AN ABSTRACT OF THE THESIS OF

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(Name of student) (Degree)

in Biochemistry and Biophysics presented on June 27, 1972
(Major) (Date)

Title: SUBUNIT STRUCTURE OF CANCER MAGISTER HEMOCYANIN

Abstract approved: Redacted for Privacy
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The molecular weight of the polypeptide chain from Cancer magister has been determined by several techniques. SDS-polyacrylamide gel electrophoresis gave one band corresponding to a molecular weight of 78,000 daltons. Gel filtration of reduced and alkylated hemocyanin on a 6 M GuHCl - 6% agarose column gave only one peak. The elution position of the peak corresponded to a molecular weight of 70,000 daltons. Sedimentation equilibrium studies in concentrated GuHCl showed the hemocyanin to be homogeneous. Depending on the ϕ' chosen for calculation a molecular weight of 71,400 - 77,400 daltons was calculated for the polypeptide chain molecular weight. Amino-terminal analysis gave one mole of aspartic acid per 82,000 grams of protein. No other amino-terminal group was found.

Dissociation of 25 S and 16 S particles at pH 9.9 gave 5 S

particles. Only one band was found on electrophoresis of the 5 S particles. Reassociation of the 5 S species in the presence of Mg^{2+} ions gave mainly 16 S particles with some 25 S particles formed and some 5 S species remaining. Electrophoresis of the reassociated hemocyanin showed four components to be present. The major band had the same mobility whether 16 S or 25 S particles were used as the starting material. Thus the 16 S particle (480, 000 daltons) must be formed from six identical or nearly identical 5 S particles of molecular weight 78, 000 daltons.

Subunit Structure of *Cancer magister* Hemocyanin

by

Donald Ellis Carpenter

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

June 1973

APPROVED:

Redacted for Privacy

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Date thesis is presented June 22, 1972

Typed by Ilene Anderton for Donald Ellis Carpenter

ACKNOWLEDGEMENTS

I wish to express my appreciation to Professor K. E. Van Holde for his guidance during the course of this study.

I wish to thank Ms. Susan J. Roxby for her help with the sedimentation studies and Mr. Robert Howard for help with the amino acid analysis.

In particular I would like to acknowledge appreciation to Dr. Robert Roxby for many an interesting discussion concerning hemocyanins and other pertinent matters.

Thanks also goes to the Public Health Service for their support.

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SUBUNIT STRUCTURE OF CANCER MAGISTER HEMOCYANIN

INTRODUCTION

Hemocyanin is a very large respiratory protein found in the hemolymph of molluscs and arthropods. The molecular weight of the native protein may vary between 450,000 to about 9,000,000 daltons depending on the species. A protein of this size must be made up of many subunits. The arthropod and molluscan hemocyanin are similar in many ways, but the weight per mole of copper is quite different. Copper analysis suggests a minimum polypeptide molecular weight of 34,000 - 39,000 daltons for arthropod hemocyanin and 24,000 - 26,000 daltons for molluscan hemocyanins. The amino acid composition of the arthropod hemocyanin has been found to be similar to that of the molluscan hemocyanin (Ghiretti-Magaldi et al., 1966). While the circular dichroism data indicate fundamental differences in the oxygen binding site between molluscan and arthropod hemocyanin, it is difficult to see how a unit of 25,000 daltons is related to one of 39,000 daltons.

Many of the earlier studies on hemocyanin dealt with the oxygen binding and the effect of pH on this binding. Unfortunately, little was known about the relationship of the subunits as affected by pH, oxygen, and divalent cations. Several years ago studies were

initiated in this laboratory to define the important role of these factors on the subunits of C. magister hemocyanin.

This thesis deals with two questions:

1. How large is the polypeptide chain?
2. Can the 25 S structure found in the hemolymph be reformed from the subunits?

LITERATURE REVIEW[1/]

The hemocyanin can exist in several states of aggregation. The arthropod hemocyanins generally exist as structures with a sedimentation coefficient of 5, 16, and 25 S. Several arthropod hemocyanins, however, can also exist in states with sedimentation coefficients of about 24 and 60 S. The molluscan hemocyanins, however, are generally much larger, existing in states with sedimentation coeffi-cents of 11, 19, 60, and 100 S and in some cases even 130 S (Erickson-Quensel and Svedberg, 1943). The higher aggregation states are usually present near neutral pH. At higher pH they are dissociated into smaller units. This dissociation can be affected by such things as divalent cations and oxygen tension.

Dissociation Properties of Hemocyanin

The dissociation properties of a hemocyanin representing an arthropod (C. magister) and those of a mollusc (L. pealei) will be reviewed.

Hemocyanins are characterized by their ability to dissociate to smaller subunits at high pH. The 25 S hemocyanin of C. magister is stable to approximately pH 8.5 where it starts to dissociate into

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[1] For a recent review on the subject see Van Holde and Van Bruggen, 1971.

smaller species (16 S and 5 S). When the pH is increased the hemocyanin dissociates entirely to 5 S species. If one adds 0.01 M Mg^{2+} ions the 25 S species is stabilized and no significant dissociation occurs up to a pH of 10.5. This is not entirely an ionic strength effect since 0.3 M KCl does not stabilize the structure. Dissociation to 5 S occurs around pH 9 (Ellerton, Carpenter and Van Holde, 1970).

The 60 S hemocyanin of Loligo pealei is stable to about pH 8 - 8.5 whereupon it dissociates to 20 S. There is a gradual decrease to 11S as the pH is increased further (Van Holde and Cohen, 1964b). In the presence of 0.01 M Mg^{2+} ions the 60 S subunit dissociates to smaller subunits between pH 7 and 8. As the pH is further increased reassociation to 60 S species occurs. This 60 S species is then stabilized to beyond pH 10. It has been shown that this dissociation in the presence of Mg^{2+} ion and the subsequent reassociation is linked to the amount of oxygen bound to the subunits and is not pH dependent per se (DePhillips et al., 1969).

Reassociation of Subunits

With Eriphia spinifrons once the 5 S subunits are produced they are not able to reassociate (Di Giamberardino, 1967). It was hypothesized that this could be due to a removal of a component required for association or to heterogeneity of the subunits. This is similar to the problem in C. magister, hemocyanin, where in the absence of

divalent cations at a pH above 9, the 5 S is formed (Ellerton et al., 1970). If the pH is then lowered to 7, 16 S species are formed. If 0.01 M Mg^{2+} ions are then added a very small amount of 25 S is formed. If more Mg^{2+} (up to 1 M) is added there is no increase in the amount of 25 S nor is there an increase with time or with temperature changes. The hemocyanin from H. americanus can be readily interconverted between 16 S and 25 S particles (Morimoto and Kegeles, 1970). By addition of calcium ions the 25 S species was favored.

Reassociation from smaller subunits to larger ones can be mediated by divalent cations and in some cases by lowering of the pH. The reassociation of the subunits of L. pealei has been found to have a temperature dependency with association occurring at high temperatures. This is another case like TMV where entropy increases on association due to the water released. If this occurs one expects that the volume of the system would decrease upon dissociation. This was found to be the case as the volume decreases from 0.74 ml/mg for the native protein to 0.71 ml/mg for the subunits (Van Holde and Cohen, 1964b).

Subunit Size of Hemocyanin

Based on copper analysis, assuming one copper per chain, a theoretical polypeptide weight of 25,000 and 36,000 daltons is

calculated for molluscan and arthropod hemocyanins, respectively. In the quest to get the subunit molecular weight many techniques have been employed. Table 1 summarizes these molecular weight determinations.

Molluscan Hemocyanin. L. pealei hemocyanin at pH 10.6 gave a molecular weight of 385,000 daltons (Van Holde and Cohen, 1964b). H. pomatia at high pH gave a molecular weight of 45,000 daltons (Konings, Siezen, and Gruber, 1969). With succinylated H. pomatia hemocyanin a value of 470,000 - 500,000 daltons was obtained by sedimentation/viscosity and sedimentation/diffusion (Konings et al., 1969). In 6 M GuHCl, 460,000 daltons was obtained. Sedimentation/diffusion studies with 1% SDS treated hemocyanin gave a molecular weight of 230,000 daltons.

Formic acid treated hemocyanin from H. pomatia found species with weights of 25,000, 50,000 and 100,000 daltons on 1% SDS-8 M urea gels. Most of the protein on these gels were of high molecular weight. Amino-terminal analysis of H. pomatia hemocyanin gave 1 mole of Arginine per 25,000 grams of protein (Dijk et al., 1970). Results from O. vulgarus hemocyanin (acetylated and cyanoethylated) gave molecular weights of 25,000 and 100,000 daltons. This was done using a calibrated 0.1% SDS sephadex column (Ghiretti-Magaldi et al., 1972).

Recent results from this laboratory show the B. canaliculatum hemocyanin to dissociate to 300,000 - 320,000 daltons in 6 M GuHCl (Roxby, 1972).

Table 1. Summary of molecular weight determination of polypeptide chains from various hemocyanin sources.

Treatment	Molecular weight	Method	Reference
		Molluscan	
H. pomatia (α)			
succinylation	470, 000	S/D	Konings et al., 1969
	500, 000	S/η	Dijk, 1971
6 M GuHCl	460, 000	S°	Dijk, 1971
1% SDS	230, 000	S/D	Dijk, 1971
1 M Formic	63, 000	S/D	Dijk, 1971
	49, 000	S/	Dijk, 1971
1 M Formic (reduced and alkylated)	68, 000	S/D	Dijk, 1970
1% SDS, 8 M urea	25, 000, 50, 000, 100, 000	acrylamida gel	Dijk, 1970
FDNB	25, 000	N-terminal analysis	Dijk, 1970
High pH	450, 000	S/D	Konings et al., 1969
L. pealei			
High pH	390, 000	S. E.	Van Holde and Cohen, 1964
O. vulgarus			
0. 1% SDS (acetylated and cyanoethylated)	100, 000,	Column	Ghiretti-Magaldi et al., 1972

Table 1. Continued

Treatment	Molecular Weight	Method	Reference
		Arthropoda	
E. spinifrons			
High pH	80, 000	Light scattering	Di Giamberardino 1967
J. lalandii			
High pH	88, 000	Archibald	Nichol *et al.*,1968
H. americanus	34, 000	based on peptide mapping	Pickett *et al.*, 1966
C. magister			
High pH	78, 000	S. E.	Ellerton *et al.*, 1970
C. moenas			
1% SDS, acetylated	25, 000, 50, 000, 75, 000	SDS-column	Ghiretti-Magaldi *et al.*, 1972
L. polyphemus			
High pH	65, 000	S. E.	Van Holde *et al.*,1971

Arthropod Hemocyanin. The molecular weight for the polypeptide chain in arthropod hemocyanins is as poorly defined as that of the molluscan chain. It has been reported that the hemocyanin from the lobster, H. americanus, can be dissociated into subunits of 35,000 daltons. The evidence for this is: the S value dropped from 4 for the native hemocyanin at high pH to 2.3 for the succinylated hemocyanin (Pickett et al., 1966). This was said to correspond to a molecular weight of 35,000 daltons. But this decrease in S could also be attributed to an increase in the frictional coefficient caused by the charged groups introduced onto the hemocyanin. The best evidence for 35,000 dalton chains is based on tryptic digests. On mapping 31 peptide spots were obtained, 14 containing arginyl and 17 lysyl (from amino acid analysis 15 lysine and 15 arginine were found per 34,000 grams of protein). But one could have a single chain with a duplicated region such as in γ-globulin (Hill et al., 1966; and Singer and Doolittle, 1966). Molecular weights ranging from 68,000 - 90,000 daltons have been obtained for the 5 S hemocyanin from other species (Moore et al., 1968; Di Giamberardino, 1967; Pickett et al., 1966).

From a recent work on the hemocyanin from Carcinus moenas two end groups were found for acid pH-SDS treated hemocyanin: aspartic acid and glycine (Ghiretti-Magaldi et al., 1970). This does give evidence for two different polypeptide chains but since the results were not quantitated the question of subunit molecular

weight still remains.

By gel filtration on a calibrated G-200 column in the presence of 0.1% SDS, acetylated hemocyanin from C. moenas gave subunits of 75,000, 50,000 and 25,000 daltons (Salvato et al., 1972). Relative amounts of the subunits produced were not disclosed. Thus it is not clear whether the observed 50,000 and 25,000 dalton subunits represent an appreciable fraction of the total protein or a minor contaminant.

The molecular weight of the polypeptide chain is not well defined. Most data point to the arthropod 5 S subunit being heterogeneous but between 68,000 and 90,000 daltons. But there are reports of subunits as small as 25,000 daltons. Results from molluscan hemocyanin vary from 25,000 daltons to 500,000 daltons. It is not clear whether the 25,000 dalton subunits are artifacts from covalent bond breakage or are the actual subunits.

MATERIALS AND METHODS

Preparation of Hemocyanin

The hemocyanin was prepared as described earlier (Ellerton, et al., 1970) but with the following exception. When a pure 25 S or 16 S hemocyanin sample was desired, the mixture of 25 S and 16 S hemocyanin was separated on a Bio-Gel A-5 M column equilibrated with 0.1 I Tris, pH 7.0 and 0.01 M $MgCl_2$. Figure 1 shows a typical elution pattern from the Bio-Gel A-5 M column and Figure 2 shows the schlieren pattern from the purified 25 S particles. A more sensitive criterion for the homogeneity of the hemocyanin is shown in Figure 3. The ln c against dr^2 plot from a sedimentation equilibrium run gave a fairly straight line with the slope corresponding to a molecular weight of 939,000 daltons. For all the work presented in this paper the starting material was purified in this manner.

Polyacrylamide Gel Electrophoresis on Native Hemocyanin

The method described by Ornstein (1964) and Davis (1964) was used. The discontinuous method was used with the high pH buffers. At pH 7.5 the gel buffer was 0.1 I Tris. The gels (7.5%) had a ratio of acrylamide to N, N'-bismethyleneacrylamide of 30:1.

All reagents were of analytical quality. The acrylamide was

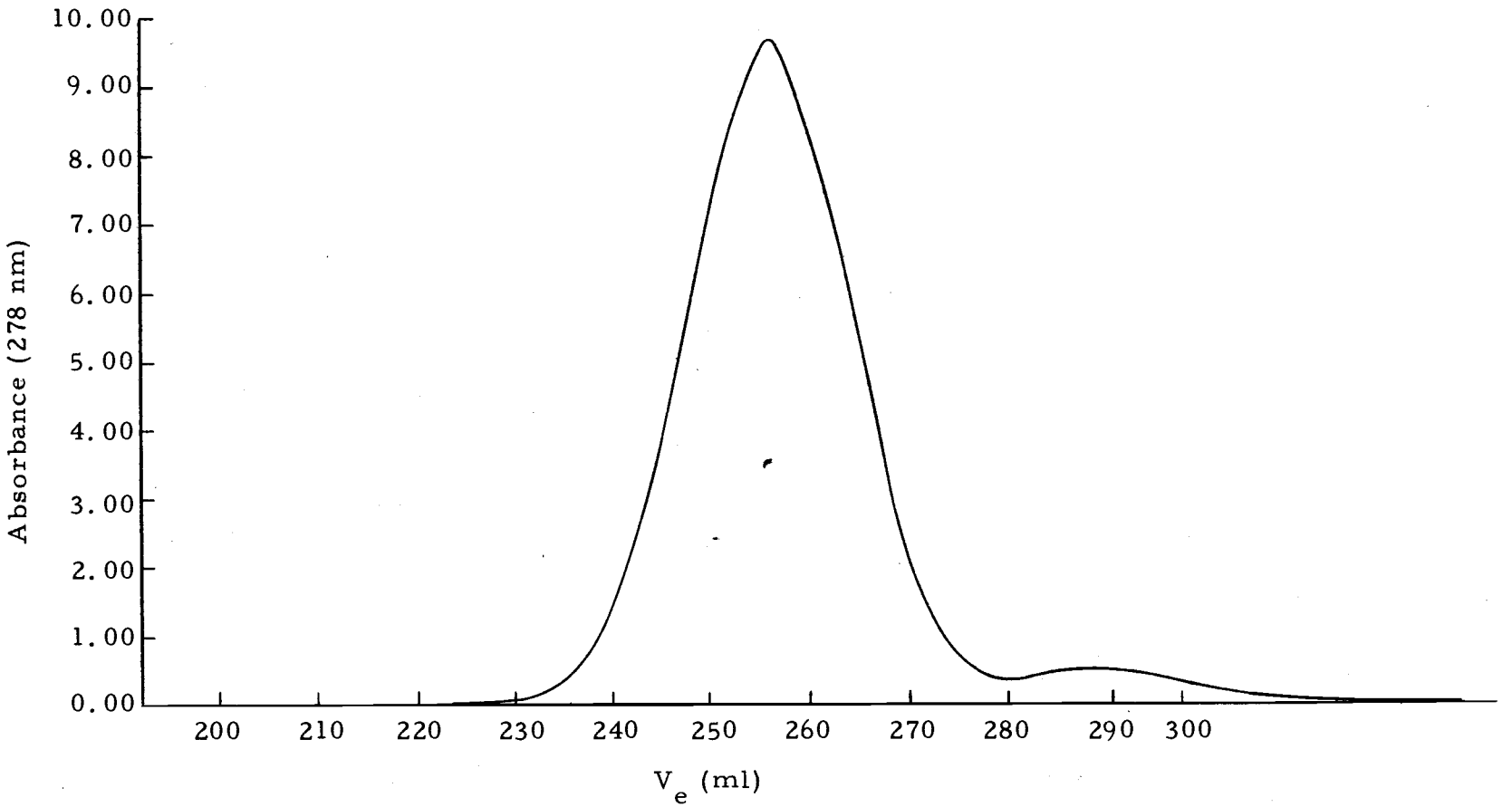

Figure 1. Elution pattern of hemocyanin from Biol-Gel A-5M equilibrated with pH 7.07 tris 0.1I, 0.01M $MgCl_2$. Hemocyanin at a concentration of 38 mg/ml, 3ml total volume was put on the column.

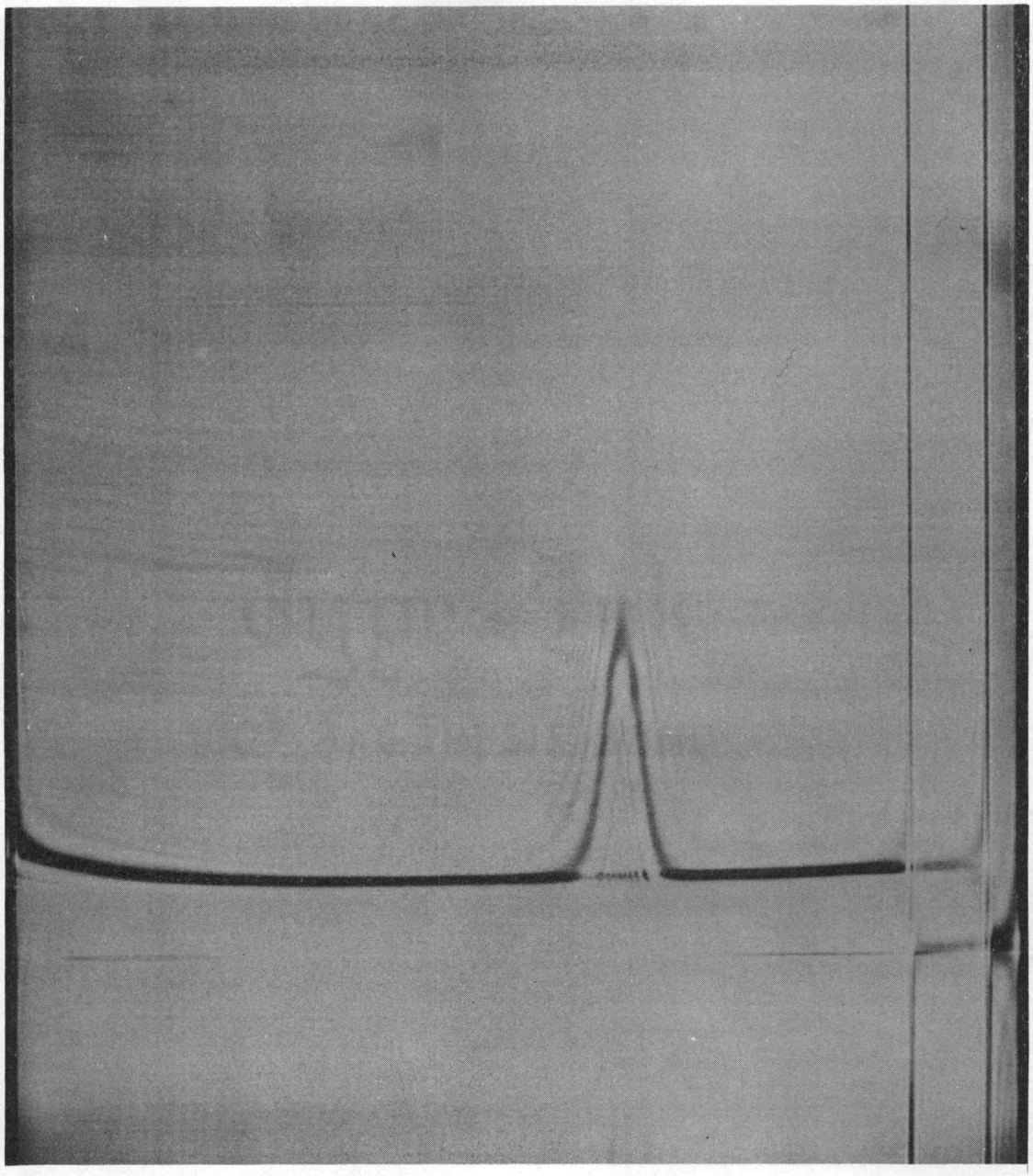

Figure 2. Sedimentation diagram of C. magister hemocyanin after purification through Bio-Gel A-5 M column (Figure 1A). Sedimented for 24 minutes at a rotor speed of 40,345 rpm.

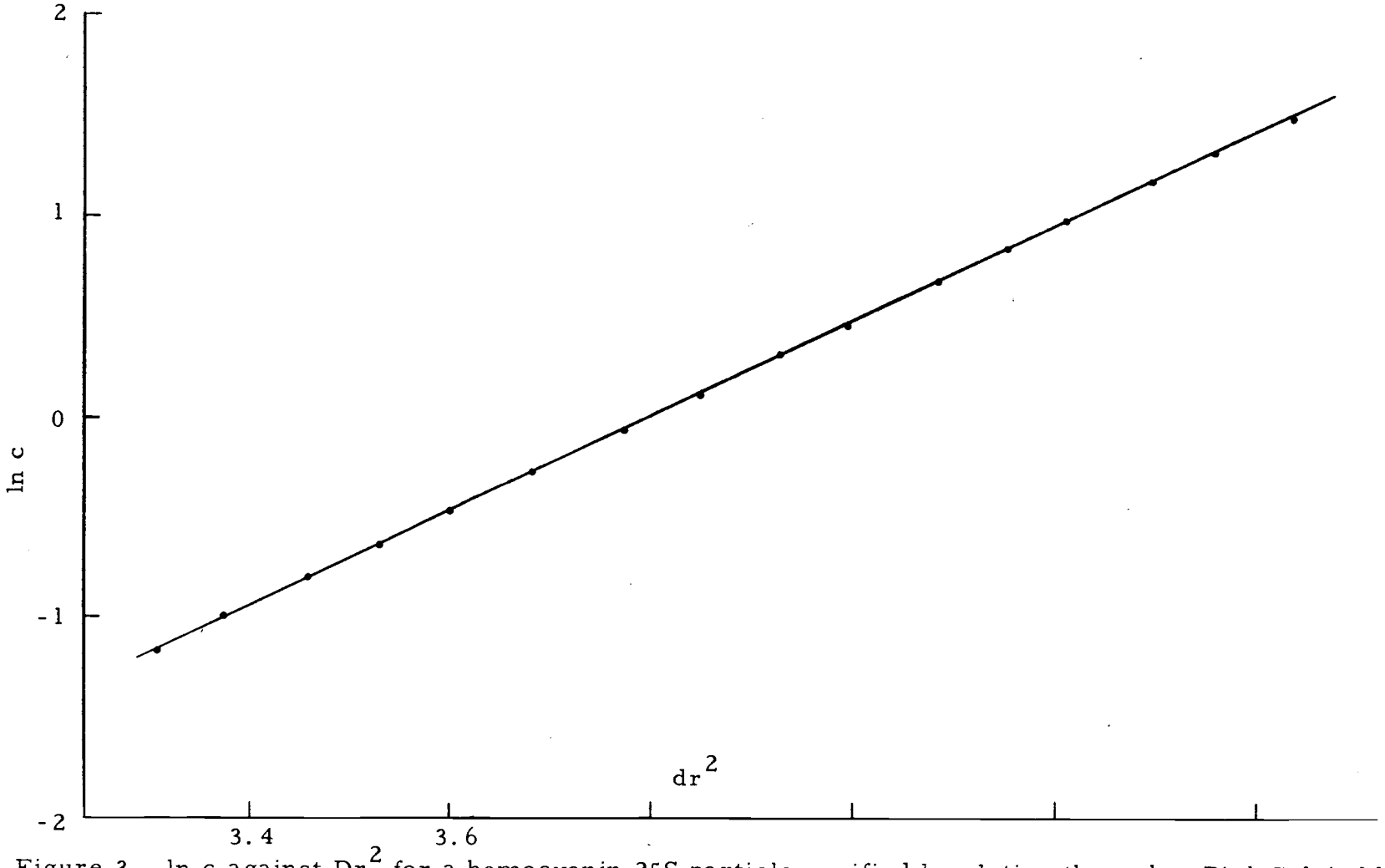

Figure 3. ln c against Dr^2 for a hemocyanin 25S particle purified by elution through a Biol-Gel A-5M column (see Figure 1A). Centrifuged for 50 hours at 8054 rpm. A least squares fit of the ln c versus dr^2 plot gave a molecular weight of 939,000 daltons.

recrystallized from chloroform and N,N'-methylenebisacrylamide was recrystallized from acetone.

Amino Acid Analysis

Pure 25 S hemocyanin was dialyzed exhaustively against distilled water. Aliquots taken for analysis contained about 0.3 mg of protein. The samples were taken to dryness and hydrolyzed with constant boiling (5.7 N) HCl in sealed and evacuated ampoules in a refluxing open air toluene bath (110°C). Times of hydrolysis were 20, 40, 67 and 140 hours.

Cysteine and cystine were determined as cysteic acid by treating 0.3 mg of protein with 30λ dimethyl sulfoxide and 3 ml of constant boiling HCl (Spencer and Wold, 1969). Hydrolysis time was 21 hours.

Amino acid analysis was done on a modified Spinco 120 B (Beckman) amino acid analyzer. Standards run before and after the protein analysis gave the same yield within the error of the measurement.

Tryptophan, which was destroyed under the hydrolytic conditions used, was determined by spectrophotometric means in 6 M GuHCl solution (Edelhoch, 1967). In this method the absorption spectrum of a known concentration of hemocyanin in 6 M GuHCl and 0.03 M in phosphate buffer, pH 6.5 was obtained.

Qualitative Amino-Terminal Analysis

The method described by Levy (1954) was used for the amino-terminal analysis of hemocyanin. Purified 25 S hemocyanin (15-40 mg) was used for each analysis. Hydrolysis was carried out using constant boiling HCl in a sealed and evacuated tube at 110°C. Times of hydrolysis were varied between 4 and 24 hours. In addition a four hour hydrolysis in concentrated HCl was performed to check for the presence of DNP-glycine and DNP-proline. Both of these derivatives are destroyed under more rigorous conditions. A third hemocyanin sample was oxidized with performic acid (Sanger and Thompson, 1953, and Schram et al., 1965) and the aqueous phase carefully examined for DNP-cysteic acid. All extractions and chromatography were carried out as described by Fraenkel-Conrat, Harris, and Levy (1955).

The ether extracts were chromatographed in two-dimensions (Whatman #1 paper). The first dimension used a "toluene" solvent system. DNP-amino acid standards (Sigma Chemical Company) were spotted on both sides of the unknown spot and chromatography in the second dimension utilized 1.5 M phosphate, pH 6. Under these conditions, DNP-glutamate and DNP-aspartate cannot be distinguished. Thus a one-dimensional chromatogram (Whatman #3 MM paper) eluted with tert-amyl alcohol-phthalate, pH 5 was used. This gave a good separation of DNP-glutamate and DNP-aspartate.

A two-dimensional system was also used for the aqueous phase (Whatman #52 paper). n-butanol-acetic acid-water (4:1:5) was used for the first dimension and m-cresol-phenol, borate, pH 9.3 for the second dimension. DNP-amino acid standards were applied before running in the second dimension. All chromatograms were examined under ultraviolet light to detect any minor spots which might be present.

Quantitative Amino-Terminal Analysis

The method used was basically that described by Beale and Whitehead (1962) and Dijk et al. (1970). The rationale was to derivitize the N-terminal amino acid with ^{3}H-DNP and to use the ^{14}C-DNP-amino acid as a recovery control. The ^{14}C-DNP-amino acids were made as described by Levy (1954) and were found to be chromatographically pure. The control was to correct for chromatography losses, extraction losses and possibly some loss from acid hydrolysis. The specific activity of the ^{3}H-FDNB was determined by reaction with a standard solution of aspartic acid using ^{14}C-DNP-aspartic acid as a control. This was checked with a standard solution of leucine. The same specific activity was obtained. About 200 μg of hemocyanin was reacted with a 100 x molar excess of ^{3}H-FDNB (based on 78,000 μg/μmole for hemocyanin). The reaction was allowed to proceed for three hours at room temperature, after which the excess

^{3}H-FDNB was removed by extraction with ether. At this stage a known amount of ^{14}C-DNP aspartic acid was added to the reaction mixture. The solution was evacuated to remove ethanol. Concentrated HCl was added to make the solution 6 N in HCl. The sample, sealed in an evacuated ampoule was hydrolyzed at 110° C. The hydrolysis times varied between 4 and 24 hours. Extraction and evaporation was done as usual. In the first experiements, the "toluene" and "phosphate" two-dimensional chromatographic systems as described above were used. But the high molarity of phosphate (1.5M) lead to problems of solubility in the scintillation fluor. Thus, this system of chromatography was abandoned and the tert-amyl alcohol-phthalate, pH 6 system was used. The spot corresponding to the N-terminal derivative was excised and eluted 3 times with 2 ml of water. The solution was freed of paper fibers by filtration and evaporated to dryness in a scintillation vial. Water (200 λ) was added to solubilize the DNP-amino acid and 13 ml of scintillation fluor was added prior to counting. Counting was performed on a Beckman Scintillation Counter, LS-230, set up for a dual label experiment. At least 10, 000 counts were obtained in each channel. Counting efficiencies were obtained by the internal method: first spiking with a known amount of ^{3}H-toluene, counting and then spiking with a known amount of ^{14}C-toluene and counting under the same conditions. Both toluene standards were calibrated by the National Bureau of Standards.

The Scintillation fluor, Aquasol, was obtained from New England Nuclear and was used because of its good counting efficiency for tritium. The labeled reagents, 1-fluoro-2, 4-dinitrobenzene-C-14 (U) and 1-fluoro-2, 4-dinitrobenzene-3, 5, 6-H-3 were obtained from Amersham/Searle Corporation.

SDS-Polyacrylamide Gel Electrophoresis

The SDS-polyacrylamide gel electrophoresis experiments were performed as described by Weber and Osborn (1969). Hemocyanin samples were made 1% in SDS, 1% in β-mercaptoethanol and 0.1 M in phosphate, pH 7. The samples were then pretreated by one of two methods: heating at 90° C for 2 minutes or incubation at 37° C for 2 hours. Following the pretreatment the samples were dialyzed against 0.1% SDS, 0.1% β-mercaptoethanol, and 0.1 M phosphate, pH 7 for several hours.

The gels, made in the usual manner, were 10% in total acrylamide and had a ratio of acrylamide to N, N'-bismethyleneacrylamide of 30:1. Protein standards used to calibrate the gels were myosin (gift from Dr. W. Harrington) bovine serum albumin (Sigma Chemical Company), ovalbumin (Sigma Chemical Company) and myoglobin (Mann Research Laboratory). Densitometric scans of the gels were made with a Gilford Linear Transport system utilizing a Beckman DU power supply and light source.

Gel Filtration in 6 M GuHCl-Agarose Column

The method used was that described by Fish et al. (1969). Blue dextran (Sigma Chemical Company) was used as a marker for the void volume (V_o). It was fractionated by running through a Bio-Gel A-5 M column, 0.1 I Tris, pH 7. The front part of the blue dextran peak was collected and used as a marker for the void volume. DNP-glycine (Sigma Chemical Company) was used to measure the amount of solvent contained within and without the gel matrix (V_i). The proteins used in calibration of the column were bovine serum albumin (67, 600 daltons), ovalbumin (43, 000 daltons) and lysozyme (14, 300 daltons). All standards were obtained from Sigma Chemical Company. The protein standards and hemocyanin were reduced and alkylated in 6 M GuHCl (as described under sedimentation studies) prior to layering on the column. Standardization runs were made before and after the hemocyanin runs. All tubes were weighed before and after sample collection to obtain the elution position.

Sedimentation Studies

Protein (0.25-2 mg/ml) was dialyzed against 6.2 M GuHCl, pH 5. If reducing agent was present (0.1 M β-mercaptoethanol or 0.05 M dithiothreitol) the protein was dialyzed into 6 M GuHCl, pH 7-8. For alkylated protein samples, the protein was first reduced

at pH 8.5 for 4 hours, alkylated with iodoacetate and the pH reduced to 6 prior to dialysis and ultracentrifugation. For several samples the GuHCl concentration was raised to 7.5 M but the sample preparation remained the same. In all cases samples were dialyzed for more than three days. Solvent density was determined by refractometric measurements (Kawahara and Tanford, 1966). The GuHCl was purchased from Heico, Inc. The iodoacetate (Eastman Organic Chemicals) was recrystallized from petroleum ether.

A Spinco Model E ultracentrifuge equipped with adjustable interference optics, an electronic speed control and temperature control was used in all sedimentation studies.

Ultracentrifugation was carried out using the high-speed technique of Yphantis (1964) and the overlay technique as described by Chervenka (1970). The overlay technique has the advantage of automatically matching the menescii, reducing the time to equilibrium and reducing the speed needed to deplete the meniscus. To facilitate matching the meniscii for the Yphantis technique, a microliter syringe with a cheney adaptor was used. All runs with a solvent or meniscii missmatch were terminated. Equilibrium was assumed when no fringe change occurred over an 8-12 hour period. Water baselines were obtained after each run.

Interference patterns (two frames, five fringes per frame) were read on a Nikon 6C comparater equipped with a digitizer (L. and W.

Electronics) which recorded on punched tape the data to be analyzed. Intermediate plots of lnc versus dr^2 were obtained on a Hewlett-Packard x-y plotter before proceeding with analysis.

A point-by-point least squares quadratic fit of the ln c versus dr^2 data was performed. The span of the fit could be varied, but two to five points on either side of the central point were normally used. This enabled the calculation of M_w^a, M_n^a, and M_z^a as a function of concentration in the cell.

Extinction Coefficient of Hemocyanin in 6 M GuHCl

About 0.3 grams of hemocyanin solution (75 mg/ml) was diluted to a volume of 25 ml with 6 M GuHCl. The spectrum obtained was compared with that given by a known dilution of the protein into Tris 0.01 I, pH 7. The absorbancy index $E_{1cm}^{1\%}$ at 277 nm was 14.28. This gives a corrected $\frac{\Delta\rho}{c_2}$ = 0.191 and a ϕ' = 0.708 in 6 M GuHCl solutions (Ellerton et al.,1970).

RESULTS

Reassociation of Hemocyanin

Hemocyanin purified as described above when run on acrylamide gel pH 7.5 (Tris) gave one band for the pure 25 S species and on a separate gel one band for the purified 16 S species. Sedimentation velocities run on the same solutions showed only one component for each. When the samples were dialyzed against bicarbonate buffer, pH 10.0 (No Mg^{2+}) sedimentation velocity showed only 5 S particles to be present. When acrylamide gels were run at pH 9.93, only one band was present. The same mobility (within 1%) was obtained using both the 16 S species and the 25 S species as starting material. When the solution was dialyzed back to pH 7.5 and made 0.01 M in Mg^{2+}, sedimentation velocity showed mostly a 16 S component with a small amount of 25 S and some 5 S particles remaining. Electrophoresis at the same pH with 0.01 M Mg^{2+} ions gave four bands: one with the same mobility as the 25 S, a second with the same mobility as the 16 S and two faster moving bands. When the sample at pH 10.0 (No Mg^{2+}, 5 S from sedimentation velocity) was dialyzed to pH 7.5 (No Mg^{2+}) sedimentation velocity showed mainly 16 S particles with some 5 S particles remaining. Electrophoresis at that pH gave 3 bands: the main band corresponding

to 16 S (95%) and two faster moving bands. The 16 S as purified from the Bio-Gel A-5 M column had the same mobility (within 4%) as the main band from the reassociated hemocyanin. The 16 S species reassociated from what had been pure 25 S starting material had the same mobility as the 16 S starting material. Table 2 gives the motilities calculated for the various states of aggregation.

Amino Acid Analysis

Table 3 shows the amino acid data for C. magister hemocyanin. A high percentage of the residues are acidic (aspartic + glutamic >25%) but there is only a very small amount of half cystine, 3 moles/ 78,000 gm of protein.

Table 4 compares data from other hemocyanins with those from C. magister. All are quite similar with the arthropods having a somewhat lower half cystine content.

Partial Specific Volume

The partial specific volume of C. magister hemocyanin as calculated from amino acid analysis and their respective partial volumes was found to be 0.725 ml/mg (Cohn and Edsall, 1943). This is in good agreement with the value found from density

Table 2. Table of mobilities[a/] for native, dissociated and reassociated hemocyanin from gel electrophoresis.

Mobility	Sample
0.075 (± 0.002)	native 25 S, pH 7.5
0.175 (± 0.006)	native 16 S, pH 7.5
0.635	25 S sample dissociated to 5 S. pH 9.9
0.630	16 S sample dissociated to 5 S, pH 9.9
0.075	25 S sample dissociated at pH 9.9 and reassociated at pH 7.5, 0.01 M Mg^{2+}
0.165	
0.383	
0.443	
0.047	16 S sample dissociated at pH 9.9 and reassociated at pH 7.5, 0.01 M Mg^{2+}
0.172	
0.393	
0.453	

[a/] $$\text{mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

Table 3. Amino acid composition of hemocyanin from C. magister.

Amino Acid	Hours of Hydrolysis 20 μmole	40 μmole	67 μmole	140 μmole	Average[a]	Weight %	Amino Acid residues per 78,000 gms protein[b]	Nearest integer per 78,000 gms
Lysine	.0359	.0367	.0350	.0368	.0361	5.24	31.9	32
Histidine	.0459	.0481	.0455	.0462	.0464	7.20	40.9	41
Ammonia	.0845	.0902	.0912	.0956				
Arginine	.0353	.0364	.0333	.0360	.0352	6.22	31.1	31
Aspartic Acid	.103	.116	.109	.105	.108	14.07	95.4	95
Threonine	.0371	.0375	.0342	.0265	.0411	4.71	36.3	36
Serine	.0404	.0362	.0318	.0206	.0450	4.05	36.3	36
Glutamic Acid	.0736	.0777	.0770	.0766	.0771	11.27	68.1	68
Proline	.0366	.0430	.0366	.0337	.0356	3.92	31.5	32
Glycine	.0439	.0458	.0397	.0450	.0449	2.90	39.6	40
Alanine	.0421	.0442	.0426	.0432	.0430	3.46	38.0	38
Half Cystine					.00366	0.428	2.6	3
Valine	.0500	.0547	.0503	.0539	.0543	6.09	47.9	48
Methionine	.0188	.0117		.0167	.0178	2.65	15.8	16
Isoleucine	.0330	.0353	.0328	.0337	.0337	4.31	29.7	30
Leucine	.0550	.0450	.0542	.0555	.0552	7.07	48.7	49
Tyrosine	.0313	.0296	.158	.0287	.0332	6.14	29.3	29
Phenylalanine	.0456	.0512	.0435	.0429	.0440	7.33	38.8	39
Tryptophan					.0139	2.93	12.3	12

[a] Data were averaged, extropolated to zero time, or value at maximum recovery used. Half cystine was determined as cysteic acid after oxidation by DMSO. Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967).

[b] Based on copper analysis of 2 Cu/78,000.gm.

Table 4. Comparison of amino acid data for hemocyanin from various sources. (Weight percent)

Amino Acid	O. vulgarus[a]	M. trunculus[a]	O. macropus[a]	C. magister	H. vulgurus[a]	E. spinifrons[a]	C. sapidus[a]	P. vulgarus[a]
Lysine	5.03	4.93	5.28	5.24	4.93	4.32	5.14	4.75
Histidine	6.07	7.48	6.54	7.20	7.37	7.43	6.90	7.62
Arginine	5.01	6.07	5.72	6.22	6.11	5.97	6.39	6.48
Tryptophan	2.47	2.63	2.07	2.93	1.74	2.42	1.98	2.04
Aspartic Acid	11.08	11.81	11.04	14.07	12.19	12.48	13.51	14.41
Threonine	4.45	4.30	5.16	4.71	4.95	4.44	4.50	4.20
Serine	3.60	3.67	3.92	4.05	3.05	3.90	3.48	2.86
Glutamic Acid	10.25	12.41	10.92	11.27	11.77	10.61	12.36	12.12
Proline	4.58	4.42	4.47	3.92	3.57	3.63	4.06	3.89
Glycine	2.50	3.04	2.76	2.90	2.73	2.71	3.29	2.95
Alanine	3.98	4.08	3.97	3.46	3.44	3.56	4.27	3.34
Half Cystine	2.02	1.29	1.69	0.428	0.64	0.45	0.85	0.70
Valine	5.10	4.92	4.75	6.09	5.27	5.46	5.78	5.25
Methionine	2.84	2.48	2.70	2.65	2.45	2.56	2.80	3.24
Isoleucine	4.86	4.02	5.29	4.31	4.53	4.02	4.84	4.76
Leucine	8.52	8.51	8.97	7.07	6.79	6.87	7.20	6.78
Tyrosine	5.86	5.47	6.01	6.14	4.60	4.98	5.73	4.66
Phenylalanine	7.03	7.73	6.89	7.33	6.80	7.21	7.04	6.89

[a] Data from Ghiretti-Magaldi et al., 1966.

measurements, 0.728 ml/gm.

Qualitative Amino-Terminal Analysis

The only N-terminal DNP-derivative found in the ether extracted phase was DNP-aspartate. No N-terminal DNP-amino acids were found in the aqueous phase. A separate experiment was performed to check for the presence of DNP-arginine but none was found.[2] Hydrolysis with concentrated HCl gave no evidence for DNP-glycine or DNP-proline. No DNP-cysteic acid was found from the performic acid oxidized protein.

As mentioned above DNP-aspartate and DNP-glutamate overlap in the "toluene" and 1.5 M "phosphate" chromatographic systems. To distinguish between these two possibilities chromatography with tert-amyl alcohol-phthalate, pH 5, was performed. The DNP-terminal amino acid ran with the DNP-aspartate standard which was well separated from the DNP-glutamate standard.

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[2] In some chromatographic solvent systems DNP-arginine runs with ε-DNP-lysine. Thus some ambiguity could exist since ε-DNP-lysine is almost always present. However, DNP-arginine gives a positive stain with the Sakaguchi test (Acher and Crocker, 1952), and can be detected in spite of the ε-DNP-lysine. With C. magister hemocyanin all such staining attempts proved to be negative.

Table 5. Sample calculation for FDNB method of quantitative N-terminal analysis.

μg Hecy	Specific activity of ^{3}HFDNB (μc/μmole)	Amount of ^{14}C-DNP-Aspartate added (DPM)		^{14}C channel net CPM	^{3}H channel net CPM
162.9	0.3391	437.2	N-terminal + control sample	85.0	302.2
			^{3}H toluene added 16,169 dPM	85.0	6534
			^{14}C toluene added 4,960 dPM	2741	9037

Counting efficiency in ^{14}C channel	dPM ^{14}C	% ^{14}C-Asp recovered	Counting efficiency of ^{14}C in ^{3}H channel	CPM of ^{14}C in ^{3}H channel	CPM of ^{3}H before ^{3}H-toluene spike
0.5354	158.8	36.32	0.5045	80.1	222.1

CPM of ^{3}H after ^{3}H-toluene spike	Counting efficiency of ^{3}H	dPM ^{3}H (recovered)	μc ^{3}H (recovered)	μc ^{3}H (reacted)	μmoles DNP-Asp-3
6454	0.3784	586.9	0.2644×10^{-3}	0.728×10^{-3}	$2.147 \times 10^{-}$

$$\frac{\mu g \text{ protein}}{\mu \text{mole DNP-Asp}}$$

75,900

Quantative Amino-Terminal Analysis

Table 5 shows a sample calculation for the N-terminal analysis and Table 6 shows an average of 5 experiments. After 4 hours of hydrolysis only 0.3 moles of end group were released per 78,000 grams of protein. But after 8 hours, hydrolysis was essentially complete and gave 0.95 ± 0.26 moles of DNP-aspartate per 78,000 grams of protein. All the results from hydrolysis lasting between 8-24 hours gave 82,000 ± 18,000 grams of protein per mole of DNP-aspartate.

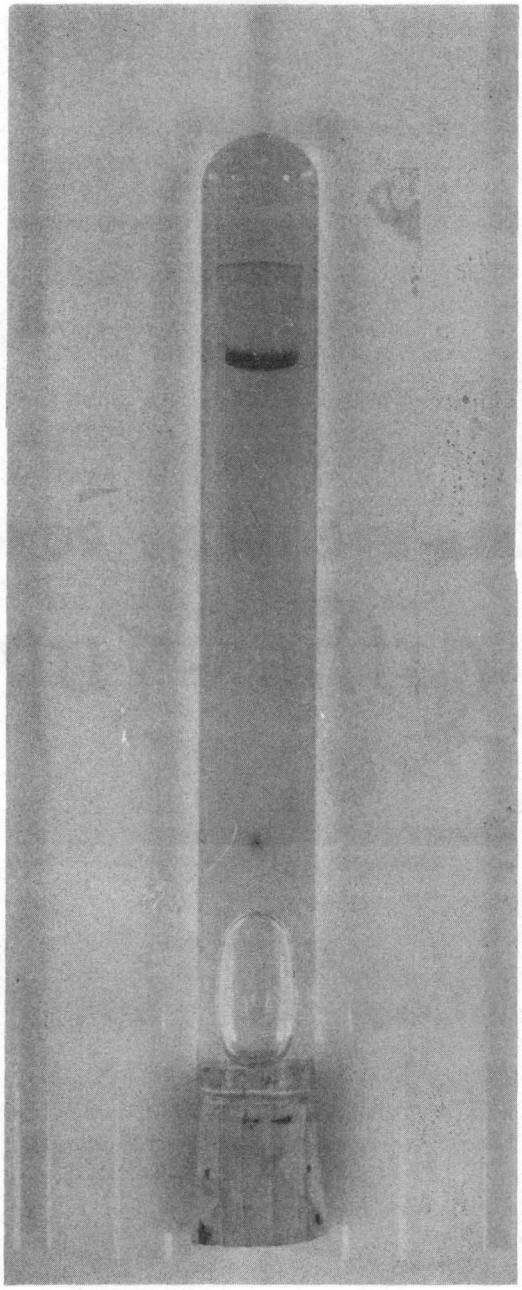
Table 6. Summary of results from quantative N-terminal analysis.

Hydrolysis time (hrs)	4 hours	8-24 hours
gm/mole	265,000	82,000 ± 18,000
Moles of end group 78,000 g of protein	0.30	0.95 ± 0.26

SDS-Polyacrylamide Gel Electrophoresis

Results from several experiments gave only one band corresponding to a molecular weight of 78,000 daltons. No heavy material was left at the top of the gel (see Figure 4). No difference was observed whether the sample was heated at 90° C for two minutes in 1% SDS, 1% β-mercaptoethanol or incubated at 37° C for two hours prior to dialysis. Figure 5 shows a densitometer scan of a typical

Figure 4. Photograph of hemocyanin on an SDS-polyacrylamide gel.



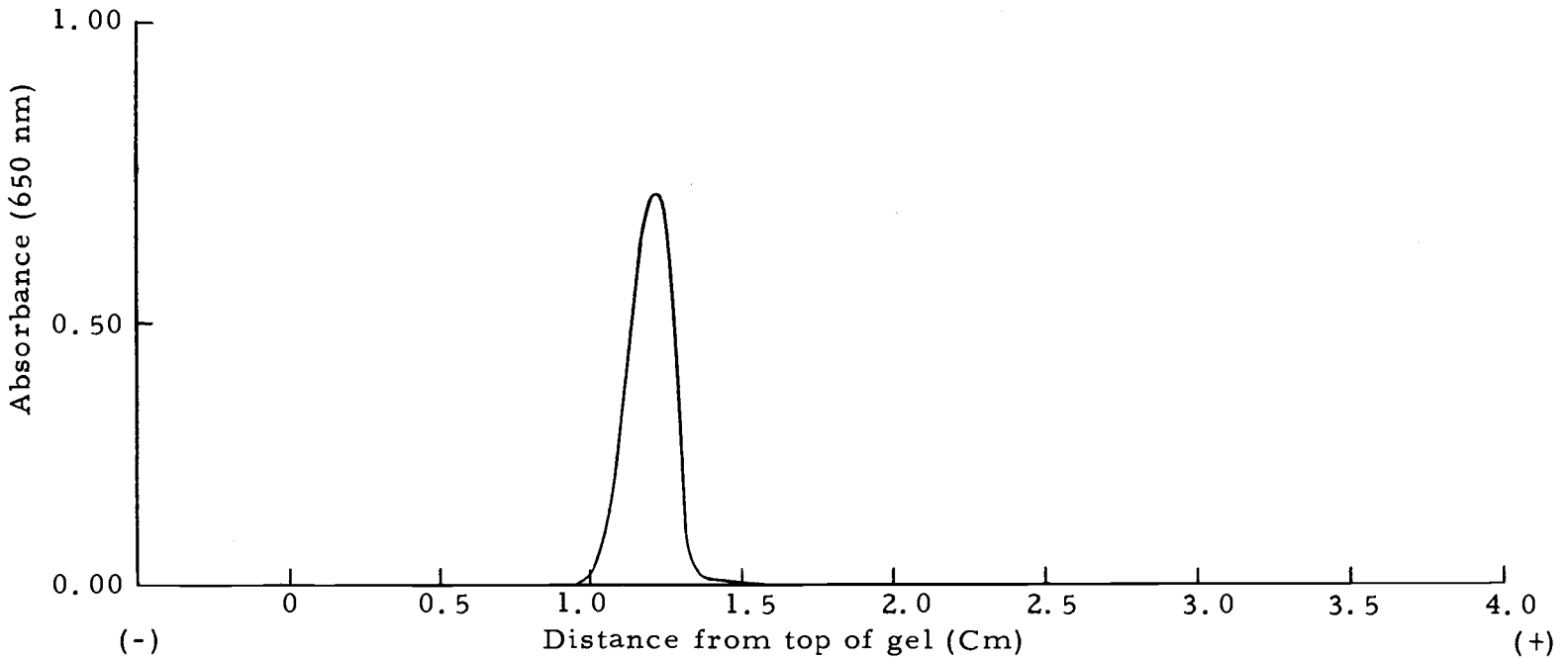
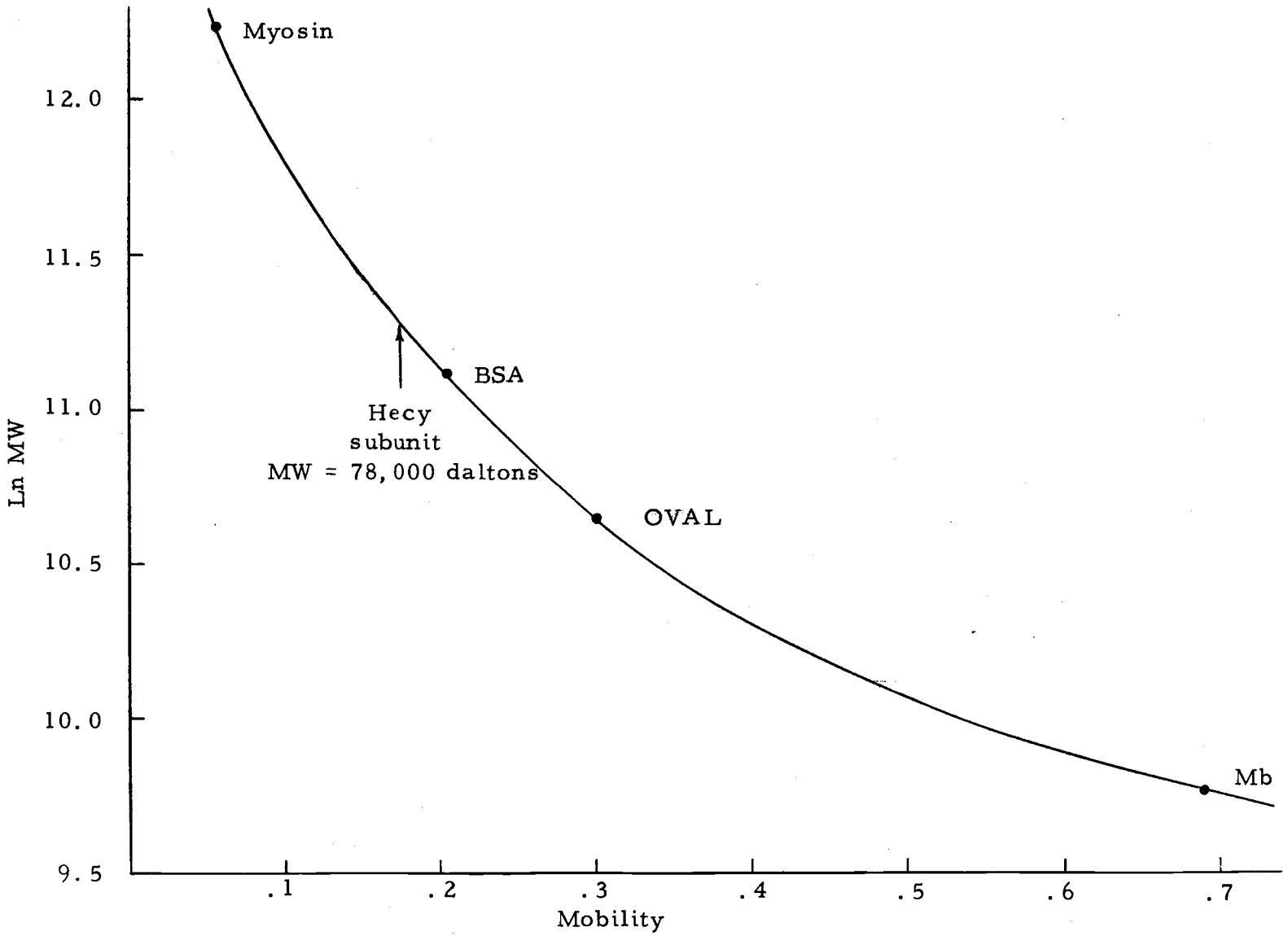


Figure 5. Densitometric tracing of hemocyanin on 0.1% SDS-10% polyacrylamide gel. The hemocyanin was reduced and incubated at 37°C for 2 hours with 1% SDS and 1% β-mercaptoethanol prior to dialysis against 0.1% SDS and 0.1% B-mercaptoethanol.

Figure 6. Calibration curve of 10% polyacrylamide gels (0.1% SDS, 0.1% β-mercaptoethanol). Protein standards were myosin (212,000 daltons), bovine serum albumin (67,600 daltons), ovalbumin (43,000 daltons) and myoglobin (17,200 daltons). The arrow shows the mobility of the hemocyanin subunit.


Myosin
BSA
Hecy
subunit
MW = 78,000 daltons
OVAL
Mb
12.0
11.5
11.0
10.5
10.0
9.5
Ln MW
.1
.2
.3
.4
.5
.6
.7
Mobility

SDS-polyacrylamide gel. Figure 6 gives the calibration curve with the arrow showing the mobility of the hemocyanin subunit.

Gel Filtration on 6 M GuHCl-Agarose Column

The elution profile of reduced and alkylated hemocyanin in the presence of 6 M GuHCl on an agarose column is shown in Figure 7. The position at which the hemocyanin was eluted corresponds to a molecular weight of approximately 70,000 daltons. The column was calibrated before and after the hemocyanin run. The calibration curve and the position at which the hemocyanin polypeptide chains were eluted are shown in Figure 8.

Sedimentation Studies

All the experiments in concentrated GuHCl solutions were characterized by downward curvature in the ln c versus dr^2 plots. This is due to the nonideality in GuHCl solutions and is expected. This is reflected in a linear plot with a positive slope for a plot of $\frac{1}{M}$ a versus concentration. In some experiments nonlinearity below three fringes was observed. The nonlinearity below about three fringes was tested in terms of heterogeneity of the sample. If a model of 72,000 daltons (92.3% by weight) and 6,000 daltons (7.7%) and with a virial coefficient of 3.9×10^{-7} mole/gram-fringe is assumed, one calculates a $\frac{1}{M_w}$a as found by the solid line (Figure 9). This is clearly different

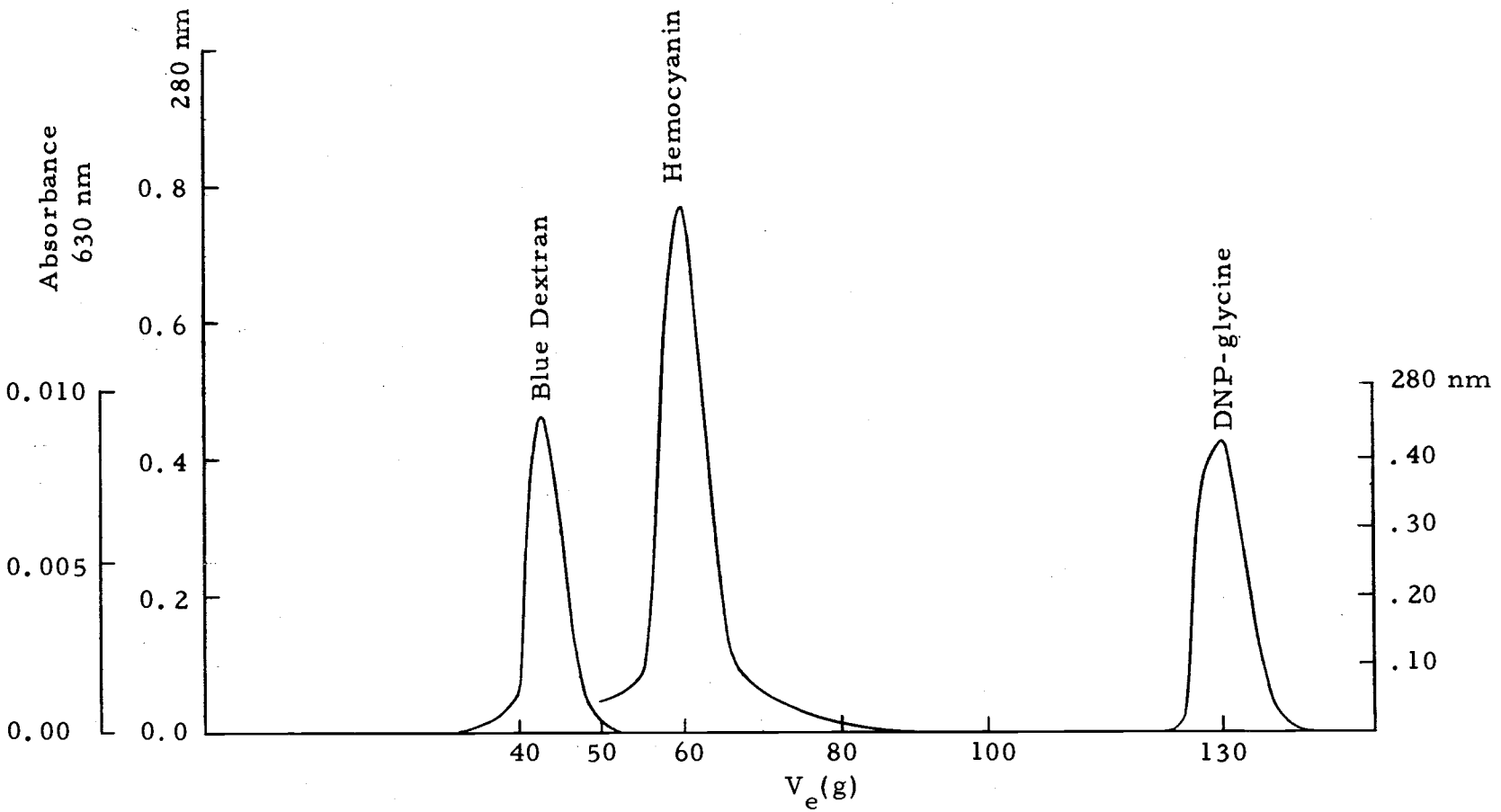


Figure 7. Elution profile of reduced and alkylated hemocyanin from a 6M GuHCl - 6% agarose column.

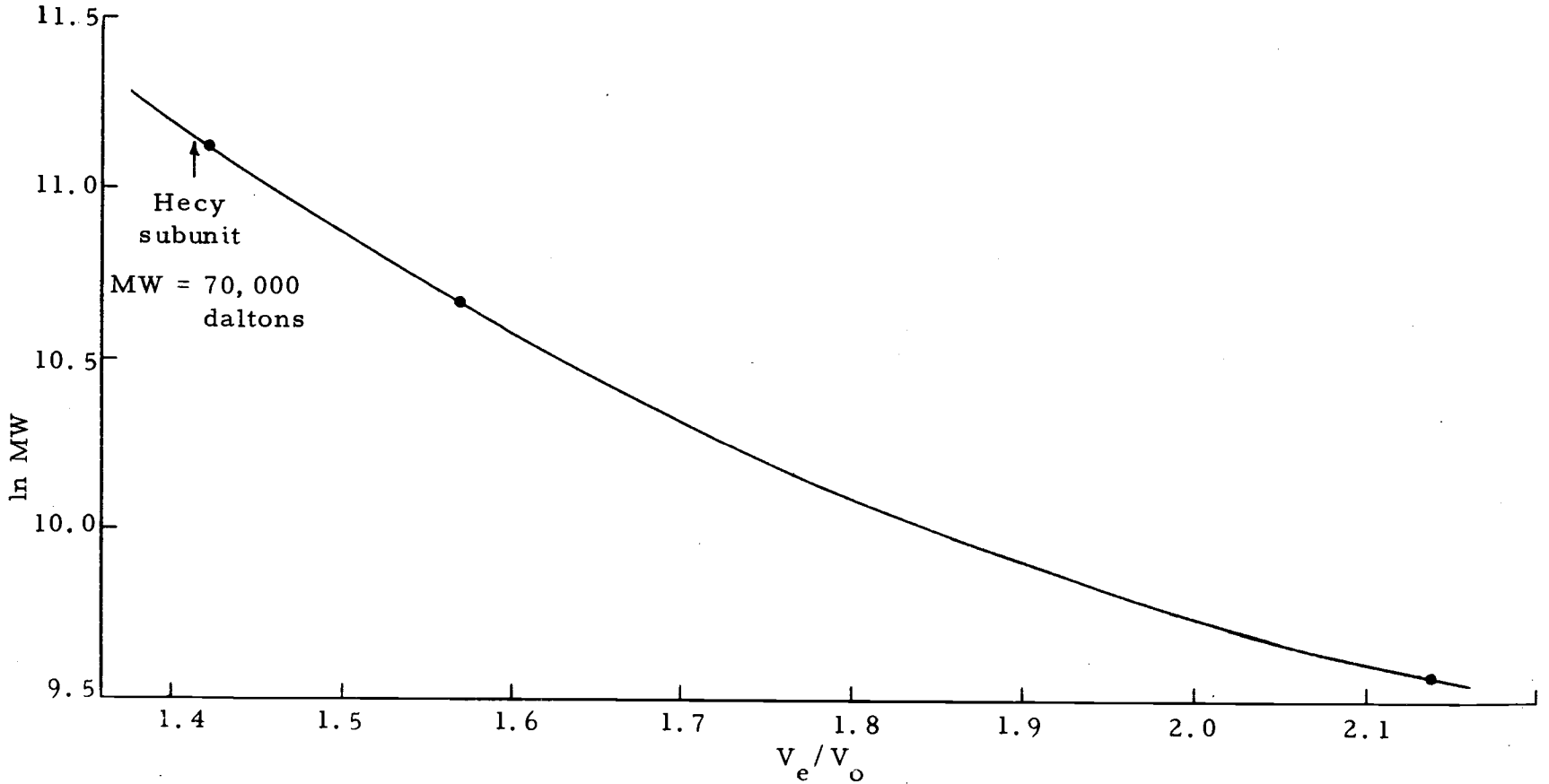
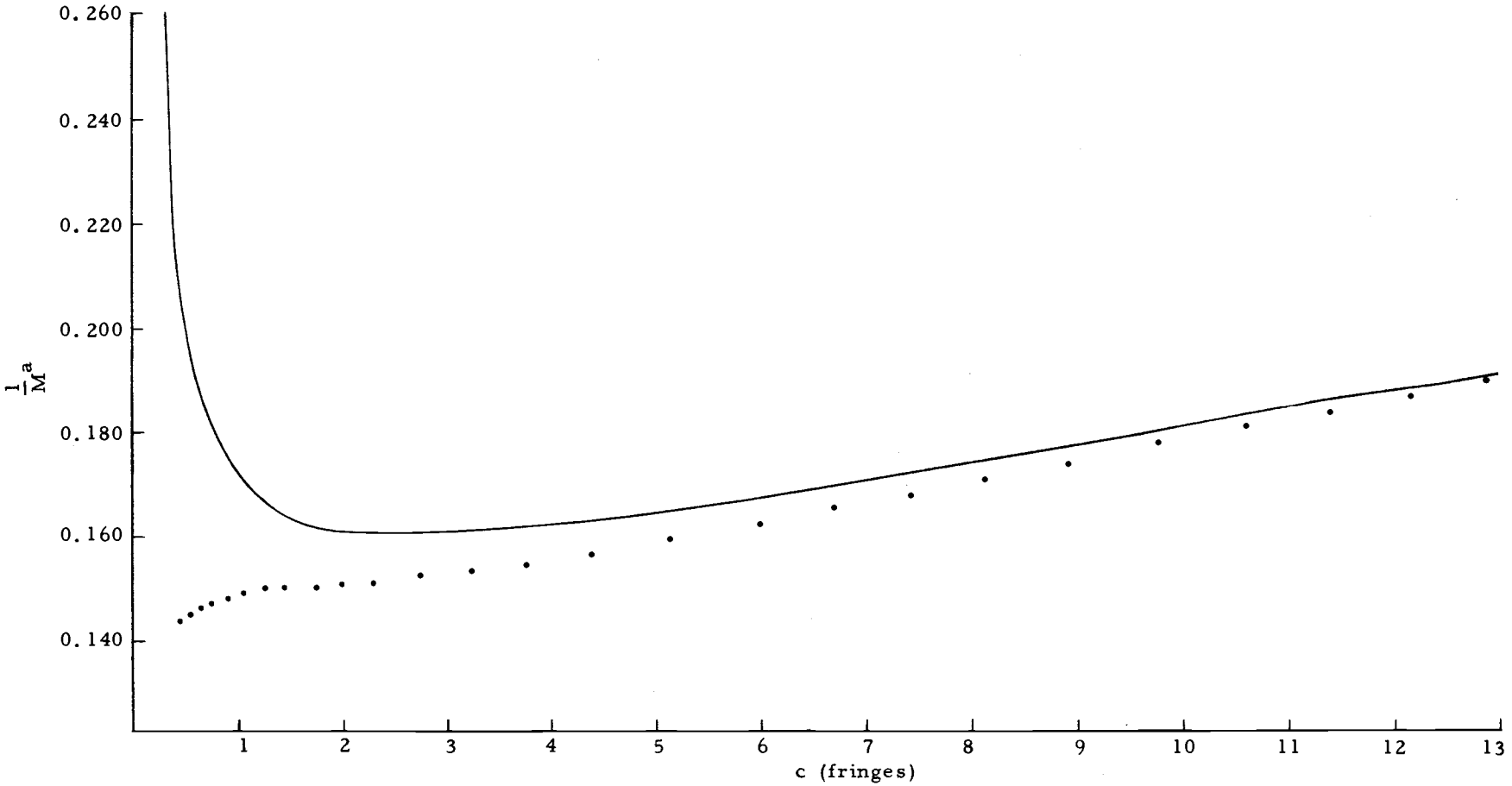


Figure 8. Calibration curve of 6M GuHCl 6% agarose column. The reduced and alkylated protein standards were bovine serum albumin (67, 600 daltons), ovalbumin (43, 000 daltons) and lysozyme (14, 300 daltons). The arrow marks the V_e / V_o where the hemocyanin polypeptide chains were eluted.

Figure 9. Reciprocal apparent weight average molecular weights versus concentration (fringes). The line gives the $\frac{1}{M_w}a$ expected for an $M_1 = 72,000$ (92.3% by weight) and $M_2 = 6,000$ (7.7%) each with a virial coefficient of 3.9×10^{-7} mole/gram fringe. The data points are the $\frac{1}{M_w}a$ versus concentration obtained from hemocyanin in 6.2 M GuHCl, 0.1 M β-mercaptoethanol. The ϕ' used in the calculation was 0.708 ml/gm.


0.260
0.240
0.220
0.200
0.180
0.160
0.140
$\frac{1}{M^a}$
1
2
3
4
5
6
7
8
9
10
11
12
13
c (fringes)

from the experimental points. If one assumes a model consisting of 72, 000 dalton species contaminated with 36, 000 dalton species one can calculate the percent contaminant. Using the expression for M_w

$$M_w = \frac{M_1C_1 + M_2C_2}{C_T}$$

and a virial coefficient of 3.9×10^{-7} mole/gm-fringe one can find the amount of 36, 000 dalton species present in the cell. By integration across the cell the total amount of 36, 000 component present would be about 0.5%.

One can calculate the M_w^a expected for M_w = 71, 400 daltons and a virial coefficient of 3.9×10^{-7} moles /gm-fringe. By comparison of the (M_w^a) predicted with that observed from a typical experiment one can find the percent deviation from the observed value. See Figure 10.

The deviation of the predicted values from that of the experimental points is less than 3.5% in the region where the data has the most error. Thus, within the limits of the high speed sedimentation equilibrium technique in concentrated GuHCl solutions, the sample appears to be homogeneous.

We have used two methods to deal with nonideality: extrapolotion of the reciprocal apparent molecular weights to zero concentration and by use of $2M_{n,c} - M_{w,c}$. For a nonideal homogeneous system extrapolation of $\frac{1}{M_w}a$, $\frac{1}{M_n}a$, and $\frac{1}{M_z}a$ to C = 0 gives $\frac{1}{M_w}$, $\frac{1}{M_n}$ and

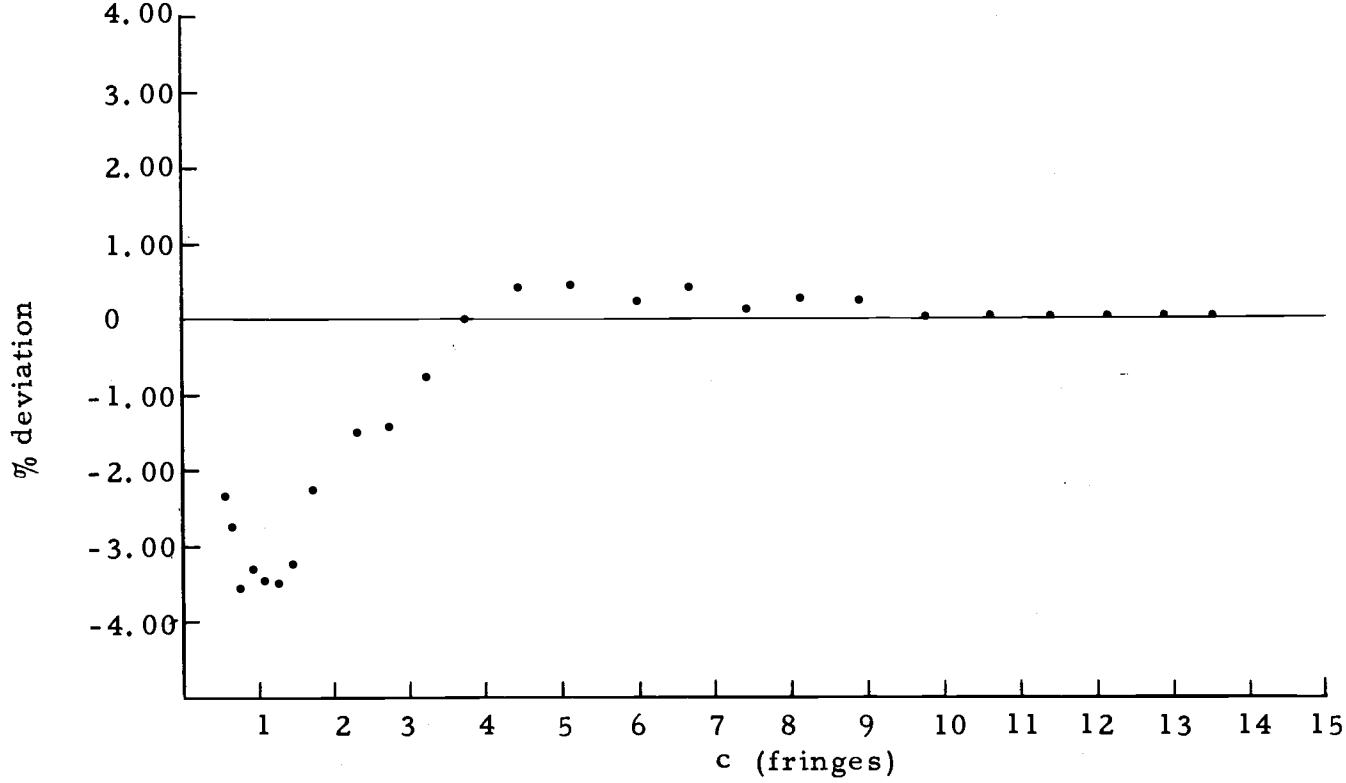


Figure 10. Percentage deviation of the observed M_w in Figure 8 from the M_w of a model calculated for M = 71,600 and B= 3.86×10^{-7} mole/gm fringe. $\frac{M_{obs} - M_{pred}}{M_{pred}} = \%$ deviation

$\frac{1}{M_z}$ (see Munk and Cox, 1972 and Roarke and Yphantis, 1969, if heterogeneous). Figures 11 and 12 show the reciprocal molecular weight distribution for a hemocyanin sample in 6.2 M and 7.5 M GuHCl respectively. The slopes of the lines thus obtained are B and 0.5 B for the $\frac{1}{M_w}a$ and $\frac{1}{M_n}a$ plots. The slope from the plot of $\frac{1}{M_z}a$ versus concentration is not quite as straight forward. By using the equation

$$M_z^a = M_z \left(\frac{M_w^a}{M_w}\right)^2 \quad \text{(Van Holde et al., 1969)}$$

and the expression for M_w

$$\frac{1}{M_w}a = \frac{1}{M_w} + Bc$$

one can obtain

$$\frac{1}{M_z}a = \frac{1}{M_z} + 2Bc\frac{M_w}{M_z} + B^2\frac{M_w^2}{M_z}c^2$$

if the sample is homogeneous then

$$M_w = M_z$$

and

$$\frac{1}{M_z}a = \frac{1}{M_z} + 2Bc + B^2 M c^2$$

Thus for moderate concentrations a linear plot is expected with a limiting slope of 2B. Table 7 summarizes the data for the various molecular weight averages extrapolated to c = 0 and the virial coefficients as found from the slope. The virial coefficients are about what one

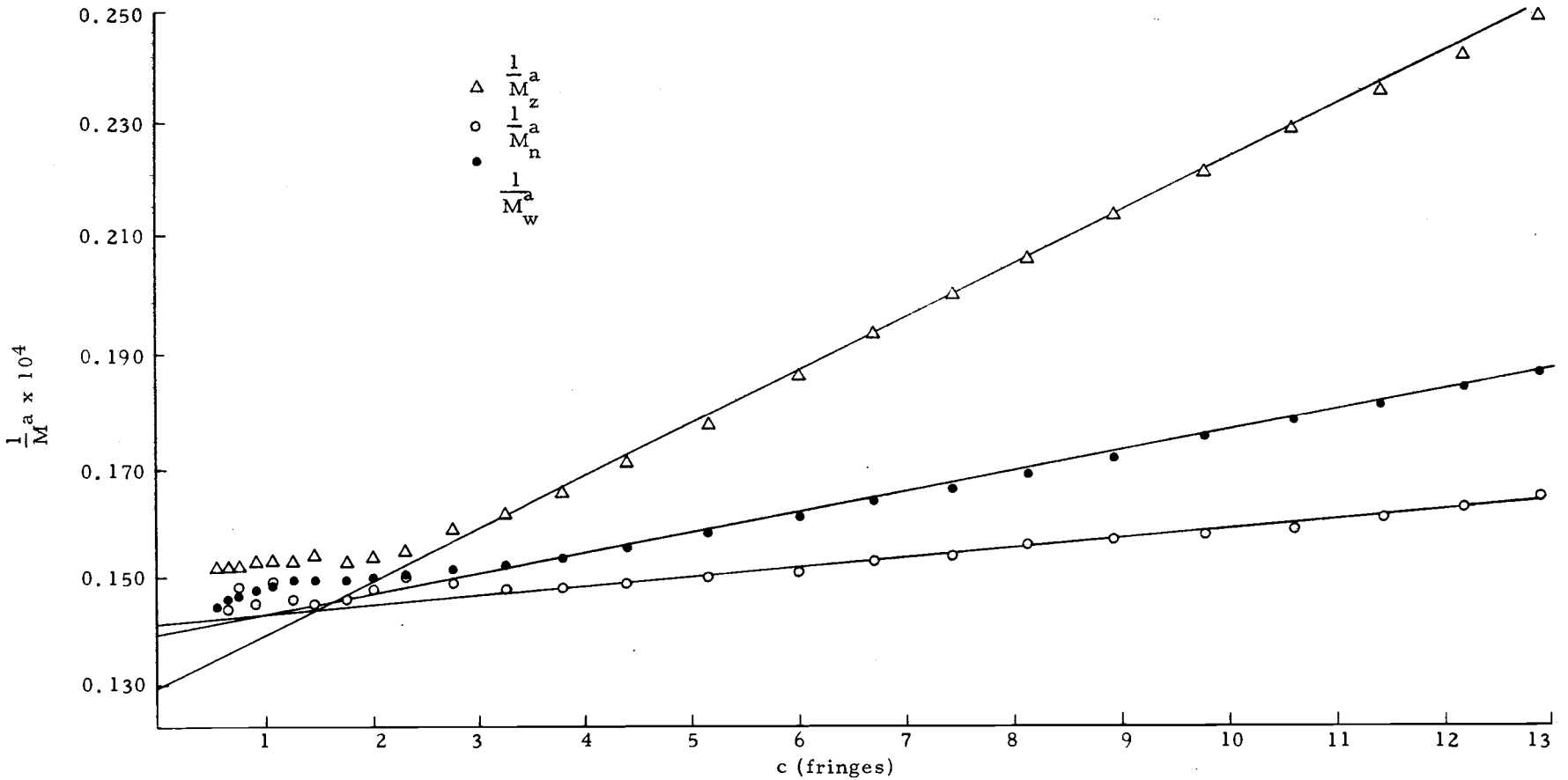
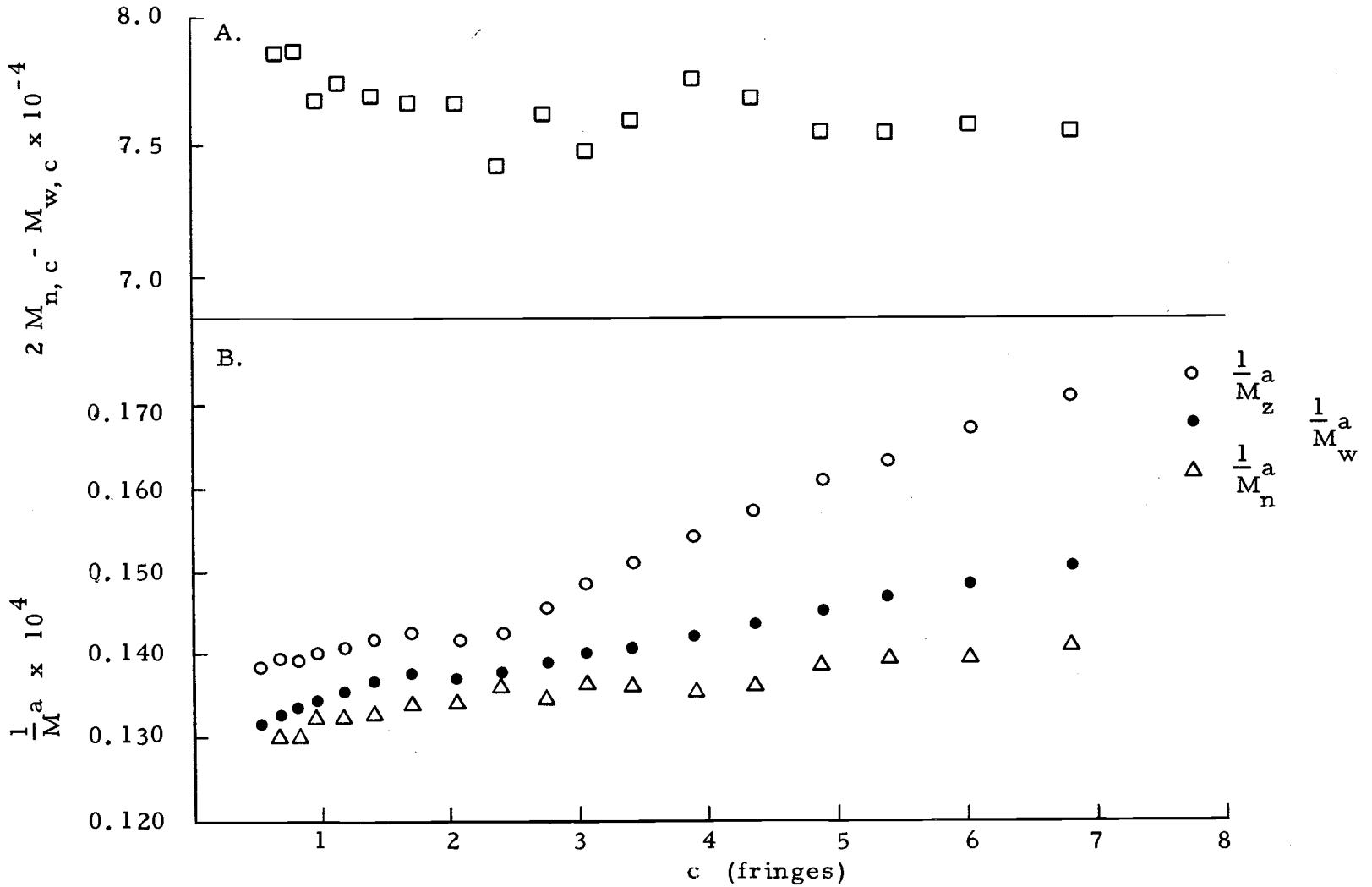


Figure 11. Reciprocal apparent molecular weight distributions versus concentration of hemocyanin in 6.2 M GuHCl, 0.1 M β-mercaptoethanol. The ϕ' used in this calculation was 0.708 ml/g.

Figure 12. Molecular weight distribution in 7.5M GuHCl with no reducing agent. Figure 10A shows 2 $M_{n,c} - M_{w,c}$ versus concentration in fringe units. Figure 10B gives the reciprocal apparent molecular weight averages against concentration. The ϕ' used in this calculation was 0.728 ml/g.


A.
B.
8.0
7.5
7.0
$2\,M_{n,c} - M_{w,c} \times 10^{-4}$
0.170
0.160
0.150
0.140
0.130
0.120
$\frac{1}{M}^a \times 10^4$
$\frac{1}{M_z}^a$
$\frac{1}{M_w}^a$
$\frac{1}{M_n}^a$
1
2
3
4
5
6
7
8
c (fringes)

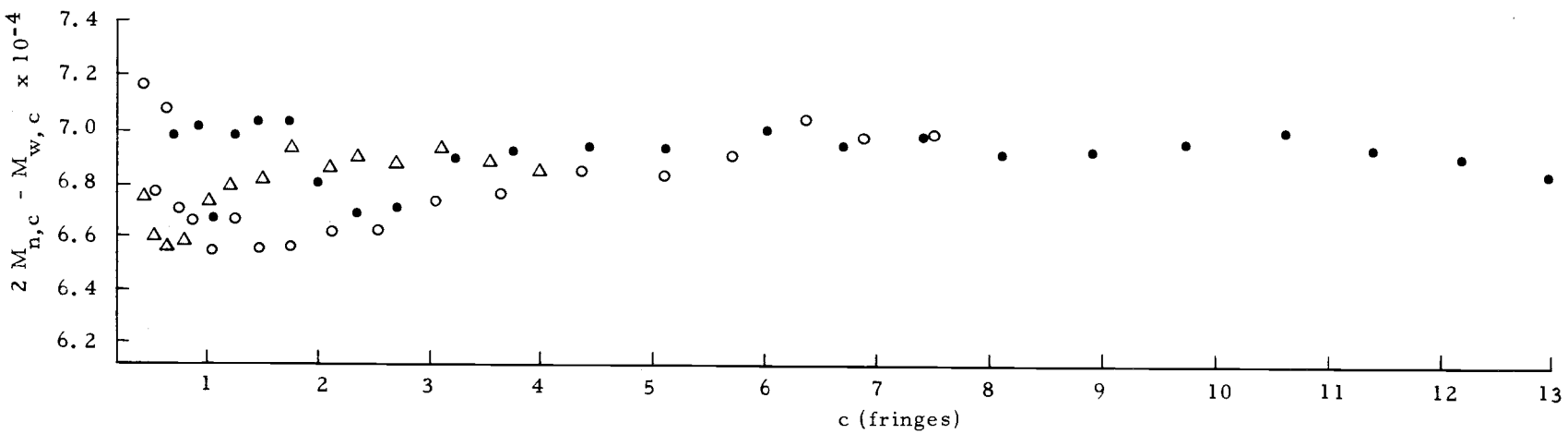


Figure 13. This shows the combined data of 2 $M_{n,c}$ - $M_{w,c}$ against concentration (fringes) for three separate experiments. All were in 6M GuHCl. Δ Hemocyanin was reduced and alkylated with iodoacetate, rotor speed 34,000 rpm. o No reducing agent was present in this experiment, rotor speed 30, 219 rpm. ● Hemocyanin was reduced with 0.1 M β-mercaptoethanol, rotor speed 28,160 rpm. The ϕ used in these calculation was 0.708 ml/g.

would expect for a flexible polymer in a good solvent (Tanford, 1961, Lapanje and Tanford, 1967).

Figure 9 shows a plot of $2 M_{n,c} - M_{w,c}$ versus concentration for three separate experiments. A weighted average of the data gives a value of 69,700 daltons. At each point the virial coefficients for $M_{n,c}$ and $M_{w,c}$ should cancel, if not upward curvature would be seen. If the sample was heterogeneous downward curvature would be present (Yphantis, 1964).

In order to reflect preferential interaction of GuHCl with proteins, Casassa and Eisenberg (1964) have defined an apparent quality ϕ'. The ϕ' is analogous to a partial specific volume for a two component system.

$$\phi' = \frac{1}{\rho_o} \left(1 - \frac{\Delta\rho}{c_2}\right)$$

where $\frac{\Delta\rho}{c_2}$ is the reduced density increment at low protein concentration and dialysis equilibrium and ρ_o is the solvent density. Since

$$\frac{d \ln c_2}{dr^2} = \frac{\omega^2}{2RT} M_2 (1 - \phi' \rho)$$

an error of 1% in ϕ' would give an error of 4% in M_2.

Most proteins studied very carefully show a 0.01 - 0.012 ml/gm decrease in ϕ' in going from dilute salt solution to 6 M GuHCl (Tanford, 1967; Reisler and Eisenberg, 1969). With a $\phi' = 0.718$ ml/gm, $M_{w(c=0)} = 75,900$ daltons, if $\phi' = 0.708$ ml/gm, $M_{w(c=0)} =$

71, 400 daltons. Reisler and Eisenberg have shown that for 7 M GuHCl the ϕ' for aldolase is 0.002 ml/gm less than in dilute salt solution (see also Seery et al., 1967). If we use a ϕ' of 0.728 ml/gm $M_{w(c=0)}$ = 77, 400 daltons for hemocyanin in 7.5 M GuHCl.

Thus it is clear that the subunits in 6.2 - 7.5 M GuHCl with or without reducing agent are homogeneous within measurement. Depending on the ϕ' used an M_w = 71, 400 - 77, 400 daltons is obtained.

Table 7. Summary of polypeptide molecular weight determination in concentrated GuHCl solutions

# of Expts	M GuHCl	Reducing agent[a/]	ϕ'	x 10^{-3} gm/mole $(M_n)c=0$	$(M_w)c=0$	$(M_z)c=0$	$2M_n - M_w$	x 10^{7} mole/gm-fringe $B(M_w)$	$B(M_n)$	$B(M_z)$
5	6.2 M	yes	0.708	68.9	70.6	73.2	69.1	5.61 (±.6)	4.43 (±1.7)	5.30 (±2.3)
			0.718	73.2	75.0	77.8	73.4			
3	6.2 M	No	0.708	68.9	72.7	77.8	67.6	2.22	0.52	3.3
			0.718	73.2	77.3	82.7	71.9			
2	7.5 M	yes	0.708	64.3	65.4	79.0	62.6	9.34	7.56	13.0
			0.728	76.4	77.8	(95.0)	74.4			
			0.708	65.4	65.6	66.1	65.7			
1	7.5 M	no	0.728	76.4	76.6	77.2	76.7	3.64	3.72	4.00
Ave[b/]	6.2 M		0.708	68.9 (±1.9)	71.4 (±1.5)	74.9 (±3.2)	67.0 (±3.0)			
	6.2 M		0.718	73.2 (±2.3)	75.9 (± .7)	79.6	72.5 (±2.9)			
	7.5 M		0.728	76.4	77.4	74.4	75.2			

[a/] If reducing agent was used results using 0.1Mβ-mercaptoethanol, 0.05 M dithiothreitol or alkylated hemocyanin in concentrated GuHCl solutions were combined.

[b/] In the average the combined results with and without reducing agent were used. The numbers in parenthesis are ± standard deviation.

DISCUSSION

Reversibility of Dissociation

In attempts to reverse the dissociation only 16 S species were formed to any extent (Ellerton et al., 1970). Solutions containing mainly 25 S particles at pH 7 were dialyzed to pH values between 9 and 11 and redialyzed back to pH values between 7 and 8.8. Reassociation occurred only to the 16 S species. When Mg^{2+} ions were added (normally to a concentration of 0.01 M but in a few instances up to 1 M) a small amount (10%) of 25 S particles were obtained. In order to test the possibility that a dialyzable cofactor (small ions, peptides, etc.) could have been lost when dialyzed at high pH the 25 S particles were carefully titrated to high pH. Sedimentation velocity showed only the presence of 5 S species. Upon addition of Mg^{2+} ions or titrating back to pH 7 and adding Mg^{2+} ions (to 0.01 M) reassociation occurred only to the 16 S component with a small amount of 25 S component.

From electron micrographs of arthropod hemocyanin two different projections were observed for the 16 S particles (Van Bruggen et al., 1963). Therefore the possibility that we have two different 16 S particles (one composed of an "A" type 5 S particle and the other a "B" type) going together to form a 25 S particle was explored.

After dissociation, the probability of 6 "A" type 5 S particles associating and finding an all "B" type 16 S particle is quite low.[3/] A series of electrophoresis experiments was performed to determine if there are electrophoretically distinguishable 16 S or 5 S species. However, only one band was obtained for the 5 S species on acrylamide gels. The 5 S species had the same mobility whether it originated from purified 16 S or 25 S particles. When the 5 S was reassociated to 16 S particles the reassociated 16 S particles had the same mobility as the 16 S particles purified from the hemolymph.

This problem is similar to the hemocyanin from E. spinifrons (Di Giamberardino, 1967) where 16 S species cannot be formed from 5 S species. In the original serum 16 S and 25 S hemocyanin are present. The difficulty of converting the 16 S to the 25 S hemocyanin is pointed out by the fact that they can be readily separated on an agarose column or by sucrose density centrifugation. It is unlikely that a small dialyzable substance is required since titration to high pH and back again does not produce 25 S hemocyanin. The possibility still exists that there are more than one type of 5 S species but that they are not electrophoretically distinguishable. For example chains could differ in amino acids which are not charged at pH 9.9. Experiments done at different pH's and peptide mapping may clarify this point.

[3/] The probability of forming an A_6 type 16 S particle is 1/256.

Molecular Weight of Polypeptide Chain

Despite our suspicions to the contrary and reports to the contrary, we find by several independent methods that the molecular weight for the C. magister hemocyanin is between 70,000-80,000 daltons. Preliminary experiments in which 70% formic acid or 50% acetic acid was the denaturant gave completely non-reproducible results. Sedimentation equilibrium of formic acid treated hemocyanin showed the presence of small molecular weight material (10,000-18,000 daltons) along with that of a large molecular weight (>38,000 daltons). Determinations carried out at different formic acid concentrations for varying lengths of time and temperature were tried. In all cases smaller molecular weight pieces were present with the larger pieces. Attempts at SDS-Acrylamide gel electrophoresis on the formic acid treated hemocyanin gave equally discouraging results. Many bands were present varying from 30,000 to about 80,000 daltons. The number and position of the bands were different from experiment to experiment. A report on SDS-urea-polyacrylamide gels on formic acid treated H. pomatia hemocyanin (Dijk et al., 1971) also found smaller molecular weight bands. Bands corresponding to a molecular weight of 25,000-50,000 daltons and multiples thereof were found. But most of the protein was found to be of high molecular weight. Similar results were obtained in this

laboratory on the molluscan hemocyanin from B. canaliculatum (Roxby, 1972).

It is well known that aspartyl residues are extremely labile to acid hydrolysis (Partridge and Davis, 1950; Schroeder et al., 1963) and hemocyanin contains between 11-14% by weight aspartic acid. At the present time we do not know whether the smaller molecular weight components from formic acid treatment are due to labile aspartyl residues or to another sensitive covalent linkage.

SDS-polyacrylamide gel electrophoresis experiments on the C. magister hemocyanin which had not been pretreated with formic or acetic acid were straight forward. Only one band was observed corresponding to a molecular weight of 78,000 daltons. No faster moving bands were present and no higher molecular weight material was present.

Gel filtration of the reduced and alkylated C. magister hemocyanin on a 6 M GuHCl-agarose column gave only one peak corresponding to a molecular weight of about 70,000 daltons. No peaks corresponding to lower molecular weight material was observed. On the 6% agarose column a very small error in the elution position makes a larger error in the molecular weight determination in the 65,000 - 80,000 dalton region. With our 6% agarose column, a 0.7% error, (1/4 of a tube) in the elution position (in the high molecular weight

range) would change the molecular weight by 11%.

The sedimentation equilibrium results in concentrated GuHCl solutions show that within the limit of the technique the subunits are quite homogeneous. Hetereogeneity would introduce curvature into a plot of $\frac{1}{M}a$ versus concentration (especially in the low concentration region) and reduce the positive slope due to nonideality. As can be seen from the model calculation for 7% of a low molecular weight material (6, 000 daltons) and 93 % (72, 000 daltons, see Figure 9) pronounced curvature is introduced into the plot. The small amount of deviation from linearity below three fringes observed in some runs was less than 3. 5% from the expected molecular weight for those concentrations. In experiments performed at higher concentrations and speeds where the effect of heterogenity is greater, a linear relationship was still obtained in a plot of $\frac{1}{M}a$ versus concentration. The second virial coefficient (5.6×10^{-7} mole/gm-fringe for 6.2 M GuHCl with reducing agent) obtained from the slope of the $\frac{1}{M_w}a$ versus concentration is about what one expects for a random coil in a good solvent (3.6×10^{-7} mole/gm-fringe; see Tanford, 1961).

A plot of $2 M_{n,c} - M_{w,c}$ versus concentration will give downward curvature if the sample is heterogeneous (Yphantis, 1964). Although there is some scatter in the low concentration region of the plot (Figure 13) the $2 M_{n,c} - M_{w,c}$ remains fairly constant over a

wide concentration range. This constancy also shows that to a large extent the virial coefficients in the $M_{n,c}$ and $M_{w,c}$ have cancelled. The slope from plots of the reciprocal molecular weights should give 0.5 B, B and 2 B for a plot of $\frac{1}{M_n}a$, $\frac{1}{M_w}a$, and $\frac{1}{M_z}a$ respectively. Within the error of measurement this relationship was found to hold.

By using an experimentally determined $\phi' = 0.708$ ml/gm for hemocyanin in 6.2 M GuHCl, a molecular weight of 71,400 daltons is calculated. If the ϕ' in 6.2 M GuHCl is 0.718 ml/gm a molecular weight of 75,900 daltons is calculated. A decrease of 0.01 - .012 ml/gm from the $\bar{v}$ for proteins in dilute salt solutions is generally observed for the ϕ' in 6 M GuHCl (Reisler and Eisenberg, 1969). In 7 M GuHCl Reisler and Eisenberg (1969) found the ϕ' to be about 0.002 ml/gm less than that for the native protein. Thus if a $\phi' = 0.728$ is used for hemocyanin in 7.5 M GuHCl, a molecular weight of 77,400 daltons is obtained.

Since only aspartic acid was found for an amino-terminal amino acid, this suggests that the chains are identical. There is the possibility, of course, that two different chains could have aspartic acid as an amino-terminal or that other amino-terminal groups are blocked either through cyclization or acetylation. The quantitative results lend further support to the polypeptide chain being about 80,000 daltons. Table 8 summarizes the subunit molecular weight determinations by the various methods.

Table 8. Summary of subunit molecular weight determinations by various methods.

Method	M. W. gm/mole
Sedimentation equilibrium in 6.2 M GuHCl (ϕ'=0.708-0.718)	71,400-75,900 ± 1,600
Gel filtration in 6 M GuHCl	70,000 ± 16,000
SDS-acrylamide gel electrophoresis	78,000 ± 2,000
N-terminal analysis	82,000 ± 18,000

We are now faced with the problem of relatedness between the arthropod and molluscan hemocyanin. Ghiretti-Magaldi et al.,(1966) have shown the amino acid composition of the arthropod hemocyanin to be very similar to those for the molluscan hemocyanin (see Table 4). It is now quite clear that C. magister hemocyanin contains one oxygen binding site per polypeptide chain of 78, 000 daltons (Ellerton et al., 1970). Studies on the 5 S particles (78, 000 daltons) at pH 10. 6 indicate that this unit can bind oxygen. . The most recent evidence for the smallest polypeptide chain of a molluscan hemocyanin B. canaliculatum suggests a weight of 300, 000-320, 000 daltons. On the basis of 50, 000 daltons per oxygen binding site there must be about six such sites per polypeptide chain. This is difficult to rationalize on the basis of gene duplication since four native units of 78, 000 daltons would make a 310, 000 daltonunitof 6 active sites. Circular dichroism data indicate fundamental differences in the oxygen binding site for arthropod and molluscan hemocyanin (Nickerson and Van Holde, 1971). This along with the weight per binding site suggests the two types of hemocyanin have evolved along parallel lines.

Clearly the question of relatedness must await the sequencing of the hemocyanin. In the case of at least one arthropod, C. magister,

the fundamental polypeptide chain weight is about 78, 000 daltons.[4/] Thus the 16 S component of C. magister must be composed of six similar polypeptide chains, each with an oxygen binding site.

[4/] Another arthropod, Calianassa has been shown to have a polypeptide chain of molecular weight 75, 000-80, 000 daltons (Roxby, 1972).

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APPENDIX

APPENDIX

Abbreviations

CPM	Counts Per Minute
DNP	Dinitrophenyl
DPM	Disintegrations Per Minute
FDNB	1-Fluoro-2, 4-Dinitrobenzene
GuHCl	Guanidine Hydrochloride
Hecy	Hemocyanin
SDS	Sodium Dodecyl Sulfate
BSA	Bovine Serum Albumin
OVAL	Ovalbumin
Mb	Myoglobin
V_e	Elution Volume
DMSO	Dimethylsulfoxide
I	Ionic Strength