Amino Acid Composition, Amino-Terminal Analysis, and Subunit Structure of *Cancer magister* Hemocyanin†

Donald E. Carpenter‡ and K. E. Van Holde*

ABSTRACT: The size of the polypeptide chains of the hemocyanin from the Dungeness crab, *Cancer magister*, has been determined in a number of ways: (1) Sedimentation equilibrium in concentrated guanidine hydrochloride (Gdn·HCl), with or without reducing agents, indicates a homogeneous or nearly homogeneous material with $M_w \cong 77{,}000$. (2) Gel filtration in 6 M Gdn·HCl yields a single peak, corresponding to a molecular weight of about 70,000. (3) Quantitative amino-terminal analysis shows 1 aspartic end group/80,000 molecular weight. (4) High-resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis indicates that there are two chains, of molecular weights about 76,000 and 83,000 daltons, present in roughly equal quantities. Since no smaller components are shown in any of these experiments, we conclude that these represent the polypeptide chains from which the hemocyanin aggregates are constructed. The most satisfactory model for all of the results involves two nearly equal chains, with molecular weights in the neighborhood of 75,000 and 80,000, respectively. These must have the same end group. Dissociation of the 16S hexamers and 25S dodecamers found in the hemolymph, at pH >10, yields functional 5S particles that correspond to the individual chains. Reassociation can be carried out readily to the 16S stage, but only small amounts of 25S particles are formed. Polyacrylamide gel electrophoresis reveals no difference between the 5S subunits obtained from native and reassociated 16S particles nor any difference between native and reassociated 16S particles. Possible effects of subunit heterogeneity on the reassociation process are discussed.

Although hemocyanin is used as an oxygen carrier by both molluscs and arthropods, many differences between the two kinds of hemocyanin have been found. Electron microscopy (Levin, 1963a–c; Van Bruggen *et al.*, 1963; Fernandez-Moran *et al.*, 1966) and sedimentation analysis (Eriksson-Quensel and Svedberg, 1936; see also Van Holde and Van Bruggen, 1971) indicate different patterns of subunit aggregation. Further, circular dichroism data (Nickerson and Van Holde, 1971) indicate fundamental differences in the oxygen-binding site between molluscan and arthropod hemocyanins. A fundamental, unresolved question is: what are the polypeptide chain weights in these proteins? Even though the amino acid compositions are quite similar (Ghiretti-Magaldi *et al.*, 1966) copper analyses suggests a minimum polypeptide weight of 25,000 daltons for molluscan hemocyanin and about 38,000 daltons for arthropod hemocyanin. Attempts to dissociate molluscan hemocyanins by denaturing solvents, high pH, etc. generally yield values in the range of 230,000–500,000 daltons (Van Holde and Cohen, 1964; Konings *et al.*, 1969a,b). In apparent contradiction to this, components as small as 25,000 daltons have been reported for *Helix pomatia* (Dijk *et al.*, 1970) and *Octopus vulgarus* hemocyanin, when samples were treated with 70% formic acid (Salvato *et al.*, 1972).

A 5S subunit of arthropod hemocyanin is observed at pH values greater than 10. Molecular weight estimates for this component fall between 68,000 and 90,000 depending on the species (Di Giamberardino, 1967; Moore *et al.*, 1968; Ellerton *et al.*, 1970). But the existence of smaller units of 25,000 daltons (Salvato *et al.*, 1972) and 35,000 daltons (Pickett *et al.*, 1966) has been claimed for *Carcinus maenas* and *Homarus americanus*, respectively. In brief, the question of the size of the polypeptide chains in hemocyanins is still confused.

In our earlier studies (Ellerton *et al.*, 1970) we found the smallest functional unit of *Cancer magister* hemocyanin to have, at pH 10.6, a sedimentation velocity of $s^0_{20,w} = 5.7$ S. High-speed sedimentation equilibrium experiments under these conditions indicated that the material was nearly homogeneous, with a weight-average molecular weight of about 78,000 over most of the cell, but with a decrease to about 68,000 at the meniscus. This suggested to us that there might be some tendency toward dissociation to smaller subunits. Accordingly, we decided to attempt to determine the polypeptide chain weights by a number of chemical and physical techniques.

While this work was being prepared for publication, we learned of some recent sodium dodecyl sulfate acrylamide gel electrophoresis studies of *C. magister* hemocyanin by Loehr and Mason (1971, 1973). They report two chains, one about 76,000, the other about 84,000. As will be shown, we have confirmed this result, and demonstrate that this is consistent with a variety of other chemical and physical measurements. The evidence is now strong that no smaller chains are present.

Perplexing results have been obtained in attempts to reassociate the 5S subunits of arthropod hemocyanins to the larger aggregates that are found in the hemolymph. For example, *Cancer magister* hemocyanin occurs *in vivo* mainly in the form of a 25S dodecamer of the 5S particles, together with a small amount of 16S hexamers. But after dissociation, reversion to conditions where the 25S particles are stable leads to almost quantitative formation of 16S particles, with only a small fraction of 25S component (Ellerton *et al.*, 1970).

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received February 14, 1973.* Supported by a Public Health Service Grant (No. HE-12326). D. E. C. was the recipient of a Public Health Service traineeship. Taken in part from a thesis submitted by D. E. C. for partial fullfillment of the requirements for the Doctor of Philosophy degree, Department of Biochemistry and Biophysics, Oregon State University, 1972.

‡ Present address: Veterans Administration Hospital, Dallas, Texas 75216.

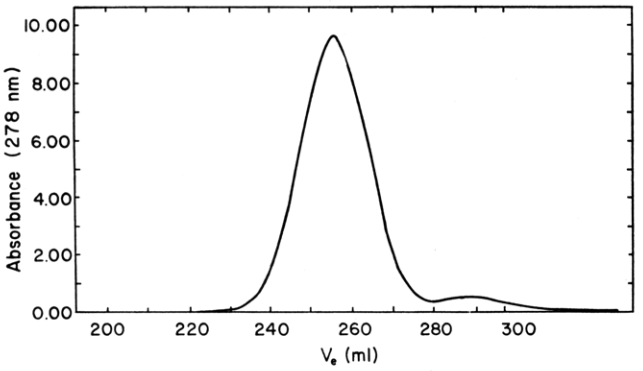

FIGURE 1: Elution pattern of hemocyanin from Bio-Gel A-5M equilibrated with Tris buffer, pH 7.07, 0.1 *I* and 0.01 M $MgCl_2$. Hemocyanin (38 mg/ml and 3.5-ml total volume) was layered onto the column. The large peak is 25S hemocyanin and the trailing peak is 16S hemocyanin.

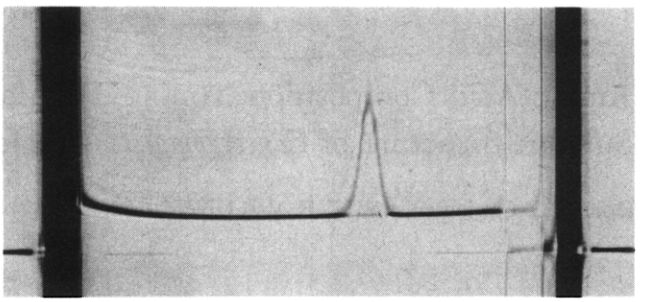

FIGURE 2: Sedimentation diagram of *C. magister* hemocyanin after purification through a Bio-Gel A-5M column. Sample sedimented for 24 min at a rotor velocity of 40,345 rpm, at 22°. Phase-plate angle 65°, concentration approximately 3 mg/ml in 0.1 *I* Tris buffer (pH 7.0), 0.01 M Mg^{2+}.

Experimental Section

Preparation of Hemocyanin. The hemocyanin was prepared as described earlier (Ellerton *et al.*, 1970) but with the following difference: when a pure 25S or 16S hemocyanin sample was desired, the mixture containing both 25S and 16S was separated on a Bio-Gel A-5M (200–400 mesh)[1] column (2.5 × 100 cm) equilibrated with 0.1 *I*[2] Tris (pH 7.0) and 0.01 M in $MgCl_2$. Figure 1 shows a typical elution pattern from the Bio-Gel A-5M column and Figure 2 shows the schlieren pattern of the purified 25S species. Sedimentation equilibrium showed the 25S species to be homogeneous, with a molecular weight of 939,000 daltons from the slope of the ln *c vs.* Δr^2 plot. This is in excellent agreement with our earlier results (Ellerton *et al.*, 1970). A plot of $M_w{}^a$ *vs.* concentration showed the sample to be only slightly nonideal. Electrophoresis of the purified 25S and 16S species at pH 7.5 gave one band for each.

For all the work presented in this paper the starting material was purified in this manner.

Polyacrylamide Gel Electrophoresis of Native Hemocyanin. An electrophoresis technique similar to that described by Hjerten *et al.* (1965) was used. At pH 9.9, 0.1 *I* bicarbonate buffer was used while at pH 7.5, 0.1 *I* Tris buffer, 0.01 M Mg^{2+} was used. The gels (7.5%) had a ratio of acrylamide to *N,N'*-methylenebisacrylamide of 30:1. About 10–20 μg of protein was applied to each gel and migration was in the direction of the cathode.

All reagents were of analytical quality. The acrylamide was recrystallized from chloroform and *N,N'*-methylenebisacrylamide was recrystallized from acetone.

Amino Acid Analysis. The amino acid analysis was carried out according to Spackman *et al.* (1958). Cysteine and cystine were determined as cysteic acid by treating 0.3 mg of protein with 30 μl of dimethyl sulfoxide and 3 ml of constant-boiling HCl (Spencer and Wold, 1969). Hydrolysis time was 21 hr.

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

Tryptophan, which was destroyed under the hydrolytic conditions used, was determined by spectrophotometric means in 6 M Gdn·HCl solution (Edelhoch, 1967).

Qualitative Amino-Terminal Analysis. The Nbs_2 method described by Fraenkel-Conrat *et al.* (1955) was used for the amino-terminal analyses. Hydrolysis was carried out using constant-boiling HCl in an evacuated and sealed tube at 110°. Times of hydrolysis were varied between 4 and 24 hr. In addition a 4-hr hydrolysis in concentrated HCl was performed to check for the presence of Dnp-glycine and Dnp-proline. Another hemocyanin sample was oxidized with performic acid (Sanger and Thompson, 1953) and the aqueous phase carefully examined for Dnp-cysteic acid.

Quantitative Amino-Terminal Analyses. The method used was basically that described by Beale and Whitehead (1962) and Dijk *et al.* (1970). The [^{14}C]Dnp-amino acids were made as described by Levy (1954) and were found to be chromatographically pure. The specific activity of the [^{3}H]Nbs_2 was determined by reaction with a standard solution of aspartic acid using [^{14}C]Dnp-aspartic acid as a control. This was checked using a standard solution of leucine. The same specific activity was obtained using a standard solution of either aspartic acid or leucine.

About 200 μg of hemocyanin was reacted with a 100× molar excess of [^{3}H]Nbs_2 (based on 78,000 g/mol). The reaction was allowed to proceed for 3 hr at room temperature after which the excess Nbs_2 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 and concentrated HCl was added to make the solution 6 N in HCl. The sample, sealed in an evacuated ampoule, was hydrolyzed at 110° for times between 4 and 24 hr. Extraction and evaporation were done in the usual manner. Chromatography was performed using the *tert*-amyl alcohol–phthalate, pH 6, system. The "toluene" and 1.5 or 2 M phosphate chromatographic systems were not used for the quantitative end-group determination because of the insolubility of phosphate in the scintillation fluor. Higher amounts of water required to solubilize the phosphate gave a very low counting efficiency for tritium. The spot corresponding to the amino-terminal derivative was excised and eluted three times with 2 ml of water. The solution was freed of fibers by filtration and evaporated to dryness in a scintillation vial at 37° under vacuum. Water (200 μl) was added to solubilize the Dnp-amino acid and 13 ml of Aquasol (New England Nuclear) scintillation fluor was added prior to counting. Counting was

[1] When run under the same conditions using 100–200 mesh gel A-5m, one asymmetric peak was obtained.

[2] Abbreviations used are: *I*, ionic strength; Nbs_2, fluoro-2,4-dinitrobenzene; Dnp, dinitrophenyl-; Gdn·HCl, guanidine hydrochloride; Temed, tetramethylenediamine.

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 had been calibrated by the National Bureau of Standards.

The labeled reagents, [U-^{14}C]1-fluoro-2,4-dinitrobenzene and [3,5-^{3}H]1-fluoro-2,4-dinitrobenzene, were obtained from Amersham/Searle Corp.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. The sodium dodecyl sulfate polyacryalmide gel electrophoresis experiments were performed in two ways. SYSTEM 1. Initial experiments were done by the procedure of Weber and Osborn (1969). Hemocyanin samples were made 1% in sodium dodecyl sulfate, 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° for 2 min or incubating at 37° for 2 hr.

Following the pretreatment the samples were dialyzed against 0.1% sodium dodecyl sulfate, 0.1% β-mercaptoethanol, and 0.1 M phosphate (pH 7) for several hours. Usually 5–15 μg of protein was applied to the 10% gels.

Protein standards used to calibrate the gels were myosin (gift from Dr. W. Harrington, 212,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons, Sigma Chemical Co.), and myoglobin (17,100 daltons, Mann Research Laboratory).

SYSTEM 2. After we learned of the results of Loehr and Mason (1971) we carried out further experiments using their procedures at pH 8.0. The gel solutions contained 0.35 M Tris-sulfate, 8% acrylamide, 0.2% bisacrylamide, 0.06% Temed, 0.05% ammonium persulfate, 0.2% sodium dodecyl sulfate, and 0.001 M EDTA. The tank buffer contained 0.03 M Tris-acetate, 0.2% sodium dodecyl sulfate, and 0.001 M EDTA. Proteins were dissolved in 0.03 M Tris-sulfate, 40% sucrose, 1% sodium dodecyl sulfate, and 1% mercaptoethanol, and heated 10 min at 65°. Electrophoresis was carried out in a water cooled (11°) slab with 14 channels, at 200 mA for about 6 hr. The slab was stained with Coomassie Blue, by the technique of Fairbanks *et al.* (1971).

Gel Filtration on a 6 M Gdn·HCl–6% Agarose Column. The method used was that described by Fish *et al.* (1969). Blue dextran (Sigma Chemical Co.), a marker for the void volume (V_0), was fractionated prior to use. Dnp-glycine was used as the marker for the measurement of solvent volume. The proteins used in calibration of the column were bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), and lysozyme (17,100 daltons). All were obtained from Sigma Chemical Co. The proteins were reduced and alkylated in the presence of Gdn·HCl as described under sedimentation studies and dialyzed prior to layering on the column. Calibration runs were made before and after the hemocyanin runs. All tubes were weighed before and after sample collection to facilitate obtaining the correct elution position. The column dimensions were 90 × 1.5 cm.

Sedimentation Studies. SAMPLE PREPARATION. Protein (0.25–2 mg/ml) was dialyzed against 6.2 M Gdn·HCl (pH 4–5). If reducing agent was present (0.1 M β-mercaptoethanol or 0.05 M dithiothreitol), the protein was dialyzed into 6.2 M Gdn·HCl (pH 7–8). For alkylated protein samples, the protein was first reduced at pH 8.5 for 4 hr and alkylated with iodoacetate and the pH reduced to 6 prior to dialysis and centrifugation. For several samples the Gdn·HCl concentration was raised to 7.5 M, but the sample preparation remained the same. In all cases samples were dialyzed at least 3 days.

Solvent densities were determined by refractometric measurements (Kawahara and Tanford, 1966). The Gdn·HCl was purchased from Heico, Inc. The iodoacetate (Eastman Organic Chemicals) was recrystallized from petroleum ether.

SEDIMENTATION. Sedimentation equilibrium was carried out using the high-speed technique of Yphantis (1964) or the overlay technique as described by Chervenka (1970). The ultracentrifuge was aligned according to recently published procedures (Richards *et al.*, 1971a,b). It was focused at the two-thirds level for sapphire windows. Aluminum- or charcoal-filled epon double-sector centerpieces with sapphire windows were employed. To facilitate matching of the menisci for the Yphantis technique, a microliter syringe with a Cheney adapter was used. All runs with a meniscus mismatch were terminated. Equilibrium was assumed when no fringe change occurred over a several hour period. Water base lines were obtained after each run.

DATA ANALYSIS. Interference patterns (five fringes per frame, 40–65 radial positions) were read on a Nikon 6-C comparator equipped with a digitizer (L. and W. Electronics) which recorded on punched paper tape the data to be analyzed. Intermediate plots of ln C *vs.* Δr^2 were obtained on a Hewlett-Packard X–Y plotter before proceeding with analysis. The data were analyzed with a program written by Dr. R. D. Dyson. The program performs a point by point least-squares quadratic fit of the ln C *vs.* Δr^2 data. Two to five points on either side of the central point were used for the span. This enables the calculation of the apparent weight-, number-, and z-average molecular weights, M_w^a, M_n^a, and M_z^a, as a function of concentration in the cell.

The reciprocals of these average molecular weights were plotted against concentration and extrapolated to $C = 0$ according to the following equations

$$1/M_n^a = 1/M_n + 0.5BC \quad (1)$$

$$1/M_w^a = 1/M_w + BC \quad (2)$$

By using the expression for M_z^a from Van Holde *et al.* (1969)

$$M_z^a = M_z\left(\frac{M_w^a}{M_w}\right)^2 \quad (3)$$

and for $1/M_w^a$, eq 2, one obtains

$$1/M_z^a = 1/M_z + 2\frac{M_w}{M_z}BC + \left(\frac{M_w}{M_z}\right)^2 B^2C^2 \quad (4)$$

Thus for moderate concentrations and homogeneous solutions one expects a limiting slope of $2B$ for a graph of $1/M_z^a$ *vs.* C (see Munk and Cox, 1972; Roark and Yphantis, 1969).

Results

Dissociation and Reassociation of Hemocyanin. Hemocyanin, purified as described above, when run on acrylamide gel, pH 7.5 (0.1 I Tris, 0.01 M Mg^{2+}), gave one band for the pure 25S species and on a separate gel gave one band for the purified 16S species. When the samples were dialyzed against 0.1 I bicarbonate buffer, pH 9.93 (no Mg^{2+}), sedimentation velocity showed only 5S subunits to be present. The same

TABLE I: Amino Acid Composition of Hemocyanin from *C. magister*.[a]

Amino Acid	μmol Hours of Hydrolysis 20	40	67	140	Av[b]	Wt %	Amino Acid Residues/ 78,000 g of Protein	Nearest Integer/ 78,000 g
Lysine	0.0359	0.0367	0.0350	0.0368	0.0361	5.24	31.9	32
Histidine	0.0459	0.0481	0.0455	0.0462	0.0464	7.20	40.9	41
Ammonia	0.0845	0.0902	0.0912	0.0956				
Arginine	0.0353	0.0364	0.0333	0.0360	0.0352	6.22	31.1	31
Aspartic acid	0.103	0.116	0.109	0.105	0.108	14.07	95.4	95
Threonine	0.0371	0.0375	0.0342	0.0265	0.0411c	4.71	36.3	36
Serine	0.0404	0.0362	0.0318	0.0206	0.0450c	4.05	36.3	36
Glutamic acid	0.0736	0.0777	0.0770	0.0766	0.0771	11.27	68.1	68
Proline	0.0366	0.0430	0.0366	0.0337	0.0356	3.92	31.5	32
Glycine	0.0439	0.0458	0.0397	0.0450	0.0449	2.90	39.6	40
Alanine	0.0421	0.0442	0.0426	0.0432	0.0430	3.46	38.0	38
Cysteine					0.00036	0.428	2.6	3
Valine	0.0500	0.0547	0.0503	0.0539	0.0543d	6.09	47.9	48
Methionine	0.0188	0.0117		0.0167	0.0178d	2.65	15.8	16
Isoleucine	0.0330	0.0353	0.0328	0.0337	0.0337	4.31	29.7	30
Leucine	0.0550	0.0450	0.0542	0.0555	0.0552d	7.07	48.7	49
Tyrosine	0.0313	0.0296	0.158	0.0287	0.0332e	6.14	29.3	29
Phenylalanine	0.0456	0.0512	0.0435	0.0429	0.0449	7.33	38.8	39
Tryptophan					0.0139	2.93	12.3	12

[a] A small amount of hexosamine was present in all hydrolysates. [b] Data were averaged, extrapolated to zero time (*c*), or value at maximum recovery used (*d*). Half-cystine was determined as cysteic acid after oxidation by Me_2SO. Tryptophan was determined spectrophotometrically by the method of Edelhoch (1967). [e] Value at 67 hr not used in average.

mobility (within 1%) was obtained for 5S subunits prepared using either the 16S or 25S species as the starting material. When the solution was dialyzed back to pH 7.5, 0.01 M Mg^{2+}, sedimentation velocity showed mostly a 16S component with a small amount of 25S and a small amount of more slowly sedimenting material. Electrophoresis in the same buffer system gave four bands: one with the same mobility as the 25S, and second with the same mobility as the 16S, and two faint faster moving bands. When the sample at pH 9.93 (5S from sedimentation velocity) was dialyzed to pH 7.5 (no Mg^{2+}) sedimentation velocity showed mainly 16S species with some smaller particles remaining. Electrophoresis under these conditions gave three bands: the main band corresponding to 16S (95%) and two faint faster moving bands. The 16S as purified from the column had the same mobility (within 4%) as the main band from the reassociated hemocyanin. The 16S species reassociated from what had been pure 25S starting material had the same mobility as the 16S starting material. Figure 3 depicts graphically the results of these experiments.

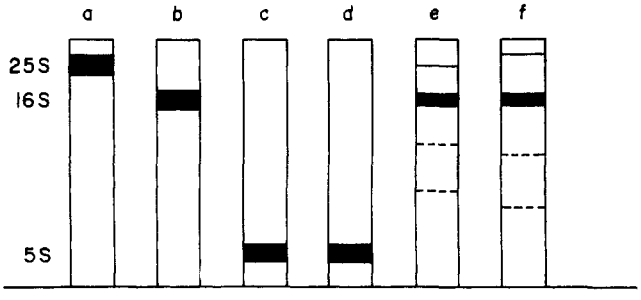

FIGURE 3: Polyacrylamide gel electrophoresis of hemocyanin. The S values along the left-hand side were the approximate values found by sedimentation velocity prior to electrophoresis. About 10–20 μg were loaded on each gel. Top is the anode. Electrophoresis is from top to bottom: (a) pure 25S, (b) pure 16S, (c) 25S sample dissociated to 5S subunits at pH 9.9, (d) 16S sample dissociated to 5S subunits at pH 9.9, (e) 5S subunits from c reassociated at pH 7.5, (f) 5S subunits from d reassociated at pH 7.5.

Amino Acid Analysis. Table I presents the amino acid composition data for *C. magister* hemocyanin. The amino acid composition is very similar to that of other hemocyanins (Ghiretti-Magaldi *et al.*, 1966). Aspartic and glutamic acid (probably partially in the amide forms) constitute a major fraction of the residues (about 25%), but there is only a small amount of cysteine; about 3 mol is found per 78,000 g of protein. The partial specific volume as calculated from amino acid analysis and residue volumes was found to be 0.725 ml/g (Cohn and Edsall, 1943). This is in excellent agreement with the value for the native 25S species as found from density measurements, 0.728 g/cm[3] (Ellerton *et al.*, 1970).

Qualitative Amino-Terminal Analysis. The only amino-terminal Dnp derivative found in the ether extracted phase was Dnp-aspartic acid. No amino-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.[3] Hydrolysis with concentrated HCl for 4 hr

[3] 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 ε-Dnp-lysine. With *C. magister* hemocyanin all such staining attempts proved to be negative.

gave no evidence for Dnp-glycine or Dnp-proline. No Dnp-cysteic acid was found from the performic acid oxidized protein. Dnp-aspartic acid and Dnp-glutamic acid 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-amino-terminal amino acid ran with the Dnp-aspartic acid standard which was well separated from the Dnp-glutamic acid standard. This was confirmed by chromatography in the "toluene" and 2.0 M phosphate system.

Quantitative Amino-Terminal Analysis. After 4-hr hydrolysis only 0.3 mol of end group was released per 78,000 g of protein. But after 8 hr, hydrolysis was essentially complete and an average of five experiments gave 0.95 mol of Dnp-aspartate/78,000 g of protein.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. SYSTEM 1. At pH 7.0 in 0.1 M phosphate, several experiments gave, in each case, only one band corresponding to a molecular weight of 78,000 daltons. No heavy material was left at the top of the gel and no smaller molecular weight material was observed (see Figure 4A). No difference was observed whether the sample was heated at 90° for 2 min or incubated for 2 hr at 37°.

SYSTEM 2. At pH 8.0, in 0.35 M Tris, two bands were always observed, in agreement with the results of Loehr and Mason (1971, 1973). As Figure 4B shows, these bands were well resolved, and appear to be of nearly equal intensity. Very similar patterns were obtained from a purified 25S fraction, from a fraction containing about 70% 16S, 30% 25S, and from a mixture of the two. Our results thus not only confirm but also extend those of Loehr and Mason who worked with hemocyanin purified by preparative ultracentrifugation, which does not lead to complete separation of the 25S and 16S components. The molecular weights, as judged from the mobilities relative to standards, were about 76,000 and 83,000, in good agreement with the results of Loehr and Mason (1972). We should be somewhat cautious, however, in interpreting these mobility differences as molecular weight differences since hemocyanins are glycoproteins, and glycoproteins are known to sometimes behave anomalously in such experiments. However, *C. magister* hemocyanin contains only about 1% carbohydrate (Loehr and Mason, 1973).

In this case as well, no smaller or larger components were observed, even on heavily loaded gels.

Gel Filtration on 6 M Gdn·HCl–Agarose Column. Purified hemocyanin eluted as a single, sharp symmetrical peak when passed through a 6% agarose column in the presence of 6 M Gdn·HCl. The position at which the hemocyanin was eluted corresponds to a molecular weight of 70,000, as judged from the elution position of standards. With our 6% agarose column a very small error in the elution position (in the higher molecular weight range) makes a large error in the molecular weight. Thus the value of 70,000 must be regarded as approximate.

Sedimentation Equilibrium in Gdn·HCl. A fundamental difficulty in obtaining absolute molecular weight values from sedimentation equilibrium experiments in Gdn·HCl arises from the preferential interaction of Gdn·HCl with proteins. As Casassa and Eisenberg (1964) have shown, this can be formally handled by replacing the anhydrous partial specific volume with an apparent specific volume, defined as

$$\phi' = \frac{1}{\rho^0}\left[1 - \frac{\Delta\rho}{C}\right] \qquad (5)$$

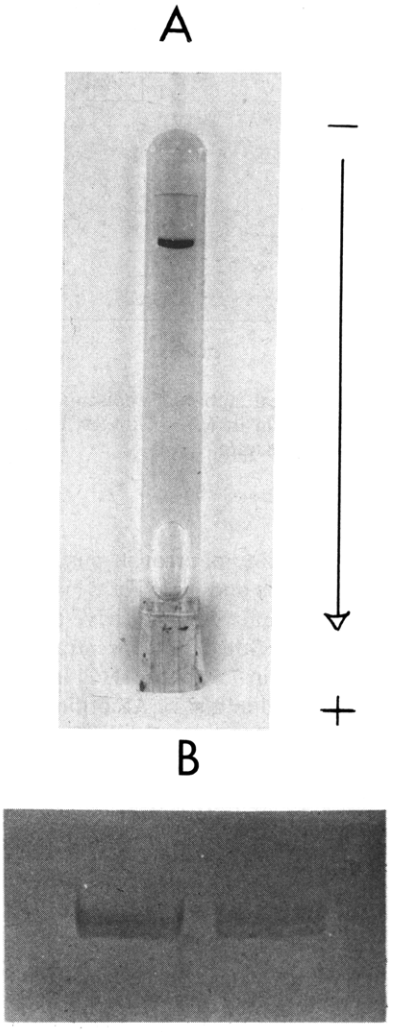

FIGURE 4: (A) Photograph of hemocyanin subunits on a sodium dodecyl sulfate polyacrylamide gel (system I). A loading concentration of 13 μg was used. The cathode is at the top. Direction of electrophoresis is from top to bottom. (B) Enlarged photograph of part of a sodium dodecyl sulfate polyacrylamide gel slab (system II). The cathode is above the top of the photograph. On the left is purified 25S material, on the right a fraction containing 70% 16 S, 30% 25 S. The doublet character is typical of results with *Cancer* hemocyanin in this system.

where $\Delta\rho$ is the difference in density between the solution and the solvent (of density ρ_0) with which it is in dialysis equilibrium. However, it is difficult to make the necessary measurements with the precision which is wanted, especially since a 1% error in ϕ' leads to about 4% error in molecular weight. We have previously reported (Ellerton *et al.*, 1970) values of ϕ' for *C. magister* hemocyanin in 6.2 M Gdn·HCl. These values, which averaged about 0.700 ml/g, are subject to correction, for we have found that the protein concentrations were incorrectly determined. Correcting for the change in extinction coefficient which occurs when hemocyanin is dissolved in 6 M Gdn·HCl instead of buffer, we now find $\phi' = 0.708$. This value is still appreciably lower than the value of about 0.73 found for *C. magister* hemocyanin in buffer solutions (Ellerton *et al.*, 1970). Reisler and Eisenberg (1969) and Hade and Tanford (1967) have found that ϕ' for many proteins in 6.0 M Gdn·HCl is between 0.01 and 0.02 ml per g smaller than in aqueous buffer.

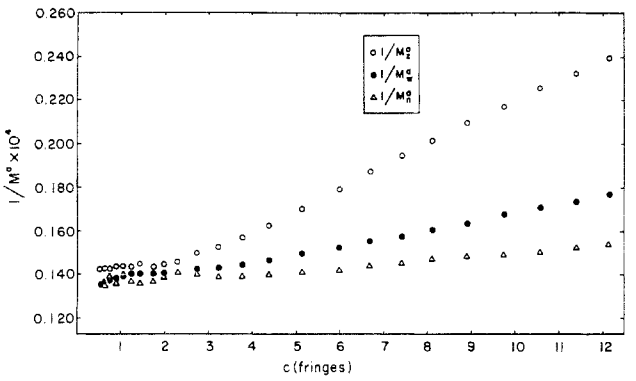

FIGURE 5: The reciprocal apparent molecular weights *vs.* concentration for hemocyanin in 6.2 M Gdn·HCl–0.1 M β-mercaptoethanol. ϕ' used was 0.718 ml/g.

TABLE II: Sedimentation Equilibrium Results[a] in Gdn·HCl.

Gdn·HCl (M)	No. of Expt	ϕ' (ml/g)	$M_n \times 10^{-3}$	$M_w \times 10^{-3}$	$M_z \times 10^{-3}$	$(2M_n - M_w) \times 10^{-3}$
6.2	8	0.708	68.9	71.4	74.9	67.0
		0.718	73.2	75.9	79.6	72.5
7.5	3	0.728	76.4	77.4	74.4	75.2

[a] Values have been averaged. Since results in the presence of reducing agent or with alkylated SH groups are not distinguishable, all have been included in the averages. The standard deviations in the different sets of experiments average about 2.0×10^3 dalton for M_n, 1.6×10^3 dalton for M_w, 3.3×10^3 dalton for M_z, 3.0×10^3 dalton for $2M_n - M_w$.

Realizing the possibility of error in our ϕ' measurements, we have used both 0.708 and 0.718 for ϕ' in 6.0 M Gdn·HCl. Furthermore, Reisler and Eisenberg have demonstrated that ϕ' may depend upon Gdn·HCl concentration, and that at least in some cases, ϕ' in 7.5 M Gdn·HCl is indistinguishable from the value obtained in buffer. Accordingly, we have used a value of $\phi' = 0.728$ for 7.5 M Gdn·HCl experiments. The effect of these various choices is seen in Table II. It is clear that because of the ambiguity in ϕ', the absolute accuracy of our results is less than the precision.

All the experiments in concentrated Gdn·HCl solutions were characterized by downward curvature in the ln C *vs.* Δr^2 plot. This is due to the nonideality in Gdn·HCl and is expected. This downward curvature along with scatter in the data points may tend to mask heterogeneity. A clearer picture is obtained by graphing the reciprocals of the apparent average molecular weights *vs.* concentration, as suggested by eq 1–4. For a nonideal homogeneous solution a straight line with a positive slope is expected for plots of $1/M_w^a$ or $1/M_n^a$ *vs.* concentration; at low concentrations, a graph of $1/M_z^a$ *vs.* C should also be linear. Heterogeneity introduces substantial curvature into such graphs in the low concentration region and reduces the overall slope.

Typical results from our experiments are shown in Figures 5 and 6, which represent data obtained in 6.2 and 7.5 M Gdn·HCl, respectively. It will be noted that in every case the graphs are nearly linear, except that there is some deviation at low concentrations (corresponding to points near the meniscus). This could easily be explained as reflecting a slight heterogeneity: If one simply takes the limiting value from the curves, in Figure 5 for example, a molecular weight of about 72,000 is indicated. On the other hand, linear extrapolation of the data from the high concentration region to $C = 0$ yields $M \cong 80{,}000$. While the data could thus be said to support the existence of two slightly different components, the deviations from linearity are scarcely greater than we frequently observe and attribute to experimental error. Thus, straight lines can be drawn through each set of data points in Figures 5 and 6, yielding in each case molecular weights of about 75,000, with no deviations of points from the lines of more than a few per cent. The best that can be said, then, is that the data are *consistent* with either a homogeneous substance of molecular weight about 75,000, or with a mixture of two or more species with molecular weights lying closely about this mean.

As a further test of the homogeneity, or near homogeneity of the samples, the quantity $2M_n^a - M_w^a$ was calculated for several experiments. Such data are shown in Figure 6B and Figure 7. The fact that nearly horizontal lines are obtained indicates that most of the variation in apparent molecular weights arises from the nonideality, which approximately cancels in the quantity $2M_n^a - M_w^a$ (Yphantis, 1964).

Table II summarizes the data for the various molecular weight averages extrapolated to $C = 0$. Regardless of the choice of ϕ', the values lie almost entirely in the range 70,000–80,000, and indicate little, if any, heterogeneity. Values of the second virial coefficient were also obtained. Approximately the same values were obtained from $1/M_n^a$, $1/M_w^a$ and $1/M_z^a$ graphs, and averaged about 4×10^{-7} mol/g·fringe.

Experiments were conducted both with and without the addition of mercaptoethanol, and in some cases on reduced, alkylated protein, at both Gdn·HCl concentrations. Addition of the reducing agent or alkylation had no significant effect on the results, as is demonstrated in Figure 7.

In summary, all of these sedimentation equilibrium data are consistent with a homogeneous, or nearly homogeneous, substance of molecular weight in the range 70,000–80,000. The data would be consistent with a model which involves equal numbers of two kinds of chains in this general weight range, as is suggested by the sodium dodecyl sulfate gel electrophoresis experiments.

Discussion

Despite our earlier expectations, and reports of results with other arthropod hemocyanins that might suggest the contrary, we feel that the evidence presented here definitely establishes that the polypeptide chains of *C. magister* hemocyanin are no smaller than 70,000–80,000 daltons. It would appear from the high resolution sodium dodecyl sulfate gel experiments that there are two kinds of chains, differing in weight by a few thousand daltons, and present in roughly equal quantities. The band doubling cannot represent an artifact of the electrophoresis, since Loehr and Mason report that each component when excised from the gel and reelectrophoresed ran as a single band. Our use of purified 25S and partially purified 16S materials shows that the two sodium dodecyl sulfate gel components do not correspond to these two structural components. Finally, Loehr and Mason have carried out electrophoresis on samples from single animals, with the same result. This rules out the possibility of genetic variability within the population as an explanation. We are left with the conclusion that *C. magister* hemocyanins are built up from two kinds of

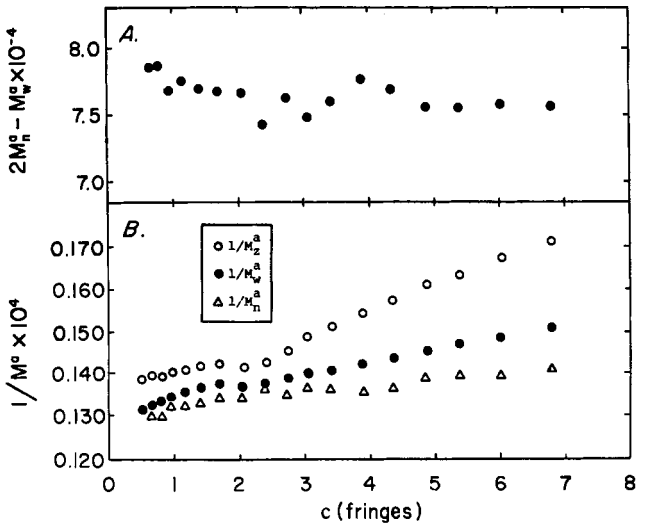

FIGURE 6: Sedimentation equilibrium data for hemocyanin in 7.5 M Gdn·HCl. Part A gives the $2M_n^a - M_w^a$ values. The ϕ' used in calculation was 0.728 ml/g. Part B gives the reciprocal apparent molecular weight averages.

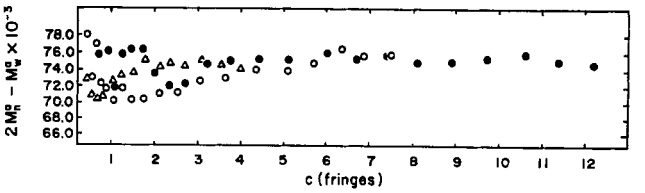

FIGURE 7: The data for three separate experiments expressed as graphs of $2M_n^a - M_w^a$ *vs. c*. All were 6.2 M Gdn·HCl: (○) hemocyanin, reduced and alkylated with iodoacetate, rotor velocity 34,000 rpm; (○) no reducing agent present, rotor velocity 30,219 rpm; (●) 0.1 M β-mercaptoethanol present, rotor velocity 28,160 rpm. The ϕ' used in calculation was 0.718 ml/g.

polypeptide chains. Both chains must have the same N-terminal group, aspartic acid. Furthermore, since resolution is not obtained in polyacrylamide gel electrophoresis at pH 9.9, the chains cannot differ greatly in charged groups. A two-chain model is certainly consistent, within the expected error of measurement, with the results of the Gdn·HCl column and Gdn·HCl sedimentation equilibrium data presented herein. The model is at least roughly consistent with our previous sedimentation equilibrium studies at pH 10.6. There we found a molecular weight which varied from about 68,000 to 78,000 across the cell. While this variation is slightly different than would be expected if the weights of the two chains were 76,000 and 83,000, any discrepancy depends largely upon the most imprecise data, that obtained from the meniscus region. We do not believe that the data available so far allow one to specify the chain weights better than 75,000 ± 5000 and 80,000 ± 5000. The uncertainty in the best of the methods (sedimentation equilibrium) becomes greater than the usual ±3% (Yphantis, 1964) when two such components are involved.

These results require that we reconsider published reports that claim the existence of smaller subunits of arthropod hemocyanins, keeping in mind that there may be fundamental species differences.

First, we must consider the results of Pickett *et al.* (1966). Sedimentation velocity studies of hemocyanin from *H. americanus* showed a drop in sedimentation coefficient from 4S for the native protein to 2.3S for the succinylated protein. But this decrease in S can probably be attributed to an increase in the frictional coefficient due either to expansion or unfolding of the hemocyanin molecule (Habeeb 1967). Peptide mapping of the *H. americanus* hemocyanin (Pickett *et al.*, 1966) gave a number of peptides consistent with a chain of 35,000 daltons. However, one could also have a single chain with a duplicated region, as in α-globulin (Hill *et al.*, 1966; Singer and Doolittle, 1966). In this case one would obtain about half the polypeptide spots expected.

End-group analysis on the hemocyanin from *Carcinus maenas* indicates two dissimilar polypeptide chains (Ghiretti-Magaldi *et al.*, 1970). The results, however, were not quantitated. Sodium dodecyl sulfate gel filtration of the acetylated hemocyanin from *C. maenas* indicates subunits with molecular weights of 25,000, 50,000, and 75,000 daltons (Salvato *et al.*, 1972). From their results, it is not clear whether the smaller components represent an appreciable portion of the protein or if they are a minor component or an artifact.

Other workers (Dijk *et al.*, 1970; Dijk, 1971) have reported smaller fragments in molluscan hemocyanins, which had been treated with strong acids (*e.g.*, 70% formic acid). For *Helix pomatia*, bands corresponding to 25,000 and 50,000 daltons were observed in sodium dodecyl sulfate–urea polyacrylamide gels following such treatment, but most of the material was of higher molecular weight. We too have seen evidence for low molecular weight components in sedimentation equilibrium studies of *C. magister* hemocyanin which had been treated with 70% formic acid or 50% acetic acid. Similarly, a wide variety of low molecular weight components were seen in sodium dodecyl sulfate gel electrophoresis of samples so treated. However, the results were not reproducible, and appeared to depend upon the time and temperature of treatment. It is our opinion that these must represent hydrolytic fragments, produced by the acid hydrolysis of especially sensitive peptide bonds. Any explanation that considers such fragments as natural constituents of the hemocyanin will have to rationalize the complete failure of powerful dissociating agents (Gdn·HCl, sodium dodecyl sulfate, with or without reducing agents) to effect dissociation, as well as the existence of only one chain end per 80,000 daltons.

The existence of two kinds of polypeptide chains in *C. magister* hemocynain may shed light on the difficulty we have experienced in reassociating from 5S to 25S particles. Suppose that a particular arrangement (or set of arrangements) of two kinds of subunits is necessary in the 16S hexamer to allow it to further associate to the 25S stage. If we begin with a mixture of the subunits at high pH, and either by lowering pH or increasing [Mg^{2+}], produce 16S particles, these might well represent a random assortment of the many possible spatial and compositional isomers. In this assortment, only a small fraction (the value depending upon the stringency of the requirements) would be able to further dimerize to yield 25S particles. Qualitatively, this is just what we observe.

The fact that we cannot resolve isomers of 25S or 16S particles on acrylamide gel electrophoresis is not contradictory, for we have also been unable to resolve the 5S subunit types in this way. Apparently, the two chains do not differ significantly in net charge. Further investigation of this idea will require some means of separating polypeptide chains in a manner that retains their native conformation.

In this connection, it is of interest that Busselen (1970) found that the dissociated hemocyanin from *C. meanas* reassociated to give products that were electrophoretically

distinguishable from the native hemocyanin. For this same hemocyanin, two N-terminal groups have been found (Ghiretti-Magaldi *et al.*, 1970). Thus, *C. meanas* may represent a case in which the two types of polypeptide chain differ more from one another than they do in *C. magister*.

Acknowledgments

We express our appreciation to Mr. Robert Howard for help in performing the amino acid analyses and to Mrs. Susan Roxby for her help in the operation of the ultracentrifuge.

References

Acher, R., and Crocker, R. (1952), *Biochim. Biophys. Acta 9*, 704.

Beale, D., and Whitehead, J. K. (1962), *in* Tritium in the Physical and Biological Sciences, Hartcup, G. R. M., Ed., Vol. 1, Vienna, International Atomic Energy Agency, p 179.

Busselen, P. (1970), *Arch. Biochem. Biophys. 137*, 415.

Casassa, E. F., and Eisenberg, H. (1964), *Advan. Protein Chem. 19*, 287.

Chervenka, C. H. (1970), *Anal. Biochem. 34*, 24.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 686.

Di Giamberardino, L. (1967), *Arch. Biochem. Biophys. 118*, 273.

Dijk, J. (1971), Ph.D. Dissertation, Groningen, The University of Groningen.

Dijk, J., Brouwer, M., Coert, A., and Gruber, M. (1970), *Biochim. Biophys. Acta 221*, 467.

Edelhoch, H. (1967), *Biochemistry 6*, 1948.

Ellerton, H. D., Carpenter, D. E., and Van Holde, K. E. (1970), *Biochemistry 9*, 2225.

Eriksson-Quensel, I. B., and Svedberg, T. (1936), *Biol. Bull. 71*, 498.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry 10*, 2606.

Fernandez-Moran, H., Van Bruggen, E. F. J., and Ohtsuki, M. (1966), *J. Mol. Biol. 16*, 191.

Fish, W. W., Mann, K. G., and Tanford, C. (1969), *J. Biol. Chem. 244*, 4989.

Fraenkel-Conrat, H. J., Harris, I., and Levy, A. L. (1955), *Methods Biochem. Anal. 2*, 359.

Ghiretti-Magaldi, A., Nuzzolo, C., and Ghiretti, F. (1966), *Biochemistry 5*, 1943.

Ghiretti-Magaldi, A., Salvato, B., and Albergoni, V. (1970), *Boll. Soc. Ital. Biol. Sper. 46*, 943.

Habeeb, A. F. S. A. (1967), *Arch. Biochem. Biophys. 121*, 652.

Hade, E. P. K., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 5034.

Hill, R. L., Delaney, R., Fellows, R. E., and Lebovitz, H. E. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1762.

Hjerten, S., Jerstedt, S., and Tiselius, A. (1965), *Anal. Biochem. 11*, 219.

Kawahara, K., and Tanford, C. (1966), *J. Biol. Chem. 241*, 3228.

Konings, W. N., Dijk, J., Wichertjes, T., Beuvery, E. C., and Gruber, M. (1969a), *Biochim. Biophys. Acta 188*, 43.

Konings, W. N., Siezen, R., and Gruber, M. (1969b), *Biochim. Biophys. Acta 194*, 376.

Lapanje, S., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 5030.

Levin, O. (1963a), *Ark. Kemi 21*, 1.

Levin, O. (1963b), *Ark. Kemi 21*, 15.

Levin, O. (1963c), *Diss. Abstr. No. 24*.

Levy, A. L. (1954), *Nature (London) 174*, 126.

Loehr, J. S., and Mason, H. S. (1971), *EMBO Conf. Hemocyanins, Louvain, Belgium.*

Loehr, J. S., and Mason, H. S. (1973), *Biochem. Biophys. Res. Commun.* (in press).

Moore, C. H., Henderson, R. W., and Nichol, L. N. (1968), *Biochemistry 7*, 4075.

Munk, P., and Cox, D. J. (1972), *Biochemistry 11*, 687.

Nickerson, K. W., and Van Holde, K. E. (1971), *Comp. Biochem. Physiol. 393*, 855.

Pickett, S. M., Riggs, A. F., and Larimer, J. L. (1966), *Science 151*, 1005.

Reisler, E., and Eisenberg, H. (1969), *Biochemistry 8*, 4572.

Richards, E. G., Teller, D. C., Hoagland, V. D., Jr., Haschemeyer, R. H., and Schachman, H. K. (1971a), *Anal. Biochem. 41*, 215.

Richards, E. G., Teller, D., and Schachman, H. K. (1971b), *Anal. Biochem. 41*, 189.

Roark, D. E., and Yphantis, D. A. (1969), *Ann. N. Y. Acad. Sci. 163*, 245.

Salvato, B., Sartore, S., Rizzotti, M., and Ghiretti-Magaldi, A. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 22*, 5.

Sanger, E., and Thompson, E. O. P. (1953), *Biochem. J. 53*, 353.

Singer, S. J., and Doolittle, R. F. (1966), *Science 153*, 13.

Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.

Spencer, R. L., and Wold, F. (1969), *Anal. Biochem. 32*, 185.

Van Bruggen, E. F. J., Schuiten, V., Weibenga, E. H., and Gruber, M. (1963), *J. Mol. Biol. 7*, 249.

Van Holde, K. E., and Cohen, L. B. (1964), *Biochemistry 3*, 1803.

Van Holde, K. E., Rossetti, G. P., and Dyson, R. D. (1969), *Ann. N. Y. Acad. Sci. 163*, 279.

Van Holde, K. E., and Van Bruggen, E. F. J. (1971), in Subunits in Biological Systems, Timasheff, S. N., and Fasman, G. D., Ed., New York, N. Y., Marcel Dekker.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.