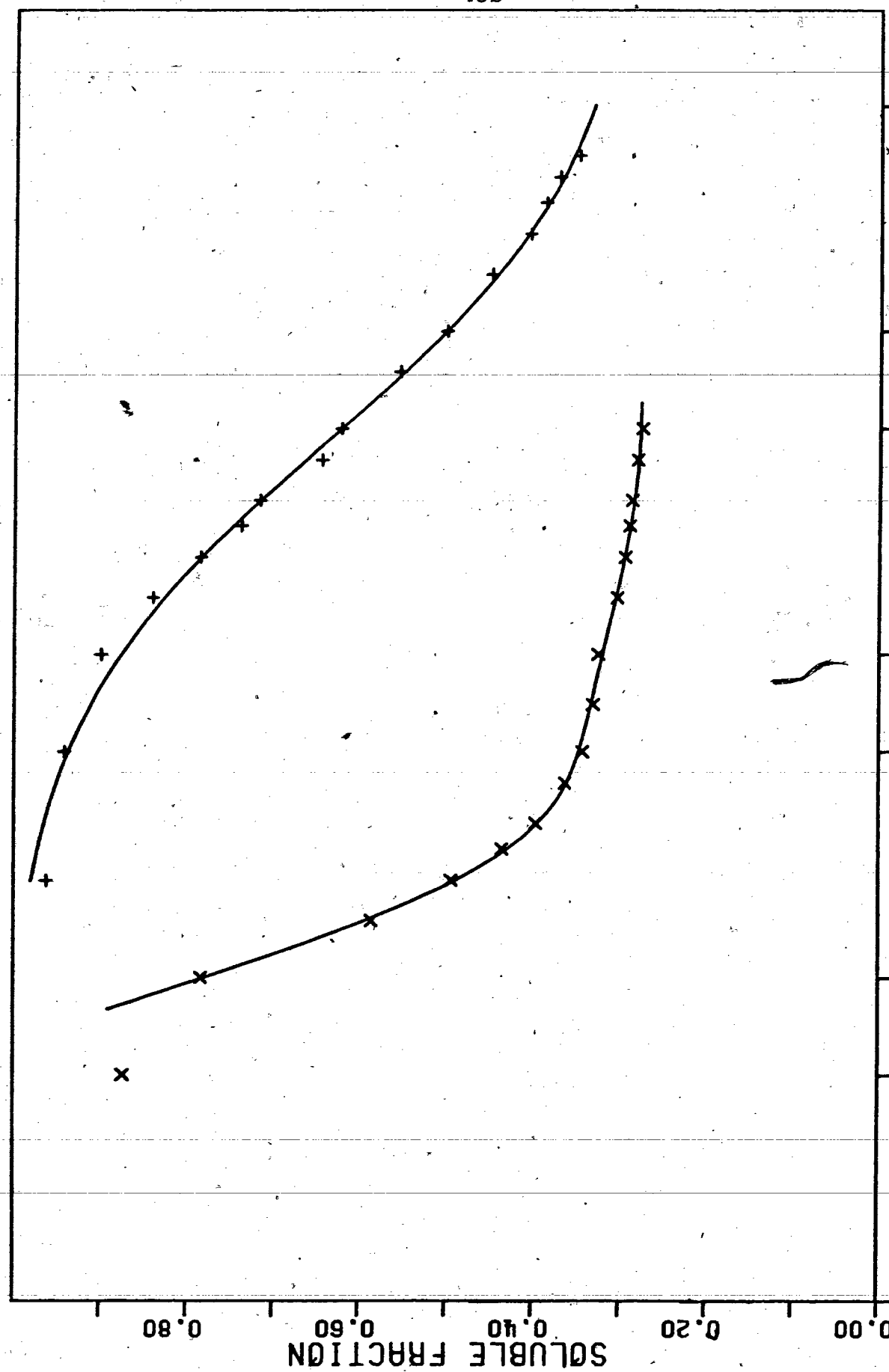

SOLUBLE FRACTION
0.00
0.20
0.40
0.60
0.80
TIME (MINUTES)
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0

Table 4 - Parameters and statistics for least-squares fits to the alkaline denaturation of carboxyhemoglobin. Comparable parameters and statistics from a control experiment with oxyhemoglobin are included. The data used for these fits is plotted in Fig. 10. The reaction conditions used in these experiments (described in the legend of Fig. 10) were significantly more alkaline than the conditions used for all of the other experiments. Errors and statistics are explained in Table 1.

HEMOGLOBIN DERIVATIVE	APPLIED MODEL	LINEAR PARAMETERS	NON-LINEAR PARAMETERS	df	FIT STATISTICS
CO-HEMOGLOBIN	$B_0 + B_1\exp(-K_1 t)$	$B_0 = 0.379 \pm 0.011$ $B_1 = 0.599 \pm 0.015$	$K_1 = 0.0180 \pm 0.0012$ (T_1 = 38.5 min)	13	SD = 0.0199 R^2 = 0.99236
CO-HEMOGLOBIN	$B_0 + B_1/(1+K_1 t)$	$B_0 = 0.270 \pm 0.010$ $= 0.741 \pm 0.010$	$K_1 = 0.0220 \pm 0.0013$ (T_1 = 38.5 min)	13	SD = 0.0113 R^2 = 0.99755
OXYHEMOGLOBIN	$B_0 + B_1\exp(-K_1 t)$ $+ B_2\exp(-K_1 t)$	$B_0 = 0.25 \pm 0.18$ $B_1 = 0.841 \pm 0.047$ $B_2 = 0.08 \pm 0.15$	$K_1 = 0.760 \pm 0.090$ (T_1 = 0.912 min) $K_2 = 0.023 \pm 0.084$ (T_2 = 31 min)	11	SD = 0.0231 R^2 = 0.9884

mixture indicating the presence of O_2, which was likely an impurity in the technical grade CO used. Since the colour change appeared to parallel the denaturation profile, oxidation may be a prerequisite for precipitation of denatured carboxyhemoglobin. In the complete absence of O_2, denatured carboxyhemoglobin might not precipitate at all with the low salt procedure.

Mercurated Hemoglobins

Modifying all of the cysteine sulfhydryl groups by addition of 8 equivalents of $HgCl_2$ per tetramer (a 2 equivalent excess) reduced the alkaline denaturation rate 23 fold (Fig. 11, Table 5) whereas mercuration of only the 2 most reactive sulfhydryl groups by addition of 2 equivalents of $HgCl_2$ per tetramer decreased stability about 30% (Fig. 12, Table 5). These results indicate that modification of the interfacial cysteines but not the reactive F9β cysteines increases the stability of oxyhemoglobin to alkali.

In contrast to the effects of $HgCl_2$, blocking all of the sulfhydryl groups by adding excess PMB causes an increase in the alkaline denaturation rate as well as the reaction order (Fig. 11, Table 4). The denaturation profile for PMB titrated hemoglobin is found to fit a single component second order model far better than the corresponding first order model (Table 5).

Figure 11.- The denaturation kinetics of oxyhemoglobin treated with 8 equivalents of $HgCl_2$ per tetramer (x) and 12 equivalents of p-hydroxymercuribenzoate per tetramer (*). Untreated oxyhemoglobin (+) is included for comparison. The kinetics were determined by the direct absorbance assay method. The solid lines represent least-squares fits based on the models and parameters depicted in Table 5 (using the second-order model for the PMB-hemoglobin data).

UNDENATURED FRACTION
0.00
0.20
0.40
0.60
0.80
TIME (MINUTES)
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0

Figure 12.- The denaturation kinetics of oxyhemoglobin (x) and methemoglobin (+) treated with 2 equivalents of $HgCl_2$ per tetramer as assessed with the low salt assay method. First-order models were applied to both sets of data (solid lines). The fit parameters obtained for oxyhemoglobin are presented in Table 5.

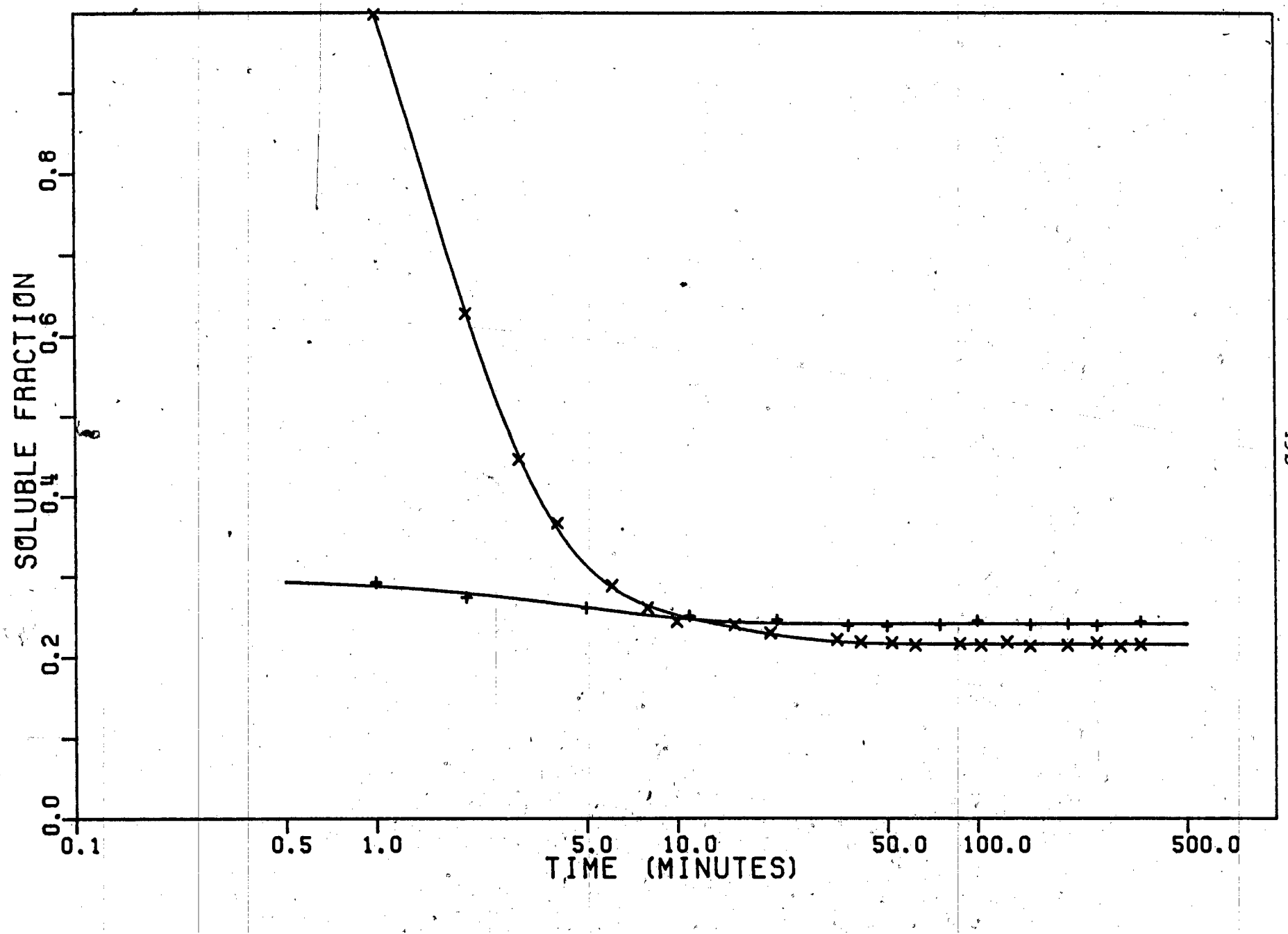

SOLUBLE FRACTION
0.0
0.2
0.4
0.6
0.8
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0
TIME (MINUTES)

Table 5 - The effects of mercuration on the alkaline denaturation kinetics of oxyhemoglobin. The first 4 fits represent application of first-order reaction kinetics to the denaturation of oxyhemoglobin that was untreated, and treated with 2 equivalents of $HgCl_2$ per tetramer, 8 equivalents of $HgCl_2$ per tetramer and 12 equivalents of PMB per tetramer. The last fit represents application of a second-order model to the PMB-hemoglobin data. The hemoglobin treated with 2 equivalents of $HgCl_2$ per tetramer was examined with the low salt assay method while the direct absorbance method was used for all of the other derivatives. The k_n values are in min^{-1}. Errors and statistics are explained in Table 1.

TREATMENT	FIT MODEL	LINEAR PARAMETERS	NON-LINEAR PARAMETERS	df	FIT STATISTICS
UNTREATED	$B_1 \exp(-k_1 t)$	$B_1 = 1.011 \pm 0.014$	$k_1 = 0.3467 \pm 0.0059$ ($T_1 = 2.00$ min)	12	SD = 0.00876 $R^2 = 0.99864$
+ 2 $HgCl_2$	$B_0 + B_1 \exp(-k_1 t)$	$B_0 = 0.2216 \pm 0.0027$ $B_1 = 1.042 \pm 0.074$	$k_1 = 0.485 \pm 0.028$ ($T_1 = 1.43$ min)	17	SD = 0.0106 $R^2 = 0.99057$
+ 8 $HgCl_2$	$B_1 \exp(-k_1 t)$	$B_1 = 0.9845 \pm 0.0072$	$k_1 = 0.0147 \pm 0.0003$ ($T_1 = 47.1$ min)	10	SD = 0.0126 $R^2 = 0.99861$
+ 12 PMB	$B_1 \exp(-k_1 t)$	$B_1 = 0.934 \pm 0.050$	$k_1 = 0.550 \pm 0.051$ ($T_1 = 1.26$ min)	17	SD = 0.0547 $R^2 = 0.9538$
+ 12 PMB	$B_1/(1+k_1 t)$	$B_1 = 0.9947 \pm 0.0061$	$k_1 = 2.339 \pm 0.023$ ($T_1 = 0.427$ min)	17	SD = 0.00128 $R^2 = 0.9999317$

Treatment of methemoglobin with 2 equivalents of $HgCl_2$ per tetramer was observed to cause complete precipitation without any exposure to alkali. As an additional test of whether the intermediate observed with methemoglobin denaturation and that obtained with oxyhemoglobin are the same, samples of oxyhemoglobin and methemoglobin were each treated with $HgCl_2$ prior to testing the alkaline denaturation reaction. The precipitated methemoglobin dissolved immediately on adding the alkaline buffer. The low salt assay profiles show that in both cases the intermediate is rendered insoluble in low salt buffer by mercuration (Fig. 12).

Sulfhydryl Oxidation

The effects of alkali on the sulfhydryl titre of oxyhemoglobin and methemoglobin is depicted in Fig. 13. Loss of PMB-reactive sulfhydryls is clearly more pronounced with oxyhemoglobin, and proceeds as 2 distinct rate components. The first of these components is similar although about 40% slower than the fast component obtained with the low salt assay (Table 6, Table 2) and accounts for 2 of the sulfhydryl groups (Fig. 13). The remaining 4 sulfhydryl groups react much more slowly with a rate constant which is too small to coincide with the slow component observed with the low salt assay.

Figure 13.- The kinetics for the loss of free sulfhydryl groups by oxyhemoglobin (+) and methemoglobin (x). Free sulfhydryl groups are determined by the absorbance increase at 250 nm due to reaction with p-hydroxy-mercuribenzoate. The data points were fit with two first order components and a constant parameter in the case of oxyhemoglobin and were connected by line segments in the case of methemoglobin. The kinetic parameters obtained for the oxyhemoglobin data are presented in Table 6.

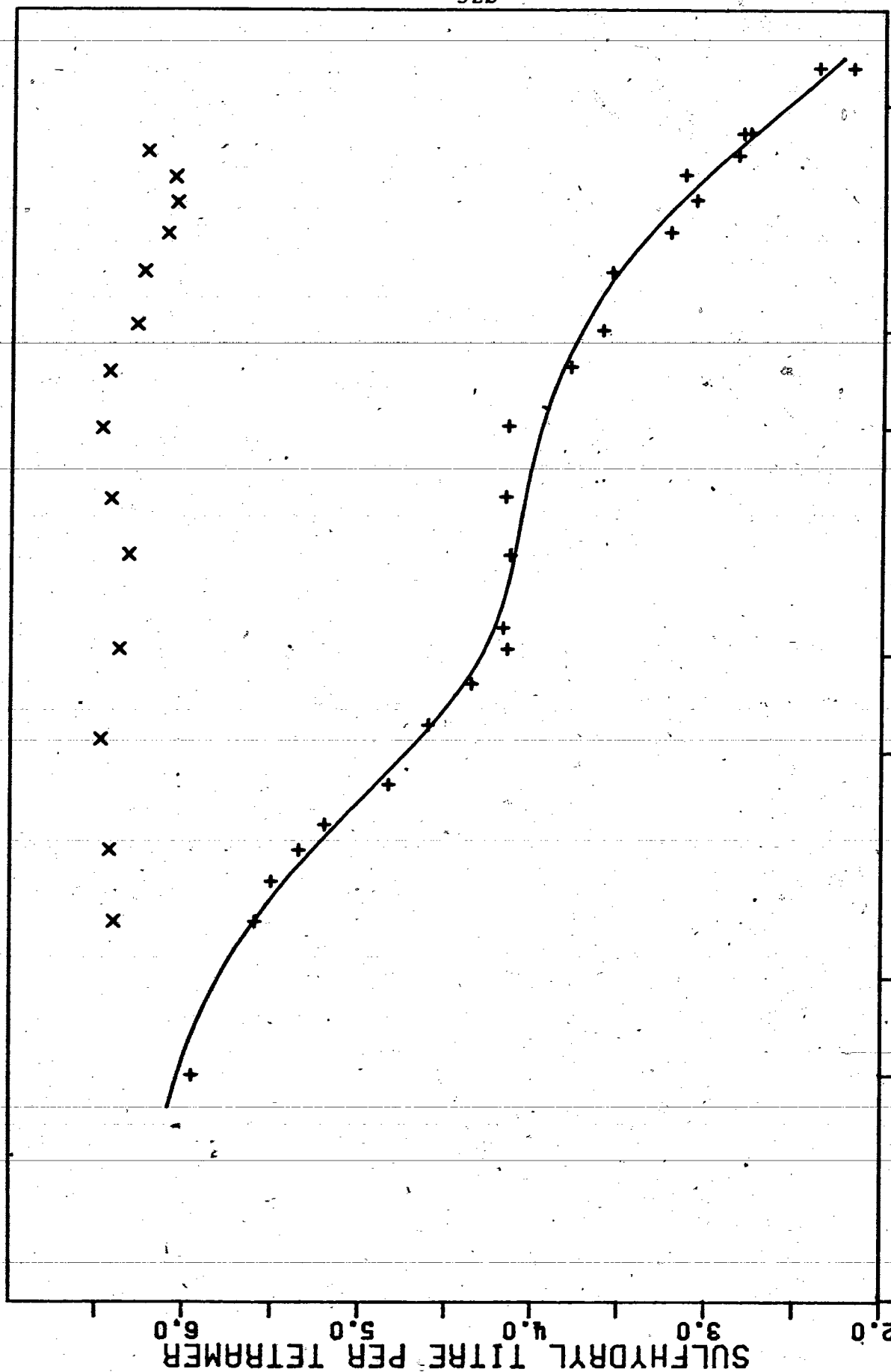

SULFHYDRYL TITRE PER TETRAMER
2.0
3.0
4.0
5.0
6.0
0.1
0.5
1.0
5.0
10.0
50.0
100.0
500.0
TIME (MINUTES)

Table 6 - The kinetics for the oxidation of sulfhydryl groups during the alkaline denaturation of oxyhemoglobin. The two fits represent two-component least squares fits to the sulfhydryl oxidation data plotted in Fig. 13. They differ only by the inclusion of an added constant term in the first fit. The k_n values are in min^{-1}.

FIT MODEL	LINEAR PARAMETERS	NON-LINEAR PARAMETERS	FIT STATISTICS
$B_0 + B_1\exp(-k_1t) + B_2\exp(-k_2t)$	$B_0 = 1.61 \pm 0.36$ $B_1 = 2.09 \pm 0.11$ $B_2 = 2.59 \pm 0.33$	$k_1 = 0.265 \pm 0.031$ $(T_1 = 2.62$ min) $k_2 = 0.00201 \pm 0.00049$ $(T_2 = 345$ min)	SD = 0.0988 $R^2 = 0.99282$ df = 19
$B_1\exp(-k_1t) + B_2\exp(-k_2t)$	$B_1 = 2.14 \pm 0.12$ $B_2 = 4.125 \pm 0.051$	$k_1 = 0.243 \pm 0.028$ $(T_1 = 2.85$ min) $k_2 = 0.00096 \pm 0.00005$ $(T_2 = 721$ min)	SD = 0.108 $R^2 = 0.99092$ df = 20

The products of alkaline denaturation of oxyhemoglobin and methemoglobin were examined by SDS gel electrophoresis. The presence of hemoglobin dimers, trimers and tetramers as well as two types of monomers were observed with oxyhemoglobin whereas only a single monomer band was observed with methemoglobin. The monomer band obtained with methemoglobin corresponds with the slower monomer band obtained with oxyhemoglobin. All of the additional oxyhemoglobin bands could be eliminated by treating the samples with 2-mercaptoethanol demonstrating that they arise by disulfide formation. This includes the faster monomer band which probably had an intrachain disulfide link. It is clear that oxidation of sulfhydryl groups to disulfides occurs during denaturation of oxyhemoglobin, however the observed bands may not represent the exact disulfide composition of the original reaction mixture since disulfide linkages could have exchanged during preparation of the electrophoresis samples.

DISCUSSION

In the Introduction I proposed a reaction model which accounts for the two reactions observed for alkaline denaturation of oxyhemoglobin with the low salt assay procedure (Fig. 4). The fast reaction is limited by conversion of $\alpha_1\beta_1$ dimers into monomers in accord with Perutz's model. The subsequent rapid unfolding of the oxyhemoglobin monomers leads to production of superoxide anion or hydrogen peroxide and oxidation of some of the, as yet undenatured, hemoglobin. This methemoglobin then denatures by the second pathway yielding a hemichrome intermediate. The slow reaction is the further alteration of this intermediate.

The above reaction scheme is supported by both my results and previous publications. For the complete reaction scheme to be correct each of the following details must be correct.

1. Monomerization is the rate limiting step for alkaline denaturation of oxyhemoglobin by the first pathway.

2. Alkaline denaturation of methemoglobin has a different rate limiting step.

3. Superoxide anions or hydrogen peroxide result from denaturation of oxyhemoglobin.

4. The superoxide or peroxide oxidizes the heme of some of the native oxyhemoglobin.

5. The rate limiting step for the slow reaction observed with oxyhemoglobin is the same as that for methemoglobin.

The Rate Limiting Step for the First Pathway

Recall that, in Perutz's model, ionization and subsequent hydration of the two cysteine sulfhydryls buried within the $\alpha_1\beta_1$ interface of human hemoglobin A accelerates denaturation of oxyhemoglobin by impairing reassociation of free monomers. If this is true, the mercuration of these cysteines should have a substantial effect on the denaturation kinetics. Whether denaturation is increased or decreased, however, will depend on whether the mercural disrupts the $\alpha_1\beta_1$ interface more or less than ionization of the two cysteines. Mercuration of both interfacial cysteines with $HgCl_2$ was found to decrease the denaturation rate more than 20 fold whereas similar treatment with the larger mercural, PMB, was found to increase it approximately 4 fold (Table 5, Fig. 12). This high sensitivity to mercuration of the interfacial sulfhydryl groups supports Perutz's idea that monomerization is the rate limiting step.

The requirement for monomerization is also supported by studies of the formation of subunit hybrids. When human CO-hemoglobin and canine CO-hemoglobin are mixed at pH 11.0, hybrids are formed in which the α subunits of one combine with the β subunits of the other (Robinson et al, 1960). In concurrence with the postulated role for the two interfacial cysteines, both hybridization and alkaline denaturation occur less rapidly with human hemoglobin F, which has only one such cysteine, than with hemoglobin A (Charlwood et al, 1960). In addition, both hybridization and alkaline denaturation of hemoglobin F are enhanced by 1 M KI (Tomita et al, 1973) implying that both processes are limited by monomerization.

The high susceptibility of deoxyhemoglobin to alkali (Fig. 5) demonstrates that autoxidation is not a prerequisite to alkaline denaturation of ferrous hemoglobins. The absence of O_2 and the presence of the reducing agent $Na_2S_2O_4$ prevent heme oxidation. The first order rate constant was only about 7% larger than that observed for oxyhemoglobin (Table 3). This parallels the observations of Haurowitz and co-workers (1954) that the deoxyhemoglobins of rat, rabbit and cow are slightly less stable than the corresponding oxyhemoglobins. In these studies, species differences had a much larger impact on the denaturation kinetics than the presence or absence of oxygen.

The similar sensitivity of deoxyhemoglobin and oxyhemoglobin denaturation to the amino acid sequence is consistent with the idea that both derivatives share the same rate determining step.

The slight stabilizing effect of oxygen is greatly surpassed by that of carbon-monoxide (Fig. 10, Table 3) suggesting that CO either slows the rate determining monomerization or slows another normally rapid step to the point that it becomes rate limiting. Since the structure of the $\alpha_1\beta_1$ interface of CO hemoglobin should be similar to that of oxyhemoglobin (Heidner et al, 1976) the latter explanation is more likely. Because of the very high affinity of CO for ferrous heme, this ligand would be expected to slow formation of hemochromes, i.e. it would effectively impair access of a near-by histidine imidazole to the ligand binding site. The conversion of hemoglobin monomers to hemochrome then becomes rate limiting for alkaline denaturation of this derivative.

The oxidation of hemoglobin sulfhydryl groups in alkali consists of two, probably parallel, reactions (Fig. 13, Table 6). The faster reaction of 2 sulfhydryl groups per tetramer shortly follows the loss of native oxyhemoglobin suggesting that the rate is limited by the supply of oxidant formed in the first pathway. A slower reaction involves at least 2 more sulfhydryls. The similarity of this separation of sulfhydryl reactivity with that of the native hemoglobin (Cecil et al, 1962) suggests that

the $\alpha_1\beta_1$ interfaces of the hemichromes resulting from alkaline denaturation of oxyhemoglobin may be intact. However, this apparently contradictory result may have another explanation. The separation of sulfhydryl reactivity could as well be due to the tertiary structure of the monomers or to the involvement of different oxidants in the two reactions.

The Rate Limiting Step for Methemoglobin

Although monomerization may limit the alkaline denaturation of ferrous hemoglobin derivatives, it appears not to do so for methemoglobin. Methemoglobin denatures rapidly at pH values from 10.0 to 10.5 (Anusiem et al, 1974) whereas oxyhemoglobin requires pH values greater than 11.5 to achieve similar denaturation rates at the same temperatures. If ionization of the buried cysteines is critical to the denaturation of both oxyhemoglobin and methemoglobin the the pKs for these cysteines must be much lower in methemoglobin. Because the environment of these cysteines is almost identical in the two valence forms, it is more likely that their ionization is not critical to the methemoglobin denaturation mechanism.

At pH 11.7 and 25°C, methemoglobin becomes insoluble with the high salt assay method before the first measurement (within 30 sec) implying that the denaturation half time is less than

0.1 min. The half time for oxyhemoglobin under the same reaction conditions is about 2 min. To be consistent with a monomerization rate determining step the methemoglobin dimers would have to dissociate at least 20 fold faster than the oxyhemoglobin dimers.

This difference in dissociation rate is too large in view of the structural similarity between the two derivatives. The reaction of PMB with the interfacial sulfhydryl groups of both oxyhemoglobin and methemoglobin at pH 10.5 and 10°C appears to be limited by dissociation of dimers into monomers (Currel et al, 1974). This reaction is found to be only 67% faster with methemoglobin than with oxyhemoglobin under the same conditions. The stabilities of the $\alpha_1\beta_1$ interface of methemoglobin and oxyhemoglobin are thus too similar to account for the wide difference in alkali susceptability of the two proteins. Denaturation of methemoglobin must therefore have a different rate determining step.

Most of the well-studied unstable hemoglobin variants have disruptive amino acid substitutions or deletions near the β-chain heme (Perutz and Lehmann, 1968) and are known to have a reduced affinity for heme and a higher susceptibility to autoxidation (Jacob et al, 1970). Methemoglobins prepared from these variants are much less stable than those prepared from the

native protein (Rachmilewitz et al, 1971). The low heme affinity has led some authors to speculate that expulsion of the heme is the rate limiting step (Jacob et al, 1968; Jacob et al, 1970). This hypothesis is supported by the heme expulsion observed in the acid denaturation of methemoglobin (Polet et al, 1969) and the heme deficiencies in precipitates of many unstable variants including hemoglobin Koln (Val FG5β - Met) Zurich (His E7β - Arg) and Hammersmith (Phe CD1β - Ser) (Jacob et al, 1970). The electrophoretic pattern of these variants is characterized by multiple bands which become a single band with the addition of hemin. The observation that cyanide inhibits both denaturation of hemoglobin Koln and the exchange of heme between methemoglobins (Jacob et al, 1968) also supports this heme expulsion hypothesis. Since normal methemoglobin is stabilized by cyanide (Rieder, 1970), movement or expulsion of the heme may also explain its instability.

My own observations on the denaturation of oxyhemoglobin and methemoglobin mercurated at the F9β cysteines with $HgCl_2$ are similar to those reported for the unstable hemoglobin variants. The modified oxyhemoglobin A does not precipitate at neutral pH, but oxidation to methemoglobin results in rapid precipitation similar to the results reported for unstable genetic variants (Rieder, 1970). Although other sulfhydryl reagents such as

N-ethyl maleimide do not cause immediate denaturation of methemoglobin, they do cause increased heme exchange and reduced stability with respect to unmodified methemoglobin (Bunn et al, 1968).

In view of the instability of methemoglobin mercurated at the F9β cysteine, this cysteine may perform a critical role in the alkaline denaturation of methemoglobin similar to the buried cysteines discussed in Perutz's model. Since this cysteine is more exposed, as demonstrated by its higher reactivity towards sulfhydryl reagents, it likely has a lower pK than the buried cysteines. The instability of methemoglobin at pH values where oxyhemoglobin is relatively stable might be attributed to greater sensitivity to ionization of this cysteine. Ferrous hemoglobins may withstand this ionization because of their higher affinity for heme (Bunn et al, 1968). Since methemoglobin tolerates addition of larger sulfhydryl reagents to the F9β cysteine, including PMB (Olson et al, 1976), its sensitivity to $HgCl_2$ must depend on some other property of the $HgCl_2$ adduct than its size. The hypothesized role for this cysteine might be tested by blocking its ionization with other sulfhydryl reagents and checking for increased alkaline stability.

Formation of Superoxide and Oxidation of Oxyhemoglobin

The inability of the oxygen of native oxyhemoglobin to dissociate as superoxide anion is attributed to steric constraints applied to the ligand by the polypeptide structure (Wallace et al, 1974). Unfolding of the polypeptide during denaturation should release these constraints and allow this anion to dissociate. Methemoglobin is incapable of similarly forming superoxide. Formation of superoxide has been demonstrated in the autoxidation of tetrameric oxyhemoglobin (Misra et al, 1972) and isolated subunits (Brunori et al, 1975).

Superoxide anion and its reduction products, hydrogen peroxide and hydroxyl radicals are capable of oxidizing sulfhydryl groups (Armstrong et al, 1978) and the vinyl side chains of heme (Rothschild, 1960) as well as the heme iron. Therefore the reduction in the sulfhydryl titre (Fig. 13) and the formation of disulfide-linked monomers observed by SDS gel electrophoresis provide good evidence that these agents are formed during alkaline denaturation of oxyhemoglobin. As expected, sulfhydryl oxidation did not occur with methemoglobin.

The slow shift in the visible spectrum of the hemichromes derived from oxyhemoglobin denaturation (Fig. 5) was probably the result of oxidative changes. It coincides with the slowest rate component observed for sulfhydryl oxidation (Fig. 13) and

presumably shares the same rate determining step. The fact that this particular shift does not match the smaller shift observed for methemoglobin (Fig. 9) and that no shifts occur with deoxyhemoglobin supports the involvement of oxidation.

Both superoxide anion and hydrogen peroxide are implicated in the autoxidation of oxyhemoglobin at pH 7 (Lynch et al, 1976; Sutton et al, 1976). Superoxide is thought to act as a reducing agent in this process by reducing heme-bound oxygen which then dissociates as peroxide leaving the heme iron in the ferric state (Lynch et al, 1976). The second-order rate constant for this reaction, 4×10^3 M^{-1} sec^{-1} (Sutton et al, 1976), suggests a half time of about 1 sec for 0.2 mM (heme) oxyhemoglobin and the same concentration of superoxide. Superoxide also reduces methemoglobin under the same conditions with a second-order rate constant of 6×10^3 M^{-1} sec^{-1} (Sutton et al, 1976) and reacts with itself, with a rate constant of 4.5×10^5 M^{-1} sec^{-1} (Behar et al, 1970). Therefore net oxidation of oxyhemoglobin by superoxide is negligible at pH 7. However, the rate of dismutation of superoxide is severely reduced in alkali (Behar et al, 1970) and should become kinetically insignificant with respect to the other reactions. Similarly, the rate of reduction of methemoglobin by superoxide should be low since only trace levels of native methemoglobin are anticipated in the reaction

mixture. Oxidation of significant amounts of oxyhemoglobin in alkali prior to denaturation is thus a predictable consequence of the presence of superoxide.

The Rate Limiting Step for the Slow Reaction

The slow reactions of both methemoglobin and oxyhemoglobin (Fig. 8) appear to involve the same reaction intermediate and rate limiting step. The strongest evidence is that their rate constants are nearly identical (Table 3) whether first-order or second-order models are compared. A further indication that the same intermediate is involved is that in both cases the intermediate is precipitated under the conditions of the high salt assay. Further, when the initial samples are treated with 2 equivalents of $HgCl_2$ per tetramer, the modified intermediate precipitates under low salt conditions in both cases.

A lower amount of the intermediate formed in the second pathway was observed with oxyhemoglobin than with methemoglobin (Fig. 8, Table 3). This is predicted by my model. In the case of oxyhemoglobin, and not methemoglobin, the first pathway competes for the native protein and therefore less protein would enter the second pathway. The model also correctly predicts that the slow reaction should not be observed for the denaturation of deoxyhemoglobin.

With the hope of showing a spectral identity of the intermediate obtained for both oxyhemoglobin and methemoglobin in spite of background absorption due to the other components of the reaction mixture, spectral shifts occurring during the slow reaction were compared. In the case of methemoglobin denaturation a small shift in the spectrum of the intermediate (Fig. 9) appears to correlate with the slow reaction (Fig. 8). However any similar shift in the spectrum after oxyhemoglobin denaturation was masked by a large spectral change which had slower kinetics (Fig. 5). The latter change roughly correlated with the slowest of the two components in the oxidation of sulfhydryl groups (Fig. 13). As previously mentioned, this change was probably due to oxidative changes which only occur in the reaction mixture with oxyhemoglobin denaturation. Thus, I was unable to prove or disprove the spectral identity of the intermediates in the two cases, however, in view of the other strong similarities in the intermediates, a common intermediate hemichrome is likely.

Denaturation and Precipitation

Two characteristics of the reaction profiles obtained with the low salt assay procedure, (1) the initial time lag in the denaturation and (2) the final level of soluble protein are not explained by my reaction scheme. Since the final concentration

of soluble protein was always the same regardless of the initial hemoglobin concentration, it cannot simply be attributed to a soluble component of the reaction mixture. Also since the precipitated protein is not in equilibrium with the soluble fraction, the final soluble level cannot be due to partial solubility of the denatured protein. The results suggest that the aggregation and precipitation reaction is effectively irreversible and highly concentration dependent. Both the initial time lag in the reaction profile and the final level of soluble protein can then be explained as situations where the concentration of denatured protein in the precipitation buffer becomes too low for precipitation to occur. Both phenomena may then be regarded as artifacts of the low salt assay method which are not relevant to my reaction model.

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