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HEMERYTHRIN DISSOCIATION-ASSOCIATION
STUDIES BY SEPHADEX CHROMATOGRAPHY

by

APPAGARI LAXMINARASIMHA RAO

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

FEBRUARY

1969

LIFE

Ayyagari L. N. Rao was born in Mogallu, Andhra State, India on December 30, 1940.

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The author also wishes to thank the members of the Department of Biochemistry and Biophysics and his fellow students for the encouragement given and for the exchange of ideas during this research project.

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CHAPTER I

INTRODUCTION

The concept of quaternary structure for proteins mainly came from molecular weight investigations of Svedberg and collaborators. This fourth structural order of a protein represents the association of several polypeptide chains without the involvement of covalent bonds. Each individual chain known as a subunit or monomer has its own primary, secondary, and tertiary structures. The number of proteins composed of subunits is rather large and a fairly complete list of such proteins was published recently (1). If we assume that for each subunit only the sequence of amino acids is specified genetically, then the rest of the assembly process in a macromolecule must be assumed to follow automatically. The actual structure is only one of many that can be realized and thermodynamically stable under physiological conditions. Electrostatic, hydrogen and hydrophobic bonds may all be involved in forming a subunit association complex, and the relative contribution of each kind of bond will vary from protein to protein. The arrangement of the subunits may determine the active center and the reactivity of the protein. A study of the assembly process of the subunits should reveal information about the stability of the macromolecule and the affect of quaternary structure on the protein. Casper (2) using Tobacco Masaic Virus Protein as a model, investigated the stability of

the different arrangements of subunits. Oosawa Kasai (3) presented a theory of linear and helical aggregations of molecules from their studies on G-F transformation of Actin. Monod et. al. (4) have attempted to explain the allosteric interactions in proteins on the basis of changes in the quaternary structure, i.e. alterations in the bonding between identical subunits. They also studied the different arrangements for the association of the subunits. However, the association behavior of the subunits could not explain the functional properties of some proteins completely. Hence, the dissociation phenomena of the macromolecules was pursued by many workers to gain information about the bonds between the subunits. The common method employed was the use of reagents which can reversibly dissociate the protein into its subunits, and the process of dissociation was then evaluated under various conditions to yield information about the forces responsible for holding the subunits together.

Riethel (5) reviewed the dissociation-association behavior of many proteins and concluded that biological activity is related to the degree of association or dissociation. In such cases, biological regulation can be visualized if a dynamic equilibrium is assumed to be existing between the associated and the dissociated forms of the protein in vivo. In vitro, the study of a dissociation equilibrium can be used to gain infor-

mation about the type of bonding between the subunits in polymeric proteins when the experimental conditions are varied systematically. The dissociating agents like urea, guanidinium salts and detergents often force the equilibrium to the monomeric species, but their use in dissociation is less desirable, since the protein also undergoes denaturation. Ansevin and Lauffer (6) and Guidotti and Craig (7) demonstrated that a number of proteins such as TMV protein, hemoglobin, cytochrome-c, etc. undergo association and dissociation as a function of concentration, the monomeric species being favored at low concentrations. Dilution is a mild method for dissociating proteins, but complete conversion to monomeric species occurs only at very low protein concentrations. Hence measurement of dissociation upon dilution becomes limited to methods by 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 can still be used in this range of concentration. However, ultracentrifuges require complicated absorption optics and are very expensive instruments. Therefore, the gel filtration technique has been chosen by many workers to determine the molecular weight changes in proteins. Winzor and Scheraga (8) in 1963 have experimentally shown

for the first time in gel filtration all the theoretically predicted transport features for chromatography. Since then, the polymerization of several enzymes has been studied by gel filtration (8-10). Chun and co-workers (11) have accumulated evidence for the coexistence of monomeric, polymeric and intermediate species in proteins. Gel filtration technique has now become a common method for determining homogeneity of proteins. Because of the many advantages of the gel filtration technique, it was decided to employ this technique in the study of dissociation-association properties of hemerythrin.

Hemerythrin, the non-heme iron protein of *Golfingia Gouldii* worms, represents an ideal system for investigating subunit-subunit interactions, since this interaction is influenced by various iron coordinating ligands and sulfhydryl blocking reagents. The native protein has been found to contain 8 sulfhydryl groups, 16 equivalents of iron, per molecular weight of 107.00 (12). Dissociation into monomers with molecular weight of 13,500 occurs when the sulfhydryl groups are blocked with organic mercurials of N-ethyl-maleimide (13). These subunits are found to be identical from sequence studies (14). The monomer, therefore, has one cysteinyl residue and two equivalents of iron atoms, which can hold one molecule of oxygen. The oxygen can be replaced, both in the octameric and monomeric forms, with various

iron coordinating ligands such as azide, thiocyanate, chloride, fluoride and other anions (15). In the absence of such coordinating ligands, depending on the pH, the site is most likely occupied by either water or hydroxyl ions. The reactivity of the sulfhydryl groups and the dissociability of the protein has been found to be different in the various liganded forms. The effect of the iron coordinating ligands on the reactivity of the cysteinyl residues of hemerythrin (15) is an example of cooperative interaction, since it has been shown that the sulfhydryl groups and the iron atoms are at separate sites in the molecule. The mechanism of the interaction could be interpreted by two models, one involving a conformational change and the other the dissociation of the protein, both resulting in a reactive form of the molecule. Evidence has been collected mainly in favor of the dissociation model which essentially has three assumptions: (1) the octamer is in a dynamic equilibrium with the monomer, (2) the iron coordinating anions shift the equilibrium towards the monomer, (3) the monomer is the reactive form of the protein.

Only the evidence for the first two assumptions will be examined here since the last assumption of the model is being actively investigated by other workers in our laboratory (16, 17).

The existence of a dynamic equilibrium has been proven by two

different methods. The hybridization experiments involved 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 (18) and, therefore, the monomers and the octamers are in a dynamic equilibrium. Although the coexistence 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 (19). In these studies, the amount of monomeric form appeared to be the function of the ligand in agreement with the second assumption of the model. Two systems were used, one containing the iron coordinating ligand, thiocyanate, and the other no coordinating anion. At each concentration studied, more monomer was found in thiocyanate containing protein solutions. The effect of the presence or absence of the iron coordinating anion on dissociation of hemerythrin is also demonstrated by ligand binding studies (20). The second assumption of the dissociation model can be expressed in affinity terms, as that the monomer has a greater affinity for the ligand than the octamer. Therefore, by dilution the binding of the ligand should increase since

more monomers are being formed. The binding studies showed an increase in apparent binding affinity by dilution and this further validates the second assumption of the model.

Both in the ultracentrifugation and binding experiments, the effects of only one anion, thiocyanate, was studied which is a non-biological ligand and may catalyze the formation of monomers with oxidized SH groups not capable to reassociate. The dissociation with ligands other than thiocyanate occurs to any measurable extent at very low concentrations, where the operation of ultracentrifuges becomes cumbersome. In order to obtain information about the bonds that are holding together the monomeric units in hemerythrin, dissociation has to be affected under various conditions. This again is difficult to study by ultracentrifugation. To prove the dynamic nature of the octamer-monomer equilibrium, the reversibility of the dissociation equilibrium must be demonstrated. An additional problem associated with ultracentrifugation experiments is the high pressure which develops in the cell in the system being studied. The pressure may shift the equilibrium point and also influence the rate of equilibration (21).

The work described in this thesis deals with the study of the dissociation equilibrium of hemerythrin by gel filtration. The effect of dilution on the protein is investigated to evaluate the equilibrium con-

stant. The elution patterns are compared with the ultracentrifugation results, to test the effects of high pressures on the equilibrium position. Reversibility of the equilibrium is tested and also the effect of various ligands on the hemerythrin dissociation equilibrium. In order to be able to define this equilibrium, the temperature dependence of it is studied and the thermodynamic parameters are evaluated.

CHAPTER II

MATERIALS AND METHODS

MATERIALS

Blue Dextran, Pharmacia Fine Chemicals Co., lot 4474

Bovine Serum Albumin, Armour Pharmaceutical Co., No. 2266, lot
A69702

Cacodylic Acid, Fisher Scientific Co., No. A-82, lot 773403

DEAE Anion Exchanger (Cellex), Bio-Rad Laboratories, Control No.
3765

α -Chymotrypsin, CalBiochem, No. 23089, lot 72313

Cytochrome-c, Mann Research Laboratories, Inc., No. 1581

Hydrochloric Acid-Concentrated, Mallinckrodt Chemical Works, No.
2612, lot JNS

Hydroxylamine Hydrochloride, Mallinckrodt Chemical Works, No. 5258

Myoglobin, Nutritional Biochemical Corporation, Control No. 1930

Ortho-Phenanthroline, J. T. Baker Chemical Co., No. T-170

Ovalbumin, Nutritional Biochemical Corporation, Control No. 4790

Potassium Perchlorate, J. T. Baker Chemical Co., No. 3220

Potassium Thiocyanate, J. T. Baker Chemical Co., No. 3326

Salyrganic Acid, Nutritional Biochemical Corporation

Sephadex G-25 (Coarse), Pharmacia Fine Chemicals, Inc., lot 9338

Sephadex G-100, Pharmacia Fine Chemicals, Inc., lot 8080

Sodium Azide, Matheson, Coleman and Bell Co., No. 2781, lot SX299

Sodium Chloride, A. R. Grade, Mallinckrodt Chemical Works, No. 7581,
lot SXS

Sodium Fluoride, J. T. Baker Chemical Co., No. 3688, lot 24061

Sodium Hydroxide, J. T. Baker Chemical Co., No. 3727

Sulfuric Acid (Concentrated), J. T. Baker Chemical Co., No. 9681

Tris (Hydroxy-methyl) Amino Methane, Reagent Grade, Sigma Chemical
Co., lot 288.5010

PREPARATION OF CRYSTALLINE OXYHEMERYTHRIN

The method of Klotz et. al. (22) was followed in the preparation of oxyhemerythrin. Live worms (*Golfingia Gouldii*) which were supplied by the Marine Biological Laboratories, Woods Hole, Massachusetts, were cut open in the tail and the coelomic fluid was collected. The erythrocytes were washed and centrifuged several times with 2.5% saline solution before laking them with distilled water. The cell debris was removed by centrifugation, and the supernatant was dialyzed against 20% ethanol for six hours. The red crystals of oxyhemerythrin were then stored in cold after centrifugation.

CONVERSION OF OXYHEMERYTHRIN INTO VARIOUS COORDINATION COMPLEXES

The method for converting the oxyhemerythrin into various stable coordination complexes, which has been reported earlier by Nagy and Klotz (15), has been followed in this investigation. This method is based upon the fact that the iron in hemerythrin forms coordination complexes with anions such as chloride, fluoride, thiocyanate, azide, etc. Overnight dialysis of oxyhemerythrin against 0.1 M solution of the particular ion, except in the case of chloride ion, was sufficient for the complete conversion into the ligand complex. The chloride complex was prepared by the addition of a small amount of sodium fluoride till the violet-pink color of the oxyhemerythrin changed to a pale yellow color of methemerythrin fluoride complex, prior to the dialysis against 0.5 M sodium chloride solution for 48-72 hours. Similarly, the aquo form was prepared by converting the oxyhemerythrin into the fluoride complex before the repeated dialysis for 4-5 days against Tris-cacodylate buffer (0.01 M; pH 7.0). The absorption spectra of the various preparations were recorded on a Beckman double beam recording spectrophotometer and were compared with those reported in the literature (15). In these preparations, the Tris-cacodylate buffer (0.01 M; pH 7.0) contained the ligand in sufficient amounts to insure complete

conversion of oxyhemerythrin into the ligand complexes (15).

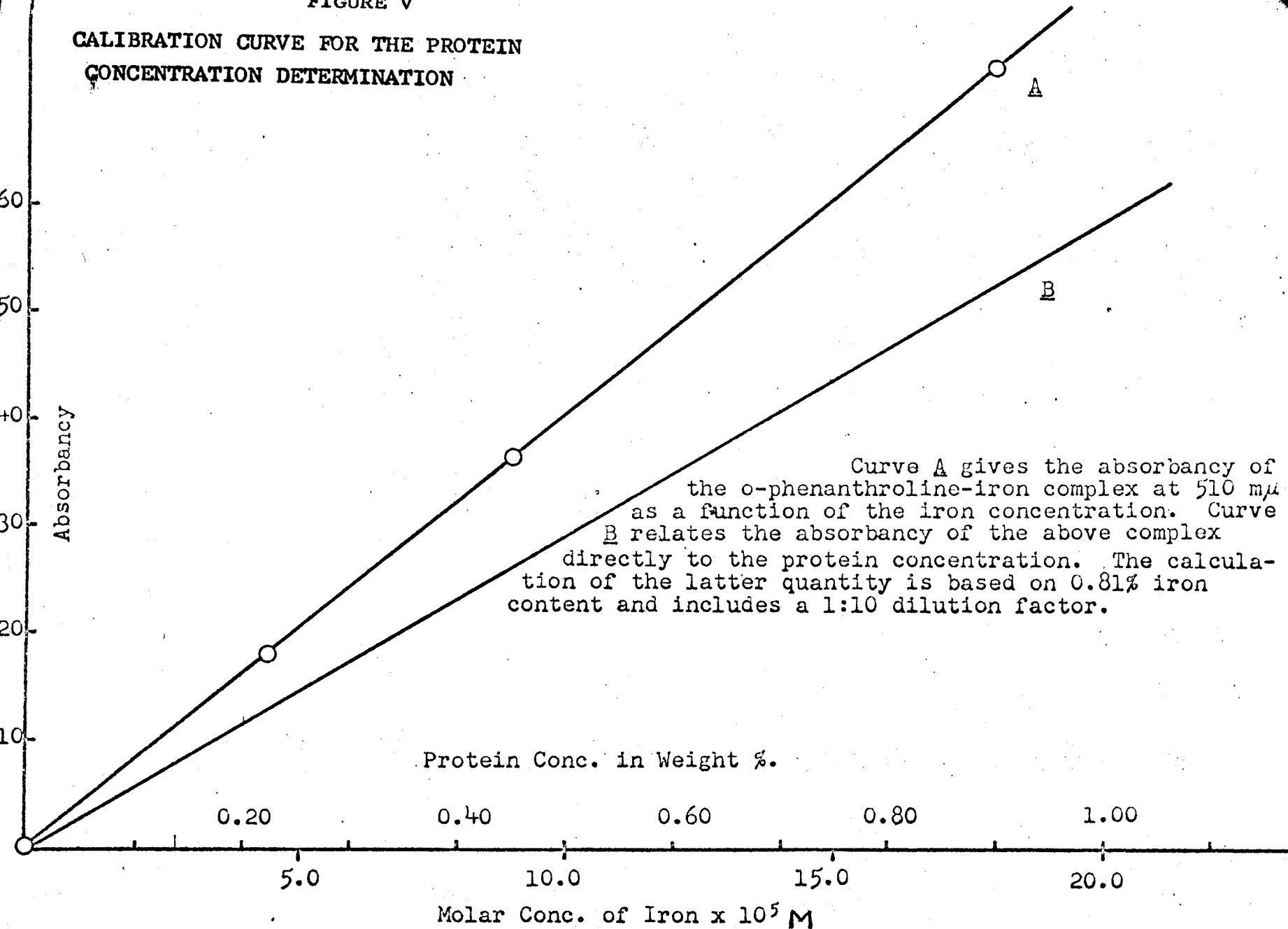
PROTEIN DETERMINATION BY IRON ANALYSIS

The iron content of the hemerythrin solutions was determined by the ortho-phenanthroline method (23,24). A 1:10 dilution of the protein solution was made by adding 2.7 ml distilled water to 0.3 ml of hemerythrin. To this diluted protein 5.0 ml of 0.25% ortho-phenanthroline made in 0.01 N H_2SO_4 and 0.1 ml of 0.88 M $NH_2-OH-HCl$ made in H_2O were added. The iron which is quantitatively released in the acidic environment was spectrophotometrically read at 510 m μ as an iron-ortho-phenanthroline complex. From the absorbance of the iron-complex, the concentration of the protein was obtained with the aid of the calibration graph (20) shown in Figure 5.

Hr CONCENTRATION DETERMINATION FROM THE MOLECULAR EXTINCTION COEFFICIENTS

Nagy (25) had earlier reported a specific absorbance of 2.73 for hemerythrin at 280 m μ . The protein solutions were read in a 1.0 cm cell against the dialysate at 280 m μ and the concentrations were calculated from the specific absorbance. At this wave length the tryptophane, tyrosine and phenylalanine residues in the protein specifically

FIGURE V

CALIBRATION CURVE FOR THE PROTEIN
CONCENTRATION DETERMINATION

absorb and hence, the protein concentrations determined at 280 m μ reflect the total protein absorbance. The near UV and visual peaks which, in general, appear at 330 and 500 m μ depend on the ligand concentration.

PREPARATION OF THE SEPHADEX G-100 COLUMN AND GENERAL EXPERIMENTAL PROCEDURE

A small amount of water is added into the vertically mounted Chromatronix column (0.9 cm; 63.9 cm) and the outlet plunger was closed by the help of a teflon plug. A 9 mm medium porosity disc was deaerated and placed into the column to serve as an additional bed support. A 1-2 cm height of swollen Sephadex G-25 coarse gel was added to protect the bed support from the fine Sephadex G-100 gel particles.

The swollen Sephadex G-100 gel was prepared by swelling 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 out 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 inlet plunger bed support consisted of a 10 micron filter membrane and a 40 micron (pore size) teflon cloth to facilitate uniform application of the sample was then introduced into the column. The excess solvent was removed by gently pushing the plunger in till 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 was filled with buffer. Compressed air at a pressure of 80 P.S.I.G. was fed into the pump. The connections were made by high pressure teflon tubing (0.063" O.D., 0.031" I.D.) and Chromatronix Cheminert fittings which allowed pressure tight joints and insured uniform flow. The column jacket was connected to the constant temperature circulator to maintain a constant temperature during the experiments. The Sephadex G-100 column, the Chromatronix CMP-1 pump and the circulator are shown in Figure 1. The gel particles were washed several hours to allow the gel bed to come to an equilibrium height.

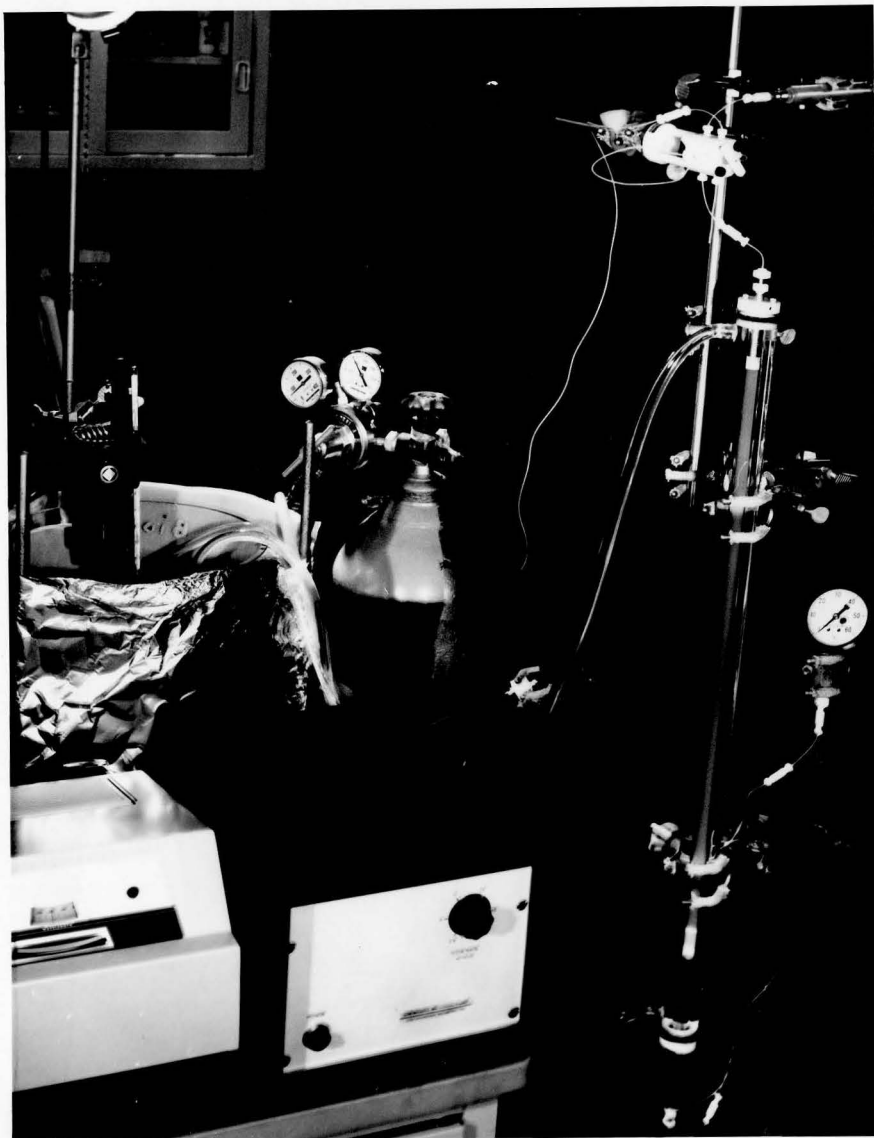
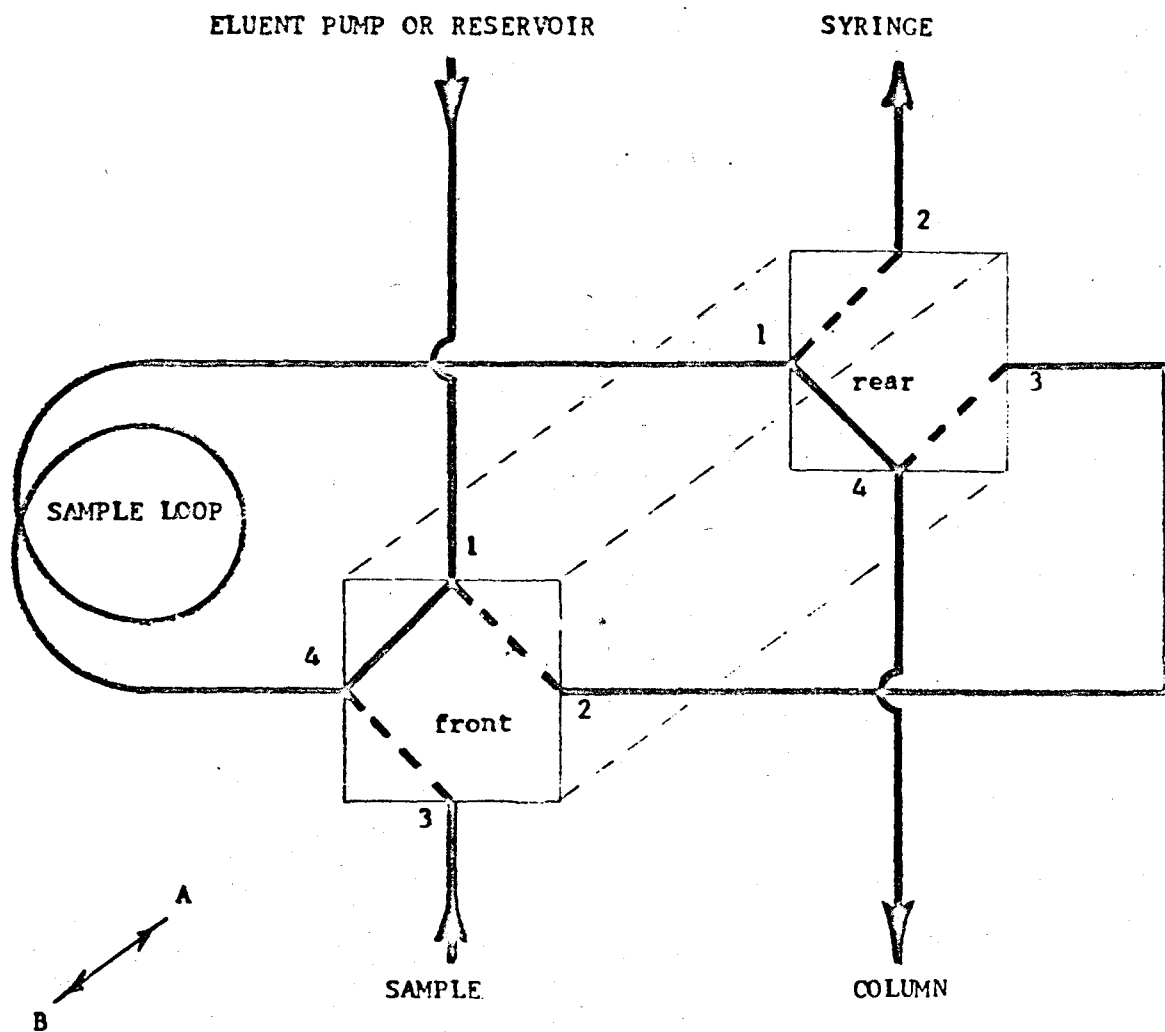


FIGURE 1

The Column and the Sample Application Set-Up

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 2. 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 ml light path) by a 20 cm teflon tubing (0.1 ml volume) and the effluent solution was measured in a 10 ml burette. The flow cell is shown in Figure 3. Depending on the concentration of the sample solution, the wave length was chosen at which the absorbance value is between 0.1 to 1.0 O.D. 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 on a 10 inch linear-log recorder and the elution profiles were examined. The Beckman DB-G spectrophotometer, linear-log 10 inch recorder and the scale expansion attachment are shown in Figure 4. The concentration of the stock protein solution was determined by both iron analysis and from the absorbance at 280 m μ . The protein was diluted very accurately, such that the hemerythrin concentration was in



SOLID LINES: INJECT SAMPLE (position A)
 DOTTED LINES: LOAD SAMPLE LOOP (position B)

FIGURE 2

Portal Configuration of the Sample Injection Valve

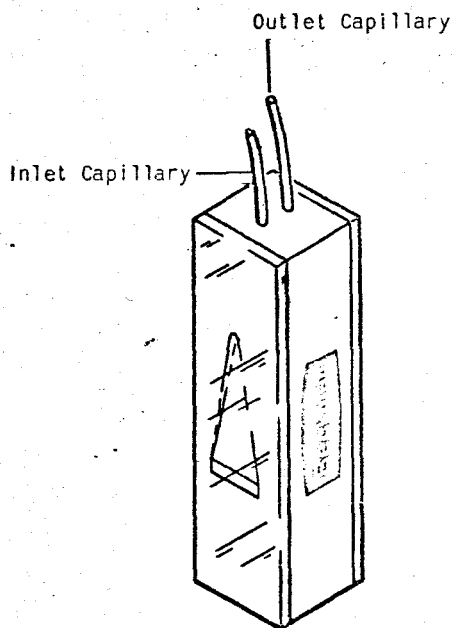
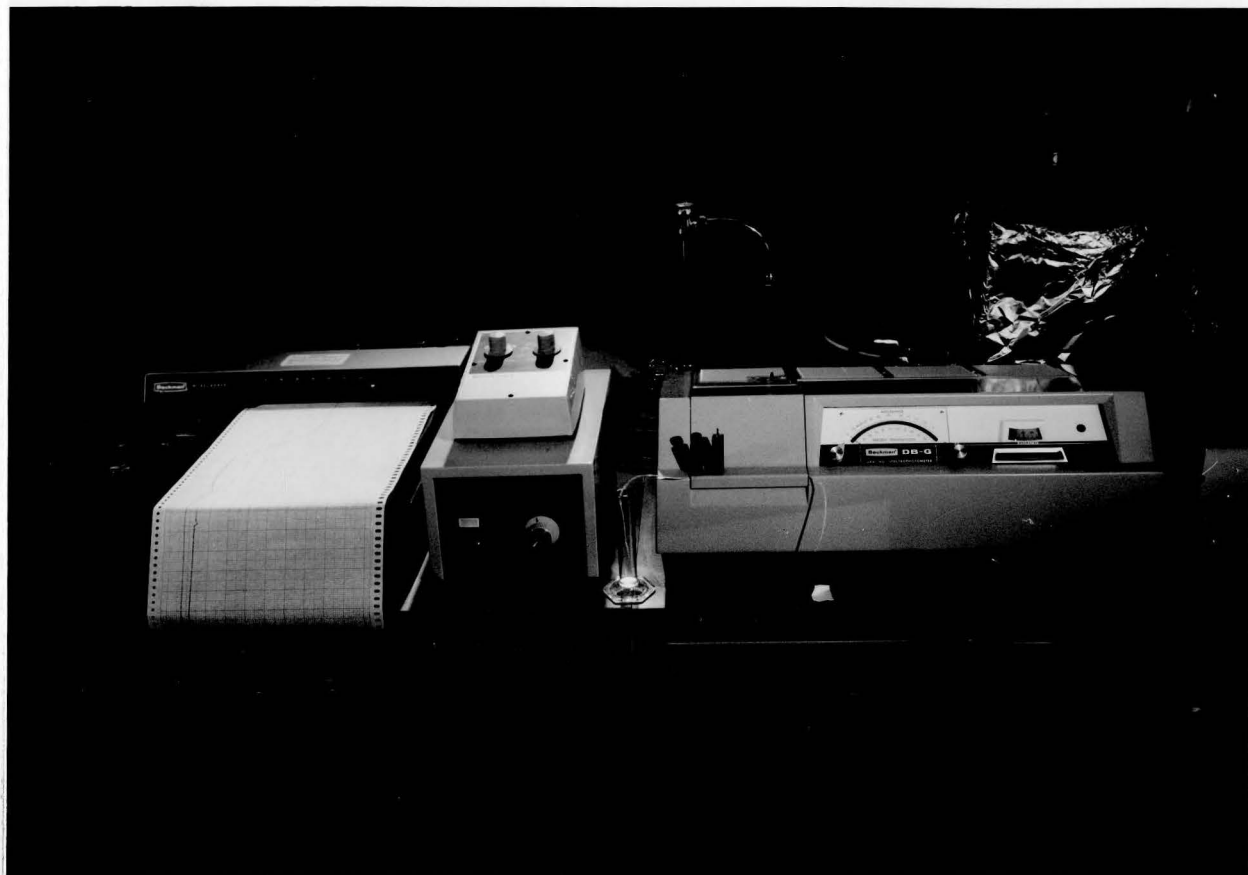


FIGURE 3

Beckman Liquid Microaperture Flow-Cell

the range of 100 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$. By using a syringe pump, the loops were filled with the diluted protein sample (10 μl of 100 $\mu\text{g/ml}$ was sufficient to produce an absorbance plateau of 0.27).



mp, where its absorbance was 0.27. At the same wavelength, a 10 $\mu\text{g/ml}$ solution would yield a 0.027 absorbance. When the solution was monitored at 210 mp, where its absorbance was 0.3. The scale expansion was 10 to 1.

FIGURE 4

The Monitoring Set-Up of the Proteins with a Beckman Double Beam (DB-G) Spectrophotometer

the range of 100 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$. By using a micro-injection pump, teflon loops were filled with this diluted protein sample whose volume (20 ml) was sufficient to produce an absorbance plateau in the elution diagram. To avoid mixing of the sample with the solvent during sample application, an empty loop (5 ml volume) was also connected to the sample loops. To obtain the void volume of the column in each experiment, 0.1 ml (1 mg/ml) of blue dextran was introduced into the inlet of the sample loops. The sample loops were connected to the sample injection valve and the protein sample along with the blue dextran was applied on the column by pushing "in" the push bar of the valve. After the 20 ml sample was applied on the column, the push bar is pulled "out". Frontal analysis was carried out to gain information about the rate of dissociation in addition to amount of dissociation. 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 $\mu\text{g/ml}$ solution was monitored at 280 $\text{m}\mu$, where its absorbance was 0.27. At the same wave length a 10 $\mu\text{g/ml}$ solution would yield a 0.027 absorbance value and hence, such a solution was monitored at 210 $\text{m}\mu$, 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 $\text{m}\mu$. Amplifications of 10 to

25 fold were necessary for solutions in the range of 1-2 $\mu\text{g/ml}$ protein concentration. The elution pattern was recorded at a chart speed of 12 inches/hour. Since, with the sample injection valve, the sample is applied on the column without any change in flow rate, the pattern was then evaluated for elution volumes. Elution volumes were obtained from the inflection points (half heights) of the rising part of the elution diagrams. The weight average molecular weights are extrapolated from the calibration graph of the G-100 column. Experiments with different ligands were carried out after equilibrating the column each time in the particular ligand containing buffer.

RECONCENTRATION OF DILUTE HEMERYTHRIN-CHLORIDE SOLUTION BY DEAE-ANION EXCHANGE CHROMATOGRAPHY

DEAE-Cellulose anion exchange resin was washed several times with deionized water and the fine particles were decanted off. This resin was packed into a 35 ml medium porosity fritted disc funnel and was allowed to soak for 30 minutes in a solution containing 1 N NaOH and 2 N NaCl. With little suction from an aspirator, the resin was then washed with triple distilled water many times and then suspended in 1 N HCl for a few minutes. The acid was then washed off with distilled water and this recycling procedure was repeated till the resin became

white. The resin was then allowed to soak in 0.01 M Tris-cacodylate buffer (pH 7.0) for a few minutes. One liter of 10 mg/ml of Hr-Cl^- complex was made in 0.01 M NaCl solution. Tris and cacodylic acid were added to 25 ml of this solution so that the pH of the buffer and the ionic strength was adjusted to 7.0 and 0.01 M respectively. This solution was used for determining the amount of dissociation in the 10 mg/ml protein. To the rest of the solution, tris and cacodylic acid were added so that the pH of the solution was 8.1 and the ionic strength was 0.01 M. This solution was then passed through the resin under very mild suction and the absorbed protein was eluted out with 10 ml of 0.01 M Tris-cacodylate buffer (pH 7.0) containing 0.5 M NaCl. The reconcentration procedure was carried out in the cold room. The reconcentrated protein was then used to check the reversibility of the dissociation equilibrium of hemerythrin.

CHAPTER III

EXPERIMENTAL RESULTS

Calibration of the Sephadex G-100 Column with Marker Proteins

The elution volume of a protein species is dependent on its molecular weight in gel filtration. To determine the molecular weights from the elution volumes on a column, the column has to be calibrated for this purpose, i.e. the elution volumes of the test substances have to be correlated with molecular weights. Because of the great differences in the structure of proteins, the relation between molecular size and molecular weight is different, for each type of protein. The substances used for calibration purposes should, therefore, be closely related to the substances to be studied.

Since hemerythrin is the protein which is being investigated for dissociation, marker proteins which have their molecular weights between the octameric molecular weight of 107,000 and the monomeric hemerythrin value of 13,500 were used for calibrating the Sephadex G-100 column. In each case, 0.5 ml of the protein solution whose concentration was 2 mg/ml was applied on the column and the elution volume was obtained. The reduced elution volumes (V_e/V_o) of the proteins, the wave lengths at which they were monitored, and the molecular weights of the proteins are tabulated in Table I. Calibration set I was used for experiments carried out at a flow rate of 12 ml/hr and set II

was used for experiments at 24 ml/hr flow rate. The void volume of the column was obtained by applying a 0.5 ml of 1 mg/ml Blue Dextran solution and measuring the elution volume corresponding to the maximum absorption of the solute at 400 m μ . The reduced elution volumes of the proteins are calculated from the elution volumes and the dead volume of the column.

TABLE I

CALIBRATION DATA OF SEPHADEX G-100 COLUMN AT 25° C.

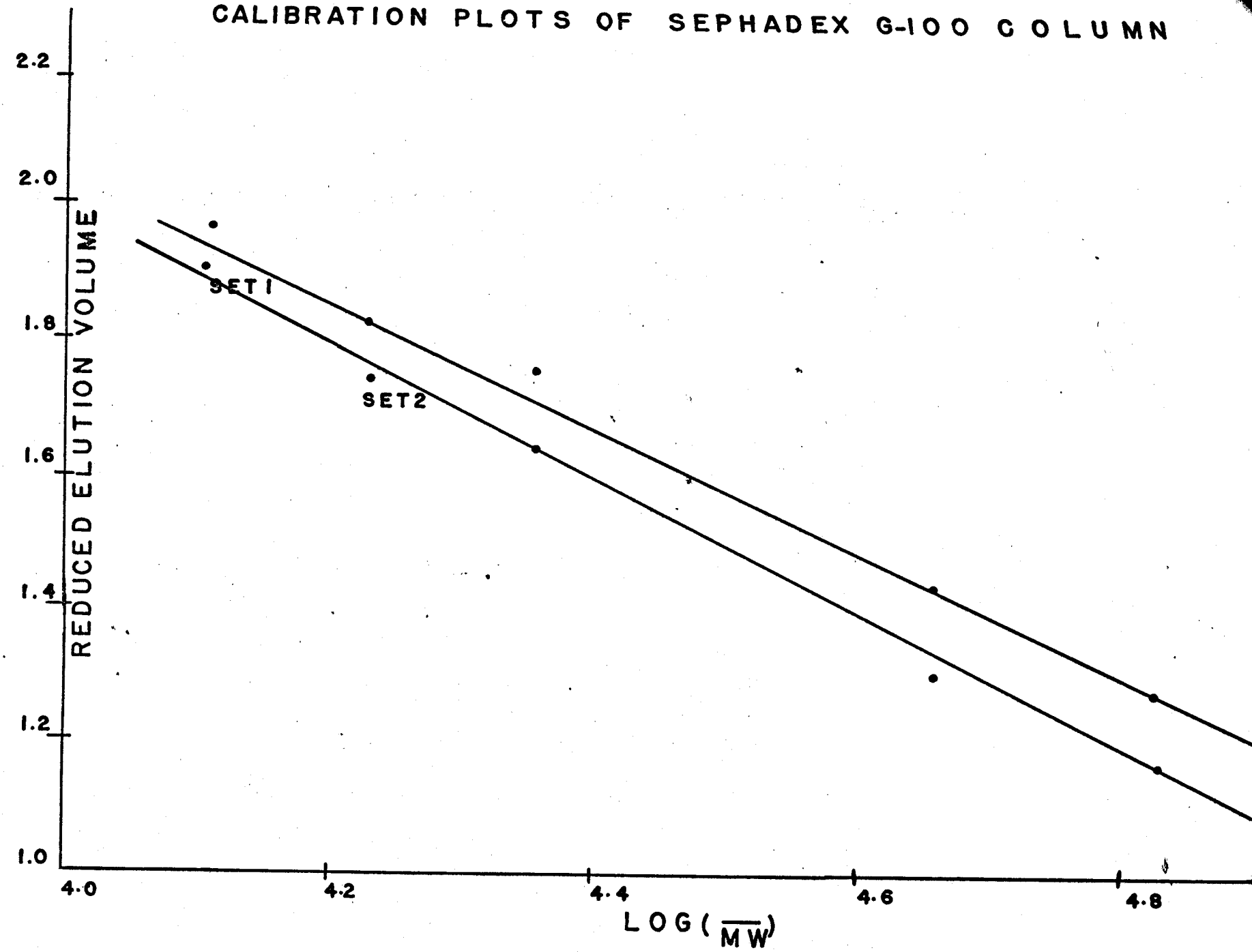
Marker Protein	Molecular Weight (M)	Wavelength m μ	Reduced Elution Volumes*		log M
			Set I	Set II	
Cytochrome-c	13,000	412	1.96	1.90	4.1139
Myoglobin	17,000	412	1.82	1.72	4.2304
α -Chymotrypsin	22,500	230	1.78	1.63	4.3522
Ovalbumin	45,000	230	1.41	1.29	4.6532
Bovine Serum Albumin	70,000	280	1.28	1.16	4.8451

*Set I carried out at a flow rate of 12 ml/hr.

Set II carried out at a flow rate of 24 ml/hr.

A plot of the reduced elution volumes vs. the logarithms of the molecular weights of the marker proteins appears in Figure 6. It was

FIGURE VI
CALIBRATION PLOTS OF SEPHADEX G-100 COLUMN



found that the calibrations were different in the two sets and that a linear relation exists between the reduced elution volumes and the logarithms of the molecular weights.

The reproducibility of the elution volumes obtained was tested by carrying out three chromatographic runs with Bovine Serum Albumin (1 mg/ml). Elution volumes and the extrapolated molecular weights from Set II are shown in Table II.

TABLE II

REPRODUCIBILITY OF ELUTION VOLUME (TESTED WITH BSA^{*})

<u>Dead Volume</u>	<u>Elution Volume</u>	<u>Mean Deviation</u>	<u>Molecular Weight</u>	<u>Mean Deviation</u>
14.2 ml	16.36 ml		66,830	
14.2 ml	16.64 ml	± 0.10 ml	63,100	$\pm 1,500$
14.2 ml	16.48 ml		66,070	

* BSA concentration of 1.0 mg/ml, monitored at 280 m μ .

Therefore, a mean deviation of ± 0.1 ml in the elution volumes should be considered in all the experiments carried out in this investigation.

EXPERIMENTS 1-19: Effect of Dilution in the Hemerythrin Dissociation Equilibrium.

Figure 7 shows the elution patterns obtained at various dilutions of hemerythrin azide complex. The octameric form and the monomeric form are represented by the chromatographic runs carried out at 8,000 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ respectively. For reference purposes, the elution patterns obtained with mercurial treated protein and with blue dextran are also included in the figure. The elution volumes and the concentrations of the hemerythrin solutions appear in Table III.

TABLE III

EFFECT OF DILUTION ON HEMERYTHRIN AZIDE COMPLEX
IN 0.01 M TRIS-CACODYLATE BUFFER AT pH 7.0

Expt. No.	Protein Conc. * gm/l	Elution Volume ml	Void Volume ml
1	8.0	16.0	13.60
2	0.3	18.2	13.60
3	0.03	23.5	13.60
4	0.01	27.1	13.60
5	0.002	29.1	13.60
6	0.3**	29.3	13.60

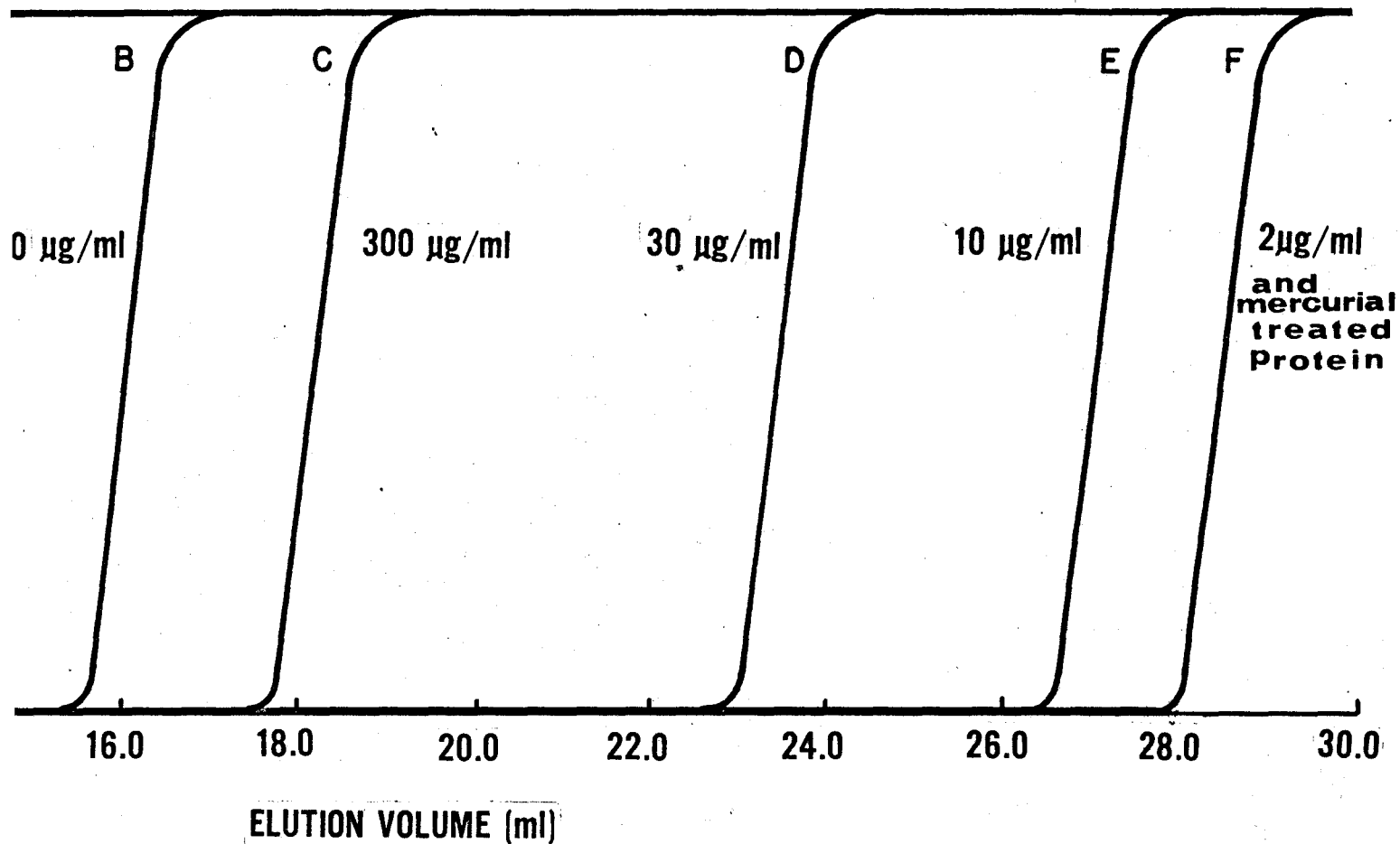
* Hr solutions obtained by dilution of the same stock solution.

** Salyrganic acid treated protein.

FIGURE VII

F ELUTION VOLUMES ON DILUTION WITH Hr-N_3^- COMPLEX

plateaus are generalized for the purpose of this diagram.
 iments, the wave lengths and scale expansions used were different.
 l. scale); B. (500 $\text{m}\mu$, std. scale); C. (295 $\text{m}\mu$, std. scale);
 d. scale); E. (210 $\text{m}\mu$, 2 x scale expansion); F. (210 $\text{m}\mu$, 10 x scale expansion).



It was observed that as the concentration of the protein is decreased, the elution volume progressively increased, indicating that the weight average molecular weight of the species is being shifted toward the monomeric value. At all concentrations, only a single elution boundary was observed. At the lowest concentration of the series (2 $\mu\text{g/ml}$), the dissociation into monomers was almost complete, insofar as the elution volume obtained at this concentration differed less than that found with the salyrganic acid treated protein.

Although the working range for separation of proteins for Sephadex G-100 columns can extend to a molecular weight of about 150,000, Andrews (26) observed that a strict linear relationship was possible only in the range of 5,000 to 60,000. Hence, experiments were only carried out at concentrations at which the column had a linear resolution. Cumulative data of the dissociation experiments with different hemerythrin azide preparations at various intervals of time are tabulated in Table IV. The derivation of the equation used for calculating the equilibrium constant (K'_8) appears in Appendix I. By using this equation, the equilibrium constant at each concentration was evaluated from the weight average molecular weight and the concentration of the protein species. As the weight average molecular weight of the protein species reaches either the octameric or monomeric weights,

TABLE IV

CUMULATIVE DATA OF DILUTION EXPERIMENTS AT 25° C WITH
HEMERYTHRIN-AZIDE COMPLEX (IN TRIS-CACODYLATE BUFFER
AT 0.01 M AND pH 7.0) TO CALCULATE THE EQUILIBRIUM CONSTANT

Expt. No.	Hemerythrin Preparation**	Protein Conc. gm/liter	Dead Volume (V _o)(ml)	Elution Volume (V _e)(ml)	Reduced Elution Volumes V _e /V _o	Weight Average Mol. Weight* (Mw)	Equilibrium Constant (K' _g) in Monomeric Units moles/liter
7	B	0.110	12.95	16.50	1.35	55,000	5.72 x 10 ⁻³⁸
8	C	0.070	13.35	18.80	1.41	47,300	6.99 x 10 ⁻³⁹
9	A	0.060	13.60	19.40	1.425	45,700	3.85 x 10 ⁻⁴⁰
10	B	0.011	13.20	23.95	1.815	17,380	4.47 x 10 ⁻⁴²
11	C	0.010	13.60	24.75	1.82	17,180	2.47 x 10 ⁻⁴²

*Weight average molecular weights are obtained by using calibration set I.

**A.B. and C. are different stock solutions of hemerythrin-azide complex.

the equation used for solving K'_8 loses its significance. Therefore, the gel filtration experiments run at a concentration of 10 $\mu\text{g/ml}$ at which the dissociation was found to be approximately 90% can not be relied on to yield the true value of K'_8 .

Another set of dissociation experiments were carried out in a concentration range where the average molecular weight of the resulting protein mixture ranged from 25,000 to 60,000. The hemerythrin stock solutions were made fresh before each dilution to avoid oxidation of the sulfhydryl groups. The crystals of oxyhemerythrin were dissolved in 2-3 ml of Tris-cacodylate buffer containing 0.1 M KSCN and the solution was centrifuged at 2,000 rpm (0°C) for 15 minutes. The concentration of the solution was determined from the extinction coefficient at 280 $\text{m}\mu$ and also by iron analysis. Suitable dilutions were made with 0.01 M Tris-cacodylate buffer (pH 7.0) containing thiocyanate. The samples were applied and the elution patterns were recorded by the usual procedure. A flow rate of 24 ml/hr was maintained during these gel chromatography experiments. The elution data at the various concentrations and the calculated values of the equilibrium constant are shown in Table V. The elution profiles obtained in these experiments appear in Figure 8. From this set of experiments also, it can be seen that the retardation of the protein species increased by dilution

CHANGE OF ELUTION VOLUMES WITH HEMERYTHRIN-SCN⁻ COMPLEX

* Experiments were recorded with 5 fold scale expansion.
(Flow rate - 24 ml/hr)

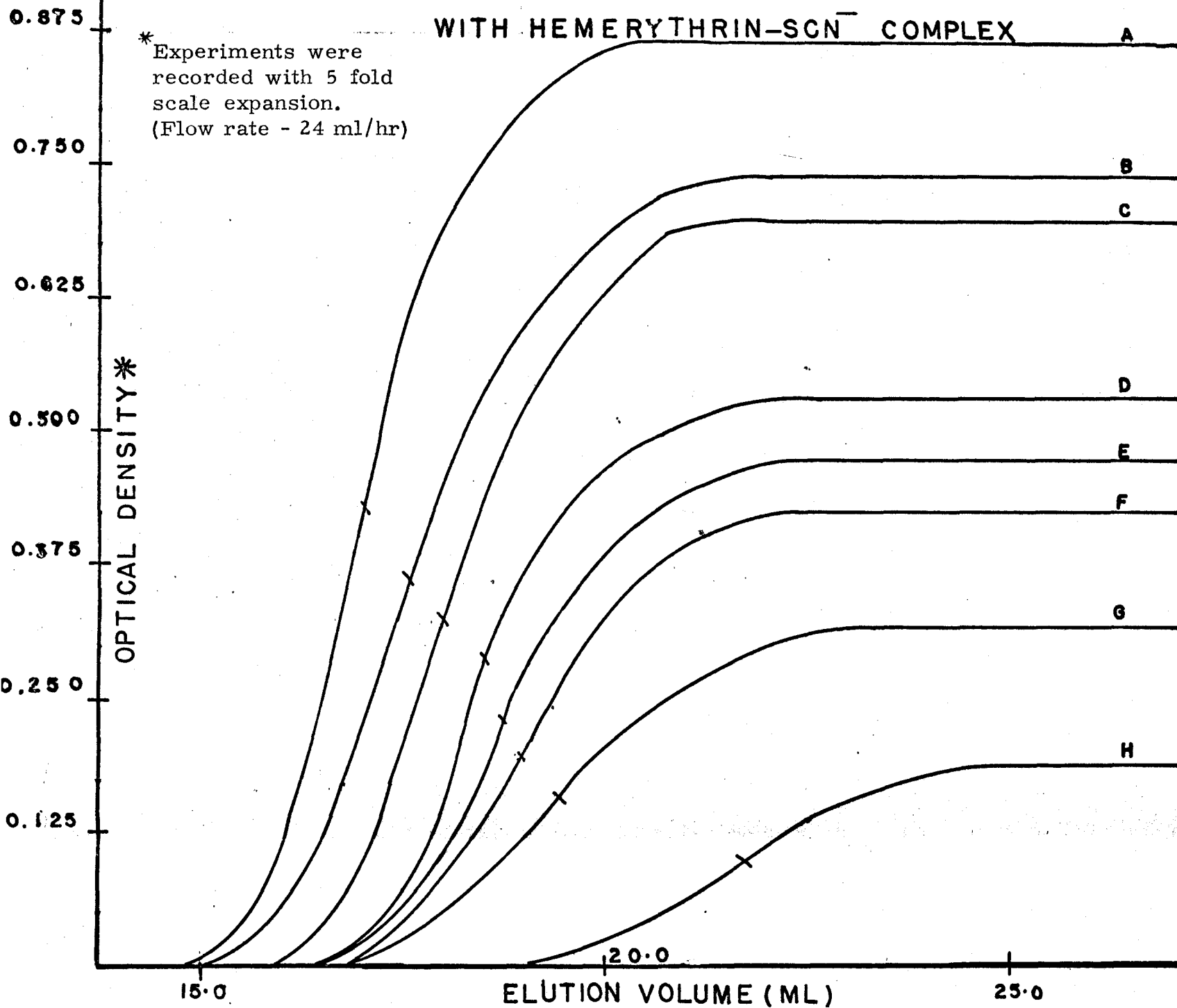


TABLE V

CUMULATIVE DATA OF THE EFFECT OF DILUTION ON Hr-SCN⁻ COMPLEX

Expt. No.	Protein Conc. gm/liter	Dead Volume (V _o)(ml)	Elution Volume (V _e)(ml)	Reduced Elution Volumes V _e /V _o	Weight Average Mol. Weight ^{**} (Mw)	Equilibrium Constant (K' ₈) in Monomeric Units moles/liter
12	0.0775 [*]	14.2	17.15	1.208	59,570	2.007 x 10 ⁻³⁹
13	0.0675	14.2	17.62	1.241	54,950	1.76 x 10 ⁻³⁹
14	0.0620 [*]	14.2	17.97	1.265	51,350	1.81 x 10 ⁻³⁹
15	0.0517 [*]	14.2	18.62	1.314	46,770	1.06 x 10 ⁻³⁹
16	0.0450	14.2	18.79	1.323	45,710	4.75 x 10 ⁻⁴⁰
17	0.0338	14.2	18.90	1.331	44,670	7.52 x 10 ⁻⁴¹
18	0.0270	14.2	19.48	1.377	40,270	3.15 x 10 ⁻⁴¹
19	0.018	14.2	21.64	1.531	28,510	1.18 x 10 ⁻⁴¹

* Dilutions were made from a stock of 3.1 mg/ml Hr-SCN⁻ solution. The rest of the dilutions were made from another stock solution of 2.9 mg/ml concentration.

** Weight average molecular weights are obtained from extrapolation of V_e/V_o from set II calibration curve.

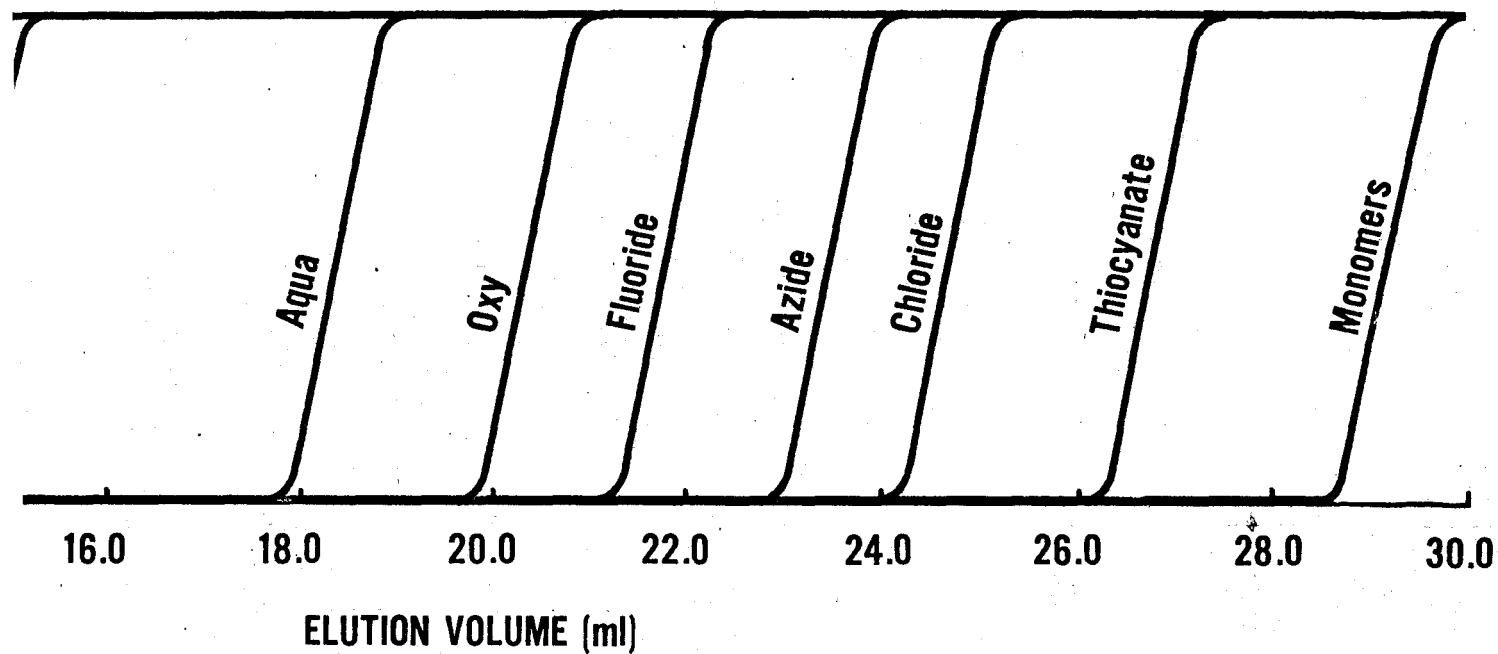
and only a single elution boundary was obtained at each concentration. The variation in K'_8 values produced by artificially introducing errors in the concentration and average molecular weight terms of the equilibrium constant equation was very small. The calculations used are shown in Appendix II. A mean value of 10^{-40} moles/l of monomers was obtained from the values of equilibrium constant of this set which showed a ± 15 fold variation.

EXPERIMENTS 20-25: Effect of Various Ligands on Hemerythrin
Dissociation.

The effect of various ligands on the dissociation equilibrium was studied by carrying out the chromatographic runs at an identical protein concentration. Figure 9 shows the representative elution patterns obtained with various ligands. The elution volumes, the average molecular weights, and the calculated values of K'_8 appear in Table VI. It has been found that the various ligands influence the dissociation equilibrium to differing degrees. The highest dissociations were produced with thiocyanate and chloride forms, whereas the aquo and oxy forms yielded the lowest dissociations. In each case, however, only a single boundary was observed. The effect of the different ligands on the dissociation can be seen by the change in elution volumes obtained in

FIGURE 1X

LIGAND-BINDING ON HEMERYTHRIN DISSOCIATION



these experiments.

TABLE VI

CUMULATIVE DATA ON THE EFFECT OF VARIOUS
LIGANDS OF HEMERYTHRIN DISSOCIATION (IN pH
7.0 AND 0.01 M TRIS-CACODYLATE BUFFER).

Expt. No.	Ligand	Conc. gm/l	V _o ml	V _e ml	Wt. Ave. Mol. Weight* (Mw)	K' ₈ Moles/l of monomer
20	SCN ⁻	0.040	13.6	25.23	15,490	7.361×10^{-38}
21	Cl ⁻	0.0255	13.6	23.55	21,130	4.873×10^{-40}
22	N ₃ ⁻	0.0234	13.6	22.70	27,540	7.00×10^{-41}
23	F ⁻	0.0243	13.6	21.35	33,000	3.84×10^{-41}
24	Oxy	0.0250	13.6	20.15	40,740	1.604×10^{-41}
25	Aquo	0.0250	13.6	18.70	54,320	1.611×10^{-42}

* Calibration Set I was used for obtaining weight average molecular weight.

EXPERIMENTS 25-28: Effect of a Specific Non-Coordinating Anion on Hemerythrin Dissociation.

Recently, it has been observed by Darnall and coworkers (27) that certain specific anions which bind to hemerythrin at a site different than the iron locus affect the reactivity of the sulfhydryl groups.

Perchlorate ion, in particular, has been shown to have a protective effect against dissociation. That is, in the presence of perchlorate ion, the observed amount of dissociation was smaller than in the absence of the anion. The effect of perchlorate was tested in this study on both coordinated and non-coordinated hemerythrin complexes. The perchlorate ion (0.1 M) containing buffer was used to equilibrate the column prior to these dissociation experiments. The protein solutions were also diluted with this same buffer and the usual procedure was carried out. The elution data obtained in these experiments and the calculated values of the equilibrium constant are tabulated in Table VII. These observations indicate that the perchlorate ion by binding to hemerythrin decreases the amount of dissociation. This finding is in agreement with the earlier reported results of Darnall et. al. (27).

EXPERIMENTS 29-31: Reconcentration of Dilute Protein as an Indication of the Reversibility of the Hemerythrin Dissociation Equilibrium.

Although the reversibility of the dissociation equilibrium has been proven by the appearance of a single elution boundary in all the experiments of this investigation, it was further tested by attempting to reconcentrate diluted hemerythrin solutions. For this purpose, three experiments were carried out with the chloride complex. The reconcentration procedure as explained in Chapter II was followed. The concen-

TABLE VII

DATA ON THE EFFECT OF PERCHLORATE ION ON HEMERYTHRIN DISSOCIATION

Expt. No.	Experimental Conditions	V _e ml	V ₀ ml	Wt. Ave. Mol. Weight* (Mw)	Protein Conc. gm/liter	K' ₈ Moles/l of monomer
26	SCN ⁻	19.6	12.9	35,900	0.048	3.48 x 10 ⁻³⁹
27	SCN ⁻ KClO ₄	19.4	13.25	41,200	0.048	2.4 x 10 ⁻³⁹
25	Aquo	18.7	13.6	54,320	0.025	1.6 x 10 ⁻⁴²
28	Aquo- KClO ₄	16.5	12.9	65,800	0.025	2.13 x 10 ⁻⁴³

* Calibration Set I was used for extrapolation of weight average molecular weight.

trated protein was diluted so that a 150 $\mu\text{g/ml}$ solution was obtained which was then eluted and the elution profile was compared with that of the undiluted (150 $\mu\text{g/ml}$) solution. Figure 10 shows the elution patterns of the diluted (10 $\mu\text{g/ml}$), undiluted, and reconcentrated protein solutions. The elution data of these experiments is tabulated in Table VIII.

TABLE VIII

ELUTION DATA OF RECONCENTRATION EXPERIMENTS WITH
HEMERYTHRIN CHLORIDE COMPLEX AT 8° C.

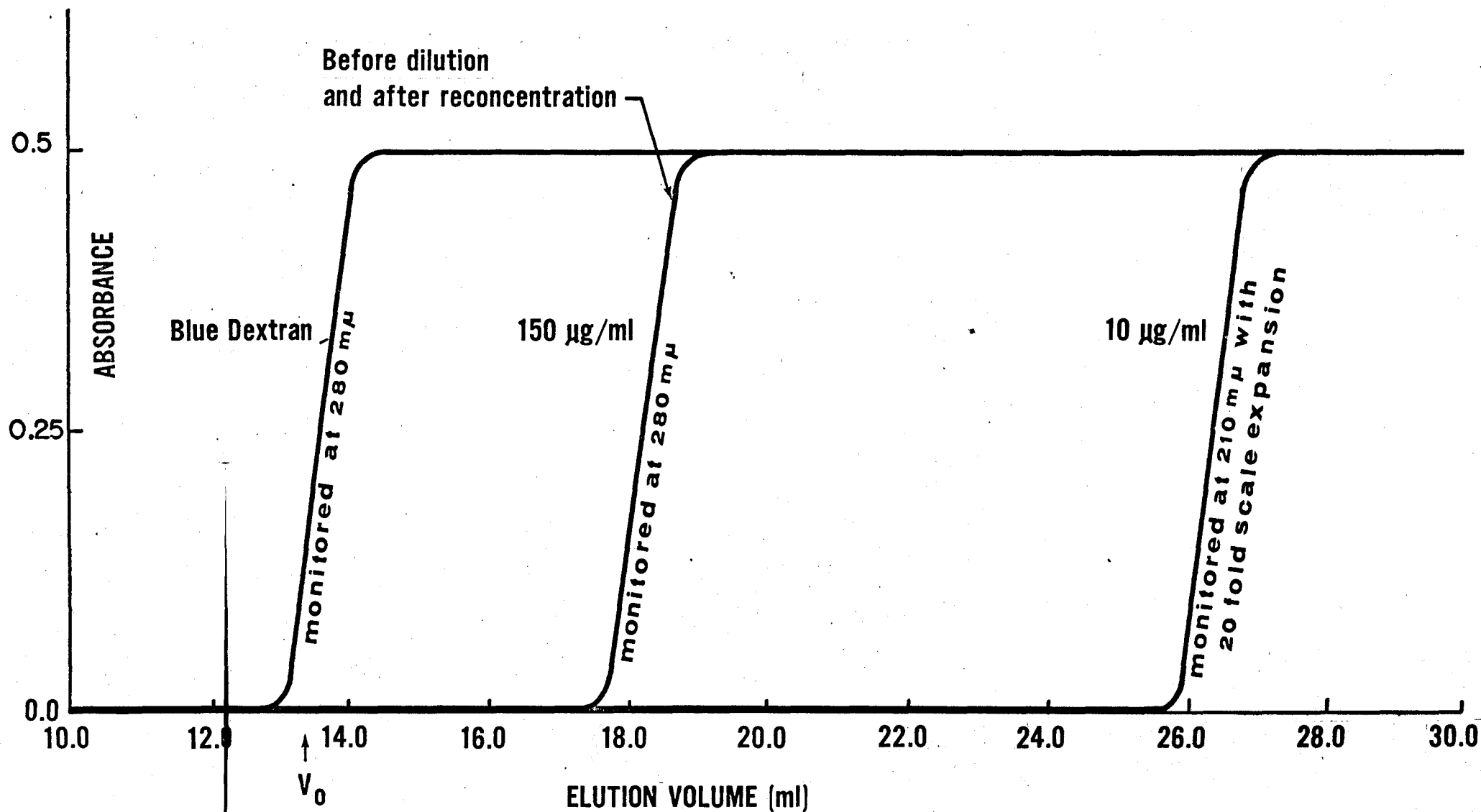
Expt. No.	Protein Soln. gm/liter	V _e ml	V ₀ ml	Wt. Ave. Mol. Weight* (Mw)
29	0.01 diluted	26.40	13.6	18,800
30	0.15 reconcentrated	18.80	13.6	49,000
31	0.15 undiluted	18.8	13.6	49,000

* Calibration Set I was used to obtain weight average molecular weight.

It was found that the weight average molecular weight shifted from 18,800 to 49,000 in the reconcentration of the diluted protein. This observation further confirms the reversibility of the hemerythrin dissociation equilibrium.

FIGURE X

CHANGE OF ELUTION VOLUMES ON CONCENTRATION WITH $Hr-Cl^-$ COMPLEX



EXPERIMENTS 32-35: Study of Hemerythrin Dissociation Equilibrium as a Function of Temperature.

Whitaker (28) earlier observed that the ratio of the elution volume to void volume can be influenced by a change in experimental temperature. However, very little change in the elution volumes of the marker proteins used in the calibration was observed for the temperature change of 5° C to 35° C. Experiments have been carried out at different temperatures from 5° C to 35° C and the dissociation of hemerythrin thiocyanate complex at each temperature was observed. A constant temperature circulator along with a Neslab portable bath cooler was used to maintain the temperature of the dissociation experiments. In all these experiments, a 50 µg/ml hemerythrin thiocyanate complex made in pH 7.0, 0.01 M Tris-cacodylate buffer was used. The elution volumes average molecular weight and the temperatures maintained in the experiments are shown in Table IX.

From the elution data of these experiments, it can be seen that an increase in temperature facilitates dissociation.

TABLE IX

DATA ON THE EFFECT OF TEMPERATURE ON
THE DISSOCIATION OF Hr-SCN^- COMPLEX

Conc. gm/l	V _e ml	V ₀ ml	Temperature °K	Wt. Ave. Mol. Wt. (Mw)	K' ₈ x 10 ⁻³⁹ (moles/l of monomer)
0.05	18.0	12.6	278	46,800	0.841
0.05	18.9	12.6	288	42,170	1.75
0.05	19.15	12.75	298	41,700	1.88
0.05	20.4	12.9	308	35,900	4.58

* Calibration Set I was used for extrapolating average molecular weight.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

It has been shown that dissociation experiments can be carried out in a wide range of concentration by using gel filtration technique. However, to determine the thermodynamic equilibrium constant, the best data can be obtained with close to 50% dissociation. Another limiting factor in these experiments was due to the fact that for Sephadex G-100 a linear relationship between the elution volume and the logarithm of the molecular weight is obeyed only in the range of 5,000 to 60,000. The equilibrium constants calculated from the dilution experimental data showed a 15 fold variation from the mean value of 10^{-40} moles/liter of monomer. The nature of the hemerythrin complex used in these chromatographic runs can not be definitely described, since the oxyhemerythrin might not have been completely converted to the thiocyanate form before the protein was eluted (time less than 45 min.).

Dissociation studies carried out during the present investigation indicated that the dissociability of the protein differs with the various hemerythrin ligand complexes. In general, higher dissociation was observed with the ligands more strongly bound to the protein and this further confirmed the earlier reported effect of coordinating ligands on the dissociability of the protein. This is a direct proof of the second

assumption of the dissociation model. Thiocyanate ion has been found to be most effective in destabilizing the native conformations of many different macromolecules like DNA (29), collagen (30), gelatin (31) and myosin (21, 32). This effect has been explained on the basis of salting "in" properties of this ion (33). Whether the action of thiocyanate on hemerythrin is due to specific binding or to this general salting "in" effect or both cannot be decided at this point. The protective effect of perchlorate ion observed in this investigation is in agreement with the reported findings of Darnall and coworkers (27). Experiments of these workers indicated that a single perchlorate ion is bound at a single site on each subunit. Since the degree of dissociation is lower in the presence of perchlorate, the protective effect might be explained on the basis of a higher affinity for this anion by the octameric protein.

The rate of equilibration of the dissociation of octameric hemerythrin can be estimated from the shape of the elution diagrams and elution times. From the elution profile and the length of the column, the number of theoretical plates can be obtained while the elution time indicates how long the protein remains on each of these plates. In a zonal type experiment with oxyhemerythrin, tangents were drawn to the inflection points of the elution curve to obtain the width of the peak. This value was then used to calculate the total number of theoretical plates of the column by using the equation derived by Morris

and Morris (34) which can be written as

$$P = \left[\frac{4V_e}{W} \right]^2$$

where P = total number of theoretical plates

V_e = elution volume of the protein (14.00 ml)

W = width of the elution peak (1.6 cm)

The total number of theoretical plates calculated was, therefore, 1225.

Since the bed height of the column was 600 mm, the height equivalent

to theoretical plate (HETP) in millimeters was 0.49 mm. This value

of HETP compares well to the reported values of 0.2 to 0.8 mm for

gel filtration by Determann (35). From the total number of plates and

the elution time, the time spent by the protein on each theoretical plate

(t_p) can be calculated. Since the elution of the protein occurred at a

constant flow rate of 12 ml/hr, the total time spent on the column or

elution time is

$$t_c = \frac{14.00}{12} \times 60 \times 60 = 4200 \text{ seconds}$$

and therefore

$$t_p = \frac{t_c}{P} = \frac{4200}{1225} = 3.4 \text{ seconds}$$

In all the experiments of this investigation with a few exceptions (explained later), only a single elution boundary was observed. This can

occur only if the monomeric species attains equilibrium with the octameric species rapidly compared to the time (3.4 sec.) the protein remains on each theoretical plate of the column. Such rapidly equilibrating species are eluted at a volume corresponding to the weight average molecular weight of the two species. On the other hand, if the time required to achieve equilibrium between octameric and monomeric species is large, compared to the time the protein spends on each theoretical plate, two boundaries will be seen in the elution diagram, one corresponding to the monomeric protein and the other corresponding to the octameric species. In other words, on each theoretical plate, the monomers will be retarded progressively from the octamers resulting in two separate boundaries which in frontal analysis will appear as two plateaus, one due to the octamer and one due to the octamer plus monomers. The experimental findings of this research indicate that the rate of equilibration of hemerythrin dissociation is rapid because separation of the equilibrium mixture of octamers and monomers was not achieved. Such a fast equilibrium was, indeed, assumed in the model proposed for explaining the all or none dissociation of octameric hemerythrin by mercurials. However, ultracentrifugation studies by Klapper (19) indicated that dilute solutions of hemerythrin gave rise to two boundaries, apparently separating the octameric and monomeric forms. Since the reversible aggregation of the monomers has not been

demonstrated, the possibility that the monomers were denatured and thus did not participate in the equilibrium cannot be excluded. In a few experiments, especially with the azide and thiocyanate forms, a second plateau corresponding to the monomers was also observed in the gel filtration studies. The amount of monomer appeared to increase with time. Even in these cases, the first boundary shifted with dilution corresponding to average molecular weights much less than 100,000 as would be expected if the equilibrium were slow. Klapper and coworkers (19) by applying Gilbert's theory (36, 37, 38) to their observed sedimentation data in which they found two boundaries, concluded that the rate of equilibration is slow for hemerythrin dissociation.

This discrepancy between the findings of the present investigation and that of Klapper may be explained on the basis of the Le Chatelier Brown principle which predicts how a system in chemical equilibrium reacts to any stress or strain applied on the system. Accordingly, if the dissociation involves a change in partial specific volume, the dissociation equilibrium in the presence of a pressure stress, is affected in such a way so as to retard the increase in volume. Kegeles et. al. (39) earlier discussed the theoretical basis for this effect and Josephs and Harrington (21) recently presented experimental evidence from studies on myosin polymerization. From these supporting observations, it may

be concluded that if the formation of the transition state complex during dissociation of hemerythrin involves an increase in volume, the rate of equilibration might be retarded in the ultracentrifuge under high pressures.

The hemerythrin dissociation experiments carried out as a function of temperature showed that temperature aids dissociation. From this study, the standard state enthalpy change was obtained. Taking a form of the Gibbs-Helmholtz equation and the relationship

$$\Delta F^{\circ} = -RT \ln K'_8$$

one obtains the equation (40)

$$\frac{d (\ln K'_8)}{dT} = \frac{\Delta H^{\circ}}{RT^2}$$

which on integration becomes

$$\ln K'_8 = \frac{-\Delta H^{\circ}}{RT} + c$$

where c is an integration constant. According to this equation, a plot of $\log K'_8$ vs. $1/T$ should be a straight line with slope equal to

$-\Delta H^{\circ}/2.303R$. The data for this plot is shown in Table X. The slope was obtained from the plot which is shown in Figure 11.

TABLE X

EQUILIBRIUM CONSTANT DATA FOR ENTHALPY CHANGE CALCULATIONS

$T^{\circ}K$	$1/T$	$*M_w$	$K'_8 \times 10^{-39}$ <u>moles/l of monomer</u>	$\log K'_8$
278	0.00359	46,800	0.8428	-39.0743
288	0.00347	42,170	1.746	-38.7579
298	0.00336	41,700	1.883	-38.7254
308	0.00325	35,900	4.365	-38.3339

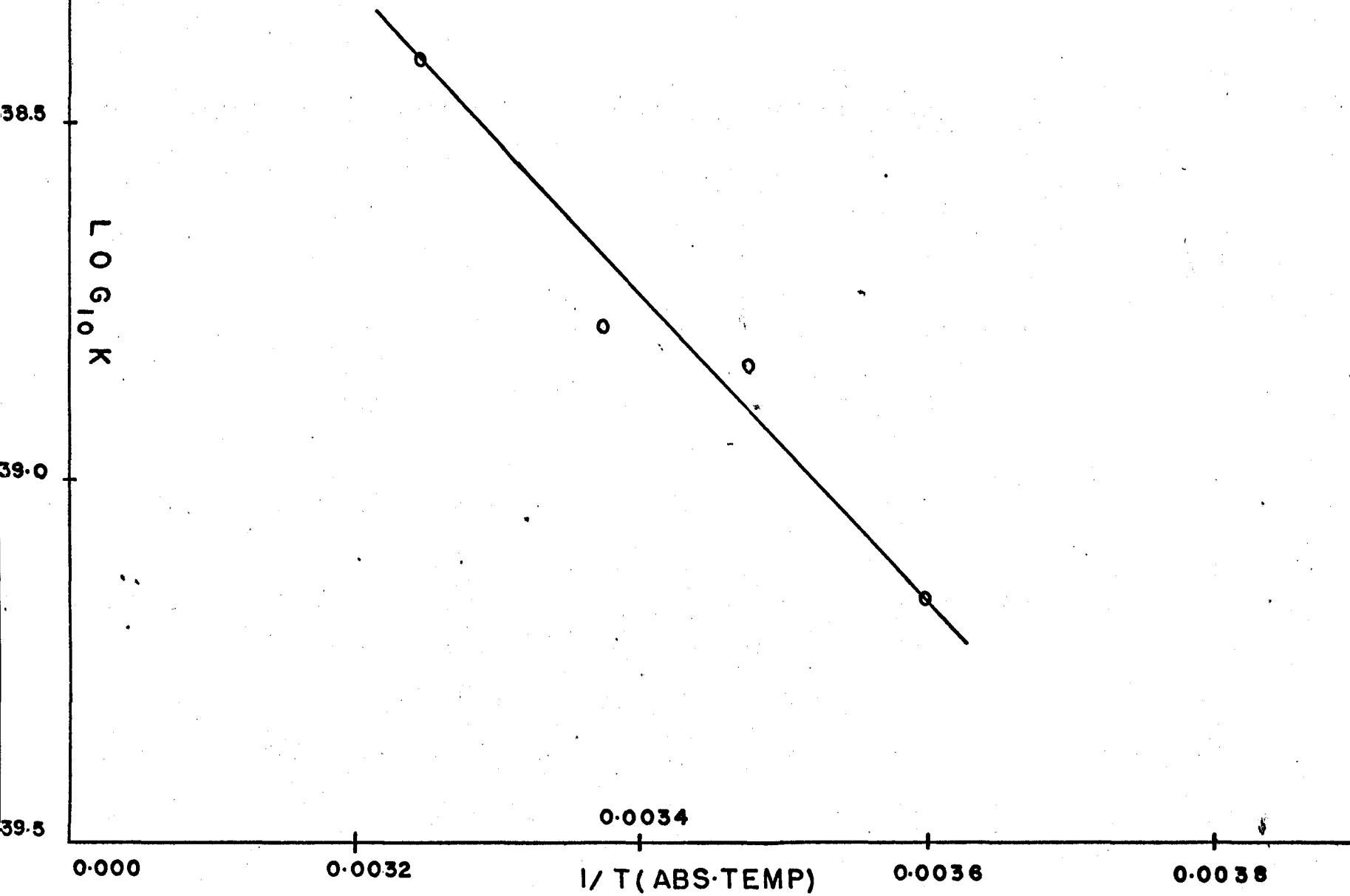
*Weight average molecular weight obtained from Calibration Set I.

A straight line was obtained when $\log K'_8$ was plotted against $1/T$.

$$\begin{aligned}
 \Delta H^{\circ} &= -2.303R \times \text{slope} \\
 &= -4.57 \times \frac{0.44}{-0.0002} \\
 &= +10.05 \text{ kcal./mole}
 \end{aligned}$$

The standard state enthalpy change (ΔH°) of the dissociation equilibrium has a positive sign which indicates that the dissociation is favored by an increase in temperature.

PLOT OF $\text{LOG } (K'_8)$
AGAINST $1/T$



From a general point of view, it can be realized that the value of ΔH° , the overall heat, expresses the difference in the energies of all bonds broken and all bonds newly formed in the dissociation system. Since a positive value of ΔH° was obtained for the dissociation of hemerythrin, it can be concluded that more bonds are being broken than those being formed. The process of association should be opposed by excess thermal motion and hence, an increase in temperature should facilitate the dissociation of hemerythrin which has been found to be the case.

The dissociation reaction at standard state conditions is considered as that of an octamer at a concentration of one mole per liter and at unit activity dissociating into monomers which are also at one mole per liter concentration and unit activity. Thus both ΔH° and ΔF° refer to energy values involving the aggregate of eight subunits. For valid comparison with other proteins, these values should be divided by eight to obtain information pertaining to a single subunit.

The dissociation constant for hemerythrin thiocyanate complex obtained in the linear resolution range of the column was 10^{-40} moles/l in monomeric units at 25° C. The standard state free energy change of the reaction was obtained from the relation

$$\Delta F^{\circ} = -RT \ln K'_8$$

so that

$$\begin{aligned}\Delta F^{\circ} &= -1.987 \times 298 \times 2.303 \times \log (1.0 \times 10) \\ &= 83.7 \text{ kcal./mole}\end{aligned}$$

For each subunit, ΔF° is equal to 83.7/8 or 10.45 kcal/mole.

Similarly, ΔH° for each subunit is equal to 10.05/8 or 1.26 kcal/mole.

From these values, the standard state entropy change is evaluated by the relation

$$\Delta S^{\circ} = \frac{\Delta H^{\circ} - \Delta F^{\circ}}{T}$$

which at 25° C becomes

$$\begin{aligned}\Delta S^{\circ} &= (1,260 - 10,450)/293 \\ &= -31.40 \text{ entropy units per mole}\end{aligned}$$

This negative entropy change value obtained at 25° C indicates a non-spontaneous breakage of bonds.

Since the thermodynamic parameters thus calculated were obtained by dissociations carried out only in the linear portion of the calibration of the Sephadex G-100 column, the validity of the equilibrium constant equation has to be tested at other ranges of dissociation. Sephadex gels or Bio-gels which have higher resolving power can be used for this purpose. The trailing boundaries in some experiments also indicate that

intermediate species may be involved in the dissociation equilibrium. This possibility can be investigated by using columns of different porosity and evaluate the number of components by analyzing the molecular sieve coefficients thus obtained. The usefulness of the gel filtration technique as employed in this investigation can be extended to obtain additional information about the bonds which hold the subunits together in a polymeric protein, by studying the dissociation at varying conditions of pH, ionic strength and with various organic solvents. Olson (41) explained that the dissociation to monomers under acid conditions is due to the electrostatic repulsion brought about by an increased net cationic charge, sufficiently strong enough to break the hydrogen bonds. Under alkaline conditions, he postulated that the protein would have a net anionic charge and hence, electrostatic repulsion will not be of sufficient strength to overcome the hydrogen bonds. Casper (42) proposed that dissociation can occur at higher concentrations and higher temperatures as the pH is increased. In view of these predictions, dissociation studies at various values of pH and at different temperatures may yield useful information about the hydrogen bonds in the octamer. Studies at different concentrations of urea can also explain the role of hydrogen bond in dissociation. If the dissociation of hemerythrin is either increased or decreased by the variation of the ionic strength of the solutions, knowledge about the electrostatic forces in the octamer

can be tested. Ionic strength of the medium also reveals information about hydrophobic bonds. An increase in ionic strength of the medium would strengthen the hydrophobic bonds by decreasing the stability of the nonpolar groups. Hence, increased dissociation might be observed in hemerythrin if the hydrophobic bonds are contributing to the forces that are holding the subunits together. Additional information on hydrophobic bonds can be obtained by a study of the dissociation in different solvents such as dioxane, benzene, and other nonpolar solvents. If a higher dissociation is observed relative to that obtained in aqueous media, then the hydrophobic nature of the intrasubunit bonds may be taken into consideration. The entropy change for forming a hydrophobic bond is positive, since the water molecules which are ordered in the vicinity of the nonpolar groups will assume a more random arrangement. Therefore, a more careful evaluation of the entropy change found in this investigation will have to be made in view of the changes of the bound water that may occur during dissociation.

SUMMARY

In the course of the investigation reported here, the value of Sephadex gel filtration in studying association-dissociation equilibria of proteins has been clearly demonstrated. This technique is simple, versatile, and relatively inexpensive. With the aid of a double beam Beckman DBG Spectrophotometer which affords the selection of any wave length between 210 and 700 m μ , low volume micro flow-cells, and a recorder with scale expansion, it was possible to use protein concentrations as low as 1 μ g/ml. Also, more sensitive detection systems may be employed when needed. Conditions such as temperature, ionic strength, solvent composition can be easily varied and thus, information may be obtained about the strength and the nature of bonds holding the subunits together.

On the basis of these studies, two conclusions have been reached. One of these is that the ligands influence the degree of dissociation as predicted earlier (15) and found from ligand binding studies (20). The equilibrium constant varied from 10^{-42} in the case of aquohemerythrin to 10^{-40} when chloride ions were present. Such a variation is outside of the experimental error observed with gel filtration.

The second conclusion that can be made with certitude is related to the rate of equilibration. The single elution boundary observed at

all concentrations and elution volumes can be explained only in terms of a fast, reversible equilibrium. The reversibility was further demonstrated by reconcentrating the dilute hemerythrin solutions containing a high proportion of monomers. Such a procedure resulted in a shift of the single boundary toward lower elution volumes and higher weight average molecular weights. The existence of such a rapid equilibrium is contradictory to the finding of Klapper (19) that the octameric and monomeric species produced upon dilution can be separated in the ultracentrifuge. Whether this discrepancy is due to formation of denatured monomers under the conditions the ultracentrifuge experiments were carried out or due to the effect of pressures on the equilibrium during high speed centrifugation cannot be decided from gel filtration studies.

Preliminary experiments also indicate that the dissociation equilibrium is dependent on the temperature with a ΔH° of 1.3 kcal per mole.

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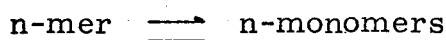
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APPENDIX I

DERIVATION OF THE EQUATION USED FOR CALCULATION OF EQUILIBRIUM CONSTANT.

The derivations for obtaining the dissociation constant of Hr dissociation equilibrium were evaluated from an adoption of the equations derived for a polymer dissociation by Rao and Kegeles (43).

Equation for the over-all dissociation of an n-mer into n-monomers can be written as



The equilibrium constant for such an equilibrium will be

$$K'n = \frac{(C_m)^n}{C_p} \dots\dots 1$$

where C_p = weight concentration of n-mer

C_m = weight concentration of monomer

If only monomers and polymers are assumed to be present in the equilibrium, then the weight average molecular weight can be represented as

$$\begin{aligned} \bar{M}_w &= \frac{\sum_i C_i M_i}{\sum_i C_i} \\ &= \frac{C_m M_m + C_p M_p}{C_m + C_p} \dots\dots 2 \end{aligned}$$

$$C_o = \text{total concentration} = C_m + C_p$$

$$. \quad C_p = C_o - C_m$$

$$. . \quad M_p = n \times M_m$$

Equation can be written as

$$\bar{M}_w = \frac{C_m M_m + M_p C_p}{C_o}$$

$$\bar{M}_w = \frac{C_m M_m + C_p \times n M_m}{C_o}$$

$$\bar{M}_w = \frac{C_m M_m + (C_p \times n \times M_m) + (C_m \times n \times M_m - C_m \times n \times M_m)}{C_o}$$

$$\bar{M}_w = \frac{C_m M_m + (C_p \cdot n \cdot M_m + C_m \cdot n \cdot M_m) - C_m \cdot n \cdot M_m}{C_o}$$

$$\bar{M}_w = \frac{C_m M_m + C_o \times n \times M_m - C_m \times n \times M_m}{C_o}$$

$$= \frac{C_o \times n \times M_m - (n-1) C_m M_m}{C_o}$$

i.e.

$$Co \times \bar{M}_w = Co \times n \cdot M_m - (n-1) C_m M_m$$

$$(n-1) C_m M_m = Co \times n \times M_m - Co \times \bar{M}_w$$

$$= Co (n \times M_m - \bar{M}_w)$$

$$C_m = \frac{Co (n \times M_m - \bar{M}_w)}{(n-1) M_m}$$

Similarly an expression for C_p can be obtained as follows:

$$\bar{M}_w = \frac{C_m M_m + C_p M_p}{Co}$$

$$= \frac{C_p M_p + C_m M_m + (M_m \times C_p - M_m \times C_p)}{Co}$$

$$= \frac{C_p \times n \times M_m + M_m (C_p + C_m) - M_m \times C_p}{Co}$$

$$\therefore (M_p = n \times M_m)$$

$$\bar{M}_w = \frac{C_p \times n \times M_m + Co \times M_m - M_m \times C_p}{Co}$$

$$= \frac{C_p \times M_m (n-1) + Co \times M_m}{Co}$$

$$Co\bar{M}w = CpMm (n-1) + CoMm$$

$$Cp (n-1) Mm = Co\bar{M}w - CoMm$$

$$= Co (\bar{M}w - Mm)$$

$$Cp = \frac{Co (Mw - Mm)}{(n-1) Mm}$$

By substituting these expressions for Cm and Cp in equation 1.

$$\begin{aligned} K'n &= \frac{(Cm)^n}{Cp} = \frac{\left[\frac{Co (n \times Mm - \bar{M}w)}{(n-1) \times Mm} \right]^n}{\frac{Co (\bar{M}w - Mm)}{(n-1) \times Mm}} \\ &= \frac{Co^n \times (n \times Mm - \bar{M}w)^n}{\left[(n-1) Mm \right]^n} \times \frac{\left[(n-1) \times Mm \right]}{Co (\bar{M}w - Mm)} \\ &= \frac{(Co)^{n-1}}{(n-1) Mm^{n-1}} \times \frac{(n \times Mm - \bar{M}w)^n}{(\bar{M}w - Mm)} \\ K'n &= \frac{(Co)^{n-1}}{(n-1) Mm} \times \frac{(nMm - \bar{M}w)^n}{(\bar{M}w - Mm)} \dots 3 \end{aligned}$$

(in gm/l)

In the case of hemerythrin dissociation

$$n = 8 \quad M_m = 13,500 \quad M_p = 107,000$$

and C_0 is the plateau concentration.

In equation (3), if (C_0) were to be expressed in mols/wt of monomeric units, the equation for the dissociation constant will have to include a new term, i.e.

$$K'_8 = \frac{(C_0)^{8-1}}{(M_m)^{8-1}} \times \frac{1}{(8-1)M_m} \times \frac{(8 \times M_m - \bar{M}_w)^8}{(\bar{M}_w - M_m)} \dots 4$$

In this equation, except K'_8 all other quantities are experimentally obtainable.

APPENDIX II

CHANGES IN EQUILIBRIUM CONSTANT BY INTRODUCTION OF ERRORS IN ELUTION VOLUME AND CONCENTRATION TERMS.

Concentration gm/l	V_e ml	K'_8 moles/l of monomer	Remarks
0.034	18.9	7.5×10^{-41}	Neglegible change in K'_8 by introducing error equal to mean deviation in V_e .
0.034	18.8 (0.5% error)	6.6×10^{-41}	
0.067	17.62	1.8×10^{-39}	Neglegible change in K'_8 by introducing 5% error in dilution of protein.
0.065 (5% error)	17.62	1.4×10^{-39}	
0.067	17.62	1.8×10^{-39}	Two fold change in K'_8 by introducing 5% error in dilution plus 1% error in measurement of elu- tion volume.
0.065 (5% error)	17.42 (1% error)	1.0×10^{-39}	
0.067	17.62	1.8×10^{-39}	Fifteen fold change in K'_8 by introducing 15% error in dilution of protein.
0.060 (15% error)	17.62	2.6×10^{-40}	

APPROVAL SHEET

The thesis submitted by Ayyagari L. N. Rao has been read and approved by the thesis director.

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

This thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

January 20, 1969
Date

Heem Kewala Nag
Signature of Advisor