MECHANISM OF THE REACTION OF HEMERYTHRIN WITH P-MERCURIBENZOATE

A Thesis

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by

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TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	iv
LIST OF ILLUSTRATIONS	v
ABSTRACT	vi
I. INTRODUCTION	1
A. Description of Protein	1
B. Cooperative Interaction	3
C. Models for Cooperative Interaction and Substantiating Experiments	4
D. Testing the Third Assumption	7
II. EXPERIMENTAL PROCEDURES	10
A. Materials	10
B. Preparation of Reagents	10
C. Concentration of Solutions	14
D. Procedure for Kinetic Studies	19
E. Determination of the Rate Constants	21
III. EXPERIMENTAL RESULTS AND DISCUSSION	26
A. Fluoride Binding to Iron	26
B. Consequences of Protein Conformational Changes	26
C. Concentration of the Sulfhydryl Groups and Iron	29
D. Rate Constants	30
IV. SUMMARY	38
APPENDIX	40
BIBLIOGRAPHY	66

LIST OF TABLES

Table		Page
I.	EXTINCTION COEFFICIENTS FROM SULFHYDRYL TITRATIONS.	41
II.	AVERAGE EXTINCTION COEFFICIENTS AT 250 mu . .	42
III.	APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 1.164×10^{-4} M.	43
IV.	APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 3.88×10^{-5} M	44
V.	APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 1.16×10^{-5} M	45
VI.	APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 2.33×10^{-6} M	46
VII.	APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 3.88×10^{-5} M IN THE PRESENCE OF BENZOIC ACID.	47
VIII.	APPARENT FIRST ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 2.33×10^{-6} M IN THE PRESENCE OF BROMIDE. .	48
IX.	FIRST AND SECOND ORDER RATE CONSTANTS CALCULATED FROM THE APPARENT FIRST ORDER RATE CONSTANTS MEASURED AT EXCESS PMB . . .	49

LIST OF ILLUSTRATIONS

Figure		Page
1.	SULFHYDRYL TITRATION WITH PMB.	50
2.	DECREASE IN ABSORPTION OF AQUO-METHEMERYTHRIN WITH FLUORIDE ADDITION.	52
3.	PLOT OF FIRST ORDER RATE EQUATION TO FIND k_1 .	54
4.	PLOT OF SECOND ORDER RATE EQUATION TO FIND k_2.	56
5.	VARIATION OF THE FIRST ORDER RATE CONSTANT WITH INITIAL PMB CONCENTRATION	58
6.	VARIATION OF THE SECOND ORDER RATE CONSTANT WITH INITIAL PMB CONCENTRATION	60
7.	VARIATION OF THE FIRST ORDER RATE CONSTANTS WITH PMB IN THE PRESENCE AND ABSENCE OF BENZOIC ACID.	62
8.	VARIATION OF THE FIRST ORDER RATE CONSTANTS WITH EXCESS PMB.	64

ABSTRACT

The reaction kinetics of p-mercuribenzoate with the sulfhydryl groups of the protein hemerythrin were investigated. A mechanism is proposed in which the organic mercurial binds at two sites, one of which is not a sulfhydryl group. From the dependence of the reaction rates on the protein concentration it is further concluded that the reactivity of the sulfhydryl groups depends on the protein's state of aggregation.

I. INTRODUCTION

A. Description of the Protein

Hemerythrin, a non-heme, iron-containing protein, is distributed among four different phyla, the primary one being sipunculids. (3). This is an oxygen carrying protein found to combine reversibly with oxygen, (16), and therefore believed to serve a respiratory function (3).

The hemerythrin isolated from Golfingia gouldii (also known as Phascolosoma gouldii) has a molecular weight of 107,000 (14), contains 16 moles of iron per mole of protein, and binds 8 molecules of oxygen (1) (14) (15). Evidence also indicates that there are 8 moles of sulfhydryl groups per mole of protein (7,4).

Hemerythrin is comprised of eight subunits having a molecular weight of 13,500 (7). Sequence studies have indicated that all the subunits are the same. (13) Thus, each subunit contains two iron atoms (capable of binding one molecule of oxygen) and one sulfhydryl group.

Similar to other iron-containing proteins, the iron can be oxidized to Fe (III) which in turn is capable of combining with a number of coordinating ligands; e.g. N_3^-, SON^-, OCN^-, OI^-, F^-, OH^-, and CN^- (8). Coordination

of the iron is reflected by characteristic changes in the visible and ultraviolet spectrum of the protein. No spectral change is noted with buffer solutions of bicarbonate, Tris, acetate, cacodylate, or sulfate, suggesting, as expected, that these ions do not coordinate with iron. In such cases water or a protein residue would bind to the iron.

The characteristic spectral changes which occur with the addition of coordinating ligand to the aquomethemerythrin have provided a convenient means for studying the nature of the iron-ligand bond. Upon complete saturation, the stoichiometry of azide (8) and thiocyanate (12) has been measured to be one anion bound to two iron atoms.

Various evidence indicates that the octamer is in dissociative equilibrium with its subunits. Manwell (17) showed the mixing of two genetically distinct hemerythrins, obtained from individual worms, resulted in hybridization of the two. Keresztes-Nagy et.al. (9) found that hybridization of a native and a succinylated hemerythrin (with new electrophoretic properties) produced an octamer with intermediate electrophoretic mobility. The occurrence of hybridization suggests that the octamers are in equilibrium with a smaller sized subunit.

Further evidence for a dissociative equilibrium was found from sedimentation studies. If the octamer

is in equilibrium with the monomeric subunit, dilution of the protein should result in a relative increase of the monomer at the expense of the octamer. Using the analytical ultracentrifuge, Klapper, Barlow and Klotz (10) have shown dilution of hemerythrin causes the appearance of a new boundary with the sedimentation properties of the monomer, with the concomitant diminution of the boundary due to the octamer. The presence of only these two boundaries suggests that the monomer and octamer are the only species involved in the equilibrium.

Reaction of the protein sulfhydryl groups with mercaptan-blocking reagents results in protein dissociation. Keresztes-Nagy, et. al. (7) have shown that this dissociation occurs by an "all-or-none" mechanism. As soon as one mercurial molecule is bound to one site on a protein molecule, the other sites also bind and dissociate into subunits. Upon removing the blocked sulfhydryl groups of the subunits with cysteine ethyl ester, it was possible to reaggregate the protein back to octamer with a ninety per cent yield. (7)

B. Cooperative Interaction

Keresztes-Nagy and Klotz (8) discovered that the environment of the iron atoms in hemerythrin effects the reactivity of the sulfhydryl group. Sedimentation studies revealed that in the absence of a coordinating

ligand, the sulfhydryl groups of aquo-methemerythrin react extremely slowly with sulfhydryl reagents. In the presence of a ligand, the reaction of the protein with sulfhydryl reagents proceeds rapidly. Therefore, a ligand coordinated to iron enhances the reactivity of the sulfhydryl groups.

In the absence of ligands, the low reactivity of the sulfhydryl group may be due to coordination with the iron (11). It is possible that ligands could replace the sulfhydryl group at the iron site, releasing the sulfhydryl group for combination with sulfhydryl reagents. However, the reaction of the sulfhydryl group with an organic mercurial in the absence of any external ligand causes no change in that portion of the protein's spectrum due to the iron. This suggests that the sulfhydryl group is not in the vicinity of the iron, but exists at a separate locus. The enhanced reactivity of the sulfhydryl groups when the environment about the iron is changed is an example of cooperative interactions: a reaction at one site of the protein effects the reactivity of a second site (8).

C. Models for Cooperative Interaction and Substantiating Experiments

Two molecular interpretations of the cooperative interaction in hemerythrin have been outlined by Klapper, Barlow, and Klotz (10). Both models assume that two protein forms are in equilibrium with one another. The

sulfhydryls of one of these forms are relatively unreactive; those of the other react readily. If coordination of external ligands to iron shifts the protein equilibrium from the unreactive to the reactive form, cooperative effects could be explained.

In the first or "dissociation" model, originally proposed by Keresztes-Nagy and Klotz (8), it is assumed that:

(1) The octameric protein is in equilibrium with a small amount of monomer.

(2) The monomer has a greater affinity for iron coordinating anions than does the octamer, and therefore anions shift the octamer $\rightleftharpoons$ monomer equilibrium to the right.

(3) The sulfhydryl groups of the monomer are more reactive than the sulfhydryl groups of the octamer.

The second or "conformational" model proposes that the binding of the ligand to the octamer generates a conformational rearrangement without dissociation of the protein. The rearrangement exposes the sulfhydryl groups or places them in such a position as to stimulate reactivity.

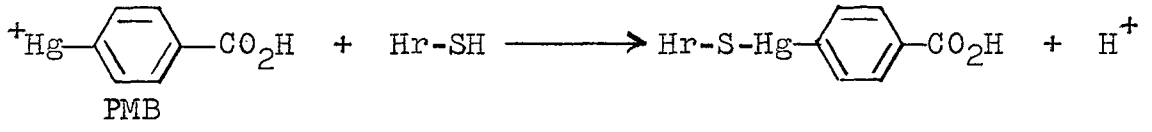
Since both models are able to explain the cooperative interactions, experiments have been performed in order to test one of the alternatives, the dissociation model.

Evidence for the first assumption, the octamer-monomer equilibrium, has been presented in the hybridization experiments (9) (17) and sedimentation studies (10).

The binding constants of azide to mercurial-produced monomer and to octamer, cited by Keresztes-Nagy and Klotz (8) indicate that the monomer has a higher binding constant. This comparison of the binding constants is open to criticism, because the monomer was prepared by chemical modification. Monomer with a non-blocked sulfhydryl group may have different properties. Therefore, another experiment was performed, measuring the binding constant of thiocyanate. As ultracentrifuge experiments have shown, the octamer is in dissociative equilibrium with the monomer. Upon dilution of the protein, more monomer is formed. If the binding constants of thiocyanate to the iron are measured at increasing dilution of protein, the apparent binding constant should begin reflecting the binding of the monomer to the ligand. If the monomer does have a greater affinity for the ligand, dilution of the protein should result in an increase of the apparent binding constant. The binding curves measured by Klapper and Klotz (12) indicated an increase in the binding affinity as the protein is diluted. These results substantiate that the monomer has a higher affinity for the anion.

D. Testing the Third Assumption

The third assumption, the greater reactivity of the monomer's sulfhydryl group, has been tested using kinetic studies (11). The reaction between p-mercuribenzoate (PMB) and the chloride form of methemerythrin was studied by measuring the formation of the sulfur mercury bond, which can be observed by an increase in absorption at 250 mu.



Variation of the mercurial over a thirteen-fold range at a constant protein concentration produced no changes in the reaction rate. An apparent first order rate constant was obtained. This suggests that the rate limiting step must be one which does not involve the mercurial.

These results of the kinetic studies neither verify the dissociation model nor reject the conformational model. In both models a first order rate limiting step may be proposed.

In the dissociation model one may consider a sequence of reactions in which the rate limiting step is the octamer-monomer dissociation; thus first order kinetics would be found.

$$Hr_8 \overset{\text{Slow}}{\rightleftharpoons} 8\ \text{Hr-SH}$$

$$\text{Hr-SH} + \text{HgR} \xrightarrow{\text{fast}} \text{Hr-S-HgR}$$

On the other hand, in the conformational model, the rate limiting step might be the conformational change of the protein. This also does not involve the mercurial. The mercurial reaction with the activated octamer would be a fast reaction, not involved in the rate limiting step.

$$Hr_8 \overset{\text{slow}}{\rightleftharpoons} (Hr\text{-}SH)_8^*$$

$$(Hr\text{-}SH)_8^* + HgR \xrightarrow{\text{fast}} Hr\text{-}S\text{-}HgR$$

Further kinetic data is required to prove the third assumption of the dissociation model.

Since octameric methemerythrin is in dissociative equilibrium with the monomer, decreasing the hemerythrin concentration will therefore produce monomers. If the second order rate constants of hemerythrin reacting with PMB are measured at increasing dilution of the protein, the apparent rate constants should begin reflecting the rate of the monomer. If the monomer is the more reactive species, as model one predicts in the third assumption, dilution of the protein should result in an increase in the apparent second order rate constant.

However, if the conformational model is valid, dilution would not promote the reaction and the apparent second order rate constant would not increase with protein dilution.

The present research is concerned with measurements of the first and second order rate constants for the reaction of methemerythrin with p-mercuribenzoate (PMB) at increasing dilution of hemerythrin, in order to test the third assumption of the dissociation model.

II. EXPERIMENTAL PROCEDURES

A. Materials

p-mercuribenzoic acid was purchased from the Sigma Chemical Co., Phascolosoma Gouldii were obtained from Marine Biological Laboratories, Woods Hole, Massachusetts. All other chemicals were purchased from general commercial sources.

B. Preparation of Reagents

Crystalline oxyhemerythrin and methemerythrin were prepared at 5° C by procedures described by Klotz et. al. (14, 16). The procedure outlined below includes some modifications which were made.

Each batch of hemerythrin was prepared from the coelomic fluid of approximately 100 worms (Phascolosoma gouldii). The worms were washed in 3.1 per cent NaCl, the coelom slit, and the fluid drained into an ice-cooled beaker. The fluid was allowed to clot for about fifteen minutes and then filtered through glass wool. The hemerythrin-containing cells were centrifuged at 17,500 r.p.m. for thirty minutes twice: once to remove the supernatant and again after washing the cells with 3.1% NaCl. A volume of 0.4% NaCl twice the volume of the cells was

added. The solution was laked overnight, covered with a 1:1 mixture of benzene and ether (approximately one ml.).

After centrifuging for thirty minutes at 17,500 r.p.m., to remove cell debris the clear solution was dialyzed overnight against 0.4% NaCl. If no precipitate appeared, the protein was crystallized overnight against an 80:20 mixture of 0.4% NaCl and 95% ethanol. (If precipitation had occurred the suspension was centrifuged to remove the contaminating solid before the crystallization step.) The crystals were centrifuged for fifteen minutes at 10,000 r.p.m., and the supernatant and milky coating were removed. The crystalline protein was redissolved in a minimum volume of 0.4% NaCl (75-100 ml.) and then recrystallized. The purified crystals were redissolved in 0.4% NaCl, and a 0.1 ml. aliquot was removed. The remaining oxy-hemerythrin was dialyzed against one liter of 1.0 M NaCl for three hours.

The aliquot of oxyhemerythrin was diluted 1:10, and the optical density of the diluted sample was read at 500 mu (where oxyhemerythrin has an extinction coefficient of 1100 cm. l. mole-Fe^{-1} (8)) to determine the concentration of iron in the oxyhemerythrin solution. In order to oxidize the iron in the protein, a two-fold excess of potassium ferricyanide was added to the hemerythrin solution. The solution was kept at room

temperature for half an hour and then was placed in the refrigerator overnight.

In order to remove ferri- and ferrocyanide, the methemerythrin was passed through a column of Dowex 1-X8, 200-400 mesh (washed by the method of Hartree (6) and used in the chloride form).

The methemerythrin was first dialyzed seven hours against 1 liter 0.05 M EDTA (brought to pH 7.0 with NaOH) and then two times against one liter 0.05 M Tris-Acetate buffer, pH 7.0. A small sample of the aquomethemerythrin was removed and diluted one tenth. The absorption of the sample was read at 355 mu to determine the approximate concentration of the hemerythrin solution. The extinction coefficient of aquo-methemerythrin at this wavelength is 3220 cm. l. mole-iron^{-1} (8).

(At this point, if aquo-methemerythrin crystals were desired, the solution was dialyzed against 0.4% $NaNO_3$: 95% ethanol in the ratio 95:5.)

Because the kinetic studies described later were performed with fluoride as the ligand bound to the iron, and the aquo form is not as stable as the fluoride form, the protein was dialyzed three times against 0.05 M Tris-Acetate containing 0.1 M NaF, pH 7.0 and stored.

All of the above operations for the preparation of the protein were done at 3-5° C.

The Tris-Acetate buffer was prepared as follows. A 0.2 M Tris and a 0.2 M acetic acid solution (made with the assumption that glacial acetic acid is 17.4 M), were combined to form a pH 7.0 solution. The buffer was diluted 1:4 with water to yield the 0.05 M Tris-Acetate solution. This buffer, pH 7.05 was the base solvent for all solutions used in titrations and kinetics. From it buffers containing 0.1 M F^-, 0.1 M F^- plus 0.05 M Br^-, and 0.1 M F^- plus 8 M urea were made. New buffer was prepared every two weeks, due to pH changes on standing.

Solutions of p-mercuribenzoate (PMB) were prepared either the night before or the day they were to be used, depending on the concentration desired. For solutions with a concentration of approximately 5×10^{-4} M to 1×10^{-3} M, excess PMB was added to 20-50 ml. of the appropriate buffer and allowed to stand overnight, allowing the excess PMB to settle. The clear solution was drawn off and its concentration determined. For higher concentrations of PMB, solutions had to be prepared the day of use. In order to remove excess PMB, centrifugation at 20,000 r.p.m. for one hour was employed. However, two hours of centrifuging sometimes proved less effective in removing excess PMB then settling overnight.

All solutions were passed through glass filters to remove dust.

C. Concentration of Solutions

The concentration of PMB solutions were determined by diluting an aliquot 1:50 and reading the absorption of the diluted sample at 232 mu. The absorption of the buffer was subtracted from that of the sample. The extinction coefficient cited by Boyer (2), 1.69×10^4, was used for determining the concentrations of PMB in 0.1 M F^-, 0.05 M Tris-Acetate and 0.05 M Tris-Acetate buffers. In 0.05 M NaBr, PMB was found to have an extinction coefficient of 1.94×10^4.

The concentration of the sulfhydryl groups in the protein was determined as follows. When the protein solution was prepared, the approximate iron content was found for the aquo-methemerythrin. Knowing that for every two iron atoms, the protein contains one sulfhydryl group, the sulfhydryl content can be approximated. Because the originally prepared protein is too concentrated to study, various dilutions are made for the kinetics. For each specific dilution, the approximate sulfhydryl concentration must be calculated. The exact value is found by a sulfhydryl titration with the diluted protein.

All the titrations of the protein were performed with a fixed concentration of protein and variation of the titrant, PMB. Volumetric flasks and microburets having an accuracy of greater than 0.5% were used. For the following concentrations of diluted protein, the

volumetric flasks and spectrophotometric cells employed are indicated: 1×10^{-4} M SH, 5 ml. flasks, 0.5 mm. cells; 4×10^{-5} M SH, 5 ml. flasks, 1 cm. cells; 1×10^{-5} M SH, 25 ml. flasks, 2 cm. cells; 1×10^{-6} M SH, 100 ml. flasks, 10 cm. cells.

An aliquot of the concentrated protein was added to each flask with the appropriate microburet. The flasks were placed in a 5° C environment for an hour, and removed individually to add a predetermined amount of PMB. Approximately five flasks contained increasing concentrations of PMB less than the SH content, and six contained increasing excess of PMB. Seven times excess was used for 1×10^{-4} M SH, 10 times excess for 1×10^{-5} M SH, and 20 times excess for 1×10^{-6} M SH. Two flasks were prepared with diluted protein only.

Following addition of PMB and final dilution with the appropriate buffer, the flasks were replaced in the 5° C environment, to prevent protein denaturation. Once filled, all the flasks were set in a 10° C water bath for 10 to 14 hours. Absorption readings were made on a Cary 16 at 250 mu in the appropriate cell. Zero reading was made in the same cell with water prior to the reading of each flask. Figure 1 contains the diagram of a typical titration.

This titration has a two-fold purpose. The first is the determination of the sulfhydryl concentration.

The second is the calculation of the total absorption change expected for the complete reaction of the protein with PMB at the various PMB to protein ratios.

The slopes of the two lines in Figure 1 and their y intercepts were determined by least squares. The concentration of PMB at which the two lines intercept is the concentration of the protein sulfhydryl groups. The slope of the line at low PMB concentration divided by cell length is the change in the molar extinction coefficient during the formation of the protein-mercurial complex plus the molar extinction coefficient of PMB. The slope of the line at excess PMB concentrations divided by cell length is the molar extinction coefficient of PMB alone at 250 mu. The difference between the two slopes is the molar extinction coefficient change during the formation of the protein-mercurial complex multiplied by the cell length.

From the calculated $\Delta\varepsilon$, the change in absorption for any amount of PMB reacting with the protein can be determined. For PMB in excess of the protein sulfhydryl groups, all of the sulfhydryl groups will react, limiting the absorption change. Therefore the change in absorption at 250 mu due to complex formation would be:

$$\Delta A_T = \Delta\varepsilon \text{ (Concentration of Protein)(Cell Length)}$$

For concentrations of PMB less than the protein, all of the PMB would be in the protein-PMB complex.

Therefore, the total change in absorption at 250 mu would be:

$$\Delta A_T = \Delta \varepsilon \text{ (Concentration of PMB) (Cell Length)}$$

In order to determine the iron content of methemerythrin a cyanide titration was first tried, since it was believed that cyanide had a high affinity for iron. The titration was first done at pH 8.0 in a Tris-Acetate buffer to minimize formation of HCN. The absorption was read at the characteristic cyanide peaks, 493 mu and 374 mu. No apparent change in absorption was observed at these peaks, up to a 1:1 addition of cyanide to iron.

There are two possible reasons for this result. At pH 8.0 more hydroxy-methemerythrin than expected might be present. Because hydroxy-methemrythrin has a maximum at 362 mu with a similar extinction coefficient to cyanide-methemerythrin, the differences between the two extinction coefficients at 374 mu at this pH may be too insignificant to produce absorption changes. On the other hand cyanide may not complex strongly with iron, and therefore a large excess of cyanide is required for complete binding of iron.

To verify this, a cyanide titration was done in a pH 6.8 Tris-EDTA buffer. A two to one addition of cyanide yielded a small decrease in absorption at 374 mu.

But a marked decrease in absorption was noted with the addition of crystalline potassium cyanide. Thus it appears that cyanide does not bind strongly to the iron in hemerythrin. This was substantiated by a difference spectrum of aquo-methemerythrin with and without potassium cyanide. The spectra of the two were identical. Low concentrations of cyanide are therefore unable to replace the ligand coordinated to the iron in aquo-methemerythrin.

The iron content of methemerythrin was therefore determined directly by slightly modified procedure of Yonetani (19). An aliquot containing 6.25 to 62.5 ug of iron was transferred to a 25 ml. volumetric flask. The sample was dissolved in 0.5 ml. concentrated sulfuric acid and heated gently. At the charring point a few drops of 30% hydrogen peroxide were occasionally added. The sample was heated until a colorless liquid was obtained. It was then diluted with two ml. distilled water, heated slowly, and evaporated to white fumes. The flask was cooled, and the solution diluted with another two ml. of water. This was followed by heating again to white fumes, cooling, and diluting with six ml. distilled water. The solution was heated to boiling and diluted to about ten ml. with distilled water.

To the above solution, two ml. of 0.25% 1,10-phenanthroline, one ml. fresh hydroquinone (1.0%), and five ml. of sodium citrate (25%) were added, in that order.

The pH was adjusted to 3.5 or 4.0 with a one to one dilution of concentrated ammonium hydroxide. Final dilution to 25 ml. was made with distilled water. After mixing well, the flasks were allowed to stand one hour. Two blanks, containing no iron, were prepared in the same manner. The absorption of the solutions was read at 508 mu.

A standard iron solution was prepared from ferrous ammonium sulfate. In order to prevent formation of ferrous hydroxide, 2.5 ml. of concentrated sulfuric acid were added per liter of initial solution. The standard, 1.0×10^{-1} M was diluted 5 ml. to one liter to produce a final standard solution containing 28 ug iron per ml.. Iron determinations with five standard iron solutions gave an extinction coefficient for the iron complex as 0.447 $mumole^{-1}$ $cm.^{-1}$. Yonetani's figure, corrected for differences in the procedure, was 0.459 $umole^{-1}cm^{-1}$ (19). These values are consistant considering modifications in the procedure which could not be corrected for.

D. Procedure for Kinetic Studies

The kinetics of the reaction between PMB and methemerythrin were followed spectrophotometrically on a Cary 16 at 250 mu (2). The temperature of the cell was held constant at $14.0 \pm 0.1^{o}C$ by passing water from a water bath, held at about 10^{o} C, through the cell holder. A thermistor calibrated to 0.01^{o} C was employed for measurement

of the solution temperature within the cell.

Daily labwork was begun by filling a cell to be used for the runs with water, and measuring its temperature with the thermistor. The bath temperature was adjusted until the temperature of the cell read 14.0 ± 0.1° C.

Protein to be used in the reaction was partially diluted to a known volume 12 hours before with filtered buffer and allowed to stand at 5° 0. Dilution to the final concentration was made in the cell with addition of buffer and PMB. However, prior to PMB addition, the protein-buffer solution remained in the cell for one hour when 2, 5, and 10 cm. cells were used, and one-half hour for the smaller cells. This time was generally long enough for the temperature of the solution to reach equilibrium. In the larger cells, where a longer time period was sometimes required, the thermistor was washed with distilled water, dried, and placed in the solution to verify that the temperature was 14.0 ± 0.1° 0 before starting the reaction. A volume of PMB of known concentration, calculated as described previously, was then added to the cell with a Hamilton syringe or a pipet. The cell was shaken by hand and immediately returned to the cell holder. The rate of the reaction indicated by the increase of absorption at 250 mu was followed to 100% completion for small ratios of PMB to protein, and to 60-80% completion for larger ratios.

At the time the protein sulfhydryl concentration had been calculated, the total change in absorption for the reaction had also been calculated. Thus, one could estimate when the reaction was 60-80% completed.

For each reaction, the readings of optical density for the first six minutes were extrapolated to time zero. The extrapolated reading at time zero was taken as A_o. The change in absorption calculated for the initial concentration of PMB and methemerythrin sulfhydryls in the cells (explained previously when determining sulfhydryl content) was added to A_o to find A_∞ . For reactions taken to completion the calculated A_∞ was in close agreement with the observed A_∞ .

The readings of absorbance, A_t, along with A_o and A_∞ , were fit into the equations /17/ and /21/ to find the first and second order rate constants. Calculations were made with an Olivetti Programma. The left hand side of the equation was plotted versus t and the slope of the best straight line was k, the rate constant. In the event of a slightly curved line, the slope was taken at 60% of the reaction.

E. Determination of the Rate Constants

In a reaction involving one reactant in the rate determining step, the rate constant k is determined from the differential equation,

$$/1/ \quad -\frac{dc}{dt} = k [c]^n$$

where t is time, $[c]$ is the concentration of the reactant, and n is the order of the reaction. If n = 1, integration of /1/ from zero to t produces:

$$/2/ \quad \ln \frac{c_o}{c_t} = kt$$

Since $c_o - p = c_t$, where p is the product,

$$/3/ \quad \ln \frac{c_o}{c_o - p} = kt$$

If the logarithm of $c_o/c_o - p$ is plotted against t, a straight line of slope k is obtained.

For a reaction between two reactants, a and b, the rate determining step may be dependent upon only one reactant and follow the kinetics of a first order reaction /3/, or may be second order, first order in both a and b. If $a + b \longrightarrow p$, then,

$$/4/ \quad \frac{dp}{dt} = k [a] [b]$$

Integration yields:

$$/5/ \quad \frac{1}{b_o - a_o} \ln \frac{a_o (b_o - p)}{b_o (a_o - p)} = kt$$

Again a plot of the left hand of the equation versus time would form a straight line with slope k.

Since the reaction of hemerythrin and PMB could be either first or second order, both plots were employed.

However, the concentrations of hemerythrin, PMB, and product were measured indirectly by absorption changes. Equations /3/ and /5/ therefore had to be put into more applicable forms, which are derived below.

We know that if a + b$\longrightarrow$p, and all species absorb at the wavelength used, then:

$$/6/ \quad A_o = \varepsilon_a a_o + \varepsilon_b b_o$$

$$/7/ \quad A_t = \varepsilon_a a_t + \varepsilon_b b_t + \varepsilon_p p_t$$

$$/8/ \quad A_\infty = \varepsilon_a a_\infty + \varepsilon_b b_\infty + \varepsilon_p p_\infty$$

where A is absorption; ε_a, ε_b, and ε_p are extinction coefficients of reactants a and b, and product p, respectively; and subscripts o, t, and ∞ refer to zero time, an arbitrary time during the course of the reaction, and time at the completion of the reaction, respectively.

$$/9/ \quad a_o - p_t = a_t$$

$$/10/ \quad b_o - p_t = b_t$$

$$/11/ \quad a_o - p_\infty = a_\infty$$

$$/12/ \quad b_o - p_\infty = b_\infty$$

Combining equations /6/ through /12/ yields:

$$/13/ \quad A_t - A_o = p_t (\varepsilon_p - \varepsilon_a - \varepsilon_b)$$

$$/14/ \quad A_\infty - A_o = p_\infty (\varepsilon_p - \varepsilon_a - \varepsilon_b)$$

Subtracting /13/ from /14/ yields:

$$/15/ \quad A_\infty - A_t = (p_\infty - p_t)\ (\varepsilon_p - \varepsilon_a - \varepsilon_b)$$

The first order rate equation is formed by dividing /14/ by /15/.

$$/16/ \quad \frac{A_\infty - A_o}{A_\infty - A_t} = \frac{p_\infty}{p_\infty - p_t}$$

If $b_o \gg a_o$, $p_\infty = a_o$, and if $a_o \gg b_o$, $p_\infty = b_o$.
Equation /16/ substituted into /3/ gives:

$$/17/ \quad \ln \frac{A_\infty - A_o}{A_\infty - A_t} = kt$$

Equation /17/ is the equation used for the calculation of the first order rate constant k.

For the second order rate constant, equation /13/ is substituted into /5/ to give:

$$/18/ \quad \frac{1}{b_o - a_o} \ln \frac{a_o}{b_o} \cdot \frac{b_o - \dfrac{A_t - A_o}{\varepsilon_p - \varepsilon_a - \varepsilon_b}}{a_o - \dfrac{A_t - A_o}{\varepsilon_p - \varepsilon_a - \varepsilon_b}} = kt$$

Substituting /14/ into /18/ produces:

$$/19/ \quad \frac{1}{b_o - a_o} \ln \frac{a_o}{b_o} \cdot \frac{b_o - \dfrac{A_t - A_o}{A_\infty - A_o} \cdot p_\infty}{a_o - \dfrac{A_t - A_o}{A_\infty - A_o} \cdot p_\infty} = kt$$

Rearrangement yields:

$$/20/\quad \frac{1}{b_o - a_o} \ln \frac{a_o}{b_o} \cdot \frac{(A_\infty - A_o)b_o - (A_t - A_o)(p_\infty)}{(A_\infty - A_o)a_o - (A_t - A_o)(p_\infty)} = kt$$

If $b_o \gg a_o$, $p_\infty = a_o$. Therefore, p can be substituted into /20/ to give:

$$/21/\quad \frac{1}{b_o - a_o} \ln \frac{(A_\infty - A_o)b_o - (A_t - A_o)a_o}{b_o (A_\infty - A_t)} = kt$$

If $a_o \gg b_o$, $p_\infty = b_o$, and the equation is the same form as /21/, but a_o and b_o are interchanged. Thus, equation /21/ is used for calculation of the second order rate constant k. The reactant in larger quantity is b_o, the one of smaller concentration is a_o. A plot is made of the left hand side of the equation against t , and the slope of the best straight line is k.

III. EXPERIMENTAL RESULTS AND DISCUSSION

A. Fluoride Binding to Iron

Aquo-methemerythrin has a characteristic peak at 355 mu, while fluoro-methemerythrin has characteristic peaks at 317 mu and 362 mu. A comparison of their spectrum (9) indicates that the largest differences in the extinction coefficients are near 355 mu. This peak was used for determining the extent of binding of fluoride to iron in a one-tenth molar fluoride solution. The decrease in absorption with increasing fluoride is shown in Figure 2. The curve suggests that in a 0.1 M fluoride solution the iron in methemerythrin is at least ninety percent saturated with fluoride.

B. Consequences of Protein Conformational Changes

Because the protein absorbs at 250 mu, any conformational changes it undergoes during the reaction might be observed as a change in absorption at 250 mu. Such a change would effect the increase in absorption used to measure the sulfhydryl reaction. In order to determine contributions due to protein conformational changes, the extinction coefficients of the various species in the reaction mixture were determined.

Titrations were performed in 0.1 M F^-, 0.05 M Tris-Acetate buffer plus 8 M urea and in 0.1 M F^-, 0.05 M Tris-Acetate. The extinction coefficient of PMB and the change in extinction coefficient during the protein-mercurial complex formation were obtained as discussed in the methods section and are listed in Table I. As indicated in the table, the extinction coefficients are independent of protein concentration.

The observed change in absorption is due to the decrease in absorption when protein and PMB are consumed in the reaction and the increase in absorption resulting from the formation of the complex. When the protein and PMB are in a one to one ratio:

$$/22/ \quad \Delta A = A_{p\text{-}PMB} - A_{protein} - A_{PMB}$$

This may also be written in the form of extinction coefficients.

$$/23/ \quad \Delta\varepsilon = \varepsilon_{p\text{-}PMB} - \varepsilon_{protein} - \varepsilon_{PMB}$$

The respective values for $\Delta\varepsilon$, ε_{PMB}, and $\varepsilon_{protein}$, may be substituted into /23/ to find the extinction coefficient of the protein-PMB complex in both fluoride and urea. (Table II)

Assuming that the absorption of the protein-PMB complex is composed of two additive parts, the sulfur-PMB grouping and the rest of the protein, then:

$$/24/ \quad \varepsilon_{p\text{-PMB}} = \varepsilon_{protein} + \varepsilon_{S\text{-PMB}}$$

If we further assume that the extinction coefficient of the protein does not change with formation of the complex, the extinction coefficient for the S-PMB complex can be calculated (Table II).

Boyer (2) has determined the change in extinction coefficient with the sulfhydryl of cysteine at pH 7.0 in an acetate buffer. In that buffer system PMB should have the same extinction coefficient as in Tris-Acetate or fluoride Tris-Acetate. Since the absorption of cysteine is small at 250 mu, the extinction coefficient for cysteine-PMB may be estimated from his data. (Table II)

The value calculated from Boyer's data is within experimental error of the value for the calculated extinction coefficient of the -S-PMB grouping in the protein dissolved in fluoride. This suggests that the assumption that the protein absorption at 250 mu does not change when its sulfhydryl groups react with PMB is valid. Therefore, the data suggests that those conformational changes which occur during the reaction are not reflected by a change in absorption at 250 mu. The differences between the extinction coefficients of the -S-PMB complex when protein is in urea and fluoride may be due to environmental differences. It is known that PMB absorbs differently in urea and in water.

Therefore it is likely that -S-PMB would have a different extinction coefficient in the urea environment than in the water environment. One must remember, however, that the similarities between the extinction coefficients of -S-PMB for protein and cysteine may be fortuitous. The absorption of the protein in fluoride could undergo a change when the protein-mercurial complex is formed, but this could be compensated by a change in the extinction coefficient of the -S-PMB complex. For these reasons no definitive conclusions can be drawn. Another important point found in Table I, is the fact that the extinction coefficient of PMB alone and in the presence of protein is the same as the extinction coefficient in the absence of protein. This suggests that either PMB binds only at the sulfhydryl site, or that if there is binding at a second site, only the reaction at the sulfhydryl site causes an absorbancy change at 250 mu.

D. Concentration of Sulfhydryl Groups and Iron

In Table I, three protein concentrations are marked with an asterisk. These sulfhydryl titrations were done on the same protein solution. The average concentration of the sulfhydryl groups was 1.86×10^{-3} M. An iron determination performed on the same protein showed the iron content was 3.35×10^{-3} M. The protein therefore contains 1.80 iron atoms for every sulfhydryl group.

These results are consistant within experimental error with the iron to sulfhydryl ratio 2:1 reported by Keresztes-Nagy and Klotz (9).

D. Rate Constants

The first and second order rate constants for the reaction of PMB with hemerythrin were calculated for various concentrations of PMB at four different hemerythrin concentrations. (See Tables III, IV, V, VI) The values for the apparent first and second order rate constants were obtained from the slopes of graphs, such as the ones in Figures 3 and 4.

The variations of the apparent rate constants listed in Tables III, IV, V, and VI indicate this is not a simple first or second order reaction. The first order rate constant decreases to an approximately one to one ratio of PMB to protein sulfhydryls, and then increases with excess PMB. (See Figure 5) The second order rate constant decreases with increasing PMB concentration and then begins to level off. (Figure 6) The data suggests that PMB is definitely involved in the rate determining step.

The data suggests the formation of a PMB-protein complex at a site other than the sulfhydryl. With this assumption, the following mechanism may be written:

$$(1)\quad \mathrm{Hr} + \mathrm{PMB} \overset{\text{fast}}{\rightleftharpoons} \mathrm{PMB\text{-}Hr\text{-}SH}$$

(2) PMB-Hr-SH + Hr $\xrightarrow{k_1}$ Hr-S-HgR + Hr

(3) PMB + Hr $\xrightarrow{k_2}$ Hr-S-HgR

(4) PMB-Hr-SH + PMB $\xrightarrow{k_3}$ Hr-S-HgR + PMB or PMB-Hr-S-HgR

(5) PMB-Hr-SH $\xrightarrow{k_4}$ Hr-S-HgR

As the reactant ratio departs from one to one, the free hemerythrin or PMB increases and the rate would also increase.

If the initial binding of PMB to protein is correct and the benzoate portion of PMB is binding to a second site on the protein, an excess of benzoic acid should displace the PMB and therefore change the reaction kinetics.

The reaction of PMB with fluoromethemerythrin was performed in the presence of 3×10^{-4} M benzoic acid. Since the protein concentration was 3.88×10^{-5} M, nine times excess benzoic acid seemed sufficient to replace the PMB at the second site. Table VII contains the rate constants for the reactions of hemerythrin and PMB in the benzoic acid. When the first and second order rate constants are compared with rates of the reaction under identical conditions but without benzoic acid (Table IV), no appreciable differences are found. This is apparent in Figure 7, where the concentration of PMB is plotted against the first order rate constants for both cases. If PMB binds to another site, benzoic acid is not capable of replacing it.

These results suggest that if PMB is bound to the second site on the protein, it is attached through the mercury atom. In such a case, it is probably bound to the hemerythrin at a nitrogen or oxygen atom. If the mercury were blocked by an ion which binds more strongly than oxygen or nitrogen, but less than sulfur, the reaction would be forced to proceed through,

$$(3)\quad \text{PMB} + \text{HrSH} \xrightarrow{k_2} \text{Hr-S-HgR}$$

and the reaction rate should increase.

Bromide is an ion which binds strongly enough to mercury to prevent complex formation at a second site on the protein (18). Bromide binds less strongly to iron than fluoride (18) and therefore should not coordinate with the protein iron in the presence of fluoride. Kinetics were performed at one protein concentration under previous conditions, but in the presence of 0.05 M bromide. The first order rate constants were calculated and are listed in Table VIII. A comparison of these rate constants with those in Table VI shows that the overall rate of the reaction was increased for concentrations of PMB below and above the protein sulfhydryl concentration. The bromide enhancement suggests that PMB may be bound at a second site on the protein through the mercury atom.

The decrease in the second order rate constants with increasing PMB concentration in the presence of

fluoride (Figure 6) suggests that PMB is inhibiting the overall reaction when it forms a complex with the protein, (found in reaction (1)). There are two forms of inhibition: one, binding would decrease the rate of the sulfhydryl reaction; two, binding would lower the apparent concentration of PMB and thus lower the apparent rate. The form of inhibition can become apparent by studying the variation of the first order rate constants (Figure 5). The minimum of the first order rate constants suggests that a tightly bound complex exists between one PMB molecule and one protein monomer. When the protein sulfhydryl is in excess, the first order rate constants are high, even though some type of inhibition exists, as found in the increased rate with bromide. Above a one to one ratio of PMB to protein, the first order rate constants are extremely low, even though free PMB should be available for the reaction with the sulfhydryl groups. If the inhibition were due to a decrease in the apparent PMB concentration, first order rate constants at ratios of PMB to protein sulfhydryls greater than two should be larger that the rate constants for ratios less than one. As Figure 5 indicates, rates for up to ten times excess PMB do not exceed or even approach the first order rates for PMB: sulfhydryl ratios less than one. Therefore, it appears that the decrease in reaction rate must be due to a decrease in the sulfhydryl reactivity.

If we accept that there is binding between PMB and protein as the data suggests, at excess PMB the protein should exist completely in the bound form. In such a case reactions (4) and (5) would be the only significant reactions occurring. The rate of the overall reaction for the mechanism would be:

$$\frac{dp}{dt} = k_3 \quad (\text{PMB-HrSH}) \quad (\text{PMB}) + k_4 \ (\text{PMB-HrSH})$$

from which it follows that:

$$k_{app.} = k_3 \ (\text{PMB}) + k_4$$

Thus, plotting the apparent first order rate constant versus PMB concentration should yield a straight line with a slope equal to k_3 and the intercept equal to k_4. The plot of $k_{app.}$ versus the initial PMB to initial sulfhydryl ratio is shown in Figure 8, and is a straight line as predicted. The rate constants from the graph are given in Table IX.

The experimental error in the rate constants is indicated in Table IX. It is primarily due to the difficulty in obtaining an accurate A_o and thus A_∞ when the PMB was in excess.

These results indicate that the overall reaction in excess PMB is comprised of a first and a second order reaction. The first order reaction could be the reaction (5) proposed in the mechanism.

(5) $\text{PMB-HrSH} \xrightarrow{k_4} \text{Hr-S-HgR}$

Because the rate constant does not vary with protein concentration, it appears that the ratio of octamer and monomer, which decreases upon dilution does not effect the rate of this reaction. Therefore, the proposed reaction could have a conformational rearrangement of the complex as the rate limiting step.

From Figure 7 we find that the second order rate constants at excess PMB increase with dilution of the protein. If the mechanism is correct, then the rate constants would be for the reaction:

(4) $\text{PMB-HrSH} + \text{PMB} \xrightarrow{k_3} \text{PMB-Hr-S-HgR or PMB} + \text{Hr-S-HgR}$

Dilution of the protein increases the monomer at the expense of the octamer. If fluoride has the same effect on the dissociative equilibrium as thiocyanate, measured by Klapper, Barlow, and Klotz (11), the protein concentrations from the highest to lowest contain approximately 8, 14, 31, and 100 percent monomer. Therefore the PMB-protein complex would be composed almost entirely of octamer at the highest protein concentration and 100 per cent monomer at the lowest protein concentration. Since k_2 increases with dilution, our model indicates that the monomer is more reactive than the octamer in the protein-PMB complex. Moreover, the large difference between the rate

constants of the highest and lowest concentration suggests that the octamer does not react significantly. This data therefore supports the third assumption of the dissociation model, that the monomer is more reactive than the octamer.

In Figures 3 and 4 the appearance of the steep slope in the first few minutes suggests that an initial fast reaction occurs in the PMB-hemerythrin reaction. The fast reaction may be due to the initial equilibrium in the proposed mechanism. The sulfhydryl groups of the unbound protein, which is not inhibited by PMB, could react very quickly and produce the fast initial change in absorption. Once a sufficient amount of PMB has complexed with the protein, the system will reach equilibrium, and the fast reaction appears to cease. However the fast initial absorption change could also be due to absorption of the PMB-protein complex itself. In either case studies of the kinetics in the presence of bromide should eliminate the fast reaction if it is due to the formation of the protein-PMB complex.

If the PMB-protein complex does absorb, kinetics would be altered significantly, since the rate of the sulfhydryl reaction is measured by the change in absorption. If the protein-PMB complex formation produces a change in the extinction coefficient, one would expect a protein-PMB complex with reacted sulfhydryl groups (PMB-Hr-S-HgR) to perform in a similar manner. From sulfhydryl titrations

it is known that if the reacted complex (which is a possible product in reaction (4)) exists as a final product in the reaction between excess PMB and hemerythrin, it does not produce an absorption change. The extinction coefficient of PMB in the presence of the reacted protein is the same as the extinction coefficient of PMB alone. Therefore if it can be determined that the reacted complex does exist as a final product, it can be assumed that the formation of the PMB-protein complex does not produce an absorption change.

IV. SUMMARY

Klapper, Barlow and Klotz proposed two mechanisms for the reaction of the sulfhydryl groups of hemerythrin with sulfhydryl reagent to explain cooperative interactions. In the dissociation model, three assumptions are made: (1) an octamer-monomer equilibrium exists; (2) the ligand bound to the iron has a higher binding affinity for the monomer and shifts the equilibrium to the right; (3) the monomer is more reactive than the octamer. The first two assumptions have been proven. The purpose of this investigation was to study the kinetics of the reaction between the hemerythrin and p-mercuribenzoate and determine if the third assumption holds true.

The first and second order rate constants were measured at four different protein concentrations. The first order rate constants within a given protein concentration were found to decrease sharply up to a one to one ratio of PMB to protein and then slowly increase. The second order rate constants decreased with increasing PMB and slowly leveled off when excess PMB was added to the protein. These results suggested a mechanism which assumed binding of PMB to a second site on the protein, which inhibits the sulfhydryl reaction. A plot of the first order

rate constants showed that with excess PMB, a second and a first order reaction occur simultaneously. The rate constant of the first order reaction was found to be constant throughout the protein concentrations. The first order reaction is therefore unaffected by a monomer or octamer concentration, and the rate limiting step could be the rearrangement of the protein-mercurial complex.

The apparent second order rate constants increased with dilution of the protein. Since a higher percentage of monomer is present in the less concentrated protein, the increase in the rate constant is most likely due to the greater reactivity of the monomer than the octamer. This suggests that the third assumption is correct.

From experiments with benzoic acid, it was found that PMB does not bind to the protein through the benzoic acid portion of the molecule. The binding must therefore occur between the mercury and nitrogen or oxygen in the protein. Preliminary studies have been done in the presence of bromide which suggests that binding does occur in this manner.

APPENDIX

TABLE I

EXTINCTION COEFFICIENTS FROM SULFHYDRYL TITRATIONS

Protein Sulfhydryl Concentration M x 10^4		$\Delta\varepsilon + \varepsilon_{PMB}$ x 10^{-3}	ε_{PMB} x 10^{-3}	$\Delta\varepsilon$ x 10^{-3}	Conditions
* 18.6		13.72	4.71	9.01	10 hr.
1.276		12.96	5.26	7.70	10 hr.
1.226		12.99	5.54	7.45	10 hr.
1.16		12.93	4.93	8.00	14 hr.
0.129		12.91	4.95	7.96	10 hr.
0.119		13.35	4.69	8.66	14 hr.
0.114		12.70	4.77	7.93	14 hr.
0.0114		13.13	4.93	8.20	14 hr.
	Ave.	13.00 $\pm$ 0.08	4.95 $\pm$ 0.09	8.05	
* 18.1		9.95	6.56	3.39	8 M urea, 2 hr.
* 19.1		10.03	6.68	3.35	8 M urea, 2 hr.
	Ave.	9.99	6.62	3.37	
			4.86		PMB alone in buffer
			4.88		PMB alone in buffer
			6.62		PMB alone in 8 M urea

All solutions were kept in a water bath at 10°C for the time indicated, and contained 0.1 M F^-, 0.05 M Tris-Acetate, pH 7.0.

*Triplicate sulfhydryl titrations on the same protein solution.

TABLE II

AVERAGE EXTINCTION COEFFICIENTS AT 250 mu

	$\Delta\varepsilon$ x 10^{-3}	ε_{PMB} x 10^{-3}	$\varepsilon_{protein}$ x 10^{-3}	$\varepsilon_{p\text{-}PMB}$ x 10^{-3}	$\varepsilon_{S\text{-}PMB}$ x 10^{-3}	
Methemerythrin in Fluoride	8.05	4.95	15.8	28.8	13.00	1
Methemerythrin in Urea	3.37	6.62	13.8	23.8	9.99	1
Cysteine	7.6	4.95	--	--	12.6	3

1. Calculated on the basis of the assumption given in equation [24], and on the assumption that protein absorption does not change at 250 mu during the reaction with PMB.

2. Obtained from Boyer (2).

3. Calculated from the relationship

$$\Delta\varepsilon = \varepsilon_{cys\text{-}PMB} - \varepsilon_{PMB}$$

TABLE III

APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 1.164×10^{-4} M

Initial PMB Concentration	Average k_1	Average k_2	PMB / protein
7.64×10^{-4}	0.0135 min^{-1}	19.2 l $mole^{-1}$ min^{-1}	6.56
6.53×10^{-4}	0.0123 min^{-1}	21.4 l $mole^{-1}$ min^{-1}	5.61
5.01×10^{-4}	0.0105 min^{-1}	23.5 l $mole^{-1}$ min^{-1}	4.30
4.01×10^{-4}	0.00939 min^{-1}	23.6 l $mole^{-1}$ min^{-1}	3.44
2.18×10^{-4}	0.00670 min^{-1}	47.6 l $mole^{-1}$ min^{-1}	1.89
1.17×10^{-4}	0.00376 min^{-1}	85.7 l $mole^{-1}$ min^{-1}	1.01
8.06×10^{-5}	0.0119 min^{-1}	164 l $mole^{-1}$ min^{-1}	0.692
4.85×10^{-5}	0.0401 min^{-1}	538 l $mole^{-1}$ min^{-1}	0.417

The rate constants were calculated as described previously. Kinetics were performed in 0.05 M Tris-Acetate, 0.1 M NaF, pH 7.0 at 14.0 ± 0.1 °C.

TABLE IV

APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 3.88×10^{-5} M

Initial PMB Concentration	Average k_1	Average k_2	$\frac{\text{PMB}}{\text{protein}}$
3.88×10^{-4}	0.0148 min^{-1}	40.5 l $mole^{-1}$ min^{-1}	10.00
3.03×10^{-4}	0.0135 min^{-1}	48.2 l $mole^{-1}$ min^{-1}	7.80
2.12×10^{-4}	0.0130 min^{-1}	69.6 l $mole^{-1}$ min^{-1}	5.47
1.53×10^{-4}	0.0120 min^{-1}	94.9 l $mole^{-1}$ min^{-1}	3.94
7.44×10^{-5}	0.0102 min^{-1}	198 l $mole^{-1}$ min^{-1}	1.92
3.13×10^{-5}	0.0103 min^{-1}	502 l $mole^{-1}$ min^{-1}	0.805
2.42×10^{-5}	0.0158 min^{-1}	653 l $mole^{-1}$ min^{-1}	0.622
1.77×10^{-5}	0.0380 min^{-1}	1300 l $mole^{-1}$ min^{-1}	0.457
1.21×10^{-5}	0.0868 min^{-1}	2790 l $mole^{-1}$ min^{-1}	0.311

The rate constants were calculated as described previously in the methods section. Kinetics were performed in 0.05 M Tris-Acetate, 0.1 M NaF, pH 7.0 at 14.0 $\pm$ 0.1 ^{o}C.

TABLE V

APPARENT EIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 1.16×10^{-5} M

Initial PMB Concentration	Average k_1	Average k_2	PMB / protein
1.02×10^{-4}	0.0189 min^{-1}	201 l $mole^{-1}$ min^{-1}	8.71
8.11×10^{-5}	0.0157 min^{-1}	206 l $mole^{-1}$ min^{-1}	6.96
6.11×10^{-5}	0.0140 min^{-1}	262 l $mole^{-1}$ min^{-1}	5.24
4.05×10^{-5}	0.0109 min^{-1}	316 l $mole^{-1}$ min^{-1}	3.48
2.16×10^{-5}	0.00835 min^{-1}	540 l $mole^{-1}$ min^{-1}	1.86
9.73×10^{-6}	0.00891 min^{-1}	1420 l $mole^{-1}$ min^{-1}	0.836
7.78×10^{-6}	0.0141 min^{-1}	2060 l $mole^{-1}$ min^{-1}	0.669
5.84×10^{-6}	0.0265 min^{-1}	3310 l $mole^{-1}$ min^{-1}	0.590
3.73×10^{-6}	0.0587 min^{-1}	6240 l $mole^{-1}$ min^{-1}	0.320

The rate constants were calculated as described previously in the methods section. Kinetics were performed in 0.05 M Tris-Acetate, 0.1 M NaF, pH 7.0 at 14.0 $\pm$ 0.1 °C.

TABLE VI

APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 2.33×10^{-6} M

Initial PMB Concentration	Average k_1	Average k_2	PMB / protein
4.49×10^{-5}	0.0286 min^{-1}	689 l $mole^{-1}$ min^{-1}	19.29
3.29×10^{-5}	0.0214 min^{-1}	697 l $mole^{-1}$ min^{-1}	14.15
2.03×10^{-5}	0.0174 min^{-1}	896 l $mole^{-1}$ min^{-1}	8.72
1.22×10^{-5}	0.0120 min^{-1}	1,110 l $mole^{-1}$ min^{-1}	5.24
7.92×10^{-6}	0.00814 min^{-1}	1,230 l $mole^{-1}$ min^{-1}	3.40
4.19×10^{-6}	0.00715 min^{-1}	2,450 l $mole^{-1}$ min^{-1}	1.80
2.10×10^{-6}	0.00696 min^{-1}	5,610 l $mole^{-1}$ min^{-1}	0.900
1.58×10^{-6}	0.0122 min^{-1}	7,610 l $mole^{-1}$ min^{-1}	0.680
1.19×10^{-6}	0.0215 min^{-1}	12,800 l $mole^{-1}$ min^{-1}	0.510
7.92×10^{-7}	0.0397 min^{-1}	20,200 l $mole^{-1}$ min^{-1}	0.340

The rate constants were calculated as described previously in the methods section. Kinetics were performed in 0.05 Tris-Acetate, 0.1 M NaF, pH 7.0 at 14.0 ± 0.1 °C.

TABLE VII

APPARENT FIRST AND SECOND ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 3.88×10^{-5} M IN THE PRESENCE OF BENZOIC ACID

Initial PMB Concentration	Average k_1	Average k_2	$\frac{PMB}{protein}$
3.11×10^{-5}	0.0108 min^{-1}	540 l $mole^{-1}$ min^{-1}	0.804
1.84×10^{-5}	0.0293 min^{-1}	1160 l $mole^{-1}$ min^{-1}	0.474
1.20×10^{-5}	0.0702 min^{-1}	3070 l $mole^{-1}$ min^{-1}	0.308
5.52×10^{-6}	0.282 min^{-1}	8960 l $mole^{-1}$ min^{-1}	0.142

The rate constants were calculated as described previously in the methods section. Kinetics were performed in 0.05 M Tris-Acetate, 0.1 M NaF, 3×10^{-4} M benzoic acid, pH 7.0 at 14.0 ± 0.1 °C.

TABLE VIII

APPARENT FIRST ORDER RATE CONSTANTS FOR THE PROTEIN SULFHYDRYL CONCENTRATION 2.33×10^{-6} M IN THE PRESENCE OF BROMIDE

Initial PMB Concentration	Average k_1	$\frac{\text{PMB}}{\text{protein}}$
3.59×10^{-5}	0.0376 min^{-1}	15.57
1.88×10^{-5}	0.0215 min^{-1}	8.12
3.93×10^{-6}	0.0229 min^{-1}	1.69
7.06×10^{-7}	0.100 min^{-1}	0.303

The rate constants were calculated as described previously in the methods section. Kinetics were performed in 0.05 M Tris-Acetate, 0.1 M NaF, 0.05 M NaBr, pH 7.0 at 14.0 ± 0.1 °C.

TABLE IX

FIRST AND SECOND ORDER RATE CONSTANTS CALCULATED FROM THE APPARENT FIRST ORDER RATE CONSTANTS MEASURED AT EXCESS PMB

Protein Sulfhydryl Concentration	k_2	k_1
1.16×10^{-4}	$11.4 \pm 0.5\%$ 1 $mole^{-1} min^{-1}$	0.0048 min^{-1}
3.88×10^{-5}	$13.6 \pm 1\%$ 1 $mole^{-1} min^{-1}$	0.0096 min^{-1}
1.16×10^{-5}	$129 \pm 1\%$ 1 $mole^{-1} min^{-1}$	0.0057 min^{-1}
2.33×10^{-6}	$524 \pm 3.5\%$ 1 $mole^{-1} min^{-1}$	0.0051 min^{-1}
		0.0063 ± 0.0013

The rate constants, which are the slopes of the lines and their intercepts in Figure 8, were calculated by least squares. The standard deviation of the k_{app} was used to determine the percent error in k_2.

Figure 1

SULFHYDRYL TITRATION WITH PMB

The titration was performed at 250 mu in 0.05 M Tris-Acetate, 0.1 M NaF, pH 7.0 at 14.0 °C.

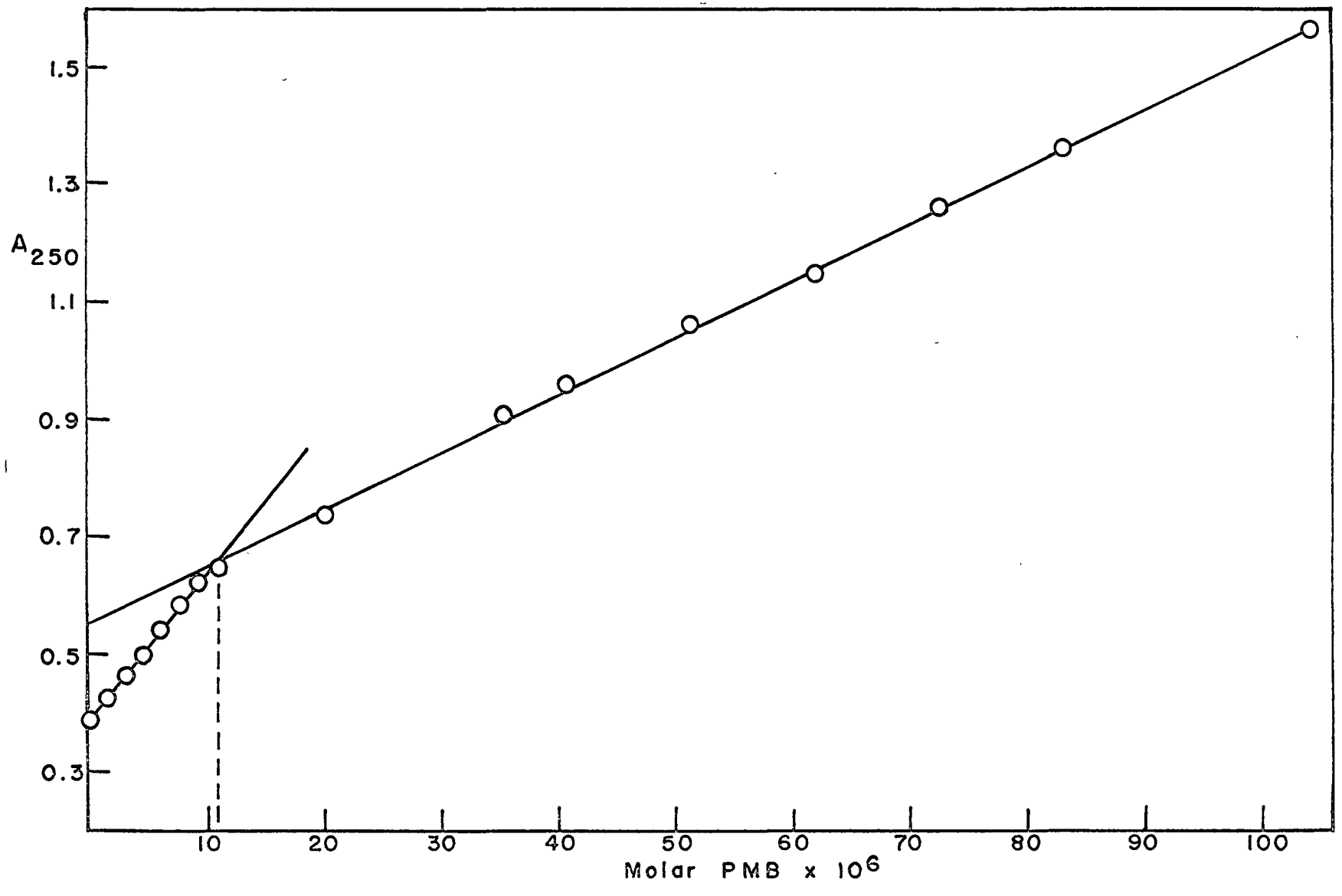

1.5
1.3
A_{250}
1.1
0.9
0.7
0.5
0.3
10
20
30
40
50
60
70
80
90
100
Molar PMB x 10^6

Figure 2

DECREASE IN ABSORPTION OF AQUO-METHEMERYTHRIN WITH FLUORIDE ADDITION

Titration was performed on a protein sulfhydryl concentration 1.4×10^{-5} M in 0.05 M Tris-Acetate, pH 7.0 at 14.0 °C.

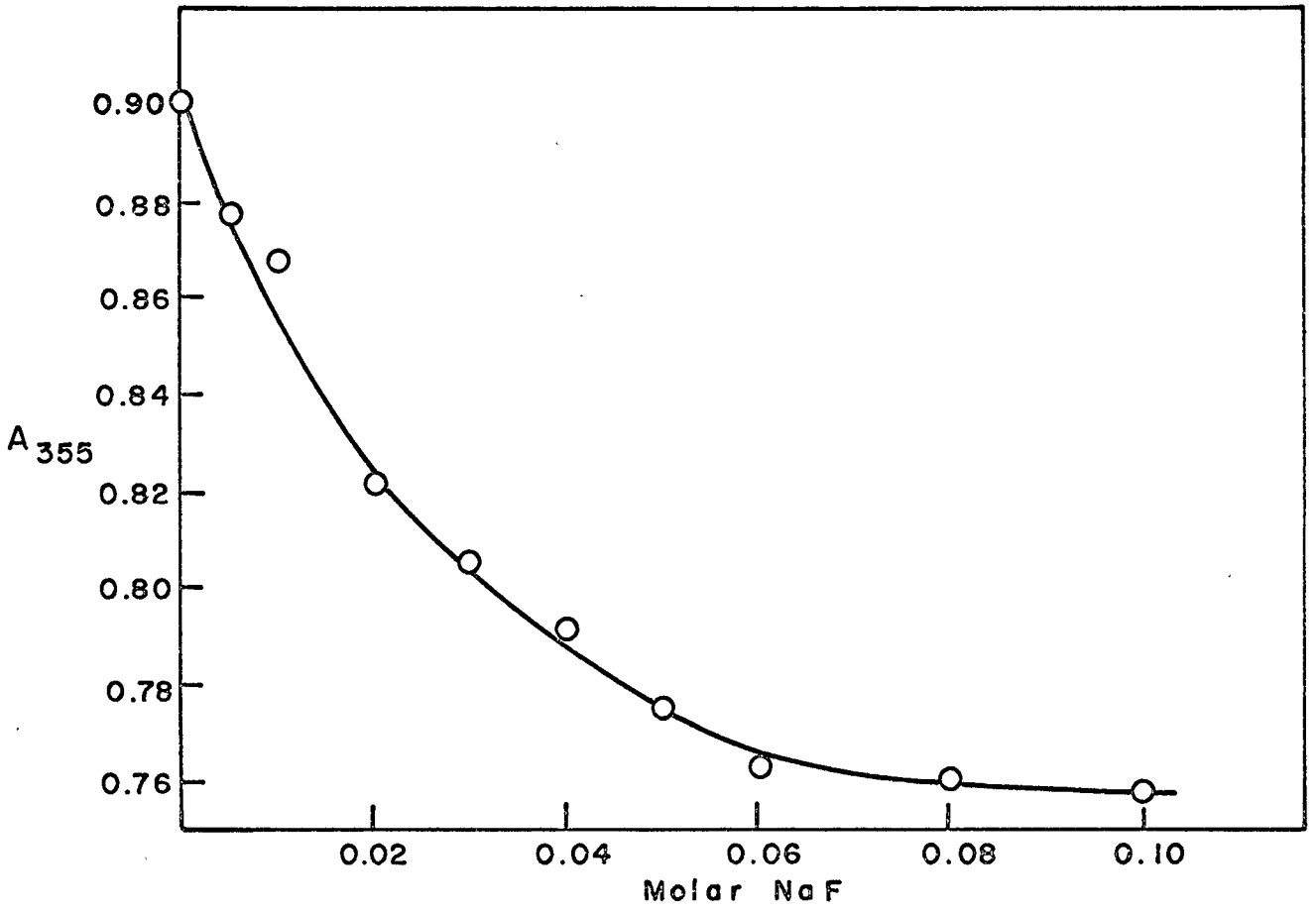

0.90
0.88
0.86
0.84
A 355
0.82
0.80
0.78
0.76
0.02
0.04
0.06
0.08
0.10
Molar NaF

Figure 3

PLOT OF FIRST ORDER RATE EQUATION

TO FIND k_1

The left hand side of equation /17/ was plotted versus time. Slope of line is k_1. The kinetics were performed in 0.05 M Tris-Acetate, 0.I M Fluoride, pH 7.0 at 14.0 °C. Sulfhydryl concentration was 3.88×10^{-5} M; PMB concentration was 7.44×10^{-5} M.

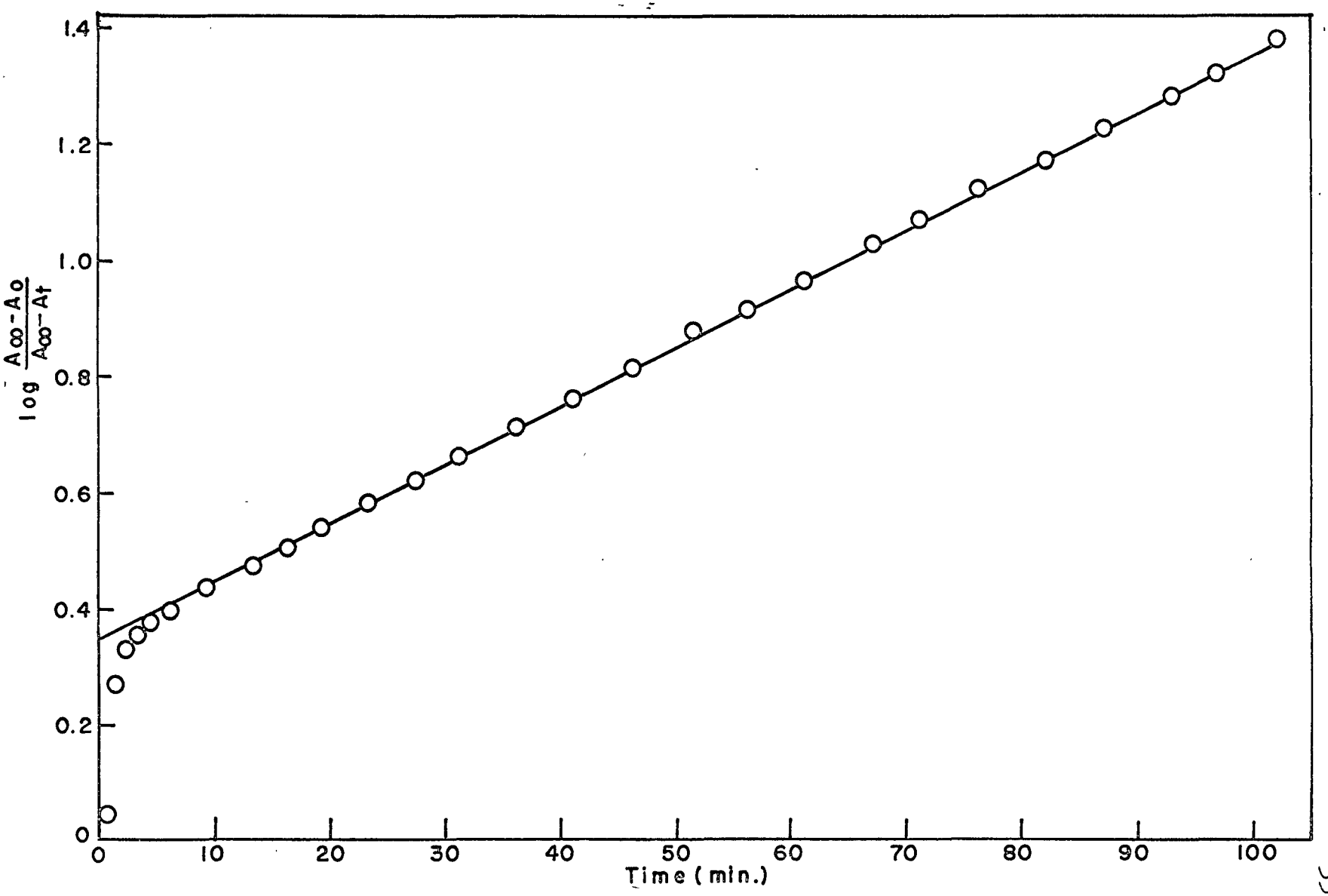

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
$\log \frac{A_\infty - A_0}{A_\infty - A_t}$
0
10
20
30
40
50
60
70
80
90
100
Time (min.)

Figure 4

PLOT OF SECOND ORDER RATE EQUATION

TO FIND k_2

The left hand side of equation /21/ was plotted versus time. Slope of line is k_2. The kinetics were performed in 0.05 M Tris-Acetate, 0.1 M Fluoride, pH 7.0 at 14.0 °C. Sulfhydryl concentration was 3.88×10^{-5} M; PMB was 7.44×10^{-5} M.

$$\left[\frac{1}{b_o - a_o} \ln \frac{(A\infty - A_o)(b_o) - (A_t - A_o)a_o}{b_o\,(A\infty - A_t)}\right] \times 10^{-3}$$

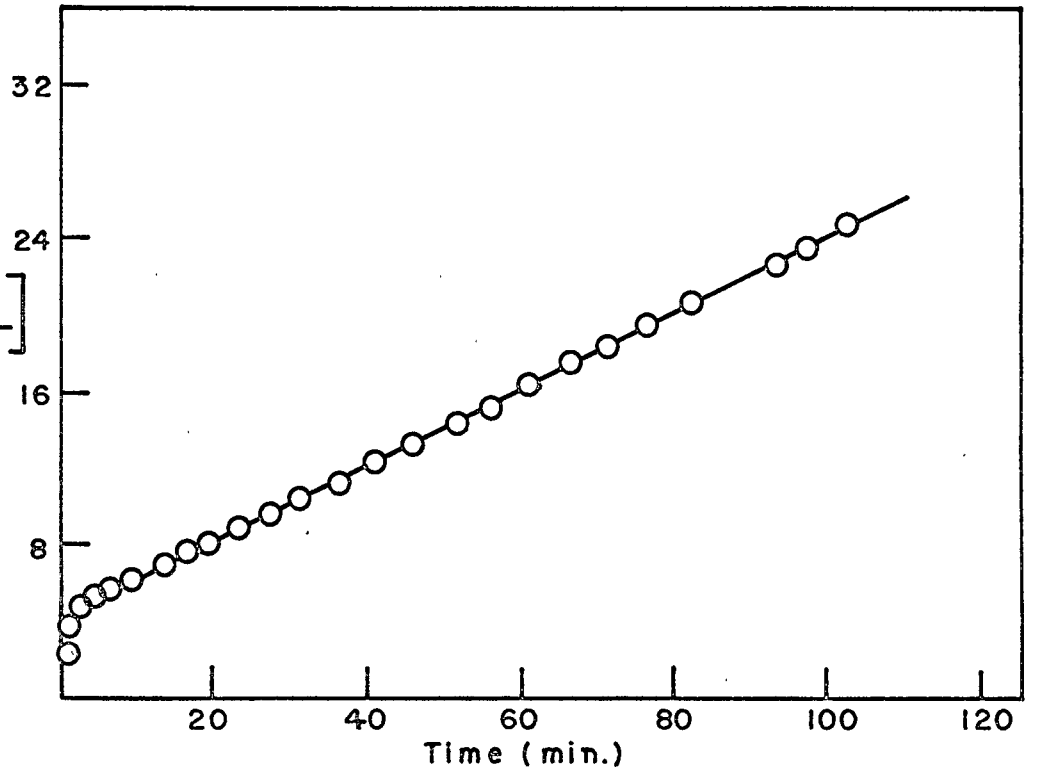


Figure 5

VARIATION OF THE FIRST ORDER RATE CONSTANT WITH INITIAL PMB CONCENTRATION

The apparent first order rate constants from Table IV, protein sulfhydryl concentration 3.88×10^{-5} M, are plotted for the various ratios of protein to PMB.

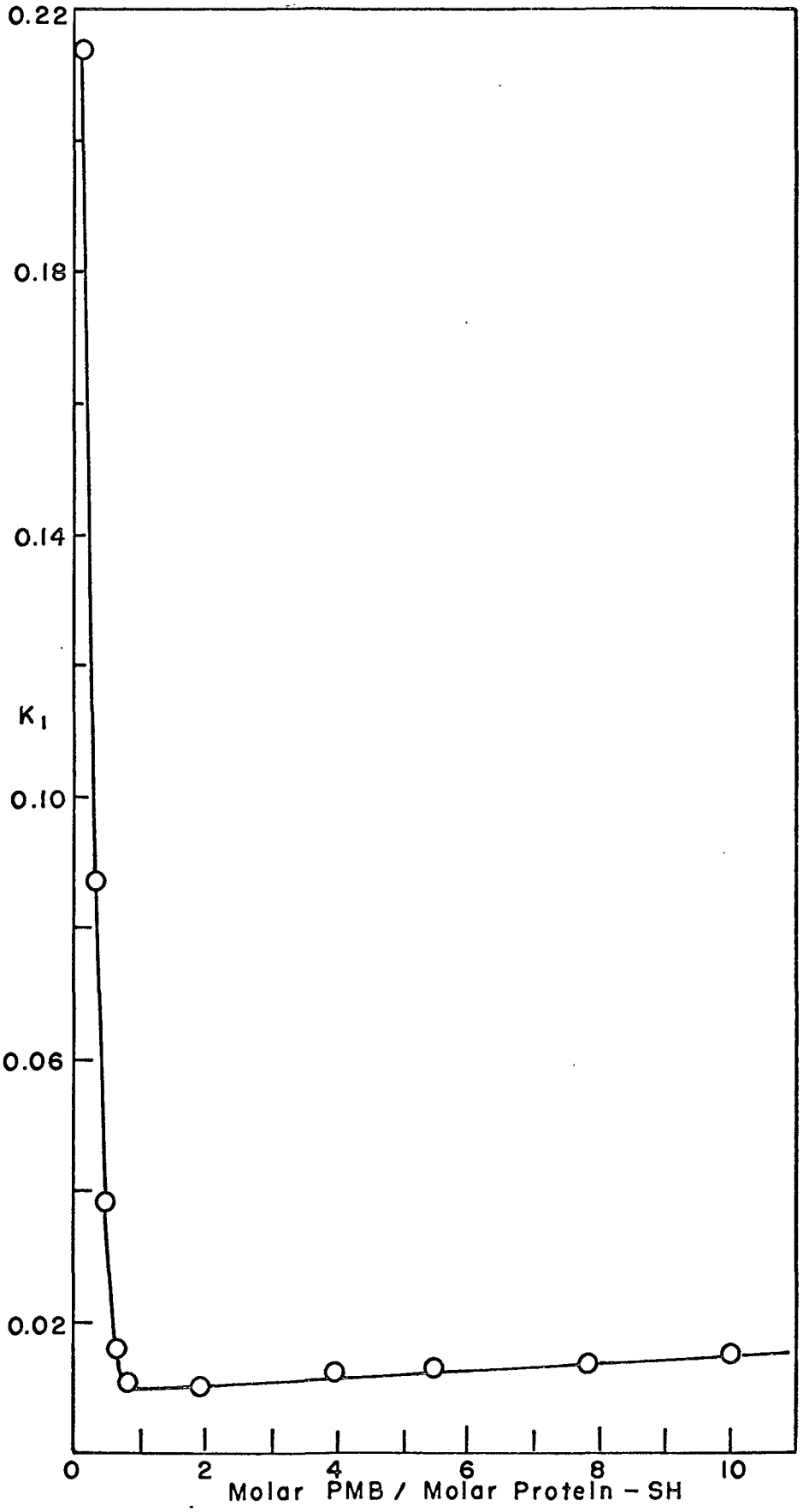

0.22
0.18
0.14
K_I
0.10
0.06
0.02
0
2
4
6
8
10
Molar PMB / Molar Protein – SH

Figure 6

VARIATION OF THE SECOND ORDER RATE CONSTANT WITH INITIAL PMB CONCENTRATION

The apparent second order rate constants from Table IV, protein sulfhydryl concentration 3.88×10^{-5} M, are plotted for the various ratios of protein to PMB.

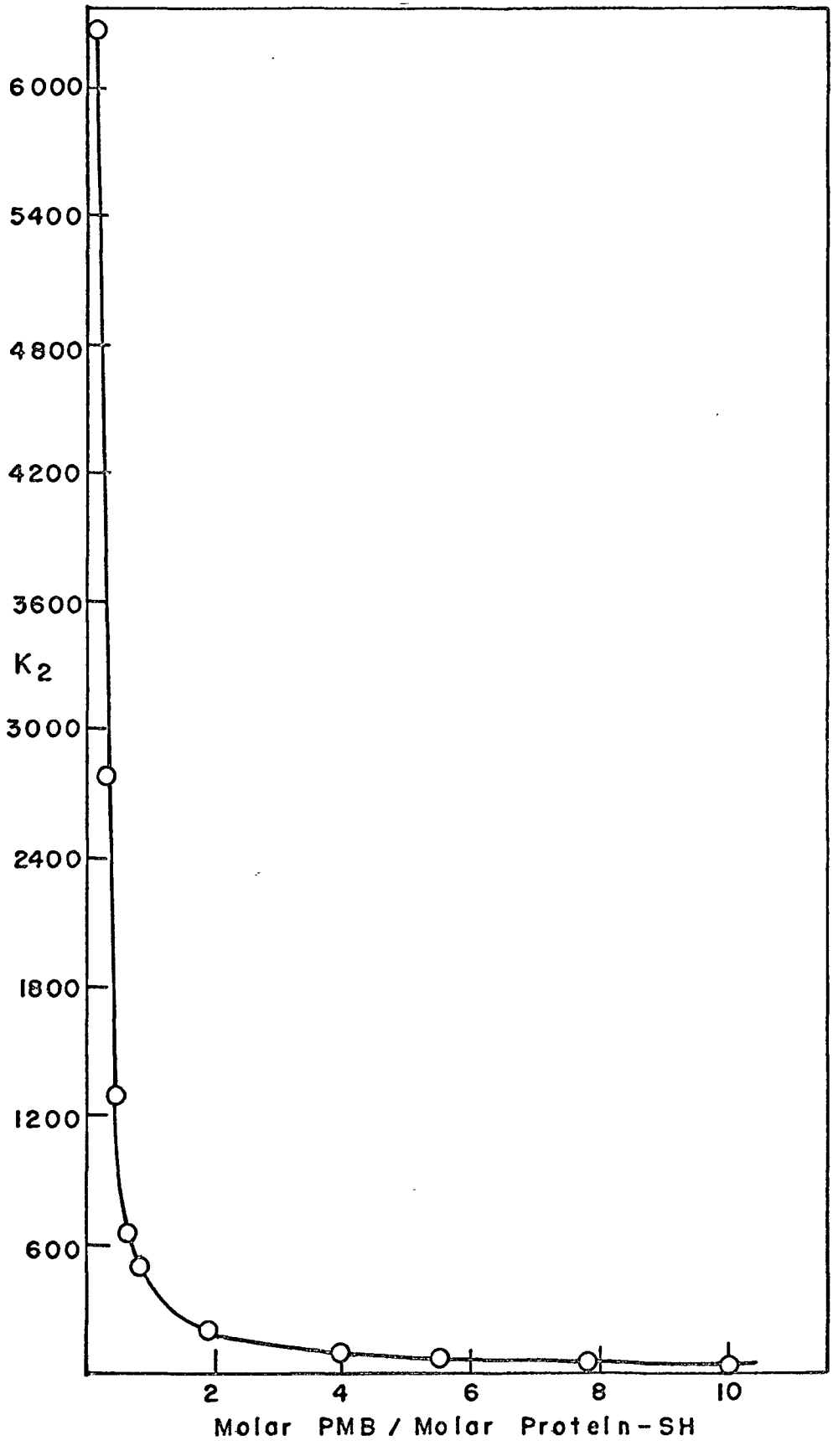

6000
5400
4800
4200
3600
K2
3000
2400
1800
1200
600
2
4
6
8
10
Molar PMB / Molar Protein-SH

Figure 7

VARIATION OF THE FIRST ORDER RATE CONSTANTS WITH PMB IN THE PRESENCE AND ABSENCE OF BENZOIC ACID

The apparent rate constants and ratios of PMB to protein sulfhydryl concentration are found in Tahles IV and VII, for the protein concentrations 3.88×10^{-5} M. ○ Presence of 3×10^{-4} M benzoic acid; Δ absence of benzoic acid.

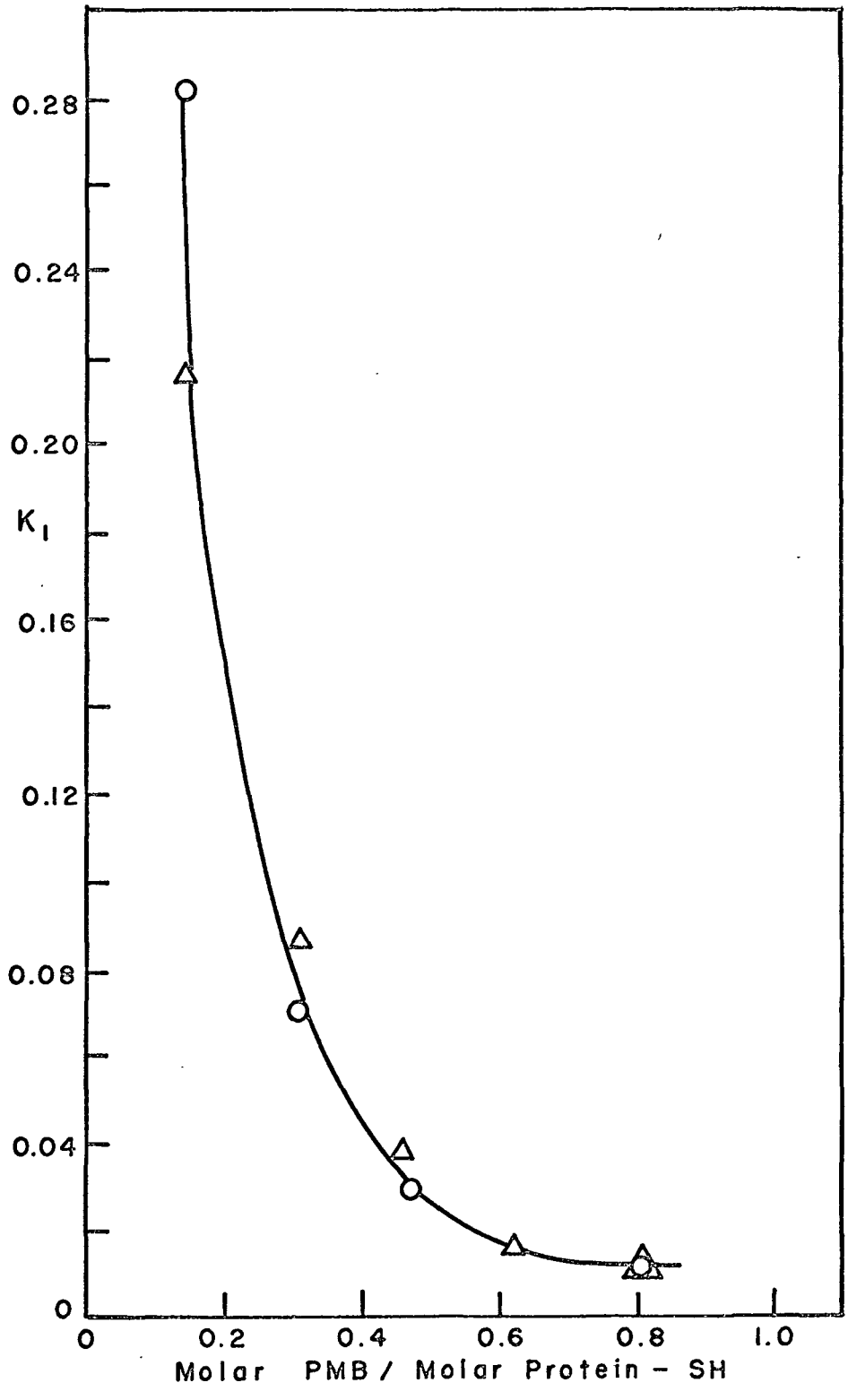
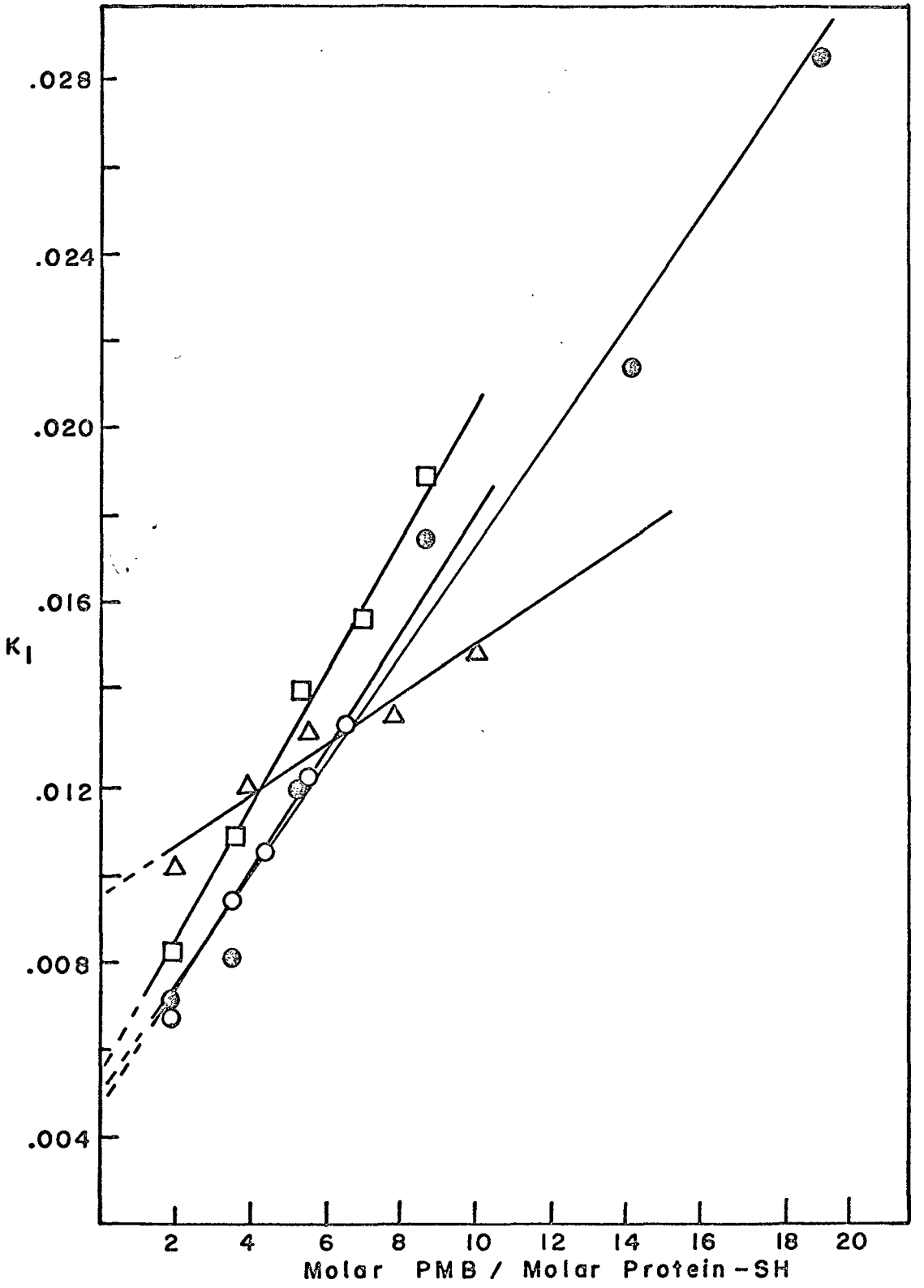

0.28
0.24
0.20
K_I
0.16
0.12
0.08
0.04
0
0
0.2
0.4
0.6
0.8
1.0
Molar PMB / Molar Protein - SH

Figure 8

VARIATION OF THE FIRST ORDER RATE CONSTANTS WITH EXCESS PMB

The apparent first order rate constants and ratios of PMB to protein sulfhydryl concentration are located in Tables III, IV, V, and VI. ○ Sulfhydryl concentration 1.16×10^{-4} M; △ sulfhydryl concentration 3.88×10^{-5} M; □ sulfhydryl concentration 1.16×10^{-5} M; ● sulfhydryl concentration 2.33×10^{-6} M.


.028
.024
.020
.016
.012
.008
.004
K_I
2
4
6
8
10
12
14
16
18
20
Molar PMB / Molar Protein-SH

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