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Analytical Gel Chromatographic Studies on Hemerythrin

Ayyagari Laxminarasimha Rao

Loyola University Chicago

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ANALYTICAL GEL CHROMATOGRAPHIC STUDIES ON HEMERYTHRIN

BY

AYYAGARI LAXMINARASIMHA RAO

A THESIS SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF LOYOLA UNIVERSITY IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

FEBRUARY
1972

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ABSTRACT

Abstract of the dissertation entitled ANALYTICAL GEL CHROMATOGRAPHIC STUDIES ON HEMERYTHRIN submitted by Ayyagari Laxminarasimha Rao in partial fulfillment of the requirements for the degree of Doctor of Philosophy, February, 1972, Loyola University of Chicago.

This investigation involved the study of protein-protein and protein-small ion interactions of hemerythrin by analytical gel chromatography. Hemerythrin is a non-heme iron-containing oxygen carrier found in certain marine worms. It is composed of eight identical subunits and can bind oxygen and other ligands such as azide, thiocyanate, chloride ions, etc. On treatment with sulfhydryl blocking reagents or on dilution this protein dissociates into monomers. The effects of dilution, various anions, and the mercurial paramercuribenzoate (pMB) on the dissociation equilibrium was examined by frontal and zonal gel chromatographic procedures. The major conclusions and original contributions of these studies may be summarized as follows:

The dissociation constant as obtained by frontal gel filtration analysis is $1 \times 10^{-42} \pm 2$ moles/liter of monomer, which value is in good agreement with previous ultracentrifugation\(^1\) and ion-binding data\(^2\). However, in contrast to the reported slow equilibration\(^3\) during sedimentation velocity experiments, frontal gel chromatographic results clearly demonstrated rapid equilibration of the octameric and monomeric species. It is possible that such a discrepancy is the result of the pressure generated during high speed ultracentrifugation on the dissociation equilibrium.

Iron-coordinating anions, as expected from earlier studies\(^4\), specifically shift hemerythrin dissociation equilibrium toward the monomeric form of the protein. Although these effects were relatively minor, they could be easily detected by changes in the elution volumes. In addition, the anions were effective in changing the degree of dissociation even at low ligand concentrations, at which no spectral change therefore no iron coordination occurs. Subsequent Hummel and Dreyer type\(^5\) gel filtration
experiments with labelled ions revealed direct binding of ligands to hemerythrin. Thus it appears that non-iron binding of ions is responsible for the observed changes in the dissociation behavior of hemerythrin.

Specific application of zonal gel filtration enabled the direct comparison of the rate of reaction of pMB with sulfhydryl groups of hemerythrin and the resulting rate of dissociation into subunits. The results indicate that the octameric form of the protein is the reactive species. The reaction proceeds probably in a random fashion followed by reequilibration and dissociation.

Combining the stopped flow technique with frontal gel chromatography the mercurial was shown to bind to hemerythrin. It was also found that the bound pMB could further react with the sulfhydryl groups in the protein. These observations about the mercurial-hemerythrin interactions have important bearing on the understanding of the reported all-or-none mechanism of the above reaction.

In conclusion this investigation demonstrates the utility of analytical gel filtration for studies on protein interactions. Although it is an empirical procedure, it appears to be accurate, sensitive, versatile, rapid and inexpensive.

LIFE

Ayyagari L.N. Rao was born in Mogallu, Andhra State, India on December 30, 1940. He graduated from Andhra Vidyalaya High School, Hyderabad, India in April, 1955. He received his B.Sc. degree from Bombay University, India in June, 1960. In 1965, he was awarded the M.Sc. degree in Biochemistry by the same University. He began his graduate work in the Department of Biochemistry and Biophysics of Loyola University School of Medicine in September, 1966, from where he earned the M.S. degree in 1969. He has been a graduate assistant in the department from September, 1966 to September, 1970, and December, 1970 to June, 1971.

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He is an associate member of the American Chemical Society, AAAS and the Biophysical Society.
This dissertation is gratefully dedicated to my benefactors:

My Father, A. Sunderama Rao,
My Uncle, A. Sambasiva Rao,
and My Cousin, A. V. Chalapati Rao,

for their help and prodding in the pursuit of my doctoral degree.

Without their true affection and understanding, I would not have been able to obtain graduate education in the U.S.A.
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I would like to express my appreciation and thanks to my research advisor, Dr. Steven Keresztes-Nagy. His encouragement has helped me to surmount the many scientific and personal obstacles which have occurred during the preparation of this dissertation.

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LIST OF ABBREVIATIONS

ATP: adenosine-5'-triphosphate
HETP: height equivalent to theoretical plate
Hr: hemerythrin
I.D: internal diameter
O.D: outer diameter
P.S.I: pressure per square inch
P.S.I.U: gauge pressure per square inch
PPO: 2, 5-diphenyloxazole
-SH: sulfhydryl group
Tris-cac: trishydroxymethane-cacodylic acid
pMB: paramercuribenzoate
CHAPTER I
INTRODUCTION

Most biochemical research is aimed at gaining new insights into fundamental biological processes through the correlation of the structure and function of macromolecules. Some classic problems in which such a correlation has aided the understanding of the underlying principles of biochemistry are the genetic code, the behavior of membranes, and the regulation of enzymes. The last problem particularly has been studied extensively due to the physiological significance of enzymes in controlling the many complex degradative and synthetic pathways. Metabolism is also regulated through other biochemical functions, such as transport and supply of small ions and metabolites to the various tissues across membranes, and energy (ATP) production. In addition to the role of enzyme control, proteins have been implicated in most of these diverse physiological processes. Thus, studies on the structure-function relationship of proteins have become increasingly important in recent years. Because of the abundant literature available on enzyme regulation, the present investigation will be restricted to studies on only certain aspects of nonenzyme-protein interactions. In both enzymic and nonenzymic proteins the type of regulation exerted depends on the type of interaction with the protein structure.
Protein structure in its present form of understanding consists of four levels of organization: primary, secondary, tertiary, and quaternary levels. The covalent attachment of the amino acids in the polypeptide chain or chains in a protein is termed the primary structure. The secondary structure consists of any folding or twisting of the primary sequence due to hydrogen bonding between the individual amino acids. Forces such as electrostatic attraction or repulsion cause further winding or looping of the polypeptide chain, and this three-dimensional arrangement is called the tertiary structure or conformation of the protein. Quaternary structure is observed only in proteins which contain more than a single polypeptide chain and is due to the association of the individual chains through non-covalent bonding. In nonpolymeric proteins, such as serum albumins, interactions with small ions, neutral molecules, etc. are generally observed as tertiary level changes. However, in polymeric proteins these interactions are observed as quaternary-structure changes, or changes in the association or dissociation of the polypeptide chains. Since the association and dissociation processes are characterized by molecular-weight changes, polymeric-protein interactions at the quaternary level may be followed with techniques commonly used for measurement of molecular weights.

Among these techniques, ultracentrifugation and electrophoresis have been most frequently used for protein-interaction studies. Such studies are now also being carried out with gel-chromatographic technique, which had been applied so far mainly for the fractionation and purification of
macromolecules. Recent applications of both ultracentrifugation and gel chromatography indicate their usefulness in measuring protein dissociation caused by interactions with solutes (Schachman and Edelstein, 1966; Fairclough and Fruton, 1966; Wood and Cooper, 1970). The commonly used depolymerizing agents (urea, guanidinium salts, and detergents) often force the equilibrium irreversibly to the monomeric species, and their use in dissociation studies is less desirable, since the protein may also undergo denaturation. Ansevin and Lauffer (1959) and Guidotti and Craig (1963) demonstrated that a number of polymeric proteins such as tobacco mosaic virus protein, hemoglobin, cytochrome, etc. undergo association and dissociation as a function of concentration, the monomeric species being favored at low concentrations. Dilution, a mild method, can be used in dissociating polymeric proteins, but complete conversion to monomeric species is possible only at very low protein concentrations. Hence, the measurement of dissociation upon dilution becomes limited to methods in which changes in the molecular weights at very low concentrations can be detected. These concentration ranges are usually below the operating limits of conventional osmometers and light-scattering instruments. Both ultracentrifugation and gel-filtration techniques have been shown to be useful at lower ranges of protein concentrations (Klapper et al., 1966; Winzor et al., 1967). However, ultracentrifuges require complicated absorption optics and are very expensive instruments. Therefore, gel-filtration technique is being chosen by many workers to study the molecular-weight changes in proteins. Winzor and Scheraga (1963)
have experimentally shown for the first time all the theoretically predicted transport features for gel chromatography. Since then, the dissociation-association phenomenon of several polymeric proteins has been studied by gel-chromatographic technique (Kakiuchi et al., 1964; Cowman and Swaisgood, 1966; Henn and Ackers, 1969; Winzor et al., 1967). Gel chromatography is, also, a rapid method for studying homogeneity of proteins, and as such can be used for studying many aspects of protein interactions. Kellett (1967), in a critical review of the methodology, has listed the major advantages of gel filtration over ultracentrifugation for the study of rapidly interacting systems. In addition to such advantages, the flexibility and simplicity of operation of gel-filtration technique make it the method of choice in this investigation whose major emphasis will be on interactions at the quaternary level of the protein structure.

Interactions at this fourth level have been studied in some important biological proteins such as hemoglobins, hemocyanins, and hemerythrins, all of which function as oxygen carriers. Among these, the hemerythrins are unique in that they provide an almost perfect model to study subunit interactions since these proteins consist of identical subunits (Groskopf et al., 1966), and possess distinct allosteric sites (Keresztes-Nagy and Klotz, 1965) for binding with oxygen (Klotz and Klotz, 1955), anions (Keresztes-Nagy and Klotz, 1965), and mercurials (Keresztes-Nagy and Klotz, 1963). In addition, the macromolecular properties of hemerythrins have been well characterized (Keresztes-Nagy and Klotz, 1965).
Therefore, hemerythrin will be used as a prototype macromolecule in the present investigation of protein interactions.

Hemerythrin, the non-heme oxygen-carrying protein of sipunculid worms, may be considered as a primitive hemoglobin. It is found in the blood of these worms circulating through a minute vascular network (\(4 \mu l\) capacity) and a large coelomic cavity (2-3 ml capacity) located in the tail portion of the animals. In contrast, hemoglobin, the most important respiratory pigment of higher vertebrates, is found in very well developed vascular systems. Hemoglobin is also known to consist of interacting subunits which give rise to a complex oxygen-transport function, but unlike in hemerythrin, these subunits are nonidentical.

Some structure-function correlations have already been carried out in hemerythrins isolated from various species of sipunculid worms (Klapper and Klotz, 1968a; Langerman and Klotz, 1969; Duke et al., 1971).

During these studies, hemerythrin of the sipunculid worm, *Golfingia gouldii*, has been found to have a molecular weight of 107,000 (Klotz and Keresztes-Nagy, 1963) and to contain sixteen iron atoms, which bind with 8 moles of oxygen (Klotz and Klotz, 1955; Boeri and Ghiretti-Magaldi, 1957; Klotz and Keresztes-Nagy, 1963). Sulfhydryl-group titrations with mercurial reagents (Keresztes-Nagy and Klotz, 1963) showed that unreacted sulfhydryl groups are necessary for the structural integrity of hemerythrin, since this reaction caused a dissociation of the protein into eight subunits. These subunits have been shown to be identical (each containing two iron atoms, one sul-
fhydryl group, and a molecular weight of 13,500) by both ultracentri-
fugation (Klotz and Keresztes-Nagy, 1963), and sequence studies (Groskopf et.al., 1966). Keresztes-Nagy and Klotz (1965) showed that the sulfhydryl group-mercurial reaction of hemerythrin may be affected by exchanging the oxygen at the iron atoms with anions (ligands). Such anionic coordina-
tion to iron has also been observed in other iron-containing proteins.
In hemerythrin, coordination to the iron was seen in both the octameric and monomeric states (Keresztes-Nagy and Klotz, 1965). The strength and stoichiometry of the coordination by the various anions has been obtained from the absorption spectra of the complexes. In both octameric and monomeric forms of hemerythrin the iron-ligand ratio was calculated to be 2:1 (Keresztes-Nagy and Klotz, 1965). These studies also show that each ligand affects the properties of the sulfhydryl groups to a different extent. In addition, the binding of the anion to the iron was observed to be stronger in the protein whose sulfhydryl groups had been previously blocked (Keresztes-Nagy and Klotz, 1965). This interdependence of two different sites in a protein molecule is an example of the cooperative or allosteric interactions which have been demonstrated earlier through the classical studies on aspartate transcarbamylase (Monod, et.al., 1965).
Keresztes-Nagy and Klotz (1965) have postulated two models to explain the observed allosteric interactions of hemerythrin: A reactive form of the protein is assumed to result from both the 'conformational model' and the 'dissociation model'. Evidence is being collected through this and other investigations (Cress, 1971) for the correct model which can explain
the features of the observed cooperativity between the sulfhydryl and iron sites in hemerythrin. Klapper and Klotz (1968a) represent these two models as shown in figure 1.

According to the conformation model, the iron-binding ligands presumably produce change in the conformation of the native octameric protein to a more reactive form. In contrast, the dissociation model postulates that the iron-binding ligands produce changes in the equilibrium to produce stable reactive monomers. The assumptions made in this model are: 1) a dynamic equilibrium exists between the native octameric and monomeric molecules; 2) the anion coordination to the iron shifts this equilibrium towards the monomeric protein; and 3) monomeric molecules are the reactive species. The first two assumptions have been examined by several workers (Keresztes-Nagy and Klotz, 1965; Klapper et al., 1966; Klapper and Klotz, 1968a) in an attempt to establish the correct model for the hemerythrin interactions. In these studies, evidence was found both for the equilibrium and the effect of coordinating anions on the equilibrium. However, the nature of the reactive protein species has not yet been well defined; hence the available data (Egan, 1969; Duke et al., 1971) could not be explained on the basis of the dissociation model.

In a general manner, the above studies can be classified into three broad types. 1) Subunit-subunit interactions (association-dissociation equilibrium) in hemerythrin; 2) Anionic interactions at specific sites in the protein; 3) Sulfhydryl group-mercurial reagent interactions. These guidelines have been kept in mind in the designing of experiments
Fig. 1. Schematic representation of the "conformation" and "dissociation" models.
in the present investigation so that the obtained results can be inte-
grated with earlier results to arrive at a working model. The status of
earlier work will now be briefly reviewed in order to evaluate the specific
problems that have been undertaken in this study.

1). First, the evidence for the existence of an association-
dissociation equilibrium in hemerythrin will be considered. Both ultra-
centrifugation (Klapper et.al., 1966) and hybridization (Keresztes-Nagy
et.al., 1965) methods were employed. The hybridization experiments in-
volved electrophoretic studies on succinylated and native hemerythrin.
Succinylation results in molecules with two additional negative charges
for each reacted-lysine residue. If hybridization occurs between the
succinylated and native proteins, octamers with intermediate electrophoretic mobility should be found. Such hybridization has been shown
experimentally (Keresztes-Nagy et.al., 1965), which proves that the mono-
mers and the octamers are in a dynamic equilibrium. Although the co-
existence of octameric and monomeric forms has been shown by high-speed-
ultracentrifugation experiments, the reversibility and, thus, the dynamic
nature of the equilibrium has not been demonstrated (Klapper et.al., 1966).
In addition, these workers interpreted their sedimentation data to be
indicative of a slow rate of dissociation. However, a controversial
problem with ultracentrifugation experiments is the so-called 'pressure
effect', which is postulated to cause changes in the partial-molal
volume of the protein species under equilibrium (Joseph and Harrington,
1966; Ikkai and Ooi, 1969; Penniston, 1971). Even though Langerman
10.

(1969) discounts the effect of pressure on the dissociation of hemerythrin, it is imperative to compare the sedimentation data with other techniques. Our preliminary experiments (Rao and Keresztes-Nagy, 1968) were designed to make such a comparison, and in contrast to the ultracentrifugation data of Klapper et.al. (1966), a fast rate of equilibrium was observed. But only the advancing profiles of frontal-gel-chromatographic-elution patterns were analyzed for this comparison. Recently Ackers (1970), outlined how to use the trailing boundaries in frontal-elution-chromatography experiments to obtain a better comparison with sedimentation velocity data. Accordingly, the present studies on subunit interactions (dissociation-association equilibrium) were examined both for the existence of an equilibrium and the rate of attainment of equilibrium.

2-a) Evidence for the effect of anions on the dissociation equilibrium has also been obtained by two different methods. Klapper and other workers (1966) used two hemerythrin systems, one containing the iron-coordinating ligand, thiocyanate, and the other no coordinating ion, in their ultracentrifugation studies. In these studies, they showed that the amount of monomeric form appeared to be the function of the presence or absence of the ligand. At each concentration studied, more monomer was found in the thiocyanate-coordinated protein solutions. The ion-binding procedure of Klapper and Klotz (1968b), also demonstrated that the dissociation of hemerythrin is affected by the presence or absence of iron-coordinating ions. The 'dissociation model', if expressed in terms of
affinity, assumes that the monomer has a greater affinity for the ligand than the octameric protein. In other words, the binding of the ligand should increase with dilution since more monomers are being formed. The binding studies showed that such an increase in the apparent binding affinity does occur by dilution.

Both in the ultracentrifugation and binding experiments, the effects of only one ion, thiocyanate, was studied. This anion is a non-biological ligand which may catalyze the formation of monomers having oxidized sulfhydryl groups incapable of reassociation. The extent of dissociation in the presence of anions other than thiocyanate is small at high protein concentrations. The operation of the ultracentrifuge is rather cumbersome at low concentrations. Only recently, Langerman and Klotz (1969) applied improved Schlieren optics for dissociation studies on hemerythrin azide by the sedimentation-equilibrium method. Gel-chromatographic technique may be an easier (operationally) procedure for studying anionic interactions and hence it was one of the aspects examined in our preliminary studies (Rao and Keresztes-Nagy, 1968). The shift of the dissociation equilibrium by the iron-coordinating ligands from an un-reactive (native) state to a reactive state (in which the sulfhydryl groups are more accessible) should be apparent through an increase in the reactivity and dissociability of the protein. Indeed such an increase has been found in the reactivity from kinetic studies (Keresztes-Nagy and Klotz, 1965; Fransioli et.al., 1968), and in the dissociability from gel-chromatographic experiments (Rao and Keresztes-Nagy, 1968). Both studies showed
that the magnitude of the increase depends on the nature of the coordinating anion. In general, greater dissociation was observed with ligands more strongly bound to the protein, and accordingly, the anions can be arranged in the order of increasing effect on the dissociation as \( \text{H}_2\text{O}, \text{O}_2\text{, F}^-, \text{N}_3^-, \text{Cl}^- \) and SCN\(^-\).

2-b). The first indication that iron-coordinating anions may also affect some site(s) other than iron atoms came from electrophoretic studies of Keresztes-Nagy and Klotz (1965). These investigators reported that the isoelectric point of methemerythrin is shifted in the presence of chloride ions. More recently, Fransioli (1969) observed an increase in the rate of reaction of sulfhydryl groups or oxyhemerythrin with mercurial, as the level of chloride ion in the reaction mixture is increased. Both the above findings have been explained on the basis of an interaction between the sulfhydryl groups and a second anion-binding site in the protein, most probably to its side chains. Fransioli (1969) also postulated from -SH group reaction studies that the second site has a greater effect on the behavior of sulfhydryl group, compared to the effect resulting from iron coordination. This finding can lead to a hypothesis in which the ligand effects can be considered as those arising primarily from non-iron bindings. Changes in the coordination spectra may accordingly be assumed to occur later in time due to subsequent chloride binding to the iron atoms. Such a hypothesis appears tenable in view of parallel observations of Darnall and coworkers (1968) with some specific ions such as perchlorate, nitrate and sulfate. Garbett et al. (1971a) recently further substantiated the existence of non-iron interacting sites for these ions.
in the hemerythrin molecule. They calculated the binding affinities of the iron-coordinating ions, thiocyanate, azide, and chloride in the absence of perchlorate ions. These values are found to be higher than earlier results of Keresztes-Nagy and Klotz (1965), which were obtained with perchlorate-containing hemerythrin solutions. Garbett et al. (1971a) similarly obtained lower values of binding constant for thiocyanate in the presence of perchlorate and nitrate ions. The conclusion of these findings is that some specific ions, such as perchlorate and nitrate ions, interact at non-iron sites in hemerythrin. These sites may possibly be identical to chloride non-iron binding sites. Therefore, we have checked this possibility with direct binding experiments on Sephadex G-25 columns in the present investigation.

Both Darnall et al. (1968) and Garbett and his associates (1971a) reported that the association properties of hemerythrin are altered by non-coordinating ions, perchlorate, nitrate, and sulfate. An ultracentrifugation method was used for observing the dissociation behavior of oxyhemerythrin in the presence and absence of perchlorate ions. The amount of dissociation was shown to be decreased by these ions. For reasons mentioned before, we have also compared this ultracentrifugation data with our gel-chromatographic experimental results.

We extended the work on non-coordinating ions (Garbett et al., 1971a) to coordinating ions, mainly to examine the effects of iron-complexing ions (such as chloride and thiocyanate) at other possible sites in the hemerythrin molecule. Furthermore, one may ask the question, what will be the effects of such non-iron binding on the molecular
properties of hemerythrin? This question has already been answered in part by Fransioli (1969), who showed that the reactivity of sulfhydryl groups increases as the chloride level is raised. Therefore, one of the aims of this study is to provide further information for understanding anionic interactions. Accordingly, we have studied the dissociation of oxyhemerythrin at various levels of chloride-ion concentration and examined the behavior of chloride-hemerythrin complex prepared by different procedures. Also, the rate of iron-coordination was simultaneously observed and correlated with changes in dissociation.

3). Sulfhydryl-group interactions with mercurial reagents have been studied in many proteins, and understandably so, since sulfhydryl groups have been implicated in such diverse functions as catalytic activity (Boyer, 1959), active-site binding (Coombs et.al., 1964; Riggs, 1952; 1959), and structural stability (Madsen, 1956). Hemerythrin has been no exception for its share of interest, especially because controversy still exists regarding the behavior of sulfhydryl groups with mercurials. Most of the work attempted to delineate the mechanism of oxygen transport, and the so-called 'all-or-none' behavior of sulfhydryl groups.

a). The frequent presence of sulfhydryl groups in the active sites of enzymes suggests their involvement also in hemerythrin. Klotz et.al. (1957) attributed the direct involvement of sulfhydryl groups in the binding of oxygen from their observations on the iron: sulfhydryl group and the sulfhydryl group : oxygen ratios. These investigators, hence, postulated iron-sulfur linkages of the type found in some other nonheme-
iron proteins (Brumby et al., 1965). But, later studies (Klotz and Keresztes-Nagy, 1963; Keresztes-Nagy, 1965) showed that the iron and sulfhydryl groups of hemerythrin are at different sites. In the absence of urea, the sulfhydryl groups reacted with mercurials and the protein subsequently dissociated with an accompanied loss of oxygen. Also, no iron was found to be released during this reaction. Darnall and coworkers (1968) have shown that the -SH groups and iron atoms in hemerythrin are in the vicinity of each other. Recent Mossbauer spectroscopic studies (Garbett et al., 1971b) suggest that the iron atoms carry oxygen in the form of a peroxo-group (Figure 2).

\[ \text{(III)} \quad \text{(III)} \]
\[ \text{Fe} \quad \text{Fe} \]
\[ \text{O} \quad \text{O} \quad \text{H} \]
\[ \text{Protein} \]

Figure 2. Fe - O₂ binding.

A further examination of the role of -SH groups in oxygen binding was carried out by Fransioli (1969). These studies showed that the rate of oxygen release is much slower than the rate of the reaction between the sulfhydryl groups and mercurial, which indicated that there is no direct role of -SH groups in oxygen transport of hemerythrin. However, no comparison was made of the rates of dissociation and the oxygen release. For this purpose, we compared the rate of oxygen release and the rate of
monomer formation during the mercurial -SH group reaction. This comparison allowed the evaluation of the indirect participation of the sulfhydryl groups in the oxygen binding.

b). In addition to any possible role (direct or indirect) in catalytic functions such as oxygen transport, the sulfhydryl groups have been shown to be of importance in maintaining the polymeric structure of proteins. Of greater interest is the 'all-or-none' manner in which sulfhydryl groups of some proteins react with the mercurial reagents. Such behavior was originally observed by Madsen and Cori (1956) in their classical studies on phosphorylase. Takenaka and Schwert (1956) observed the 'all-or-none' reaction with muscle lactic dehydrogenase. In hemerythrin, similar behavior was shown by Keresztes-Nagy and Klotz (1963) from studies with p-chloromercuribenzoate, a mercurial reagent. Experimentally, the 'all-or-none' dissociation is considered as a 1:1 linear relationship between the percent of dissociation and the extent of reaction of the sulfhydryl groups. Such a relationship means that at any instant in the reaction with the mercurial, undissociated but already reacted octameric species do not exist, and that the rate of reaction is identical with the rate of subsequent dissociation. In spite of numerous studies by various research groups (Egan, 1969; Klapper and Klotz, 1968b; Klapper, 1970; Duke et.al., 1971), the mechanism of the 'all-or-none' reaction of hemerythrin sulfhydryl groups is not well understood. Any contribution to the understanding of the sulfhydryl-group interactions will also be important in choosing the correct model for the allosteric interactions in hemerythrin.
Hence, it was logical to investigate the nature of reactive species of hemerythrin. For this purpose, comparative studies on the rate of reaction and dissociation have been carried out in the present study.

It should be noted here that Egan (1969) has postulated a prereaction-binding step by mercurial in explaining the observed rates of reaction of sulfhydryl groups of different hemerythrin-ligand complexes. Similarly Duke et al. (1971) also reported a rapid binding by mercurial at non-sulfhydryl sites in the hemerythrin molecule. However, neither of these studies conclusively proved whether the mercurial-binding step occurs before or after the reaction with the sulfhydryl groups. Therefore, by employing rapid-mixing procedures, we have examined the nature of the pre-binding step with gel-chromatographic technique.

In view of the many facets of hemerythrin interactions that have been studied during the course of this investigation, the specific problems will be reiterated here. I.) The characterization of the dissociation equilibrium. II.) The quantitative features of anion binding at iron and non-iron sites. III.) The examination of the postulates offered for the 'all-or-none' behavior of hemerythrin sulfhydryl groups.
CHAPTER II

MATERIALS AND METHODS

MATERIALS:

All chemicals and reagents were obtained from commercial sources and were used without further purification or modification. The marker proteins were also purchased from commercial sources.

Both Phoenix (Texas) and Chromatronix (California) columns have been used. The sizes and modifications (if any) are mentioned in the various experimental procedures.

Buffer solutions were made from commercial Tris (hydroxymethyl) aminomethane (pK 8.2) and cacodylic acid (dimethyl arsenic acid, pK 6.2).

Chlorine-36 (essentially carrier free) was obtained from General Nuclear Inc., Houston, Texas, in the form of hydrochloric acid.

Golfingia gouldii were purchased from Marine Biological Laboratories, Woods Hole, Mass.

Isolation and purification of hemerythrin:

Preparation of oxyhemerythrin was carried out in the cold room using the following procedure, adapted from the original method of Klotz et.al. (1957). Live worms were slit in the abdomen and the coelomic fluid was collected. Visceral debris was removed along with fibrin by filtering through glasswool after whipping with a glass rod for several minutes.
The filtrate was transferred into glass centrifuge tubes and centrifuged for 20 minutes at 0°C and 2,000 rpm. The supernatant plasma was discarded and the erythrocytes were washed and centrifuged with 2.5% cold saline solution until the supernatant was clear. The intervening white layer of fat was removed and discarded before laking the cells in triple-distilled water at 5°C. The cell debris was removed by centrifugation at 0°C and 2,000 rpm for 2 hours. The supernatant, which contains the oxygen-carrying protein of the worms, was transferred to plastic centrifuge tubes for centrifuging at 30,000 rpm and 0°C for 2 hours. This process removed the remaining fat from the supernatant solution, which was then dialyzed against one liter of cold 20% ethanol for 12 hours. The red crystals of oxyhemerythrin so obtained were washed 3-4 times with the dialysate and the supernatant was discarded. The centrifuge tube containing the crystalline protein was then stoppered and frozen till further use.

Conversion of oxyhemerythrin into coordination complexes:

**Aquomethemerythrin:** 40-50 mg of crystalline oxyhemerythrin were dissolved in 10 ml of 0.01 M pH 7.0, Tris-cacodylic acid buffer. The solution was centrifuged for 15 minutes at 0°C and 10,000 rpm and any undissolved solid material was discarded. A small amount (5-10 mg) of sodium fluoride was added until the violet-pink color of the oxyhemerythrin solution changed to the pale yellow color of methemerythrin fluoride complex. The protein was dialyzed against a liter of 0.01M, pH 7.0 Tris-cacodylate buffer for 6 hours. The dialysate solution was changed 3-4
times, at 4-6 hour intervals. The resulting aquoprotein was then examined in a Beckman DB-G spectrophotometer, for comparison with the previously reported absorption spectra (Keresztes-Nagy and Klotz, 1965).

Other complexes of hemerythrin:

The aquohemerythrin prepared as mentioned above, was converted into the chloro complex by dialyzing against Tris-cacodylate buffers containing 0.05M sodium chloride before the final dialysis against 0.5M Cl⁻.

For the series of experiments carried out in increasing concentrations of chloride ion, the oxyprotein solution was equilibrated at room temperature with the buffer solution containing the chloride ion for 15-25 minutes.

Similarly, in the experiments with non-iron-coordinating ions (ClO₄⁻, Br⁻, CH₃COO⁻) equilibration was performed with buffer solution containing 0.1M of the ion to be used.

Preparation of pMB solution.

40-50 mg of p-chloromercuribenzoate was dissolved in 20 ml of 0.01M, pH 7.0, Tris-cacodylate buffer by vigorous stirring. The solution is centrifuged at 0°C and 10,000 rpm for 15-20 minutes, to remove any undissolved pMB, and other solid impurities. The supernatant was then made up to volume to give the required concentration with the Tris-cacodylate buffer. The solution was stored at 0-5°C in an amber-colored container to protect against light.
The concentration of the pMB solution was obtained from its extinction coefficient \((4.8 \times 10^3\), Boyer, 1954) at 250 nm.

Determination of hemerythrin concentration by iron analysis and from molar-extinction coefficients:

The iron content of the hemerythrin solutions was determined by the ortho-phenanthroline method. A 1:10 dilution of the protein solution was made by adding 2.70 ml distilled water to 0.30 ml of hemerythrin. To this diluted protein, 5.0 ml of 0.88 M hydroxylamine hydrochloride were added. The iron, which is quantitatively released in the acidic environment, was spectrophotometrically read at 510 nm as an iron-ortho-phenanthroline complex. From the absorbance of the iron-complex, the concentration of the protein was obtained with the aid of a calibration graph (Keresztes-Nagy, 1962). Keresztes-Nagy and Klotz (1965) had earlier reported a specific absorbance of 2.73 for hemerythrin at 280 nm. Accordingly, the protein solutions were read in a 1.0 cm cell against the dialysate at 280 nm and the concentrations were calculated from the specific absorbance. At this wave length, the tryptophan, tyrosine, and phenylalanine residues in the protein specifically absorb and hence, the protein concentrations determined at 280 nm reflect the total protein absorbance. The near UV and visible peaks which, in general, appear between 330 and 500 nm depend on the ligand concentration.

General chromatographic procedures for dissociation studies:

A small amount of water is added to a vertically mounted Chroma-
tronix column [1.5 cm (internal diameter) X 65 cm] and the outlet plunger was closed with a teflon plug. A 1-2 cm height of swollen Sephadex G-25 coarse gel was added to protect the bed support.

The Sephadex gel was prepared by soaking the dry Sephadex in an excess amount of Tris-cacodylate buffer (0.01 M; pH 7.0) for three days after the removal of fine particles by decantation. The column was filled with the slurry (2:1 ratio of buffer to gel particles) and after the settling of some gel particles, the outlet plunger was opened and the buffer was allowed to flow slowly. The remaining slurry was then added so that the column was finally filled to a bed height of approximately 50 cm, taking care that the gel particles were never allowed to settle completely before the addition of more slurry. The outlet was closed off again and the jacket was installed. The bed support of the inlet plunger consisted of a 10 micron filter membrane; a 40 micron (pore size) teflon cloth was then introduced into the top of the column. The bed support ensures the uniform application of sample solutions. The excess solvent was removed by gently pushing the plunger into the column until it was horizontally flush with the gel particles. The inlet plunger was fixed in this position by tightening the screws on the collar of the plunger. The inlet-plunger tubing was connected to the sample injection valve. The sample injection valve, in turn, was connected to the Chromatronix pump, which had previously been purged with compressed air and the solvent reservoir filled with buffer. Compressed air at a pressure of 80 guage pressure per square inch (P.S.I.G.) was fed into the pump. The
23.

connections were made by high-pressure teflon tubing (0.138 cm O.D., 0.068 I.D.) and Chromatronix-Cheminert fittings which allowed pressure-tight joints and insured uniform flow. The gel particles were washed several hours so that the gel bed could come to an equilibrium height. The sample injection valve had two alternate flow paths, and the solvent could be switched from one to another by the push bar on the valve. The portal configuration of the sample injection valve appears in Figure 3.

A brass pressure gauge (0-50 P.S.I.G.) was connected via a "tee" to the tubing which fed the solvent to the injection valve. The gauge was isolated from the system to avoid the introduction of dead volume in the inlet tubing of the column. Both 12 ml/hr. and 24 ml/hr. flow rates were used in the investigation. The outlet plunger was connected to a Beckman flow cell (0.3 ml capacity, 0.1 cm light path) by 20 cm teflon tubing (0.1 ml-dead volume) and the effluent solution was measured in a 10 ml burette. The concentration of the sample solution determined the wavelength chosen, such that the absorbance value was between 0.1 to 1.0 absorbance units. At low concentrations where the absorbance values are below 0.1, a scale-expansion unit was used to amplify the absorbances. The absorbances were recorded with a 10 inch linear-log recorder. The stock protein was diluted very accurately to obtain the required hemerythrin concentration. With the aid of the laboratory vacuum, teflon loops were filled with this diluted-protein sample whose volume (20-30 ml) was sufficient to produce an absorbance plateau in the elution profile. The solution profile obtained from such large samples is called frontal-elution chromatograms. The mixing of the sample with the solvent during sample
SOLID LINES: INJECT SAMPLE (position A)
DOTTED LINES: LOAD SAMPLE (position B)

FIGURE 3
Portal Configuration of the Sample Injection Valve
application was avoided by also connecting an empty loop (0.8 ml volume) at the end of the sample loop. The exclusion volume of the column in each experiment was obtained by introducing 0.1 ml of blue dextran (1 mg/ml) into the sample loop. The sample loop was then connected to the injection valve and the protein sample together with the blue dextran was applied on the column by pushing "in" the push bar of the valve. After the sample was applied on the column, the push bar is pulled "out". The wave length at which the protein was monitored and the scale expansion for recording the absorbances were selected according to the protein concentration. For example, a 100 µg/ml solution was monitored at 280 nm, where its absorbance was 0.27. At the same wave length a 10 µg/ml solution would yield a 0.027 absorbance value and hence, such a solution was monitored at 210 nm, where it gave an absorbance of 0.3. The scale expansion unit was used for solutions which have low absorbance values when measured at 210 nm. Amplifications of 2 to 5-fold were used for solutions in the range of 10-25 µg/ml protein concentration. The elution pattern was recorded at a chart speed of 12 inches/hour. Since with the sample injection valve, the sample was applied on the column without any change in flow rate, the elution profiles could be directly evaluated for centroid volumes ($\bar{V}$).

In the experiments in which the effect of various anions was under consideration, the column is preequilibrated with the buffer solution containing the particular ion. The concentration of the ion used was 0.1 M except in the case of chloride ion, which was varied from 0.1 to 0.5 M.
All the dissociation experiments were maintained at 25° with a Braun Thermomix II regulator. Schematically, the monitoring set up is shown in Figure 4.

Column calibration procedure:

Marker proteins having molecular weights in the range between the hemerythrin octameric and monomeric values of 107,000 and 13,500 were used for calibration purposes. Elution volumes were calculated from the inflection points \( \frac{1}{2} C_0 \), the plateau concentration) of elution diagrams which were obtained by the application of 15 ml samples at a concentration of 1-2 mg/ml.

The exclusion volume \( (V_o) \), and the total bed volume \( (V_t) \) were derived through the application of 15-20 ml (mg/ml) of blue dextran and potassium chromate \( (K_2CrO_4) \) solutions to the column. The internal volume \( (V_i) \) of the column was calculated from the relation \( V_i = V_t - V_o \). The partition coefficients (diffusion coefficients) are defined as \( \frac{V_e - V_o}{V_i} \) (Appendix I) and were evaluated from the elution volume values of the standard marker proteins.

Treatment of elution data:

The weight-average partition coefficients \( (\bar{C}_w) \) for hemerythrin at the various protein concentrations corresponding to the plateau region of the elution diagram were obtained by measuring the centroid position \( \bar{V} \) of the ascending edges of the elution boundaries according to the relationship derived by Ackers and Thompson (1965): 

\[
\bar{C}_w = \frac{\bar{V} - V_o}{V_i},
\]

where 

\[
\bar{V} = \frac{1}{C_0} \int_0^{C_0} Vdc.
\]

First moments (Longworth, 1943) of the
Fig. 4. Schematic of the monitoring set up for Sephadex Columns.
leading edges were used as the centroid volumes in the frontal elution experiments at different protein concentrations. Sample calculations are shown in Appendix II.

The properties of the elution patterns were examined in terms of Gilbert's postulates (1955) for interacting systems as outlined by Winzor and Scheraga (1963). To facilitate comparison of our data with previous sedimentation velocity studies (Klapper et al., 1966), we prepared first derivative curves of the elution patterns, and plotted versus the elution rates [the ratio of the elution volume ($\bar{V}$) of the derivative peaks to the total volume of the column ($V_t$)] of the slow and fast components of the trailing boundaries against the plateau protein concentrations.

Gel-filtration studies on the rates of dissociation and reaction of hemerythrin - SH groups with pMB.

The reaction was started in 4.0 ml spectrophotometric cuvettes by mixing hemerythrin and pMB in a 1:2 mole ratio, the actual concentrations being $6.0 \times 10^{-5}$ and $1.2 \times 10^{-4}$ M respectively. The progress of the mercaptide-bond formation was followed by measuring the increase of absorbance at 250 nm in a Beckman DU spectrophotometer (Boyer, 1954). At appropriate intervals, 0.10 ml aliquots were removed from the cuvette and injected through a Chromatronix valve to the top of a 1.2 x 60 cm Chromatronix column packed with Sephadex G-75 gel. The column was water-jacketed so that the temperature was maintained at 8°C. A constant flow-rate of 60.0 ml per hour was assured by a Chromatronix-surgeless-positive
displacement pump. The effluent was monitored at 250 nm by a DBG Beckman double-beam spectrophotometer equipped with 0.3 ml volume, 1.0 cm light-path flow cell. The temperature of the column and the effluent were maintained constant with a Wilkins-Anderson low temperature bath. Schematically, the monitoring set up is identical to that represented in Fig. 4.

**Spectrophotometric-titration method for measurement of the rate of reaction between pMB and hemerythrin-SH groups.**

The specific absorbance of 250 nm of the mercaptide-bond formation (Boyer, 1954) was followed in a Beckman DU spectrophotometer. The cell compartment was kept at 8° with the aid of a Neslab Tanison circulation thermostat (Model T 23) in conjunction with a Neslab portable bath cooler (Model PBC-4). The reaction was started by addition of pMB solution into a 3.0 ml hemerythrin sample and absorbance readings were taken against a blank protein solution to which the same volume of buffer as that of the mercurial was added.

The extent of dissociation at various time intervals of the reaction between pMB and the hemerythrin-SH groups was measured by making an additional reaction solution, as explained above, from which aliquots could be removed at various time intervals.

The final state of the reaction mixtures, at different ratios of mercurial and the protein, was estimated by allowing the samples to equilibrate for several (6-8) hours at room temperature. The percent dissociation was obtained from the areas of the octameric and monomeric peaks monitored at 280 nm.
The end point of the reaction in all the above experiments was evaluated from the known extinction coefficient of the mercaptide bond 
\( (7.2 \times 10^3, \text{Fransioli}, 1969) \).

**Studies on mercurial-hemerythrin binding with rapid-mixing procedures:**

A 0.9 x 35 Sephadex, G-25 (coarse) column was used to demonstrate mercurial-hemerythrin binding, in a procedure similar to Hummel and Dreyer (1962). The column was preequilibrated with the desired level of pMB before a 0.2 ml protein sample was applied with the Chromatronix injection valve. A flow-rate of 60 ml/hr was maintained with the CMP-1 pump, and the effluent was monitored in a microflow-cell (0.3 ml capacity, 1 cm light-path) at 250 nm.

In a similar manner, the binding was measured by frontal analysis in which the protein and pMB are mixed instantaneously before application to the column. A 0.4 x 50 cm microcolumn (Chromatronix) was packed with swollen Sephadex G-25 (coarse). The temperature of the column was maintained at 8°C by circulating ice water using the Braun Thermonix II regulator. Continuous mixing of the mercurial and the hemerythrin solution was achieved in a 0.3 ml glass mixing chamber by using both CMP-1 pump (Chromatronix) and a syringe pump at 12 ml/hr. The reaction mixture was applied on the column through the outlet of the mixing chamber and the effluent was continuously measured at 250 nm as explained earlier. Fig. 5 shows diagramatically the arrangement by which the mixing was achieved during these experiments.
Fig. 5 shows diagramatically the arrangement by which mixing was achieved during binding experiments.
Measurement of the rate of conversion of oxyhemerythrin in the presence of chloride ions:

The oxyprotein was diluted into the buffer containing the required amount of chloride, and the rate of conversion of the absorption spectra to that of the chloro-complex was measured in a Cary-15 spectrophotometer. A blank containing the same concentration of chloride ion was used to measure the appearance of the characteristic 385 nm peak and the 370 nm trough in the spectra of the oxy-protein. The conversion was assumed to be 100% when the oxy-protein diluted into the chloride ions developed the same spectra as that of a chloro-complex obtained through dialysis procedure (both solutions of identical protein concentration). At high protein concentration, 1 cm quartz cells were used; and 10 cm quartz cells were used to obtain spectra of equal magnitude at low protein concentrations.

determination of chloride-hemerythrin binding:

This procedure is identical to that of the mercurial-hemerythrin binding, except in the monitoring of the effluent. A 0.9 x 25 cm column was packed with Sephadex G-25 (coarse) gel and equilibrated with 0.003 M chloride containing 0.01 M Tris-cacodylate buffer (pH 7.0). 1.0 ml oxyhemerythrin, made in the same buffer, was applied through the injection valve. The chloride in each 0.8 ml fraction was measured (Schales and Schales, 1941) by titrating an aliquot with Hg(NO₃)₂ to a purple-violet-colored end point. Diphenyl carbazone (4% alcohol) indicator and 0.1 N HNO₃ were used in these titrations with a Micro-Metric syringe pump. After
a few preliminary experiments, the above procedure was modified to estimate more conveniently the concentration of bound and free chloride. Instead of having the chloride level in the protein sample identical to that of the eluting buffer, higher levels were used to prepare the oxyhemerythrin solutions. Also, the amount of sample applied on the column was increased to 1.7 ml to obtain larger areas of protein-bound chloride. The excess chloride was measured from the peak area at the elution position of the salt. Various concentrations of the protein were applied to obtain the number of moles of chloride bound to each mole of oxy-hemerythrin. These values were then utilized in a Scatchard plot (Scatchard, 1949) to evaluate both the number of binding sites and the strength of this binding.

Scintillation-counting procedure using $^{36}\text{Cl}$:

In this procedure, the equilibrating buffer contained a small amount of labelled chloride ($^{36}\text{Cl}$). The concentration of the stock radioisotope, $^{36}\text{Cl}$, was determined with standard Hg(NO$_3$)$_2$ solution after the acidic isotope solution (~4 N H$^{36}\text{Cl}$) was neutralized to pH 7.0 with concentrated NaOH. An aliquot of this stock Na$^{36}\text{Cl}$ was then added to the buffer such that the labelled chloride represents approximately one half of the total chloride (0.0018 M $^{36}\text{Cl}$ in 0.003 M total chloride). Oxy-hemerythrin samples at various protein concentrations were prepared by using 0.003M labelled chloride in a total of 0.006 M chloride. 25-drop fractions were collected directly into glass scintillation vials and 10.0 ml of the scintillation toluene-PPO solution (5g PPO -1 liter toluene - 200 ml Biosolv-BB5-3, Beckman) was added to each fraction. The amounts of bound and free
chloride was measured from the radioactivity of the fractions with a Beckman LS-250 Scintillation spectrometer (counting conditions: gain 360 Ext. Std. 0.765; variable discriminator 0-8.12). The counts (CPM) were corrected for quenching by the protein from a quenching curve.

A small aliquot of the stock was diluted to contain $5 \times 10^{-2}$ μci/ml, and 0.1 ml of this dilution was added to each of several vials containing 10 ml of the fluor. Various concentrations of oxy-hemerythrin were added (0.5 ml) to these vials and counted for 10 minutes in the spectrometer. The counting efficiencies at each protein concentration were determined from the reduction in the counts ($\approx 10,000$ cpm) of the blank vial which contained no protein. From the External Standard Ratio (ESR) values the quenching curve could be plotted, from which the % efficiency was evaluated in measuring the isotope of each fraction obtained in the binding studies.
CHAPTER III

EXPERIMENTAL RESULTS

A. Calibration of Sephadex Columns:

The elution volume obtained in either a zonal or frontal chromatographic experiment may be utilized in evaluating both the weight-average molecular weight and the average molecular radius of the macromolecular species under study. But first, the elution-volume value has to be converted into a more useful parameter, the partition coefficient. This term in turn is related to some of the physical characteristics of standard marker proteins. In recent literature, the partition coefficient is also known as the molecular-sieve coefficient (\(\sigma\)) and is defined as shown in Chapter II. The experimentally derived elution volumes and the calculated sieve coefficients of the marker proteins are listed in Table I. Calibration graphs were obtained by plotting the sieve coefficients against the logarithm of the molecular weights of the marker proteins. These plots (Figs. 6 (G-75) and 7 (G-100) are linear (\(\sigma = A \log M + B\)), as predicted by established chromatography theories (see Appendix I). The values of the calibration constants \(A\) and \(B\) were 0.675 and 3.320 for the G-75 column, and 0.625 and 3.168 for the G-100 column.

The weight-average molecular weights of hemerythrin solutions were then obtained by extrapolation of the experimentally derived elution volume (molecular sieve coefficient, \(\sigma\)) to its corresponding molecular
Fig. 6. Calibration plot of Sephadex G-75 Column
Fig. 7. Calibration Plot of G-100 Column
TABLE I

Calibration data of Sephadex G-75 and G-100 columns at 25°C.

<table>
<thead>
<tr>
<th>Marker protein</th>
<th>Elution volume in ml.</th>
<th>Molecular-sieve coefficient</th>
<th>Weight-average Molecular Weight (M)</th>
<th>log (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-75</td>
<td>G-100</td>
<td>G-75(^1)</td>
<td>G-100(^2)</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>31.80</td>
<td>25.95</td>
<td>0.048</td>
<td>0.15</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>35.90</td>
<td>29.80</td>
<td>0.130</td>
<td>0.25</td>
</tr>
<tr>
<td>(\alpha)-chymotrypsinogen</td>
<td>48.70</td>
<td>37.10</td>
<td>0.385</td>
<td>0.44</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>52.40</td>
<td>39.80</td>
<td>0.451</td>
<td>0.51</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>56.70</td>
<td>43.25</td>
<td>0.545</td>
<td>0.60</td>
</tr>
</tbody>
</table>

1. Exclusion volume \((V_0)\) and internal volume \((V_i)\) are 29.4 and 50.0 ml respectively.

2. Exclusion volume and internal volume are 20.2 and 38.4 ml respectively.


weight in the calibration graphs.

Conjugated proteins and highly asymmetric macromolecules are known to deviate from a linear relationship between the molecular weight and elution volume. For this reason, many workers (Ackers, 1964; Siegel and Monty, 1965; Giddings et al., 1968) believe that the molecular size rather than the molecular weight is best correlated with the elution volume.

Ackers (1967) derived a linear relationship between the molecular radius, \(a\), and the inverse-error-function complement of the column sieve coefficient, \((\text{erfc}^{-1} \sigma)\): \(a = a_0 + b_0 \ (\text{erfc}^{-1} \sigma)\), in which \(a_0\) and \(b_0\) are
column-calibration constants (not the same as A and B). Such a relation assumes that the gel pores can accommodate all random types of molecular sizes. The experimentally derived sieve coefficients are converted into their inverse-error-function complements (sample conversion shown in Appendix III), which are then plotted against molecular radii of the marker proteins. Table II lists the values of erfc⁻¹σ and the molecular radii; Fig. 8 illustrates the linear relationship between these two parameters. The column-calibration constants, a₀ and b₀, were 8.3 and 19 for the G-75 column and 7.5 and 24.4 for the G-100 column.

**TABLE II**

Calibration data in relation to molecular size

<table>
<thead>
<tr>
<th>Marker protein</th>
<th>Sieve coefficient (σ)</th>
<th>(1-σ)</th>
<th>erfc⁻¹σ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-75</td>
<td>G-100</td>
<td>G-75</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>0.048</td>
<td>0.150</td>
<td>0.952</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>0.130</td>
<td>0.250</td>
<td>0.870</td>
</tr>
<tr>
<td>α-chymotrypsin</td>
<td>0.385</td>
<td>0.440</td>
<td>0.615</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>0.450</td>
<td>0.510</td>
<td>0.550</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>0.545</td>
<td>0.600</td>
<td>0.455</td>
</tr>
</tbody>
</table>

From the column calibration constants a₀ and b₀, the octameric and monomeric molecular radii were obtained. On the G-75 column those were 15.0 ± 0.5 Å° and 42.6 ± 0.5 Å°, and on the G-100 column 16.2 ± 0.5 Å° and 35.4 ± 0.5 Å° respectively.
Fig. 8. Calibration plots of G-75 and G-100 columns on the basis of molecular radius.
A general note on the dissociation experiments: The results of the various dissociation experiments as presented in this chapter are organized on a logical rather than a chronological basis. The compilation might appear discontinuous, especially because both Sephadex G-75 and G-100 columns have been used for the study of hemerythrin interactions. In addition, different ligand complexes of hemerythrin, were also used in this study.

The reproducibility of the elution-volume values of experiments similar to those in the present study had been previously (Rao, 1969) reported to be on the order of ± 0.10 ml (± 1000 in molecular weight value). We are aware of the small deviation (a 100 fold deviation) in the equilibrium constant data due to such errors in the measurement of the elution volumes (Appendix VI).

B. 1. Dissociation Experiments with Hemerythrin-azide on a Sephadex G-100 Column:

This set of experiments was performed to demonstrate the usefulness of analytical-gel-chromatography method. Therefore the elution diagrams obtained at various concentrations of hemerythrin (see Chapter II for actual procedure) were examined only for the dissociation behavior of the protein. The position of the elution at any concentration was evaluated from the centroid volume of the leading boundary of the frontal-elution experiment (see Appendix I for related Theory and Appendix II for a sample derivation of the centroid volume). The data from these experiments are presented in Table III. The sieve coefficients of the completely
dissociated and undissociated hemerythrin were obtained from the elution position of a 500 µg/ml hemerythrin-azide solution treated with salyrganic acid (mersalyl acid, a mercurial reagent) and a 8000 µg/ml hemerythrin-azide solution. At these and other concentrations of hemerythrin, only the advancing elution profiles were recorded, and are shown in Fig. 9. Since the sieve coefficient is a diffusion parameter, the amount of hemerythrin depolymerization may be qualitatively deduced by comparing the sieve coefficients at the various concentrations with those of the completely dissociated and undissociated forms. The data in Table III demonstrate

**TABLE III**

Effect of dilution on the dissociation of hemerythrin-azide as observed on a G-100 column. 1

<table>
<thead>
<tr>
<th>Hemerythrin concentration g/l</th>
<th>Centroid volume, $\bar{V}$ (ml)</th>
<th>Weight-average sieve coefficient ($\sigma_w$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.000</td>
<td>24.25</td>
<td>0.105</td>
</tr>
<tr>
<td>0.333</td>
<td>27.20</td>
<td>0.182</td>
</tr>
<tr>
<td>0.300</td>
<td>27.35</td>
<td>0.186</td>
</tr>
<tr>
<td>0.073</td>
<td>28.55</td>
<td>0.218</td>
</tr>
<tr>
<td>0.060</td>
<td>29.70</td>
<td>0.247</td>
</tr>
<tr>
<td>0.025</td>
<td>30.80</td>
<td>0.276</td>
</tr>
<tr>
<td>0.011</td>
<td>35.25</td>
<td>0.391</td>
</tr>
<tr>
<td>0.500 + salyrganic acid</td>
<td>43.75</td>
<td>0.613</td>
</tr>
</tbody>
</table>

1. Column size 1.1 x 55 cm; $V_o = 20.2$ ml; $V_i = 38.4$ ml; Flow-rate 12 ml hour; Temperature 25°C; 0.01 M, pH 7.0 Tris-cacodylate buffer was used.
Fig. 9. Change of elution volumes on dilution with hemerythrin-azide complex observed on a G-100 Column.
that the value of the sieve coefficient is increased as the protein concentration is lowered. This observation can also be seen from the advancing boundaries of the elution profiles (see Fig. 9). The elution position shifted with decreasing concentration towards the monomeric (completely dissociated) elution position. Only a single plateau was found in the ascending elution profiles of these experiments (Fig. 9).

B.2. Dissociation experiments with Hemerythin-thiocyanate on a G-100 column.

The preceding experimental results indicated that the elution position of a frontal-gel-chromatographic diagram can be used to obtain qualitative information on the dissociation status of hemerythin. These experiments were performed primarily to examine the use of gel chromatography for the study of the dissociation process in hemerythin; therefore, the extent of dissociation has not been estimated. For a comparison of the extent of dissociation under various conditions of concentration, ligands, and depolymerizing reagents, such estimations become essential. Since the elution volume (molecular-sieve coefficient) is related to the weight-average molecular weight of the system (Ackers and Thompson, 1965), this parameter has been used in the next set of experiments.

The extent of dissociation was quantitatively examined in the thiocyanate-hemerythin complex at different protein concentrations. The elution position is obtained from the centroid volumes of the ascending profiles. Weight-average molecular weight (MW) at each protein concentration was extrapolated from the calibration graph, Fig. 7. The percent
of dissociation was then calculated from these values and from the known octameric (Mp) and monomeric (Mm) hemerythrin molecular weights by using the following equation:

$$\bar{MW} = \frac{C_m M_m + C_n M_n}{C_m + C_n}$$

where $C_m + C_n = C_0$, the plateau concentration (concentration of protein applied on the column). Intermediate species between monomers and octamers are assumed not to exist. Table IV lists the elution volumes and the calculated percent of dissociation (Appendix IV shows a sample calculation).

**TABLE IV**

Effect of dilution on the dissociation of hemerythrin-thiocyanate complex.

<table>
<thead>
<tr>
<th>Protein Concentration (g/l)</th>
<th>Centroid Volume, $V_2$ (ml)</th>
<th>Weight-average Molecular weight, $\bar{MW}$</th>
<th>Percent Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.328</td>
<td>27.0</td>
<td>72,400</td>
<td>37.6</td>
</tr>
<tr>
<td>0.105</td>
<td>29.1</td>
<td>50,120</td>
<td>61.2</td>
</tr>
<tr>
<td>0.072</td>
<td>29.9</td>
<td>42,650</td>
<td>69.2</td>
</tr>
<tr>
<td>0.047</td>
<td>30.6</td>
<td>37,600</td>
<td>74.5</td>
</tr>
<tr>
<td>0.026</td>
<td>32.4</td>
<td>26,900</td>
<td>85.8</td>
</tr>
</tbody>
</table>

1. Prepared by overnight dialysis against 0.1 M KSCN\textsuperscript{−} containing 0.01 M, pH 7.0 Tris-cacodylate buffer. Dialysate used for column elution.

2. A G-100 (1.0 x 55 cm) column of exclusion volume ($V_0$) = 17.3 ml was used in these experiments.
Experimental data with the oxyprotein were obtained in the same way as that of hemerythrin-thiocyanate-complex. Recent reviewers of chromatographic theories (Winzer, 1969; Ackers, 1970) suggest the use of both advancing and trailing boundaries of frontal elution profiles. Accordingly, the centroid volume of the ascending boundary is used to obtain the weight-average molecular weight of the protein species, and the descending boundary information is used to reveal the type of equilibrium in hemerythrin. In 1955, Gilbert (1955) presented a theory for the behavior of rapidly interacting (dissociating) systems on transport methods. In 1963, Winzor and Scherega (1963) showed experimentally all the postulates of this theory; since then gel-chromatographic data have been checked for these postulates by many investigators.

With oxy-hemerythrin, a lower range of protein concentrations had to be used since at higher concentrations the extent of the dissociation was small, and it was difficult to observe the changes in the elution volume. Table V lists the centroid volumes of the ascending boundaries and shows that the value of $\bar{V}$ increased with increasing dilution. This finding indicates that the weight-average molecular weight decreased with decreasing protein concentrations (Winzor, 1969).

The weight-average molecular weight was estimated for each protein concentration from the weight-average partition coefficients and the calibration constants of the column. From the weight-average molecular
weights, the percent dissociation was calculated, with the assumption that only monomers and octamers were present. The dissociation constant, \( K \), was obtained from the leading edges of the elution boundaries from the equation derived by Ackers and Thompson (1965):

\[
K = \frac{(\alpha)^n x (C_0)^n - 1}{1 - \alpha} \quad (1),
\]

where \( C_0 \) is the concentration of protein in the plateau region, \( n \) is the stoichiometry of dissociation (assumed to be 8), \( \alpha = (\bar{\sigma}_w - \sigma_n) / (\sigma_m - \sigma_n) \), \( \bar{\sigma}_w \) is the weight-average partition coefficient, and \( \sigma_n \) and \( \sigma_m \) are the sieve coefficients of the octamer and monomer respectively. The values of the octameric and monomeric sieve coefficients were obtained by using the calibration equation \( \sigma = -A \log M + B \). For the G-100 column, the values of \( A \) (0.625) and \( B \) (3.168) are known (Chapter II); by substitution \( \sigma_n \) and \( \sigma_m \) were calculated to be 0.025 and 0.587 respectively. The dissociation constants at the various protein concentrations were calculated by using equation 1 and the values so obtained are also listed in Table V.

Winzor and Scheraga (1963) have shown that information about the rate with which the dissociation-association equilibrium is achieved can be obtained from the overall shape of the elution curves, and from the analysis of the trailing boundaries. When the first derivative of the trailing edge of the elution profiles was plotted against elution volume, two peaks were evident (Fig. 10). The peak which emerged first was called the fast component of the trailing boundary, while the peak
Fig. 10. Concentration derivative curves of the trailing edges of the elution profiles at various concentrations of Hr- oxy complex, on a G-100 Column.
TABLE V
Dissociation behavior of oxyhemerythrin$^1$ on a G-100 column.

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Centroid Volume, $V$ (ml)</th>
<th>Molecular-sieve Coefficient, $\sigma_w$</th>
<th>Percent Dissociation</th>
<th>Dissociation constant in monomeric units$^3$ $K$ (moles/l.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>27.8</td>
<td>0.148</td>
<td>22.06</td>
<td>$2.13 \times 10^{-41}$</td>
</tr>
<tr>
<td>0.080</td>
<td>28.1</td>
<td>0.155</td>
<td>25.10</td>
<td>$2.71 \times 10^{-42}$</td>
</tr>
<tr>
<td>0.040</td>
<td>32.2</td>
<td>0.251</td>
<td>46.25</td>
<td>$2.49 \times 10^{-42}$</td>
</tr>
<tr>
<td>0.030</td>
<td>34.4</td>
<td>0.303</td>
<td>58.40</td>
<td>$2.09 \times 10^{-42}$</td>
</tr>
<tr>
<td>0.023</td>
<td>33.0</td>
<td>0.270</td>
<td>50.70</td>
<td>$2.12 \times 10^{-43}$</td>
</tr>
<tr>
<td>0.016</td>
<td>38.0</td>
<td>0.389</td>
<td>80.40</td>
<td>$3.22 \times 10^{-43}$</td>
</tr>
<tr>
<td>0.010</td>
<td>39.7</td>
<td>0.428</td>
<td>87.80</td>
<td>$3.41 \times 10^{-44}$</td>
</tr>
</tbody>
</table>

1. Prepared in 0.1 M chloride containing 0.01 M, pH 7.0 Tris-cacodylate buffer solution.
2. Column size 1.1 x 55 cm; $V_0 = 21.5$ ml; $V_1 = 42.5$ ml.
3. A sample calculation of $K$ is presented in Appendix V.

which emerged last was called the slow component. A plot of the elution protein concentrations revealed that the elution rate of the slow component was independent of protein concentration, while that of the fast component decreases with decreasing protein concentration (Fig. 11). Such behavior has been predicted for rapidly equilibrating systems (Winzor and Scheraga, 1963) on the basis of Gilbert's theory (Gilbert, 1955). Ackers and Thompson (1965) suggested that the trailing boundaries
Fig. 11. The variation of elution rates of the trailing boundaries with protein concentration. Experiments on G-100 column with oxyhemerythrin.
can be further used to calculate the dissociation constant of the equilibrating species. For these calculations, one must first estimate the position of the minimum in the derivative curve of the trailing boundary. Together with the values of the octameric \( \sigma_n \) and monomeric \( \sigma_m \) sieve coefficients, the elution volume at the minimum position \( V_{\min} \) can then be used in the following equation (Ackers and Thompson, 1965):

\[
K = \frac{n \left( C_{\min} \right)^{n-1}}{\lambda \left( 1 + \frac{\lambda}{n} \right)^{n-1}}
\]

(2)

where \( \lambda = (\sigma_m - v'_{\min}) / (v'_{\min} - \sigma_n) \) and \( C_{\min} \) is the concentration at the minimum position of the trailing-boundary-derivative curve. \( v'_{\min} \) has been defined by Ackers and Steare (1967b) as

\[
v'_{\min} = \frac{V_{\min} - V_0 - S}{V_i}
\]

where \( S \) is the volume of the protein sample applied on the column.

However, dissociation constants from the trailing boundaries could not be calculated for oxyhemerythrin, because the position of the minimum was difficult to measure in the derivative curves. This difficulty may be due to the profiles obtained on the G-100 column or to the nature of the hemerythrin complex used in these studies.

Therefore, the next set of dissociation experiments was performed on a Sephadex G-75 column, so that the trailing boundaries would not be as diffuse as those on a G-100 column. Also, a greater dissociating complex, hemerythrin-azide (Rao and Keresztes-Nagy, 1968) was used.
C. Dissociation Experiments with Hemerythrin-azide Complex on a G-75 Column.

The G-75 column, besides giving less diffuse trailing boundaries as mentioned above, allows higher flow-rates to be used, and this shortens the experimental time. Andrews (1964) showed that the useful range of resolution for G-75 columns can be extended only to a maximum molecular weight of 70,000. Hence, lower protein concentrations (and therefore lower weight-average molecular weights) were also used for hemerythrin-azide complex. The elution profiles of these dissociation experiments are shown in Fig. 12, and the first derivative curves of that trailing boundaries in Fig. 13. The sieve coefficients were calculated from the centroid volumes of the ascending boundaries, and the weight-average molecular weight was obtained from the calibration graph (Fig. 6), at each protein concentration by extrapolation. Ackers and Steere's graphical procedure (1967) was employed for the evaluation or calculation of the molecular-sieve coefficient of the monomer. Accordingly, the various values of $\sigma_w$ are plotted against the protein concentration, and the value of $\sigma_w$ at zero protein concentration (Fig. 14) is used as $\sigma_m$ (0.62). An $\sigma_n$ of 0.0105 was calculated from the relationship $\sigma_n = \sigma_m - A \log i$, where A (0.675) is the column-calibration constant, and i is the number of subunits. The dissociation constants calculated from these parameters of the leading boundaries are shown in Table VI.

The advancing boundaries on these experiments were comparatively sharper than the diffuse trailing boundaries of the elution profiles,
TABLE VI

Effect of Dilution on the Dissociation of the Hemerythrin-Azide Complex at 25°C.

(Analysis of the Leading Boundary)

<table>
<thead>
<tr>
<th>Protein concentration (g/l)</th>
<th>Centroid volume ( \langle V \rangle ) ml</th>
<th>Weight-average coefficient ( c_w )</th>
<th>Dissociation Constant ( K ) (moles/l)</th>
<th>Weight-average molecular weight ( M_w )</th>
<th>Per cent dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.166</td>
<td>35.70</td>
<td>0.1257</td>
<td>( 3.248 \times 10^{-41} )</td>
<td>53,900</td>
<td>57.80</td>
</tr>
<tr>
<td>0.100</td>
<td>36.30</td>
<td>0.1377</td>
<td>( 5.501 \times 10^{-42} )</td>
<td>51,760</td>
<td>59.52</td>
</tr>
<tr>
<td>0.080</td>
<td>37.40</td>
<td>0.1597</td>
<td>( 4.419 \times 10^{-42} )</td>
<td>48,100</td>
<td>63.29</td>
</tr>
<tr>
<td>0.060</td>
<td>37.70</td>
<td>0.1657</td>
<td>( 8.368 \times 10^{-43} )</td>
<td>47,100</td>
<td>64.51</td>
</tr>
<tr>
<td>0.040</td>
<td>39.50</td>
<td>0.2016</td>
<td>( 2.749 \times 10^{-43} )</td>
<td>41,600</td>
<td>70.42</td>
</tr>
<tr>
<td>0.030</td>
<td>41.90</td>
<td>0.2495</td>
<td>( 2.515 \times 10^{-43} )</td>
<td>35,400</td>
<td>76.92</td>
</tr>
<tr>
<td>0.010</td>
<td>54.70</td>
<td>0.5050</td>
<td>( 1.244 \times 10^{-43} )</td>
<td>14,800</td>
<td>98.62</td>
</tr>
</tbody>
</table>

1. G-75 column size (2.2 x 59.5 cm); \( V_0 \) is 29.4 ml; \( V_i \) is 50.1 ml; 0.01 M, pH 7.0 Tris-cacodylate buffer containing 0.1 M azide was used.

2. Evaluated from Eq. 1 in terms of moles/l.

3. Derived values from the calibration equation, \( \sigma = -0.675 \log M + 3.320 \).
Fig. 12. Elution diagrams obtained at various concentrations of hemerythrin-azide complex on a G-75 Column.
Fig. 13. First difference curves of the trailing boundaries of the elution diagrams shown in Fig. 12. \( \bar{V} \) is obtained through a geometrical analysis (Longsworth, 1943) as the first moment of the gradient curve of the advancing boundary. The peak positions of the fast and slow peaks are utilized to obtain the migration rates. \( V_{min} \) is the position of the minimum in the gradient curve.
Fig. 14. Plot of the weight average sieve coefficient ($\bar{\sigma}_w$) against the protein concentration ($C_0$) -- Hr.-$N_3^-$ experiments on G-75 column.
as in the case of the oxyhemerythrin experiments on the G-100 column. However, it was still possible to estimate the minimum position in most of the derivative curves (Fig. 13) of the trailing boundaries, unlike that in the oxyhemerythrin boundaries.

Ackers and Thompson's procedure (1965) for the stoichiometry of dissociation (n) was used according to the following equation:

\[ n = \frac{3 V'_{\text{min}} - \sigma_m - 2 \sigma_n}{3 V'_{\text{min}} - 2 \sigma_m - \sigma_n} \]  

(3)

The values of 'n' so obtained are presented in Table VII. From the position of the minimum in the derivative curves of hemerythrin-azide dissociation experiments, the dissociation constants were also evaluated (Table VII).

The trailing-boundary derivative curves were examined for the rate of equilibrium in hemerythrin-azide complex. And, according to Gilbert's postulates (1955), a rapid rate of equilibrium is observed in these experiments (Fig. 15). Fig. 16 shows the trailing boundary-derivative curve of 2 µg/ml hemerythrin-azide solution in comparison to the curve obtained at 20 µg/ml concentration. Only a single slow peak (in the derivative curve) is observed at the 2 µg/ml protein concentration.

As indicated earlier, the column-partition parameter, the sieve coefficient, can also be evaluated in terms of the size of the molecular species being studied. By using the calibration equation \( a = a_0 + b_0 \, [\text{erfc}^{-1}(\sigma)] \), we obtained the molecular radii of the monomeric and octameric forms of hemerythrin. Table VIII contains these values. It was of interest to compare such values with those obtained on a Sephadex
### TABLE VII

Effect of Dilution on the Dissociation of the Hemerythrin-Azide Complex at 25°C.

(Analysis of the Trailing Boundary)

<table>
<thead>
<tr>
<th>Protein concentration (g/l)</th>
<th>Elution volume ( V_{\text{min}} ) (ml)</th>
<th>Protein concentration at the minimum in the derivative curve ( C_{\text{min}} ) (g/l)</th>
<th>Dissociation constant ( K ) (moles/l)</th>
<th>Stoichiometry of dissociation ( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.166</td>
<td>68.5</td>
<td>0.00806</td>
<td>( 1.74 \times 10^{-43} )</td>
<td>---</td>
</tr>
<tr>
<td>0.100</td>
<td>71.5</td>
<td>0.00787</td>
<td>( 5.63 \times 10^{-43} )</td>
<td>11.0</td>
</tr>
<tr>
<td>0.080</td>
<td>72.5</td>
<td>0.00879</td>
<td>( 1.15 \times 10^{-42} )</td>
<td>6.0</td>
</tr>
<tr>
<td>0.060</td>
<td>73.0</td>
<td>0.00806</td>
<td>( 6.40 \times 10^{-43} )</td>
<td>5.0 ( \pm ) 1.0</td>
</tr>
<tr>
<td>0.040</td>
<td>71.8</td>
<td>0.00550</td>
<td>( 2.73 \times 10^{-44} )</td>
<td>10.00</td>
</tr>
<tr>
<td>0.030</td>
<td>71.5</td>
<td>0.00623</td>
<td>( 5.99 \times 10^{-44} )</td>
<td>11.00</td>
</tr>
<tr>
<td>0.010</td>
<td>73.0</td>
<td>0.00623</td>
<td>( 5.64 \times 10^{-44} )</td>
<td>5.0</td>
</tr>
</tbody>
</table>

a. \( V_0 = 29.4 \) ml, \( V_1 = 50.1 \) ml and sample applied = 20.0 ml.

b. Derived from Eq. 2 in terms of moles/l.

c. Evaluated from Eq. 3.
Fig. 15. The variation of elution rates of the trailing boundaries with protein concentration - Hr. N₃ experiments on a G-75 Column.
Fig. 16. First derivative curves of the advancing and trailing boundaries obtained in frontal experiments with 200 µg/ml and 2 µg/ml of Hr. N₃⁻ on a G-75 Column.
G-100 column. For this purpose, the sieve coefficients of the same ligand-complex (Table III data), hemerythrin-azide, were used to calculate the molecular radii of the polymeric and monomeric species. The obtained results are also shown in Table VIII.

**TABLE VIII**

Data on monomeric and octameric molecular radii of hemerythrin-azide complex.

<table>
<thead>
<tr>
<th></th>
<th>G-75 Column</th>
<th>G-100 Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomeric sieve coefficient, ( \sigma_m )</td>
<td>0.620</td>
<td>0.6130</td>
</tr>
<tr>
<td>Octameric sieve coefficient, ( \sigma_n )</td>
<td>0.0105</td>
<td>0.1053</td>
</tr>
<tr>
<td>( l - \sigma_m )</td>
<td>0.380</td>
<td>0.3870</td>
</tr>
<tr>
<td>( l - \sigma_n )</td>
<td>0.9895</td>
<td>0.8947</td>
</tr>
<tr>
<td>( \text{erfc}^{-1} \sigma_m )</td>
<td>0.3507</td>
<td>0.3577</td>
</tr>
<tr>
<td>( \text{erfc}^{-1} \sigma_n )</td>
<td>1.809</td>
<td>1.145</td>
</tr>
<tr>
<td>Calibration constant, ( a_0 )</td>
<td>8.3</td>
<td>7.5</td>
</tr>
<tr>
<td>Calibration constant, ( b_0 )</td>
<td>19</td>
<td>24.4</td>
</tr>
<tr>
<td>Molecular radius of monomer in ( \AA )</td>
<td>15.0 \pm 0.5</td>
<td>16.2 \pm 0.5</td>
</tr>
<tr>
<td>Molecular radius of octamer in ( \AA )</td>
<td>42.7 \pm 0.5</td>
<td>35.4 \pm 0.5</td>
</tr>
</tbody>
</table>

The close agreement (within 1.2 \( \AA \)) between the values of monomeric molecular radius on the two columns indicates that the gel particles are inert to the protein. Therefore, the data obtained with linear resolution on both G-75 and G-100 columns can be used for comparison purposes.
D. Examination of the Rate of Equilibrium on the Theoretical Plate

Concept:

At this point, it may be recalled that there is a contradiction in the findings of this investigation and those of Klapper et al. (1966) about the rate of hemerythrin equilibrium. With this discrepancy in mind, we examined some of the aged (90 days after preparation of the protein) solutions of hemerythrin-thiocyanate. The elution profile at a protein concentration of 68 µg/ml is shown in Fig. 17. One can see the presence of a peak at the beginning of the descending portion of the elution diagram, which may represent the monomers whose sulfhydryl groups have been oxidized. Such 'crippled' monomers have also been reported by Langerman (1969) by the disc-electrophoresis method. The change of an -SH group to a SO$_3^-$ group is attributed to the oxidizing properties of the thiocyanate ion. The above dissociation experiment with aged hemerythrin-thiocyanate solution indicates that the consideration of the nature of the ligand is also important in the interpretation of elution boundaries for the rate of equilibrium. Therefore, the dissociation of oxyhemerythrin was examined at various concentrations, and without the solubilizing salt, sodium chloride, a known iron-complexing ligand. At very low protein concentrations (< 20 µg/ml), the boundary shape is different than those obtained with hemerythrin-thiocyanate and oxyhemerythrin (no salt) at higher protein concentration (68 µg/ml). Figures 18 and 19 show the elution profiles at 20 µg/ml and 68 µg/ml concentrations respectively. The behavior at low oxyhemerythrin concentrations may be
Fig. 17. Elution profile of a 68 µg/ml Hr-SCN\textsuperscript{-} preparation (> 90 day old) on a G-75 Column.
Fig. 18. Elution profile of a 20 µg/ml oxyhemerythrin solution on a G-75 Column.
Fig. 19. Elution profile of a 68 µg/ml oxyhemerythrin solution on a G-75 Column.
interpreted as that of an intermediate type of equilibrium. Recently, further experiments (Tan and Keresztes-Nagy, 1971) confirmed this intermediate rate of equilibration for oxyhemerythrin at very low concentrations.

Information about the rate of equilibration of the dissociation of octameric hemerythrin may also be derived from the spread of the derivative curve of the leading boundary, and from the elution time. From the spread of the derivative curve and the length of the column, one can obtain the number of theoretical plates; from the elution time one can estimate how long the protein remains on each of these plates. In a frontal-elution-type experiment, the spread of the derivative curve is measured as the width of the base of the peak. This value is then used to calculate the total number of theoretical plates of the column by using the equation derived by Morris and Morris (1963):

\[
P = \left(\frac{4V_e}{W}\right)^2,
\]

where

- \( P \) = total number of theoretical plates
- \( V_e \) = elution volume of the protein
- \( W \) = width of the elution peak

These parameters were evaluated for the dissociation experiment of hemerythrin-thiocyanate (68 \( \mu \text{g/ml} \), Fig. 17), and the oxyhemerythrin experiment (68 \( \mu \text{g/ml} \), Fig. 19). Table IX contains the values of these parameters. The values of height equivalent to theoretical plate (HETP) were obtained from the column-bed heights, and were found to be 0.33 mm.
for G-100 and 0.45 mm for the G-75 column. These HETP values agree with the reported values of 0.2 to 0.8 mm for gel chromatography by Determann (1968).

**TABLE IX**

Experimental data of oxyhemerythrin and thiocyanate complex to evaluate HETP

<table>
<thead>
<tr>
<th>Sephadex type</th>
<th>Height of the column bed height</th>
<th>Elution volume</th>
<th>Flow-rate</th>
<th>Elution time</th>
<th>Width of the derivative peak</th>
<th>P</th>
<th>HETP</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxy- Hr</td>
<td>550 mm</td>
<td>40.6 ml</td>
<td>24 ml/hr</td>
<td>6960 seconds</td>
<td>4 cm</td>
<td>1650</td>
<td>0.33 mm</td>
</tr>
<tr>
<td>Hr - SCN⁻</td>
<td>564 mm</td>
<td>75.0 ml</td>
<td>60 ml/hr</td>
<td>4500 seconds</td>
<td>8 cm</td>
<td>1306</td>
<td>0.45 mm</td>
</tr>
</tbody>
</table>

1 Obtained from the peak position of the leading boundary-derivative curve.

Since the elution of the protein was carried out at constant flow-rate, the time spent on each theoretical plate by the protein can be calculated from the total elution volume. For the oxyhemerythrin, this time was 4.3 seconds; for the thiocyanate, 3.5 seconds. A fast type of equilibrium behavior can be observed only if the monomeric species attain equilibrium with the octameric species much faster than the time (3.5 - 4.3 sec.) the protein remains on each theoretical plate of the column.
On the other hand, a slow type of equilibrium behavior can be observed if the time required to achieve equilibrium between the octameric and monomeric species is large compared to the time the protein remains on each theoretical plate. In the latter case, two boundaries should be seen in the ascending portion of the elution diagrams. In other words, on each theoretical plate, the monomers will be retarded successively from the octamers, so that two separate plateaus appear in the elution profiles. Such a situation has been observed in the elution profile of a 25 µg/ml oxyhemerythrin at 0.1 M ionic strength and 5° (Tan and Keresztes-Nagy, 1971).

As shown in Table IX, the flow-rates were different in each dissociation experiment, but the time spent on every theoretical plate was found to be almost the same (3.5 and 4.3 seconds). Since flow-rates greater than 60 ml/hr cannot be used with higher grades of Sephadex gels (G-50), gel chromatography limits further comparison on the basis of elution time.

E. Observation of the Effect of Iron-Coordinating Ligands, SCN⁻, N₃⁻ and O₂.

A re-examination of the dissociation data presented so far, reveals that the nature of the ligand affects the depolymerizing behavior of hemerythrin. Although the study of the effect of coordinating anions on the dissociation properties of hemerythrin was not one of the aims of this investigation, a comparison of the available data (Table X) was made for verification of previous findings (Rao and Keresztes-Nagy, 1968).
These data indeed show that iron-binding ligands alter the dissociation behavior of hemerythrin, and that the thiocyanate complex dissociates to a greater extent than the azide complex at each protein concentration. This behavior is especially evident at the lowest protein concentration. In view of this finding, we next examined the dissociation properties of hemerythrin whose iron atoms were combined with its natural ligand oxygen, instead of coordinating anions. The results are tabulated in Table XI.

The percent of dissociation at all concentrations as seen in Table XI is low, compared to the values obtained with hemerythrin-coordinating anion complexes. One may recall here that the useful range of resolution in the calibration graph of G-75 to be 3,000 - 60,000. The weight-
TABLE XI

Effect of dilution on the dissociation of oxyhemerythrin\(^1\) with a G-75 column (2.2 x 59.5 cm)

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Centroid Volume(^2), (V) (ml)</th>
<th>Weight-average Molecular weight</th>
<th>Percent Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.176</td>
<td>36.0</td>
<td>102,000</td>
<td>5</td>
</tr>
<tr>
<td>0.100</td>
<td>39.7</td>
<td>93,300</td>
<td>13</td>
</tr>
<tr>
<td>0.086</td>
<td>40.4</td>
<td>88,900</td>
<td>20</td>
</tr>
<tr>
<td>0.068</td>
<td>40.6</td>
<td>87,000</td>
<td>23</td>
</tr>
<tr>
<td>0.040</td>
<td>41.4</td>
<td>81,300</td>
<td>28</td>
</tr>
<tr>
<td>0.034</td>
<td>42.0</td>
<td>78,500</td>
<td>31</td>
</tr>
<tr>
<td>0.027</td>
<td>42.3</td>
<td>71,000</td>
<td>39</td>
</tr>
<tr>
<td>0.020</td>
<td>two apparent plateaus in the ascending boundary</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Prepared in 0.01 M Tris-cacodylate (pH 7.0) buffer.
2. The exclusion volume \((V_0) = 33.7 \text{ ml.}\)

Average molecular weights obtained in these experiments are obviously in the non-linear portion of the G-75 column calibration plot. Therefore, the calculated percent of dissociation must not be considered as an absolute value at any oxyhemerythrin concentration.
F. Studies on the Specific Effect of Chloride Ion:

Early in the dissociation studies with oxyhemerythrin, it was noticed that some of the molecular properties (percent of dissociation) were altered by the presence or absence of sodium chloride in the eluent. However, this finding was not totally surprising, because the kinetic properties of the hemerythrin sulfhydryl groups had been found to depend not only on the presence of chloride ion, but also on its concentration (Fransioli, 1969). Hence the dissociation behavior of oxyhemerythrin was studied in the presence of 0.5 M chloride ion. The results of these experiments are presented in Table XII.

**TABLE XII**

Dissociation studies of oxyhemerythrin in the presence of 0.5 M Cl⁻ on a G-75 column (2.2 x 59.5 cm).

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Weight-average Molecular Weight</th>
<th>Percent Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.147</td>
<td>72,800</td>
<td>37.3</td>
</tr>
<tr>
<td>0.098</td>
<td>68,860</td>
<td>41.4</td>
</tr>
<tr>
<td>0.089</td>
<td>62,800</td>
<td>48.4</td>
</tr>
<tr>
<td>0.064</td>
<td>58,210</td>
<td>52.8</td>
</tr>
<tr>
<td>0.035</td>
<td>41,700</td>
<td>70.2</td>
</tr>
</tbody>
</table>

1. Made in 0.01 M Tris-cacodylate (pH 7.0) buffer containing 0.5 M Cl⁻.
2. \( V_0 = 33.7 \) ml.
As shown in Table XII, a greater amount of dissociation was observed in the presence of 0.5 M chloride ion, in comparison with the oxy-hemerythrin data (Table XI). In order to explain this finding, we compared in Table XIII the extent of dissociation of oxyhemerythrin in the presence of no chloride (0.1 M buffer), 0.1 M chloride (0.01 M buffer), and 0.5 M chloride (0.01 M buffer).

### TABLE XIII

Comparative data\(^1\) on oxyhemerythrin dissociation in various experimental conditions.

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Conditions of Dissociation Experiment</th>
<th>Percent dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>Oxy.Hr (in 0.01 M buffer) + 0.1 M chloride</td>
<td>22</td>
</tr>
<tr>
<td>0.089</td>
<td>Oxy.Hr (in 0.1 M buffer) + no chloride</td>
<td>20</td>
</tr>
<tr>
<td>0.098</td>
<td>Oxy.Hr (in 0.01 M buffer) + 0.5 M chloride</td>
<td>41</td>
</tr>
<tr>
<td>0.100</td>
<td>Oxy.Hr (in 0.01 M buffer) + no chloride</td>
<td>13</td>
</tr>
<tr>
<td>0.023</td>
<td>Oxy.Hr (in 0.01 M buffer) + 0.1 M chloride</td>
<td>51</td>
</tr>
<tr>
<td>0.027</td>
<td>Oxy.Hr (in 0.01 M buffer) + no chloride</td>
<td>39</td>
</tr>
</tbody>
</table>

1. Compiled from Tables V, XI and XII.
These data indicate that as the chloride concentration is increased to 0.5, the depolymerization of hemerythrin increases, a finding in agreement with kinetic data of Fransioli (1969). However, one must also consider the effects on hemerythrin dissociation due solely to the increase in the ionic strength of the solutions. For this purpose, the data obtained with oxyhemerythrin made in 0.1 M buffer solution and in the absence of chloride ion are also included in the above Table XIII. Some increase in the percent of dissociation was found, which was most likely due to the increase in ionic strength from 0.01 M to 0.1 M and the lower protein concentration used (0.089 mg/ml instead of 0.100 mg/ml). Therefore, any further increase in the dissociation in 0.1 M chloride can only be explained on the basis of specific effects by the chloride ion. In spite of higher protein concentration (0.125 mg/ml) an increase in the dissociation was observed in 0.1 M chloride. Similarly, the increase in the percent of dissociation in the presence of 0.5 M chloride, must be due to the specific effects of chloride ion and not because of the increase in the total ionic strength. The dissociation was examined at 0.5 M chloride, since this is the level of chloride at which the iron-chloride coordination is known to be not less than 90%. For a further understanding of the experimental data listed in Table XIII, one must, therefore, observe the extent of coordination that can occur in oxyhemerythrin in the mere presence of chloride ion. Such an observation can be easily carried out by following the changes in the iron-coordination spectra of oxyhemerythrin (See Chapter II). As shown in Fig. 20,
Fig. 20. Spectral Conversion of a 40 µg/ml protein in 0.5 M chloride ion.

1. Intermediate stages
2. Chloro complex spectra
3. 380 nm
the spectral changes progress with time and the final coordination spectra of the oxyhemerythrin in 0.5 M chloride was found to be exactly identical with that of a chloride-hemerythrin complex made by the standard dialysis procedure (Keresztes-Nagy and Klotz, 1965). In spite of the identical spectral nature of these two hemerythrin complexes, the possibility that they might possess dissimilar dissociation properties cannot be ruled out.

The percent dissociation of a 40 µg/ml oxyhemerythrin solution at three different stages of spectral conversion was therefore examined. Table XIV lists the experimental conditions and the results of these experiments.

**TABLE XIV**

Dissociation behavior of a 40 µg/ml oxyhemerythrin\(^1\) in 0.1 M chloride ion.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Time after dilution into chloride (mts)</th>
<th>Centroid Volume(^2) (ml)</th>
<th>Weight-Average Molecular Weight</th>
<th>Percent Dissociation</th>
<th>Percent Spectral Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample applied at 60 ml/hr immediately after preparation</td>
<td>43</td>
<td>42.5</td>
<td>55,100</td>
<td>56.0</td>
<td>36</td>
</tr>
<tr>
<td>Sample applied at 24 ml/hr immediately after preparation</td>
<td>85</td>
<td>42.6</td>
<td>54,500</td>
<td>56.4</td>
<td>67</td>
</tr>
<tr>
<td>Sample applied at 24 ml/hr one hour after preparation</td>
<td>140</td>
<td>42.4</td>
<td>56,000</td>
<td>55.8</td>
<td>100</td>
</tr>
</tbody>
</table>

1. Prepared in 0.01 M (pH 7.0) Tris-calcodylate buffer medium.

2. \(V_0 = 32.0\) ml. A G-75 column (2.2 x 49.5 cm) is used.
At all the three levels of spectral conversion, the amount of dissociation was found to be almost identical. This finding suggests that the dissociation property of oxyhemerythrin is independent of the status of iron coordination.

Hence, from these data (Table XIV), the effect of chloride may be interpreted as that of a secondary non-iron binding. Such a secondary chloride binding step has been postulated previously by Keresztes-Nagy and Klotz (1965), and Fransioli (1969). According to this postulate, the amount of dissociation should increase until all the chloride-binding sites are saturated. Indeed, in the presence of 0.5 M chloride ion, where most of the binding sites should be saturated, a higher percent of dissociation was found (Table XIII). One would also expect the rate of iron-chloride coordination to be higher at higher chloride levels. Fig. 21 shows the results obtained in 0.1 M and 0.5 M chloride levels. As expected, the rate of conversion at 0.5 M chloride was found to be faster than that of a oxyhemerythrin in 0.1 M chloride ion.

Similarly, observation of the rate of spectral conversion of different oxyhemerythrin solutions in the presence of 0.5 M chloride might also be useful. Fig. 22, shows that the rate of conversion decreases at higher protein concentrations. This finding can be interpreted as the result of a dissociation effect rather than a mass effect, since in the latter case the conversion of the more concentrated protein would be faster than that of a less concentrated oxyhemerythrin.
Fig. 21. Effect of chloride on percent spectral conversion of oxy-Hr. to Hr.–chloride complex.
Fig. 22. Effect of protein concentration on percent spectral conversion of oxy Hr. to chloro complex.
So far, we have considered experimental data which strongly indicate the binding of chloride ion at sites other than the iron atoms in oxyhemerythrin. One may also deduce from the observed independence of the dissociation effect (in relation to the amount of iron-chloride coordination, Table XIV), that the non-iron sites bind with greater speed. In addition, this binding has been shown to have a greater effect on the -SH group reactivity, than the iron-chloride binding (Fransioli, 1969).

However, direct evidence for such a fast chloride binding at sites other than the iron atoms is lacking. Therefore, this investigation sought to provide the evidence from direct binding experiments on G-25 columns. But before the results of these experiments are examined, it might be worthwhile to examine the evidence obtained from some experiments performed as an extension on the observations of the coordination spectral conversion studies.

As indicated earlier, the dissociation properties of oxyhemerythrin are affected by chloride ions independently of the changes at the iron atoms (oxygen release and iron-chloride coordination). Nonetheless, does the conformation (in relation to dissociation properties) remain the same after complete iron-chloride coordination (100% spectral conversion)? An answer to this question is provided by the data in Table XV. The amount of dissociation of a 46.5 µg/ml oxyhemerythrin in 0.5 M chloride has been examined, long after complete oxy-chlorohemerythrin spectral conversion was achieved.
TABLE XV

Dissociation studies\(^1\) of a 46.5 µg/ml oxyhemerythrin in 0.5 M chloride in relation to time.

<table>
<thead>
<tr>
<th>Time after sample preparation (Hours)</th>
<th>Centroid Volume$^1$, $\overline{V}$ (ml)</th>
<th>Weight-Average Molecular Weight</th>
<th>Percent of Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>43.5</td>
<td>55,500</td>
<td>55.9</td>
</tr>
<tr>
<td>27.5</td>
<td>43.4</td>
<td>55,000</td>
<td>56.0</td>
</tr>
<tr>
<td>91.5</td>
<td>43.6</td>
<td>56,000</td>
<td>55.8</td>
</tr>
</tbody>
</table>

1. As observed on a G-75 column with a $V_o = 33.0$ ml.

The apparent constancy in the weight-average molecular weight, as shown in the above Table (XV), indicates that no further changes in the dissociation (and probably, conformation) occur after the initial effect by the chloride ions.


From the experimental results of the chloride-effect on the dissociation behavior of hemerythrin, the following observations can be made: (1) chloride ion affects the dissociation behavior independent of coordination with the iron atoms of hemerythrin, (2) the conformational changes, (dissociation effects) are stable for at least a period of 90 hours.
With respect to earlier evidence (Keresztes-Nagy and Klotz, 1965; Fransioli, 1969; Table X, This chapter), of chloride effects on the dissociability and reactivity of sulfhydryl groups through iron-coordination, what do those observations mean? In other words, are the conformations which arise from chloride-iron binding and from chloride binding at other sites in the protein moiety one and the same? The fact that their coordination spectra became identical with time does not indicate identical conformations.

Indirect evidence that the conformations are not the same has already been furnished by some of our experiments with the thiocyanate-hemerythrin complex. Originally, these experiments were performed to obtain a hemerythrin-thiocyanate complex whose sulfhydryl groups are in the reduced state, since thiocyanate ion tends to oxidize the sulfhydryl groups during dialysis and with time. We tried to prepare a complex with this ion by simple dilution of the oxyprotein into the thiocyanate-containing buffer. Due to the rapid exchange of oxygen with thiocyanate ion, a hemerythrin complex having the same spectral characteristics as that derived from the dialysis procedure (Keresztes-Nagy and Klotz, 1965), was obtained within 20-30 minutes after dilution. The percent of dissociation of one such hemerythrin-thiocyanate preparation was examined at several protein concentrations. The results are shown in Table XVI.
TABLE XVI

Dissociation behavior\(^1\) of a thiocyanate-hemerythrin complex obtained by dilution procedure\(^2\).

<table>
<thead>
<tr>
<th>Percent Concentration</th>
<th>Weight-average Molecular weight</th>
<th>Percent of Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0775</td>
<td>59,570</td>
<td>50.75</td>
</tr>
<tr>
<td>0.0450</td>
<td>45,710</td>
<td>65.63</td>
</tr>
<tr>
<td>0.0270</td>
<td>40,270</td>
<td>71.38</td>
</tr>
</tbody>
</table>

1. Observed on a G-100 (2.0 x 50.6 cm) column with \(V_0 = 14.20\) ml.
2. Made in 0.1 M KSCN containing 0.01 M, pH 7.0, Tris-cacodylate buffer.

Similarly, the dissociation behavior of the hemerythrin-thiocyanate complex prepared by the dialysis procedure was also examined. These results are listed in Table XVII.

TABLE XVII

Dissociation behavior\(^1\) of a hemerythrin-thiocyanate complex obtained through the dialysis procedure\(^2\).

<table>
<thead>
<tr>
<th>Protein Concentration (mg/ml)</th>
<th>Weight-Average Molecular Weight</th>
<th>Percent of Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0720</td>
<td>42,650</td>
<td>69.2</td>
</tr>
<tr>
<td>0.0475</td>
<td>37,600</td>
<td>74.5</td>
</tr>
<tr>
<td>0.0260</td>
<td>26,900</td>
<td>85.8</td>
</tr>
</tbody>
</table>

1. Observed on a G-100 (2.0 x 50.6 cm) column with a \(V_0\) of 14.20 ml.
2. 0.01 M, pH 7.0 Tris-cacodylate buffer containing 0.1 M KSCN was used.
As can be seen from Tables XV and XVI, at each (almost identical) concentration, the percent dissociation of the thiocyanate complex obtained by the dialysis procedure was significantly different from that of the complex obtained by the direct dilution method. Note that both these preparations possessed identical iron-coordination spectra. The changes in the dissociation properties could have been brought about by thiocyanate linkages at sites other than the iron atoms.

The above results, therefore, show that identical coordination spectra of hemerythrin (proteins) do not necessarily represent the identical nature of their conformations.

Returning to the question concerning the nature of the chloro-hemerythrin complex prepared by different procedures, it is entirely possibly that, irrespective of identical coordination spectra, the conformations may be different.

Fig. 23 shows the two different methods by which chloro complex was obtained. The oxygen of the oxyform is rapidly exchanged with fluoride ions, as shown in scheme II, and the complex is then dialized against chloride-containing buffer to yield a chloro complex. Aqua form is the intermediate-ligand complex in this procedure. The aquo-Hr has been found to produce the same chloride complex, either by dialysis or dilution with 0.5 M chloride, since they showed identical dissociation behavior at identical protein concentrations, as shown in Table XVIII.
Fig. 23. Shows two different procedures through which a hemerythrin chloride complex containing identical absorption spectra can be obtained from oxy-hemerythrin. The oxygen is exchanged directly in Scheme I with 0.5 M chloride ion, whereas 0.1 M fluoride ions are used in Scheme II.
TABLE XVIII
Comparison of the dissociation behavior of chlorohemerythrin obtained through the aquo complex.

<table>
<thead>
<tr>
<th>Protein Preparation</th>
<th>Protein Concentration (µg/ml)</th>
<th>Weight-average Molecular Average</th>
<th>% Dissociation</th>
<th>% Spectral Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqua-Hr + 0.5 M Cl⁻ (immediately after dilution)</td>
<td>23</td>
<td>44,700</td>
<td>68</td>
<td>5 - 10%</td>
</tr>
<tr>
<td>Aqua-Hr + 0.5 M Cl⁻ (38 hrs after dilution)</td>
<td>23</td>
<td>43,700</td>
<td>69</td>
<td>100%</td>
</tr>
<tr>
<td>Aqua-Hrs + 0.5 M Cl⁻ (67 Hrs after dilution)</td>
<td>23</td>
<td>43,150</td>
<td>69</td>
<td>100%</td>
</tr>
<tr>
<td>Aqua-Hr - dialysis with 0.5 M Cl⁻</td>
<td>22</td>
<td>42,700</td>
<td>70</td>
<td>100%</td>
</tr>
</tbody>
</table>

1. Observed on a G-75 column (2.2 x 49.5 cm) with $V_0 = 33.7$ ml.

However, the chloride complex obtained by the scheme I (Fig. 23) exhibited different dissociation behavior, even though it had the same spectral characteristics as that obtained through the upper scheme. Table XIX lists the percent dissociation observed with protein complexes produced by both the methods.
TABLE XIX
Comparative data obtained on a chlorohemerythrin prepared by two different procedures.

<table>
<thead>
<tr>
<th>Protein Preparation</th>
<th>Concentration (µg/ml)</th>
<th>Weight-Average Molecular Weight</th>
<th>% Dissociation</th>
<th>% Spectral Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aquo-Hr</td>
<td>40</td>
<td>76,000</td>
<td>33</td>
<td>---</td>
</tr>
<tr>
<td>Aquo-Hr + 0.5 M Cl⁻ (upper scheme)</td>
<td>40</td>
<td>42,200</td>
<td>68</td>
<td>100</td>
</tr>
<tr>
<td>oxy-Hr + 0.5 M Cl⁻ (lower scheme)</td>
<td>40</td>
<td>54,950</td>
<td>56</td>
<td>100</td>
</tr>
<tr>
<td>oxy-Hr</td>
<td>40</td>
<td>82,200</td>
<td>28</td>
<td>---</td>
</tr>
</tbody>
</table>

1. Observed on a G-75 (2.2 x 49.5 cm) column with V₀ = 33.7 ml.

Therefore, the results of the dissociation experiments carried out on hemerythrin complexes (with SCN⁻ and Cl⁻) prepared by different methods can be summarized together with the results on anionic effects as follows: the dissociation behavior is dependent not only on the absence or presence of a particular ion (and its amount), but also by the method of preparation.

H. Studies on the Effect of Some Specific Non-Coordinating Anions:

Before presenting the chloride binding data, the effects of some known non-iron coordinating ions (such as perchlorate, nitrate, sulfate) on the dissociation behavior of oxyhemerythrin will be
considered. This is done in order to call attention to the similarity in the present observations with non-iron chloride bindings and the specific binding by perchlorate and other ions, also at non-iron sites (Darnall et al., 1968; Garbett et al., 1971a).

Darnall and coworkers (1968) have shown with ultracentrifugation technique that the non-coordinating ions (perchlorate, nitrate, and sulfate) reduce the dissociation of hemerythrin octamer. Previously, we have confirmed the effect of perchlorate ion on dissociation by gel chromatographic experiments (Rao, 1969). Now, two more ions, acetate and bromide, have been examined for their effects on the dissociation of oxyhemerythrin. The concentration of the ion used was 0.1 M, and the protein was diluted into the ion-containing buffer to a concentration of approximately 25 µg/ml. Table XX lists the results of these dissociation experiments. For comparison purposes, the observed extent of dissociation of a 22 µg/ml aquo-Hr is also included in this table.

Recently, Garbett et al. (1971a) have provided evidence that perchlorate ion (more than the other non-coordinating ions, nitrate and sulfate) decreases the dissociation of hemerythrin by binding to, most probably, the histidine residues in the protein. From the results in Table XX, acetate and bromide apparently do not affect the properties the same way as the perchlorate ion.

It is hoped that the binding-experimental data can be presented here in perspective with the specific binding of non-coordinating ions.
TABLE XX

Effects of some specific non-coordinating ions on the dissociation of oxy-hemerythrin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein Concentration (mg/ml)</th>
<th>Weight-average Molecular Weight</th>
<th>Percent of Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxy-Hr (0.01 M Tris-cac buffer)</td>
<td>27</td>
<td>71,000</td>
<td>38.5</td>
</tr>
<tr>
<td>Aquo-Hr (0.01 M Tris-cac buffer)</td>
<td>25</td>
<td>54,300</td>
<td>56.4</td>
</tr>
<tr>
<td>oxy-Hr + 0.1 M perchlorate</td>
<td>22</td>
<td>54,320</td>
<td>56.4</td>
</tr>
<tr>
<td>oxy-Hr + 0.1 M acetate</td>
<td>25</td>
<td>35,500</td>
<td>76.5</td>
</tr>
<tr>
<td>oxy-Hr + 0.1 M bromide</td>
<td>25</td>
<td>37,140</td>
<td>74.7</td>
</tr>
</tbody>
</table>

1. As observed on a G-75 (2.2 x 49.5 cm) column of $V_o = 28.2$ ml.

I. Direct Experimental Evidence for Binding by Chloride at Non-Iron Sites before Coordination with the Iron Atoms:

Initially, we monitored at 220 nm the depletion of chloride in the effluent of a pre-equilibrated G-25 column. These experiments were carried out in a manner similar to those performed originally by Hummel and Dreyer (1962). But the high absorption of the buffer salts, even at an ionic-strength of $10^{-3}$, prevented the observation of the ligand-protein peak and the characteristic ligand trough in the base level of chloride. In these experiments, the oxyhemerythrin was allowed to
equilibrate with the chloride-containing buffer for about ten minutes before the sample was applied to the similarly-equilibrated G-25 column. Since direct monitoring with a spectrophotometer was almost impossible, aliquots from the column effluent were assayed for chloride ion. The titration data of aliquots from binding experiments at 0.003 M (Cl⁻) are shown in Fig. 24 a and b. The appearance of a trough at the salt-elution position represented chloride-protein binding, since equilibrium conditions were maintained.

In order to have a Cl⁻ concentration high enough to measure by titration, we used a higher level of chloride in the sample preparation, than in the equilibrating buffer. The chloride level of the sample was chosen so that the unbound-chloride concentration would be higher than the chloride concentration in the column equilibration solution. Accordingly, at equilibrium, the unbound chloride will appear as a peak instead of a trough. With this procedure, the depletion in the ligand peak was measured at various protein concentrations by a comparison with the area of a sample containing only the chloride. From these measurements the moles of chloride bound to each mole of oxyhemerythrin (the r values) were obtained. Table XXI lists such values.

The value of r, a measure of binding, varied from 1-3. This variation was attributed to the inaccuracy of the titration procedure, rather than to a variation in the amount of binding, because the free Cl⁻ concentration was identical (3 x 10⁻³M), in all experiments. The depletion of the ligand peak could not be correlated with the ligand bound to the protein, since the titrant [Hg(NO₃)₂] could react with the sulfhydryl groups in the oxyhemerythrin.
Figs. 24 a and b show the titration profile of an oxyhemerythrin solution on a G-25 Column equilibrated with 0.003 M Cl⁻.

a. Blank protein sample

b. Protein + chloride (same amount as that of the column equilibration)

Conditions: Protein concentration = 7.8 µg/ml. Sample = 0.8 ml. 1 µg/ml of Hg(NO₃)₂ = 0.01 µ moles of chloride ion.
### TABLE XXI

Experimental data of chloride-hemerythrin binding by the Hg(NO$_3$)$_2$ titration procedure

<table>
<thead>
<tr>
<th>Oxy-hemerythrin concentration (mg/ml)</th>
<th>Amount of chloride depleted (µmoles)</th>
<th>Number of moles of chloride mole of oxy-hemerythrin (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>0.54</td>
<td>1.14</td>
</tr>
<tr>
<td>29</td>
<td>0.45</td>
<td>0.72</td>
</tr>
<tr>
<td>24</td>
<td>0.38</td>
<td>0.42</td>
</tr>
<tr>
<td>22*</td>
<td>0.35</td>
<td>1.15</td>
</tr>
<tr>
<td>17</td>
<td>0.27</td>
<td>0.21</td>
</tr>
<tr>
<td>16.5*</td>
<td>0.26</td>
<td>0.75</td>
</tr>
<tr>
<td>12.6</td>
<td>0.20</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Experimental conditions: The G-25 column was equilibrated with 0.003 M (Cl$^-$)
Blank chloride sample contained a 0.006 M Cl$^-$.
Sample size 1.7 ml; Aliquots from titration 100 µl.
Each fraction collected = 0.5 ml

* A different stock oxyhemerythrin solution was used in these experiments.

Because of these limitations of the method, we adopted a scintillation-counting procedure using labelled chloride. A series of binding experiments were performed at various oxyhemerythrin concentrations, and
the bound chloride was measured from the activity associated with the protein fraction. Fig. 25 shows the results of these experiments. The amount of activity appearing at the protein elution position is shown to increase with increasing hemerythrin concentration. The counts were corrected for protein quenching. The quenching curve obtained in the range of concentrations used in these experiments is presented in Fig. 26. The data in Table XXII indicate that there was no significant variation in the extent of binding over the range of protein concentrations employed in these experiments. The results also indicate that the measurements of bound chloride are reliable and reproducible by the scintillation-counting procedure.

**TABLE XXII**

Data on the chloride-hemerythrin binding by using labelled chlorine-36.

<table>
<thead>
<tr>
<th>oxy-hemerythrin concentration (mg/ml)</th>
<th>µmoles of octamer</th>
<th>µmoles of chloride bound</th>
<th>moles of chloride bound moles of octameric protein (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>0.78</td>
<td>1.24</td>
<td>1.59</td>
</tr>
<tr>
<td>34</td>
<td>0.54</td>
<td>0.9</td>
<td>1.66 ± 0.2</td>
</tr>
<tr>
<td>34</td>
<td>0.54</td>
<td>0.9</td>
<td>1.66</td>
</tr>
<tr>
<td>18.5</td>
<td>0.29</td>
<td>0.464</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Thus far, two major aspects of hemerythrin interactions have been examined: (1) The association-dissociation equilibrium. (2) Anionic (iron-coordinating as well as non-iron coordinating) effects on the equilibrium.
Fig. 25. Binding experiments with oxyhemerythrin on a G-25 Column with chlorine-36.

Experimental conditions: Sample = 1.7 ml; Flow-rate = 120 ml/hr. Column saturation buffer = 0.01 Tris-cacodylate containing 0.003 M chloride ($^{36}$Cl = 0.0018 M); Temperature = 25° C. Protein sample contained 0.006 M chloride (0.003 M Cl-36).
Fig. 26. Quenching curve obtained in the range of 18.5 to 50 mg/ml protein concentration. Counting conditions may be found in 'Methods'.
Since the sulfhydryl groups of hemerythrin have been previously shown (Keresztes-Nagy and Klotz, 1965; Egan, 1969; Duke, et al., 1971) to affect both of the above interactions, an examination of the nature of the sulfhydryl-mercurial reaction and its influence on the observed equilibrium and anion binding was logical, if not absolutely essential.

Furthermore the use of gel chromatography for such an examination appeared to be advantageous since the reactants could possibly be separated from the reaction mixture as compared to the conventional spectrophotometric procedure (Boyer, 1954).

Therefore, the sulfhydryl group-mercurial interactions were next studied with gel-chromatographic technique.

J. Studies on the Nature of Mercurial-Hemerythrin Interactions:

(1) **Binding of pMB to hemerythrin.**

A slowly-reacting complex of hemerythrin, such as the oxyhemerythrin was used and the chromatographic elution was carried out at 5 - 8°C with excess mercurial concentrations. A 0.9 x 35 cm Sephadex G-25 column pre-equilibrated with pH 7.0; 0.01 M Tris-cacodylate buffer which was also used for the elution of 0.2 ml samples.

Fig. 27 shows the resolution between oxyhemerythrin (2.7 x 10^{-5} M) and NaN_3^{-} sample. The elution was monitored at 250 nm and the area under the protein peak was measured and found to be 7.3 sq. cms. A 60 ml/hr flow rate was used for rapid measurements of mercurial-protein reaction. The exclusion volume, measured with a blue dextran sample, was found to be 7.0 ml and the elution positions of the protein and salt were 8.0 ml.
Fig. 27. Elution of hemerythrin and NaN$_3$ on a G-25 Column.
Sample = 0.2 ml. NaN$_3$ = 1 % w/v. Hr-Cl$^-$ = 2.7 x 10$^{-5}$ M.
and 22.5 ml respectively. Fig. 28 represents the elution profile obtained by the application of $2.7 \times 10^{-5}$ M oxyhemerythrin sample on the column pre-equilibrated with $8 \times 10^{-5}$ M pMB-containing buffer. The area under the protein peak was found to have increased to 105 sq. cms. However, the trough following the protein peak could not be used to measure the amount of pMB removed, as is generally done in binding experiments of this type, primarily due to the appearance of a second positive peak. For the examination of this peak, we applied a blank sample (0.2 ml) containing 0.1 M NaCl in 0.01 M Tris-cacodylate buffer on the column. Fig. 29 shows the elution pattern obtained, which contained a trough and a significant positive peak. In addition, the application of 0.2 ml buffer solution with no Cl$^-$ produced a very diffuse trough. These experiments indicate that the NaCl added to solubilize the original oxyhemerythrin solution retarded the pMB, on the Sephadex column, probably through a non-specific binding.

For this reason, further experiments were carried out with oxyhemerythrin solutions prepared without the addition of NaCl. The areas of the elution peaks were calibrated by the application of three different concentrations of oxyprotein. The areas were obtained by the use of a polar planimeter, from which a linear relationship was found between the amount of protein applied and the area of the elution peak. Blank protein elution peaks obtained at 250 nm with $1.25$ and $2.5 \times 10^{-5}$ M oxyhemerythrin concentrations were measured and used for the evaluation of binding of pMB. The extent of binding was obtained at three column equilibrating levels of mercurial concentration by applying 0.4 ml
Fig. 28. Elution profile obtained by the application of oxyhemerythrin (2.7 x 10^{-5} M) on a G-25 Column saturated with pMB (8 x 10^{-5} M). Sample = 0.2 ml, Flow-rate = 60 ml/hr.
Fig. 29. Elution profile obtained by the application of 0.2 ml of 0.1 M NaCl on the G-25 column saturated with $8 \times 10^{-5}$ M pMB solution. Flow-rate=60 ml/hr. 0.01 M Tris-cacodylate buffer was used.
protein solutions. Parallel kinetic experiments were performed at 250 nm in a DU-spectrophotometer, whose cell compartment was maintained at 8°C. The end point and the extent of reaction at the protein elution time were calculated from these observations. The protein elution occurred at 9.4 minutes, at which time the extent of reaction was found to be less than 40%. Accordingly, any area increase in addition to that due to pMB-Hr reaction was evaluated after the appropriate conversion of the protein peak area in terms of mercaptide-bound formation using the extinction coefficients of pMB, protein, and the pMB-SH reaction.

Table XXIII lists the values for pMB binding obtained in these experiments at 25% reaction time. For the calculation of these binding values one mole of pMB is assumed to completely saturate one mole of oxyhemerythrin (monomer).

<table>
<thead>
<tr>
<th>Ratio of pMB : Hr-SH</th>
<th>Moles of pMB bound per Moles of monomeric Hr (r)</th>
<th>Ratio of pMB : Hr-SH</th>
<th>Moles of pMB bound per Moles of monomeric Hr (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4 : 1</td>
<td>--</td>
<td>1.2 : 1</td>
<td>--</td>
</tr>
<tr>
<td>(3x10⁻⁵M : 1.25x10⁻⁵M)</td>
<td></td>
<td>(3x10⁻⁵M : 2.5x10⁻⁵M)</td>
<td></td>
</tr>
<tr>
<td>4.8 : 1</td>
<td>0.24</td>
<td>2.4 : 1</td>
<td>0.06</td>
</tr>
<tr>
<td>(6x10⁻⁵M : 1.25x10⁻⁵M)</td>
<td></td>
<td>(6x10⁻⁵M : 2.5x10⁻⁵M)</td>
<td></td>
</tr>
<tr>
<td>9.6 : 1</td>
<td>0.9</td>
<td>4.8 : 1</td>
<td>0.56</td>
</tr>
<tr>
<td>(12x10⁻⁵M : 1.25x10⁻⁵M)</td>
<td></td>
<td>(12x10⁻⁵M ; 2.5x10⁻⁵M)</td>
<td></td>
</tr>
</tbody>
</table>

1. Complete saturation is assumed when one mole of pMB binds to one mole of hemerythrin (if n = 1, then 100% saturation at r = 1).
It is clear from these results that binding of pMB to hemerythrin also can be observed as an increase in the area of the protein peak. In zonal type of experiments, the total increase in area represents both binding and reaction. Hence it was difficult to recognize the progress in reaction resulting from pMB binding to oxy-Hr. Therefore, frontal-chromatographic experiments were performed in an attempt to find out if the bound fraction of pMB can lead to any further reaction of the hemerythrin -SH groups. For this purpose, precooled pMB and hemerythrin solutions are pumped into a mixing chamber, as explained in 'Experimental Methods'. Constant mixing was allowed with the help of a small spinbar and the reaction was initiated by pumping both pMB and the hemerythrin chloride at the same instant. The eluting buffer contained 0.1 M NaCl so as to obtain a resolution between the protein and the mercurial elutions. Just before the elution of the pMB, the flow was stopped but the monitoring of the solution in the flow-cell was continued. This continuation permitted the measurement of the reaction in the absence of any free pMB; hence, any further increase in the absorbance at 250 nm will be directly proportional to the mercaptide-bound formation, the bound mercurial being utilized in the process. The temperature of the cell compartment was allowed to rise to 25°C. The amount of reaction by the bound pMB was evaluated from the final absorbance value. Representative elution profiles are shown in Fig. 30. The increase in the absorbance after the stoppage of flow as illustrated in this Fig. indicates that additional reaction has occurred. Since free pMB could
Fig. 30. Elution profiles of frontal elution experiments on a G-25 column (0.4 OD x 50 cm) saturated with pMB (7 x 10^{-5} M). The protein (Hr-Cl^- = 8.5 x 10^{-5} M) was pumped into the mixing chamber (0.3 ml) at a flow-rate of 12 ml/hr by the Chromatronix pump from a 0.5 ml sample loop. The pMB was injected by the syringe pump at 12 ml/hr. The temperature was allowed to rise up to 25° after the flow was stopped and the reaction was measured at 250 nm for 80 minutes.
not be present in the flow-cell with the protein the further reaction must be due to bound fraction of the mercurial.

(2) **Comparison of the rate of dissociation and the rate of reaction on Sephadex G-75 column.**

In the binding experiments explained so far, the dissociation state of the protein was not known and hence it was difficult to attribute the pMB binding completely to the octameric species. However, if correlation could be made between the extent of reaction and percent dissociation, it will be possible to find out which form is the reactive species of hemerythrin. This is also important in deciding about the correct model as previously mentioned. For the purpose, a 6-hour reaction-time sample was applied to a Sephadex G-75 column and monitored according to the procedure explained in Chapter II. The column length was selected such that the octamers were resolved from monomers. The elution profile obtained in such an experiment is shown in Fig. 31a. The percent reaction was calculated from the decrease in the elution peak (250 nm) area of a blank-pMB solution. Similarly, the percent dissociation was obtained from the octameric and monomeric peak areas. (See Appendix VII for these calculations). The results indicated that while the extent of reaction is 83%, only 60% dissociation was observed. Such an observation suggests that the octamers are reactive species. Further proof was obtained from detailed comparative studies on the rate of reaction and dissociation.
Fig. 31 a. Elution patterns obtained on a 1/2 x 23" Sephadex G-75 column with a 0.1 ml mixture of $6.0 \times 10^{-5}$ M oxyhemerythrin and $1.2 \times 10^{-4}$ M pMB at a reaction time of 360 minutes.

b. Elution profiles at 90 and 406 minutes.
Reaction mixtures at various time intervals after the pMB and oxyhemerythrin have been mixed in a 2:1 ratio, were applied on the G-75 column. The elution peaks for the octamer, monomer, and un-reacted pMB were 25.2, 44.2 and 70.5 ml respectively. Representative elution patterns at the 90 and 406 minute sampling times are shown in Fig. 31b. As the experiment proceeds, monomers are formed and pMB is used up. From the known extinction coefficients of the protein (20.5 x 10^3) and pMB (4.8 x 10^3) as well as of the extinction coefficient due to the mercaptide-bond formation (7.2 x 10^3), one is able to calculate the extent of the reaction and the degree of dissociation by measuring the areas under the various peaks. The decrease of the area under the pMB peak represents the extent of the reaction; the end point is taken when the area has diminished to half of its original value, since the reagent is used in a two-fold excess. The combined area under the monomeric and octameric peaks increases, not only by this amount, but also by the amount of the additional absorption of the mercaptide bond formed. Assuming that the protein under the monomeric peak is all reacted, one can calculate the degree of dissociation, as well as the percent reacted protein present under the octameric peak. Data obtained this way are shown in Table XXIV.

As can be seen, the percent reaction calculated from area measurements correlates well with that obtained from the increase of absorbance at 250 nm observed directly in the spectrophotometer. The rates of reaction and dissociation obtained as above are shown in Fig. 32.
Fig. 32 Comparison of the rate of reaction of oxygenated hemerythrin with pMB and the rate of dissociation into monomers. Both rates were obtained from analyses of gel chromatograms (Fig. 31b). The rate of release of oxygen is also shown.
In contrast to these kinetic-type experiments, the final state of the reaction conforms to the original observation (Keresztes-Nagy and Klotz, 1963) that there is a linear relationship between the degree of dissociation and the per cent of sulfhydryl groups titrated, as shown in Fig. 33. These data were obtained (see Chapter II for detailed procedure) with the chloride form of hemerythrin, which reacts and dissociates rapidly, and hence gel-filtration experiments show only the final state of the reaction. It can be seen that, when the ratio of protein -SH groups to pMB is 4:1, the area under the octameric peak is three times larger than the area of the monomeric peak. When this ratio is 4:2, the extent of dissociation is 50 percent, since the areas under the two peaks are nearly identical.
Fig. 33. Elution patterns obtained with two different reaction mixtures of chloro-hemerythrin and pMB. The hemerythrin and pMB concentrations in a, are $1.2 \times 10^{-4}$ M and $3.0 \times 10^{-5}$ M and in b, are $1.2 \times 10^{-4}$ M and $6.0 \times 10^{-5}$ M.
The observed rate of reaction was also compared with the rate of release of oxygen from oxyhemerythrin. The monitoring was carried out at 500 nm as explained in Chapter II, and the theoretical 100% oxygen loss was evaluated from the extinction coefficient of aquo complex being formed through oxygen release. The % release was then computed from this expected absorbance of aquo form, and the absorbance of the initial oxyhemerythrin. The rate of release of oxygen found in such a manner is also plotted in Fig. 32.
The present investigation has been undertaken with the basic aim of studying protein interactions with gel chromatography. Therefore, we have used this technique wherever possible, in exploring the molecular properties of the model protein, hemerythrin, as outlined in the preceding chapter. Before the interpretation of the observed data on hemerythrin, the advantages and disadvantages of the employed analytical method will be considered.

In view of the extensive reviews available on frontal-gel chromatography (Ackers and Steere, 1967; Winzor, 1969; Ackers, 1970), we will discuss here only some of its salient features. The first, and major, advantage of frontal elution procedure for the hemerythrin dissociation experiments, is that the elution volume is obtained as a steady-state parameter (Gilbert, 1966). In the leading boundary of a frontal-elution experiment the concentration of the protein rises from zero to a maximum, $C_0$. Addition of a further volume of protein (concentration, $C_0$) at the plateau region results in the elution of protein whose concentration is identical to that applied on the column. Therefore, the column is in a steady-state condition. Hence, the amount of protein on the column ($\bar{V} \times C_0$) is a steady-state parameter. Winzor (1969) used 'steady-state' data to derive equilibrium constants.
by rationalizing similar derivations of Archibald (1947) for the ultracentrifuge.

The other advantages of the employed chromatographic technique pertain to the sample application and direct monitoring of the protein species. Large samples could be applied by the Chromatronix injection valve without changes in flow-rate. Thus any possible stress (pressure) effects on the Sephadex gel particles (which are known to exhibit anomalous resolution characteristics at high hydrostatic pressures; Determann, 1969) were eliminated. Direct monitoring of the effluent through the flow-cell could be carried out even at microgram levels of protein, by using 10-20 fold scale expansion on the linear-log recorder. However, because of the high electrical noise to signal ratios at large scale expansions, only 2-5 expansion of the 0.0 - 1.0 absorbance scale is recommended.

On the other hand, gel-chromatographic procedure could also be used in conjunction with other detection methods, such as enzymatic assay or radioactive-labelling procedures. We have already employed the latter method in some of our studies (anion-hemerythrin interactions) by using radioactive chlorine-36. The enzymatic-assay procedure was recently shown to be useful at less than microgram concentrations of protein (Keresztes-Nagy and Orman, 1971). Studies at such low levels of protein are almost impossible with light scattering and ultracentrifugation. Keeping in mind these general comments about gel chromatography, experimental data obtained on hemerythrin interactions will now be discussed.
A. Hemerythrin Subunit - Subunit Interactions (Association-Dissociation Equilibrium.)

Preliminary experiments on the G-100 column (Tables III and IV) illustrate the usefulness of frontal-chromatographic technique to the study of dissociation equilibrium in hemerythrin. These studies also indicate the existence of a dissociation equilibrium, since the ascending elution boundary was found to shift with protein concentration. Such behavior has been previously ascribed to a reversible equilibrium by Ackers and Thompson (1965). The reversibility of hemerythrin-dissociation equilibrium has already been established with ion-exchange and gel-filtration procedures (Rao and Keresztes-Nagy, 1968).

For a comparison of the dissociation studies of hemerythrin by frontal-gel chromatography with sedimentation-velocity experiments (Klapper et al., 1966), one must analyze the trailing boundaries of the elution profiles. The boundaries obtained with the Sephadex G-100 column were too diffuse, and the minimum position in the derivative curve could not be easily distinguished. Therefore, the major part of the comparison was carried out with results obtained on a Sephadex G-75 column.

Tables V and VI, list the calculated values of 'K' from results obtained on this column with oxy-hemerythrin and hemerythrin-azide complexes. A careful inspection of the results presented in these Tables reveals that, in addition to a variation, there appears to be a
trend in the values of dissociation constant for the equilibrium in hemerythrin. At low protein concentrations the value of $'K'$ was found to be comparatively smaller than the values at higher concentrations. We, at the present time, attribute this trend to the difficulty in obtaining the absolute values of elution volume (sieve coefficient). Langerman and Klotz (1969) showed that a 2 percent experimental error in the calculation of monomeric hemerythrin concentration would cause a 100 percent variation in the value of dissociation constant. Similarly, we considered a 15 percent error in the measurement of protein concentration, and the value of the dissociation constant was calculated to be fifteen-fold higher than the value of $'K'$ without the error (Appendix VI). Errors in the measurement of low protein concentration could sometimes amount to 15-20 percent; accordingly, the values of dissociation constant would reflect the corresponding variation in $'K'$. In addition, this constant is dependent to the 8th power on the concentration of monomeric species. Therefore, the observed variation in $'K'$ does not seem to be excessive in view of such considerations.

The exclusion properties of G-75 required the use of a range of hemerythrin concentrations low enough to yield weight-average molecular weights of less than 60,000. In general, the range of hemerythrin concentrations employed in these experiments was lower than that used in other studies (Klapper et al., 1966; Klapper and Klotz, 1968b, Langerman and Klotz, 1969). Lower values of $'K'$ were obtained, most probably, because of the range of hemerythrin concentration used in our studies. These values, together with the values derived from
sedimentation-velocity, sedimentation-equilibrium, and ion-binding studies are presented in Table XXV. As shown in this Table, the value of the dissociation constant obtained in the present study, seems to be in general agreement with the values obtained by other methods. Recently, lactate dehydrogenase has been shown to have an octamer–monomer system with a dissociation constant in the range of $10^{-32}$ (Millar et al., 1969). This value may be considered to be in agreement with the hemerythrin-dissociation constant. In addition, a recent compilation of equilibrium constants of subunit association of various proteins (Klotz et al., 1970), indicates that gel-chromatographic method can provide results comparable to those obtained by the ultracentrifugation technique.

A comparison of the results presented in Tables VI and VII also shows that the values of 'K' vary depending on whether they are obtained from analysis of the elution profile's trailing or leading boundary. This disparity is primarily due to difficulty in judging the correct position of the minimum ($\bar{V}_{\text{min}}$) in the derivative curves. As can be seen from Eq. 2, small errors in the measurement of $\bar{V}_{\text{min}}$ (and therefore of $C_{\text{min}}$) can lead to large errors in the value of 'K'. Cox (1971) has reported similar difficulties in detecting the exact position of the gradient minimum in computer simulations of ultracentrifugation studies. In spite of such possible errors, the derived values of 'K' from the leading and trailing boundaries are in reasonably good agreement (Tables VI and VII).
### TABLE XXV
Comparative Data on the Dissociation Constant of Hemerythrin from Various Methods

<table>
<thead>
<tr>
<th>Range of Protein concentration (g/l)</th>
<th>Method</th>
<th>Ligand</th>
<th>Average value of dissociation constant (K) in monomeric units (moles/l)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(4.1 - 0.025)</td>
<td>Sedimentation velocity</td>
<td>SCN⁻</td>
<td>$6.77 \times 10^{-39}$</td>
<td>(Klapper et al., 1966)</td>
</tr>
<tr>
<td>(0.957 - 0.029)</td>
<td>Ion-binding</td>
<td>SCN⁻</td>
<td>$2.50 \times 10^{-37}$</td>
<td>(Klapper and Klotz, 1968b)</td>
</tr>
<tr>
<td>(0.920 - 0.055)</td>
<td>Sedimentation equilibrium</td>
<td>N₃⁻</td>
<td>$3.10 \times 10^{-36}$</td>
<td>(Langerman and Klotz, 1969)</td>
</tr>
<tr>
<td>(0.166 - 0.010)</td>
<td>Frontal gel chromatography</td>
<td>N₃⁻</td>
<td>$6.26 \times 10^{-42}$</td>
<td></td>
</tr>
<tr>
<td>(0.125 - 0.010)</td>
<td>Frontal gel chromatography</td>
<td>Oxygen</td>
<td>$2.50 \times 10^{-42}$</td>
<td></td>
</tr>
</tbody>
</table>
The general agreement between our values of 'K' and those obtained with other methods becomes more obvious if one considers the thermodynamic parameter, free energy change (\( \Delta G^\circ \)), for the dissociation equilibrium. \( \Delta G^\circ \) is obtained from the relation \( \Delta G^\circ = RT \ln K \) and was found to be positive and small (6.70 Kcal/mole of monomer). Langerman and Klotz reported a standard free-energy change of 6 Kcal/mole for the formation of one mole of monomer from octamer. Therefore, we can conclude that the observed variation in the value of 'K' is not a real variation, and hence gel-chromatographic procedure appears to have an accuracy equal to that of the other methods.

However, our earlier temperature-effect studies (Rao, 1969) indicated that the gel-chromatographic method to be a more sensitive technique than ultracentrifugation. We observed a small enthalpy change of +1.3 Kcal/mole of monomer for the octamer dissociation. Langerman and Klotz (1969) could not observe any changes in the dissociation of hemerythrin with temperature and hence reported no enthalpy changes for the process. Only recently, by the use of microcalorimetric procedure, Langerman and Sturtevant (1971) were able to measure the enthalpy changes for the hemerythrin equilibrium. They reported a value of 1 ± 0.5 Kcal/mole of monomer, which agrees remarkably well with our findings.

The value of the dissociation constant is not the only information which can be obtained from analysis of the trailing edges of the elution patterns. One can also estimate the stoichiometry of dissociation (n) and analyze the boundaries in terms of Gilbert's theory.
(1955). As can be seen from Table VII, the values of 'n' obtained varied between 5 and 12. This variation was also due to difficulties inherent in the estimation of the position of $V_{\text{min}}$. However, the average of all values of 'n' in Table VII is $8.0 \pm 1.0$ which justifies our earlier assumption of 'n' equal to 8. These results provide the first direct experimental evidence that the dissociation of hemerythrin is an all-or-none process and does not involve significant stable concentrations of any intermediates. Similar previous evidence was derived from sedimentation-equilibrium studies (Langerman and Klotz, 1969) and was based on the fit of experimental data to theoretical equations for an octamer-monomer equilibrium.

In studies on interacting-protein systems at equilibrium, the behavior of the elution profiles obtained in frontal-gel chromatography will be dependent on both the equilibrium position and on the rate of re-equilibration relative to the rate of separation of the interacting protein species. The presence of a dissociation equilibrium in native hemerythrin has already been established (Keresztes-Nagy and Klotz, 1965, Klapper et al., 1966). In contrast to these studies, gel-chromatography method has been shown not to affect the reversibility of the equilibrium (Rao and Keresztes-Nagy, 1971). Therefore, both the rate of equilibration and the equilibrium position were observed with gel chromatography. The dissociation equilibrium of hemerythrin may be represented as $(H_r)_8 \stackrel{k_f}{\leftrightarrow} k_r 8 H_r$, where $k_f$ and $k_r$ are the forward and reverse rate constants. In a slow rate of re-equilibration ($k_f$ and $k_r$
are small) after the shift caused by dilution is complete, both the octameric and monomeric components would emerge at their respective elution positions and the proportion of the respective components would depend on the time required for elution.

However, if $k_f$ and $k_r$ are very large, equilibrium is achieved rapidly in comparison to the elution time. This results in only a partial or no resolution between the equilibrating components. The advancing boundary of a frontal elution experiment in such a system will be hyper-sharp and correspondingly the trailing boundary will be diffuse compared with that of a non-dissociating protein elution profile. In a general manner, the hyper-sharpening effect may be said to be caused by the tendency for the larger molecular species to move faster than the dissociated species. A continuous concentration distribution has to be assumed in the advancing boundary as it begins to migrate through the column. At each concentration level, the amounts of monomer and polymer are readjusted; accordingly, at any particular moment, due to diffusion, the region ahead of the mean position becomes lower in concentration than the region behind it. Therefore, the species behind the mean region migrate faster and the species ahead of it migrate slower. Thus in the newly acquired position, the polymeric species are in an environment of lowered concentration which causes further dissociation. Again, readjustment between the polymeric and monomeric species occurs and the process is continually repeated, with the final effect of partial opposition to diffusion spreading. This situation results in a boundary
which is considerably sharper than that for an ideal solute with the
same elution volume. This effect operates in a reverse manner on the
trailing boundary. Here the effect of dispersion into a region of
lowered concentration seems to broaden the trailing edge by promoting
dissociation. Diffusion aids this process and thus non-enantiographic
(non-symmetrical) elution profiles are considered to be characteristic
of a rapidly equilibrating systems.

In a third possible situation, both $k_f$ and $k_r$ have values nearly
equal to the time for separation in chromatography experiments, whereby
the patterns become complicated.

Excellent theoretical discussions on gel-chromatographic data of
rapidly interacting systems are presently available in which equations
of free migration were adapted to frontal-elution parameters (Gilbert,
1966; Nichol et al., 1967; Ackers and Thompson, 1965; Ackers, 1967b).
The equations which have been used in the calculation of dissociation
data are listed in Appendix I. The qualitative aspects of boundary
shapes of associating systems have been experimentally shown by Winzor
and Scheraga (1963), by employing the first-derivative curves to
demonstrate small deviations from ideal enantiographic (symmetrical)
elution profiles. Bethune and Kegeles (1961) have also put forward a
theory for a rapidly polymerizing system involving aggregates greater
than dimers. For such a system, they predict that there should be in-
flection points (bimodality) in the trailing edge of the elution pattern.
This has been experimentally confirmed by other workers (Winzor and Scheraga, 1963; Ackers and Thompson, 1965). On the basis of both of these theories, the behavior of the frontal-elution profiles of the hemerythrin-dissociation experiments prove that the native equilibrium is of a rapid nature in relation to the elution time. In the frontal-chromatography experiments of hemerythrin-azide on the G-75 column, a single leading boundary was always observed, whereas two boundaries would be expected for a slowly equilibrating system. In addition, the trailing boundaries show only partial resolution, which agrees with the Gilbert theory (1955, 1959) for rapidly equilibrating systems. Furthermore, the fast and slow components of the trailing boundaries should not be identified as octameric and monomeric components (as was done in the sedimentation on velocity experiments of Klapper et.al., 1966). This is evident since the elution rate of the fast peak is dependent on protein concentration (see Figs. 11 and 15), as is also predicted by theory (Gilbert, 1955, 1959).

It can be seen from figures 10 and 11 that a rapid equilibrium is present in oxyhemerythrin also, which finding is in agreement with the rate observed with hemerythrin-azide complex. Since, protein-protein interactions, in general, have been known to be relatively fast, the hemerythrin dissociation equilibrium would be expected to be fast. Our results, indeed, confirm such an expectation.

Although the gel-chromatographic data we have obtained is in agreement with the results of other workers (Klapper and Klotz, 1968b,
Langerman and Klotz, 1969) on the dissociation constant 'K' and the stoichiometry of dissociation (n), there is a contradiction in regard to the rate of hemerythrin association-dissociation equilibrium. Earlier (Klapper et.al., 1966) it has been reported that the rate with which equilibrium is achieved was relatively slow under almost identical experimental conditions of temperature, pH, and ionic strength as those employed in this present study. These investigators claimed that the fast and slow components could be completely resolved; that the sedimentation constant of the fast component was independent of the protein concentration; and that the area under the slow sedimenting peak was constant. From such observations, they concluded that the attainment of equilibrium between the octameric and monomeric species is relatively slow. Consequently, these two components are expected to be separable on the basis of molecular weight in transport experiments.

In contrast, as indicated earlier, a rapid type of equilibrium was found in our gel-chromatographic experiments in which the equilibrating components could not be separated.

One can speculate about the discrepancy between the gel-filtration and ultracentrifugation studies. It is possible that in the ultracentrifuge the dissociation of hemerythrin could become irreversible owing to either oxidation or denaturation of the monomeric species. Unfortunately, the reversibility of dissociation under the conditions of ultracentrifugation has not yet been experimentally demonstrated, as was done with the gel-filtration method (Rao, 1969). Langerman and
Klotz (1969) reported that the partial-specific volume of octamer and monomer was essentially identical. Therefore, we hypothesize a transition state in the dissociation of octamer to monomer as an alternate explanation for the observed discrepancy. According to this hypothesis, the nature of the change in the partial-specific volume would be reflected in a decrease in the rate of equilibration at pressures generated during high-speed ultracentrifugation (Josephs and Harrington, 1966). Finally, the possibility cannot be ruled out that partial resolution of octameric and monomeric hemerythrin in the ultracentrifuge has been mistakenly identified as complete separation of the two components.

In some gel-chromatographic experiments elution-behavior characteristic of other types (intermediate and slow) of equilibrium have also been observed. The elution profile of a 20 µg/ml oxyhemerythrin (Fig. 18) was found to be different in both the advancing and trailing boundaries. This behavior corresponds to an intermediate rate of equilibrium. Recently, other investigators (Tan and Keresztes-Nagy, 1971) have shown such an intermediate equilibrium in oxyhemerythrin solutions at low protein concentrations (< 25 µg/ml) and low pH conditions (between 5 and 6).

Furthermore, these workers (Tan and Keresztes-Nagy, 1971) could also show, in preliminary experiments with oxyhemerythrin at higher ionic strength (0.25 µ) and low temperature (10°) conditions, the requirements of a slow rate of equilibrium (Fig. 34). Accordingly, two distinct plateau regions were observed in the leading boundary,
Fig. 34. Effect of ionic strength and temperature on the dissociation of oxy-hemerythrin (Tan and Keresztes-Nagy, 1971).

A. Hr concentration 22 μg/ml; 0.01 M; pH 7.0; 25°
B. Hr concentration 22 μg/ml; 0.25 M; pH 7.0; 10°
(as observed on a G-75 column).
amd complete resolution of both trailing and leading boundaries into two components could be achieved. The elution volumes of these components corresponded to the octameric and monomeric species. Such experiments indicate that gel-chromatography technique can be used for studying the various types of equilibrium. From this observation, we conclude that the contradiction with the ultracentrifuge experimental finding is not due to an artifact of the frontal-elution-gel-chromatographic procedure.

The calculations of the time spent by the protein species on each theoretical plate of both G-75 and G-100 columns enabled the evaluation of hemerythrin equilibrium in terms of resolution time. Accordingly, the equilibration between the oxy-hemerythrin subunits and octamers is found to be faster than 4.3 seconds. Similarly, the hemerythrin-thiocyanate and hemerythrin-azide equilibria (rapid rate) are faster than 3.5 seconds (Table IX). The equilibration (intermediate) of oxyhemerythrin monomers and octamers at concentrations less than 20 µg/ml is probably equal to the resolution time on each theoretical plate of the column (3-4 seconds). Likewise, the equilibration (slow rate) of the 25 µg/ml oxy-hemerythrin in 0.25 µ and 10° (Tan and Keresztes-Nagy, 1971) would have to be slower than 3-4 seconds. In view of such short intervals of time allowed on each theoretical plate in a G-75 or G-100 column, it is difficult to reconcile the slow nature of the equilibrium observed by Klapper and other workers (1966), with the sedimentation-velocity procedure.
As explained in Chapter III, the sieve coefficients are best correlated with the size parameter, molecular shape, and hence such a relation was obtained in an empirical calibration of both G-75 and G-100 columns with standard-marker protein molecules. Representative plots are shown in Fig. 8 in which the linear relationship \( a = a_0 + b_0 \text{erfc}^{-1}(\sigma) \) was observed. At very low hemerythrin concentrations, the equilibrium is shifted toward monomers, as shown by the higher values of sieve coefficient. The radius of monomeric hemerythrin was derived by the above linear relationship between the molecular radius and the sieve coefficient. The values so obtained are 15.0 Å on G-75 and 16.2 Å on G-100 columns. The close agreement between these values was unexpected since exponents of ultracentrifugation and light-scattering technique have suggested interactions between the column media and the protein being studied. Certainly the inert nature of column media (Sephadex) is indicated by our results on molecular radii of monomers.

If we can assume that the monomeric hemerythrin is a sphere (Keresztes-Nagy and Klotz, 1965), an entirely plausible assumption for protein subunits, the molecular weight of the hydrated subunit may be evaluated by the equation \( M_{\text{max}} = \frac{4\pi Na^3}{3\bar{v}} \). From the partial-specific volume, \( \bar{v} \) (0.735, Keresztes-Nagy, 1962), and \( N \), Avogadro's number, the value calculated for \( M_{\text{max}} \) is 14,620. This value is, at best, an upper limit since both molecular asymmetry and the amount of hydration have to be considered.
The apparent molecular radius of the hemerythrin octamer molecule was also evaluated from the calibration equation to be 42.6 Å and 35.4 Å respectively. Due to the non-linear resolution of Sephadex G-75 at high molecular weights (Andrews, 1964), it is most likely that the resolution will also be non-linear at high molecular radii (beyond 36 Å or 70,000 MW). Therefore, we have used only the value obtained with the G-100 column in the calculation of octameric molecular weight. The octameric molecular weight of 113,000 derived from the G-100 column data is in agreement with the earlier reported value of 107,000 (Keresztes-Nagy and Klotz, 1963).

For most proteins, the partial-specific volume lies in the range of 0.71 - 0.75, but the assumption that the frictional ratio (a measure of hydration, and molecular shape) is the same for hemerythrin and marker protein is less justified. Bovine serum albumin is now known not to be a spherical molecule, but rather an ellipsoid (Squire et al., 1968), and it is likely that other marker proteins also may not be spherical molecules.

Both Ackers (1970) and Winzor (1969) suggest the complementary use of data obtained from two different analytical methods. Accordingly, the diffusion coefficient was calculated from gel-filtration value for the molecular radius (a) and the sedimentation coefficient ($S_{20,w}$) obtained on ultracentrifuge (Keresztes-Nagy, 1962). The reported values of octameric and monomeric sedimentation coefficients
are $6.8 \times 10^{-13}$ sec and $2.1 \times 10^{-13}$ sec. These values were used together with the diffusion coefficients of octamer and monomer which were calculated from the equation

$$D_{20,\omega} = \frac{RT}{N_\omega \eta a},$$

where $\eta$ is the viscosity, $T$ the absolute temperature, and $R$ is the gas constant. A value of $5.17 \times 10^{-7}$ cm$^2$/sec was obtained for the diffusion coefficient of the octamer, and a value of $7.61 \times 10^{-5}$ cm$^2$/sec was obtained for the monomeric diffusion coefficient. The anhydrous molecular weights were derived from the relation

$$M = \frac{RT S_{20,\omega}}{D_{20,\omega} (1-\gamma f)},$$

where $\gamma$ represents the density of water at 20°C, and $\gamma$ the partial-specific volume. The molecular-weight values so obtained are 101,000 and 14,600 for the octameric and monomeric hemerythrin respectively. The previously established values of 107,000 and 13,500 (Keresztes-Nagy and Klotz, 1965) agree well with these values. Any variation of the derived values can be explained on the basis that the estimated value of the Stokes radius depends not only on the precision of the elution-volume measurement, but also on the accuracy of the calibration data of marker proteins.

In spite of an adequate correlation between sieve coefficients and molecular Stokes radii, the most common application of gel filtration has been to estimate the molecular weight from the primary
experimental parameter, the elution volume. This is illustrated by the vast amount of literature on protein molecular weights derived from elution volumes; for many molecular species, direct correlation with molecular weights was observed. In the present study also, good correlation has been found between the molecular weight of the dissociated hemerythrin obtained from calibration graph of marker proteins, and the previously determined value of 13,500 (Klotz and Keresztes-Nagy, 1963). As indicated earlier, the equilibrium between the octameric and monomeric hemerythrin is a rapid process; consequently, at any protein concentration the weight-average molecular weight can be used to calculate the percent dissociation, which is a measure of the shift in the equilibrium. Accordingly, the measurement of weight-average molecular weights at various conditions (which might influence the dissociation-equilibrium of hemerythrin) has been further utilized in understanding the nature of ligand-protein interactions.


That iron-binding ligands do indeed influence the degree of dissociation was demonstrated previously by sedimentation-velocity experiments (Klapper et al., 1966) and ion-binding studies (Klapper and Klotz, 1968b). In the present investigation, three different ligands were examined at various protein concentrations. The data in Table X shows that the dissociability of the protein differs with the various hemerythrin-anion complexes. At an identical protein
concentration of 100 µg/ml, thiocyanate complex was 61.2% dissociated, the azide form 59.5%, and the oxy form 13% (Table XI). Further comparison of the extent of dissociation of these three complexes at 30 µg/ml concentration also indicates that the presence of ligands shifts the equilibrium toward liganded monomers. This behavior agrees with earlier experiments on ligand effect with hemerythrin-azide complex (Keresztes-Nagy and Klotz, 1965), in which monomer had greater affinity for the ligand (L) than the octamer. This stronger binding of the monomeric protein can be explained by the following scheme:

\[
\begin{align*}
(HrO_2)_8 + 8L & \rightleftharpoons K' 8 Hr L + 8 O_2 \\
(HrO_2)_8 + 8L & \rightleftharpoons K 8 Hr O_2 \\
& + + \\
& \downarrow \quad \downarrow \\
8O_2 + (HrL)_8 & \rightleftharpoons 8 Hr L + 8 O_2
\end{align*}
\]

In accordance with such a scheme, iron-complexing ligands have been shown to affect the reactivity of the sulfhydryl groups with mercurial reagents (Fransioli, 1969). Similarly in the present investigation, the effects of ligands on the dissociability have been demonstrated. A study of the ligand effect of various ions was not intentionally pursued, and hence results obtained on both G-75 and G-100 columns will be used. Such a compilation would be justifiable if only the data obtained in the linear portion of the calibration graphs are
utilized. An examination of those results, listed in Table X, reveals that the effect on the dissociation of octameric hemerythrin depends on the nature of complexing ligand. Accordingly, different degrees of dissociation were found with the oxythiocyanate and azide complexes at almost identical concentrations. In contrast, no difference was observed between the dissociation of azide and aquo complexes of hemerythrin (Langerman and Klotz, 1969) by sedimentation-equilibrium technique. This disadvantage of ultracentrifugation once again confirms our contention that gel-chromatographic procedure is well suited for protein interaction studies.

Our studies on the various hemerythrin complexes also indicate that thiocyanate ion influences the dissociation of hemerythrin to the greatest extent. This observation parallels the finding of greater destabilization of the native macromolecular ion formations of DNA (Hamaguchi and Geidusche, 1962), collagen (Gustavson, 1956), gelatin (Bello et al., 1956) and myosin (Tonomura et al., 1962), in the presence of thiocyanate ions. In these cases, the destabilizing effect was explained on the basis of the "salting-in" properties of SCN\(^-\) (Schmidt, 1938). Such general "salting-in" effects of ligands may have to be considered, even in hemerythrin. The effect of SCN\(^-\) on the dissociation has been so far attributed only to the specific binding at the iron site. Nevertheless, thicyanate binding at other sites might also alter the dissociation. Therefore, one has to distinguish the effects due to coordination and possible non-specific binding (non-
covalent linkages, as those postulated for "salting-in" effects) for the interpretation of effects on dissociation. We tried to achieve this distinction by studying the changes in dissociation in the presence of thiocyanate ion which occur before iron coordination. But, the rate of thiocyanate coordination (measured by the conversion of oxy-hemerythrin absorption spectra to that of a thiocyanate-hemerythrin complex) was found to be very rapid. Therefore, the effect of thiocyanate ion on the dissociation could not be studied before the iron-thiocyanate complexing occurred.

B.2. Effect of Method of Preparation on the Dissociation Properties.

To our surprise, we found that hemerythrin solutions prepared by dilution into thiocyanate-containing buffers exhibited a dissociation behavior different than the thiocyanate-hemerythrin complex obtained by dialysis of oxyhemerythrin itself. Table XVI gives the extent of dissociation at three different protein concentrations. The complex prepared by direct exchange dissociated to a lesser extent than the complex prepared by dialysis (Table XVII). A further comparison with the dissociation of oxyprotein (Table XI, 27 µg/ml) reveals that the preparation obtained by the direct exchange has greater dissociation. These results suggest that thiocyanate ions can also alter the conformation (by non-covalent linkages) to produce a form different than that produced in the dialysis procedure. At the present time, we do not know if these conformations would become identical with time.
This unexpected effect of thiocyanate on the dissociation properties of hemerythrin presented a serious problem because it raised questions about the nature of stock oxyhemerythrin solutions. These were routinely being made with sodium chloride. The properties of the protein so obtained might possibly have been affected by chloride ions. Therefore, the extent of dissociation was examined in the presence and absence of chloride ions. These results were compared with those obtained from an oxy-hemerythrin solution made in 0.1 M buffer. Data presented in Table XIII clearly show that the protein preparations made in the presence of chloride ions exhibited higher amount of dissociation. In general, the dissociation was found to depend on the chloride concentration. Accordingly, the dissociation of oxyhemerythrin containing 0.1 M chloride was higher than that of oxyhemerythrin itself and lower than that of oxyprotein in 0.5 M Cl⁻ (Table XIII). Since we did not have a control experiment at 0.5 M buffer, the effect of ionic strength cannot be deduced from the observed dissociation at 0.5 M Cl⁻.

In the above experiments, the extent of iron coordination and the effect of time on the altered conformations are not known. Therefore, the rate of chloride-iron complexing has been examined. Even though the rate of the oxy to chloro-Hr spectral change is slower than that of the oxy to thiocyanate-complex, considerable conversion occurs in the presence of chloride ions. The conversion-rate studies at 0.1 and 0.5 M chloride levels indicate the conversion to be dependent on the ionic strength (Fig. 21). Especially at the lower protein concentrations, the rate of oxy to chloro-spectral conversion was observed
to be fast (Fig. 22). This finding suggests that the conversion is a
dissociation effect and not a mass effect, since in the latter case the
rate of conversion at high protein concentration would have to be faster
than at low concentrations. The faster rate at low concentrations may
be attributed to an easier accessibility of iron atoms in the monomeric
species (This point is also made in the discussion of mercurial-
hemerythrin interaction studies.)


The oxyprotein whose properties have been altered by chloride
ions does not seem to undergo further conformational changes with time,
since the amount of dissociation remained constant (See Table XV).
Furthermore, the dissociation behavior was observed to be independent
of the amount of iron-chloride coordination (spectral conversion,
Table XIV). Therefore, the effect of chloride was ascribed to non-
covalent binding which is independent of iron-chloride linkage. How-
ever, the effect of chloride may also be explained on the basis of
changes in the solvent structure. For this reason, we performed direct
binding experiments with oxyhemerythrin on Sephadex (G-25) columns.

Initially, titration procedure (Schales and Schales, 1941) was
employed for estimating the chloride levels in the effluent of the
zonal-chromatographic-binding experiments (Hummel and Dreyer, 1962).
Chloride was detected at the elution position of oxyhemerythrin, which
was an indication of the presence of chloride-protein complex. But,
the extent of binding could not be accurately measured mainly because
of the titrant used in these experiments. The mercuric ion of the titrant, Hg(NO$_3$)$_2$, could react with the sulfhydryl groups to produce errors in the titration values (Table XXI). A more reliable method of evaluating the binding ratios was therefore developed. This was a scintillation-counting procedure which measured the amount of chloride in the effluent. At various protein concentrations, the amount of chloride binding was measured and excellent reproducibility was attained (See Table XXII). At a free chloride concentration of 0.003 M, 1.6 moles of chloride were found to complex with oxyhemerythrin. At this level of chloride ion, coordination to the iron-atoms can be considered negligible (Keresztes-Nagy and Klotz, 1965). Therefore, we concluded from the observed results (at 0.003 M chloride) that binding at non-iron sites is preferred to the iron-chloride binding.

B. 4. Nature of the Chloride Binding Site:

Non-iron binding of chloride to oxyhemerythrin most probably, occurs at the free imidazole, e-amino and guanido groups of the basic amino acids. Recent work (on dissociation and reactivity of hemerythrin) on some specific non-coordinating ions (Garbett et.al., 1971a) suggest that the arginines near the cysteine residue may be involved in binding these ions. At this point, we are unable to distinguish this non-coordinating ion-binding site from the chloride-binding site. In all likelihood, both of these sites are identical; the protective effects of non-coordinating ions such as perchlorate, nitrate, and sulfate
arising because of their bigger ionic size in comparison with that of chloride. Therefore, the arginine residues No. 48 and No. 49 (See Fig. 35), by virtue of the very close proximity to cysteine (No. 50) and other amino acids of the active site, may be assumed to be the chloride-binding sites. As mentioned earlier these amino acids are already implicated in binding with perchlorate ions. (Garbett et al., 1971a).

Preliminary experiments on the extent of dissociation of hemerythrin in the presence of 0.1 M perchlorate, acetate, and bromide ions did not provide any new information about the postulated binding site for chloride and non-coordinating ions. However, the percent dissociation of oxy-hemerythrin in the presence of perchlorate was found to be identical to that of an aquo-hemerythrin at a protein concentration of 27 µg/ml (Table XIX). This observation agrees with previous work of Darnall et al. (1968), who showed that the circular dichroic spectra (and therefore presumably the conformation) obtained in the presence of 0.1 M perchlorate ion is essentially the same as that of metaquohemerythrin. Bromide ion has been recently shown to coordinate with the iron atoms of hemerythrin (Garbett et al., 1969); therefore, the effect on the dissociation may be similar to those of the other iron-coordinating ions (Keresztes-Nagy and Klotz, 1965). The greater percent of dissociation in the presence of acetate ion is probably an electrostatic effect. Chiancone and Gilbert (1965) postulated such electrostatic effects for the dissociation of oxyhemoglobin in the presence of acetic acid.
Fig. 35. The amino acid sequence of monomeric hemerythrin.
The mechanism of the allosteric effects of iron-binding anions has been interpreted by two models (Keresztes-Nagy and Klotz, 1965); one involves a conformational change, while the other involves the dissociation of the protein, but both result in the formation of a reactive form of the molecule. Previous evidence has mainly favored the dissociation model (Klapper et al., 1966; Langerman, 1969), but more recent experiments suggest a mechanism which may involve both conformational change and dissociation. This evidence will be considered in greater detail in the discussion of hemerythrin-mercurial interactions.

Recognizing the effects of chloride through a non-iron-binding site, both on the reactivity and dissociability, one may propose that subtle, but stable, conformational changes are caused in the presence of this ion. This proposal is supported by the experimental results with chloro-complex made through dialysis and direct-exchange procedures (Fig. 23). The dialysis procedure involves the release of oxygen with fluoride ion (0.1 M F-) and the further conversion of the resulting aquo-hemerythrin by the dialysis against 0.5 M chloride. The dissociation behavior of this protein was found to be different than that made by dilution of the protein in the presence of 0.5 M chloride (Table XIX). Interestingly, these two preparations of hemerythrin possess identical spectral characteristics. Results presented in Table XVIII, show that once the character of iron is affected (such as by the formation of aquo complex), further changes (such as dialysis or the presence of
0.5 M chloride) bring about the same effect (e.g. dissociation). These results can be rationalized by assuming that chloride in the absence of fluoride preferentially binds at a site other than the iron. The conformation arising from this binding appears to be stable (Table XV). Our assumption for such a preferential binding seems credible if one considers the rate of exchange of oxygen by chloride and fluoride ions. Fluoroide ions release the oxygen of oxyhemerythrin much faster, in spite of their lower affinity for the iron-atoms, in comparison with chloride ions (Duke et.al., 1971; Garbett et.al., 1971a). Such a situation arises only, if the iron atoms are available for exchange of oxygen to fluoride ions and not to the chloride ions. Recent evidence (Garbett, et.al., 1971a) does indicate that the environment around the iron atoms is essentially non-polar, and hence in an aqueous media (as that of our experimental system) the iron atoms are not exposed to the surface. If this is the case, the ions which can enter the crevice in the protein structure (to release the oxygen) will be the fluoride ions since their ionic radius is smaller than that of chloride (Weast, 1968). Accordingly, the presence of fluoride ions would force the formation of a complex with the iron atoms, in contrast to the non-iron complexing by chloride ions.

However, the above hypothesis does not explain why the rate of release of oxygen is faster with thiocyanate and azide ions than with chloride ions. Both SCN\(^{-}\) and N\(_3\)^- are larger in ionic size than chloride. Therefore, these ions may cause faster conformational changes to release
the oxygen. In an alternate explanation for the observations with F\(^-\) and Cl\(^-\), none of the oxygen atoms can be visualized projecting out of the non-polar environment of the iron. The bigger size of azide and thiocyanate ions might therefore influence the accessibility of oxygen for these ions. The faster release of oxygen by fluoride may then be attributed to specific effects of fluoride ions, such as its ability to form a non-electrolytic complex with ferric iron from ferric chloride (Hutchinson, 1959).

Interpretation of new findings on hemerythrin on the basis of physiological function has not always been possible, except in the above mentioned binding of chloride. Vascular hemerythrin transports oxygen from the high-oxygen pressure of sea water to the coelom. The coelomic hemerythrin, in turn, constitutes a transfer system between the vascular hemerythrin and the tissues (retractor muscles). The hemerythrin non-iron binding sites are probably saturated in the presence of sea-water, which is isoionic with the coelomic fluid (red corpuscles). As the retractor muscles retract, high pressures (14-79 mm PSI) are exerted on the body cavity, and the coelomic fluid is circulated (Prosser and Brown, 1961). Tissue pressures are generally lower, and in the mud at low tide the worm is undoubtedly exposed to lower oxygen concentrations. A modular role can be exerted by chloride in low O\(_2\) conditions by releasing oxygen at a faster rate. We have shown in this investigation that the rate of oxygen release is higher in the presence of chloride ions, and
that the conformation is altered by saturation (binding) with the chloride. Therefore, it is very likely that chloride does have a physiological function in the transport of oxygen by hemerythrin.

Before discussing further data obtained on hemerythrin interactions through gel-chromatographic procedures other than frontal-gel-chromatographic technique and zonal-binding experiments, the major aspects studied so far can be listed as (1) evaluation of the dissociation constant 'K'; (2) calculation of the stoichiometry of dissociation 'n'; (3) interpretation of the rate of reequilibration of the dissociation reaction; (4) estimation of the size (radius) of the octameric and monomeric molecules; (5) observation of the ligand effect by the extent of dissociation; (6) direct evidence for non-iron binding by chloride ion.

C.1. Hemerythrin-Mercurial Interactions:

The knowledge gained from the chloride binding experiments was extended to understand the much studied reaction of hemerythrin sulfhydryl groups with mercurial reagents. From spectrophotometric kinetic data, Egan (1969) proposed a pre-reaction-binding step at non-sulfhydryl groups in the protein, which is assumed to be slow and hence rate-limiting. Similarly, Duek et al. (1970) postulated rapid binding of pMB to sites other than the sulfhydryl groups from steady-state binding experiments and sedimentation velocity data. Therefore, in an effort to determine the nature of the pMB-Hr binding, gel-filtration experiments were performed in a Sephadex G-25 column. The measurement of binding in the presence of minimum reaction was facilitated by employing high column
flow-rate (60 ml/hr), low temperature (8°C) and a slower-reacting hemerythrin species (oxy-form). On a short column able to completely resolve hemerythrin and pMB, the extent of binding was calculated from the area of the peak at the protein-elution position. About 0.5 moles of pMB were found to bind with each mole of oxy-hemerythrin. The elution profile of this experiment exhibited two positive peaks; the more retarded peak was tentatively identified as that of pMB-chloride complex. Such pMB-chloride binding was not unexpected, since it may be postulated from known interactions of phenyl compounds with anions (Stengle and Baldeschwieler, 1966). However, the retardation of pMB-Cl⁻ complex in comparison with the salt-elution position of the column was interesting, if not completely surprising, since the Sephadex material has been known to bind with aromatic substances (Geolette, 1960). Accordingly, the column material might have interacted with the pMB which would delay the elution process of the pMB-chloride complex. Additional studies in this direction are being planned to quantitatively investigate pMB interactions with chloride, fluoride, etc. Results of such experiments can be expected to aid the interpretation of the kinetics of mercurial reaction with the various hemerythrin-ligand complexes.

Therefore further experiments were carried out in the absence of chloride ion. The extent of binding was evaluated at 25 percent reaction and at three levels of pMB concentrations. A higher amount of binding was observed at higher mercurial and lower protein concentrations (Table XXIII). The extent of reaction was estimated to be less than 40%
from the frontal-chromatographic concentrations at two different levels of pMB. However, binding may also contribute to the increase in absorbance of 250 nm from which the percent reaction is calculated. Furthermore, in these experiments we were not able to distinguish if the pMB binding it happens before or after the reaction with the sulfhydryl groups. Also, it is important to know if the bound pMB could then react with the sulfhydryl groups of hemerythrin.

Such knowledge about the fate of the pMB is crucial for the interpretation of the observed kinetics of pMB-hemerythrin reactions. Recently, Klapper (1970) reported that monomeric methemerythrin has 6 binding sites for pMB. These binding measurements were made 3 minutes after the mixing of pMB and protein. However, more recently, Cress (1971) observed that approximately 30 percent of the sulfhydryl-mercurial reaction is completed within one minute of mixing of the reactants. Therefore, theoretically, one can suggest some binding of pMB by the monomers in the next two minutes (which was the time interval remaining before the binding measurements). Furthermore, Klapper (1970) did not show the bound mercurial's ability to partake in the reaction with sulfhydryl groups. Any such evidence would be important for the understanding of the complex kinetics of hemerythrin-sulfhydryl and pMB reaction. In an attempt to achieve this evidence, oxyhemerythrin and pMB were rapidly mixed prior to G-25 column (0.3 x 50 cm) application and by arresting the column flow just before mercurial elution to ensure the absence of unreacted pMB. Under these conditions, any
additional reaction occurring after stopping the column elution must arise from the mercurial bound to the hemerythrin which has not yet reacted with the sulfhydryl groups. In one experiment, such an increase in the absorbance at 250 nm was observed, which indicates that the bound pMB was able to react with the sulfhydryl groups. We are presently not able to ascribe the binding of pMB as the cause for the kinetic complexities of the reaction. It should be noted, nevertheless, that the usage of the micro-bore column, and the rapid mixing of reactants in a small-volume mixing chamber with the very high flow rates (> 60 ml/hr) from two pumps, does allow the study of relatively rapid reactions between small and large molecules.

C.2. Evidence on the Mercurial-Reactive Species of Hemerythrin.

The first two hypotheses of the dissociation model (which was extended to explain the allosteryism of the iron atoms and sulfhydryl groups), the existence of a native equilibrium between the octameric and monomeric hemerythrin, and the effect of iron-binding ligands on this equilibrium have been shown to be valid by both the previous (Keresztes-Nagy et al., 1965; Klapper and Klotz, 1968; Langerman and Klotz, 1969) and the present investigations. The third hypothesis of the dissociation model, which predicts that the –SH groups of the monomeric hemerythrin are more reactive than the octamers is of considerable interest, since it's validity has not yet been conclusively proven. This hypothesis assumes that the rate of dissociation is equal to the rate of
reaction. If such is the actual case, then one has to visualize con-
formational changes arising from the mercurial binding to octamer, which
eventually expose the sulfhydryl groups to react with pMB and dissociate
immediately into subunits. In actuality, this might not be the situation
since recently Cress (1971) showed that the sulfhydryl groups of mono-
mers are not more reactive than those of octamers. Therefore, the
octameric hemerythrin are probably the reactive species. For further
proof of the octameric reactive species, we compared the rate of reaction
with the rate of dissociation.

Previously, gel chromatography was mostly limited to studies on
complex formation or binding. Therefore, the present application of
gel chromatography for observing both the dissociation and the rate of
reaction suggests its usefulness in many other unexplored biochemical
reactions.

For the past several years, Boyer's (1956) spectrophotometric-
titration procedure was used for reaction-rate measurements. Parallel
ultracentrifugation experiments (Duke et al., 1970) could only estimate
the amount of dissociation at the completion of reaction, since these
studies were time consuming. Once again, we were able to use gel chroma-
tography to study the rate of reaction as well as the rate of dissociation
were evaluated from the same elution profiles. At various stages of the
reaction, aliquots were applied on the column.
Inspection of Fig. 32 clearly indicates that the rate of dissociation falls behind the rate of reaction. Consequently at any time during the reaction, a considerable portion of the octameric species is associated with pMB, as shown on the last column in Table XXI. These findings are not in agreement with the proposition of the dissociation model (Keresztes-Nagy and Klotz, 1965) that the sulfhydryl groups are reactive only in the monomeric form, and thus dissociation is a prerequisite for the reaction of the sulfhydryl groups in hemerythrin with pMB.

C.3. The 'All-or-None' Behavior of Hemerythrin -SH Groups.

The disagreement between the extent of the reaction and the degree of dissociation is a temporary one since the latter eventually would catch up with former even in the case of the oxygenated hemerythrin. That the final state indeed corresponds to the originally observed 'all-or-none' type is demonstrated with the chloro complex of the protein (Fig. 33).

What is then the reaction mechanism which leads from reacted octamers to segregation into unreacted octamers and reacted monomers, when less than stoichiometric amounts of mercurial are used? One possibility is that the reaction of the octamers, itself, is an 'all-or-none' type resulting in two distinct populations of octameric molecules (Mechanism 1, Fig. 36); one with all eight cysteiny1 residues reacted, and another with no reacted sulfhydryl groups. This type of
Fig. 36. Possible reaction mechanisms consistent with the observed octameric intermediate species and the final "all-or-none" behavior of the sulfhydryl groups in hemerythrin. Route 1 represents the initially "all-or-none" mechanism, while route 2 corresponds to the random reaction of the sulfhydryl groups. The symbol * stands for a conformationally altered molecule.
reaction was first proposed for phosphorylase (Madsen and Cori, 1956) and may be the result of a "zipper" or "wedge" mechanism (Madsen and Gurd, 1956; Boyer, 1958) or it may be due to an isomerization of the octamers to expose all -SH groups in only one isomeric form. Such a two-state model was proposed by Monod, Wyman and Changeux (1965) to explain the allosteric behavior of enzymes. The completely reacted octamers then would undergo secondary conformational changes leading to an irreversible dissociation by a relatively slow rate. Accordingly, the overall rate of dissociation may be slower than the rate of the reaction, as is the case with oxygenated hemerythrin.

Alternatively, the 'all-or-none' final state may be the result of a second mechanism (Fig. 36) according to which the reaction itself is random; meaning that initially all the octamers react more or less to the same extent. In this case, only a single population of octameric molecules would be produced in contrast to the above discussed mechanism. The randomly reacted octamers then would dissociate into reacted and unreacted monomers, of which the latter ones would reassociate to yield completely unreacted octamers. After the initial step the two mechanisms may become undistinguishable, because, for a time, the reacted monomers may also reassociate and segregate. This is assumed since blockage of the sulfhydryl groups alone may not necessarily result in irreversible dissociation and further conformational changes might take place before the reacted octamers irreversibly depolymerize.
It may be possible to distinguish between the two mechanisms experimentally since the charges on the completely reacted, partially reacted, and completely unreacted octamers are expected to be different from one another. Hence, if a mixture of reacted and unreacted oxyhemerythrin octamers (Table XXIV, last column) cannot be resolved into two species by electrophoresis or ion-exchange chromatography, the reaction itself would be originally of the random type. However, finding two species in such a mixture, corresponding to completely reacted and unreacted octamers, would not conclusively prove mechanism 1, since this state could have come about by secondary reequilibration. From the dissociation and reaction-rate studies, it is already possible to conclude that the 'all-or-none' final state arises via the formation of reacted octamers which would dissociate into monomers with a relatively slow rate. Thus, the major conclusion from the rate studies is the definite invalidation of the third hypothesis in the dissociation model.

The role of sulfhydryl groups in the oxygen-carrying function of hemerythrin was evaluated by comparing the rate of reaction with the rate of oxygen-release (Fig. 32a). A rapid release of oxygen is observed in comparison with the rate of dissociation. Previously, Fransiol (1969) found that the rate of reaction of -SH groups and pMB is faster than the rate of oxygen release. This apparent contradiction about the oxygen release can be explained by considering both our and Fransiol's (1969) experimental conditions. We employed a lower oxyhemerythrin concentration \((6.0 \times 10^{-5} \text{ M})\) in comparison with that of previous experiments.
The ratio of pMB:Hr was almost identical in both cases (2:1). Therefore, greater amount of monomers would be present in the protein solution of our reaction mixture. If, as we mentioned earlier, the oxygen in the monomeric hemerythrin is more easily accessible than in the octamers, one can expect to observe a faster rate of \(O_2\) release at lower protein concentrations. In general, the rate of oxygen loss can be explained by implicating an indirect role for free sulfhydryl groups in the oxygen transport of hemerythrin. Accordingly, once the sulfhydryl groups are reacted with mercurial, oxygen is gradually released. Such indirect role of sulfhydryl groups in maintaining catalytic functions is not uncommon in proteins.

D. Considerations for the Working Model of Hemerythrin Interactions:

A closer examination of the rate of reaction curve (Fig. 32) shows that about 30% of the reaction occurs rapidly in the first 9-10 minutes. Such an initial fast phase of reaction has also been observed by other investigators (Egan, 1969; Duke et al., 1971, Cress, 1971). Among these, one group of workers (Duke et al., 1971) indicated that the mercurial binding at non-sulfhydryl sites is faster than with the sulfhydryl groups. Earlier hypothesis of Egan (1969) also proposes similar binding in the mode of a surface denaturation by pMB. Such a binding can be assumed to gradually unfold the molecule so that all the sulfhydryl groups are eventually exposed for reaction with pMB. The initial fast reaction may be attributed to the presence of some fast-reacting sulfhydryl species. However, recent experiments of Duke et al.
(1971) discount the hypothesis for fast-type sulfhydryl groups. These workers suggest the rapid binding of pMB at non-sulfhydryl groups as the contributing factor in the initial fast portion of the mercurial-hemerythrin reaction. Such a rapid binding of pMB may not be of any consequence, since the ability of the bound pMB to react has been already observed. (A more detailed treatment may be found in the work of Cress (1971)).

Alternatively, one can also explain the initial phase of the hemerythrin-pMB reaction on the basis of a small amount of reactive species in equilibrium with the rest of the inactive species. As the reaction proceeds, conformational change allows further reaction of the mercurial. The results of the present study indicate that the octameric hemerythrin is the reactive species. In such a hypothesis, some proportion of the octameric hemerythrin must contain the reactive -SH groups, and hence a different conformation. Anion binding is assumed to shift the equilibrium of both the reactive and unreactive octameric species towards the monomers.

From the present investigation, we conclude that any model postulated for hemerythrin interactions must involve octamers as the reactive species, and the ligand interactions at both the iron and non-iron sites. The relevance of pMB pre-reaction binding cannot yet be predicted as to its effect on the kinetics of pMB-hemerythrin sulfhydryl reaction. Therefore, further investigation of this problem can be of great importance to an understanding of the behavior of sulfhydryl groups in other biologically important proteins.
CHAPTER V

SUMMARY AND CONCLUSIONS

This investigation used gel-chromatography technique as an analytical tool to investigate certain thermodynamic and kinetic problems involving protein interactions. The effects of dilution, various anions, and the sulfhydryl-group blocking reagent, paramercuribenzoate, on the association and dissociation of the model protein, hemerythrin, were studied. The major conclusions and original contributions of this dissertation may be summarized as follows:

(1). The standard free energy for the dissociation of hemerythrin octamer into subunits is about 6 Kcal per mole of monomer; this value agrees well with previous measurements by sedimentation-equilibrium (Langerman and Klotz, 1969) and ion-binding techniques (Klapper and Klotz, 1968a).

(2). The octamers and monomers cannot be separated from their equilibrium mixture because the association-dissociation equilibrium is rapidly established. This contrasts with the sedimentation-velocity experimental finding (Klapper et.al., 1966) of a slowly established equilibrium.

(3). From analysis of elution boundaries, we have concluded that the stoichiometry of dissociation is equal to the number of subunits (i.e. 8). Thus, no stable intermediates, such as dimer or
tetramer, need be taken into account when making equilibrium calculations. A similar conclusion was reached on the basis of the sedimentation-equilibrium studies already mentioned.

(4). Iron-coordinating anions, as expected from earlier studies, specifically affect hemerythrin-dissociation equilibrium, in general favoring the monomeric form of the protein. In regard to detecting minor shifts in the degree of dissociation as the function of various parameters such as specific ligands, temperature, etc., gel filtration proved to be more sensitive than ultracentrifugation.

(5). In contrast to earlier beliefs, the binding of the anion to the metal (iron) in hemerythrin with concomitant spectral changes has been shown not to be the major factor affecting protein conformation and dissociation. These effects could be demonstrated at low ligand concentrations not connected to any spectral change, as well as higher concentrations prior to, and independent of, any spectral transitions.

(6). Subsequently, we have shown that at least chloride ion, the ligand most studied, binds to hemerythrin (with stronger affinity) at a site other than the iron.

In this study, the Hummel and Dreyer gel-chromatography procedure (1962) was used in conjunction with radioactive chloride to detect binding.

(7). The binding of pMB to hemerythrin, as opposed to its reaction with cysteiny1 residues, was shown both by the zonal-Hummel and Dreyer
technique (1962) and frontal-elution chromatography. Combining this latter procedure with stopped-flow method, we obtained evidence that the bound pMB can react with the sulfhydryl groups in hemerythrin.

(8). Finally, we have also studied the rate of reaction of pMB with the protein sulfhydryl groups and compared it with the rate of dissociation into the monomeric form. We were able to observe these two rates simultaneously, employing zonal-gel-filtration techniques. We have concluded that, contrary to previous suggestions (Keresztesy-Nagy and Klotz, 1965), it is the octameric form of the protein which reacts with the mercurial, most probably in a random fashion, followed by reequilibration and dissociation resulting in the observed all-or-none behavior.
BIBLIOGRAPHY


APPENDIX - I


The various mathematical equations which have been utilized to convert the experimental parameters ($V$, $\sigma$ etc.) into useful quantities ($K$, % dissociation) are compiled in this Appendix. Therefore, only some of the aspects (pertinent to this investigation) of chromatographic theory will be presented here. More detailed reviews of gel chromatography may be found elsewhere (Determann, 1969; Winzor, 1969; Ackers, 1970; Cann, 1970).

Among the commercially available molecular-sieve media, Sephadex beads (dextran gels, cross linked with epichlorohydrin) are most extensively used in column chromatography. Separation of substances on Sephadex columns is dependent on the extent of penetration into the gel pores (whose size can be selected from a wide variety of cross-linked Sephadexes). It is this penetrating ability which determines the migration of a solute on gel-chromatographic columns. The fundamental parameter, distribution coefficient, $K_D$, describes the penetration (equilibration) by the equation:

$$K_D = \frac{Q}{\beta c},$$

where $Q$ is the amount of solute within the gel per unit column length, $\beta$ is the gel's internal volume per unit column length, and $c$ is the concentration of solute within the void volume. We will use the nomenclature of Ackers and Thompson (1965) to denote $K_D$, with $\sigma$, the molecular sieve coefficient.
Since the sieve coefficient ($\sigma$) depends on the porosity of the gel and the size of the solute, its value can only vary from zero to unity. A very large solute with no penetration ($\sigma = 0$) is excluded at a volume which is equal to the solvent space outside of the gel beads ($V_o$). On the other hand, a very small solute penetrates completely into the gel's interior space and is therefore excluded at a volume ($V_t$) which comprises both the inside ($V_i$) and outside ($V_o$) of the gel phase. The value of $\sigma$ is, accordingly, equal to unity. A solute with intermediate penetration will be eluted from the column in a volume

$$V_e = V_o + \sigma V_i$$

where $\sigma$ can have a value between zero and unity.

**Zonal and Frontal Chromatographic Analysis:**

The essential difference in the two types of procedures is the volume of sample (ideal solute) solution applied to the column. In the frontal analysis procedure, a large volume of the sample is added to the column such that the spaces exterior to the gel media are completely replaced with the sample solution. The elution profile, accordingly, will contain a plateau region where the concentration of the sample is identical to that of the applied sample ($C_o$). The advancing boundary of the elution profile depicts the concentration increase from zero to $C_o$, whereas the descending counterpart shows the decrease in concentration from $C_o$ to zero. The elution volume of the sample may be obtained from the volume at the centroid position (the equivalent boundary position) ($\text{Longsworth, 1943; Nichol and Ogston, 1965}$).
In the zonal procedure, the small volume of the sample (ideal solute) migrates through the column to assume a Gaussian distribution curve. The elution volume of the sample corresponds to the volume at which the concentration of the sample is maximum in the elution profile. Theoretically, this position should agree with the elution volume obtained in a frontal experiment. In spite of the wide use of the zonal procedure, the frontal-elution method is recommended for fractionation and purification work. The latter procedure is recommended especially for studies on protein interactions, because of the absence of dilution effects. Furthermore, the type of protein interactions can be analyzed on the basis of existing theory (Gilbert, G. A., 1955) for the rate of equilibration and stoichiometry.

Equilibrium Nature of Gel Filtration Data:

There is still no unified theory of gel chromatography available, but most authors (Porath, 1963; Squire, 1964; Laurent and Killander, 1964) agree that it is an equilibrium procedure. Gilbert (1966) has illustrated the steady-state nature of the quantity, elution volume, which is obtained in a frontal-elution-chromatography method. Further, he has indicated that equilibrium data may be derived from elution volume from the following considerations: The amount of the solute retained in the column (at any instant) is given by multiplying the elution volume, $\bar{V}_i$, by the concentration of the solute in the inflow of the column, $\bar{W}_i$, under steady-state conditions. Kinetic factors such as re-equilibration (Andrews, 1964) within the column (caused by occlusion of the reaction
mixture by the gel phase) and the rates of diffusion of the various species in the reaction mixture to and from or within the gel phase (Ackers, 1964), cannot alter the steady state value $\bar{V}_t \times \bar{W}_t$. If the solution entering the column is already in internal equilibrium, its rate, of the restoration or disturbance in the column, can only affect the elution (concentration) profile (Longsworth and MacInnes, 1942) and not the value of $\bar{V}_t$ (Longsworth, 1943). Therefore, Gilbert (1966) concluded that equilibrium constants can be calculated from elution volume, $\bar{V}_t$, without any kinetic effects. Recent reviewers (Kellett, 1967; Winzor, 1969; Ackers, 1970) of chromatography theories consider this conclusion to be generally valid. Furthermore, the close agreement between theoretical calculations and experimentally derived equilibrium constants from elution volumes illustrates (Hammes and Schimmel, 1965; Ackers and Thompson, 1965) the validity of Gilbert's (1966) conclusion. Therefore, we have used the steady-state parameter, elution volume, in the calculation of the equilibrium constant for hemerythrin dissociation.

**Evaluation of Elution Volume:**

As mentioned earlier, frontal analysis is more useful in quantitative studies, since the observed elution volume corresponds to the applied sample concentration. In a frontal elution experiment, the 'elution' volume can be derived from the first moment of the boundary $\bar{V}$, more commonly known as the centroid position (Longsworth, 1943). In ideal systems, this position corresponds to $C_o / 2$, where $C_o$ is the plateau
concentration. In systems such as hemerythrin, which consist of association-dissociation equilibria, the elution volume position is obtained from the relation (Winzor, 1969),

\[
\bar{V} = \frac{1}{c_0} \int_0^c V dc = \frac{1}{c_0} \Sigma VdC.
\]

**Column Calibration:**

The experimental parameter, elution volume, of a solute is directly related to its molecular-sieve coefficient (partition coefficient) by the equation \( \sigma = \frac{\bar{V} - V_0}{V_i} \). It is this coefficient which is useful in the calibration of columns on the basis of molecular size or weight.

Many models (Porath, 1963; Squire, 1964; Laurent and Killander, 1964; Ackers, 1964; Albertsson, 1960; Cassasa, 1967; Giddings et al., 1968; Ackers, 1967) have been proposed to visualize the penetrable spaces within the gel matrix, among which only the statistical model of Ackers (1967) includes a variety of different geometrical shapes for the gel pores. According to this model, the total volume of gel-matrix space which can be occupied by the molecular species of radius 'a' is then expressed by the following relation:

\[
\sigma = \text{erfc} \left( \frac{a - a_o}{b_o} \right) = 1 - 2/ \pi \int_0^{a/a_o} b_o e^{-a^2} da.
\]
where $a_o$ is the position of the maximum value of the Gaussian distribution, $b_o$ is a measure of standard deviation and $\sigma$ is the sieve coefficient (Ackers, 1970). The symbol 'erfc' represents the error-function complement.

A linear form of the above equation predicts the molecular radius, $a$, to be related to the inverse-error-function complement ($\text{erfc}^{-1}$) of the sieve coefficient, $\sigma$, by the relationship $a = a_o + b_o (\text{erfc}^{-1} \sigma)$.

Such correlation has been observed for a variety of gels and molecular species in which the column calibration constants, $a_o$ and $b_o$, were obtained from the ordinate intercept and slope of the plot of $'a'$ versus $\text{erfc}^{-1} \sigma$.

Similarly, one can also relate the sieve coefficient ($\sigma$) with $M$, the molecular weight of the solute. However, the non-linear correlation between these two quantities can only be made linear over a certain range of molecular weights (and sieve coefficients). A number of workers (Andrews, 1964, 1965; Whitaker, 1963; Leach and O'Shea, 1965) employed the logarithm of molecular weight, $M$, for such empirical straightening-out procedure by using an equation,

$$\sigma = -A \log M + B,$$

where $A$ and $B$ are empirical column-calibration constants and $M$ the molecular weight of the solute species. For any gel medium, this linear relationship can only hold over a certain portion of the calibration plot between $\sigma$ and $\log (M)$. 

Equilibrium of Monomer with a Higher Polymeric Species:

In frontal gel chromatography, the elution profile varies according to the type of equilibrium between the monomer and the polymer. This effect can be visualized by the consideration of the rate of re-equilibration relative to that of separation of the species. The relative concentrations of the interacting components are obtained fairly easily from the elution profiles of slowly equilibrating systems. Identification of the two distinct boundaries in the advancing and descending elution profiles with the individual components is correct in systems where the rate of separation on the column is faster than the re-equilibration of the components. However, such identification cannot be made in systems with intermediate and rapid rates of re-equilibration.

The median bisector (Nichol and Ogston, 1965) of the leading and trailing elution boundaries may be used in the evaluation of the elution volume (and thus, the sieve coefficient) of a polymerizing system with an equilibrium rate almost identical to the rate of separation on the column.

Ackers and Thompson (1965) have shown that a sharp leading boundary, and a diffuse trailing boundary, will be obtained in the elution profile of a frontal type experiment, if the displacement by the transport process is slow in comparison with the rate of attainment of equilibrium between the molecular species. Such elution behavior had been predicted by earlier workers (Winzor and Scherage, 1963; Ackers,
1964). Ackers and Thompson (1965) have further shown that by neglecting the normal boundary dispersion of a single solute, the sieve coefficients of the individual species can be obtained from the boundary characteristics, since the centroid elution volumes depend only on the plateau concentrations and equilibrium constant.

In a multicomponent system, the equilibrium partition coefficient for total solute is a weight average of the partition coefficients of the individual components (Ackers and Thompson, 1965). Accordingly the weight average partition coefficient

\[ \bar{\sigma}_w = \sum_j \frac{c_j \bar{\sigma}_j}{\sum_j c_j} \]

where \( c_j \) is the concentration of species \( j \) and \( \bar{\sigma}_j \) is the corresponding partition coefficient.

If interactions are present between species the equilibria(um) will be shifted by a change in the total solute concentration and \( \bar{\sigma}_w \) will exhibit a concentration dependence. Ackers (1970) has deduced the relationship between \( \bar{\sigma}_w \) and centroid volume (\( \bar{V} \)) of a frontal chromatographic experiment as follows:

\[ \bar{\sigma}_w = \frac{\bar{V} - V_o}{V_i} \]

where \( V_o \) and \( V_i \) are the void and internal volume of the gel phase respectively.
The monomeric sieve coefficient designated as \( \sigma_m \) can be derived from frontal-elution experiments by employing an extrapolation procedure, in which the values of \( \text{v} \) are plotted against the concentrations at which they were obtained. \( \sigma_m \), the monomeric sieve coefficient, is derived by extrapolating this curve to zero protein concentration. Ackers and Steere (1967) have demonstrated that the relation,

\[
\sigma_n = \sigma_m - A \log i,
\]

may be used to obtain the polymeric sieve coefficient \( \sigma_n \), if 'i', the number of monomers are known.

The stoichiometry of the dissociation process of the polymer can then be evaluated by the use of the individual sieve coefficients, \( \sigma_m \) and \( \sigma_n \). In addition, one needs to know the position of the minimum of the gradient curve of the elution profile. This is obtained by plotting small concentration differences, \( \Delta C \), across the elution profile against the corresponding values of \( \Delta V \). Ackers and Thompson (1965) have used Gilbert's postulates (1955), originally derived for ultracentrifugal analysis, for the calculation of 'n', the stoichiometry of dissociation. Depending on the type of monomer \( \rightarrow \) polymer system, the elution behavior of \( nA_1 \rightarrow A_n \) can be predicted, if intermediate association forms are assumed to be unstable. If \( n = 2 \), the gradient across the trailing boundary will have a single maximum that is skewed in the direction of increasing volume. If, however, \( n \) is greater than 2, the gradient will exhibit two maxima and a single minimum. This minimum
position is defined by the equation:

\[ V'_{\text{min}} = V_0 + \left[ \frac{2}{3(n-1)} \sigma_m + \frac{n-2}{3(n-1)} \sigma_p \right] V_i + S \]

where \( S \) is the sample applied on the column. For a given column \( V'_\text{min} \) is independent of both the equilibrium constant and plateau concentration \( C_0 \), and depends only on the stoichiometry, \( n \), of the reaction. The degree of polymerization can be completed directly from the coefficients of the individual species and of the minimum by this equation for \( 'n' \).

If \( V'_\text{min} \) is defined as \( (V'_\text{min} - V_0 - S)/V_i \), then

\[ n = \frac{3 V'_\text{min} - \sigma_m - 2 \sigma_p}{3 V'_\text{min} - 2 \sigma_m - \sigma_p} \]

The practical upper limit lies in the region \( n \geq 10 \), as is also the case for the sedimentation velocity method.

Ackers (1964) has indicated that the centroid position of the hyper sharp leading boundary (of a rapid interacting system) can be related to the monomeric and octameric sieve coefficients as follows:

\[ \bar{V} = V_0 + \sigma_p + \alpha(\sigma_m - \sigma_p)V_i \]

Where \( \alpha \) is the weight fraction of monomeric species in the plateau region. This quantity is given by \( \alpha = (\sigma_w - \sigma_p)/(\sigma_m - \sigma_p) \).

From the known values of \( \alpha \) and \( 'n' \), the dissociation constant \( K \) for the \( \text{NA}_1 \leftrightarrow \text{A}_n \) equilibrium by the equation

\[ K = \frac{\alpha^n (C_0)^{n-1}}{1 - \alpha} \]

where \( C_0 \) is the solute concentration in the plateau region of the elution diagram.
Similarly, 'K' can be derived from the trailing boundary:

\[ K = \frac{n \left( C_{\text{min}} \right)^{n-1}}{\lambda + \left(1 + \frac{\lambda}{n} \right)^{n-1}} \]

where \( C_{\text{min}} \) is the concentration at the minimum position in the gradient curve and

\[ \lambda = \frac{\sigma_m - v'_\text{min}}{v'_\text{min} - \sigma_p} \]

In the case of hemerythrin dissociation, we already know that the number of subunits are 8; therefore, all the above equations could be used for the calculation of the dissociation constant, K.

If the value of 'K' were to be expressed in moles/liter of monomeric units, the equation for 'K' should include a new term, \( M_m \), the monomeric molecular weight (13,500). Hence, we divided the values of K obtained from the above equations with the quantity, \( (M_m)^{n-1} \), to express in units of moles/liter of monomer.
Calculation of the Centroid Volume from a Frontal Elution Diagram:

We have used Longsworth's (1943) procedure for this calculation. The procedure consists of dividing the first difference (gradient) curve by a number of ordinates, e.g. five, at equal intervals between the base line and the maximum, as shown in the following figure on the other scale; the abscissas, \( V'_{1} \), and \( V''_{1} \), of the ends of each ordinate, together with the corresponding abscissa, \( V'_{i.e} \), of the edge of the pattern, are then determined. The abscissa, \( \overline{V}_{1} \), of the midpoint ordinate is thus \( \frac{V'_{1} + V''_{1}}{2} \). If the distances \( \overline{V}_{1} - V'_{i.e} \) are the same for each ordinate, the gradients in the boundary are symmetrical about the ordinate through the maximum. If the distances to the centers of the ordinates are not equal, the boundary is 'skew'. If the skewness is not too great, the following relation can be used to obtain \( \overline{V} \), the centroid, or the first moment of the curve:

\[
\overline{V} = \frac{1}{1 \times \Delta V_{1}} \frac{\Delta V_{i} \left( \overline{V}_{i} - V'_{i.e} \right)}{i}
\]

where \( \Delta V_{1} \) is the length of the first ordinate, \( V''_{1} - V'_{1} \).

The curve in Fig. 37 is used to show the mathematical calculations used in obtaining the elution volume from the first difference plots.
Fig. 37. A representative first difference curve of the leading boundary obtained in a frontal elution experiment. Calculations are shown in Appendix II.
Similarly, 'K' can be derived from the trailing boundary:

\[
K = \frac{n (C_{\text{min}})^{n-1}}{\lambda + (1 + \frac{\lambda}{n})^{n-1}}
\]

where \( C_{\text{min}} \) is the concentration at the minimum position in the gradient curve and

\[
\lambda = \frac{C_m - v_{\text{min}}'}{v_{\text{min}}' - C_p}
\]

In the case of hemerythrin dissociation, we already know that the number of subunits are 8; therefore, all the above equations could be used for the calculation of the dissociation constant, \( K \).

If the value of 'K' were to be expressed in moles/liter of monomeric units, the equation for 'K' should include a new term, \( M_m \), the monomeric molecular weight(13,500). Hence, we divided the values of K obtained from the above equations with the quantity, \( (M_m)^{n-1} \), to express in units of moles/liter of monomer.
TABLE XXVI

Various terms for the calculation of the centroid volume

<table>
<thead>
<tr>
<th>$V_1'$ (ml)</th>
<th>$V_1''$ (ml)</th>
<th>$\Delta V_1 = (V_1''-V_1')$</th>
<th>$\frac{V_1'' + V_1''}{2}$</th>
<th>$\frac{V_1 - V_{i.e}}{2}$</th>
<th>$\Delta V_1 (\bar{V} - V_{i.e})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>21.0</td>
<td>31.0</td>
<td>10.0</td>
<td>52/2 = 26.0</td>
<td>21.0</td>
<td>210.0</td>
</tr>
<tr>
<td>21.5</td>
<td>28.5</td>
<td>7.0</td>
<td>50/2 = 25.0</td>
<td>20.0</td>
<td>140.0</td>
</tr>
<tr>
<td>22.0</td>
<td>27.0</td>
<td>5.0</td>
<td>49/2 = 24.5</td>
<td>19.5</td>
<td>97.5</td>
</tr>
<tr>
<td>23.0</td>
<td>26.0</td>
<td>3.0</td>
<td>49/2 = 24.5</td>
<td>19.5</td>
<td>58.5</td>
</tr>
</tbody>
</table>

$\Sigma \Delta V_1 = 25.0$

$\Sigma \Delta V_1 (\bar{V} - V_{i.e}) = 505.0$

$$\bar{V} = \frac{\Sigma \Delta V_1 (\bar{V} - V_{i.e})}{\Sigma \Delta V_1} = \frac{505}{25} = 20.20 \text{ ml} + V_{i.e}$$

since $V_{i.e} = 5.0 \text{ ml}$

$$\bar{V} = 25.20 \text{ ml}.$$
APPENDIX III

Conversion of $\sigma$ into its Inverse-Error-Function Complement ($\text{erfc}^{-1}$).

For this purpose, we have used the Table of Error Function and its Derivative, published as Applied Mathematic Series, No. 41 by the National Bureau of Standards, U.S. Department of Commerce.

The linear relation, $a = a_0 + b_0 \text{erfc}^{-1} \sigma$, is predicted from the correlation of $\sigma$, the sieve coefficient and the molecular radius '$a$', which can be expressed as:

$$\sigma = \text{erfc} \left( \frac{a-a_0}{b_0} \right) = 1 - \frac{2}{\sqrt{\pi}} \int_0^{\frac{a-a_0}{b_0}} e^{-a^2} da.$$

Therefore,

$$1 - \sigma = \frac{2}{\sqrt{\pi}} \int_0^{\frac{a-a_0}{b_0}} e^{-a^2} da.$$

If $\frac{a-a_0}{b_0}$ is denoted by the term $x$, one can then use the mathematical tables. The value of '$x$' is read off directly from the corresponding value of $\frac{2}{\sqrt{\pi}} \int_0^{x} e^{-a^2} da$.

It can be seen that $x = \text{erfc}^{-1} \sigma$ from the calibration equation.

This procedure can be understood from the following example:

$$\sigma = 1 - \sigma \text{ or } \frac{2}{\sqrt{\pi}} \int_0^{x} e^{-a^2} da \quad \text{The corresponding value of} \ x \ \text{from the Tables is}$$

0.016  0.984  = 1.703

1.703 is the value of $\text{erfc}^{-1} \sigma$, where $\sigma = 0.016$. 
APPENDIX IV

Calculation of % Dissociation from the Weight-Average Molecular Weight.

The following relation is used for this purpose:

\[
\bar{M}_w = \frac{C_p \times M_p + C_m \times M_m}{C_p + C_m},
\]

where \(C_p + C_m\) = plateau protein concentration (g/l), and \(M_m\) and \(M_p\) are monomeric and octameric molecular weights (13,500 and 107,000 respectively for hemerythrin). Example: \(\bar{M}_w\), obtained from extrapolation of \(\bar{c}_w\) (the weight-average sieve coefficient) in the calibration graph, is 74,130 ± 1,500. (Concentration of protein = 480 ± 5 µg/ml)

\[
73,130 = \frac{C_p \times 107,000 + C_m \times 13,500}{C_m + C_p}
\]

\(C_p = 2.64 \ C_m\)

\(C_m + C_p = 480 = 3.64 \ C_m\); \(C_m = \frac{480}{3.64} = 132\)

Percent dissociation = \(\frac{C_m}{C_o} \times 100\)

= \(\frac{132}{480} \times 100 = 27.4\%\).
APPENDIX V

Calculation of the Dissociation Constant from the Weight-Average Sieve Coefficient.

The equation for $K$ can be represented as:

$$K = \frac{(\alpha)^n \times (C_0)^{n-1}}{(1-\alpha) \times (M_m)^{n-1}}$$

where $\alpha$ is $\frac{\sigma_w - \sigma_p}{\sigma_m - \sigma_p}$, $n = 8$ and $M_m = 13,500$.

Example: $\sigma_w = 0.148; \sigma_m = 0.587$ and $\sigma_p = 0.025; C_0 = 0.125$ g/l.

$$\alpha = 0.221$$

$$K = \frac{(0.221)^8 \times (0.125)^7}{(1-0.221) \times (13,500)^7}$$

$$= 2.13 \times 10^{-41} \text{ moles/liter in monomeric units.}$$
### APPENDIX VI

Changes in Dissociation Constant by Introduction of Errors in Elution Volume and Concentration Terms.

<table>
<thead>
<tr>
<th>Concentration gm/l</th>
<th>$\bar{V}$ ml</th>
<th>$K$ moles/l of monomer</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.034</td>
<td>18.9</td>
<td>$7.5 \times 10^{-41}$</td>
<td>Negligible change in $K'$ by introducing error equal to mean deviation in $\bar{V}$.</td>
</tr>
<tr>
<td>0.034</td>
<td>18.8 (0.5% error)</td>
<td>$6.6 \times 10^{-41}$</td>
<td></td>
</tr>
<tr>
<td>0.067</td>
<td>17.62</td>
<td>$1.8 \times 10^{-39}$</td>
<td>Negligible change in $K'$ by introducing 5% error in the measurement of protein concentration.</td>
</tr>
<tr>
<td>0.065 (5% error)</td>
<td>17.62</td>
<td>$1.4 \times 10^{-39}$</td>
<td></td>
</tr>
<tr>
<td>0.067</td>
<td>17.62</td>
<td>$1.8 \times 10^{-39}$</td>
<td>Twofold change in $K'$ by introducing 5% error in dilution plus 1% error in measurement of elution volume.</td>
</tr>
<tr>
<td>0.065 (5% error)</td>
<td>17.42 (1% error)</td>
<td>$1.0 \times 10^{-39}$</td>
<td></td>
</tr>
<tr>
<td>0.067</td>
<td>17.62</td>
<td>$1.8 \times 10^{-39}$</td>
<td>Fifteen fold change in $K'$ by introducing 15% error in the measurement of protein concentration.</td>
</tr>
<tr>
<td>0.060 (15% error)</td>
<td>17.62</td>
<td>$2.6 \times 10^{-40}$</td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX VII

Calculation of the % Dissociation and of Reaction from Elution Peak Areas Obtained in Zonal Experiments with an Oxyhemerythrin-pMB Reaction Mixture.

\[
\text{Percent reaction} = 100 \times \frac{\text{Theoretically expected increase in area}}{\text{Octameric Peak Area} + \text{Monomeric Peak Area} - \text{Blank Protein Peak area}}
\]

Example: For a protein blank of an area 0.135 cm., the theoretically expected increase in area would be:

\[
0.135 \times \frac{33}{20} = 0.226 - 0.135 = 0.08 \text{ sq cm},
\]

since the absorbance of the protein would increase by the reaction of -SH groups and unreacted pMB. The extinction coefficients of the protein, pMB, and the mercaptide-bound formation are \(20 \times 10^3\), \(5 \times 10^3\) and \(7 \times 10^3\) respectively.

\[
\% \text{ Reaction} = 100 \times \frac{0.215 - 0.135}{0.09} = 83\%.
\]

This value can be experimentally estimated from spectrophotometric titrations. As mentioned in the text, we observed a close agreement between these two values for % reaction.

Similarly, the percent dissociation can be calculated from the relation:

\[
\% \text{ dissociation} = 100 \times \frac{\text{True monomer area}}{\text{Blank protein peak area}}
\]

since all monomers must contain pMB.

The monomeric peak in the above experiment had an area of 0.135. The true area can only be \(0.135 \times \frac{20}{33}\).

\[
\% \text{ dissociation} = 100 \times \frac{0.135 \times \frac{20}{33}}{0.135}
\]

In a similar fashion the values of % dissociation and % reaction for the experiments mentioned in Chapter III were calculated.
APPROVAL SHEET

The dissertation submitted by Ayyagari Laxminarasimha Rao has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation 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.

January 19, 1972
Date

Signature of Advisor