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Hemerythrin: Reactivity of the Sulfhydryl Group and Protein Association-Dissociation

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HEMERYTHRIN: REACTIVITY OF THE SULFHYDRYL GROUP AND PROTEIN ASSOCIATION-DISSOCIATION

By

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ABSTRACT

Hemerythrin, a nonheme, iron-containing protein, consists of eight identical subunits, each of which bear one sulfhydryl group. When these sulfhydryl groups react with p-mercuribenzoate (PMB), the native protein dissociates into subunits. This process follows an all-or-none mechanism. Apparently, when one sulfhydryl group reacts, all other sulfhydryl groups in that octamer also react. Using a spectrophotometric technique, this investigation attempted to probe the nature of the reaction of hemerythrin with PMB and the subsequent dissociation.

The kinetic data reveal that when PMB interacts with hemerythrin, about 20-40% of the sulfhydryl groups react at a rate too fast to measure, while the remainder react at a slower first order rate. Although the reaction is first order in protein only, the concentration of PMB affects the calculated rate constant. The reaction in a solution of hemerythrin monomers proceeds at about the same rate as in a solution of octamers which demonstrate that the monomer species is no more reactive than the octamer. Furthermore, light scattering measurements revealed that the dissociation of hemerythrin is a first order process with a rate constant of about one half the rate constant for the formation of the mercury-sulfur bond.

Apparently, the reaction is a random process. When PMB interacts with hemerythrin, while it forms a covalent, mercury-sulfur bond, it also induces a change in the subunit by a concomitant process which renders the monomer unable to associate into octamers. Over a period of

time, the reacting mixture reequilibrates so that all reacted subunits are nonassociating monomers and the unreacted subunits have reformed into octamers.

The kinetic data for the reaction of PMB with hemerythrin indicates a two step reaction scheme in which the first step is a slow, unimolecular change in the protein followed by the rapid formation of the mercury-sulfur bond. Therefore, the reaction scheme

$$\text{Hr} \rightleftharpoons \text{HrSH}$$

$$\text{Hr} + \text{HgBz} \rightleftharpoons (\text{HgBz})\text{HrSH}$$

$$(\text{HgBz})\text{Hr} \rightleftharpoons (\text{HgBz})\text{HrSH}$$

$$\text{HrSH} + \text{HgBz} \longrightarrow \text{HrSHgBz}$$

$$(\text{HgBz})\text{HrSH} + \text{HgBz} \longrightarrow (\text{HgBz})\text{HrSHgBz}$$

is proposed, where Hr represents an unreactive subunit of hemerythrin; HrSH, the subunit with a reactive sulfhydryl group; (HgBz)Hr and (HgBz)-HrSH, the protein species to which PMB is noncovalently bound; HrSHgBz and (HgBz)HrSHgBz, the protein with which PMB has formed a mercury-sulfur bond. This scheme assumes that the rate determining step is a small but definite change in conformation about the sulfhydryl group which makes this group reactive toward PMB and that noncovalent binding of PMB to the protein, which is known to occur, affects this change in conformation. In this reaction scheme, the fast phase of the reaction represents the depletion of the reactive species, after which the steady state conditions hold. A first order rate law can be derived by the steady state assumption for the formation of the mercury-sulfur bond.

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LIFE

Michael Charles Cress began life April 12, 1941 in San Francisco, California. He attended public and parochial schools in rural Idaho, El Paso, Texas, Pasco, Washington, and the Seattle area. While in High School, he was an active member of his schools' debate teams. He graduated from Highline Senior High School in June, 1959 and entered the University of Portland. During his undergraduate years, he was active as a reporter and columnist for the student newspaper, The Beacon. He received his B.S. Degree in Chemistry with minors in Mathematics and Philosophy in 1963.

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I. INTRODUCTION

The factors which determine the particular conformation and state of aggregation of a protein are not yet adequately established and understood. The investigation of the quaternary structure of a protein, therefore, may have heuristic value in understanding the structure, function, and nature of proteins (1). The sulfhydryl group (-SH, also known as the mercapto group) appears to have considerable importance in protein structure and function (2). The investigation of the iron-containing, nonheme protein hemerythrin, which has a single sulfhydryl group in each of its eight identical subunits, may contribute to an understanding of the role the sulfhydryl group has in protein conformation and association-dissociation equilibria.

A. The Character of Hemerythrin

Hemerythrin is an iron-contining, nonheme protein which serves an oxygen carrying function in sipinculids, a type of sea worm. Because of the nonheme iron, the hemerythrin is often thought of as a type of primitive hemoglobin. Besides members of the phylum Sipunculoidea, various species of the marine phyla, Priapulida, Brachiopoda, and Annelida, also use hemerythrins as their respiratory protein.

Early investigation of the protein which was isolated from the coelomic cavity fluid of Goldfingia gouldii (also known as Phascolosoma gouldii) indicated that hemerythrin contains sixteen gram atoms of iron

per mole of protein and upon saturation binds sixteen gram atoms of oxygen (3, 4). Keresztes-Nagy (5-10) further elucidated the character of hemerythrin, finding that it has a molecular weight of about 107,000 gm $mole^{-1}$ and that it consists of eight identical subunits each of which bear two iron ions and one sulfhydryl group. When N-ethylmaleimide or organic mercurials such as p-mercuribenzoate react with hemerythrin, dissociation of the octamer protein into its subunits occurs (7). These reagents are noted for their reactivity toward sulfhydryl groups of proteins. When added to hemerythrin in less than stoichiometric amounts, all the reagent binds to the monomer only and none of it binds to the undissociated octamer. This indicates that the reaction of these reagents with the sulfhydryl groups of hemerythrin was by an all-or-none mechanism (7). Furthermore, Keresztes-Nagy and Klotz (8) found that hemerythrin also binds certain ligand anions which are noted for their ability to form coordination complexes with iron ions. They observed that in the absence of these ligands, the sulfhydryl groups were relatively unreactive toward the mercurials and that the reactivity of the sulfhydryl groups depended on the nature of the ligand bound. Yet spetral studies showed ligand binding in an iron complex, the nature of which did not change upon reaction with mercurials or subsequent dissociation, indicating that this complex was at a site separate from that of the sulfhydryl group.

An experiment, outlined in Figure 1, illustrates the relationship be-

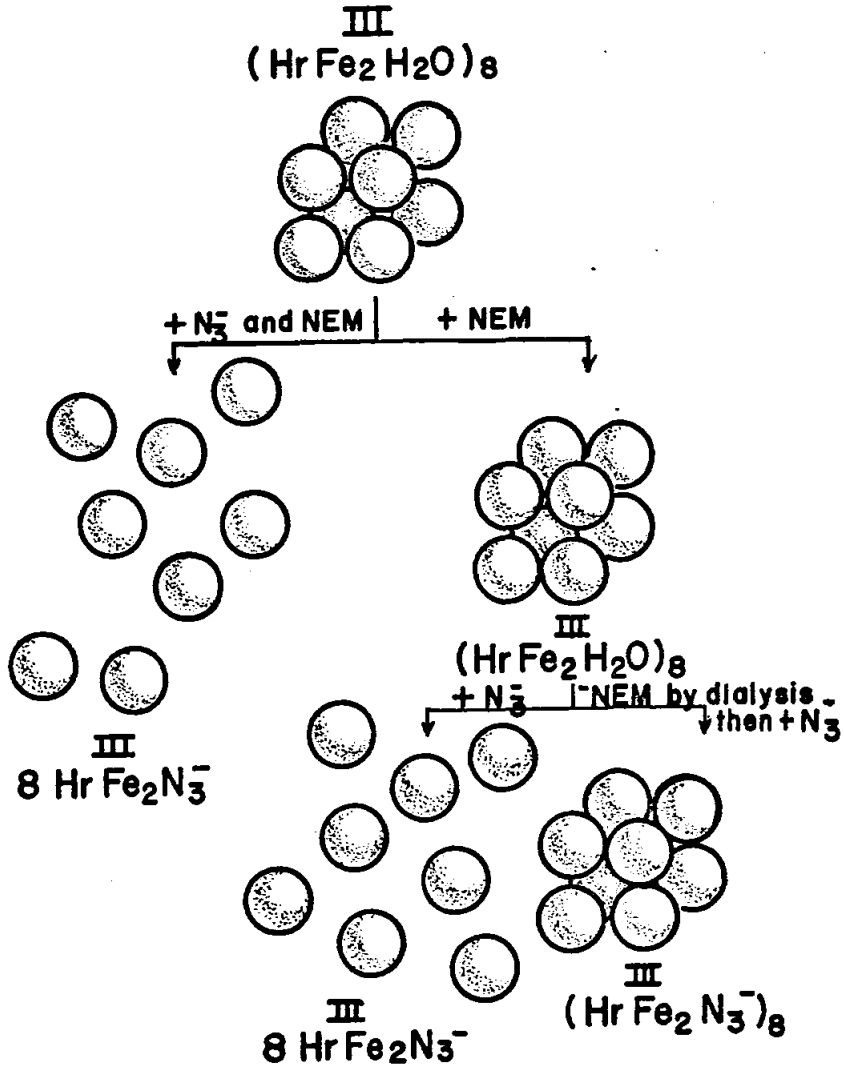

FIGURE 1: Outline of the Effect of Azide Ion on the Reactivity of Hemerythrin toward N-ethylmaleimide (NEM).

tween the reaction of sulfhydryl reagents which dissociate hemerythrin and the ligand present. Keresztes-Nagy and Klotz (9) added N-ethylmaleimide to a solution of aquomethemerythrin. (Corresponding to methemoglobin, methemerythrin designates the irreversibly deoxygenated protein containing trivalent iron. The prefex aquo- designates the presumed iron ligand.) They divided the solution and added azide ion to one half. After allowing the solutions sufficient time to react, they analyzed the solutions in an ultracentrifuge to determine whether dissociation had occured. These experiments showed that the azide solution contained only monomers while the other solution contained only octamers. To one half the undissociated hemerythrin solution, they added azide ion, but the other half, they dialysed to remove any unreacted N-etheylmaleimide. Then they added azide ion to the dialysed portion as well. The ultracentrifuge experiments revealed that the undialysed portion had dissociated but the dialysed protion had not. These experiments indicate that the ligand activates the sulfhydryl group and that both ligand and sulfhydryl reagent are required to effect dissociation.

Keresztes-Nagy and Klotz (8) developed the equilibrium model to account for the apparent interaction between the sulfhydryl group and the iron-ligand complex. This model proposes that octameric hemerythrin is in equilibrium with the monomer form. Only the sulfhydryl groups of the monomer can react with the mercurial reagent; sulfhydryl groups of the octamer are inaccessible to the reagent. The ligand is capable of

shifting the equilibrium toward the monomer, thus making the sulfhydryl groups more available to the reagent. Although this association-dissociation equilibrium model can satisfactorily explain the cooperative-interaction phenomenon, other models can also explain the phenomenon. The three assumptions which were made for the equilibrium model must be tested to determine the correct model.

Considerable evidence has been collected for two of these assumptions: first, that there is an octamer-monomer equilibrium and second, that the ligands shift the equilibrium toward the monomer. Hybridization experiments (9, 10), analytical ultracentrifuge experiments (11, 12), and Sephadex column chromatography experiments (13-15) proved the existence of the equilibrium. In this laboratory, Rao (13-15) studied the octamer-monomer equilibrium with Sephadex gel chromatography and observed that the association-dissociation equilibrium is rapidly established and that iron-combining ligands affect the equilibrium, increasing the extent of dissociation according to the series of complexes: aquo, oxy, fluoride, chloride, and thiocyanate. Ultracentrifuge and ligand binding experiments confirm this except that Klapper (11, 16, 17) found evidence of a slow equilibration. For this discrepancy, Rao (13) postualted a pressure effect in the ultracentrifuge. Langerman and Klotz (12) calculated an association constant which agrees within experimental error with that calculated by Rao (13-15) and also they agree with him (14) on the value of the standard free energy of dissociation for hemerythrin octamer into subunits which is about 6 kcal

mole^{-1} of monomer units formed. The third assumption is that the monomer sulfhydryl group be the only reactive species.

Egan (18) has considered the kinetic implication of the equilibrium model which involves the processes

$$(HrSH)_8 \rightleftharpoons 8HrSH \qquad (1)$$

$$HrSH + HgBz \longrightarrow HrSHgBz \qquad (2)$$

where HrSH represents the subunit of hemerythrin, HgBz represents the mercurial reagent which in this case is p-mercuribenzoate, and HrSHgBz represents the protein reacted with the mercurial. She concluded that the third assumption required one of two kinetic possibilities. If the equilibration (Reaction 1) were slow and Reaction 2 fast, the rate determining step would be the dissociation of octamer, the reaction would be first order in protein concentration only and first order overall. When the equilibrium is rapidly attained as Rao (13-15) has observed experimentally, then the rate determining step is the formation of the mercury-sulfur bond. Reaction 2 is first order in p-mercuribenzoate and first order in monomer units of protein. But the monomer is in equilibrium with octamer, and therefore, its concentration is a function of the eighth root of the octamer concentration and the equilibrium constant

$$[HrSH] = \sqrt[8]{K\,[(HrSH)_8]} \qquad (3)$$

Consequently, the observed overall order of the reaction would be first order in p-mercuribenzoate and essentially zero order in terms of the protein concentration.

Boyer (19) has developed a spectrophotometric technique which directly observes the formation of the mercury-sulfer bond when p-mercuribenzoate reacts with sulfhydryl groups and which thereby permits direct kinetic investigation of the sulfhydryl group. In this laboratory, Egan (18) measured the rate of formation of the mercaptide bond for the reaction of chloromethemerythrin and p-mercuribenzoate. She claimed that the reaction was second order overall, first order with respect to hemerythrin concentration and first order with respect to p-mercuribenzoate concentration. In contrast, Klapper and Klotz (16) claim to have observed that this same reaction was first order in terms of protein concentration, although they presented no data. Egan (18) claimed that she could fit her data to either first or second order rate equations. Fransioli (20), who investigated the reactivity of the sulfhydryl group of several forms of hemerythrin, and Duke, Barlow, and Klapper (21), who investigated the reaction of fluoromethemerythrin with p-mercuribenzoate, also fit their data to both first and second order rate equations. Apparently the reaction of the sulfhydryl group of hemerythrin is more complicated than Egan (18) had presumed originally. The third assumption remains unproven.

Working in this laboratory, Fransioli (20) also applied the spectrophotometric technique to the hemerythrin sulfhydryl group. She observed that the rate of reaction increases as chloride ion concentration increases. She also compared the reaction rates for various ligand forms of heme-

rythrin and observed that the ligands affect the reaction, increasing the rate according to the series: aquo, fluoride, chloride, thiocyanate, and azide. This is a series similar to that for increasing dissociation. Fransioli also directly compared the rate of oxygen release with the rate of mercury-sulfur bond formation when oxyhemerythrin reacts with PMB and she discovered that the rate of oxygen release is much slower than the rate of mercaptide formation and appears to be first order. The sulfhydryl group is not required for oxygen binding. Except for the measurement of a second order rate, her work is consistent with the equilibrium model, but nevertheless, it does not prove it.

At the same time, Klotz and associates (22-23) continued the investigation of the physicochemical properties of hemerythrin, particularly into the nature of the iron-oxygen binding site. They determined the amino acid sequence and confirmed that the subunits were identical, each having a molecular weight of 13,500 gm $mole^{-1}$ (22-24). Darnall, Garbett, and Klotz (25) discovered that certain anions binding at a noniron site affect the formation of the iron-ligand complex. Using the spectral shift observed when the hydroxy form is converted into the aquo form of the protein as a criterion for evaluating the strength of binding at the other site, they found that perchlorate and nitrate bind strongly; phosphate, weakly; sulfate, dodecylsulfate, acetate, and trichloroacetate, not at all. Cir-

cular dichroic spectra indicate that the spectral shift observed was not due to anion coordination to the iron but to anion binding at some other site. Furthermore, perchlorate decreases the reactivity of the sulfhydryl group of aquomethemerythrin and nitrate also protects but to a lesser extent. Later work by Garbett et al (26, 27) confirmed that perchlorate and certain other anions bind at two sites on the hemerythrin subunit. Of these sites, the one to which perchlorate ion binding is stronger is close to both the iron-ligand site and the cysteinyl residue. The protective effect of perchlorate on the sulfhydryl group is lost when either azide or thiocyanate is present. When anions bind, all the subunits are independent and noninteracting; there is no evidence of an allosteric interaction among them. Circular dichroic spectra indicate an α-helix content of about 75% and there is no significant change in these spectra in the UV range when ligands bind to iron, p-mercuribenzoate reacts with the sulfhydryl group, or the octamer dissociates into monomers (28).

The structure of iron-ligand and iron-oxygen complexes can be deduced from Mössbauer spectra which for hemerythrin are characteristic of Mössbauer spectra for certain model compounds (29). Garbett and coworkers (30, 31) correlated absorption, circular dichroic, and Mössbauer spectra of hemerythrin, then proposed structures for the iron complexes in hemerythrin which are illustrated in Figure 2. Oxyhemerythrin and methemerythrin contain high-spin iron(III) atoms, antiferromagnetically coupled in dimeric pairs via an oxo (O^{2-}) bridge derived from water. Oxy-

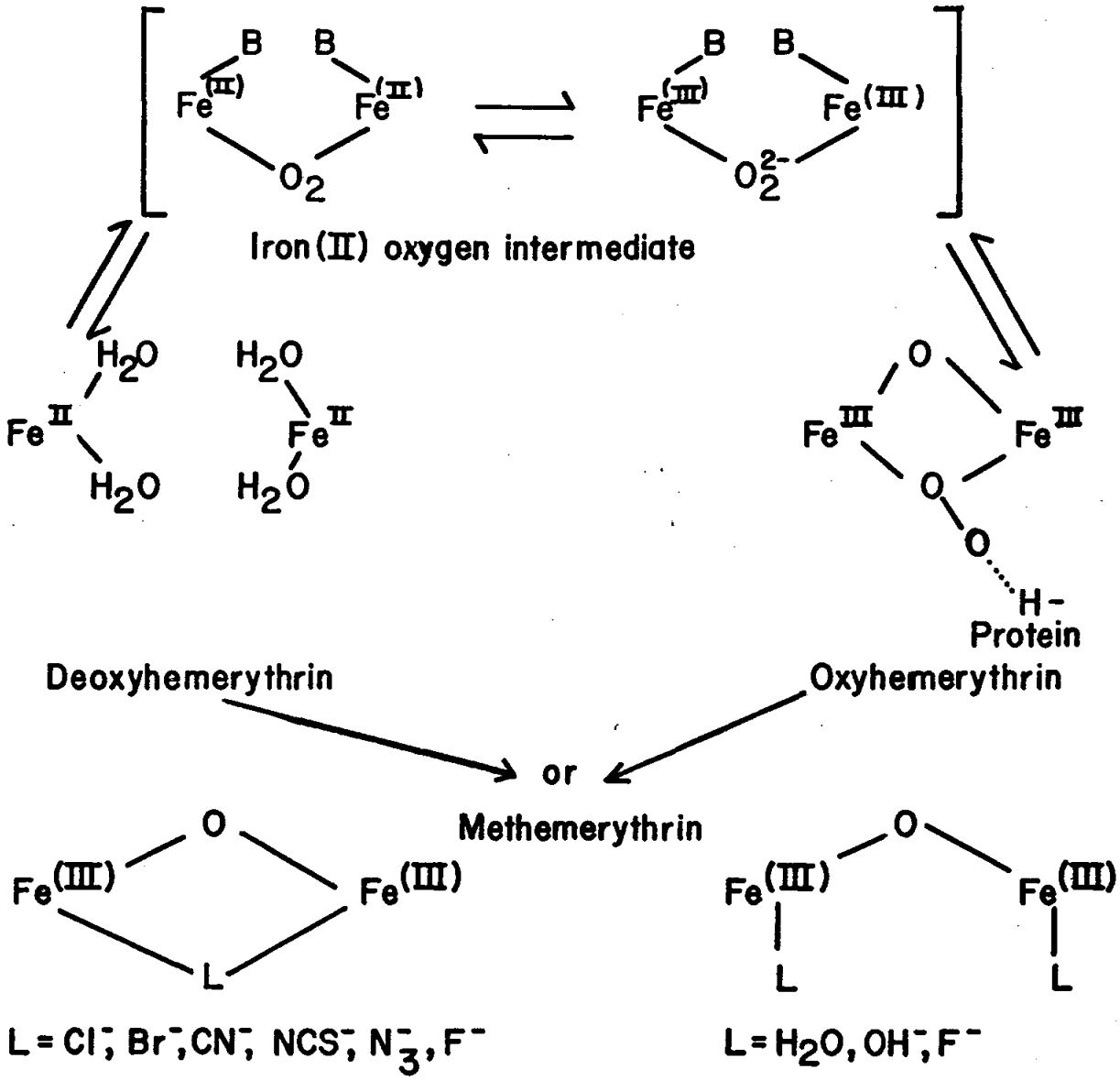

FIGURE 2: Iron-Ligand Structures in Hemerythrin.

hemerythrin also contains a second bridge formed by a peroxo (O_2^{2-}) group. For methemerythrin, when the ligand is chloride, bromide, cyanate, thiocyanate, azide, and sometimes fluoride, the ligand also acts as a bridge. When the ligand is water, hydroxide, and sometimes fluoride, however, a ligand binds to each iron(III) atom and there is no second bridge. In oxyhemerythrin, the environment of the two iron atoms differ, but in methemerythrin, their environments are the same. In deoxyhemerythrin, like methemerythrin, the two iron atoms have identical environments, but the iron atoms are present as high-spin iron(II) and there is no magnetic coupling between them. York and coworkers (33, 34) confirmed the Mössbauer spectra and obtained magnetic susceptibility measurements which support the proposed iron structures. Using tetranitromethane for chemical modification of hemerythrin, Rill and Klotz (32) demonstrated that at least some of the tyrosine side chains of the protein coordinate to the iron. Using 5-diazo-1-H-tetrazole for chemical modification, Fan and York (35) demonstrated that four histidine residues in hemerythrin coordinate to the iron. They also eliminated as possible iron ligands the ε-amino groups of lysine and the N-terminal group by reacting hemerythrin with trinitrobenzenesulfonic acid. But the entire protein-iron structure is yet unknown.

Earlier thermodynamic measurements of the association-dissociation equilibrium in hemerythrin led to the conclusion that a minor change of as little as one side chain might alter the quaternary structure (12). Langer-

man and Sturtevant (36), therefore, investigated the association with calorimetry. They discovered that on association, there is binding of additional protons so that the equilibrium may be written

$$8Hr + 8nH^{+} \rightleftharpoons [Hr(H^{+})_{n}]_{8} \qquad (4)$$

The number of protons binding per monomer (n) was not measured directly but was estimated from the calorimetric and ultracentrifuge (12) data to be 0.6 ± 0.3. Langerman and Sturtevant (36) give a summary of the thermodynamic parameters for the various reactions of hemerythrin. Apparently, for the association, the main thermodynamic driving force is entropic (ΔS^{o} = 23 eu per mole of monomer, Rao (15) obtained ΔS^{o} = -15.5 eu for dissociation). Their calorimetric data correlated with earlier observations established that the enthalpy of association is small and positive (14, 36), that the partial specific volume of the monomer and the octamer are essentially identical (12), and that the heat capacity change upon association is small and positive (36). The first of these facts suggest that hydrophobic bonds are involved in the association, but the other two facts are contrary to that expected if only hydrophobic binding occurred. A simple molecular interpretation of the association-dissociation equilibrium, therefore, is not feasible from the present knowledge.

DePhillips (37) studied the binding of oxygen to hemerythrin and from a Hill plot observed, as had Keresztes-Nagy and Klotz (8) earlier, that little cooperative interaction occurs between oxygen binding sites. He also observed that perchlorate has a profound effect on oxygen binding

which confirms the idea that the oxygen binding site, the perchlorate binding site, and the sulfhydryl group are close to each other.

Work on hemerythrin isolated from species of sipunculids other than Golfingia gouldii, though not as extensive as that of G. gouldii hemerythrin, does indicate that these other proteins are similar. For example, York and Bearden (33) observed that the Mössbauer spectra of Dendrostomun zostericulum and G. Gouldii oxyhemerythrins are identical. Oxygen binding studies gave about the same results for the hemerythrin from G. gouldii (8, 36, 37), Sipunculus nudus (38), and D. pyroides (39). Farrell and Kitto (39) characterized some macromolecular properties of D. pyroides hemerythrin and on the basis of immunodiffusion, amino acid analysis, and peptide mapping, demonstrated that this hemerythrin is closely related to the hemerythrin from G. gouldii. Bossa et al (40) likewise obtained for S. nudus hemerythrin optical rotatory dispersion curves and circular dichroic spectra which were similar to those of G. gouldii hemerythrin.

B. The Sulfhydryl Group

The availability of reagents which will specifically react with the sulfhydryl group given the proper conditions revealed the involvement of sulfhydryl groups in enzyme catalytic sites and in maintenance of subunit-subunit interactions which led to an early appreciation of the role of the sulfhydryl group in proteins. Boyer (41) and Cecil (42) have thoroughly discussed the sulfhydryl group; Glazer (43) has summarized more recent de-

velopments; and Leach (2) has presented a comprehensive guide to experimental techniques for investigating sulfhydryl groups. The extent of the role of sulfhydryl groups may be judged by the number of so-called sulfhydryl enzymes which Boyer (41) has defined as one which loses its catalytic activity when some or all of its sulfhydryl groups undergo chemical modification. He listed over one hundred such enzymes, although, as Cecil (42) has pointed out, the sulfhydryl group has been shown to be actually part of the active site for only a few of them.

The sulfhydryl group in proteins is particularly noted for the variability of its reactivity. Sulfhydryl groups may be classed as reactive for those which react at rates comperable to that of simple thiols, or unreactive for those which do not react unless considerable denaturation of the protein occurs, or any degree of reactivity between these extremes. But regardless of the reactivity of the sulfhydryl group in the intact protein, when the protein is denatured, all sulfhydryl groups have a reactivity comperable to that of simple compounds. Boyer (41) discussed the factors which might limit sulfhydryl group reactivity. Generally, the degree of reactivity is probably due to the extent the sulfhydryl group is involved in noncovalent interactions with other parts of the protein or buried within the protein structure. Glazer (43) covers the recent evidence that sulfhydryl groups are involved in hydrophobic interactions within apolar environments. This evidence includes that of Edsall (44) who investigated the ionization of cysteine and concluded that the sulfhydryl

group has little affinity for forming hydrogen bonds. Further, Perutz (45) presented the tertiary and quaternary structure of horse oxyhemoglobin and observed that the unreactive sulfhydryl groups were located in the interior of the molecule, appeared to be involved in hydrophobic bonds, and were not too important for the protein structure, but the reactive sulfhydryl group was at the surface.

Several proteins besides hemerythrin dissociate when reacted with sulfhydryl group reagents (46). Because the hemerythrin molecule in each of its eight subunits contains but one sulfhydryl group which has a reactivity that varies under different molecular conditions, it presents an ideal protein for the study of the role of the sulfhydryl group. Central to an understanding of the role of the sulfhydryl group in hemerythrin structure is a knowledge of the proper mechanism by which p-mercuribenzoate or other organomercury reagent reacts with that sulfhydryl group.

C. The Three Models

When certain proteins such as hemerythrin (7) or asparatate transcarbamylase (47, 48) react with p-mercuribenzoate or other reagent that substitutes on the sulfhydryl group, the sulfhydryl groups of that protein react by an all-or-none mechanism; either all the sulfhydryl groups of a particular protein molecule react or none of them react. Keresztes-Nagy and Klotz (7) demonstrated the all-or-none nature of the reaction in the following manner. They added salyrganic acid, an organic mercurial, to

hemerythrin solutions with a different ratio of the mercurial to protein sulfhydryl group for each solution. After the solutions had reacted, they analyzed them in an ultracentrifuge. In each solution, two components were observed, one was the hemerythrin monomer and was the same fraction of the total protein as the fraction of sulfhydryl groups titrated with the mercurial, the other component was undissociated hemerythrin octamer. There was a one-to-one relationship between percentage monomer and percentage sulfhydryl group blocked by mercurial. To explain the all-or-none phenomenon and the apparent cooperativity between sulfhydryl groups and other sites on the protein molecule that has been observed also, three models, which have been listed by Gerhart and Schachman (48), have been devised.

Madsen and coworkers (49-51) observed the reaction of muscle phosphorylase with p-mercuribenzoate and noted that attachment of mercurial to the protein, enzymic inhibition, and protein dissociation were all-or-none phenomena. Madsen and Gurd (51), therefore, devised the zipper or wedge model. Boyer (41) developed the model into its final form whereby when one sulfhydryl group on a molecule reacts, it activates the other sulfhydryl groups so that they all react almost immediately. Although Battell et al (52, 53) discovered that many of the early observations for phosphorylase were invalid, many of the hemerythrin experiments and the results from them parallel those of Madsen and coworkers (49-51).

Monod, Wyman, and Changeux (54) proposed the second model, the

allosteric model, as a general molecular theory to explain many types of protein interactions, particularly those which regulate enzymes and through them metabolic pathways. This model postulates that the protein exists in two conformations which are in equilibrium with each other. Binding of anions or ligands or attachment of chemical modifiers to a site on one form displaces the equilibrium in favor of that form. The resulting conformation is characteristic of all subunits of the protein oligomer not just those containing bound ligand. In this manner, sites on all subunits are either activated or deactivated simultaneously depending upon the nature of the effector. For hemerythrin, this model may be applied by postulating that the coordination of ligand with iron stabilizes the conformational state in which all the sulfhydryl groups in the octamer are unmasked, but which does not induce dissociation ot the protein.

Keresztes-Nagy and Klotz (8) devised the equilibrium model for hemerythrin. This third model is described in Section A of this chapter. Like the allosteric model, the equilibrium model also involves two states, one reactive, one unreactive. But the two states in equilibrium are an oligomer and its monomer subunit rather than two conformational states with the same quaternary structure.

D. The Purpose

This work attempts to determine the correct model for the reaction of hemerythrin with p-mercuribenzoate and the subsequent dissociation of

the protein. To this end, the reaction of p-mercuribenzoate with the sulfhydryl group must be better characterized. The order of the reaction must be determined with less ambiguity and the relationship between the formation of the mercury-sulfur bond and the dissociation revealed. A deeper insight into the nature of the effect of ligands and other anions on the rate of reaction should be obtained when earlier data are correlated with the data of this work. Finally, from the data obtained, a molecular model will be proposed to explain the nature of the reaction. This model should indicate a possible function of sulfhydryl groups in the protein structure and better reveal how the sulfhydryl group reagents effect protein dissociation. But most important, this thesis and its reaction model should provide a guide that points the direction for further research. Actually, we cannot consider the primary intention, which is to determine the model for the reaction of the sulfhydryl group, to be achieved until this further research is completed.

II. EXPERIMENTAL PROCEDURES

This work continues the investigation of the reaction of PMB with the sulfhydryl group of hemerythrin isolated from the coelomic cavity of Golfingia gouldii, examines the effect of certain ligands upon that reaction, and attempts to elucidate the relationship of the reaction to the consequent dissociation of the protein.

A. Materials

All chemicals used in these experiments were reagent grade obtained from commercial sources with the exception of the following. The principle mercury reagent was prepared from p-choloromercuribenzoate, sodium salt, lot 72032 obtained from Calbiochem. The salyganic acid, o - [(3-hydroxymercuri-2-methoxypropyl) - carbamoyl] phenoxyacetic acid, which was used for one set of experiments, was obtained from a sample provided by Dr. Keresztes-Nagy. Proteins used, other than hemerythrin, and the commercial sources from which they were obtained are the following:

Bovine Plasma Albumin, Armour Pharmaceutical Co., lot A69702.

Myoglobin, Nurtitional Biochemical Corp., lot 1930.

Ovalbumin, Nutritional Biochemical Corp., lot 4790.

Ribonuclease-A, Sigma Chemical Co., lot 49B-8043.

1. Preparation of crystalline hemerythrin

Crystalline hemerythrin was obtained by the established procedure of

Klotz et al (4) from live G. gouldii worms supplied by the Marine Biological Laboratories, Woods Hole, Massachusetts. In this procedure, the coelomic fluid is drained from the worms, whipped with a glass rod, and strained through glass wool to separate the fibrin. The cells are removed from the fluid and washed several times in 2.5% saline after which they are lysed in cold distilled water. The cell debris was removed by centrifugation and the supernatant was dialysed against 20% ethanol. During the dialysis, red crystals of oxyhemerythrin form. These crystals are centrifuged out, washed a few times, and stored while still wet in the stoppered centrifuge tube. These preparations were stable for at least a year when kept in a freezer. Details of the procedure are found in other theses from this laboratory (13, 18, 20) and elsewhere (4, 5).

2. Preparation of reagent solutions

All buffers used contained 0.01 M Tris-cacodylate at pH 7.0. Equimolar amounts of cacodylic acid (dimethylarsinic acid) and Tris base (2-hydroxymethyl-2-amino-1, 3-propanediol) were dissolved in distilled water along with the desired amount of ligand and the pH adjusted to 7.0 by the addition of small amounts of the appropriate constituent. The distilled water, which was used to prepare the buffers, had been further purified by passage through a Continental ion exchange deionizer.

a. Protein solutions. Oxyhemerythrin crystals were dissolved in a small amount of buffer, then solid sodium fluoride was added to a final

concentration of 1 M. Chloride ion does not dissplace the oxygen from the iron ligand site rapidly (8), whereas the fluoride ion does so within thirty minutes. The hemerythrin solution was centrifuged to remove any denatured protein and undissolved lipid material and then the solution was dialysed four times aginst buffers, increasing the amount of ligand in the buffer to the desired concentration for the last two dialyses. In most cases, the protein concentration was directly measured spectrophotometrically, based on the extinction coefficient 2.77 ml mg^{-1} cm^{-1} at 280 nm found by Keresztes-Nagy (5). These solutions were generally quite stable, although a negligible amount of denaturation occurred with time. After several weeks, a hemerythrin solution was usually recentrifuged to remove denatured protein.

b. Mercurial solutions. Solid p-chloromercuribenzoate, sodium salt was added to buffer. The solution was mixed and allowed to stand for at least two hours; then the undissolved material was removed by centrifugation. The anion associated with the mercury para to the carboxyl group depends on the pH and the buffer, therefore Boyer (19) proposed the general term p-mercuribenzoate (PMB)* for this mercurial compound in solution. The concentration of PMB had been determined in earlier work in this laboratory by titration with glutathione (18, 20), but a spectrophoto-

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* In the text, Tables, and Figures, the abbreviation PMB will be used for p-mercuribenzoate. In chemical and kinetic equations, the symbol HgBz will be used instead.

metric determination based on the molar extinction coefficient 1.69×10^4 $M^{-1}cm^{-1}$ at 232 nm found by Boyer (19) was more convenient and as accurate. The PMB solutions were kept in the dark but slowly decomposed, losing about 3% in concentration of PMB during a week. Consequently, they were frequently recentrifuged and restandardized.

B. Spectophotometric Techniques

Boyer (19) devised the technique for the spectrophotometric titration of protein sulfhydryl groups with PMB on the basis of a shift on the UV spectrum. He has demonstrated that the increase in absorbance at 250 nm is entirely due to the formation of the mercury-sulfur bond. Benesch and Benesch (55) have described this technique in detail and several reviews have discussed various aspects of the determination of sulfhydryl groups inproteins (2, 41, 56). Egan (18) and Fransioli (20) used Boyer's technique to investigate the reactivity of the sulfhydryl group of hemerythrin. They titrated chloromethemerythrin with PMB, found that one mole of hemerythrin monomer units reacts with one mole of PMB. The measured molar extinction coefficient was 7.2×10 M^{-1} cm^{-1}, a value which agreed with those obtained for the formation of the mercury-sulfur bond with cystein and ovalbumin (19) and with phosphorylase a (49). Essentially the same results were obtained by Klapper and coworkers (21, 57) for fluoromethemerythrin. Egan (18), Fransioli (20), and Duke et al (21) have demonstrated that when PMB reacts with hemerythrin, PMB forms a

covalent bond only with the lone sulfhydryl group on each subunit and that the increase in absorbance at 250 nm is solely due to the formation of that bond. These authors then monitored the absorbance at 250 nm after addition of PMB and from these data derived kinetic parameters for the reaction of the protein sulfhydryl group with PMB.

Essentially the same technique was used in these experiments. Hemerythrin stock solution, buffer, and PMB solution, in amounts varied to obtain the desired final concentrations, were mixed together in a vial or spectrometer cell. The pathlength of the cell was chosen with regard to the protein and PMB concentrations so that the total absorbance was less than 1.5 and the change in absorbance was on the order of 0.1 or greater. A cell containing a blank consisting of the reactant, hemerythrin or PMB, whichever contributed the greater amount to the total absorbance, was used in conjunction with the cell containing the reaction mixture. Before each absorbance reading, the spectrophotometer was zeroed against the blank cell, so that the absorbance actually measured was less than the absolute absorbance. Egan (18) found from her titrations that Beer's Law applied to hemerythrin-PMB solutions up to a total absorbance of about 3.0. The molar extinction coefficient for the formation of the mercury-sulfur bond remained essentially constant and it was unaffected by such a high total absorbance, most of which had been compensated by the blank. Because of the small volume of the shorter pathlength cells, for experiments requiring them, the reaction mixture was prepared in a

vial and part of it transferred into the cell.

In any event, the various reagent solutions were equilibrated at the chosen temperature before addition of PMB. A Neslab Instruments protable bath cooler and circulation system pumped a coolant through the walls of the Beckman DU spectrophotometer cell chamber and through jacketed beakers in which the reagents were kept before mixing.

When the PMB was added to the reaction solution, Parafilm was placed over the top of the container and gentle inversion mixed the reagents. At regular, convenient time intervals after the addition of PMB, the absorbance at 250 nm of the reacting solution was read, the first measurement usually being made either thirty seconds or one minute after the addition. The time of measurement was kept by an electric timer which had been started at the moment the PMB solution was pipetted into the protein solution.

Experiments with very low protein concentration required the use of 10 cm pathlength cells and a Cary-15 recording spectrophotometer. To cool this large size cell, a glass coil was devised to carry the coolant around the cell. The temperature inside the cell was checked with a thermister type theromometer, as was also done for the 1.0 and 0.5 cm cells used with the Beckman DU.

C. Light Scattering Techniques

Leonardo da Vinci attributed the blue color of the sky to the interaction of light with particles in the air and so is perhaps the first person to consider scientifically the phenomenon of light scattering (58). Since that time, light scattering or turbidity measurements have found many uses and have great potential as a tool for physio-chemical studies of biological macromolecules.

1. Theory of light scattering

Rigorous examination of electromagnetic scattering begins with the observations of Tyndall (58) in 1869 and the theoretical derivations of Lord Rayleigh (59) in 1871 which related the intensity of scattered light to particles in the air. The equation Lord Rayleigh derived for a dilute gas, which relates the intensity of light scattered (i_θ) at an agnle θ to the intensity of the incident light beam (I_o), has the form

$$i_\theta = I_o \left(\frac{8\pi^4 N\alpha^2}{\lambda' r^2} \right) (1 + \cos^2\theta) \qquad (5)$$

where N is the number of scattering particles α, their polarizability; λ', the wavelength of the light in the scattering medium; and r, the distance from the scattering medium to the observer. In 1947, Debye (60) extended the Rayleigh equation to solutions of macromolecules based on the effect of local spontaneous density fluctuations in the solution on light. Both the theory of fluctuations by Debye (60) and the exact molecular theory of scattering developed by Fixman (61) reveal a close connection

between light scattering and osmotic pressure and, for the excess scattering over that of the solvent for a solution of particles small with respect to the wavelength of incident light (λ), give the equation in the form (62, 63)

$$R_\theta = \frac{2\pi^2 n_0^2 (dn/dc)^2 R T M}{N_{av} \lambda^4 (\partial\mu/\partial c)_{T,P}} = \frac{K R T M}{(\partial\mu/\partial c)_{T,P}} \qquad (6)$$

where

$$R_\theta = \frac{r^2 i\theta}{I_\theta(1 + \cos^2\theta)} \qquad (7)$$

is the Rayleigh ratio; c, the concentration of solute in gm cm^{-3} ; N_{av}, Avagadro's number; R, the gas constant; μ the chemical potential of the solute; n_o and n, the refractive indexes of the solvent and solution respectively. The term dn/dc, known as the increment of refractive index and also expressed as $(n - n_o)/c$, is a constant for a given solute-solvent system but must be determined separately from the light scattering measurements. For small molecules, it is convenient to measure the light scattered at 90^o to the incident light where the angular term R is unity. Light scattering theory also applies to larger molecules which are not small compared with the wavelength but this aspect will not be discussed here since hemerythrin meets the requirements of equation 6. All details of light scattering which are pertinent to biological macromolecules have been discussed in review (62, 63).

The osmotic pressure (Π) is related thermodynamically to the chemical potential of the solvent by the equation

$$\Pi = \frac{\mu_o^o - \mu_o}{\bar{V}_o} \tag{8}$$

where μ_o^o and μ_o are the chemical potentials of the solvent in the pure state and in the solution respectively and $\bar{V}_o$ is the partial molal volume of the solvent. The chemical potentials of solvent and solute are related by the Gibbs-Duhem equation

$$x \left(\frac{\partial \mu}{\partial x}\right)_{T,P} = -x_o \left(\frac{\partial \mu_o}{\partial x}\right)_{T,P} \tag{9}$$

where x_o and x are the mole fractions of solvent and solute respectively. By differentiating equation 8 with respect to the mole fraction of solute (x), inserting it into equation 9, and coverting to the concentration units of gm cm^- (c), one obtains the equation

$$\left(\frac{\partial \mu}{\partial c}\right)_{T,P} = \frac{M}{c} \left(\frac{\partial \Pi}{\partial c}\right)_{T,P} \tag{10}$$

which may be substituted into equation 6 and then rearranged to give

$$\frac{Kc}{R_{90}} = \frac{1}{RT} \left(\frac{\partial \Pi}{\partial c}\right)_{T,P} \tag{11}$$

which demonstrates the relationship between osmotic pressure and scattered light intensity. Differentiating the virial-like equation for osmotic pressure

$$\frac{\Pi}{cRT} = \frac{1}{M} + Bc + Cc^2 + \ldots \tag{12}$$

and substituting into equation 11 gives the familiar light scattering equation

$$\frac{K\,c}{R_{90}} = \frac{1}{M} + 2Bc + 3Cc^2 + \ldots \tag{13}$$

which resembles equation 12 for osmotic pressure. Generally, light scattering measurements are made on solutions sufficiently dilute that the third and higher terms on the right hand side of equation 13 are

negligible. The usual practice is to plot the values of Kc/R_{90} against concentration and extrapolate back to c = 0, where the intercept is the reciprocal of the molecular weight and the slope of the line is twice B, the second virial coefficient.

Although equation 13 for scattered light resembles equation 12 for osmotic pressure and both phenomena depend in the same way on the thermodynamic properties of the system, scattered light intensity is directly proportional to molecular weight, but osmotic pressure is inversely proportional. The import of this is that the two methods are complementary. For multicomponent systems, by writing the equation for the various components and summing, with light scattering, one finds the weight average molecular weight defined as (64)

$$\overline{M}_w = \frac{\Sigma c_i M_i}{\Sigma c_i} \quad \frac{\Sigma N_i M_i^2}{\Sigma N_i M_i} \tag{14}$$

whereas by osmotic pressure, one obtains the number average molecular weight defined as

$$\overline{M}_N = \frac{\Sigma N_i M_i}{\Sigma N_i} \tag{15}$$

where N_i, M_i, and c_i are the number, molecular weight, and concentration or mass of the ith species. The two molecular weights together give a measure of the polydispersity of the system. Equation 13, therefore, is usually written

$$\frac{Kc}{R_{90}} = \frac{1}{\overline{M}_w} + 2 B c \tag{13}$$

as the typical light scattering equation.

With the developement of suitable theory and a sufficiently reliable instrument made commercially available by the design of the Brice (65), the light scattering technique became widespread for the routine characterization of biological macromolecules. Eventually, the technique was applied to study the association and siscoation of protein molecules.

2. Measurement of protein aggregation by light scattering

Since the intensity of light scattered by the constitutents dissolved in a solution is directly proportional to the weight average molecular weight of those constituents, it is possible to directly follow the course of any reaction which involves a change of the molecular weight of the reactants. Light scattering was one of the techniques first used to demonstrate the association of polymer molecules in dilute solution. During these experiments, the relative turbidity of a polyvinyl chloride-acetate copolymer was observed as a function of time after heating and cooling the solution (66).

a. Aggregating proteins. Early light scattering data for the clotting of the protein fibrinogen gave both the weight average molecular weight and the molecular length during the polymerization (67,68). Light scattering measurements of the interaction of insulin molecules produced constants for the equilibria between insulin monomer and its various polymers while also indicating which polymer predominates under differing conditions (69). Steiner (70) presented a paper on the reversible asso-

ciation processes of globular proteins in which he developed the theory for applications of light scattering as well as other methods of macromolecular physics. In a lengthy series of papers, he applied these techniques and particularly light scattering to several globular proteins involved in association processes such as insulin (71, 72) and the combination of trypsin with soybean inhibitor (73). From light scattering data, he obtained the degree of polymerization and the consecutive association constants for the reversible processes of these proteins. The light scattering technique has also been a usefull tool to measure the thermodynamic parameters of antigen-antibody reactions (74, 75). In their investigation of the molecular interactions of β-lactoglobulin, Townend and Timasheff (76-79) used nonkinetic light scattering measurements to determine that β-lactoglobulin formed tetramers, and for this tetramerization, to evaluate equilibrium constants and the thermodynamic functions (ΔH^{o}, ΔG^{o}, and ΔS^{o}).

b. Mercaptalbumin. An important application of the light scattering technique evaluated the kinetic parameters for the dimerization of human serum mercaptalbumin mediated by mercurials which reactions may be represented by the equations (80)

$$Ab\text{-}SH + HgX_2 \rightleftharpoons Ab\text{-}SHgX + H^+ + X^- \qquad (16)$$

$$\text{Ab-SH} + \text{Ab-SHgX} \underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}} (\text{Ab-S})_2\text{Hg} + \text{H}^+ + \text{X}^- \qquad (17)$$

$$(\text{Ab-S})_2\text{Hg} + \text{HgX}_2 \rightleftharpoons 2\text{Ab-SHgX} \qquad (18)$$

where Ab-SH represents the native mercaptalbumin; Ab-SHgX, the albumin monomer with sulfhdryl group reacted with mercury reagent (HgX_2); and $(Ab-S)_2Hg$, the albumin dimer. Observing the molecular weight changes that occurred with different mixtures of reactants, Edelhoch et al (80) concluded that reaction 17 was the rate determining step for the formation of dimers. Substituting the weight fraction of protein which is in the form of a dimer, defined by $D = c_2/c$, into the definition of the weight average molecular weight (equation 14) gives the relationship

$$D = \left(\frac{M_w}{M_1}\right) - 1 \qquad (19)$$

where the subscripts 1 and 2 denote the respective quantities for monomer and dimer. For a solution for which B = 0, substituting the light scattering equation into equation 16 gives

$$D = (R_{90,s}/R_{90,1}) - 1 \qquad (20)$$

where the Rayleight ratios are for solution containing a mixture of dimer and monomer ($R_{90,s}$) and for the same solvent medium containing pure monomers ($R_{90,1}$). For the case in which B is not zero, equation 20 would be more complex but since the investigators found that B remained

the same for both a solution of pure dimers as well as one of pure monomers, the value of R_{90} can be simply extrapolated back to c = 0. For equation 17, when x equals the dimer concentration; a, total albumin concentration in monomer units; b, total mercurial concentration; the rate law

$$\frac{dx}{dt} = k_d(b - x)(a - b - x) - k'_{-d}(x) \qquad (21)$$

can be easily integrated, with the apparent rate constant for dissociation, $k'_{-d} = k_{-d}[H^+][X^-] = k_d/K'$, and the apparent equilibrium constant,

$$K' = \frac{[(Ab\text{-}S)_2Hg]}{[Ab\text{-}SH][Ab\text{-}SHgX]} = \frac{K}{[H^+][X^-]} \qquad (22)$$

For those experiments which use the stochiometric ratio, a = 2b, the integration of equation 21 can be put in a form directly related to the light scattering measurements,

$$k_d = \frac{2.303}{t}\left(\frac{2M_1}{1000c}\right)\left(\frac{D_e}{1 - D_e^2}\right)\log\left[\frac{D_e(1 - D_eD)}{D_e - D}\right] \qquad (23)$$

since D = 2x/a = x/b and $D_e = x_e/b$ for the dimer at equilibrium. For dissociation,

$$k'_{-d} = \frac{2.303}{t}\left(\frac{1 - D_e}{1 + D_e}\right)\log\left(\frac{1 - D_eD}{D - D_e}\right) \qquad (24)$$

but k'_{-d} can also be calculated from the relationship $k'_{-d} = k_d/K'$, since K' is directly measureable by light scattering.

c. Phosporylase. Kinetic light scattering measurements have been of greatest value when used in conjunction with other methods. In this manner, Madsen and coworkers (49-51) investigated the interaction of muscle

phosphorylase with PMB. These authors were interested in the relationship between the processes resulting from the addition of PMB, the formation of the mercury-sulfur bond, the dissociation of phosphorylase a into subunits, and the reversible inhibition of enzymic activity. Madsen (50) monitored the effect of PMB on the turbidity of solutions of both forms of phosphorylase. The light scattering data confirmed that phosphorylase b is one half the molecular weight of phosphorylase a and the inhibited enzyme is one quarter that of phosphorylase a. For the kinetic experiments, Madsen ignored the effect of the second virial coefficient, since it could not be evaluated for the phosporylase monomer. This may have introduced a small systematic error. He treated the turbidity data obtained as a function of time after addition of PMB by a method similar to that of Edelhoch et al (80) and found that the dissociation of phosphorylase a tetramer into monomer follows first order kinetics and is much slower than the enzymic inhibition. The dissociation of phosphorylase b dimer into monomer also follows first order kinetics though the data was more variable. The measurement of the rate of formation of the mercaptide bond and the rate of enzymic inhibition were found by Madsen and Cori (49) to be second order reactions and both processes had rate constants of about the same order of magnitude.

From the combined data, Madsen and Gurd (51) deduced an all-or-none hypothesis for the mechanism of the reaction of PMB with the sulf-

hydryl groups of phosphorylase. The light scattering technique can be a useful adjunct to other kinetic techniques, for the data obtained from it can give direct insight into the aggregate structure of the macromolecule during a reaction.

3. The Sofica Light Scattering Photometer

The typical light scattering apparatus contains a light source, usually a mercury lamp, an arrangement of lenses and filters which direct a beam of monochromatic light through the sample cell, a trap for the light beam which has passed directly through the cell so that this light is not reflected back, and a photomultiplier which monitors the light scattered at some angle to the incident light beam. The photomultiplier assembly is usually on a turn-table which permits that the photomultiplier be turned to receive the light scattered at various angles. Figure 3 indicates in a block diagram the major components of the Sofica light scattering photometer, Model 42 000, which was used in this work. The manufacturers (Société Française d'Instruments de Contrôle et d'Analyses) have termed this insturment a photo gonio diffusometer. Tomimatsu et al (81) have described this instrument which is based on a design by Whippler and Scheibling (82) and compared it with the Brice type of instrument. The photo gonio diffusometer is described in detail in the Sofica instrument manual (83). Only the more important features which are relevent to this work are described here.

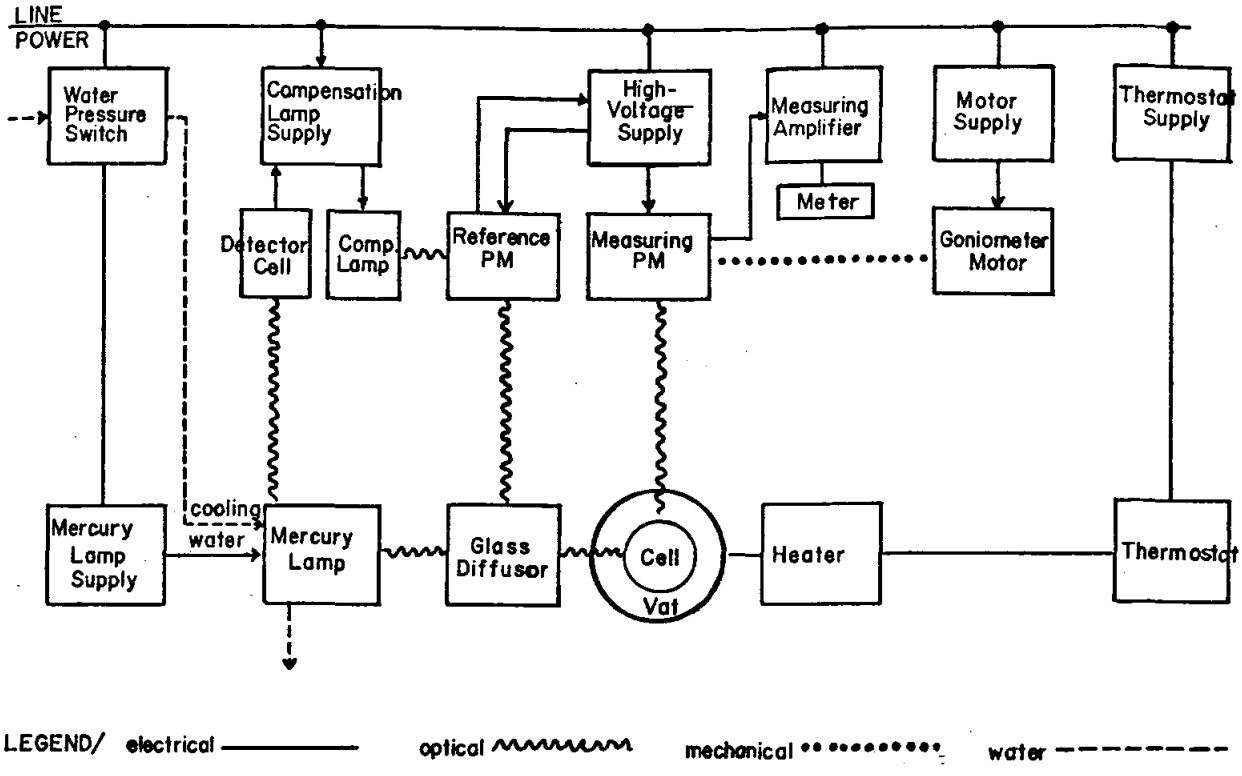


FIGURE 3: Block Diagram of the Sofica Light Scattering Photometer.

a. The reference photomultiplier. The central distinguishing feature of the Sofica instrument which involves the electronic circuitry is the reference photomultiplier which receives a small amount of light from the mercury lamp through a glass diffusor. The reference photomultiplier detects fluctuations in the incident light beam intensity and through a dynode feedback link, regulates the high voltage supplied to the two photomultipliers. This compensates for the fluctuations of light from the mercury lamp and results in a remarkable stability in the output from the measuring photomultiplier (83). In this laboratory, I observed over a period greater than four hours a maximum drift of 3% in the reading of scattered light intensity from the glass standard provided with the instrument.

b. The optical assembly and the immersion vat. The basic optical assembly is shown in schematic form in Figure 4. A water cooled mercury vapor lamp, type SP-500W, generates the light beam. A No 61 Wratten type filter for the 546 nm green line of the mercury vapor lam is supplied with the instrument and was used in all light scattering experiments. The sample cell is enclosed in a vat which is filled with a solvent that has a refractive index near that of the cell glass. This feature virtually eliminates reflections from the outer surface of the cell. When water is used as the solvent for preparing samples for light scattering, the refelction of the water-glass interface on the inside of the cell introduces

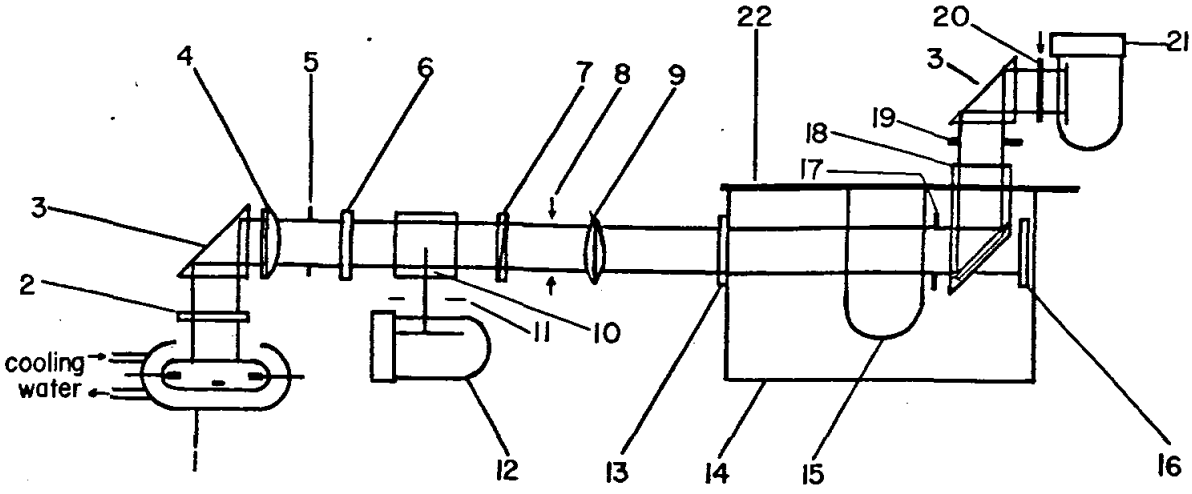


FIGURE 4: Schematic Diagram of the Optical Components of the Sofica Instrument.

1. Mercury vapor lamp.
2. Heat-absorbing glass window.
3. Total reflection prisms.
4. Converging lens.
5. Source slit.
6. Filter (546 nm).
7. Polarizer.
8. Adjustable slit.
9. Collimating lens.
10. Glass diffusor.
11. Iris diaphragm.
12. Reference photomultiplier.
13. Vat window.
14. Liquid immersion vat.
15. Sample cell.
16. Light trap.
17. Entry slit.
18. Total reflection prism (Air blade type).
19. Exit slit.
20. Shutter.
21. Measuring photomultiplier.
22. Rotating plate (Motor driven).

an error of about 1% in the molecular weight (81). The entire interior of the vat, except for the light entrance and observation windows, is blackened with a coating stable for the solvents most commonly used in the vat, benzene (which I used), toluene, or silicone oil. The light trap is a special light absorbing glass placed opposite the entrance window for the incident light beam. Reflection from either the benzene-black coating or benzene-black glass interfaces is negligible. The measuring photomultiplier and the assembly of its associated prisms, exit slit, and shutter are mounted on a rotating plate which serves as a cover of the vat and at the center of which is located the sample cell. The plate can be turned 180°, either by hand or by motor.

c. The sample cell. The cells are cylindrical and each is mounted in a collar which fits the flange around the hole in the center of the rotating plate and thereby holds the cell suspended in the light path. The standard cell has a minimum capacity of 15 ml but smaller cells are also available.

d. Temperature control. For controlling the temperature during light scattering experiments, the walls of the vat contain electrical heating coils which can raise the temperature of the vat up to about 150°C. For cooling to temperatures below that of the room, the vat walls also contain coils for the circulation of coolant. In these experiments, a Wilken-Anderson refrigerated water bath circulator was connected to these coils and during the experiments, the circulator maintained the temperature of

the vat at 13-14°C.

e. The recorder. The output of the measuring photomultiplier is sent to an amplifier and then displayed on a meter. The meter has a series of scales so that the scattered light intensity can be measured through a 200-fold range. A Photovolt Model 43 recorder was connected parallel to the meter through output jacks which were provided with the light scattering photometer.

4. Calibration of the Instrument

The output of the measuring photomultiplier, which is the actually recorded experimental parameter, the relative intensity of light scattered at an angle θ (I_θ), is amplified and read on the meter in arbitrary units. The instrument is calibrated on a relative basis which depends on a standard value of the Rayleigh ratio for benzene (R_b). The relative intensity is converted into the Rayleigh ratio by the factor R_b/I_b, where I_b is the scattered light intensity of a pure, dustfree benzene standard, so that for the Sofica instrument, equation 13 takes the form

$$\frac{Kc}{I_{90}}\left(\frac{I_b}{R_b}\right) = \frac{1}{M_w} + 2Bc \qquad (25)$$

The value taken for R_B at 546 nm was $16.3 \times 10^{-6} cm^{-1}$ determined by Carr and Zimm (84). This number agrees well with theoretically calculated values (85).

To avoid the inconvenience of keeping a fresh supply of suitable benzene standard on hand, a calibrated, flint type glass provides the

standardizing intensity (I_{gs}). The glass standard was calibrated by measuring I_{gs} and I_b under the same experimental conditions in order to obtain the ratio I_b / I_{gs}, the calibration factor for the particular glass standard and instrument. Generally, only a change in optical alignment or a change in the vat liquid will change the calibration factor. The glass standard is already mounted in a cell type holder so I_b can be conveniently obtained frequently during the course of an experiment by replacing the sample cell with the glass standard and measuring I_{gs}.

Tominatsu et al (82) have compared the Sofica and Brice-Phoenix instruments and they found that the molecular weights measured from the two instruments agree to within 2%.

5. Preparation of Solutions for Light Scattering Measurements

Since the scattering of light is very sensitive to larger particles, the presence of dust or denatured protein greatly increases the turbidity of a solution. It is essential for accurate light scattering measurements that the solutions are prepared as free of dust or large particles as is possible.

a. Cleaning glassware. All glassware was soaked in cleaning solution after which it was rinsed with filtered, distilled water. The pipets were dried in an oven but the rest of the glassware including the cells were dried in a vacuum desiccator and again rinsed with filtered benzene. The distilled water and benzene used in the rinses were filtered through

0.05 μm Millipore membranes. All glassware not in use is kept covered and every precaution taken to prevent any dust particles contaminating them.

b. Clarifying solutions. Dust is usually removed from solutions for light scattering either by filtration or by centrifugation for long periods of time. For hemerythrin, the filtration method usually gave satisfactory results. In order to clarify the various solutions, they were passed through 0.22 μm pore size Milepore membranes, with a syringe and a Millipore Swinnex-47 filter holder. For large amounts of solution, a Millipore stainless steel pressure filter holder and barrel with a 100 ml capacity was utilized, connected to a tank of compressed air.

c. Dissymmetry. Large particles scatter light more intensely in the forward direction, at an angle less than 90° to the incident light beam. For a solution of globular proteins which do not have a dimension greater than one twentieth the wavelength of the light, such as hemerythrin, equation 13 is applicable; then the extent to which this kind of solution is dust free or clean may be judged from the ratio of scattered light density at two angles symmetrical about 90°, usually 45° and 135°. The ratio I_{45}/I_{135} is known as the dissymmetry and for small globular proteins, it should be 1.03 or less for accurate molecular weight determinations. Because the kinetic measurements were based on relative values, I accepted a dissymmetry of 1.15 as sufficiently dust free for the kinetic measurements.

6. Measurement of Scattered Light Intensity

The simplest technique, when only molecular weight is the objective, involves only a stock protein solution and the buffer used for its preparation. In this procedure, 15.0 ml of buffer is pipetted into the cell and the scattered light intensity recorded at the angles of 45°, 90°, and 135°. Then a 1.0 ml aliquot of the protein stock solution is added to the cell. After each addition, the cell contents are mixed by stirring with a glass rod, and the intensity of scattered light at the three angles recorded. This process is repeated a few times so that a series of values for both I_{90} and the dissymmetry are obtained for a series of protein concentrations. The intensity readings are converted into Rayleigh ratios; then these data are plotted in the form Kc/R_{90} as a function of concentration in terms of gm cm^{-3} (c). This plot is extrapolated back to c = 0 to obtain the reciprocal of the weight average molecular weight. To check out the instrument, the molecular weight of bovine serum albumin was measured by this method. With a dissymmetry of 1.05, a value of 69,000 was obtained for this protein which is quite close to the established weight of 67,000 (86).

For the kinetic experiments, an alternate procedure recommended by the Sofica instruction manual (83) was adapted. A 25.0 ml solution was prepared for each of several protein concentration. Each solution was filtered and a 15.0 ml aliquot pipetted into the sample cell. After a preliminary light scattering measurement was taken and the dissymmetry

checked, 1.0 ml of PMB solution was added to the protein solution and at regular time intervals, the intensity of the light scattered by the solution was recorded. Readings were also taken on 15.0 ml of buffer both before and after adding 1.0 ml of PMB solution.

In both procedures, the actually measured intensity of light scattered by the solution ($I_{90,m}$) is converted into the desired intensity of light scattered by the solute by subtracting the intensity of light scattered by the solvent or buffer ($I_{90,s}$), according to the relationship

$$I_{90} = I_{90,m} - I_{90,s} \qquad (26)$$

For the kinetic experiments, the solvent was considered to be the buffer plus PMB.

For the calculation of the constant K, which by equation 6 is

$$K = \frac{2\pi n_o^2 (dn/dc)^2}{N_{AV}\,\lambda^4} \qquad (27)$$

the value of dn/dc was 0.189 ml gm^{-1} measured by Keresztes-Nagy (5) for hemerythrin.

The hemerythrin concentration was measured at the end of the experiment by the determination of iron of Fortune and Mellon (87). This determination, based on the absorbance of an o-phenanthroline-iron complex at 510 nm, was adapted to hemerythrin by Keresztes-Nagy (5) and it has been extensively used in this laboratory (13-15, 18, 20).

Several spectrophotometric experiments paralleled the light scattering experiments. A 3.0 ml aliquot of the remaining filtered heme-

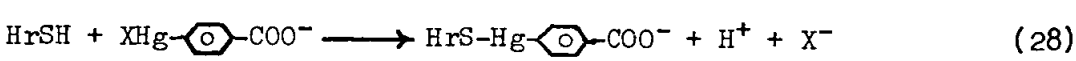
rythrin solution was mixed with 0.2 ml of the PMB solution, the same proportions as used for the light scattering experiments. Then the formation of the mercury-sulfur bond was directly observed under the same conditions as the dissociation was observed.

III. REACTIVITY OF THE SULFHYDRYL GROUP IN HEMERYTHRIN

A study of the reactivity of the sulfhydryl group has vital importance for understanding tertiary and quaternary structures of proteins. The hemerythrin sulfhydryl group is excellent for this study because there is only one cysteinyl residue per each of the eight identical subunits and the reactivity of this sulfhydryl group varies, depending upon the presence of different small anions. Presumably, the binding of these anions induces conformational changes in hemerythrin. Kinetic measurements of the formation of the sulfur-mercury bond can yield some insight into the nature of these changes and the role of the sulfhydryl group in protein structure and association.

A. Formation of the Mercury-Sulfur Bond

For the combination of PMB with hemerythrin, the formation of the mercury-sulfur bond by the reaction



$$HrSH + XHg\text{-}C_6H_4\text{-}COO^- \longrightarrow HrS\text{-}Hg\text{-}C_6H_4\text{-}COO^- + H^+ + X^- \qquad (28)$$

may be monitored by observing the increase in absorbance at 250 nm (19). Figure 5 depicts the increase in absorbance with time for a typical spectrophotometric experiment for the reaction of PMB with chloromethemerythrin. The reaction is monitored until the absorbance reaches a fairly constant value, the end point. Generally, a definite end point was achieved,

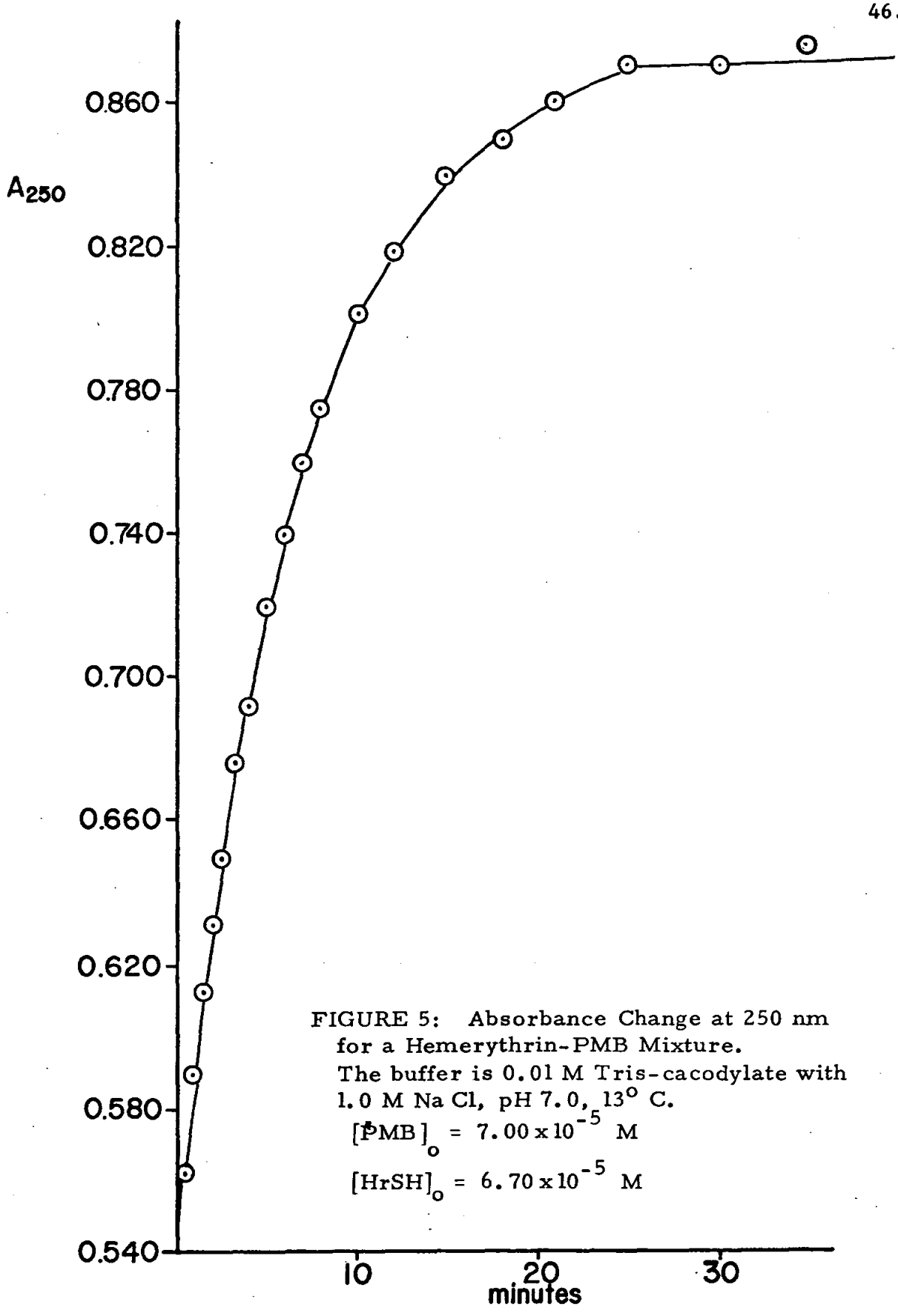


FIGURE 5: Absorbance Change at 250 nm for a Hemerythrin-PMB Mixture.
The buffer is 0.01 M Tris-cacodylate with 1.0 M Na Cl, pH 7.0, 13° C.
$[PMB]_o = 7.00 \times 10^{-5}$ M
$[HrSH]_o = 6.70 \times 10^{-5}$ M

however, in some cases, a good constant absorbance did not develope. In these latter cases, the curve was extrapolated to an estimated end point. This approach usually proved satisfactory. When the concentration of PMB exceeds that of hemerythrin in monomer units, the amount of unreacted protein sulfhydryl group is proportional to the difference between the end point absorbance (A_e) and the absorbance at time t (A_t), and therefore the concentration of protein in monomer units ([HrSH]) is given by the relationship

$$[HrSH]_t = \frac{(A_e - A_t)}{E_m} \qquad (29)$$

where E_m is the molar extinction coefficient for the mercaptide bond over the absorbance of the protein and mercurial. When the concentration of PMB is less than that of sulfhydryl group, then the right hand term of equation 29 gives instead the concentration of unreacted PMB. The concentration of the other reactant then follows from the stochiometry of equation 28 and the initial concentrations. These concentrations were used to evaluate the kinetic parameters of the reaction of PMB with the sulfhydryl group of hemerythrin.

1. Determination of Reaction Order and Treatment of Data

Although it had been reported earlier that the reaction of hemerythrin sulfhydryl groups with PMB is first order with respect to PMB concentration, the reaction being second order overall (18), later work in this laboratory indicates that it may be otherwise. Kinetic data obtained by spectrophotometry for the reaction of PMB with hemerythrin can be made

to fit either first or second order rate equations, although for one case, it may be forced fit. Table I contains the absorbance and concentration data from the experiment of Figure 5. In this experiment, the concentration of PMB and protein in monomer units were nearly equal so that pseudo-first order conditions do not apply. Figure 6 depicts the data of the experiment treated as a first order overall reaction, while Figure 7 depicts the same data treated as a second order reaction overall. Clearly, the data fit the first order plot better.

a. The reaction order by the differential method.

Sometimes, a differential treatment of the data can determine the correct reaction order as described by Benson (88). The data tabulated in Table I are so presented in Figures 8 and 9. If we consider the rate equation for nth order with one reactant

$$-\frac{dA}{dt} = kA^n \qquad (30)$$

by taking the logarithms, it can be written

$$\log\left(-\frac{dA}{dt}\right) = \log k + n \log A \qquad (31)$$

Then the slope of the line in Figure 8, which is a graph for hemerythrin according to equation 31, should give the order of protein concentration in the reaction, provided that the reaction is zero order in terms of the mercurial concentration. The slope of 1.14 is sufficiently close to unity to indicate that the reaction may be first order in hemerythrin. If the reaction were of mixed order

TABLE I: DATA FOR THE REACTION OF PMB WITH HEMERYTHRIN. Experiment of Figure 5.

$[HrSH]_o = 6.70 \times 10^{-5}$ M in monomer units.
$[PMB]_o = 7.00 \times 10^{-5}$ M.

The buffer contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0, at 13° C.

time min	A_{250}	$A_e - A_t$	[HrSH] * M X 10^5	[PMB] M X 10^5	dc/dt M sec^{-1} X 10^8
0.5	0.563	0.307	4.20	4.50	
1.0	0.590	0.280	3.84	4.14	
1.5	0.612	0.258	3.53	3.83	
2.0	0.631	0.239	3.27	3.57	
2.5	0.650	0.220	3.01	3.31	
3.3	0.677	0.193	2.64	2.94	
4.0	0.692	0.178	2.44	2.74	5.56
5.0	0.720	0.150	2.05	2.35	4.57
6.0	0.740	0.130	1.78	2.08	4.40
7.0	0.760	0.110	1.51	1.81	3.74
8.0	0.775	0.095	1.30	1.60	3.26
10.0	0.802	0.068	0.93	1.23	2.50
12.0	0.819	0.051	0.68	0.98	1.67
15.0	0.840	0.030	0.41	0.71	1.34
18.0	0.850	0.020	0.27	0.57	1.21
21.0	0.860	0.010	0.14	0.44	0.27
25.0	0.870				
30.0	0.870	Taken as end point of the reaction.			
35.0	0.875				

* in monomer units

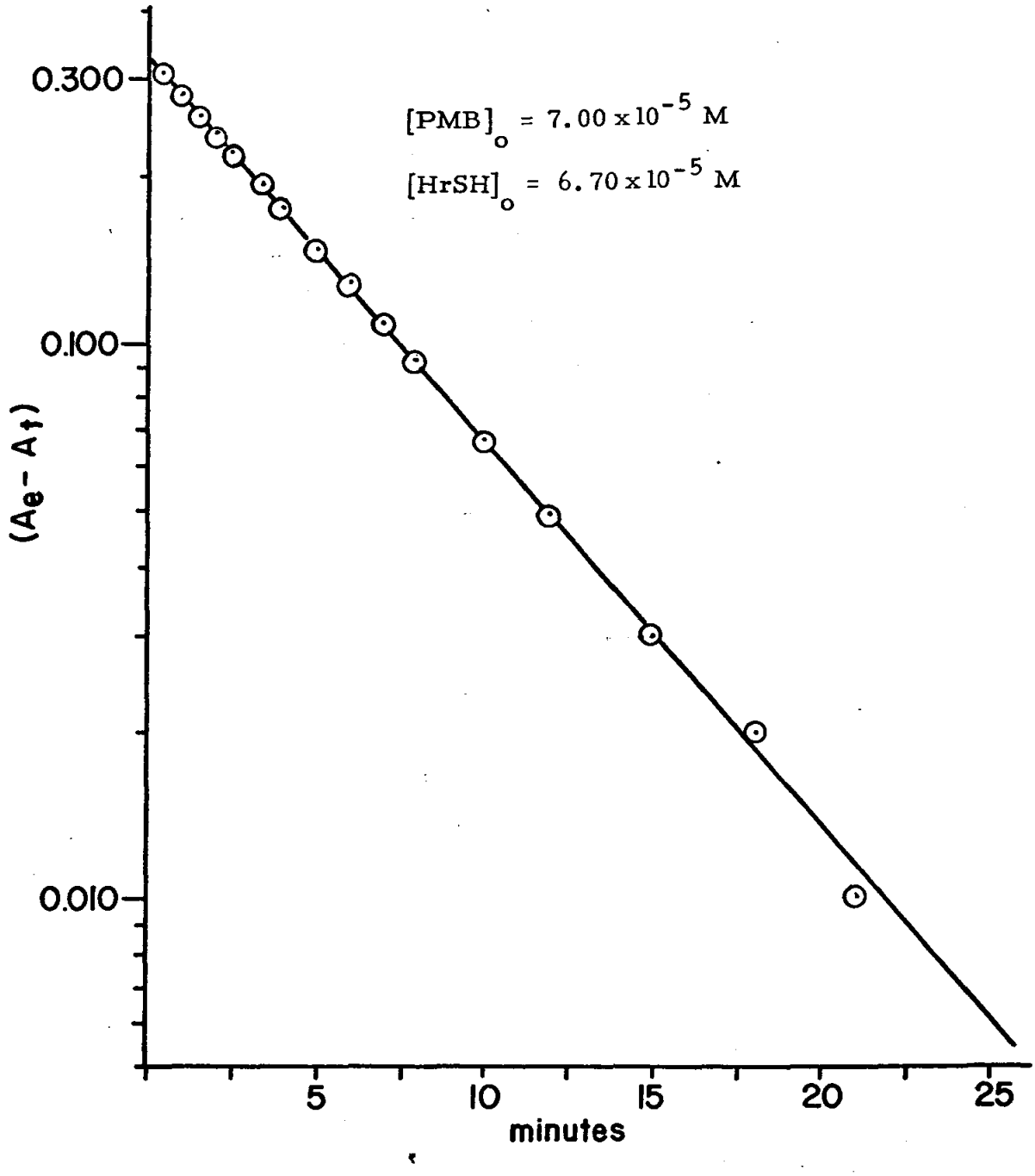


FIGURE 6: First Order Plot for The Reaction of the Sulfhydryl Groups of Hemerythrin with PMB. Data from Table I.

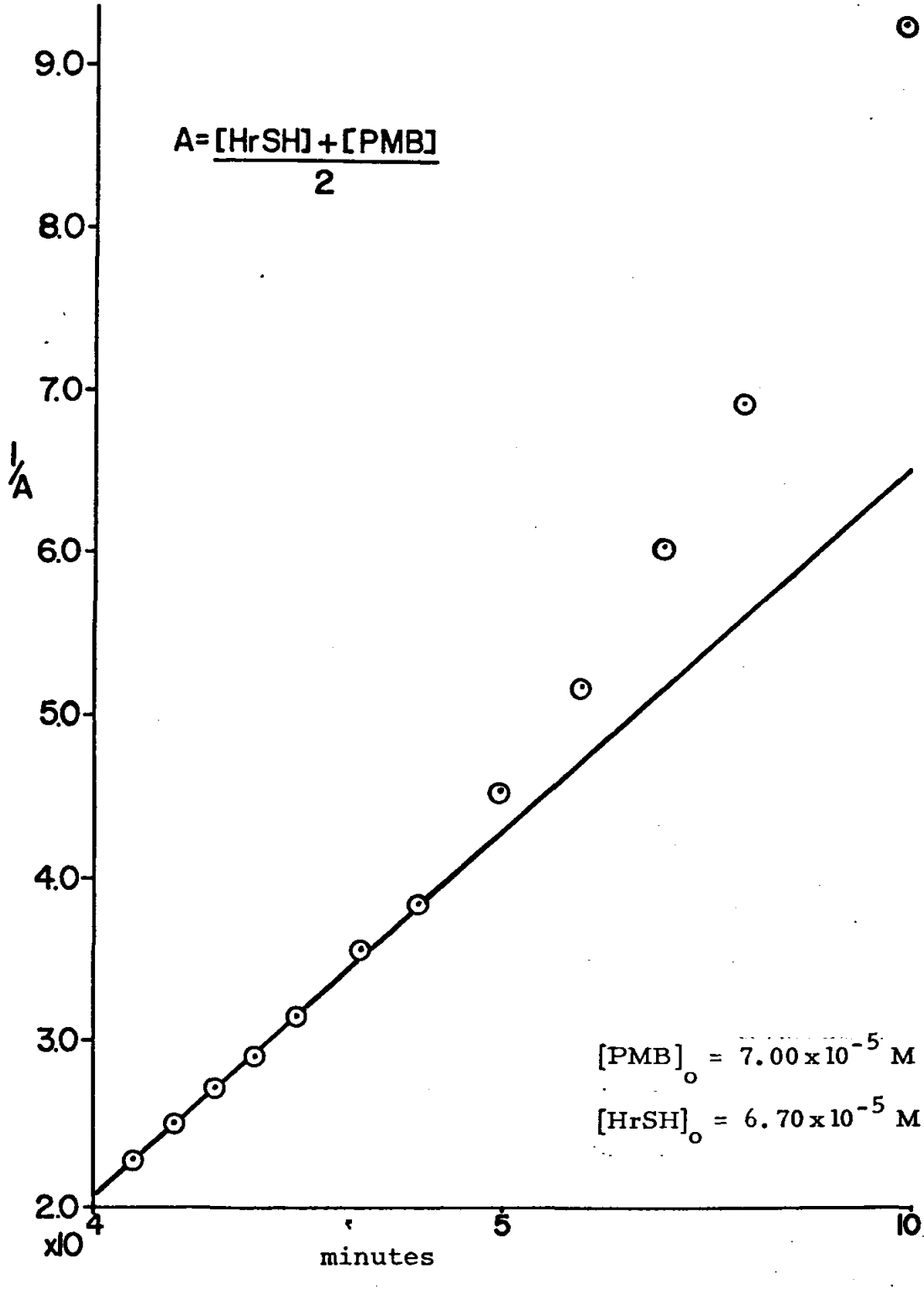


FIGURE 7: Second Order Plot for the Reaction of the Sulfhydryl Group of Hemerythrin with PMB. Data from Table I. See Appendix II for treatment of data.

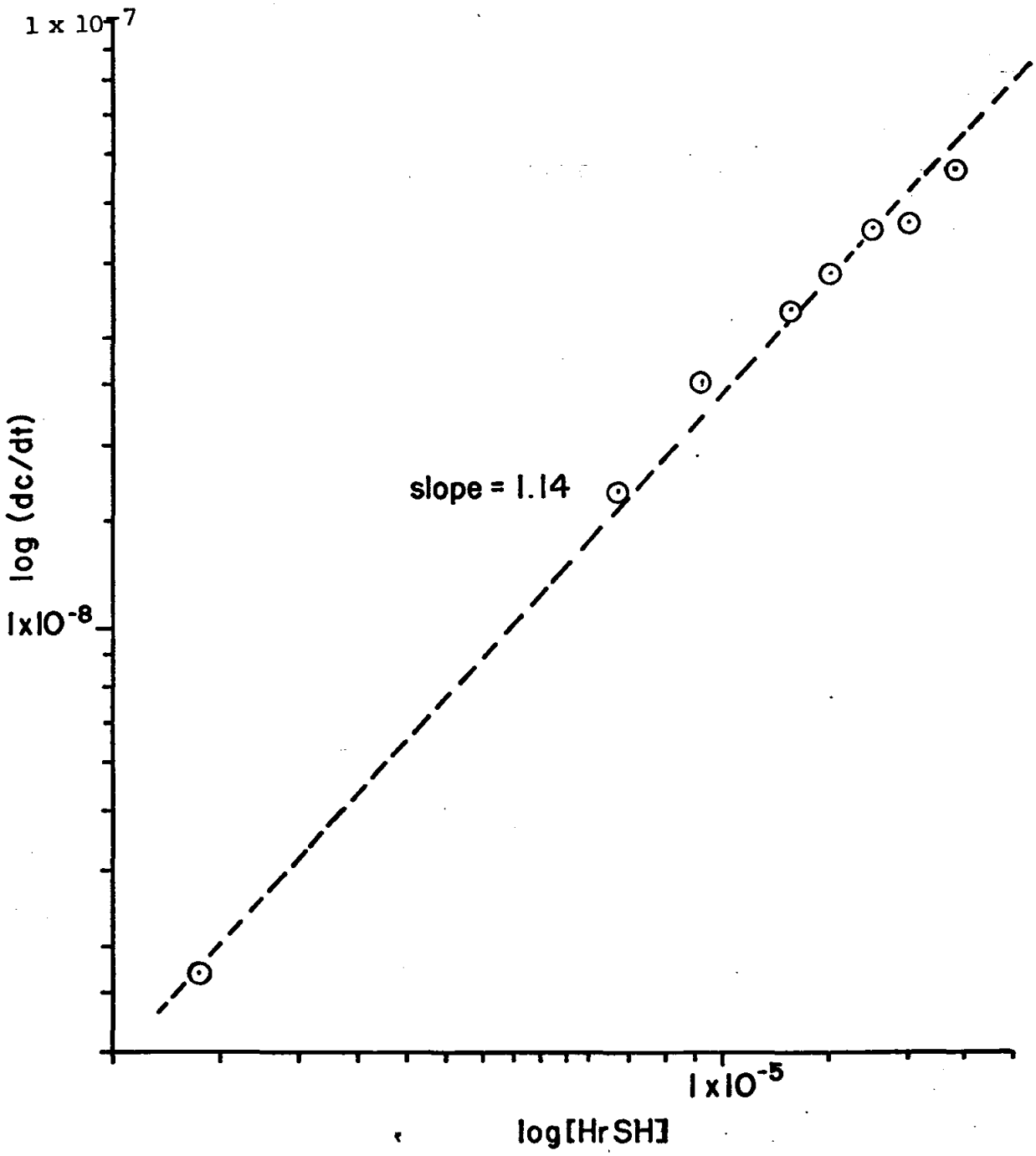


FIGURE 8: First Order Differential Plot for the Reaction of the Sulfhydryl Groups of Hemerythrin with PMB. Data from Table I.

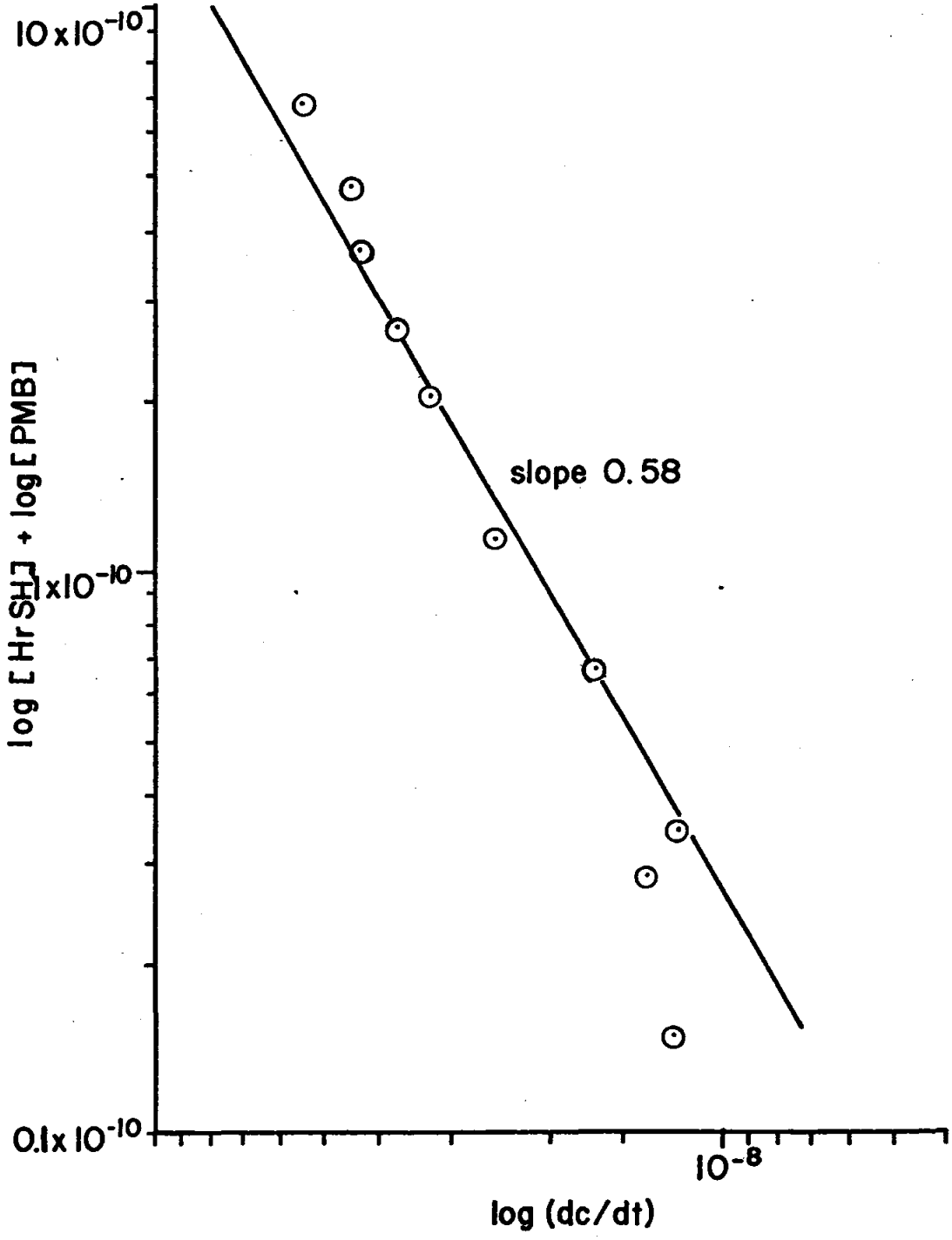


FIGURE 9: Second Order Differential Plot for the Reaction of the Sulfhydryl Group of Hemerythrin with PMB. Data from Table I.

$$- \frac{dA}{dt} = kA^{x}B^{y} \qquad (32)$$

then

$$\log \left(- \frac{dA}{dt} \right) = \log k + X \left(\log A + \frac{y}{x} \log B \right) \qquad (33)$$

and Figure 9, which is a graph according to equation 33 assuming first order for protein and first order for mercurial (x = y = 1), would give a line with a slope of one if the reaction were second order. The slope actually is 0.58 which might be expected for a first order reaction since the concentration of PMB is nearly the same as that of hemerythrin in monomer units. While this method is not very accurate, it strongly points toward the reaction having a first order rate overall. But it cannot distinguish between whether protein or mercurial is the first order reactant for this particular experiment. Furthermore, the reaction may involve nonintegral orders which might be evaluated with a more thorough analysis of the data.

b. Reaction order by evaluation of calculated rate constants.

Egan (18) has claimed that the rate of formation of mercaptide bond depends on the concentration of PMB and she has based this claim on kinetic data obtained under conditions when the reaction was presumed to be pseudofirst order in terms of PMB. Table II and Figure 10 depict an experiment in which the concentration of hemerythrin exceed that of PMB; Figure 11 depicts $\log(A_e - A_t)$ as a function of time for this experiment; Figure 12 depicts the reaction as a second order reaction overall. The

TABLE II: DATA FOR THE REACTION OF HEMERYTHRIN WITH PMB WHEN PROTEIN IS IN EXCESS. Experiment of Figure 10.

$[HrSH]_o = 6.70 \times 10^{-5}$ M in monomer units.
$[PMB]_o = 5.00 \times 10^{-5}$ M.

time min	A_{250}	$A_e - A_t$	[PMB] M X 10^5	[HrSH] * M X 10^5	[HrSH] / [PMB]
0.5	0.400	0.221	3.03	4.73	1.56
1.0	0.442	0.179	2.45	4.15	1.69
1.5	0.463	0.158	2.16	3.86	1.79
2.5	0.492	0.129	1.77	3.47	1.96
3.0	0.505	0.116	1.59	3.29	2.07
3.5	0.520	0.101	1.38	3.08	2.23
4.25	0.541	0.080	1.10	2.80	2.54
5.0	0.558	0.063	0.86	2.56	2.98
6.0	0.580	0.041	0.56	2.26	4.04
7.0	0.584	0.027	0.37	2.07	5.59
9.0	0.612	0.009	0.12	1.82	15.17
12.0	0.621				
15.0	0.621	Taken as end point of the reaction			
20.0	0.621				

* in monomer units

The buffer contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0, at 13° C.

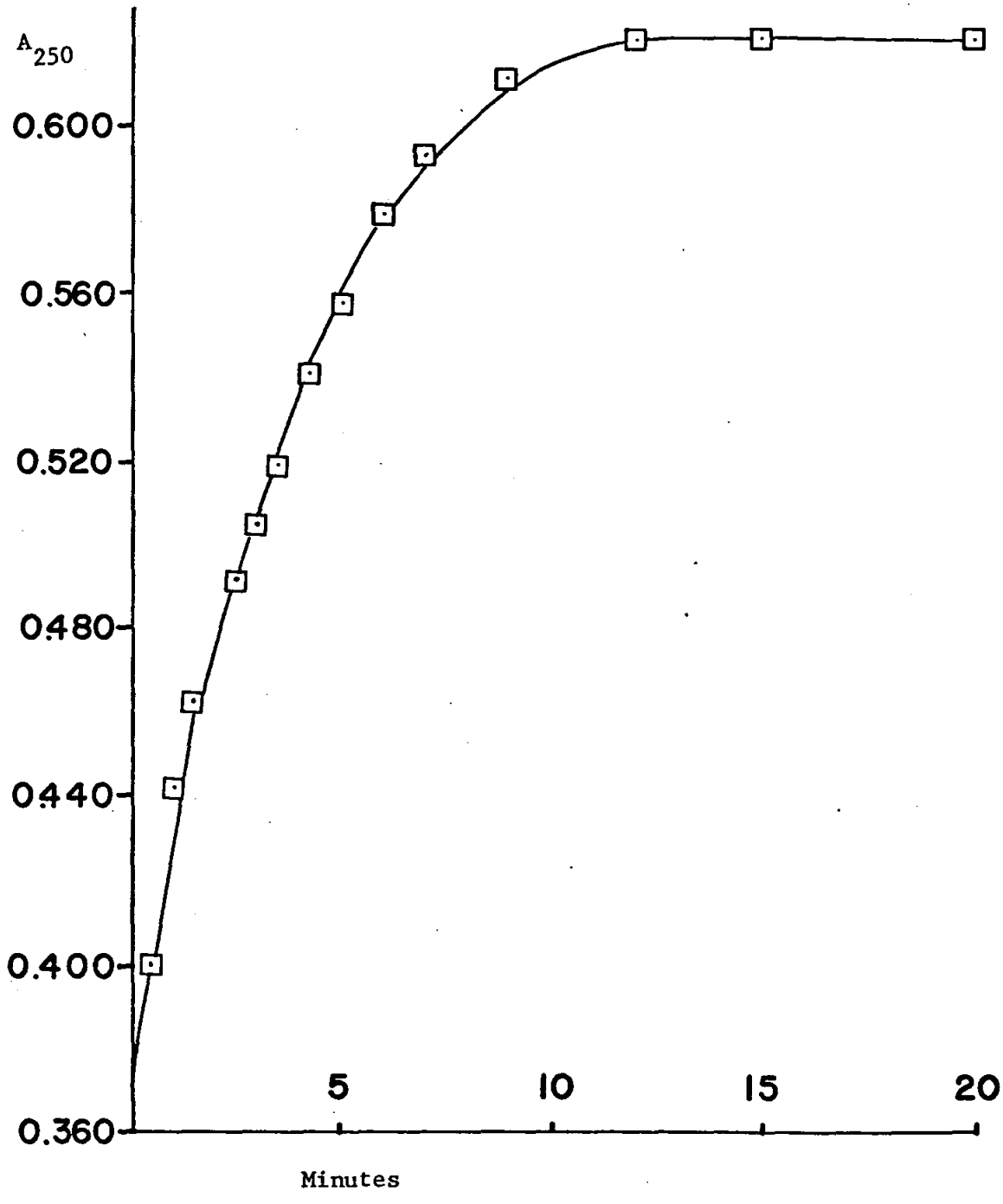


FIGURE 10: Absobance Change at 250 nm for a Hemerythrin-PMB Mixture. Hemerythrin Monomer Units in Excess of PMB. The buffer contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0, 13° C. The initial concentrations are 6.70×10^{-5} M monomer units of hemerythrin and 5.00×10^{-5} M PMB.

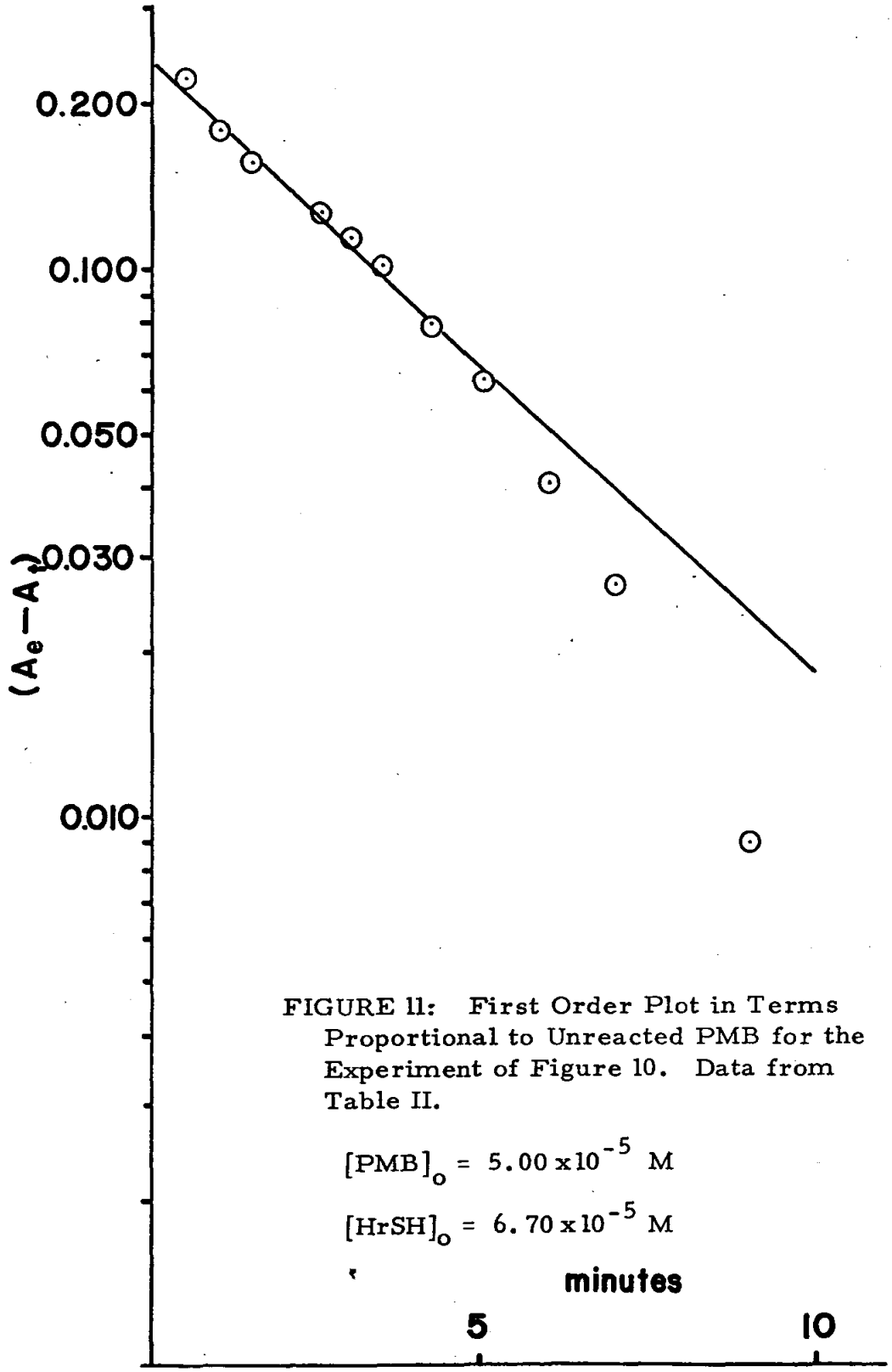


FIGURE 11: First Order Plot in Terms Proportional to Unreacted PMB for the Experiment of Figure 10. Data from Table II.

$[PMB]_o = 5.00 \times 10^{-5}$ M

$[HrSH]_o = 6.70 \times 10^{-5}$ M

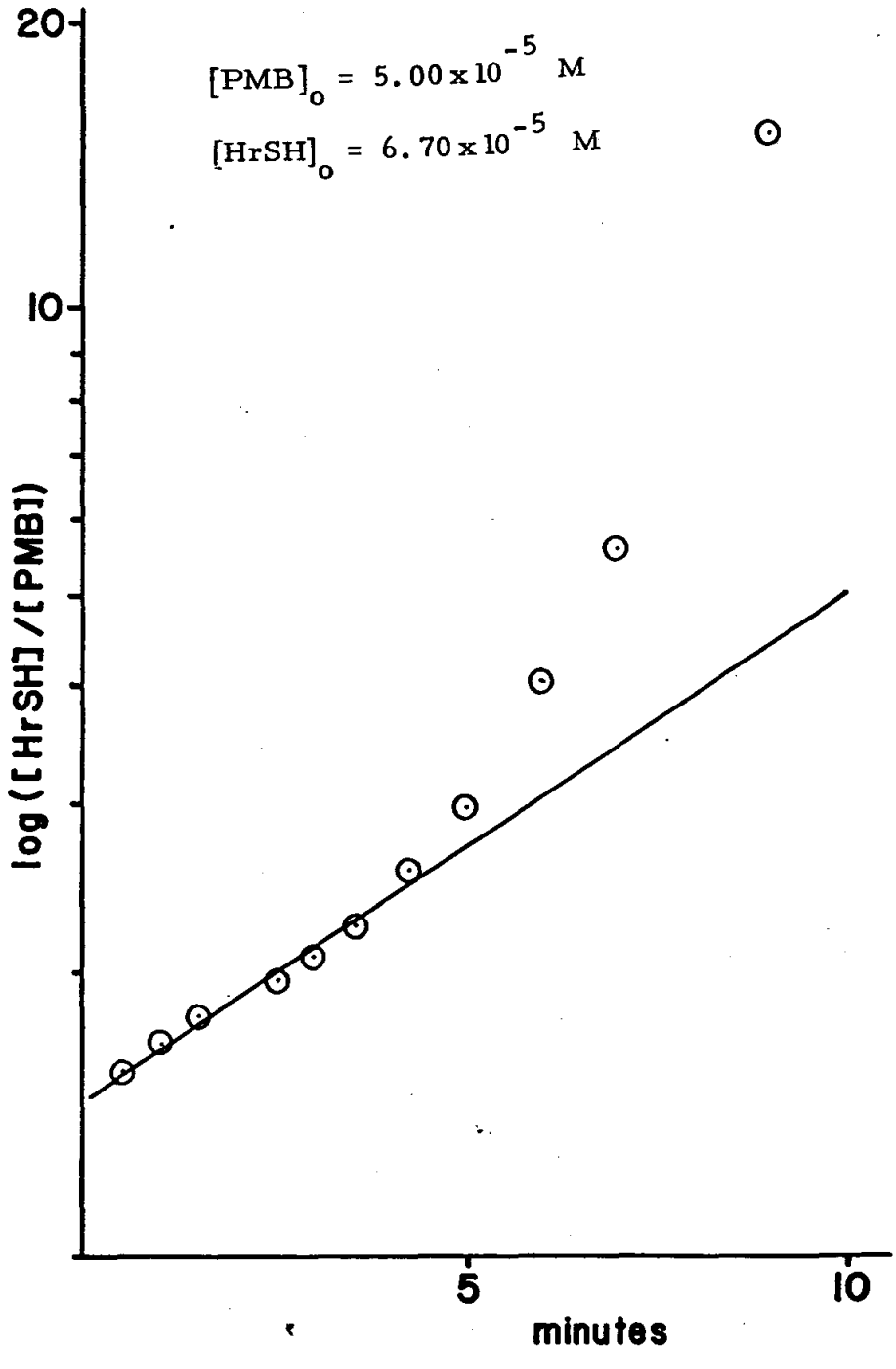


FIGURE 12: Second Order Plot for the Reaction of PMB with the Sulfhydryl Group of Hemerythrin for the Experiment of Figure 10. Data from Table II.

reaction may be first order in protein only, therefore, Figure 13 depicts the same experiment for log[HrSH] as a function of time. The first order rate constants that were calculated by both methods and the second order rate constant are presented for comparison in Table III for a series of experiments with a constant initial concentration of protein but with differing concentrations of PMB. Both the second order constant and the first order constant calculated from PMB concentration increase when the PMB concentration is decreased below the storchiometric ratio. While the rate constant calculated from hemerythrin concentration tends to decrease as initial PMB concentration decreases, this effect may be within experimental error. Rate constants calculated from PMB concentration appear to be false constants; the reaction appears to be true first order in terms of hemerythrin concentration, regardless of whether PMB is the reactant in excess.

c. Calculation of rate constants.

Figures 6, 7, 8, 11, 12 and 13 along with Table III demonstrate that the reaction is first order in terms of unreacted hemerythrin concentration in monomer units, which is the same as the unreacted sulfhydryl group concentration. The rate constant is easily found from the integrated first order rate law in the form

$$\ln[HrSH]_t = -k_1 t + \ln[HrSH]_o$$

Besides the graphical method, rate constants were evaluated by a least squares calculation for many experiments. Natural logarithms of the

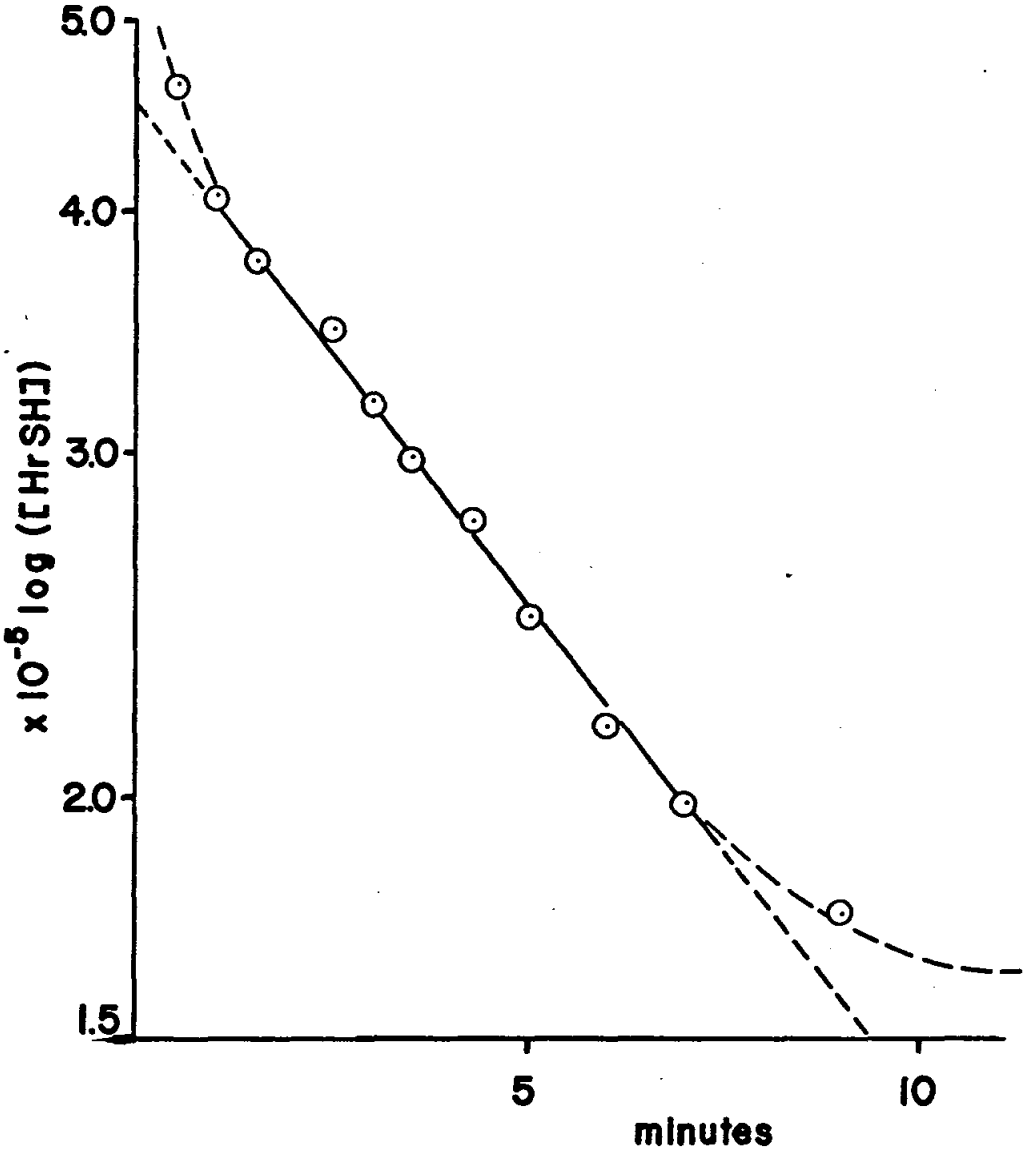


FIGURE 13: First Order Plot in Terms of Unreacted Monomers of Hemerythrin for the Experiment of Figure 10. Data from Table II.

TABLE III: FIRST AND SECOND ORDER RATE CONSTANTS FOR THE REACTION OF PMB WITH HEMERYTHRIN.

Initial Concentrations

$[PMB]_5$ M X 10^5	$[HrSH]^*_5$ M X 10^5	ratio	k_1 in sec^{-1} X 10^3 for HrSH#	k_1 in sec^{-1} X 10^3 for PMB**	k_2 M^{-1} sec^{-1}
9.97	6.60	1.5 a	2.9 ± 0.3	———	51 ± 4
6.98	6.60	1.06 a	2.8 ± 0.2	———	72 ± 3
4.98	6.60	0.75 b	2.6 ± 0.2	6.7 ± 1.1	175 ± 36
2.99	6.60	0.45 b	2.0 ± 0.1	12.0 ± 1.3	234 ± 11
4.98	9.89	0.60 c	2.0 ± 0.2	19 ± 2	374 ± 20
2.99	9.89	0.30 c	1.8 ± 0.2	40 ± 4	473 ± 25

* in monomer units.

\# Rate constant calculated in terms of hemerythrin concentration.

** Rate constant calculated in terms of PMB concentration.

a Average of two experiments.

b Average of three experiments.

c One experiment.

The buffer contained 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

concentration were taken as a function of time and used to calculate a straight line of regression with a standard program on an Olivetti Programa calculator. The negative slope of the calculated line gives the rate constant and the y intercept then is the natural logarithm of the presumed initial concentration of hemerythrin in monomer units. This initial concentration was not the same as the actual starting concentration, because of a fast reaction phase which accounted for 20 - 40% of the total reaction and which was completed before the first spectrophotometric reading could be taken. The extent of the fast reaction could be judged by comparing the calculated initial concentration with the actual starting concentration.

2. The Reaction in 1.0 M NaCl

Table IV presents again the experiments of Table III with additional experiments showning the first order rate constants, apparent initial concentrations, and percentages of the protein which reacted by the fast phase. For this set of experiments, chloromethemerythrin was the form of the protein; the buffer contained 1.0 M NaCl; the temperature of the reaction was maintained at 13°C. Apparently when PMB is the reactant in excess, the reaction follows true first order kinetics with respect to the protein concentration and has a rate constant of $2.75 \pm 0.23 \times 10^{-3}\ sec^{-1}$. With sulfhydryl group concentration in excess, the reaction is still probably first order in terms of the protein concentration, although the rate constants appear to be smaller. Since the fast reaction

TABLE IV: Calculated First Order Rate Constants for the Reaction of PMB with Chloromethemerythrin in 1.0 M NaCl and at 13° C.

Initial Concentrations

[PMB] $M \times 10^5$	[HrSH] * $M \times 10^5$	ratio	k_1 $sec^{-1} \times 10^3$	a_o# $M \times 10^5$	% fast + Reaction
6.02	2.42	2.5	2.65 ± 0.05	1.87	23%
6.02	4.11	1.5	2.34 ± 0.06	2.56	27%
6.02	4.11	1.5	2.47 ± 0.06	2.64	28%
5.16	3.42	1.5	3.06 ± 0.06	2.55	28%
5.16	3.42	1.5	2.68 ± 0.09	2.28	36%
9.97	6.60	1.5	3.22 ± 0.05	4.87	26%
9.97	6.60	1.5	2.67 ± 0.02	4.71	29%
6.98	6.60	1.06	2.68 ± 0.06	4.60	30%
6.98	6.60	1.06	3.02 ± 0.08	4.75	28%
Average of nine			2.75 ± 0.23		
4.98	6.60	0.75	2.69	4.2	35%
4.98	6.60	0.75	2.26	4.9	26%
4.98	6.60	0.75	2.82	4.3	35%
2.99	6.60	0.45	1.94	5.2	21%
2.99	6.60	0.45	2.06	5.6	15%
2.99	6.60	0.45	1.89	5.4	18%
4.98	9.89	0.6	2.03	6.9	30%
2.99	9.89	0.3	1.84	8.2	17%
3.61	6.06	0.6	2.22	3.9	36%
3.61	6.06	0.6	2.04	3.8	37%
3.61	8.48	0.4	1.72	6.1	28%
4.81	8.48	0.6	1.97	5.4	36%
2.40	6.06	0.4	1.48	4.6	26%
3.09	5.40	0.6	1.56	3.4	37%
5.0	16.7	0.3	2.10	13.6	19%
3.0	16.7	0.2	2.14	15.3	8%
4.1	8.2	0.5	1.45	5.6	32%
4.1	8.2	0.5	1.96	6.2	24%
4.1	8.2	0.5	1.38	5.9	28%
4.1	8.2	0.5	2.30	6.4	22%

* in terms of monomer units

\# Apparent initial concentration of hemerythrin.

\+ The percentage of the sulfhydryl groups which reacted by the fast phase.

The buffer contains 0.01 Tris-cacodylate at pH 7.0. For rate constants calculated by least squares, the standard deviation is included.

phase usually involves 20-40% of the total protein, those experiments in which the PMB-hemerythrin monomer ratio is about 0.30 or less may not yield valid first order rate constants.

Another series of experiments in which the protein concentration was constant while the PMB concentration varied but always kept in excess is collected in Table V. The experimental conditions are the same as previous except that the temperature for these experiments was maintained at 10°C rather than 13°C. As before, the rate constant is essentially independent of the mercurial reagent, although the rate constant is noticeably smaller for the two lower PMB concentrations.

3. Comparison of the Reactivity of Chloromethemerythrin and Fluoromethemerythrin

In order to avoid complications from the effects of high ionic strength and difficulties with the solubility of sodium fluoride, kinetic data were obtained from series of experiments in which ligand concentration in the buffer was 0.25 M.

a. Chloromethemerythrin. Table VI presents the series of experiments for chloromethemerythrin in 0.25 M NaCl. Again, as long as PMB is in excess, the rate of reaction is essentially independent of PMB concentration with an average first order rate constant of $k = 1.36 \pm 0.11 \times 10^{-3}$ sec^{-1}. When hemerythrin concentration in terms of monomer units exceeds that of PMB, the rate constants were somewhat smaller in most experiments.

TABLE V: CALCULATED FIRST ORDER RATE CONSTANTS FOR THE REACTION OF PMB WITH CHLOROMETHEMERYTHRIN IN 1.0 M NaCl AND AT 10° C.

The buffer contains 0.01 Tris-cacodylat, 1.0 M NaCl, pH 7.0. The initial concentration of hemerythrin was for all experiments 2.00 X 10^{-5} M in monomer units.

$[PMB]_o$ M X 10^5	ratio #	k_1 sec^{-1} X 10^3	a_o* M X 10^5	% fast + reaction
2.17	1.1	1.58 ± 0.04	1.33	34%
"	"	1.68 ± 0.08	1.39	30%
"	"	1.50 ± 0.02	1.32	34%
Average of three		1.59 ± 0.02		
3.61	1.8	1.66 ± 0.08	1.34	33%
"	"	1.55 ± 0.02	1.41	30%
"	"	1.74 ± 0.03	1.32	34%
Average of three		1.65 ± 0.07		
7.23	3.6	2.44 ± 0.14	1.33	34%
7.23	3.6	2.13 ± 0.07	1.26	37%
7.23	3.6	2.44 ± 0.08	1.39	30%
10.84	5.4	2.44 ± 0.11	1.54	23%
14.46	7.2	2.10 ± 0.04	1.54	23%
14.46	7.2	2.27 ± 0.06	1.45	38%
18.07	9.0	2.05 ± 0.08	1.62	20%
Average of seven		2.27 ± 0.15		

The ratio of $[PMB]_o/[HrSH]_o$

* The apparent initial concentration of hemerythrin, the portion which reacts curing the slow phase.

\+ The percentage of the sulfhydryl groups which react by the fast phase.

TABLE VI: Calculated First Order Constants for the Reaction of PMB with Chloromethemerythrin in 0.25 M NaCl and at 13° C. The buffer is 0.01 M Tris-cacodylate, pH 7.0.

Initial concentrations.

$[PMB]$ $M \times 10^5$	$[HrSH]^*$ $M \times 10^5$	ratio	k_1 $sec^{-1} \times 10^3$	$a_o^\#$ $M \times 10^5$	% Fast $^+$ Reaction
68.0	8.69	7.8	1.20 ± 0.12	5.66	35%
68.0	8.69	7.8	1.40 ± 0.07	6.44	26%
40.8	8.69	4.7	1.66 ± 0.07	8.14	7%
27.2	8.69	3.1	1.47 ± 0.14	5.88	32%
13.6	8.69	1.6	1.40 ± 0.03	7.13	18%
13.6	8.69	1.6	1.40 ± 0.02	6.76	22%
9.25	8.69	1.06	1.52 ± 0.03	6.15	36%
9.37	2.29	4.1	1.45 ± 0.05	1.90	17%
9.37	2.29	4.1	1.40 ± 0.02	1.86	19%
9.37	4.58	2.05	1.28 ± 0.03	3.79	17%
9.37	6.87	1.4	1.40 ± 0.04	4.72	31%
9.37	6.87	1.4	1.27 ± 0.02	4.84	30%
9.37	9.16	1.02	1.26 ± 0.01	5.79	37%
9.37	2.29	4.1	1.24 ± 0.02	1.81	21%
4.17	2.29	1.81	1.22 ± 0.09	1.78	22%
4.17	2.29	1.8	1.17 ± 0.05	1.79	22%
Average of sixteen			1.36 ± 1.11		
2.50	2.29	1.1	0.94 ± 0.02	1.60	30%
2.50	2.29	1.1	0.96 ± 0.01	1.60	36%
2.50	1.14	2.2	0.95 ± 0.02	0.93	18%
2.50	1.14	2.2	1.04 ± 0.04	0.88	23%
2.50	1.14	2.2	1.04 ± 0.02	0.85	25%
Average of five			0.99 ± 0.04		

* in terms of monomer units
\# apparent initial concentration of hemerythrin
\+ the percentage of sulfhydryl groups which react by the fast phase

TABLE VI: Continuation from previous page.

$[PMB]$ $M \times 10^5$	$[HrSH]^*$ $M \times 10^5$	ratio	k_1 $sec^{-1} \times 10^3$	$a_o^\#$ $M \times 10^5$	% Fast Reaction$^+$
7.92	8.69	0.9	1.39	5.6	36%
6.60	8.69	0.8	0.94	5.2	40%
6.60	8.69	0.8	0.96	5.4	38%
5.4	8.69	0.6	0.82	5.5	37%
3.96	8.69	0.46	0.61	6.05	30%
2.62	8.69	0.3	2.10	8.0	8%
1.31	8.69	0.15	1.74	8.2	6%
11.24	12.42	0.9	1.13	8.3	33%
11.24	12.42	0.9	1.00	8.8	29%
3.75	12.42	0.3	1.12	10.0	19%
9.0	12.45	0.7	0.86	9.4	25%
9.37	11.43	0.8	1.05	7.2	37%
9.37	11.43	0.8	1.14	6.4	44%
8.32	11.43	0.7	0.91	8.4	26%
8.32	16.01	0.5	0.40	11.0	31%
8.32	22.87	0.4	0.83	17.1	25%

* in terms of monomer units
\# apparent initial concentration of hemerythrin
\+ the percentage of sulfhydryl groups which react by the fast phase.

Also, the experiments with the lowest concentration of PMB gave rate constants smaller than the others even though PMB was in excess. For the PMB-hemerythrin ratios of 0.30 or less, the kinetic data is particularly suspect, not only because the fast phase of the reaction complicates the observations, but also because the hemerythrin concentration exceeds the mercurial to such an extent that zero order conditions probably prevail for the protein.

b. Fluoromethemerythrin. Parallel to the chloromethemerythrin experiments, another series examined fluoromethemerythrin in a buffer containing 0.25 M NaF. A typical experiment is described in Table VII and Figure 14. As with the chloride form, the data for a single kinetic experiment can be forced to fit both first and second order rate curves. But as Figures 15 and 16 demonstrate, the data fit a first order treatment better than second order. The calculated rate constants, both first and second order, are affected by the starting concentrations of both mercurial reagent and protein. In keeping with the calculations for chloromethemerythrin, Table VIII presents the first order rate constants for fluoromethemerythrin. The rate constant increases with increasing PMB concentration and decreases with increasing hemerythrin concentration. For fluoromethemerythrin, a smaller percentage of the total protein reacts by the fast phase than for chlorothemerythrin.

TABLE VII: DATA FOR THE REACTION OF FLOUROMETHEMERYTHRIN WITH PMB. Experiment of Figure 14.

$[PMB]_o = 4.15 \times 10^{-5}$ M.
$[HrSH]_o = 2.76 \times 10^{-5}$ M in monomer units.

time min	A_{250}	$A_e - A_t$	[HrSH] * $\times 10^5$ M	[PMB] $\times 10^5$ M	[PMB]/[HrSH]
1	0.119	0.071	1.94	3.33	1.72
3	0.124	0.066	1.81	3.20	1.77
5	0.127	0.063	1.73	3.12	1.80
8	0.130	0.060	1.64	3.03	1.85
12	0.134	0.056	1.53	2.92	1.91
16	0.136	0.054	1.48	2.87	1.94
20	0.140	0.050	1.37	2.76	2.02
25	0.145	0.045	1.23	2.62	2.13
31	0.149	0.041	1.12	2.51	2.24
35	0.152	0.038	1.04	2.43	2.34
40	0.157	0.033	0.09	2.29	2.54
46	0.160	0.030	0.82	2.21	2.70
52	0.165	0.025	0.68	2.07	3.04
58	0.167	0.023	0.63	2.02	3.21
65	0.172	0.018	0.49	1.88	3.84
72	0.174	0.016	0.44	1.83	4.16
80	0.177	0.013	0.36	1.75	4.86
90#	0.181#	0.009#	0.25#	1.65#	6.60#
100#	0.185#	0.005#	0.14#	1.53#	10.23#
110#	0.186#	0.004#	0.11#	1.50#	13.64#
120	0.190				
130	0.190	End			
140	0.190	Point			

Data for 90, 100, and 110 min excluded from kinetic calculations.

* in monomer units.

The buffer contained 0.01 M Tris-cacodylate, 0.25 M NaF, pH 7.0. At 13° C. Experiment used 0.5 cm cells.

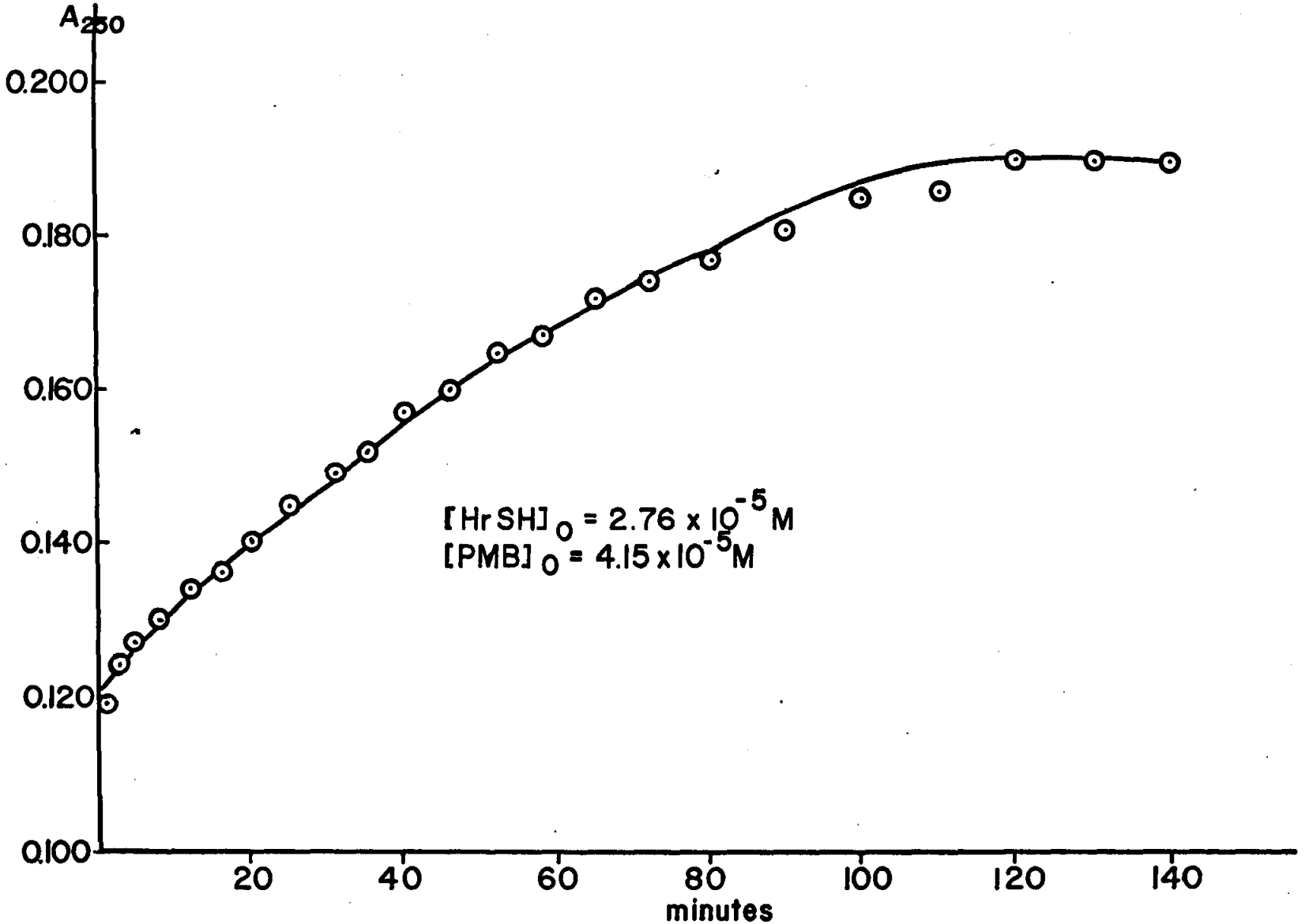


FIGURE 14: Absorbance Change at 250 nm for the Reaction of Flouromethemerythrin with PMB. The Buffer Contains 0.01 M Tris-cacodylate, 0.25 M NaF, pH 7.0, at 13° C.

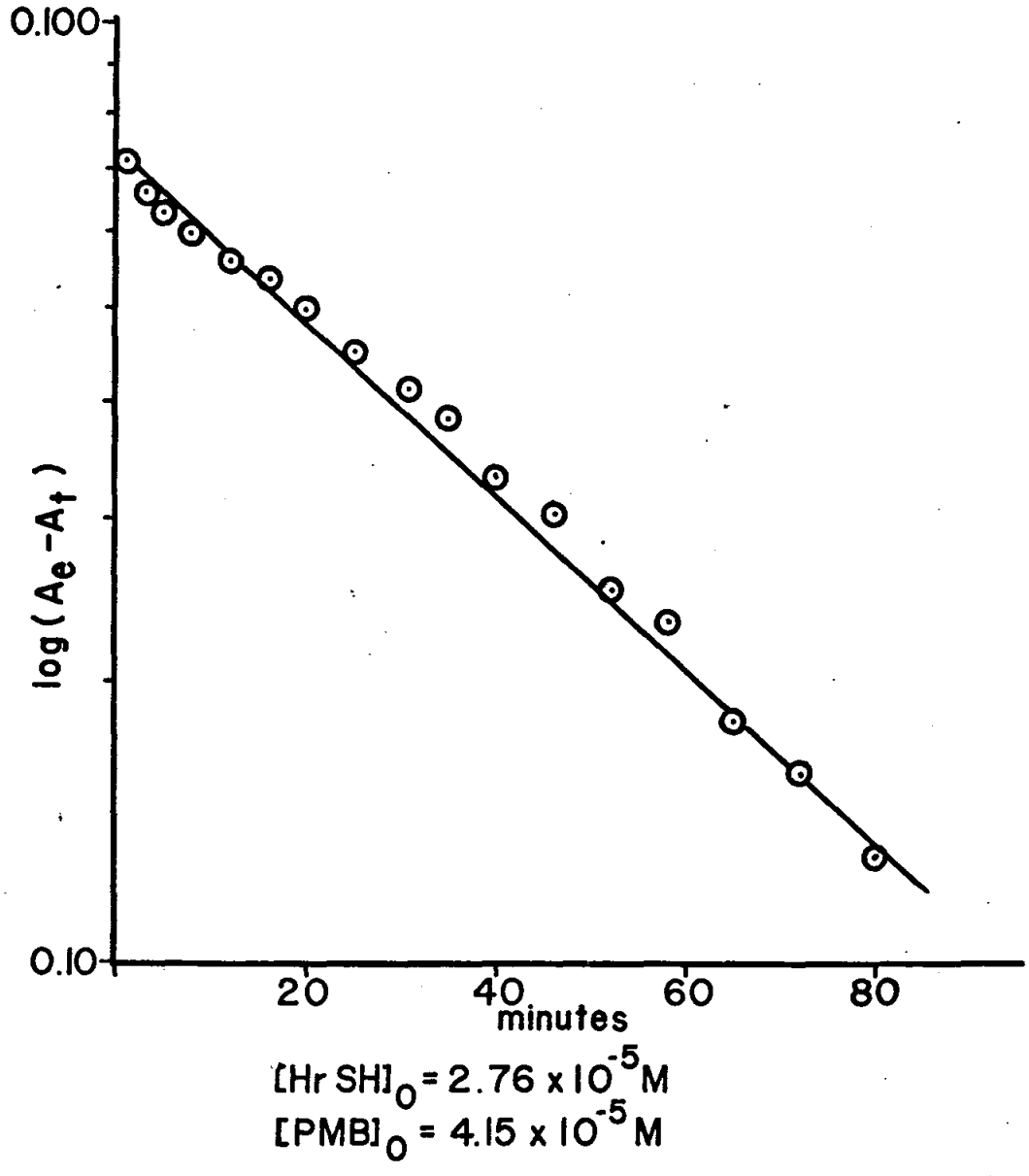


FIGURE 15: First Order Plot for the Reaction of Flouromethemerythrin with PMB for the Experiment of Figure 14. Data from Table VII.

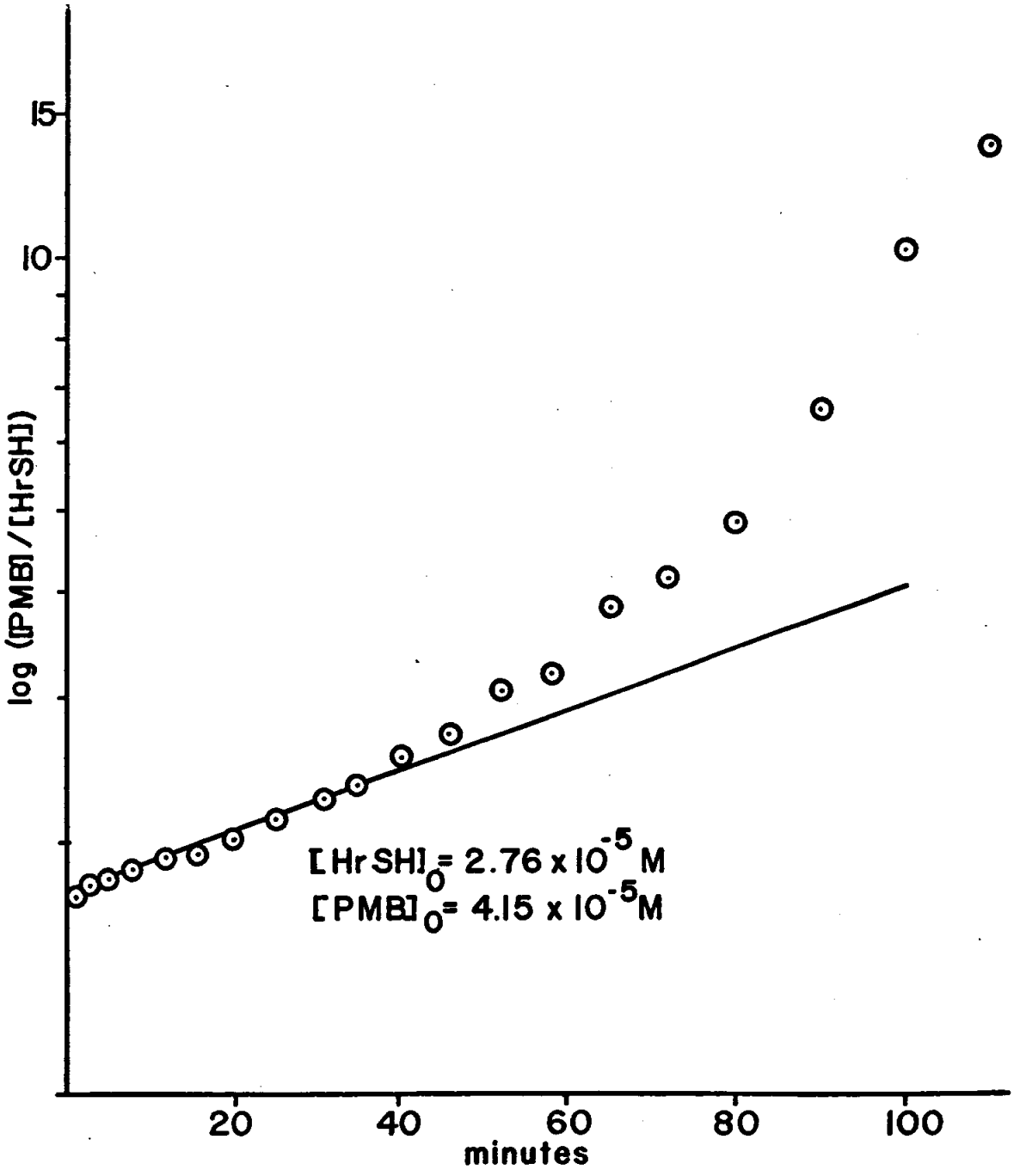


FIGURE 16: Second Order Plot for the Reaction of Flouromethemerythrin with PMB for the Experiment of Figure 14. Data from Table VII.

TABLE VIII: Calculated First Order Rate Constants for the Reaction of PMB with Flouromethemerythrin in 0.25 M NaF and at 13° C.

The fubber contains 0.01 M Tris-cacodylate at pH 7.0.

Initial Concentrations

[PMB] M X 10^5	[HrSH] * M X 10^5	ratio	k_1 sec^{-1} X 10^4	a_o# M X 10^5	% Fast Reaction
2.08	2.76	0.75	2.05 ± 0.05	1.75	14%
4.15	2.76	1.5	3.47 ± 0.07	2.02	27%
4.15	2.76	1.5	3.65 ± 0.13	2.06	25%
8.31	2.76	3.0	4.72 ± 0.13	2.48	10%
8.31	2.76	3.0	4.73 ± 0.13	1.92	30%
12.46	2.76	4.5	4.63 ± 0.05	1.91	31%
12.46	2.76	4.5	5.23 ± 0.08	2.17	21%
20.77	2.76	7.5	4.80 ± 0.07	2.35	15%
20.77	2.76	7.5	5.03 ± 0.07	2.41	13%
20.77	2.76	7.5	5.00 ± 0.08	2.78	?
12.0	5.25	2.3	4.72 ± 0.07	4.07	22%
12.0	5.25	2.3	5.00 ± 0.07	3.65	30%
12.0	5.25	2.3	5.07 ± 0.13	4.24	19%
20.0	5.25	3.8	5.47 ± 0.07	4.64	12%
20.0	5.25	3.8	5.67 ± 0.22	4.82	8%
40.0	5.25	7.6	5.90 ± 0.13	5.18	1%
40.0	5.25	7.6	5.93 ± 0.12	5.39	?
60.0	5.25	11.5	7.13 ± 0.12	6.00	?
60.0	5.25	11.5	6.20 ± 0.07	5.39	?
13.07	1.84	7.1	5.68 ± 0.13	1.68	10%
13.07	1.84	7.1	5.67 ± 0.10	1.66	11%
13.07	3.68	3.6	4.33 ± 0.07	2.91	21%
13.07	3.68	3.6	4.28 ± 0.05	3.11	16%
13.07	3.68	3.6	4.42 ± 0.05	2.93	20%
13.07	5.52	2.4	4.73 ± 0.23	4.72	14%
13.07	5.52	2.4	4.90 ± 0.17	4.57	17%
13.07	7.36	1.8	4.83 ± 0.18	5.59	24%
13.07	7.36	1.8	4.55 ± 0.20	5.75	22%
41.7	3.07	13.6	6.32 ± 0.03	3.11	?
41.7	3.07	13.6	7.41 ± 0.08	3.15	?
41.7	6.15	6.8	5.70 ± 0.08	5.79	6%
41.7	6.15	6.8	5.87 ± 0.08	5.16	16%
41.7	9.22	4.5	5.28 ± 0.08	8.28	10%
41.7	9.22	4.5	5.22 ± 0.15	8.60	12%

* in monomer units

the apparent initial concentration of hemerythrin in monomer units.

4. The Rate of Mercaptide Bond Formation for a Solution of Hemerythrin Monomers

When they proposed the equilibrium model to explain the all-or-none reaction between hemerythrin and PMB, Keresztes-Nagy and Klotz (8) postulated that the monomer form would react faster than the octamer form of hemerythrin. Accordingly, the formation of the mercury-sulfur bond was studied in a solution sufficiently dilute to contain only or largely monomeric hemerythrin. The use of a 10 cm pathlength cell with a Cary-15 spectrophotometer permitted monitoring mercaptide formation in a solution as dilute as 1.0×10^{-6} M in monomer units of protein. The gel chromatography measurements by Rao (14) indicate that in this range of concentration, hemerythrin exists in solution essentially only as monomers. Table IX presents the rate constants found for hemerythrin monomers in 1.0 M NaCl, 0.25 M NaCl, 0.025 M NaCl, and 0.25 M NaF. The reactivity of the monomer is no greater than the reactivity of the octamer form of hemerythrin. For 1.0 M NaCl ligand concentration, the average constant for the reaction of monomers is within experimental error of the average value for the reaction of octamers. For 0.25 M NaCl, the rate constants for monomeric chloromethemerythrin appear to be 25% less than the average rate constant for the chloride octamer but some octameric values are as low. The difference of about 25% in the value of the rate constants may be within the limits of experimental error. The rate constants for monomeric fluoromethemerythrin are about the same as

TABLE IX: Calculated First Order Rate Constant for the Reaction of PMB with Hemerythrin at Low Protein Concentrations. All buffers contain 0.01 M Tris-cacodylate at pH 7.0 and 13° C.

Initial Concentrations

[PMB] M X 10^6	[HrSH] * M X 10^6	ratio	k_1 sec^{-1} X 10^6	a_o# M X 10	% Fast + Reaction
In 1.0 M NaCl					
11.8	2.4	4.9	2.36 ± 0.03	2.0	17%
11.8	2.4	4.9	2.16 ± 0.08	1.9	21%
11.8	1.2	9.8	1.98 ± 0.05	1.1	3%
11.8	1.2	9.8	2.63 ± 0.14	1.0	21%
12.0	1.5	8.0	3.02 ± 0.06	0.8	47%
12.0	1.5	8.0	1.89 ± 0.05	0.9	41%
12.0	2.9	4.1	2.40 ± 0.04	2.6	10%
5.5	2.9	1.9	2.49 ± 0.05	2.3	21%
5.5	2.9	1.9	2.03 ± 0.08	2.1	28%
5.5	2.2	2.5	1.70 ± 0.06	1.3	41%
5.5	1.5	3.7	2.35 ± 0.03	0.8	48%
5.5	2.2	2.5	1.78 ± 0.05	1.4	40%
Average of Twelve			2.23 ± 0.31		
In 0.25 M NaCl			sec^{-1} X 10^3		
25.0	2.29	11	0.99 ± 0.08	2.5	?
25.0	2.29	11	1.14 ± 0.09	2.0	14%
25.0	2.29	11	0.99 ± 0.05	1.9	15%
25.0	1.14	22	1.01 ± 0.01	0.9	18%
25.0	1.14	22	0.93 ± 0.01	0.8	28%
Average of five			1.01 ± 0.05		
In 0.25 M NaF			sec^{-1} X 10^4		
8.7	1.81	4.8	3.22 ± 0.07	1.04	43%
8.7	1.81	4.8	3.32 ± 0.08	1.2	33%
8.7	1.81	4.8	3.22 ± 0.07	1.2	33%
13.0	1.81	7.2	2.87 ± 0.07	1.4	22%
13.0	1.81	7.2	3.23 ± 0.10	1.5	17%
13.0	0.9	14	4.57 ± 0.05	0.6	38%
13.0	0.9	14	4.18 ± 0.07	0.8	10%
13.0	0.9	14	3.92 ± 0.08	0.9	?
In 0.025 M NaCl			sec^{-1} X 10^4		
13.0	1.19	11	3.23 ± 0.13	0.7	38%
13.0	1.06	12	3.07 ± 0.05	0.8	27%
13.0	1.06	12	3.72 ± 0.12	0.9	12%
Average of three			3.33 ± 0.25		

* interms of monomer units.

\# apparents initial concentration of hemerythrin.

\+ the percentage of sulfhydryl groups which react by the fast phase.

lower values for the corresponding octamer form.

B. Analysis of the Data

1. First Order Kinetics for the Formation of the Mercury-Sulfur Bond

This investigation of chloromethemerythrin revealed that, when PMB reacts with the sulfhyryl group, the reaction is first order in terms of the protein concentration in monomer units. Although previous investigators (18, 20, 21) claimed that the data for a single experiment could be made to fit both first and second order rate calculations, these data clearly fit a first order curve much btter than a second order curve. Furthermore, the fact that the calculated first order rate constant is independent of PMB over a wide range of concentrations unequivocally demonstrates that the formation of the mercury-sulfur bond is a first order process. This evaluation that the reaction follows first order kinetics contradicts the earlier reports from this laboratory (18), but it agrees with Klapper and Klotz (16). In contrast, for the reaction of aquomethemerythrin, Garbett and coworkers (27) claim to have observed second order kinetics, overall first order in mercurial concentration and first order in protein concentration in monomer units.

Although the rate of reaction is independent of the PMB concentration, the initial concentration of mercurial does appear to affect the calculated rate constants in some instances. If the PMB concentration is relatively low but still in excess of hemerythrin sulfhydryl group, the reaction

proceeds at a slower rate. In Table V, the seven experiments with PMB concentration of 7.23×10^{-5} M or greater gave and average $k_1 = 2.27 \pm 0.51 \times 10^{-3}$ sec^{-1}. Yet for PMB concentrations of 3.61 and 2.17×10^{-5}M, average rate constants were 1.65 ± 0.07 and $1.59 \pm 0.07 \times 10^{-3}$ sec^{-1}, respectively. A similar pattern is noted in Table VI when the mercurial reagent is in excess for chloromethemerythrin in a buffer containing 0.25 M NaCl. For the sixteen experiments with a PMB concentration of 4.17×10^{-5}M or greater, the average value calculated is $k_1 = 1.36 \pm 0.11 \times 10^{-3}$ sec^{-1} but for five experiments with 2.50×10^{-5}M PMB, $k = 0.99 \pm 10^{-3}$ sec^{-1}. For the monomer in 0.25 M NaCl, the five experiments in Table VII have an average rate constant $k_1 = 1.01 \pm 0.05 \times 10^{-3}$ sec^{-1} which is the same as that which was calculated from the experiments with more concentrated hemerythrin but with the same mercurial concentration of 2.50×10^{-5} M. Originally, I thought that the slower rates were due to some aging process of the protein in solution or to some defect of the PMB solution, but when the PMB concentration was increased to 4.17×10^{-5} M, then the reaction proceeded at a faster rate. Apparently, the PMB concentration does affect the rate constant for the reaction, but at most concentrations used in this investigation for chloromethemerythrin, a saturation point has been reached.

For fluoromethemerythrin, the calculated rate constant increases as the initial PMB concentration is increased but the rate constant decreases as the initial hemerythrin concentration is increased. However, the magnitude of the difference in rate constants calculated from replicate

experiments is in some cases about the same as the difference in rate constants calculated for differing initial concentrations of reactants. Therefore, the trends which I claim to observe in Table VII may be dismissed as due to experimental error. On the other hand, Duke, Barlow, and Klapper (21), who observed the reaction of PMB with fluoromethemerythrin in a solution containing 0.1 M NaF, obtained first order rate constants which were the same order of magnitude as those that I obtained and they also observed the same behavior of the rate constant as the initial concentrations of the reactants were varied. But the trends they observed were more definate than those in Table VIII; for example, they observed a four-fold increase in the rate constant as the initial PMB concentration was increased twenty-fold. Consequently, the evaluation of the data from Table VIII which I have given conforms with the data published by Duke et al (21).

The first order kinetics observed for both chloromethemerythrin and fluoromethemerythrin indicate that the rate determining step must be a unimolecular process involving either the sulfhydryl group or the protein subunit. The behavior of the rate constant for the fluoride complex of hemerythrin suggests a noncovalen binding of PMB to the protein if the change in the calculated rate constant reflects the formation of a protein-PMB complex which somehow affects the rate determining step.

However, the rate of reaction is not a direct function of a PMB-protein complex but is directly proportional to hemerythrin concentration. In

Table III, the first order rate constants which were calculated in terms of mercurial concentration increase sharply as the intial PMB concentration is decreased below that of hemerythrin subunits. If the rate were a function of the PMB-protein complex and if the binding were strong, the concentration of unreacted PMB would be essentially equal to the concentration of complex. Klapper (89) examined the reaction of PMB and fluoromethemerythrin when the ratio of PMB to sulfhydryl group was less than one; he applied first order kinetic analysis to the data in a manner similar to Figure 11; he observed that the rate constant increased as PMB concentration was decreased; and he interpreted it to mean that PMB inhibits the reaction. This behavior of the so-called rate constant, however, is expected from a kinetic analysis of processes involving two reactants, the rate being first order in one, but zero order in the other reactant.* The rate of reaction is not proportional to the zero-order reactant even though it is present in less than stoichiometric amounts. A first order rate constant calculated in terms of the concentration of this reactant would appear to increase according to the ratio between the two reactants. This is what Klapper (89) observed and what is depicted in Table III. The correct calculations are in terms of hemerythrin concentration. The rate constants calculated on the basis of protein concentration are somewhat smaller than when PMB concentration exceeds hemerythrin concentration while this may be an experimental artifact, if PMB binding to the protein

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*See Appendix I

indirectly affects the rate determining step, then the smaller rate constants and the concentration effects are real.

2. The Reactivity of Monomers and the Equilibrium Model

According to the equilibrium model, if the dissociation of hemerythrin octamer were slow and the reaction of PMB with the monomer were fast, then the observed reaction order would be approximately first order in protein and zero order in PMB (18). But Rao (13-15) has demonstrated that the equilibration between octamer and monomer is rapid with regard to both gel chromatography filtration and the rate of formation of the mercury-sulfur bond in hemerythrin. Although Klapper and coworkers (11, 16, 17) have claimed a slow equilibration, Rao (13-15) is probably correct. Furthermore, the equilibrium model as proposed by Keresztes-Nagy and Klotz (8) requires that the reactivity of the monomer sulfhydryl group be greater than that of the octamer sulfhydryl group, regardless of whether the equilibrium is rapidly or slowly established. Experiments with dilute hemerythrin solutions measured the reactivity of the sulfhydryl group in the monomer species of hemerythrin. The reaction proceeded in all cases at the same rate or more slowly than that measured for a more concentrated solution of hemerythrin. The reactivity of the sulfhydryl group does not appear to be related directly to the quaternary structure. The equilibrium hypothesis in its present form, therefore, can not account for the behavior of the sulfhydryl group in hemerythrin.

With regard to fluoromethemerythrin in particular, the rate constants for the monomer are about the same as the lower values for the corresponding octamer. Therefore, the increase in the octameric rate constant with a decrease in protein concentration cannot be explained by the dissociation of the protein to give a greater portion of a supposedly more reactive monomer.

3. Protein Subunit-Subunit Interactions and the Formation of the Mercury-Sulfur Bond

Since the monomer reactivity is about the same as that of the octamer species, also there can be no major subunit-subunit interactions in the octamer which alter the reactivity of the sulfhydryl group such as the allosteric model proposed by Monod, Wyman, and Changeux (54) or the wedge model proposed by Madsen and Gurd (51) require. These models imply that the oligomeric structure constrain the subunit conformation and thereby limit its activity. For example, muscle phosphorylase b is an allosteric enzyme (90, 91). Avramovic-Zikic et al (92) investigated the sulfhydryl groups of this enzyme, found one which was correlated with enzymic inactivation, was involved in allosteric interactions, and had a reactivity which increased 100-fold when phosphorylase b was dissociated into subunits with imidazole citrate buffer.

Binding studies have found no major conformational interactions between subunits of hemerythrin which is circumstancial evidence that

there are none of these interactions affecting the reactivity of the sulfhydryl group and that they are entirely absent in the hemerythrin octamer. These measurements of the binding of small coordinating ligands to the iron site (8, 26, 27), of non coordinating anions such as perchlorate to non-iron binding sites (26), and of oxygen to the iron (8, 37) all demonstrated that the subunits are independent and noninteracting when ligands, anions, or oxygen bind to the protein. This is in keeping with ORD studies which indicate no major change in the gross secondary and teritiary structures of hemerythrin when ligands bind, when PMB reacts with the sulfhydryl group, or when the octamer dissociates into subunits (28). That a change in the conformation of the octamer could occur which would affect the sulfhydryl group and yet have no other effect that could be detected experimentally is unlikely. The only subunit-subunit interaction in hemerythrin, apparently, is the interaction involving the association-dissociation equilibrium.

4. The Fast Reaction Phase

In every kinetic experiment, a certain percentage, usually 20-40%, of the sulfhydryl groups reacted by a process so fast that it was completed before the first spectrophotometric measurement could be taken. An initial fast reaction phase has been observed by others for aquomethemerythrin (27) and fluoromethemerythrin (21). Similarly, Madsen and Cori (49) observed an initial fast reaction for phosphorylase. This fast

phase in hemerythrin could be due to a class of more reactive sulfhydryl groups. This class might arise by a special orientation of the subunit in the octamer, by a particularly reactive conformation of the protein, or by denatured protein.

Some investigators in this laboratory have suggested that the fast reaction is due to a class of subunits which have a particularly reactive position in the octamer structure. That the subunits of hemerythrin are in different environments in the native protein structure is unlikely, however, because a solution of hemerythrin monomers also reacts with a fast phase. If the fast phase were due to some of the subunits in an octamer having a more reactive environment than other subunits in the same octamer, then all subunits would have the same reactivity in a solution of monomers. One would also expect the monomers to have a different reactivity if the quaternary structure affected the rate of reaction. Furthermore, in their discussion of the quaternary structure of proteins, Klotz, Langerman, and Darnall (93) make the basic assumption that all subunits of a protein are in equivalent environments. This assumption is supported by all but one or two of the 20-25 protein of which structures are known from X-ray diffraction studies. The hemerythrin octamer is believed to be fairly compact (6), so it would seem that either the cube or square antiprism structures which Klotz et al (93) proposed for octameric proteins is applicable to hemerythrin. In these structures, every subunit has the same enviroment and the same relationship to the other

subunits of the octamer.

Duke, Barlow, and Klapper (21) report an experiment which indicates that a special reactive species is not involved in the fast reaction. They added PMB to react with approximately 30% of the sulfhydryl groups of a solution of fluoromethemerythrin, then after the reaction was completed, they added excess mercurial and found, contrary to what would have been expected if a depletion of a fast species had occurred, that the reaction kinetics upon the second addition were identical with those of a control to which no prior PMB had been added. However, the sulfhydryl group may be in one or more conformations which are in equilibrium with each other. In one conformation, a sulfhydryl group may be exposed to the solvent medium and reactive while in the other conformation, it is involved in hydrophobic interactions. Whenever the reactive species is depleted, the equilibrium restores it before the next addition of mercurial reagent.

C. The Effect of Anions or Ligands on the Rate of Mercury-Sulfur Bond Formation

Previous investigators observed a relationship between the presence of certain iron coordinating ligands and the reactivity of the sulfhydryl group of hemerythrin (8). Since the sulfhydryl group is not directly bonded to the iron, they considered the relationship an example of cooperative interaction. Ions in the solution, hoever, are known to affect the reactivity of the mercurial. Boyer (19) observing the effect of various anions

on the rate of mercaptide bond formation, postulated that the effect of ions on reaction rate is due in part to the displacement of the hydroxyl group from the mercury. Benesch and Benesch (55) on the basis of unpublished date claim strong interactions take place between several anions and PMB. A major concern of Fransioli (20) and subsequent workers in this laboratory was whether the effect of ligands was due to an action of the anion on PMB in solution or due to a binding of anion to hemerythrin which induces some change in the protein. Although there have been several attempts to differentiate between the effect of anions on the protein and on the mercurial, the precise site of action is not absolutely known. But all the date on hemerythrin and PMB taken together point to the nature of some of these effects.

The rate constants for the hemerythrin sulfhydryl group in 1.0 M NaCl, $2.75 \pm 0.25 \times 10^{-3}$ sec^{-1}, and in 0.25 M NaCl, $1.36 \pm 0.11 \times 10^{-3}$ sec^{-1}, both at 13°C, may be compared. According to the equilibrium constant of 650 M^{-1} for chloride ion binding to the iron site measured by Garbett, Darnall, and Klotz (26), over 99% of the protein is in the form of the chloride-iron complex for either ligand concentration. The binding of additional chloride at the iron site, therefore, cannot account for the increase in the value of k_1 for 1.0 M NaCl over the value of k_1 for 0.25 M NaCl. The change in reactivity may be due to either ionic strength effects, or binding of chloride ion at some other site on the protein, or some change in the conformation of the protein, or an interaction between the

chloride anion and the PMB molecule.

1. Effects of Ionic Strength

Boyer (19) found considerable variation in the rate of reaction of PMB with ovalbumin as he changed the concentration of chloride or sulfate anions present in the buffer. Investigating the effect of sulfate on hemerythrin in this laboratory, Egan (94) found no change in the rate of reaction if the ionic strength is kept constant as the sulfate concentration is increased. Boyer (19) had reported a significant decrease in rate of reaction of ovalbumin with PMB when chloride ion is added, but he had not maintained a constant ionic strength in his experiments for either chloride or sulfate. Fransioli (20) observed the effect of chloride ion on ovalbumin. When she kept the ionic strength constant, ovalbumin reacted with PMB in the presence of 0.5 M Na Cl at almost the same rate as ovalbumin and PMB without any chloride present. This indicates that the variation in reactiviey observed by Boyer (19) must be at least partially due to changes in the ionic strength.

2. Binding of Anion to Hemerythrin at a Site Other Than the Iron-Complex.

Because of the effects of ionic strength, Fransioli (20) maintained a constant ionic strength in all her experiments whenever she compared them with each other. She measured the rate of oxygen release by oxyhemerythrin after addition of PMB, both in the presence and absence of

chloride ion. In the absence of PMB, oxyhemerythrin does not readily release its oxygen, either with or without chloride ion, whereas when PMB is added, the rate of oxygen release is faster if chloride ion is present. She also compared the reactivity of the sulfhydryl group of oxyhemerythrin with that of methemerythrin over a range of chloride concentrations. An increase in chloride concentration increases the rate of mercaptide bond formation of both oxyhemerythrin and methemerythrn. The effect of chloride ion with oxyhemerythrin, and also probably to the same extent with methemerythrin, must be due to either chloride binding at a site other than the iron-ligand site or chloride interaction with the mercurial molecule. The rate constants calculated by Fransioli for both oxyhemerythrin and methemerythrin demonstrate that as the chloride ion concentration increases,this other effect is more important to the rate of reaction than the binding of ligand to the iron site.

Other investigators (14, 25) besides Fransioli (20) have demonstrated that anions binding to a site or sites other than the iron coordination site affect the reactivity of the sulfhydryl group. Using gel chromatography, Rao (14) discovered that chloride ion binds at other sites on hemerythrin with greater affinity than the ion binds to the iron complex of the protein. Furthermore, this binding of chloride at the other sites affects the association-dissociation equilibrium to a greater extent than does the ligand binding to iron, while on the other hand, it does not effect any spectral changes of the type associated with the iron-ligand binding of hemerythrin.

3. Chloromethemerythrin, Fluoromethemerythrin, and the Effect of Anions on the Conformation of the Protein

The chloride ion and the fluoride ion dissimilarly affect the formation of the mercury-sulfur bond in hemerythrin. As Fransioli (20) had also observed, both anions promote the rate of reaction, but for the same ionic concentration, the rate constant for chloride is an order of magnitude greater than for fluoride. But significantly, the kinetic data for formation of the mercury-sulfur bond for both the chloride and fluoride complexes of hemerythrin follow first order rate equations. That the reaction is first order in protein and zero order in PMB indicates that the rate determining step depends on the reactivity of the protein rather than PMB. If PMB does not exert a control on the rate determining step, then changing the reactivity of the mercurial should not affect the overall rate. Therefore, the ligand must exert its influence on the hemerythrin molecule.

Presumably, the ligand binding to the protein induces some change of conformation in the tertiary structure of the protein. In order to see if this premise could be further investigated, the reaction was performed in a solution containing 8 M urea. For these experiments, it was necessary to use potassium fluoride rather than sodium fluoride because of the solubility of the salt in 8 M urea. With either the chloride or fluoride form of methemerythrin, the reaction was too fast to measure. An attempt was made to slow down the reaction rate by diluting the reagents.

The Cary-15 spectrophotometer and 10 cm pathlength cells enabled a ten-fold reduction of both the hemerythrin and PMB concentrations, but the reaction rate was still so fast that it had reached completion before a first reading could be taken (about one and one half minutes). Since it is believed that the degree of reactivity of the sulfhydryl group depends in part inversely on the extent that the group is involved with hydrophobic interactions in the protein structure (42, 43), the complete destruction of secondary and tertiary structure should disrupt the protective effect of these interactions. Hasinoff et al (95) followed the rate of mercury-sulfur bond formation in phosphorylase b and the model compounds, glutatione and 2-mercapto-ethanol, and with a stopflow apparatus, they measured a second order rate constants with a magnitude of 10^6 M^{-1} sec^{-1} for the fastest reacting sulfhydryl group of phosphorylase b and for the model compounds. Consequently, when the protein is denatured, the increase in the reactivity of the sulfhydryl group in hemerythrin can be expected to give a rate constant with the same order of magnitude, 10^6 M^{-1} sec^{-1}. Stopflow experiments are required to determine whether the two anions still exert dissimilar effects on the reaction of PMB with the sulfhydryl group when hemerythrin is denatured. Nevertheless, the tertiary structure of the protein apparently plays a major role in regulating the reactivity of the sulfhydryl group in hemerythrin.

4. The Effects of Perchlorate Ion on Hemerythrin

Klotz and coworkers (25-27) observed that perchlorate ion inhibits both ligand binding and sulfhydryl group reactivity in hemerythrin, although perchlorate ion does not form any ligand bonds with iron. They found that perchlorate binds at two sites per subunit of which the one that binds stronger affects the ligand binding and the reactivity of the sulfhydryl group. This one, therefore, must be near both the iron binding site and the sulfhydryl group. When the protein is dissociated with N-ethylmaleimide or salyganic acid, the addition of perchlorate ion to the protein solution does not convert hydroxymethemerythrin into aquomethemerythrin, indicating that the presence of the sulfhydryl blocking group prevents the binding of perchlorate to the protective site. When azide or thiocyanate ions are present, the protective effect of perchlorate on the sulfhydryl group is lost.

5. Hemerythrin, the Theoretical Effects of Anions on PMB, and Carboxy-peptidase A

All this data on hemerythrin can be correlated and compared with the theoretical effects of anions on PMB, although as Hasinoff et al (95) have pointed out, there has been little experimental data published for ligand binding to organic mercurials. Riodan and Vallee (56) discussed the optimum conditions, such as pH and buffer composition, of a solution for the reaction of PMB with the sulfhydryl groups of proteins. They warn that high concentration of phosphate, chloride, or nitrate may retard the

reaction while a number of anions, such as pyrophosphate, sulfate, or perchlorate, may promote the reactivity of PMB. Coombs, Omote, and Vallee (96), when they elucidated the nature of the zinc binding groups of carboxypeptidase A, observed the reaction of PMB with the sulfhydryl group of that protein and noted that the reaction of PMB was strongly affected by the ions present in the reacting medium. High concentrations of phosphate, chloride, or nitrate either prevented or retarded the reaction, but perchlorate ion and Tris base facilitated it, the optimum buffer being 1.0 M Tris base, 1.0 M $NaClO_4$. These effects were ascribed to a solubilizing effect of the anion on the mercurial. But for hemerythrin, the chloride and perchlorate ions affect the reactivity of the sulfhydryl group in an opposite manner. Whether or not anions affect PMB, the opposite effects observed on the reactivity of the sulfhydryl groups of the two proteins, hemerythrin and carboxypeptidase A, require that for at least one protein, the major mechanism affecting the reactivity of the sulfhydryl group be an interaction of the anion directly with the protein. The data indicate that in hemerythrin, a major factor affecting the reactivity of the sulfhydryl group is the binding of certain ligands to the iron in the protein and the binding of certain anions to one or more sites elsewhere on the protein molecule.

D. The Model Proposed by Klapper and Coworkers for the Reaction of p-Mercuribenzoate with Fluoromethemerythrin

As previously mentioned, my data for the reaction of PMB with fluoromethremerythrin agree with those of Duke, Barlow, and Klapper (21). From their experiments for the reaction of the hemerythrin sulfhydryl group in a buffer containing 0.1 M NaF at 14° C, they obtained first order rate constants of the same order of magnitude as those in Table VIII, observed that the calculated rate constant increased with increasing PMB concentration and decreased with increasing hemerythrin concentration, and suggested that this behavior indicated the rapid binding of the mercurial to the protein at a site other than the cysteinyl residue. The trends in the rate constant that Duke et al (21) observed were more pronounced than those in Table VIII. Klapper (57) using the flow dialysis technique of Colowick and Womack (97) demonstrated that PMB will bind to hemerythrin at sites other than the sulfhydryl group and he presented data which he interpreted as meaning that PMB probably binds to protein faster than the mercury-sulfur covalent bond is formed. Klapper and his associates (21), therefore, proposed a model based on the equilibrium

$$\mathrm{BzHg} + \mathrm{HrSH} \underset{}{\overset{K}{\rightleftharpoons}} \mathrm{BzHg(HrSH)} \qquad (35)$$

where BzHg, HrSH, and BzHg(HrSH) represent PMB, a monomer unit of the native protein, and PMB-protein complex respectively, so that the

formation of the mercury-sulfur bond can proceed by the two processes

$$HgBz + HrSH \xrightarrow{k_1} HrSHgBz \quad (36)$$

$$HgBz + HgBz(HrSH) \xrightarrow{k_2} HgBz(HrSHgBz \quad (37)$$

in which HrSHgBz and HgBz(HrSHgBz) represent the formation of the mercaptide bond. From this model, the rate law for this formation of the mercury-sulfur bond (S-Hg) is

$$\frac{d[S\text{-}Hg]}{dt} = k_1 [HgBz][HrSH] + k_2[HgBz][HgBz(HrSH)] \quad (38)$$

Combining the equation for the total mercurial available for the reaction,

$$[HgBz]_T = [HgBz] + [HgBz(HrSH)] \quad (39)$$

and the equilibrium constant for equation 35,

$$K = \frac{[HgBz(HrSH)]}{[HrSH]\ [HgBz]} \quad (40)$$

and then substituting into equation 38, the rate law can be derived in the form

$$\frac{d[S\text{-}Hg]}{dt} = \left[k_1 \frac{[HrSH]}{1 + K[HrSH]} + k_2 \frac{K[HrSH]}{(1 + K[HrSH])^2} [HgBz]_T \right] [HgBz]_T \quad (41)$$

If $K[HrSH] \gg 1$, then equation 41 becomes

$$\frac{d[S\text{-}Hg]}{dt} \approx \left[\frac{k_1}{K} + \frac{k_2}{K[HrSH]} [HgBz]_T \right] [HgBz]_T = k_{app}[HgBz]_T \quad (42)$$

The condition of large K[HrSH] implies that the binding affinity of the protein is large from which Klapper and coworkers (21) claim that at excess concentrations of PMB, the concentration of native protein in monomer units ([HrSH]) will be independent of mercurial and approximately proportional to the total protein concentration and consequently

$$k_{app} \approx \left[\frac{k_1}{K} + \frac{k_2}{K} \frac{[HgBz]_T}{[Hr]_T} \right] \qquad (43)$$

where $[Hr]_T$ is the total protein concentration and is the proportionality constant. According to equation 43, the apparent rate constant would vary proportional to PMB concentration and inversely proportional to hemerythrin concentration, and would therefore appear to fit the experimental data. The investigators calculated from their data that $k_1 > k_2$ which implies that the unbound hemerythrin reacts faster than bound hemerythrin. During the fast phase, the reaction would proceed through the unbound protein form (equation 36) and as the mercurial bound to the protein completely, the reaction would then proceed through the now dominant and slower bound form reaction (equation 37).

While this model superficially seems to explain the reactivity of the sulfhydryl group in hemerythrin, equation 42 actually gives the rate law in a form pseudofirst order in terms of PMB concentration. The experimental rate constants were calculated as first order in terms of sulfhydryl group concentration. Actually, the PMB concentration for many experiments, both mine and Klapper's, so exceeded the sulfhydryl group concentration that the mercurial concentration would remain approximately constant during the course of the reaction. Under this, the pseudofirst order condition, both terms, k_{app} and $[HrBz]_T$, for the right hand side of equation 42 would be expected to remain constant throughout

the course of the reaction. Furthermore, Egan (18) and Fransioli (20) both found that when PMB was in excess, the kinetic data did not fit a first order curve in terms of the mercurial concentration. Figure 17 depicts the data for absorbance as a function of time for a typical kinetic run from this study and Table X contains the data for sulfhyrdryl group, reacted monomer unit, and PMB concentrations. Figure 18 presents data from Table X as a first order curve in terms of the sulfhydryl group while Figure 19 presents the data as a first order curve in terms of PMB for the same experiment. A least squares calculation from the data of Figure 18 produces a rate constant $4.72 \pm 0.07 \times 10^{-4}$ sec^{-1} and a half time of 24 min. The half time value is about the same as one taken directly from Figure 17. On the other hand, Figure 19 is only linear for the first 30 min. The half time from this graph is 99 min, which is longer than the period for almost the entire reaction. I have already shown that rate constants calculated on the basis of PMB concentration are invalid for the reaction with chloromethemerythrin and the same arguments apply to the unpublished data by Klapper (89) on fluoromethemerythrin. My data for the formation of the mercury-sulfur bond does not fit equation 42. Apparently, the model represented by equations 35, 36, and 37 and the rate law derived by Duke et al (21) does not explain the kinetic behavior of the fulfhydryl group in fluoromethemerythrin.

A fundamental criterion of validity for scientific postulates is that they lead to a theory which correlates empirical data and successfully

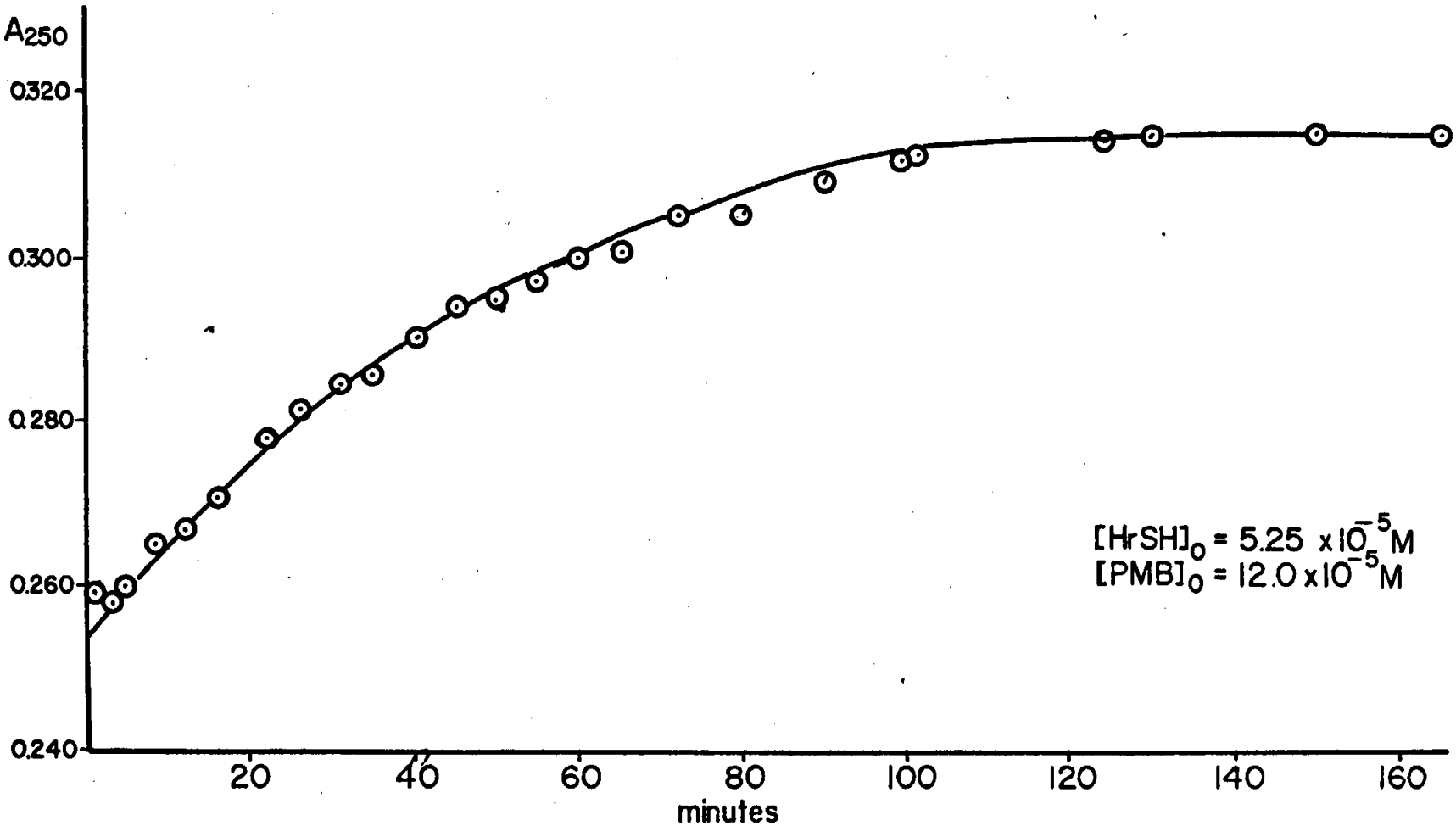

FIGURE 17: Absorbance Change at 250 nm for the Reaction of Fluoromethemerythrin with PMB. The Buffer Contains 0.01 M Tris-cacodylate, 0.25 M NaF, pH 7.0, at 13° C.

TABLE X: DATA FOR THE REACTION OF FLOUROMETHEMERYTHRIN WITH PMB. Experiment of Figure 17.

$[PMB]_o = 12.0 \times 10^{-5}$ M.
$[HrSH]_o = 5.25 \times 10^{-5}$ M in monomer units.

time min	A_{250}	$A_e - A_t$	[HrSH] * M X 10^5	[PMB] M X 10^5
3	0.258	0.052	3.56	10.31
5	0.260	0.050	3.42	10.17
8.5	0.265	0.045	3.08	9.83
12	0.267	0.043	2.94	9.69
16	0.271	0.039	2.67	9.42
22	0.278	0.032	2.19	8.94
26	0.281	0.029	1.99	8.74
31	0.285	0.025	1.71	8.46
35	0.286	0.024	1.64	8.39
40	0.290	0.020	1.37	8.12
45	0.294	0.016	1.10	7.85
50	0.295	0.015	1.03	7.78
55	0.297	0.013	0.89	7.64
60	0.300	0.010	0.68	7.43
65	0.301	0.009	0.62	7.37
90	0.310	End point of the reaction		

* in monomer units.

The buffer contained 0.01 M Tris-cacodylate, 0.25 M NaF, pH 7.0. At 13° C. Experiment used 0.2 cm cells.

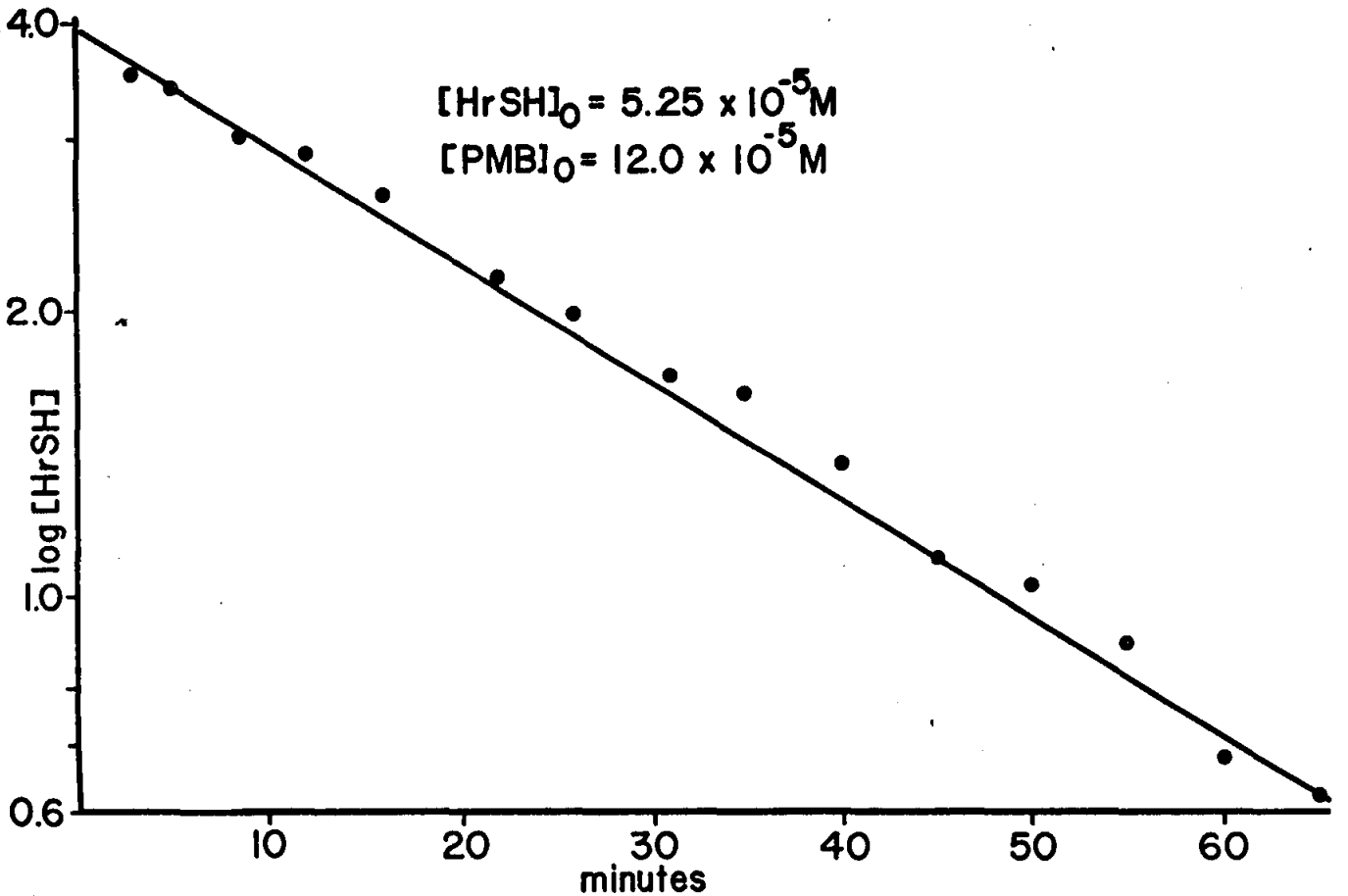

FIGURE 18: First Order Plot in Terms of Unreacted Sulfhydryl Groups for the Experiment of Figure 17. Data from Table X.

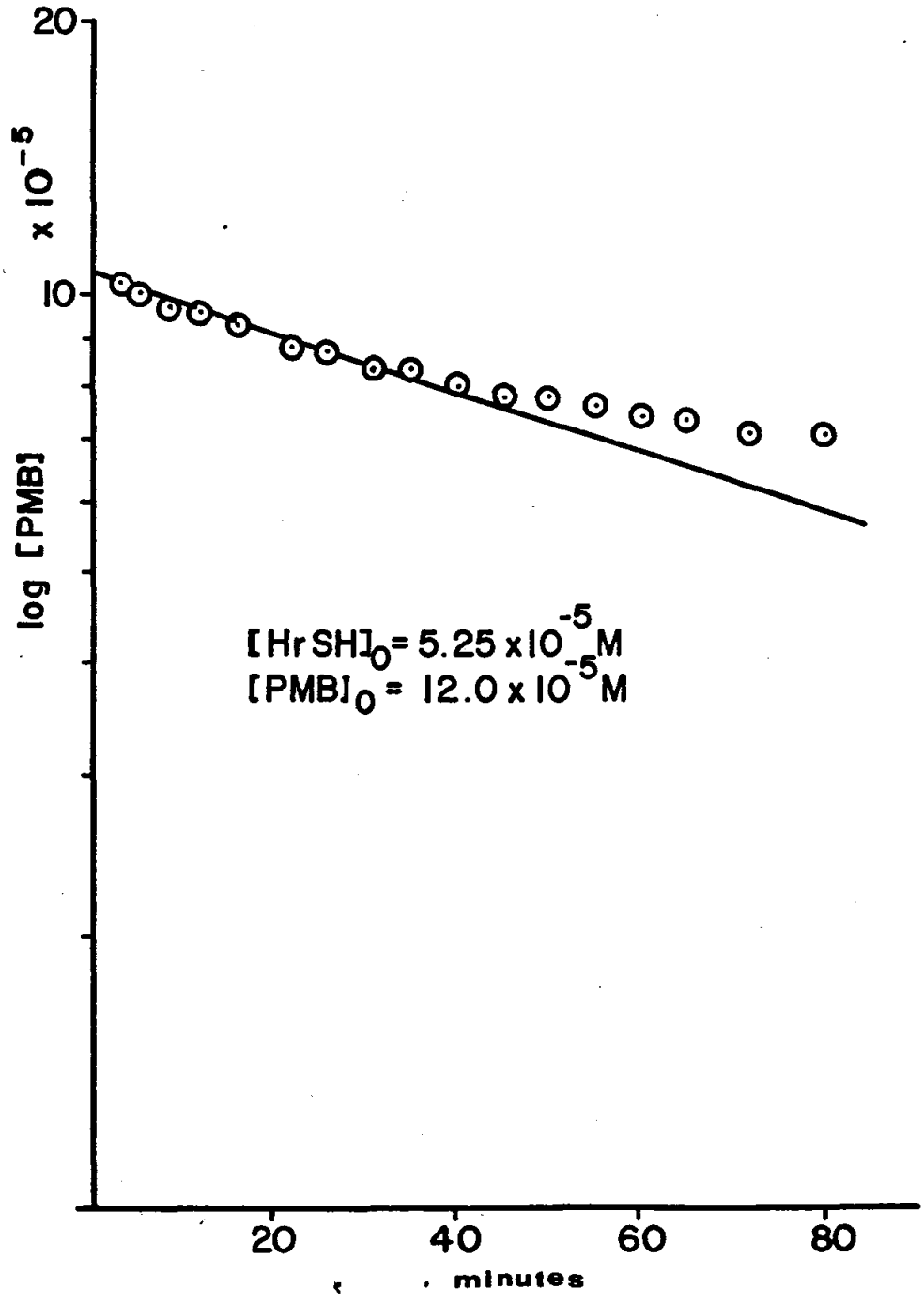

FIGURE 19: First Order Plot in Terms of PMB Concentration for the Experiment of Figure 17. Data from Table X.

predict the outcome of further experiments. Not only does equation 42 not correlate to the data, but also untenable assumptions were made in its derivation which should be considered. Klapper (57) did demonstrate that PMB binds rapidly to hemerythrin at nonsulfhydryl group sites but whether this binding affects the reactivity of the sulfhydryl group remains unproven. Presumably, a single mercurial binding site would affect the sulfhydryl group, this single site being near the sulfhydryl group. Using the graphical method of Jenkins and Taylor (98), Klapper (57) calculated 5.7 ± 0.3 binding sites per protein subunit and apparently all sites have the same intrinsic binding constant, $1.23 \pm 0.31 \times 10^{3}$ M (sic). Although he refers to this value as a binding constant which if it were the constant for equation 35 would have the units M^{-1}, Jenkins and Taylor (98) developed their method for the calculation of enzymic dissociation constants and Klapper does not make clear the exact nature of his constant. Whether a binding or a dissociation constant, in the protein concentration range used in the experiments, the value of 1.23×10^{3} M (or M^{-1}) is inconsistent with the assumption $K[HrSH] >> 1$ which was made to simplify equation 41. I attempted several calculations with the constant supplied by Klapper and never calculated $K[HrSH] >> 1$; a constant greater than $10^{5} M^{-1}$ is required.

Even if the mercurial did bind with sufficient affinity at a single site near the sulfhydryl group as Duke et al (21) seem to postulate, the assumption would not hold for all experiments.

Because

$$K = [HgBz(HrSH)]/[HrSH][HgBz],\ K[HrSH] = [HgBz(HrSH)]/[HgBz],$$

then for the assumption $K[HrSH] >> 1$ to be true, the concentration of bound mercurial must greatly exceed the free mercurial. Even with strong binding, if the concentration of total mercurial greatly exceeds the concentration of binding sites, the bound mercurial cannot exceed the free mercurial. The model calls for one binding site so that for PMB to monomer ratios of two or greater, regardless of the PMB-protein affinity, the assumption cannot hold.

The second assumption, that the free unbound and unreacted hemerythrin concentration ([HrSH]) is proportional to the total protein concentration, which assumption was made to obtain k_{app} in the form of equation 43, is based on the previous assumption of strong mercurial binding to the protein. Even if there were strong mercurial binding, the sulfhydryl groups are entirely blocked during the course of the reaction so that the concentration term [HrSH] could not remain unchanged over the entire period of the reaction. The assumption might become applicable during a steady state phase of the reaction but this condition is not specified in the derivation of equation 42. In consideration of the method of its derivation and the fact that it does not correlate with the data, equation 42 must be rejected. This model is inadequate for the observed kinetic behavior of the reaction of PMB and hemerythrin. In Chapter V, I will present a model applicable to both the fluoride and chloride forms of methemerythrin.

IV. THE DISSOCIATION OF HEMERYTHRIN

Previous investigators (6-9) have shown that hemerythrin, when treated with PMB, dissociates into eight identical subunits. This dissociation appears to proceed by an all-or-none type reaction. To explain the all-or-none phenomenon and to relate the dissociation of hemerythrin with the reactivity of the sulfhydryl groups, Keresztes-Nagy and Klotz (8) proposed the equilibrium model. This model requires that the monomer be the only reactive species. Egan (18) measured the rate of mercury-sulfur bond formation and deduced that the octamer is the reactive species. I have found that the reaction is actually first order in protein concentration and first order overall. Klapper and Klotz (16) who also claim a first order reaction for chloromethemerythrin interpret this as substantiating the equilibrium model. This would be the case, however, only if the equilibirum between octamer and monomer is slowly attained relative to the rate of formation of the mercury-sulfur bond (18). Although Klapper and coworkers (11, 16, 17) claim that the equilibrium is slowly attained, Rao (13-15) has demonstrated with gel chromatography that it is rapidly attained. With rapid equilibration, the rate of reaction is expected to be first order in terms of mercurial concentration (18). Furthermore, I did not find that dilute solutions of hemerythrin were more reactive than a solution of hemerythrin octamer which would be expected if the equilibrium model were valid, regardless of whether the equilibration is slow or rapid. A direct comparison of the rate of

mercaptide bond formation is required to determine unequivocally which species indeed reacts. Therefore, the rate of dissociation was measured by a light scattering technique.

A. The Rate of Dissociation for Hemerythrin

1. Molecular weight of the hemerythrin octamer by light scattering

For each kinetic experiment, before the addition of PMB, the intensity of scattered light for each hemerythrin sample was measured in order to obtain a value for the weight average molecular weight which can be compared with the established molecular weight for hemerythrin. This value provides a check on the calibration of the instrument and permits one to judge the reliability of that series of kinetic experiments. Using the light scattering equation for the Sofica instrument,

$$K\left(\frac{I_b}{R_b}\right)\left(\frac{c}{I_{90}}\right) = \frac{1}{\overline{M}_w} + 2Bc \qquad (25)$$

values of $K(I_b/R_b)(c/I_{90})$ were calculated for a series of experiments, are shown in Table XI, and are plotted as a function of concentration (as mg cm^{-3}) in Figure 20. If a least squares regression line is drawn through the points of Figure 20, the intercept will give a value of 112,400 gm $mole^{-1}$ for the weight average molecular weight which agrees very well for the light scattering method with the molecular weight of hemerythrin. But the line would have a negative slope which is characteristic either of net attractive forces between protein solute molecules in a

TABLE XI: DATA FOR THE MEASUREMENT OF THE MOLECULAR WEIGHT OF HEMERYTHRIN OCTAMER BY LIGHT SCATTERING

c [1] mg ml^{-1}	z *	I_{90} #	(c/I_{90}) gm cm^{-3} X 10^5	$K(I_B/R_B)(c/I_{90})$ moles gm^{-1} X 10^6
0.143	1.12	13.8	1.10	8.96‡
0.287	1.01	26.8	1.08	8.80‡
0.358	1.09	34.8	1.03	8.39
0.412	1.09	40.0	1.03	8.39
0.544	1.05	53.2	1.02	8.31
0.559	1.12	53.7	1.04	8.47
0.665	1.03	67.9	0.99	8.07
0.737	0.97	73.4	1.01	8.23
0.922	1.10	90.5	1.02	8.31
				8.31 ± 0.09

$\bar{M}_w = 120 \pm 1 \times 10^3$ gm mole^{-1}

The buffer contains 0.01 Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

* Dissymmetry = I_{45}/I_{135}

\# I_{90} is measured in arbitrary units

$K = 2.34 \times 10^{-7}$ mole cm^2 gm^{-2}

$R = 16.3 \times 10^{-6}$ cm^{-1}

‡ First two values not included in the average for the calculation of molecular weight.

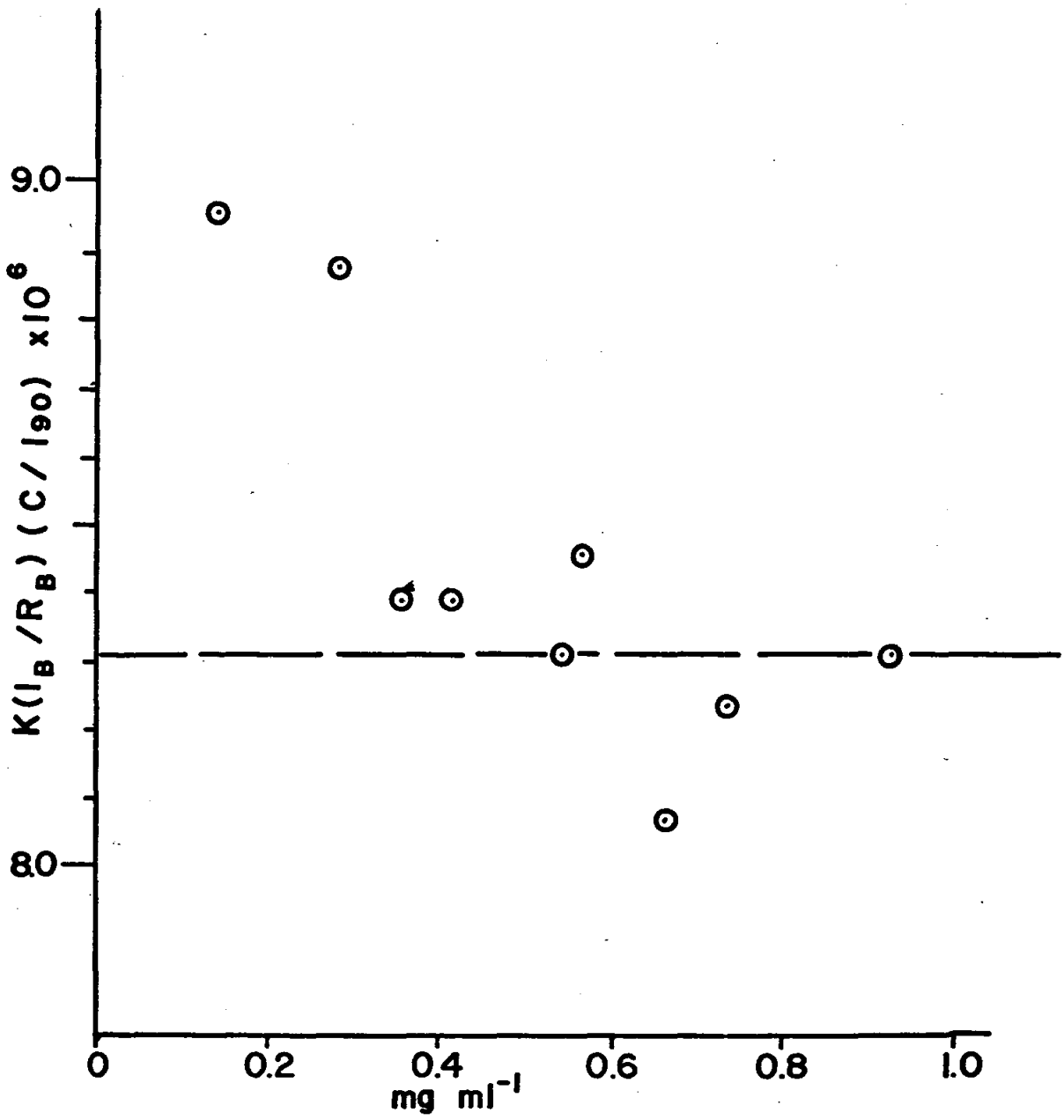

FIGURE 20: Molecular Weight Determination of Hemerythrin by Light Scattering. The Buffer Contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

solution of low ionic strength or of a protein dissociating into subunits (99). The high ionic strength entailed by the presence of 1.0 M NaCl limits the possibility to dissociation. Frontal gel chromatographic analysis of a 0.143 mg cm^{-3} solution of hemerythrin in 1.0 M NaCl on a Sephadex G-75 column indicated that at this concentration, 56% of the protein had dissociated. Light scattering measurements indicate that the protein is less dissociated, than do gel chromatogrphy experiments. Eliminating the two lowest concentration points gives a weight average molecular weight of 120,000 gm $mole^{-1}$, which is acceptable considering the dissymmetry. The slope and thereby the second virial coefficient are probably zero.

I assumed that the light scattering measurements were valid for kinetic analysis when the calculated weight average molecular weight was reasonably close to 107,000 gm $mole^{-1}$ and I accepted only those experiments. When the weight average molecular weight obtained from the experiment differed by a large amount, such as by a factor of two, from the accepted molecular weight of hemerythrin, the kinetic values obtained from these experiments differed by a significant factor from the accepted kinetic values. The unacceptable experiments were also discarded because they had a high dissymmetry which indicated that the abnormal values were due to either aging or denaturation of the protein, or to contaminating dust.

2. An attempt to measure the molecular weight of the monomer and the end point of the dissociation

a. The dissociation of hemerythrin upon addition of PMB. After the measurement of the molecular weight of the undissociated protein was taken, PMB was added to the hemerythrin sample. In Figure 21, is shown a typical measurement as a function of time after addition of the mercurial reagent of the intensity of light scattered at 90° by a solution of hemerythrin and PMB, while the data for this experiment are in Table XII. The values of I_{90} observed after a period of about 30 min which appear to be the end point of the reaction did not correspond in most cases to the expected molecular weight of the hemerythrin monomer. The observed intensities of scattered light for the presumed end points of the reaction corresponded to molecular weights ranging from about 13,000 to 70,000 gm mole $^{-1}$. Aliquots of a reacting hemerythrin-PMB mixture were taken shortly after and 30 min after the addition of PMB and placed on a Sephadex G-75 column. The aliquot taken shortly after the addition of PMB was found to contain mostly octamer and little monomer, while no octamer and only monomer was found in the 30 min aliquot.

b. The molecular weight of the monomer by light scattering. An attempt was then made to measure the molecular weight of the monomer by light scattering. The monomeric hemerythrin was prepared by adding salyrganic acid to the protein in solution and dialysing again against the

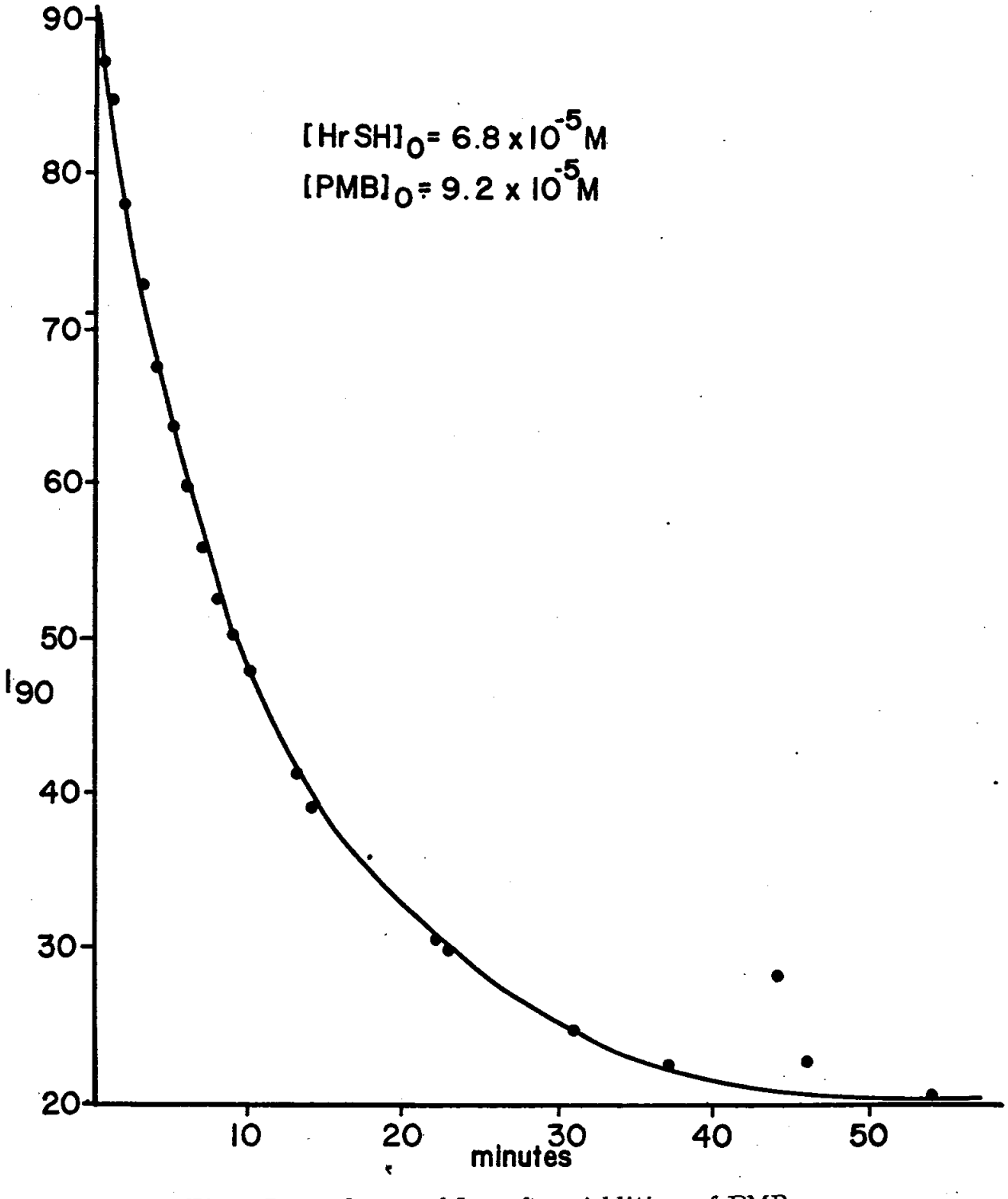

FIGURE 21: Time Dependence of I_{90} after Addition of PMB. Hemerythrin Concentration is 0.92 mg ml^{-1} in 0.01 M Tris-cacodylate Buffer Containing 1.0 M NaCl, pH 7.0 at 13° C.

TABLE XII: INTENSITY OF LIGHT SCATTERED AT 90° FOR A SOLUTION OF HEMERYTHRIN AFTER ADDITION OF PMB

The buffer contains 0.01 Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C. The initial concentrations are 6.8 X 10^{-5} M monomer units of hemerythrin and 9.2 X 10^{-5} M PMB.

time min							
0	96.8	90.8	70.4	13	47.2	41.2	20.8
0.5	93.2	87.2	66.8	14	45.1	39.1	18.7
1	90.8	84.8	64.4	22	36.8	30.8	10.4
2	84.0	78.0	57.6	23	36.0	30.0	9.6
3	79.0	73.0	52.6	30	31.0	25.0	4.6
4	73.6	67.6	47.2	31	30.8	24.8	4.4
5	69.8	63.8	43.4	37	28.4	22.4	2.0
6	66.0	60.0	39.6	44	34.3	28.3	——
7	62.0	56.0	35.6	46	28.9	22.9	——
8	58.8	52.8	32.4	54	26.4	20.4	end point
9	56.4	50.4	30.0	61	26.4	20.4	
10	54.0	48.0	27.6	buffer	6.0		

The reading at t = 0, is before addition of PMB.

buffer which was 1.0 M NaCl, 0.01 M Tris-cacodylate at pH 7.0. An aliquot of this solution was passed through a Sephadex G-75 column and thereby shown to contain only monomers. The result of the determination of molecular weight by light scattering for monomer is shown in Table XIII and Figure 22. The c = 0 intercept gives an abnormal value of 57,000 gm $mole^{-1}$ as the monomer molecular weight which indicates either that the method is invalid for a monomer solution of that the instrument is miscalibrated.

c. The molecular weights of small proteins by light scattering. In order to check instrument calibration, molecular weights were then determined by light scattering for the proteins: bovine serum albumin, ovalbumin, myoglobin, and ribonuclease. The increment of refractive index (dn/dc) used for these proteins was one that had been determined for bovine serum albumin (100). Although the increments of refractive index for proteins lie fairly close together, there is sufficient individuality so that a comon value should not be used for accurate measurements (101). Nevertheless, in this instance, only the relative measurements are of interest. The dissymmetry values of these experiments were in the same range as the dissymmetry for the molecular weight measurement of the monomer. The tabulation of molecular weights given in Table XIV indicates that the instrument is properly calibrated, however, the accuracy of the light scattering method decreases for lower molecular weights.

TABLE XIII: DATA FOR THE MEASUREMENT OF THE MOLECULAR WEIGHT OF HEMERYTHRIN MONOMER BY LIGHT SCATTERING

c mg ml^{-1}	z *	I_{90} #	(c/I_{90}) gm cm^{-3} X 10^{5}	$K(I_B/R_B)(c/I_{90})$ moles gm^{-1} X 10^{6}
0.28	1.26	12.0	2.33	18.7
0.29	1.36	14.5	2.09	16.8
0.49	1.26	21.8	2.35	18.8
0.53	1.38	28.6	1.85	14.8
0.57	1.33	26.1	2.28	18.3
0.75	1.34	36.0	2.08	16.7
0.95	1.43	44.9	2.11	16.9
1.12	1.55	50.0	2.24	18.0
1.28	1.96	58.4	2.19	17.4
				17.4 ± 1.0

* Dissymmetry = I_{45}/I_{135}
\# I_{90} is measured in arbitrary units
$K = 2.34 \times 10^{-6}$ mole cm^2 gm^{-2}
$R = 16.3 \times 10^{-6}$ cm^{-1}

The buffer contains 0.01 M Tris-cacodylate, 1.0 M NaCl pH 7.0 at 13° C.

$$\bar{M}_w = 57 \pm 3 \times 10^{3} \text{ gm mole}^{-1}$$

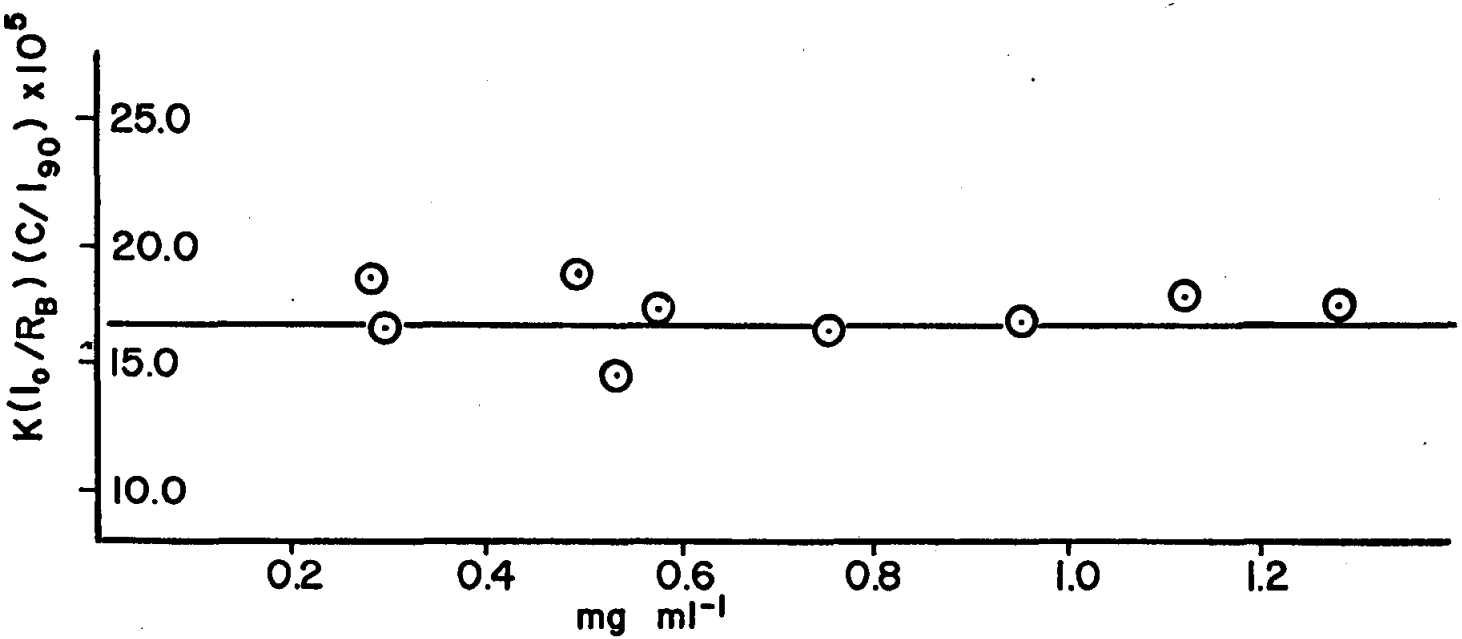

FIGURE 22: Molecular Weight Determination of Hemerythrin Monomer by Light Scattering. The Buffer Contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0, at 13° C.

TABLE XIV: MOLECULAR WEIGHTS OF SMALL PROTEINS DETERMINED BY LIGHT SCATTERING

Protein	Wt. Ave. Mol. Wt. *	z #	Established Mol. Wt. †	Per Cent Error
Bovine serum albumin	63,000	1.5	67,000	7%
Ovalbumin	50,000	1.5	45,000	11%
Myoglobin	23,000	1.7	17,800	30%
Ribonuclease	17,200	1.6	13,600	27%

* Weight Average Moledular weight by light scattering.
# Dissymmetry is average value for the determination.
† Molecular weights taken from Determann (86).

d. Possible reasons for the high molecular weights at the end point of the dissociation. Possibly, the hemerythrin monomer unfolds and poses-ses sufficiently different physical parameters, in particular the radius of gyration, so that assumptions made in calculation of the octamer molecular weight are no longer valid. Or possibly, because light scattering measurements are so sensitive to aggregation, the technique is detecting transient oligomers the lifetimes of which are too short for methods such as gel chromatography to detect. Particularly, this may explain the weight average molecular weight of 57,000 gm $mole^{-1}$ observed rather than the 13,500 gm $mole^{-1}$ value expected. Also possible, during the course of the dissociation, there is also a concomitant denaturation and aggregation of the protein. This is supported by the fact that generally the dissymmetry increases during the reaction and also the observation by myself and other investigators in this laboratory that after a period of time, the protein begins to precipitate. Consequently, the end point of the protein dissociation would not be reached because the denaturation is observed instead. Very small changes of protein denaturation and aggregation can have a noticeable effect on light scattering measurements. Evidence for denaturation and aggregation has also been observed in previous spectrophotometric experiments (20). Similar behavior was noted by Madsen and Cori (49, 50) for the reaction of PMB with phosphorylase. Nevertheless, for the initial phase of the dissociation, the denaturation

should be negligible. Light scattering measurements for this phase represent the true weight average molecular weight of the dissociating octamer.

The most likely explanation is that a certain amount of denature protein or dust was present in the reaction mixture before addition of PMB. This component would remain unchanged by the reaction. As the reaction proceeds and the octamer concentration decreases, the denatured component will still contribute the same intensity of scattered light. But since the weight average molecular weight for the entire solution is less, this contribution of the contaminating component will be proportionately greater. Therefore, the minimum value of I_{90} was usually taken as the end point of the reaction.

3. Kinetic experiments for the dissociation of hemerythrin

Since the scattered light intensity is directly proportional to weight average molecular weight and the weight average molecular weight is a measure of the extent of the dissociation, a direct evaluation of the rate of change of I_{90} was used to obtain the rate of dissociation. The weight average molecular weight is related to the concentration of the solutes by the equation

$$\overline{M}_w = \frac{\Sigma c_i M_i}{\Sigma c_i} = \frac{\Sigma m_i M_i^2}{\Sigma m_i M_i} \qquad (14)$$

where c_i, m_i represent the concentrations, in either gm cm^{-3} or moles

liter^{-1} , and the molecular weight of the ith species. For hemerythrin, this becomes

$$\bar{M}_w = \frac{[(Hr)_8]M_o^2 + [Hr]M_m^2}{[(Hr)_8]M_o + [Hr]M_m} \qquad (44)$$

where the subscripts o and m identify molecular weights of the octamer and monomer species, respectively. If X is the concentration of undissociated monomer units and a is the total concentration in monomer units so that $[Hr] = (a - X)$ and $[(Hr)_8] = (X/8)$, and since $M_o = 8M_m$, then

$$\bar{M}_w = \frac{(7X + a)M_m}{a} \qquad (45)$$

Presumably, at the end point, $X = 0$ and I_{90} is proportional to M_m (plus the contaminants). Therefore, the difference in the intensity of scattered light (I'_t), given by the equation

$$I'_t = I_{90,t} - I_{90,ep} \qquad (46)$$

where $I_{90,ep}$ is the intensity of light scattered at the end point of the dissociation, should be directly proportional to the value of X and directly proportional to the concentration ration $[(Hr)_8]_t/[(Hr)_8]_0$. These values were then graphed on a semilogrithmic plot as a function of time. The experiment of Figure 21 is so treated in Figure 23. The straight line shown in Figure 23 is indicative of a first order or pseudofirst order reaction. For these experiments, the concentration of PMB exceeded that of hemerythrin in monomer units, but in many cases not in sufficient excess to give pseudofirst order results. Intuitively, one would expect a dissociation process to be unimolecular and thereby a first order reaction.

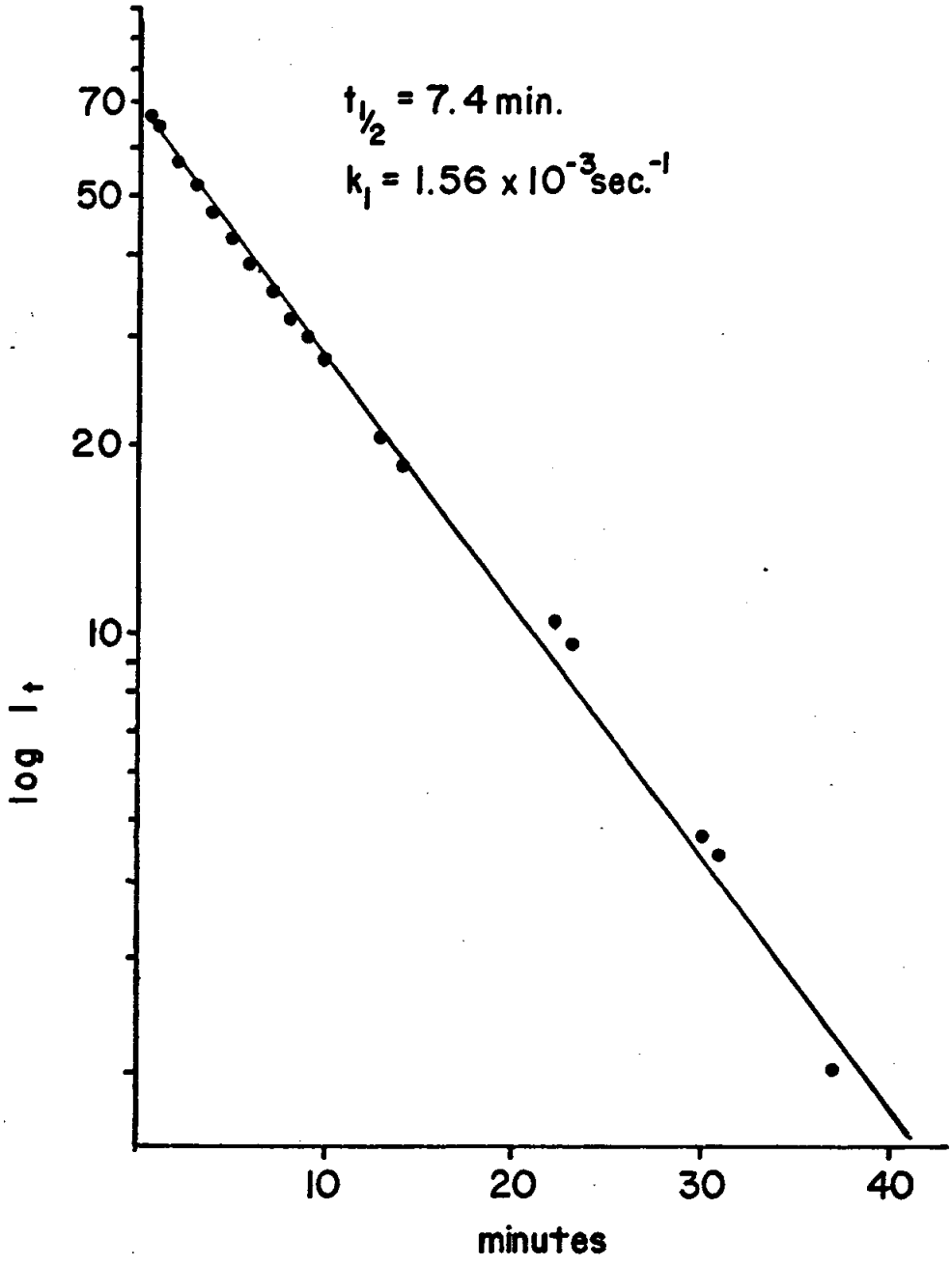

FIGURE 23: Log(I_t') as a Function of Time after Addition of PMB. Data from Table XII. Initial concentration of monomer unit of hemerythrin is 6.8×10^{-5} M, initial concentration of PMB is 9.2×10^{-5} M.

On the basis of the integrated first order rate equation

$$\ln ([(Hr)_8]_0 / [(Hr)_8]_t) = k_1 t \qquad (47)$$

the half time ($t_{\frac{1}{2}}$) for the reaction was taken as the point on the log(I'_t) plot where I'_t was equal to one half its extrapolated value at t = 0, and from this half time, a first order rate constant was calculated. If the value of the end point is to high because of the presence of dust or denatured protein, then the calculated value for the rate constant will also be too high. The rate constant obtained by this method, therefore, may not be correct but only an upper limit to the true rate constant.

Table XV is a tabulation of the various kinetic experiments, giving hemerythrin concentration in monomer units, PMB concentration, half time, and the calculated rate constant. For all these experiments, the solvent was 0.01 M Tris-cacodylate buffer (pH 7.0) with a ligand concentration of 1.0 M NaCl, while the temperature was maintained at 13°C. From these data, it appears that the hemerythrin dissociation follows a true first order rate law and the concentration of PMB does not influence the first order rate constant.

B. Kinetic Parameters from Initial Rates of Dissociation

To check the premise that the initial light scattering measurements were reliable indicators of the rate of dissociation, the rate constant was determined by a differential initial velocity method as described by

TABLE XV: HALF TIMES AND FIRST ORDER RATE CONSTANTS FROM LOG (I_t') GRAPHS FOR THE DISSOCIATION OF HEMERYTHRIN

The buffer contains 0.01 Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

Initial concentrations [PMB] M X 10^5	[HrSH] * M X 10^5	ratio	half time $t_{1/2}$ sec	k_1 sec^{-1} X 10^3
4.2	0.4	10.5	420	1.65
4.2	1.2	3.5	396	1.75
4.2	1.8	2.3	450	1.54
4.2	2.5	1.7	468	1.48
4.2	3.0	1.4	420	1.65
9.2	1.0	9.2	468	1.48
9.2	2.6	3.5	414	1.67
9.2	3.8	2.4	408	1.70
9.2	6.8	1.4	444	1.56
11.8	3.7	3.2	360	1.92
12.6	2.6	4.8	324	2.14
29.4	3.0	9.8	312	2.22
29.4	4.2	7.0	372	1.86
29.4	5.6	5.2	420	1.65
55.0	4.3	12.8	354	1.96
78.0	4.1	19.0	360	1.92
	Average		399 ± 40	1.76 ± 0.18

* in monomer units

Benson (88). Using equation 25, the values of I_{90} were converted into molecular weights. Only the first few minutes were considered; a straight line could usually be drawn through the points for about the first three minutes and the slopes of these lines are collected in Table XVI. These slopes represent the differential obtained rom equation 45.

$$\frac{d\bar{M}_w}{dt} = \frac{7M_m}{a}\frac{dX}{dt} \tag{48}$$

For the hemerythrin dissociation, a rate equation can be written in the form

$$-\frac{d[(Hr)_8]}{dt} = k_n[(Hr)_8]^n \tag{49}$$

where n is the order of the reaction in terms of octamer concentration and k_n is a rate constant which may under pseudofirst order conditions contain the concentration of PMB. Since $[(Hr)_8] = (X/8)$, by differentiating, substituting into equation 48, and rearranging, the equation

$$\frac{d[(Hr)_8]}{dt} = \frac{1}{8}\frac{dX}{dt} = \left(\frac{a}{56M_m}\right)\frac{d\bar{M}_w}{dt} \tag{50}$$

can be obtained. Also in Table XVI, the initial slopes are converted according to equation 50 into the corresponding values for the rate of change of octamer hemerythrin concentration $(-d[(Hr)_8]/dt)$. Taking the logarithm of equation 49

$$\log\left(-\frac{d[(Hr)_8]}{dt}\right) = \log(k_n) + n \log[(Hr)_8] \tag{51}$$

it is possible to use the initial rate data to evaluate both order and rate constant for the dissociation. The initial slope date for the rate of octamer concentration change as a function of concentration are plotted logarithmetically in Figure 24 according to equation 51. The slopes of both lines

TABLE XVI: INITIAL VELOCITY DATA FOR THE DISSOCIATION OF HEMERYTHRIN

Initial concentrations

[PMB] M X 10^5	[HrSH] * M X 10^5	$-\frac{dM_w}{dt}$ gm $mole^{-1}$ sec^{-1}	$-\frac{d[(Hr)_8]}{dt}$ M sec^{-1} X 10^9
Series I			
9.2	1.0	138	1.81
	2.5	147	4.84
	3.8	137	6.85
	6.4	123	10.41
Series II			
4.2	0.4	146	0.79
	1.2	137	2.14
	1.8	141	3.36
	3.0	130	5.16

* in monomer units

Note, the initial concentration is listed in terms of monomer units as is also the absissa of Figure 24. The concentration in monomer units is required by equation 50 to convert the rate of change of molecular weight ($d\overline{M}_w/dt$) into the rate of change of octamer concentration ($d[(Hr)_8]/dt$). The absissa of Figure 24 should be in terms of octamer concentration, but since if negligible dissociation has occurred, the octamer concentration is one eighth of monomer unit concentration, use of the monomer unit concentration introduces no error.

The buffer contained 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

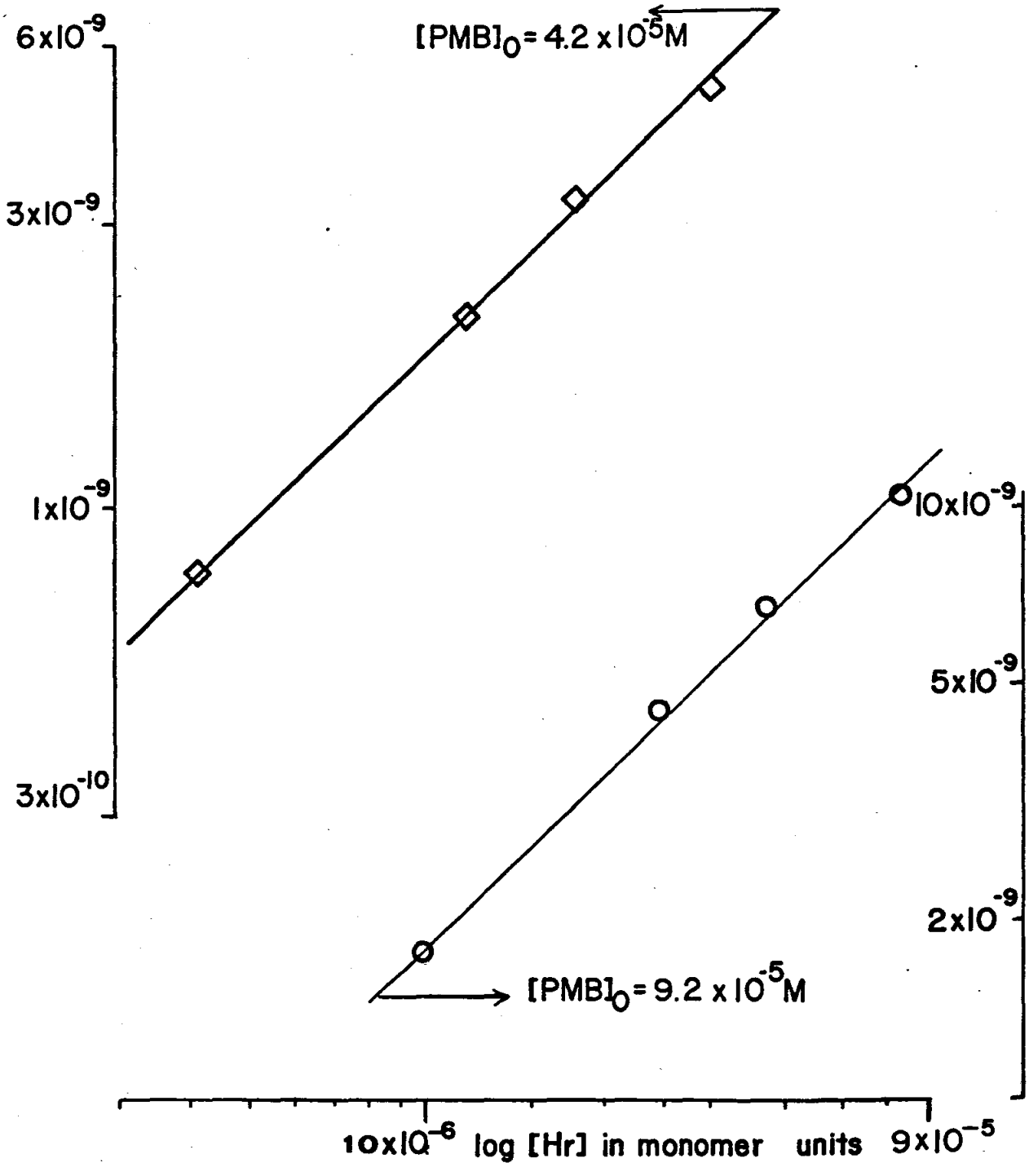

FIGURE 24: Log-Log Plot of Initial Velocity Data Taken from Table XVI. The ordinate is in terms of $d[(Hr)_8]/dt$.

in Figure 24 are 0.98 which is within experimental error of the expected value of unity for a first order reaction. The first order rate constant was then calculated, using equation 51 and taking n as one. Again, for both series, the same first order rate constant was obtained. Since the concentration of PMB differed by a factor of two for these two series of experiments, if the rate constant had been pseudofirst order (i.e. $k_1 = k_2[PMB]$), then the rate constants would have varied by the same factor of two. We can conclude that, at least for the concentration ranges examined, the dissociation of hemerythrin octamer into subunits as a consequence of the addition of PMB is dependent only on hemerythrin concentration and not directly on PMB concentration, at least in the initial period of the reaction. In Table XVII, the rate constant and half times are summarized and compared.

C. The Rate of Formation of the Mercury-Sulfur Bond Compared with the Rate of Hemerythrin Dissociation

A fundamental question concerning hemerythrin-PMB interaction was whether or not PMB reacts with the octameric or monomeric species of the protein. This issue may be resolved, in part, by whether the rate of hemerythrin dissociation is faster or slower than the rate of mercury-sulfur bond formation.

TABLE XVII: SUMMARY OF FIRST ORDER RATE CON TANTS AND HALF TIMES FOR THE DISSOCIATION OF HEMERYTHRIN

Source	$t_{1/2}$ sec	k_1 sec^{-1} X 10^3
Table XV (average of 16 measure-ments)	399 $\pm$ 40	1.76 $\pm$ 0.18
Series I	495	1.40
Series II	495	1.40

1. The rate of mercury-sulfur bond formation measured under the same conditions as for the dissociation.

The light scattering method requires a high degree of stability of the macromolecules undergoing investigation and for the hemerythrin molecule reacting with PMB, this was only achieved in a buffer containing 1.0 M NaCl. Because of the ligand affect on the protein, the mercaptide formation must be measured under the same conditions used to measure the dissociation. Accordingly, a series of spectrophotometric experiments were produced which paralleled the light scattering experiments, having the same buffer, mercurial concentration, and protein concentration. A typical experiment from this series is depicted in Figure 25; Table XVIII contains the kinetic data. By equation 29

$$[(HrSH)] = \frac{A_e - A_t}{E_m} \qquad (29)$$

the value of $(A_e - A_t)$, where A_e is the end point absorbance and A_t is the absorbance at time t, is related directly proportional to the concentration term $[(HrSH)]_t$ in the integrated first order rate equation

$$\ln[(HrSH)]_t = -k\ t + \ln[(HrSH)] \qquad (34)$$

and a plot of $\log(A_e - A_t)$ as a function of time will have a slope of $-2.303k_1$ and a y intercept equal to $\log(A_e - A_0)$; therefore, it will give the necessary kinetic information. As an example of this treatment, Figure 26 presents the data for the experiment from Table XVIII. The half time of the reaction can be readily taken directly from the graph as equal to the time at which $(A_e - A_t)$ is one half of its value extrapolated

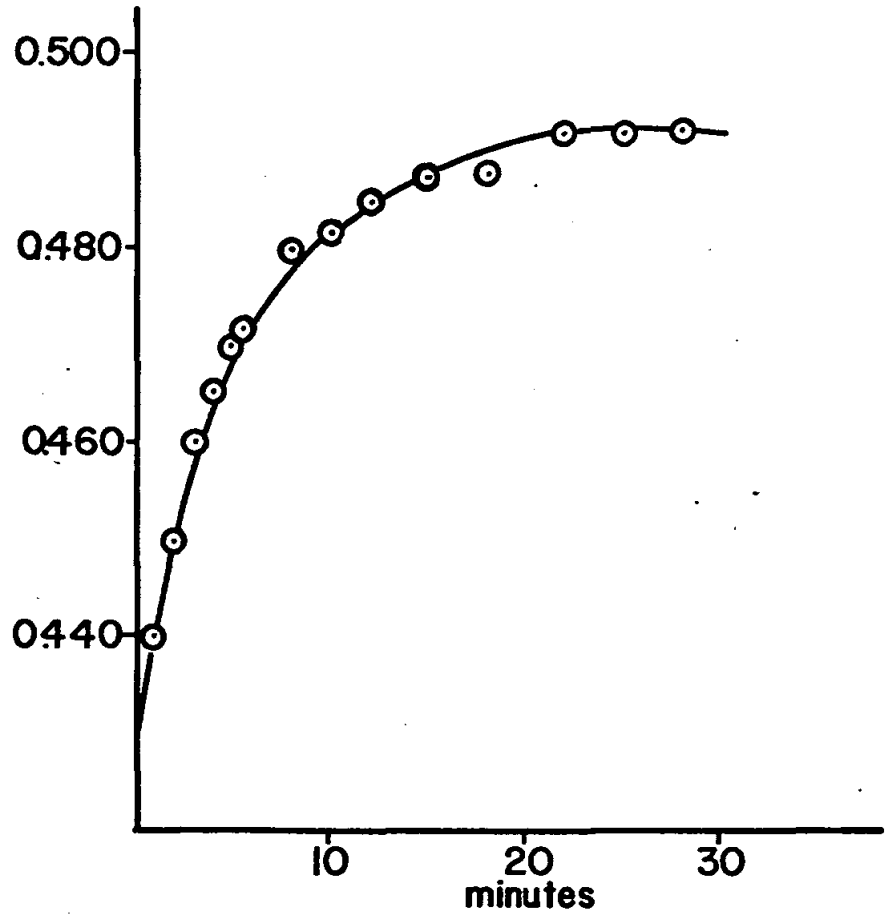

FIGURE 25: Absorbance Change at 250 nm for the Reaction of Hemerythrin with PMB. The Buffer Contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

TABLE XVIII: ABSORBANCE DATA FOR THE REACTION OF PMB WITH HEMERYTHRIN.

The buffer contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C. The initial concentrations of reactants are 1.2 X 10^{-5} M monomer units of hemerythrin and 4.2 X 10^{-5} M PMB.

time min	A_{250}	$A_e - A_t$
1	0.440	0.052
2	0.450	0.042
3	0.460	0.032
4	0.465	0.027
5	0.470	0.022
6	0.472	0.020
8	0.480	0.012
10	0.482	0.010
12	0.485	0.007
15	0.488	0.004
18	0.488	0.004
22	0.492	
25	0.492	The end point
28	0.492	

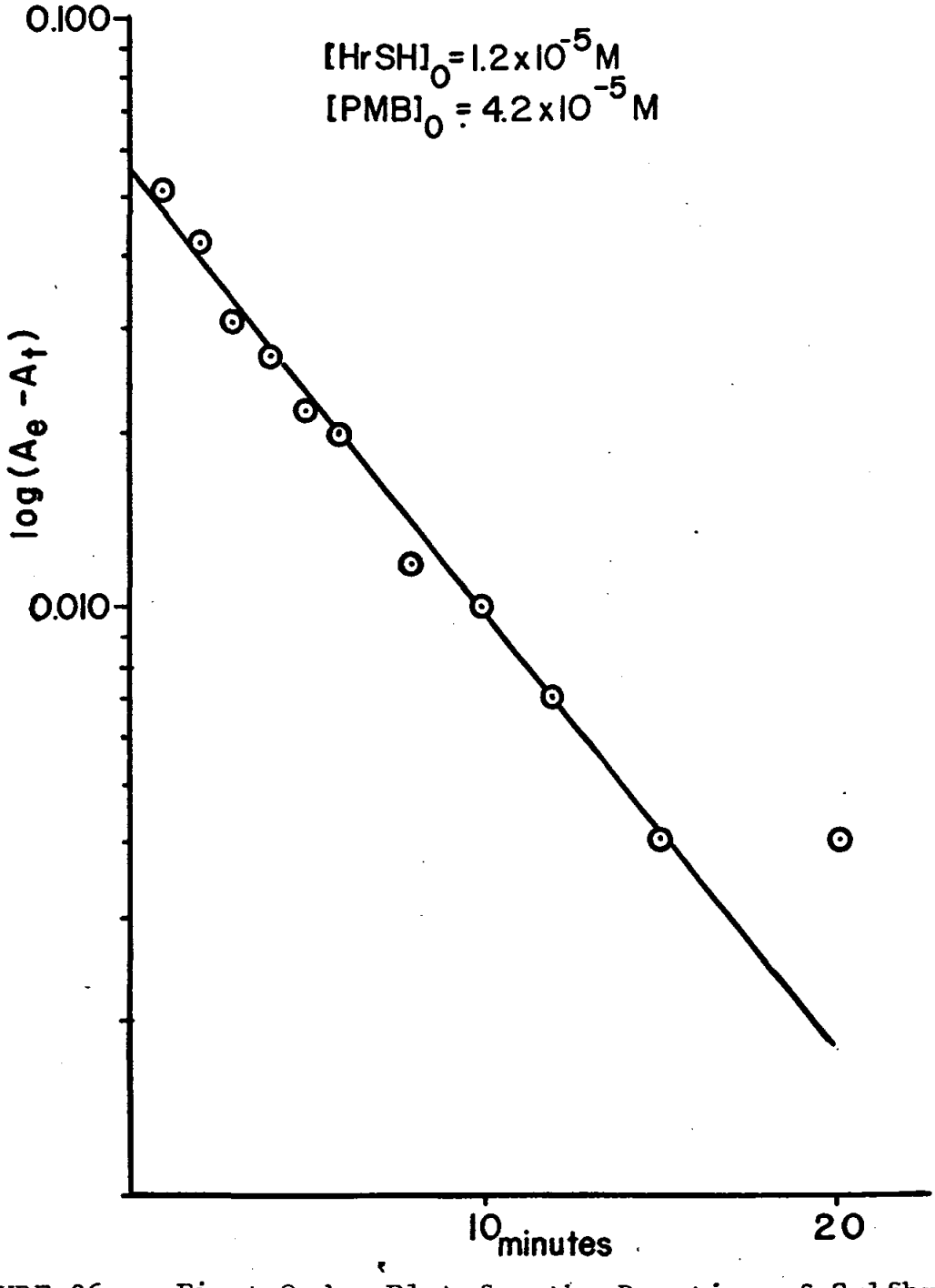

FIGURE 26: First Order Plot for the Reaction of Sulfhydryl Groups of Hemerythrin with PMB for the Experiment of Figure 25. Data Taken from Table XVIII.

to t = 0. As in the light scattering experiements, a first order rate constant was calculated from the half time. This graphical method corresponded to the graphical method used to treat the light scattering data; therefore, it is more appropriate than other methods of treating the data for direct comparison of the two processes. In Table XIX, are presented the kinetic value for the mercury-sulfur bond formation under the same conditions as the light scattering experiments. Some of the experiments in Table XIX duplicate in protein and PMB concentrations light scattering experiments found in Table XV. Others represent the duplicate of light scattering experiments which were discarded. The factors which render light scattering measurements invalid, such as dust and other contaminants, do not have as great an effect on the spectrophotometric measurements and consequently, satisfactory measurements could be obtained on the same solution which was useless for light scattering experiments.

Average values of $t_{\frac{1}{2}} = 206 \pm 11$ sec and $k_1 = 3.36 \pm 0.20 \times 10^{-3}$ sec^{-1} are obtained from the entire set of experiments in Table XIX. These values can be compared with the corresponding light scattering values for the dissociation of the protein, $t_{\frac{1}{2}} = 399 \pm 40$ sec and $k_1 = 1.76 \pm 0.18 \times 10^{-3} sec^{-1}$. Figure 27 compares the relative rates of protein dissociation and the mercury-sulfur bond formation as the percentage of total weight average molecular weight change and the percentage of unreacted sulfhydryl groups. In Figure 27, extrapolated to zero time, only 70% of the sulfhydryl groups remain unreacted, which reflects the fast reaction of

TABLE XIX: HALF TIMES AND FIRST ORDER RATE CONSTANTS FROM LOG $(A_e - A_t)$ PLOTS FOR THE FORMATION OF THE MERCURY-SULFUR BOND UNDER THE SAME CONDITIONS AS THE DISSOCIATION OF HEMERYTHRIN WAS OBSERVED

The buffer contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

Initial concentrations [PMB] M X 10^5	[HrSH] * M X 10^5	ratio	$t_{1/2}$ sec	k_1 sec^{-1} X 10^3
4.2	1.2	3.5	195	3.56
4.2	1.8	2.3	195	3.56
4.2	2.5	1.7	225	3.08
4.2	3.0	1.4	216	3.11
6.8	2.4	2.8	204	2.40
6.8	2.2	3.1	210	3.30
7.8	3.8	2.1	210	3.30
13.2	3.8	3.5	195	3.56
16.3	3.8	4.3	228	3.04
6.8	3.0	2.3	186	3.72
	Average		206 ± 11	3.36 ± 0.20

* in monomer units.

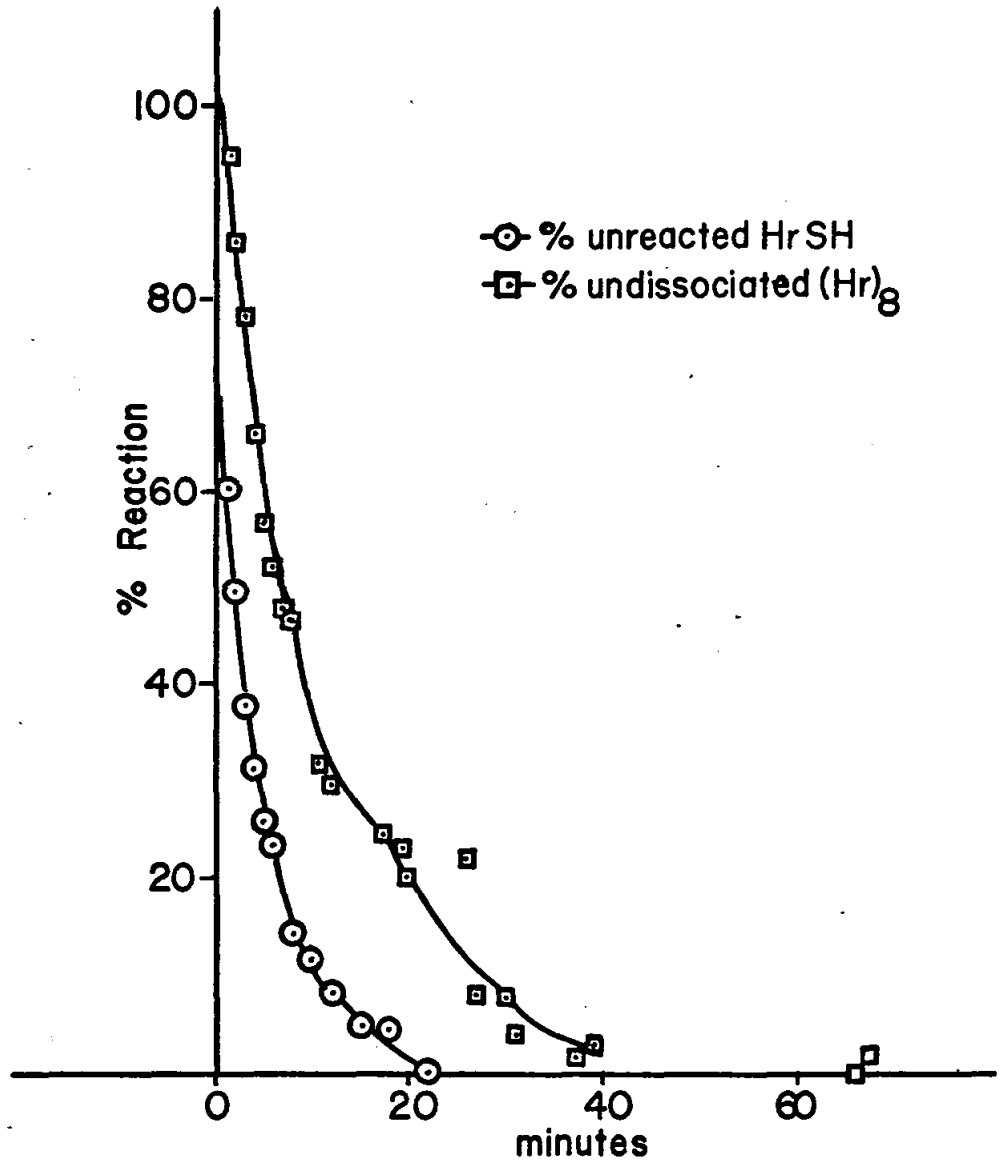

FIGURE 27: Direct Comparison of the Extent of Mercury-Sulfur Bond Formation with the Dissociation of Hemerythrin. The initial concentrations are for hemerythrin, 1.2×10^{-5} M in monomer units, and for PMB, 4.2×10^{-5} M.

approximately 30% of the protein. From either a comparison of the two rate constants or from Figure 27, these experiments demonstrate that PMB mercaptide bonds with the octamer species. The reaction of PMB with the sulfhydryl groups of the octamer has also been demonstrated by gel chromatography in this laboratory(14).

2. The equilibration model

The ratio between the two rate constants is only 1.9 however, which may be within the range of experimental error. The two rate constants by themselves are insufficient for drawing a conclusion. However, consider them with the other data, that the monomer posesses the same reactivity as the octamer, that the equilibration between octamer and monomer is rapid (13-15), that gel chromatography has duplicated the observation that sulfhydryl groups react with PMB faster than dissociation occurs (14), and that there does not appear to be any fast reaction phase for dissociation. From this then, apparently, the difference between the two rates is real and the two processes, formation of the mercury-sulfur bond and irrevsible dissociation, constitute separate actions at the molecular level. The octamer species is reactive which completely excludes the equilibrium model as previously proposed (8) as a mode for the PMB-hemerythrin reaction.

The equilibrium model may, however, be modified to still explain the all-or-none effect of the reaction. As mentioned in Section III B 3, the

mercaptide bond formation is probably a random process. Direct wedge and allosteric interactions between subunits can be excluded since the monomer species would react much faster than the octameric species if some sort of regulatory subunit-subumt interaction took place. Rao (13-15) has shown that the hemerythrin octamer-monomer equilibirum is rapidly attained. Thus, octamers are constantly and rapidly dissociating and reassociating. When PMB interacts with the sulfhydryl group of hemerythrin, a concomitant change in the subunit prevents it from reassociating after the octamer to which it belongs dissociates. In this manner, with PMB added in less than stochiometric amounts, by reequilibration of reacted and unreacted species within the time required to preform an ultracentrifuge experiment, all the PMB is bound to monomers and none is bound to octamers. The change which prevents the reassociation of the subunits is probably a small change of conformation that was induced by the binding of PMB to the protein.

A lag in the extent of dissociation behind that of the formation of mercury-sulfur bonds has been observed for other proteins. Madsen and Cori (49. 50) found that for phosphorylase, dissociation followed a first order rate law and was slower than enzymic inhibition. The enzymic inhibition was somewhat slower than mercaptide formation and both processes followed second order rate equations. Madsen and Gurd (51) originally claimed an all-or-none effect in phosphorylase but in later investigations,

Battell et al (52, 53) reexamined this enzyme, found differing reactivities for the various sulfhydryl groups, and rejected the claim of all-or-none behavior. Phosphorylase was the classic all-or-none protein but other protein but other proteins, among them fumarase (102) and the protein component of turnip yellow mosaic virus (103), react with sulfhydryl blocking reagents by all-or-none mechanisms which were expressed either by enzymic inhibition or protein dissociation. The equilibration model here presented cannot be applied to protein with more than one reacting sulfhydryl group per subunit. The evidence accumulated for other proteins, however, suggests major conformational changes occurring in these proteins. For example, Robinson et al (102) concluded from their experiments that the sulfhydryl groups of fumarase were buried within the protein molecule and situated in a hydrophobic environment. Only an extensive change in the protein conformation will expose these groups to the mercurial reagents. Similarly, Kaper and Houwing (103) reported that a certain amount of PMB binds to the protein component of turnip yellow mosaic virus before it starts to break up into subunits. Kaper and Jenifer (104) later found that the reaction of PMB with this protein induces a conformational change which exposes a large number of prototropic groups. The all-or-none effect in hemerythrin should be considered in a class different from the effect observed in other proteins because in hemerythrin, it is probably a secondary effect due to reequilibration of the qaternary

structure of the reacting protein rather than directly due to an interaction involving a change in the conformational states of the protein.

D. Dissociation of Hemerythrin When the Concentration of Protein in Monomer Units Exceeds That of PMB

The dissociation of the protein was also observed by a series of light scattering experiements in which the concentration of hemerythrin in monomer units exceeded the concentration of PMB. The data were more difficult to analyze because of uncertainties in the end points. Values of the more reliable experiments are expressed in Table XX. Until the ratio between mercurial and sulfhydryl group is decreased below 0.40, the rate constant remains within the range of experimental error for the rate constant remains within the range of experimental error for the rate constant obtained with an excess of mercurial. The increase in rate constants observed at the lower PMB-hemerythrin ratios may be an artifact from selecting incorrect end points or from complications involving the fast reaction phase for formation of the mercury-sulfur bond. The kinetic analysis which was used to calculate the rate constants in Table XX depended on the concentration of that portion of the protein which was titrated with PMB. A kinetic analysis based on the total protein concentration will yield a rate constant nearly an order of magnitude less than the constant obtained with the mercurial in excess.

TABLE XX: HALF TIMES AND RATE CONSTANTS FOR THE DISSOCIATION OF HEMERYTHRIN WHEN THE CONCENTRATION OF PROTEIN IN MONOMER UNITS EXCEEDS THAT OF PMB

The buffer contains 0.01 M Tris-cacodylate, 1.0 M NaCl, pH 7.0 at 13° C.

Initial concentrations [PMB] M X 10^5	[HrSH] * M X 10^5	ratio	$t_{1/2}$ sec	k_1 sec^{-1} X 10^3
5.1	6.0	0.8	420	1.65
3.6	6.0	0.6	480	1.44
2.5	6.0	0.4	240#	2.89#
1.5	6.0	0.25	102#	6.79#
2.9	5.0	0.6	408	1.70
2.1	5.0	0.4	384#	1.80#
1.5	2.9	0.5	444	1.56
1.1	2.9	0.3	240#	2.89#
	Average of four		438 ± 24	1.59 ± 0.09

Excluded from the average

* in monomer units

E. Some Aspects of the Kinetics of Hemerythrin Dissociation

A rigorous kinetic analysis of the dissociation of hemerythrin is difficult if at all possible. The irreversible dissociation of the protein must be considered in context with the octamer - monomer equilibrium and the formation of the mercury-sulfur bond. Likely, the rate determination of modified protein is similar to the rate determining step for the modification of the protein by PMB which would account for the similarity in the rate constants. Complicating this is the superimposed association-dissociation equilibirum. According to the gel chromatography experiments of Rao (13-15), these reversible dissociation and dissociation processes are rapid compared with the rates of sulfhydryl group reaction with PMB and irreversible dissociation of the octamer. Tan (105) is extending that investigation to evaluate the effects of temperature and ionic strength on the equilibrium. He does not yet exclude the possibility that the rate of equilibration may be slow relative to the rate of chemical modification at the temperature at which I made my observations (13°C) since most of Rao's observations were made at room temperature.

After the fast phase ends, on the average, each octamer in solution contains two subunits which have already reacted. If the equilibration is relatively slow, the octamer would remain intact until some critical number of its subunits had reacted and then the octamer would dissociate. Monomers formed by the dissociation but which had not yet reacted with

PMB could re-form into octamers but the modified subunits could not. However, if the equilibration is relatively rapid then no critical number of subunits would be involved, the octamer would dissociate spontaneously, and subunits that had already reacted with PMB would still be able to reassociate. An additional step would be required after the formation of the mercury-sulfur bond to further modify the subunit in order to prevent it from reassociating. Otherwise, the observed rate of dissociation would be the same as that for the rate of formation of the mercury-sulfur bond.

The present data is not yet sufficient to determine whether any of these interpretations is correct. We can not give an adequate kinetic analysis of the dissociation of hemerythrin without further experimentation.

V. A MODEL FOR THE REACTION OF PMB WITH HEMERYTHRIN

That PMB reacts with the sulfhydryl group of hemerythrin by a first order process indicates that the rate determining step involves a unimolecular change in the protein iteself. The rate is a function of the total unreacted protein concentration rather than that of a complex; yet, at least for fluoromethemerythrin, the concentrations of PMB and protein affect the rate constant in a manner that implies that the rate determining step somehow involves PMB-hemerythrin complex. The formation of the covalent bond between mercury and sulfur, which is the actual process that the spectrophotometer detects, appears to occur as a random process and proceeds faster than the protein dissociation. A model may be proposed which will explain the data. If the data fit, then experiments should be devised to prove the model.

A. Assumptions for the Model

The precise behavior of sulfhydryl groups in proteins when they react with PMB remains largely unknown. Consequently, assumptions must be made about the interactions of sulfhydryl groups and PMB in order to formulate a model for the reaction.

1. Conformational Changes Involving Sulfhydryl Groups

Apparently, it a sulfhydryl group is moderately reactive and situated near the surface of the molecule, the hydrophobic interactions in which it is involved control to a major extent the reactivity of the group (43). Cecil and Thomas (106) examined the less reactive sulfhydryl groups of human hemoglobin; they allowed carboxyhemoglobin to react with mercurry(II) chloride in the presence of excess sodium sulfite. Under the contiditions of the experiment, the mercury was present only either free in solution as the complex anion disulfitomercurate(II), $Hg(SO_3)_2^{2-}$, or combined with the protein by mercury-sulfur bonds. Cecil and Thomas monitored the formation of the mercaptide bond by amperometric titration of free $Hg(SO_3)_2^{2-}$. Two sulfhydryl groups react almost immediately and were ignored; the kinetics of the less reactive groups were examined. The reaction of these groups follows first order kinetics, first order in terms of the concentration of unreacted sulfhydryl groups and zero order in terms of the free $Hg(SO_3)_2^{2-}$ complex.

2. The Binding of PMB to Hemerythrin

Klapper (57) demonstrated that PMB binds noncovalently to hemerythrin, that this binding is rapid and probably faster than the formation of the covalent mercury-sulfur bond, and that this binding does not affect the absorbance at 250 nm. Rao (14) has also observed this noncovalent binding of PMB by gel chromatography. Duke et al (21) concluded that the

binding of PMB to hemerythrin probably complicates the reaction between the mercurial and the sulfhydryl group. They suggest that this binding phenomenon may be quite common and that the interpretation of PMB-protein reaction kinetics should be cautious.

3. The Assumptions

On the basis of the known binding of PMB with hemerythrin and the apparent hydrophobic interactions of sulfhydryl groups, I formulated two basic assumptions for the reaction of PMB with hemerythrin.

a. The conformational states of the sulfhydryl group in hemerythrin. For hemerythrin, I assumed that the sulfhydryl group can exist in two states which are in equilibrium, analogous to those proposed by Cecil and Thomas (106) for hemoglobin. Garbett, Darnall, and Klotz (26) have deduced that the iron environment is probably nonpolar; furthermore, they (27) also argue that a perchlorate binding site must be near to both sulfhydryl group and iron site which means that the sulfhydryl group is probably near the iron. DePhillips (37) speculated about the nature of the perchlorate binding site in hemerythrin. Considering the observations of Rifkind and Eichhorn (107, 108) on the structure of polyarginine in solution, he raised the possibility that perchlorate ion binds to two consecutive argininyl residues adjacent to the cysteinyl residue. At pH 6, polyarginine exists as a random coil but the addition of a small amount of perchlorate ion converts polyarginine into a helical structure. In the absence of perchlorate

ion, polyarginine only assumes the helical structure at pH 10.6, the pK of the guanidinium group. Rifkind (107) attributed the helical structure to the simultaneous interaction of the guanidinium group with two oxygens of the tetrahedral perchlorate anion. This ring structure neutralizes the change on the arginine side chain and so favors the coil to helix transition. If this interaction also occurs in hemerythrin, then the perchlorate ion would stabilize the conformation adjacent to the cysteinyl residue and permit the sulfhydryl group to participate more easily in hydrophobic bonds.

b. Effect of binding of PMB on the sulfhydryl group. Also, I assumed that the binding of PMB to hemerythrin induces surface denaturation of the protein, destabilizes the conformation around the sulfhydryl group, and thereby facilitates the formation of the mercury-sulfur bond. Egan (18) has discussed the idea of surface denaturation which has been suggested by work on turnip yellow mosaic virus. This virus, like hemerythrin, reacts with PMB, followed by dissociation of the protein component; a certain amount of PMB binds to the virus before it dissociates (103). Godschalk and Veldstra (46) examined the reaction of turnip yellow mosaic virus with aliphatic mercurials. From polarographic measurements of the reaction kinetics, they deduced that addition of mercurial first leads to the establishment of an absorption equilibrium, involving nonsulfhydryl sites on the surface of the virus particle, then surface denaturation makes the sulfhydryl group accessible to the mercurial.

c. Summary of the assumptions. The first assumption proposes that there is an equilibrium

$$Hr \rightleftharpoons HrSH \qquad (52)$$

where Hr represents a subunit of hemerythrin in which the sulfhydryl group is involved in hydrophobic interactions and cannot react with PMB, while HrSH represents a subunit in which the sulfhydryl group is exposed to the solvent and can react with PMB. This assumption is based on the observed first order kinetics; the rate of reaction represents a change converting the sulfhydryl group from an unreactive conformation to a reactive conformation; the fast phase represents the reaction of the accessible sulfhydryl groups which are in equilibrium with the rest of the sulfhydryl groups prior to the addition of PMB. The second assumption proposes that as a consequence of the binding equilibrium

$$HgBz + Hr \rightleftharpoons (HgBz)Hr \qquad (53)$$

where HgBz is PMB and (HgBz)Hr, the noncovalent complex of PMB and protein, the conformational change in the equilibirum

$$(HgBz)Hr \rightleftharpoons (HgBz)HrSH \qquad (54)$$

proceeds at a faster rate than does the corresponding process in equation 52. This latter assumption arises from the behavior of the rate constant for fluoromethemerythrin. With these assumptions and equations, a rate equation can be derived for the formation of the mercury-sulfur bond.

B. Mathematical Derivation of a Rate Law for the Reaction of PMB with Hemerythrin

On the basis of the basic assumptions, the equations for the interaction of PMB with hemerythrin proceeds by the reactions:

$$\mathrm{Hr} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{HrSH} \tag{52}$$

$$\mathrm{HgBz} + \mathrm{Hr} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (\mathrm{HgBz})\mathrm{Hr} \tag{53}$$

$$(\mathrm{HgBz})\mathrm{Hr} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} (\mathrm{HgBz})\mathrm{HrSH} \tag{54}$$

$$\mathrm{HrSH} + \mathrm{HgBz} \xrightarrow{k_7} \mathrm{HrSHgBz} \tag{55}$$

$$(\mathrm{HgBz})\mathrm{HrSH} + \mathrm{HgBz} \xrightarrow{k_8} (\mathrm{HgBz})\mathrm{HrSHgBz} \tag{56}$$

where HrSHgBz and (HgBz)HrSHgBz represent the subunits of hemerythrin to which PMB is covalently bound to the sulfur in the cysteinyl residue. For the formation of this product (S-Hg), the rate law may be written

$$\frac{d[(\mathrm{S\text{-}Hg})]}{dt} = \frac{-d[(\mathrm{-SH})]}{dt} \tag{57}$$

$$= K_7 [\mathrm{HrSH}][\mathrm{HgBz}] + k_8 [(\mathrm{HgBz})\mathrm{HrSH}][\mathrm{HgBz}]$$

where [(-SH)] is the concentration of unreacted sulfhydryl groups or unreacted monomer units of hemerythrin. After the fast phase of the reaction, steady state conditions arise and the concentration of reactive subunits becomes small. For them during the steady state, the rate equations can be written

$$\frac{d[(HgBz)HrSH]}{dt} = k_5[(HgBz)Hr] - k_6[(HgBz)HrSH] - k_8[(HgBz)HrSH][HgBz] = 0 \quad (58)$$

and

$$\frac{d[HrSH]}{dt} = k_1[Hr] - k_2[HrSH] - k_7[HrSH][HgBz] = 0 \quad (59)$$

from which the steady state concentrations

$$[HrSH] = \left(\frac{k_1}{k_2 + k_7[HgBz]}\right)[Hr] \quad (60)$$

and

$$[(HgBz)HrSH] = \left(\frac{k_5}{k_6 + k_8[HgBz]}\right)[(HgBz)Hr] \quad (61)$$

are obtained. Substituting these concentration terms into equation 57 gives the equation

$$\frac{d[S\text{-}Hg]}{dt} = \left(\frac{k_7 k_1}{k_2 + k_7[HgBz]}\right)[Hr][HgBz] + \left(\frac{k_8 k_5}{k_6 + k_8[HgBz]}\right)[(HgBz)Hr][HgBz] \quad (62)$$

The values of k_7 and k_8 are expected to be large and comperable to rate constants for reactive sulfhydryl groups which have been measured by Hasinoff et al (95) with a magnitude of 10^6 $M^{-1}\,sec^{-1}$. On the other hand, the values of k_2 and k_6 should be sufficiently smaller, so that $k_7[HgBz] \gg k_2$ and $k_8[HgBz] \gg k_6$. If these conditions hold, then equation 62 reduces to

$$\frac{d[S\text{-}Hg]}{dt} = -\frac{d[(-SH)]}{dt} = k_1[Hr] + k_5[(HgBz)Hr] \quad (63)$$

The concentration of unreacted sulfhydryl group, [(-SH)], is the same as the total concentration of unreacted monomer units of hemerythrin or

$$[(-SH)] = [HrSH]_T = [Hr] + [(HgBz)Hr] + [HrSH] + [(HgBz)HrSH] \quad (64)$$

but the concentrations of reactive species [HrSH] and [(HgBz)HrSH] are negligible. The other two protein species are related by the binding equilibrium (equation 53) so that

$$K = \frac{[(HgBz)Hr]}{[Hr][HgBz]} \quad (65)$$

Since the total unreacted protein is essentially the sum of [(HgBz)Hr] and [Hr], then

$$K = \frac{[(HgBz)Hr]}{([HrSH]_T - [(HgBz)Hr])[HgBz]} \quad (66)$$

which by rearranging gives

$$[(HgBz)Hr] = \left(\frac{K[HrBz]}{1 + K[HgBz]}\right)[HrSH]_T \quad (67)$$

and likewise

$$[Hr] = [HrSH]_T - [(HgBz)Hr] = \left(1 - \frac{K[HgBz]}{1 + K[HgBz]}\right)[HrSH]_T \quad (68)$$

These expressions may then be substituted into equation 63 to obtain the rate law in terms of unreacted protein

$$\frac{d[S\text{-}Hg]}{dt} = \left[k_1\left(\frac{1}{1 + K[HgBz]}\right) + k_5\left(\frac{K[HgBz]}{1 + K[HgBz]}\right)\right][HrSH]_T \quad (69)$$

The apparent rate constant that was experimentally measured coresponds to the term in brackets

$$k_{app} = \left[k_1 \left(\frac{1}{1 + K[HgBz]} \right) + k_5 \left(\frac{K[HgBz]}{1 + K[HgBz]} \right) \right] \quad (70)$$

C. Application of the Model to Hemerythrin

1. Chloromethemerythrin, PMB Concentration in Excess

If PMB binds to the protein with an affinity sufficient that $K[HgBz] \gg 1$, then

$$\frac{K[HgBz]}{1 + K[HgBz]} \approx 1 \quad (71)$$

[Hr] becomes negligible, and $[(HgBz)Hr] \approx [HrSH]_T$, so that equation 69 reduces to

$$\frac{d[S\text{-}Hg]}{dt} = - \frac{d[(-SH)]}{dt} = k_5[HrSH]_T \quad (72)$$

The observed reaction is true first order; the rate of reaction is proportional to the concentration of hemerythrin in monomer units. The rate constant (k_5) represents a first order process which is a small but definite change in the conformation of the PMB-protein complex. The kinetic data for chloromethemerythrin appear to fit equation 72. Furthermore, saturation effect is real because for the lower concentrations of PMB, the left hand term of relationship 71 becomes less than one.

2. Fluoromethemerythrin

When PMB binds to hemerythrin with less affinity than required by equation 72, then the calculated rate constant is a combination of the two rate constants for conformational change, k_1 and k_5. The two terms in parentheses in equation 70

$$k_{app} = \left[k_1 \left(\frac{1}{1 + K[HgBz]} \right) + k_5 \left(\frac{K[HgBz]}{1 + K[HgBz]} \right) \right] \qquad (70)$$

may be considered as partition functions, dividing the mercaptide formation between the two classes of reacting subunit; the class of subunit is defined by whether or not PMB is bound to it. As the free PMB concentration increases, the greater the term (K[HgBz]/1 + K[HgBz]) becomes and the greater the portion of protein to which PMB is bound. The second assumption postulates that $k_5 > k_1$, so that the greater the protion of protein bound, the greater the observed rate constant. The concentration of free PMB increases as the concentration of total mercurial is increased while it decreases as the concentration of protein is increased.

Sample calculations for various concentrations of hemerythrin and PMB taken from Table VIII revealed that although the concentration of free PMB may change by a large percentage during the course of a reaction, the partition factors will change by only a few per cent. For these calculations, the K was arbitrarily taken as 10^3 M^{-1} which is a probable value as measured by Klapper (57). The value of k_{app} as defined by equation 70 remains essentially constant during the course of an experi-

ment but k_{app} will increase as PMB concentration is increased and it will decrease as the hemerythrin concentration is increased.

3. Chloromethemerythrin When the Concentration of Monomer Units Exceeds That of PMB

When the concentration of hemerythrin in monomer units exceeds that of PMB, the calculated rate constants are smaller. Since PMB cannot bind to all of the protein, some of the reaction proceeds through the slower conformational change in protein to which no PMB is bound (k_1). The calculated rate constant is determined by equation 70 when steady state conditions hold, but when the initial PMB to monomer units ratio is 0.4 or less, then the fast phase is almost the entire reaction observed.

For experiments in which the PMB-protein ratio is about 0.7-0.9, the data usually fit the predictions of the model very well. Figure 13, which is reproduced as Figure 28, presents the first order treatment of a typical experiment of this type. The data consists of three phases. The first phase occurs prior to the establishment of steady state conditions; it is the fast phase, the depletion of the reactive conformation of the protein by reactions 55 and 56. For the experiments in which PMB was the excess reagent, this phase was always completed by the first absorbance reading, but for this experiment, the reaction proceeded at a slower rate because of the lower concentration of PMB. After the fast phase is completed, during the period of steady state, the concentration data in terms

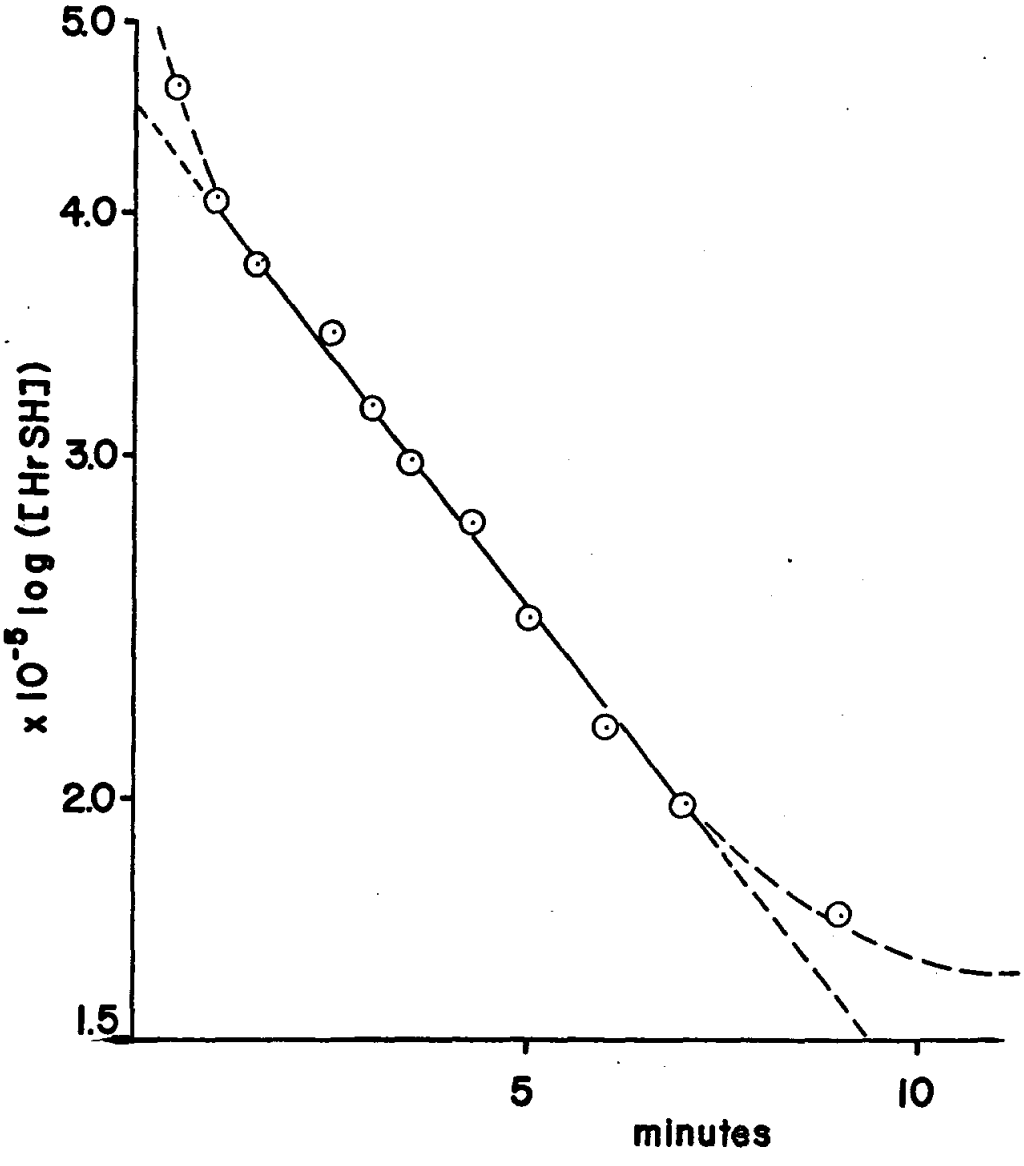

FIGURE 28: First Order Plot for the Reaction of Hemerythrin with PMB in Terms of Unreacted Protein Concentration When the Concentration of Monomer Units Exceeds That of PMB. Same Figure as Figure 13. Data from Table II.

of monomer units of hemeryrhrin follow a fist order plot, the natural log of concentration as a linear function of time. In Figure 28, 2.303 times the slope of the linear portion equals the k_{app} of equation 70. Toward the end of the reaction, the steady state ceases; the conditions $k_7 [HgBz] >> k_2$ and $k_8 [HgBz] >> k_6$ no longer hold true. The free mercurial concentration is so low that the reaction of it with protein becomes slower than the rate of conformation change, while the percentage change of the protein concentration per unit time becomes so small that the reaction proceeds almost zero order in hemerythrin.

For PMB-protein ratios less than 0.5, there is probably no second phase and the calculated rate constants probably do not correspond to the k_{app} of equation 70 but rather the rate of reaction probably follows equation 57. For these experiments, the experimental error is large because the percentage of total protein that reacts is small and the concentration data are derived from small difference in absorbance readings. These readings themselves were quickly taken during a short period of time so that few data exist for each experiment.

For a ratio of 0.3 or less, the entire reaction is probably by the fast phase. For example, in one experiment in which the initial concentration were 8.69×10^{-5}M monomer units of hemerythrin and 1.31×10^{-5} M PMB, only 15% of the sulfhydryl groups react. The data fit a second order graph with a rate constant $k_2 = 6.4 \times 10^2 M^{-1} sec^{-1}$ but a first order plot in terms of PMB concentration gives a rate constant $k_1 = 48 \times 10^{-3}$ sec^{-1}

which when dvided by the protein concentration gives a comparable second order rate constant $k = 5.5 \times 10^2\ M^{-1}sec^{-1}$. For these lower ratios then, the rate constants which appear in Tables IV and VI are probably invalid, the reaction being approximately second order in PMB and protein. The second order rate constants of about 500 $M^{-1}\,sec^{-1}$ do not necessarily correspon to k_7 and k_8 of Reactions 55 and 56 because the binding equilibrium complicates the reaction.

D. Simplifying the Model

The model which has been presented will account for both my data and that of Duke et al (21), but it is complicated. As yet, no evidence exists that the noncovalent binding of PMB to hemerythrin will affect the reactivity of sulfhydryl groups. Furthermore, there is no justification in the assumption that PMB binds to chloromethemerythrin more strongly than to fluoromethemerythin. These assumptions were required to formulate a model which applied to the observations of Duke et al (21). For my data on fluoromethemerythrin in Table VIII, I claim the same behavior in the rate constant can be observed which Duke, Barlow, and Klapper (21) had observed in their study. This observation is that the calculated rate constant increases as PMB concentration increases and it decreases as the intial concentration of hemerythrin increases. The magnitude of these changes in the rate constant, however, is comparable to the precision of measurements. Consequently, the variation in calculated

rate constants can also be attributed to experimental error.

If the initial concentration of neither reagent affects the calculated rate constant, then the second assumption is not required. A simple two step mechanism based on reactions 52 and 55,

$$Hr \rightleftharpoons HrSH \qquad (52)$$

$$HrSH + HgBz \longrightarrow HrSHgBz \qquad (55)$$

sufficiently explains my data. From these two equations, one can easily derive a simple first order relationship for steady state conditions. Having fewer assumptions, this model is more desirable. But if the trends which Duke et al (21) observe and which I claim for Table VIII are real, then the simple model would be inadequate.

The data of Duke and coworkers (21) covered a greater range of concentrations. The magnitude of the concentrations effects was greater than what the precision of their experiments would allow for experimental error. We cannot easily dismiss this data. There is, however, still another alternative. If the true order of the reaction of PMB and hemerythrin is not integral but some fractional order, then the rate constant which had been calculated on the assumption that the order was first in protein and zero in PMB would change with changing concentrations of reactants. A computor analysis of the kinetic data based on the equation

$$\log \left(- \frac{dA}{dt} \right) = \log k + x \, [\log A + (y/x) \log B] \qquad (33)$$

given earlier in Chapter III may determine nonintegral values for the reaction order (x and y in the equation for the reactants A and B). This analysis would require a certain degree of previcion in the data which may be lacking in my experiments.

E. Guidelines for Future Research

Although the model explains the assembled kinetic data, it is based on unproven assumptions. Nevertheless, these assumptions may guide further research with the object of proving or supporting them. If further research substantiates the assumptions, it will aid in the developement of concepts for structure and function in proteins. In terms of immediate possibilities, the binding of PMB to hemerythrin and the equilibrium of the fast reacting species need to be checked.

Although PMB does bind to hemerythrin, its affinity for chloromethemerythrin is unknown. The model implies that PMB binds more strongly to the chloride form of the protein than to the fluoride form. The difference in the effect of the two ligands may be mediated by the anion altering the PMB binding site, chloride facilitating PMB binding to a greater extent than fluoride. Or the ligand effect may be the result of direct binding between the ligand bound at a noniron site and the mercury portion of PMB.

One experiment not performed in this laboratory is the only evidence that a reactive species of hemerythrin is responsible for the fast phase

and that this species is in equilibirum with the other less reactive species of protein. An examination of the fast phase should be directed toward determining whether the reactive species is regenerated at a rate consistent with the measured rate constants which the model presumes to be actually for the conformational change. These experiments would be conclusive, however, by providing negative results. Positive results would not produce any greater understanding of the character of hemerythrin.

A more positive but difficult approach will be the seeking after the thermodynamic character of hemerythrin. In this laboratory, Rao (13-15) has examined the thermodynamic and kinetic aspects of the association-dissociation equilibrium with the techniques of gel chromatography; Tan (105) is carrying this research further. The kinetic data for the reaction with PMB reveal, however, that the formation of the mercury-sulfur bond may depend on states of hemerythrin which are not directly involved with the association-dissociation equilibrium. If these states may be characterized thermodynamicaly, then they may be compared to the postulates of the kinetic model.

Lumry and Biltonen (109) have reviewed and discussed various aspects of protein conformation and the relationship of these conformations to physiological function in protein. They emphasize the type of approach that they call an approach with models such as the method developed by Brandts (110) which they describe as less a theory than it is a semipirical or semiphenomenological method for analysing the changes in thermo-

dynamic quantities which occur during two-state transitions in proteins. A main difficulty in gathering thermodynamic data for hemerythrin will be detecting the presence of the two states required by equation 53 because these two states probably differ by only what has been termed a subtle change in conformation. A subtle conformational change is so called because it is hard to detect with physical methods, although it is defined operationally as a process that while accompanied by large entropy or enthalpy changes signifying it to be a cooperative process, it is not accompanied by large changes in heat capacity (109); it is not a process involving unfolding of the protein. But because these processes are difficult to detect by physical methods such as optical rotatory dispersion, finding suitable methods will probably require a trial and error approach.

An insight may be gained from a measurement of the thermodynamic functions for activationin the reaction of PMB with hemerythrin. These values may be compared with those for the reaction of PMB with small model compounds and with those for conformational change in hemerythrin or other proteins.

For a better understanding of the reaction, work with compound analogs of PMB such as aliphatic mercurials will provide a greater basis of comparison. The analog compounds should reveal information about which factors are important in the formation of the mercury-sulfur bond, what type of interactions are involved in the PMB-protein binding, and what in the cause and effect relationship for the dissociation of the protein.

A rigid approach to research is seldom feasible. A labyrinthine path is the road of science, for more typically, the results of each step in research direct the experiments for the next. This discussion is only intended to point out general guidelines not to chart the way. It therefore remains for others to provide the data which will determine whether this thesis represents a faltering step forward or a meaningless bable.

VI. SUMMARY

Hemerythrin, a nonheme, iron-containing protein, consists of eight identical subunits, each of which bear one sulfhydryl group (5-9). Upon reaction of these sulfhydryl groups with organic mercurials such as p-mercuribenzoate (PMB), the native protein dissociates into subunits (6). This dissociation appears to follow an all-or-none mechanism; adding mercurial in less than stoichiometric amounts results in all the mercurial being bound to monomers and none being bound to intact octamers (7). To explain the all-or-none behavior of the sulfhydryl groups, Keresztes-Nagy and Klotz (8) proposed the equilibirum model which postulates that the octamer of hemerythrin is in equilibirum with it monomer subunit, that the presence of iron-coordinating ligands shifts the equilibrium, and that only the monomer species of hemerythrin is reactive toward the mercurial. The first two of these postulates that there is an equilibrium between octamer and monomer species and that ligand anions affect this equilibrium have been proven (9-17).

This investigation attempted to probe the nature of the interaction of hemerythrin with PMB and the subsequent dissociation of the protein. The spectrophotometric technique developed by Boyer (19) permits a direct observation of the formation of the covalent, mercury-sulfur bond.

Using this technique, previous investigators (18, 20, 21) have examined the reactivity of the sulfhydryl group in hemerythrin, but they reported contradictory findings and failed to provide an adequate model for the behavior of the sulfhydryl group in hemerythrin. This work extended the kinetic investigation of the interaction of PMB with hemerythrin and it should provide some insight into the nature of that interaction.

A kinetic analysis of the data reveal that when PMB interacts with either chloro- or fluoro-methemerythrin, about 20 - 40% of the sulfhydryl groups present react at a rate so fast that in nearly all cases, this phase of the reaction was completed in less than one minute. The remainder of the protein sulfhydryl groups react with PMB by a first order process; the rate of reaction is solely a function of unreacted protein concentration. That the reaction follows first order kinetics rejects the earlier claim from this laboratory (18) that the reaction is second order.

Yet, although the rate of reaction is directly proportional to the concentration of unreacted monomer units of hemerythrin, which is also the concentration of sulfhydryl groups, for fluoromethemerythrin, the rate constant calculated from the kinetic data varies with the initial concentrations of the reactants. The rate constant increases as the concentration of PMB is increased and it decreases as the concentration of hemerythrin is increased. Duke et al (21) have observed the same behavior for the rate constant for fluoromethemerythrin. For chloromethemerythrin, the concentration of PMB did not significantly affect the

rate constant over most of the range of concentrations which were used in these experiments. But at the lower concentrations, the rate constants were noticeably lower which indicates that for the chloride complex of hemerythrin, the same effects occur but that a saturation point has been reached.

In a solution of hemerythrin so dilute that the monomer species of the octamer-monomer equilibirum predominates, the reaction proceeds no faster than it does for more concentrated solutions of hemerythrin which demonstrates that the monomer species of the protein is no more reactive than the octamer. Therefore, the fast reaction phase cannot be ascribed to the reaction of dissociated protein nor can the increase in rate constant for fluoromethemerythrin when the protein concentration is decreased be ascribed to further dissociation of the octamer.

That PMB reacts only with the monomer species of hemerythrin rather than the octamer is essential to the equilibrium model as proposed by Keresztes-Nagy and Klotz (8). Using a light scattering technique, I observed the actual dissociation of chloromethemerythrin upon addition of PMB. The dissociation is a first order process which has a rate constant of about one half of the rate constant for the formation of the mercury-sulfur bond. The mercurial, therefore, reacts with the octamer species and dissociation follows formation of the mercury-sulfur bond. The two rate constants are too close in magnitude for a definitive conclusion to be drawn from them alone, but since the monomer is no more

reactive than the octamer, the two observations taken together completely exclude the equilibrium model as originally proposed.

Furthermore, the reactivity of the sulfhydryl group of hemerythrin is not a function of the quaternary structure of the protein; there is no evidence of any subunit-subunit interactions which affect the reactivity of hemerythrin toward reagents which substitute on the sulfhydryl group. Apparently, the reaction is a random process. While PMB is interacting with hemerythrin and forming covalent, mercury-sulfur bonds, the mercurial is also inducing some concomitant change in the monomer which prevents the subunit from participating in the octamer-monomer equilibrium. Over a period of time then, all the reacted subunits will be requilibrated into unassociating or crippled monomers.

The calculation of the rate constant for the formation of the mercury-sulfur bond when the protein is the excess reactant, further demonstrates that the reaction is first order. The rate constants calculated by fitting either PMB or hemerythrin concentration to first order semilog graphs prove that the rate of reaction is a function of protein concentration. The data is consistent with a two step reaction scheme in which the first step is a slow, unimolecular change in hemerythrin followed by a rapid formation of the mercury-sulfur bond.

Taking all the data on the formation of the mercury-sulfur bond in hemerythrin, I assumed that the rate determining step was a small but definite change in conformation about the sulfhydryl group and that this

this change in conformation was affected by the binding of PMB to hemerythrin which is known to occur. Therefore, the reaction scheme

$$\mathrm{Hr} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{HrSH} \tag{52}$$

$$\mathrm{HgBz} + \mathrm{Hr} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (\mathrm{HgBz})\mathrm{Hr} \tag{53}$$

$$(\mathrm{HgBz})\mathrm{Hr} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} (\mathrm{HgBz})\mathrm{HrSH} \tag{54}$$

$$\mathrm{HrSH} + \mathrm{HgBz} \xrightarrow{k_7} \mathrm{HrSHgBz} \tag{55}$$

$$(\mathrm{HgBz})\mathrm{HrSH} + \mathrm{HgBz} \xrightarrow{k_8} (\mathrm{HgBz})\mathrm{HrSHgBz} \tag{56}$$

is proposed, where Hr represents an unreactive subunit of hemerythrin; HrSH, the subunit with a reactive sulfhydryl group; (HgBz)Hr and (HgBz)-HrSH, the protein species to which PMB is noncovalently bound; HrSHgBz and (HgBz)HrSHgBz, the protein with which the PMB has formed the covalent mercury-sulfur bond. The fast phase of the reaction is due to the depletion of reactive protein by reactions 55 and 56 and proceeds to the onset of steady state conditions. From this scheme under the steady state, one can derive the rate law for the formation of the mercury-sulfur bond (S-Hg) in the form

$$\frac{d[S\text{-}Hg]}{dt} = -\frac{d[HrSH]_T}{dt} \tag{69}$$

$$= \left[k_1 \left(\frac{1}{1+K[HgBz]} \right) + k_5 \left(\frac{K[HgBz]}{1+K[HgBz]} \right) \right] [HrSH]_T$$

where $[HrSH]_T$ represents the concentration of total unreacted protein or unreacted sulfhydryl group and $K = k_3/k_4$ is the binding constant for equation 53. The term in brackets is essentially a constant over the course of the reaction, so that equation 69 can be easily integrated as a first order rate law. The term in brackets is the k_{app} which is the rate constant that was calculated from the kinetic data. It would vary in the same manner as the rate constant observed for fluoromethemerythrin when the initial concentrations of PMB and protein were varied. When the noncovalent binding of PMB to the protein is very strong, then equation 69 reduces to

$$\frac{d[S\text{-}Hg]}{dt} = -\frac{d[HrSH]_T}{dt} = k_5 [HrSH]_T \tag{72}$$

This equation applies to chloromethemerythrin at those concentrations of PMB where the saturation effect appears. Consequently, the reaction scheme explains all the data for the formation of the mercury-sulfur bond but it yet remains for the assumptions to be proven.

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APPENDIX I

A FIRST ORDER PROCESS INVOLVING TWO REACTANTS

When a reaction occurs as a two step process, the slowest step may determine the overall rate of reaction. An example of this type of process is represented by the reaction scheme

$$A \longrightarrow A^* \qquad (1)$$

$$A^* + B \longrightarrow P \qquad (2)$$

where A and B are two reactants that combine to form the product P and A* is an intermediate a A which is reactive toward B. If reaction 1 is slow and reaction 2, fast, then the overall rate of reaction will be determined by the first step and the observed order of the reaction will be first in A and zero in B. If the quantity of B exceeds that of A, no problem developes in the kinetic treatment of the data; the reaction proceeds until all of A has reacted. But if the quantity of A exceeds that of B, the reaction will continue only as long as B is present. The intuitive impulse is to treat the data as if the rate were determined by the concentration of B, although this is erroneous. A reaction that is first order in A when B is the excess reactant should still be first order in A when A is the excess reactant, provided that steady state conditions still hold. Table XXI presents theoretical data for quantitites of hypothetical reactants A and B. The rate at which A reacts and disappears is directly propor-

tional to the concentration of A by the equation

$$-\frac{dA}{dt} = -\frac{dB}{dt} = k_1 A \qquad (3)$$

Although B reacts and disappears at the same rate as A, the rate of change in B is not a function of B but of A. A plot of log B as a function of time will not be linear. In Figure 29, points represent the data taken from Table XXI for the reaction of A and B at various concentrations of B less than A. Although a curved line through the points is correct, one can draw a straight line if one ignores some of the later points. In an actual laboratory experiment, a certain amount of scatter of the points is expected and also the later points are expected to have the greatest error. Consequently, it is easy for one to ignore the nonlinearity of his data and to draw a straight best fit line. The dashed lines in Figure 29 represent lines of this type; they are a forced fit of the data to a first order equation. As the initial concentration of B decreases, the false lines increase in slope giving shorter half times and larger rate constants.

In Figure 29, the solid line represents A. In an actual experiment, A would be expected to follow a first order plot on only part of the way. When the quantity of B becomes so low that reaction 2 is no longer significantly faster than Reaction 1, the first step no longer determines the rate. Reaction 2 is second order, but if A exceeds B by a sufficient amount, then the observed reaction will proceed as pseudofirst order

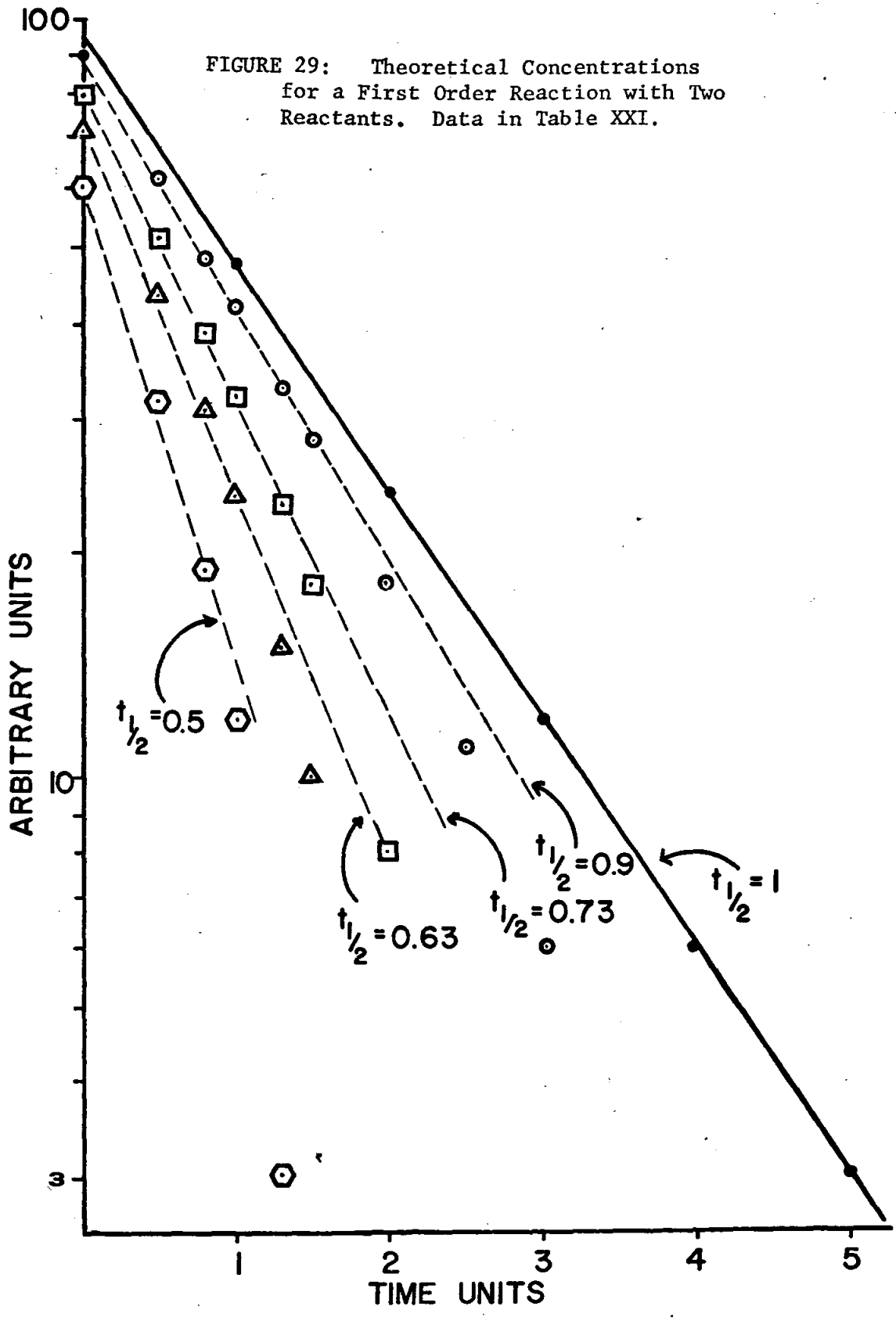
in B. Consequently, the effect of the increase in the false rate constants with decrease of the zero order reactant will be observed only at certain ratios of the two reactants, during steady state conditions for a first order reaction in A while A is the excess reactant.

TABLE XXI: CALCULATIONS FOR A THEORETICAL FIRST ORDER REACTION WHICH INVOLVES TWO REACTANTS

half times	for A	for B 1.	2.	3.	4.
0	96	90	80	72	60
0.5	68	62	52	44	32
0.8	55	49	39	31	19
1.0	48	42	32	24	12
1.3	39	33	23	15	3
1.5	34	28	18	10	-
2.0	24	18	8	-	-
2.5	17	11	1	-	-
3.0	12	6	-	-	-
4.0	6	-	-	-	-
5.0	3	-	-	-	-
$t_{\frac{1}{2}}$	1.0	0.9	0.73	0.63	0.5
k_1	k_A	1.1 k_A	1.4 k_A	1.6 k_A	2 k_A

All units are arbitrary for time and quantity of A and B.

FIGURE 29: Theoretical Concentrations for a First Order Reaction with Two Reactants. Data in Table XXI.



APPENDIX II

GRAPHICAL TREATMENT OF KINETIC DATA FOR SECOND ORDER

For a second order reaction of the type

$$A + B \longrightarrow P \qquad (1)$$

where A and B react to form product P, the normal practice is to graph the logarithm of the ratio of the two reactant concentrations as a function of time. This plot represents the integrated second order rate equation

$$\ln\left(\frac{A}{B}\right) = (A_o - B_o)\, k_2 t + \ln\left(\frac{A_o}{B_o}\right) \qquad (2)$$

where A and B represent the concentrations of the two reactants while A_o and B_o denote the initial concentrations. The rate constant (k_2) can be calculated from the slope of the line (m) in the graph by

$$k_2 = \frac{2.303m}{A_o - B_o} \qquad (3)$$

(when base ten logarithms or semi-log paper is used). If the two reactants are present in near stoichiometric amounts, however, the ratio of the two reactants remains nearly equal to one during almost the entire reaction so that an alternative method is desired.

In this method, * one can write the instantaneous concentration of

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* S. W. Benson, The Foundations of Chemical Kinetics McGraw-Hill Book Company, Inc., New York, 1960, pgs. 20-21.

B by the equation

$$B = A + \Delta \tag{4}$$

where $\Delta = A_o - B_o$, and then the rate equation becomes

$$-\frac{dA}{dt} = k_2 A (A + \Delta) \tag{5}$$

Then if one makes the substitution $A' = A + \Delta/2$, the rate equation becomes

$$-\frac{dA'}{dt} = k_2 \left(A' - \frac{\Delta}{2} \right) \left(A' + \frac{\Delta}{2} \right) \tag{6}$$

$$= k_2 \left(A'^2 - \frac{\Delta^2}{4} \right)$$

$$= k_2 A'^2 \left[1 - \left(\frac{\Delta}{2A'} \right)^2 \right] \tag{6}$$

When $\Delta \leq A_o/4$, the term in brackets varies from ≥ 0.99 to ≥ 0.96 as half of the reaction occurs; therefore, it may be considered a constant and replaced with its average value of $(1 - \Delta^2/4A'_o A'_f)$. The two A' terms represent the initial and final concentrations of A'. Then the rate equation can be easily integrated to give

$$\frac{1}{A'} - \frac{1}{A'_o} = k_2 t - \frac{\Delta^2}{4A'_o A'_f} \tag{7}$$

In a graphical representation, the plot of $1/A'$ as a function of time should give a reasonably straight line for the first 50 to 75% of the reaction.

To obtain the rate constant, one devides the slope of this line by the term $(1 - \Delta^2/4\ A'_o\ A'_f)$. The value of A' is (A + B)/2 for a reaction of the type expressed by equation 1.

APPROVAL SHEET

The dissertation submitted by Michael Charles Cress has been read and approved by the undersigned member of the faculty of the Loyola University Stritch School of Medicine who served as director of the research program.

The final copies have also been examined and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

May 22. 1972
Date

Steven Kenneth Hoeg
Signature of Advisor