

STRUCTURE AND FUNCTION OF HEMOCYANIN FROM THE ISOPODS

LIGIA EXOTICA AND LIGIA PALLASII

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by  
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A DISSERTATION

Presented to the Department of Biology  
and the Graduate School of the University of Oregon  
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Title: STRUCTURE AND FUNCTION OF HEMOCYANIN FROM THE ISOPODS  
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Hemocyanins are blue, copper-containing respiratory proteins found dissolved in the hemolymph of many molluscs and arthropods. Arthropodan hemocyanins are high molecular weight polymers assembled from subunits with molecular weights around 75,000 into multiples of six ( $M_r = 450,000$ ). Each subunit contains two copper atoms per one molecule of bound oxygen. Aggregates of one (16S), two (24S), four (36S) and eight (60S) hexamers can occur. In general, crustacean arthropods have hexamer or two hexamer aggregates in their hemolymph, thalassinid shrimps and arachnids, four hexamer, and xiphosurans, eight hexamer aggregates.

Arthropodan hemocyanins exhibit a marked degree of subunit heterogeneity. The amount of heterogeneity may parallel the degree of polymerization. One major question concerning arthropodan hemocyanin is whether different subunits have specific roles in determining the structure of the aggregates. There is some evidence that certain subunits play special roles in the assembly process, with some participating in the formation of hexamers and others linking the hexamers into higher aggregates. In some cases, the linking subunits appear to

be connected by disulfide bonds. The second major question concerns the role of different subunits in modulating the oxygen binding properties of the aggregates. Only a few studies have been done so far which demonstrate that subunits do vary in their functional properties.

Hemocyanins from the isopods Ligia exotica and Ligia pallasii appear to have little subunit heterogeneity yet form one and two hexamer aggregates like other crustacean hemocyanins. The relative simplicity of the isopod system suggested that it would be a useful model to study the role of specific subunits in the structure and function of hemocyanin.

This study describes the structural characteristics of isopod hemocyanin using a variety of chromatographic and electrophoretic techniques. The subunit compositions of the two polymeric components, 16S and 24S, are presented. The 16S molecules are assembled from only one type of subunit,  $M_1$ . The 24S molecules contain ten  $M_1$  and two  $M_2$  subunits. Evidence is presented that suggests the two  $M_2$  subunits form a  $Ca^{+2}$  sensitive, non-covalent link between the two hexamers of the 24S molecules. Dissociation studies indicate that the  $M_1$  and  $M_2$  subunits have different intermolecular binding properties.

Oxygen binding properties of 16S and 24S molecules were measured spectrophotometrically. The two types of polymers have highly developed homo- and heterotropic interactions. Both 16S and 24S molecules have similar oxygen affinities, Bohr effects of comparable magnitude and show a high degree of cooperativity with  $n_H = 3 - 5$  between pH 6.8 - 8.25. The 24S fraction, containing  $M_2$  subunits as well

as  $M_1$ , shows a slightly greater cooperativity than the 16S fraction, which contains only  $M_1$  subunits. It is apparent that of the oxygen binding properties examined, the 16S homo-hexamer, composed of apparently identical subunits, is as functionally versatile as the heterogeneous two-hexamer species, except possibly for a slight disadvantage in cooperative oxygen binding.

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## INTRODUCTION

Hemocyanins are blue, copper-containing respiratory proteins found dissolved in the hemolymph of many arthropods and molluscs. In the seventeenth century Swammerdam (1669) recorded the characteristic blue color of body fluids from certain molluscs. The name hemocyanine for this protein was coined by Fredericq in 1878. Both molluscan and arthropodan hemocyanin molecules are characteristically high molecular weight polymers which can exist in a number of discrete aggregation states depending on conditions of pH, ionic strength and divalent cations (Eriksson-Quensel and Svedberg, 1936; Van Holde and Van Bruggen, 1971). Oxygen combines reversibly and cooperatively with hemocyanin in a ratio of one oxygen molecule per two copper atoms. The structure and function of hemocyanin has been reviewed in a number of recent publications (Van Holde and Van Bruggen, 1971; Lontie and Witters, 1973; Bonaventura et al., 1977; Antonini and Chiancone, 1977; Bannister, 1977; Wood, 1980; Lamy, 1981; Mangum, 1981). Therefore, this thesis will not review all of the hemocyanin literature but will describe some of the more recent findings.

Despite general similarities, molluscan hemocyanins differ markedly from arthropodan hemocyanins with respect

to both primary and quaternary structure. The degree of homology between hemocyanins of these phyla is uncertain. The smallest subunit of a molluscan hemocyanin has an unusually high molecular weight ( $M_r = 300,000-450,000$ ) (Quitter et al., 1978 ; Gielens et al., 1979) and a sedimentation coefficient of 11S (Brouwer and Kuiper, 1973; Waxman, 1975). Based on limited proteolysis experiments, each subunit appears to be composed of a series of covalently linked oxygen binding domains (Lontie et al., 1973; Brouwer, 1975). These domains each have a molecular weight of about 50,000 and contain two copper atoms. Thus one subunit contains 7-8 oxygen binding sites. The 11S subunits are self-assembled into huge aggregates ( $M_r = 4 \times 10^6 - 9 \times 10^6$ ) which have a characteristic appearance in the electron microscope. The largest molecules ( $M_r = 9 \times 10^6$ ), which are composed of 20 subunits, appear as cylinders 35 nm in diameter x 38 nm in height with tenfold rotational symmetry and a so-called collar at each end (Van Bruggen et al., 1962; Fernandez-Moran et al., 1966; Mellema and Klug, 1972). The  $9 \times 10^6$  molecular weight molecule (100S) dissociates stepwise into 1/2 (60S) 1/10 (20S) and 1/20 (11S) units (Siezen and Van Driel, 1974). In certain classes of molluscs, hemocyanin exists in the hemolymph only as 60S molecules ( $M_r = 4.5 \times 10^6$ ); no 100S molecules are found. This has been found in both the polyplacophorans (chitons) (Ryan, M., personal communica-

tion) and the cephalopods (Van Holde and Cohen, 1964).

The smallest functional arthropodan hemocyanin subunit has a molecular weight around 75,000 ( $S_{20,w} = 5$ ) and contains two copper atoms. Thus the molecular weight per oxygen binding site is slightly larger (75,000 vs. 50,000) while the polypeptide chain is much smaller (75,000 vs. 360,000) in arthropodan than in molluscan hemocyanin. The assembly of arthropodan hemocyanin subunits also differs from that of the molluscs. The 5S subunits are assembled into multiples of six ( $M_r = 450,000$ ) with a sedimentation coefficient of 16S. Both ultracentrifugation and electron microscopy have established the 16S aggregation state as the basic building block. Aggregates of one (16S), two (24S), four (36S) and eight (60S) hexamers can occur. Electron microscopy of crustacean hemocyanin shows that the 5S subunits are roughly spherical with diameters of 5-6 nm (Schepman, 1975) while x-ray diffraction of Panulirus monomers shows a kidney shape (van Schaick et al., 1981). The 16S hexamers appear in electron micrographs as squares, rectangles and hexagons about 13 nm in diameter, while the 24S molecules look like two squares or a hexagon and square side by side. The 36S polymers appear in electron micrographs as square planar arrangements of four 16S molecules each. The 60S hemocyanin appears to be two parallel stacked 36S structures (Van Holde and Van Bruggen, 1971).

A number of models have been proposed for the arrangement of the subunits. The six monomers in the hexagon have been described as arranged in a trigonal antiprism (Wibo, 1966) or in two triangles lying in parallel planes, rotated by  $25^{\circ}$  (Schepman, 1975). It has also been suggested that the monomers occupy the corners of a regular octahedron (Klarman et al., 1979). The 24S component of Astacus hemocyanin has been investigated by small angle x-ray scattering. Based on theoretical models calculated to fit the data and electron micrograph images, Pilz et al., (1980) predict a compact arrangement of subunits within the 24S molecule.

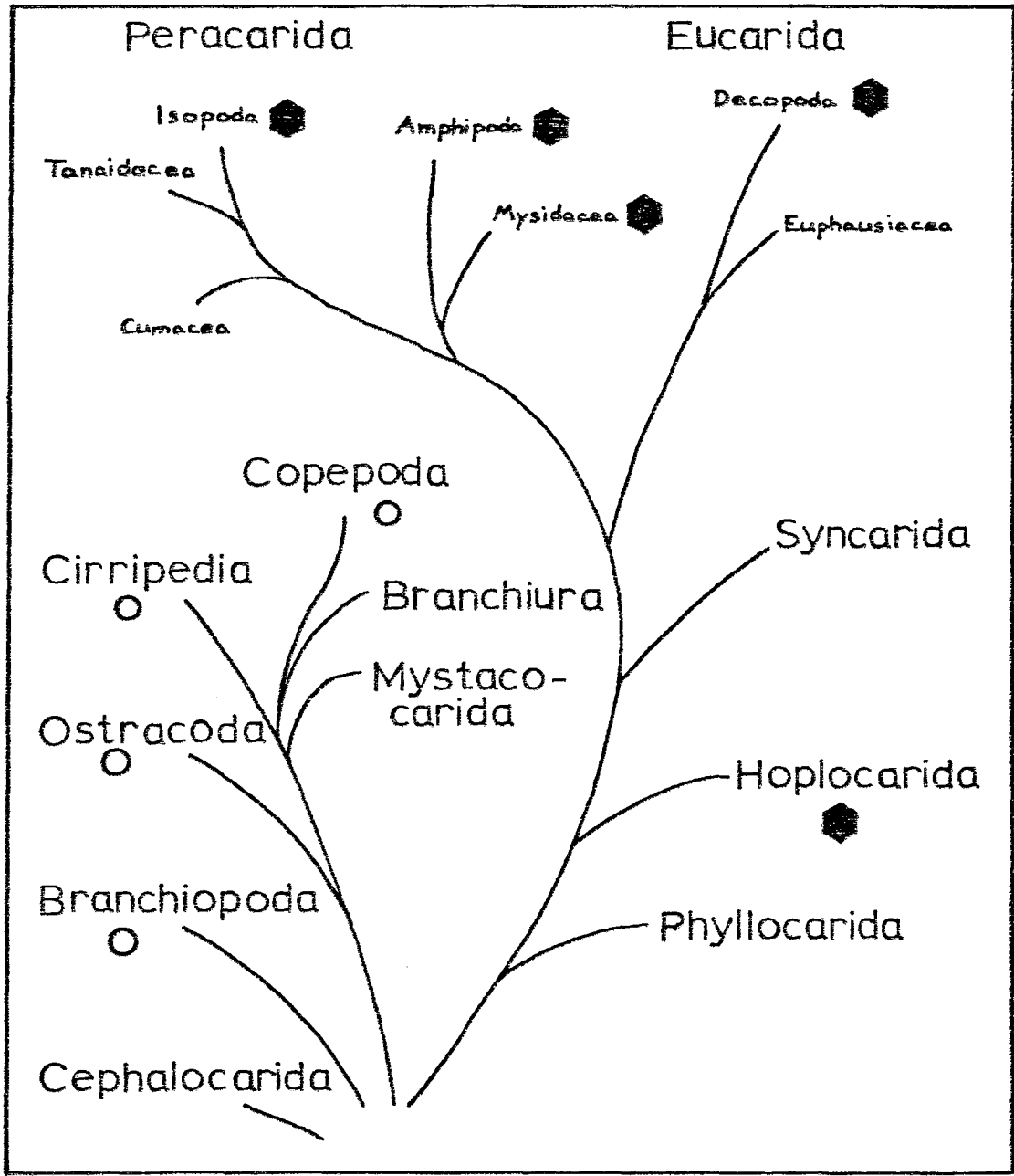
The stability of both molluscan and arthropodan hemocyanin aggregates varies with pH, divalent cations and ionic strength. In general, dissociation occurs both in the acid and alkaline regions, with pH values around neutrality favoring stability. The presence of divalent cations, especially  $\text{Ca}^{2+}$ , shifts the dissociation to higher pH values. High ionic strength, around 1M, also promotes stability. Sensitivity to these factors varies among hemocyanins from different species of arthropods, as does the reversibility of the process. Experiments on dissociation and reassembly of chelicerate hemocyanins, especially to the 5S subunit level, have been more successful than those of crustacean hemocyanins.

While many of the experiments on hemocyanin stability

have been done under non-physiological conditions, recent studies on the 39S hemocyanin of the ghost shrimp Callinassa indicate that dissociation can occur under physiological conditions of low salinity. The hemocyanin has a  $Mg^{2+}$  dependent reversible 39S-16S association (Miller, 1980).

The distribution of hemocyanin within the phylum Arthropoda appears to be restricted to the Chelicerata and the Crustacea; none has been described in the Unirami (insects, centipedes, millipedes). Usually only one size aggregate predominates within a species, and closely related species tend to have that same size aggregate. In the Chelicerata, both the Merostomata (horseshoe crabs) and the Arachnida (spiders and scorpions) contain hemocyanin. The Merostomata are the only class of arthropods which have the eight hexamer (60S) aggregates; most arachnids contain four hexamer (33-37S) aggregates, although hemocyanin of the spider Cupiennus salei exists as two hexamer (24S) and hexamer (16S) (Markl et al., 1979b). Amongst the Crustacea, only the malacostracans are known to contain hemocyanin (Fig 1 modified from Barnes, 1980). Within this class, most research has focused on the eucaridan decapods. In general, the more primitive decapods contain 16S hemocyanin under physiological conditions. However, Astacidea (crayfish and lobster) hemocyanin exists predominantly as two

Figure 1. Distribution of respiratory proteins in the Crustacea (modified from Barnes, 1980). ○, hemoglobin; ●, hemocyanin.



hexamer (24S) aggregates and Thalassinoidea (ghost shrimp) as four hexamer (36S) aggregates. The phylogenetically more advanced Brachyura or "true crabs" have two hexamer (24S) aggregates in their hemolymph (Mangum 1981; Markl et al., 1979a). Hemocyanin has also been reported in the Hoplocarida (stomatopods) (two hexamer aggregates, Svedberg 1933) and the Peracarida (isopods, amphipods and mysids) (Berthet and Berthet, 1963; Freel, 1978). Thus although certain trends are apparent in the phylogenetic distribution of multiple hexamer aggregates, the pattern is blurred by a number of exceptions.

Initial studies indicated that arthropodan hemocyanin polymers were assembled from a pool of homogeneous subunits. However, recent advances in protein chemistry have clearly demonstrated that most hemocyanins are composed of more than one type of polypeptide chain. The subunit heterogeneity is most marked in the eight hexamer aggregates of the horseshoe crabs, where 8-12 different polypeptide chains have been described (Sullivan et al., 1976; Markl et al., 1979b). Subunit heterogeneity in arachnid four hexamer hemocyanin ranges from 6 subunits in the whip-scorpion Mastigoproctus brasilianus (Markl et al., 1979b) to 8 in the scorpion Androctonus australis (Lamy et al., 1979). In crustacean hemolymph the two hexamer (24S) aggregates contain between 4-7 different polypeptide chains while the hexamers (16S)



are composed of 2-6 subunit types (for example, Markl et al., 1979a; Larson et al., 1981).

A question which arises in response to the marked degree of subunit heterogeneity in arthropodan hemocyanins is whether specific subunits might play specific roles in the structure of the polymers. A few studies indicate that different subunit types are required for the assembly process of horseshoe crab (Limulus) (Schutter et al., 1977; Bijlholt et al., 1979), scorpion (Androctonus) (Lamy et al., 1977) and crayfish (Cherax destructor) (Jeffrey et al., 1978) hemocyanins. The two hexamer hemocyanin aggregates appear to require at least one more subunit type than the hexamers, and it has been suggested that the additional subunits are dimers which link the hexamers into the larger aggregates. Such dimers have been described in the hemocyanins of Cherax destructor (Murray and Jeffrey, 1974), Cupiennus salei (Markl et al., 1979b), Astacus leptodactylus (Pilz et al., 1980) and in several other arthropodan hemocyanins (Markl et al., 1981). "Hexamer-former" subunits from one species of chelicerate hemocyanin have been combined with "linker" subunits from another species to form hybrid four hexamers and eight hexamers (Van Bruggen et al., 1980).

Another question suggested by subunit heterogeneity is whether the functional properties of the hemocyanin are modulated by different subunits. The oxygen binding

properties of arthropodan hemocyanins have been reviewed by Mangum (1981). Arthropodan hemocyanins in general demonstrate strong homotropic effects. The values of the Hill's coefficients ( $n_H$ ) of oxygen binding (calculated at 50% saturation) are typically 2-4, although  $n_H = 5-6$  for some hemocyanins. The larger aggregates often have higher oxygen affinities than their subunits although the opposite is true in some species. Oxygen affinity often increases with degree of polymerization, and since  $Ca^{2+}$  and  $Mg^{2+}$  increase polymerization, divalent cations often increase the oxygen affinity. Within physiological pHs, most hemocyanins have a normal Bohr shift. This is difficult to measure, of course, since a change in pH will often result in a change in aggregation state. In addition to protons and divalent cations,  $Cl^-$  anions also appear to be allosteric effectors (Brouwer et al., 1978). Oxygen binding experiments on isolated subunits of Limulus hemocyanin (Sullivan et al., 1974; Bonaventura et al., 1974) and Androctonus hemocyanin (Lamy et al., 1980) indicate that the oxygen affinities and the sensitivity to allosteric modulation are unique for each subunit type. Thus subunit heterogeneity may be important from a functional as well as a structural viewpoint.

This study on the hemocyanin of isopods was initiated for several reasons. Taxonomically, isopod hemocyanin is of interest. The Isopoda belong to the Peracarida, a

Superorder of crustaceans which also includes such animals as the Amphipoda (beach hoppers) and the Mysidacea (mysid shrimps). Over 40% of the crustacean species belong to the Peracarida. Despite the large number of species, almost no work has been done on hemocyanins of these small crustaceans when compared to the detailed studies of eucaridan decapod hemocyanins. The presence of hemocyanins in the hemolymph of isopods and amphipods was noted by Berthet and Berthet in 1963, and some preliminary electrophoretic studies done (Berthet et al., 1964; Wieser, 1965; Alikhan, 1971). A comparative study of Oniscoidea (terrestrial isopod) hemocyanins has presented information on pH induced dissociation, subunit molecular weight, absorption spectra, amino acid composition and immunochemical relationships between species of whole hemocyanin (Sevilla, 1977, 1978; Sevilla and Lagarrigue, 1979a). Some oxygen binding characteristics of Oniscoidea hemocyanins have also been reported (Sevilla and Lagarrigue, 1979b). A preliminary report on the giant sea roach, Bathynomus giganteus, has indicated the presence of a 16S hemocyanin in its hemolymph (Van Holde and Brenowitz, 1980).

Isopod hemocyanin is also of interest as a model system for protein structure and function. The hemocyanin of the semiterrestrial isopod Ligia exotica was suspected to contain an unusually low degree of subunit heterogeneity

(Bonaventura and Bonaventura, personal communication). Because of its possible relative simplicity, it was thought that isopod hemocyanin might be a good system with which to approach some basic questions regarding arthropodan hemocyanin structure and function. The studies described below were undertaken to answer the following questions:

1. What roles do the different subunits of Ligia hemocyanin play in the assembly of the native polymers?
2. What other factors, i.e. pH, ions, are involved in maintaining the 16S and 24S aggregation states of Ligia hemocyanin?
3. What are the differences, if any, in oxygen binding properties between the 16S and 24S components?
4. What roles do the different subunits play in the functional properties of the polymers?

This thesis presents a detailed characterization of the quaternary structure, subunit composition and functional properties of isopod hemocyanin. The first part describes the hemocyanin of Ligia exotica which exists in both a 16S and 24S aggregation state. Data are presented on various ionic interactions which influence the state of aggregation. The highly cooperative oxygen binding behavior and the pronounced Bohr effect of L. exotica hemocyanin and its dissociation products are documented. The absence of conspicuous charge heterogeneity among the 5S subunits, which

is in contrast to most other arthropod hemocyanins, makes Ligia hemocyanin a good model system for investigation of the relationship between its structure and function.

The second part of this thesis has been carried out on the hemocyanin of the large Pacific Coast isopod, Ligia pallasii. A combination of column chromatography, gel electrophoresis and other biochemical techniques indicate that L. pallasii hemolymph contains 16S and 24S hemocyanin aggregates present in a ratio of about 40% to 60%, respectively. The 16S hexamer contains only one kind of polypeptide chain,  $M_1$ . The 24S two-hexamer aggregate is made up of two polypeptide chains,  $M_1$  and  $M_2$ . The two polypeptide chains differ from one another in their electrophoretic mobilities on urea gels, sodium dodecyl sulfate gels in the absence of reducing agent, regular gels at alkaline pH, and in modified peptide maps. The 24S aggregate undergoes a  $Ca^{2+}$  dependent reversible dissociation to hexamer at neutral pH; the resulting hexamers of the 24S aggregate are each composed of five  $M_1$  and one  $M_2$  subunits. Thus the native 16S and 24S molecules are structurally distinct from one another. Dissociation properties are described which suggest that the  $M_1$  and  $M_2$  polypeptide chains have different intermolecular binding affinities.

The functional properties of the two polymeric components are similar with respect to oxygen binding affinities and Bohr effects. Both components have highly

cooperative oxygen binding, but the cooperativity of the 24S molecule is consistently higher than that of the native 16S molecule over the pH range 7.0-8.35.

PART I: LIGIA EXOTICA HEMOCYANIN

## MATERIALS AND METHODS

Ligia exotica (Roux) was collected from the sea wall on Pivers Island, NC. The animals ranged in size from 1.5 to 3 cm in length. Hemolymph was obtained from the live animal by inserting a capillary pipet between the fifth and sixth thoracic segments. The hemolymph from 100 to 150 animals was mixed with a small volume of ice-cold 0.1 ionic strength Tris-HCl buffer, pH 8.0, that was made 10 mM in  $MgCl_2$  and 0.1 M in NaCl. Phenylmethylsulfonyl fluoride (1 mM) was included in the extraction buffer to inhibit proteolytic degradation by serine proteases. The pooled hemolymph was allowed to coagulate during gentle stirring. After centrifugation at 12000g for 10 min, the supernatant was immediately applied to a 1.8 x 70 cm column of Sepharose 4B at 4°C equilibrated with the same buffer (without phenylmethylsulfonyl fluoride). Absorbance of the eluted fractions was measured at both 280 and 340 nm. All further experiments, except the electron microscopy, were performed on the Sepharose 4B purified hemocyanin. Samples were dialyzed overnight at 4°C against the appropriate buffer prior to centrifugation or oxygen binding experiments.

The Sepharose 4B column was calibrated with the following proteins: Eudistylia vancouveri chlorocruorin ( $M_r = 2.8 \times 10^6$ ) (Terwilliger et al., 1975), Lumbricus terrestris hemoglobin ( $M_r = 2.6 \times 10^6$ ) (Wiechelman and Parkhurst, 1972), Helisoma trivolvis hemoglobin ( $M_r = 1.7 \times 10^6$ ) (Terwilliger et al., 1976), Callinectes sapidus hemocyanin ( $M_r = 9.4 \times 10^5$ ) (Hamlin and Fish, 1977), Glycera dibranchiata polymeric hemoglobin ( $M_r = 1.25 \times 10^5$ ) (Seamonds et al., 1971), and bovine serum albumin.

Electron microscopy was performed with hemocyanin which had been collected in filtered seawater containing sodium tetrathionate (Eastman) and phenylmethylsulfonyl fluoride (Eastman) as proteolytic inhibitors. Prior to electron microscopy, 10  $\mu$ l of the pigment was diluted with 1 ml of 0.1 ionic strength Tris-HCl buffer, pH 7.1. The diluted blood was sprayed onto a specimen grid covered with a thick carbon film. The molecules were negatively stained with a few droplets of unbuffered 1% uranyl acetate. Pictures were taken with a Philips EM-200 electron microscope (80 kV) at a magnification of 26500X on Kodak FRP 35-mm roll film. Prints of the negatives were made at 6X magnification.

Sedimentation velocity experiments were carried out at 20°C by use of a Beckman Model E analytical ultracentrifuge equipped with a mechanical speed control and Schlieren optics. Sedimentation coefficients were corrected to standard conditions of the viscosity and density of water



according to the method of Svedberg and Pedersen (1940). Protein concentrations were 5 mg/ml, obtained from absorption measurements at 340 nm, assuming  $\epsilon$  (1%) = 2.23 for crustacean hemocyanin (Sullivan et al., 1974).

Regular disc gel electrophoresis of Ligia hemocyanin at pH 8.9 followed the procedure of Davis (1964). Electrophoresis at pH 7.4 was run in a 50 mM Tris-HCl/Tris-maleate buffer system, while at pH 10.5, a 50 mM sodium bicarbonate/sodium glycinate buffer system was used. All buffers included 10 mM ethylenediaminetetraacetic acid (EDTA). Similar results were obtained both with and without the use of a spacer gel on 7.5% gels.

Sodium dodecyl sulfate (SDS) gel electrophoresis was performed using 5, 6, and 10% acrylamide gels, with a constant ratio of acrylamide to N, N' methylenebisacrylamide of 37:1 (Weber and Osborn, 1975). Thin-slab SDS gel electrophoresis on 7.5 and 10% gels was also done (Laemmli, 1970; Studier, 1973; Ames, 1974). Prior to SDS gel electrophoresis the proteins were heated to 100°C for 1.5 min in boiling SDS incubation buffer which contained 1 mM phenylmethylsulfonyl fluoride. The 5 and 6% gels were calibrated with myosin, phosphorylase A,  $\beta$ -galactosidase, and bovine serum albumin, while the 7.5 and 10% gel markers included phosphorylase A, transferrin, bovine serum albumin, ovalbumin,  $\alpha$ -chymotrypsinogen A, sperm whale metmyoglobin, and

lysozyme (Sigma Chemicals). The gels were stained with Coomassie blue according to Fairbanks et al., (1971).

Oxygen equilibrium studies were carried out by the spectrophotometric method of Riggs and Wolbach (1956). Spectral changes were measured with a Cary 14 recording spectrophotometer. Ligand binding kinetics were determined by rapid mixing methods which have been previously described (Bonaventura et al., 1974).

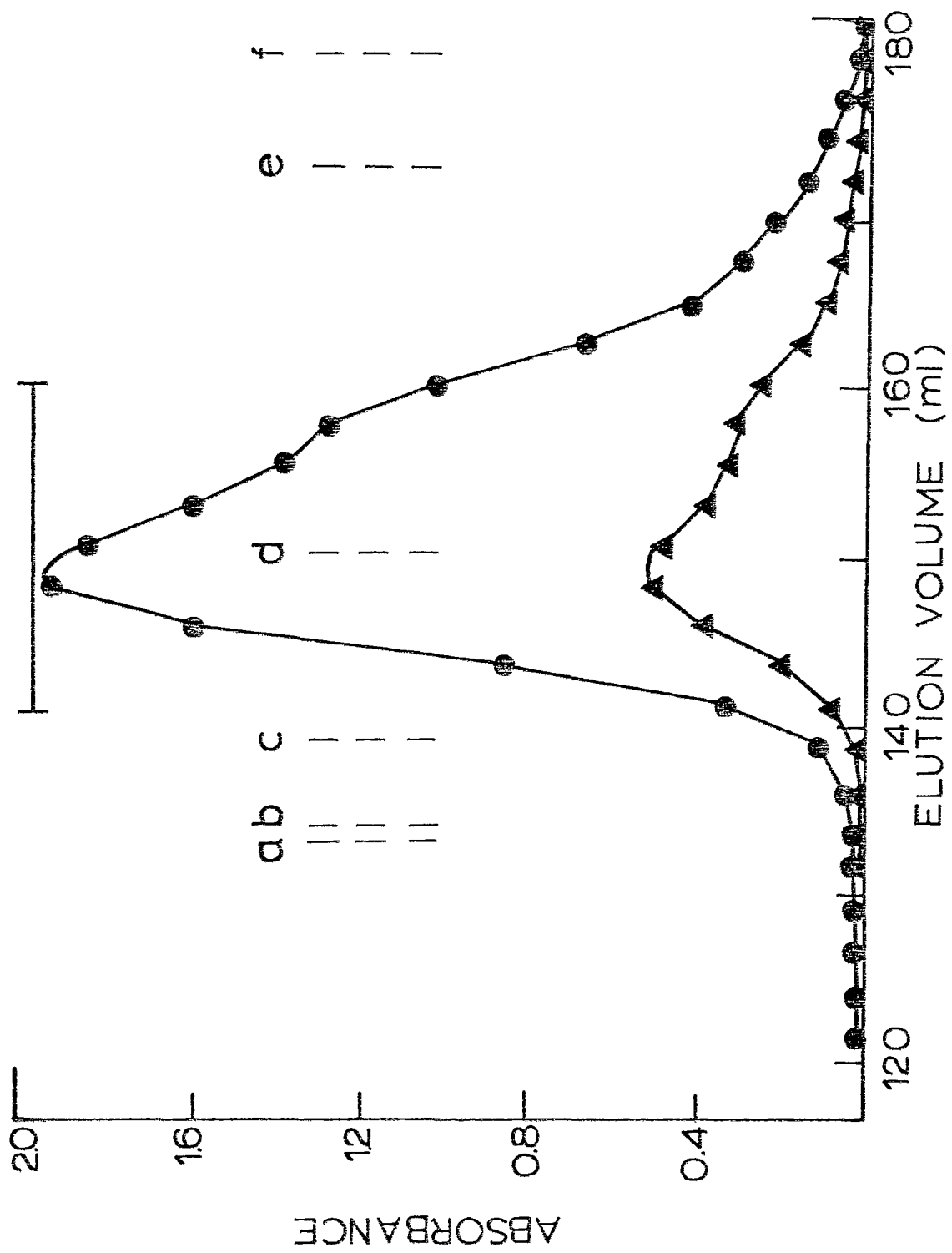
## RESULTS

### Structural Properties

Ligia hemocyanin chromatographs as a single asymmetric peak on Sepharose 4B as shown in Figure 2. As calculated from a plot of log molecular weight vs. elution volume, the main peak has an apparent molecular weight of approximately 900,000 and the trailing shoulder an apparent molecular weight of 450,000. A yellow nonhemocyanin fraction elutes in the 140,000-170,000 molecular weight range. The 900,000 and 450,000 molecular weight components are present in a ratio of about 65 to 35%, respectively. All subsequent studies were done on the pooled fractions shown under the bar in Figure 2.

Sedimentation velocity experiments from pH 7 to 9 with purified Ligia hemocyanin in the presence of 10 mM  $MgCl_2$  or  $CaCl_2$  show two molecular weight components, a major peak with  $s_{20,w}^0$  of approximately 24S and a minor peak with  $s_{20,w}^0$  equal to 16S. Only one component, corresponding to 16S material, is present at pH 9.0 if divalent cations are removed by dialysis against 0.01 ionic strength Tris-HCl, 10 mM in EDTA.

Figure 2. Elution pattern of Ligia exotica hemocyanin chromatographed on Sepharose 4B, 0.1 ionic strength Tris-HCl, pH 8.0, made 10 mM in MgCl<sub>2</sub> and 0.1 M in NaCl. Column volume 1.8 x 70<sup>2</sup> cm. Calibration proteins: (a) Eudistylia chlorocruorin, (b) Lumbricus erythrocrutorin, (c) Helisoma hemoglobin, (d) Callinectes hemocyanin, ( ) Glycera dibranchiata polymeric hemoglobin and (f) bovine serum albumin. Absorbance at 280 ( ● ) and 340 nm ( ▲ ).



An electron micrograph of Ligia hemocyanin is shown in Figure 3. Single hexagonal and square profiles are present as well as dimeric structures composed of two squares, or a hexagon and a square, joined side by side. The dimeric structures correspond to the 900,000 molecular weight species (16S). This interpretation is based on the results of column chromatography and ultracentrifugation and by analogy with the aggregation states of other crustacean hemocyanins (Van Holde & Van Bruggen, 1971; Schepman, 1975).

Dissociation of Ligia hemocyanin into lower aggregation states occurs under conditions of low ionic strength, of high pH, and in the presence of a chelating agent such as EDTA. At pH 9.0 only 16S material is observed. At pH 9.5, there is substantial dissociation into 5S monomers. When purified hemocyanin is dialyzed against 0.05 ionic strength Tris-HCl, 10 mM in EDTA, pH 9.5, and chromatographed on Sephacryl S-200, the elution profile illustrated in Figure 4 is obtained. Fraction I, about 44% of the hemocyanin, elutes with the void volume of the column. Sedimentation velocity measurements show that fraction I is predominantly 16S with a slight trace of 5S material. Fraction II, about 56% of the protein, sediments with a major  $s_{20,w}^0$  peak of 5S and a very small peak of 16S. Fraction II therefore corresponds to the 5S monomers reported for other crustacean hemocyanins. The relative percentage of fraction II

Figure 3. Electron micrograph of Ligia exotica hemo-  
cyanin, pH 7.1, negatively stained with 1%  
uranyl acetate, 159,000x.

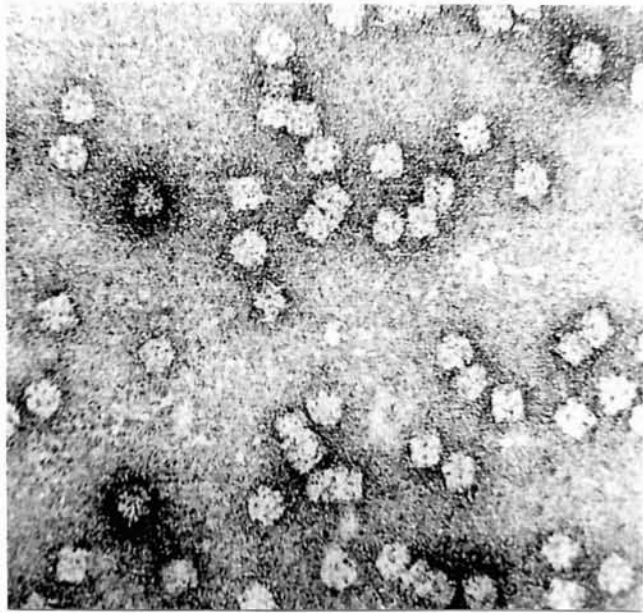
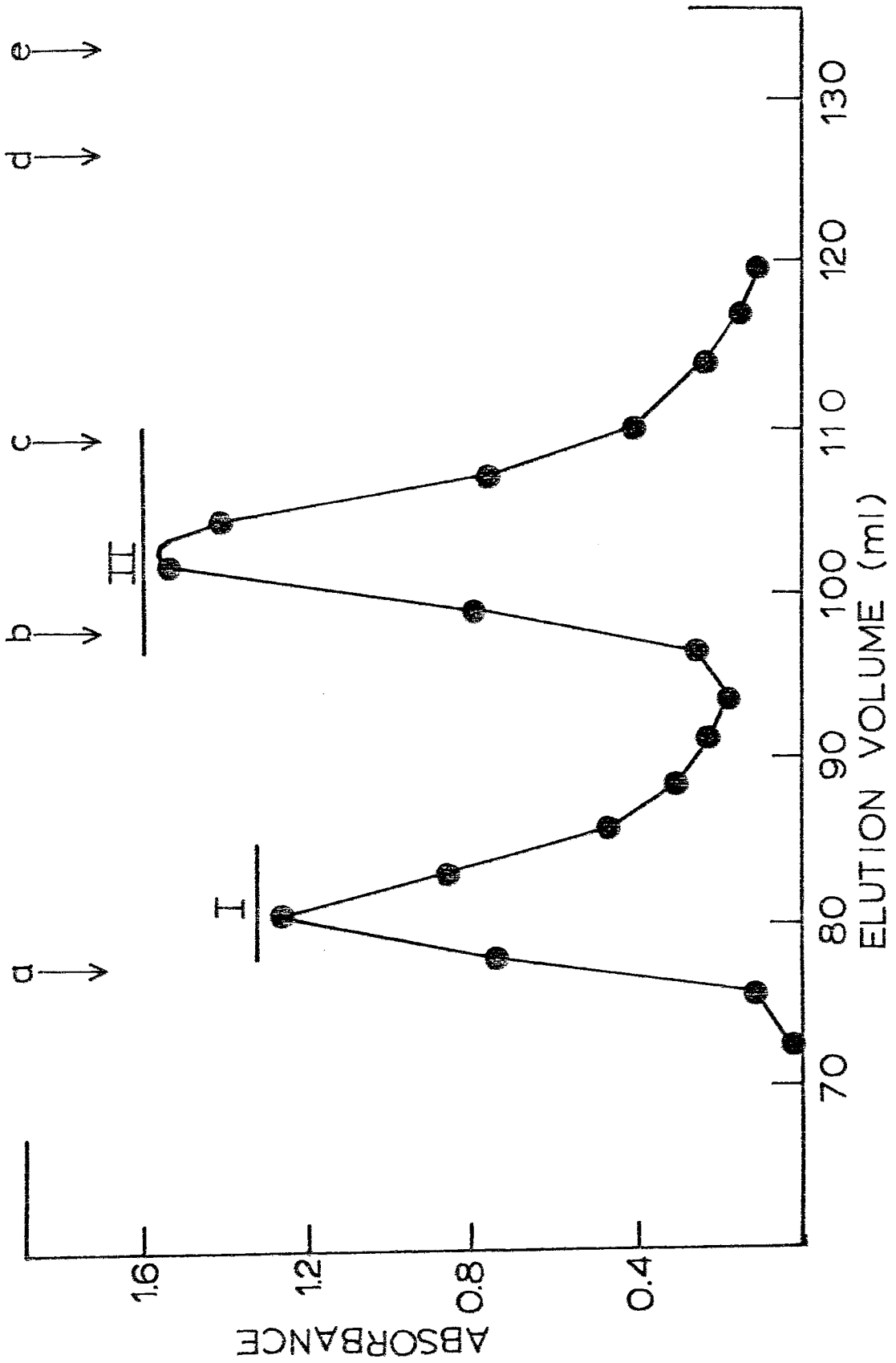




Figure 4. Elution pattern for purified Ligia exotica hemocyanin chromatographed on Sephacryl S-200 after dialysis versus 0.05 ionic strength Tris-HCl, pH 9.5, made 10 mM in EDTA. Column volume 1.4 x 110 cm; column buffer same as dialysate. Calibration proteins: (a) blue dextran, (b) bovine serum albumin, (c) ovalbumin, (d)  $\alpha$ -chymotrypsinogen A, and (e) sperm whale metmyoglobin.



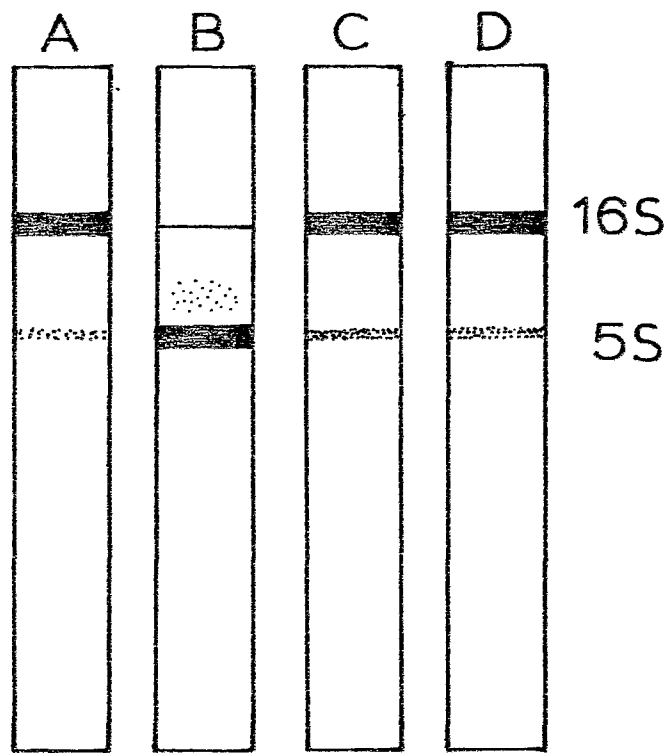
can be increased to 70% by dialysis and chromatography at pH 10.5.

When fraction I hexamers from a pH 10.5 chromatography are rechromatographed on the same column, they elute as hexamers in exactly the same position as before. Thus the proportions of the 16S and 5S populations at pH 10.5 are not established by a simple pH-dependent equilibrium. Neither 16S nor 5S fractions lose copper at pH 10.5 as judged by 280/340 nm absorbance ratios.

The 5S subunits in fraction II can be reassociated to 14-16S hexamers by simply lowering the pH of the Tris-EDTA dialysis buffer to 8.0. Attempts to reconstitute the 24S dodecamers by dialysis against 0.1 ionic strength Tris-HCl, pH 8.0, made 10 mM in  $Mg^{2+}$  and  $Ca^{2+}$  and 0.1 M in NaCl, were unsuccessful.

Disc gel electrophoresis can be used to study the distribution between 16S and 5S populations as a function of pH. Figure 5A shows that at pH 8.9 fraction I gives a single sharp band corresponding to the 16S material and a single, less intense band of 5S material. Figure 5B shows that fraction II gives one sharp major band corresponding to the 5S material, a thin single band corresponding to 16S molecules, and a diffuse stained area between the 5S and 16S material. Figure 5C shows that after dialysis at pH 8.0 fraction II gives an electrophoretic pattern just like that of fraction I hexamers. Finally, Figure 5D shows that when

Figure 5. Disc gel electrophoresis, pH 8.9, on 7.5% acrylamide gels of fractions I and II from Sephacryl S-200. (A) Fraction I, major component is 16S; (B) fraction II, major component is 5S; (C) fraction II, after dialysis at pH 8.9, major component is re-associated 16S; and (D) fractions I and II (after dialysis at pH 8.0) combined, major component is 16S. The S values indicate the approximate sedimentation velocity of the samples prior to electrophoresis.



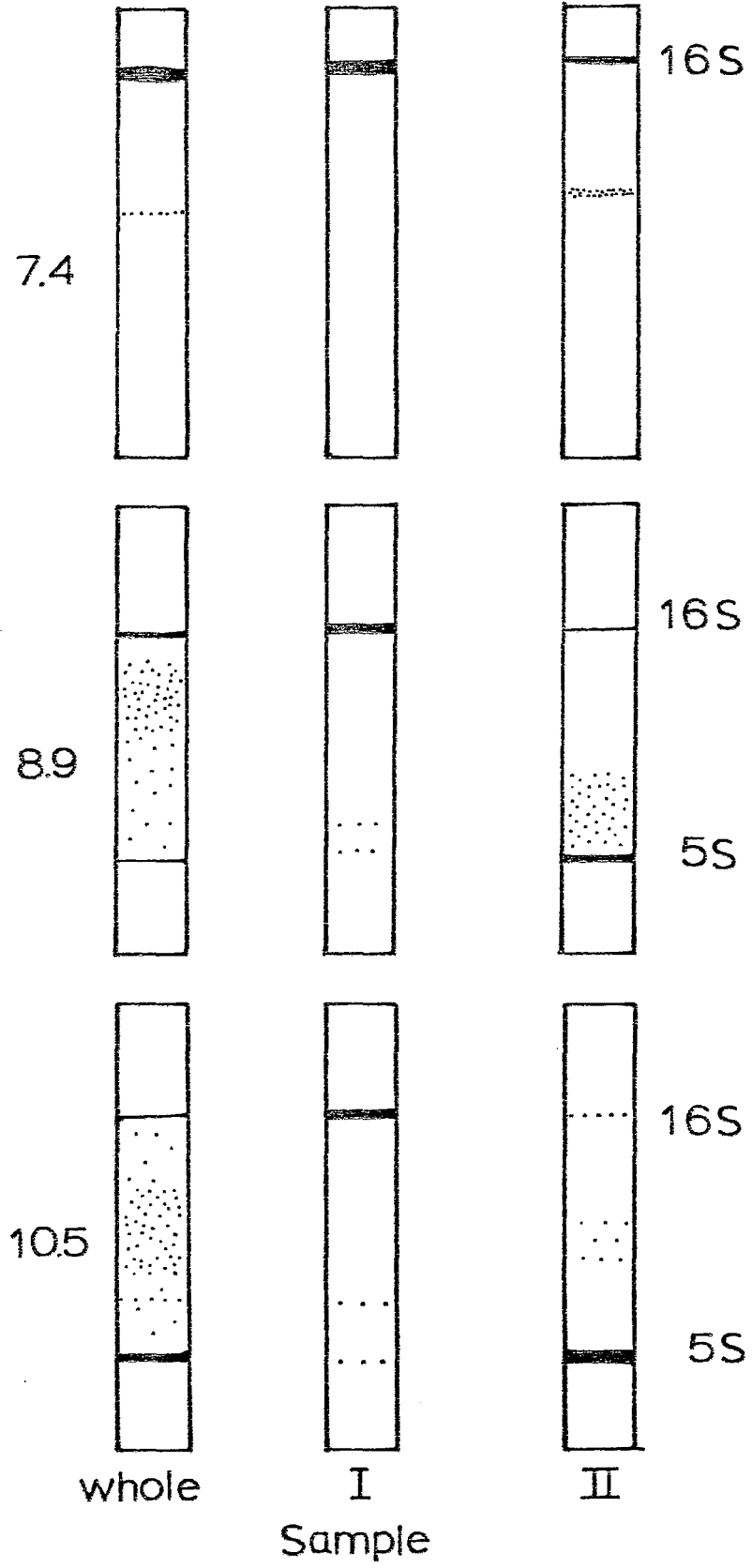
fractions I and II (after dialysis at pH 8.0) are coelectrophoresed, they give a single sharp band corresponding to 16S material (major band) and a single faint band corresponding to 5S material.

Figure 6 is a further illustration of how pH influences the aggregation state of Ligia hemocyanin. Samples of purified, unfractionated hemocyanin and fractions I and II from a pH 10.5 chromatography were electrophoresed at pH 7.4, 8.9, and 10.5. It can be seen that the proportions of bands corresponding to putative hexamer, monomer, and intermediate aggregation states vary as a function of pH. The 16S material generally predominates at pH 7.4 and the monomeric form predominates at pH 10.5. As previously noted, the hexamers isolated by chromatography at pH 10.5 do not dissociate into monomers and thus do not show a monomeric band. Although the pattern of staining due to the intermediate aggregation states varies from one electrophoresis pH to another, the 16S and 5S components consistently migrate as single, homogeneous bands.

SDS gel electrophoresis of the freshly purified pigment on 5, 6, and 10% acrylamide disc gels in tubes shows a single band of about 70,000 daltons, corresponding to the molecular weight of the smallest polypeptide chain. SDS electrophoresis of crude hemolymph removed from the animal and immediately put into boiling incubation buffer containing the proteolytic blocking agent phenylmethylsulfonyl

Figure 6. Disc gel electrophoresis of purified Ligia exotica hemocyanin (pH 8.0,  $Mg^{2+}$ ), fraction I (pH 10.5, EDTA) and fraction II (pH 10.5, EDTA). Electrophoresis buffers, pH 7.4, 8.9 and 10.5; 7.5% acrylamide gels stained with Coomassie blue.

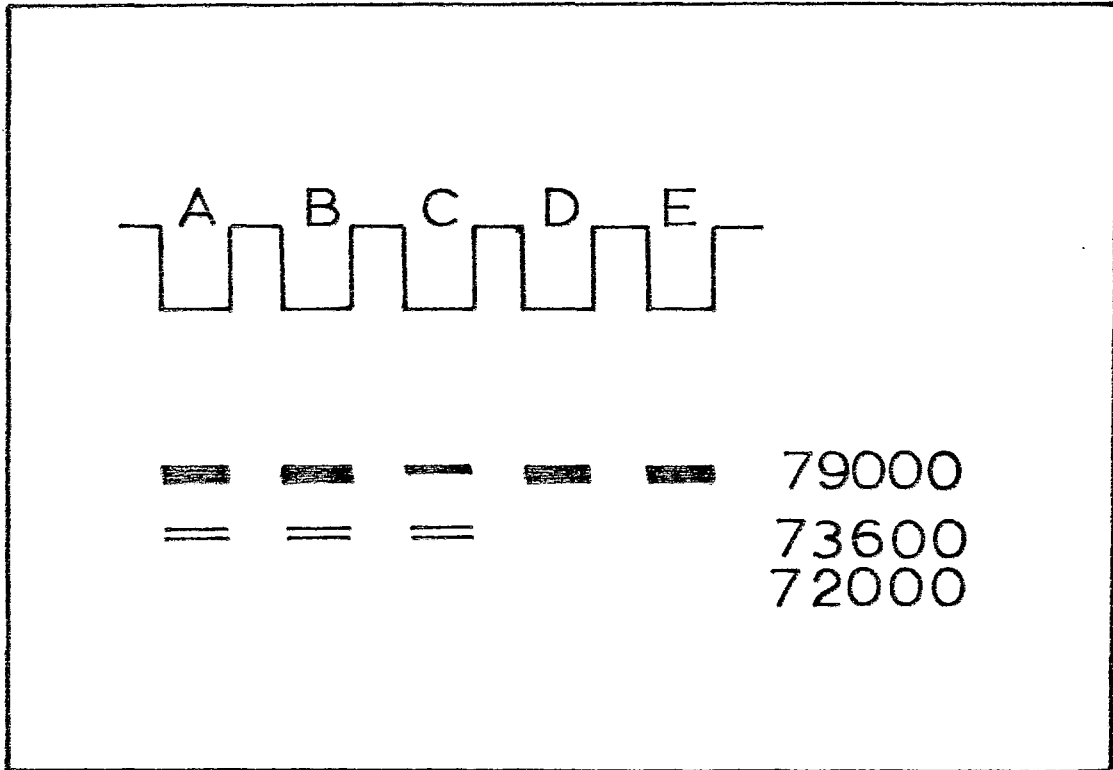
Electrophoresis pH





fluoride also gives a major band of about 70,000 daltons, although several other minor bands are present. Other studies were carried out using SDS slab gel electrophoresis. The SDS slab gels have, in general, greater resolving capability than SDS tube gels. Figure 7 summarizes these extended studies. SDS slab gel electrophoresis of purified Ligia hemocyanin on a 7.5% gel shows three bands, a major component corresponding to 79,500 daltons and two minor components of 73,600 and 72,000 daltons (Figure 7A). Fraction I hexamers obtained from both the pH 9.5 and 10.5 Sephacryl separations also show the same three bands. However, while the relative proportions of the three bands from the 16S material obtained at pH 9.5 (Figure 7B) are similar to those of the whole hemocyanin, the 16S fraction isolated at pH 10.5 (Figure 7C) shows a decrease in staining intensity of the 79,500-dalton component. The fraction II samples, obtained from chromatography at both pH 9.5 and 10.5, migrate on SDS slab gels as a single band with a molecular weight of 79,500, whether applied to the gel as 5S monomers or as reaggregated 16S hexamers (Figure 7D, E). Thus, the smaller molecular weight bands observed in SDS gels of the purified hemocyanin and the fraction I hexamers are not present in SDS gels of the fraction II monomers.

Figure 7. SDS electrophoresis of Ligia exotica hemocyanin on 7.5% slab gel. (A) Purified pigment, 24S and 16S; (B) fraction I hexamers from Sephacryl S-200, pH 9.5; (C) fraction I hexamers from Sephacryl S-200, pH 10.5; (D) fraction II monomers from Sephacryl S-200; and (E) fraction II reaggregated hexamers from Sephacryl S-200 monomers.



### Oxygen Equilibria

The pH dependence of oxygen binding by Sepharose 4B purified hemocyanin is summarized in Table I. The purified material, containing 24S and 16S molecules, shows a positive Bohr effect and a high degree of cooperativity. Values of the Hill coefficient at half-saturation,  $n_H$ , varied from 3.6 to 4.7 in the presence of  $Mg^{2+}$ . Figure 8 and Table II summarize the oxygen binding characteristics of the monomeric and hexameric forms of Ligia hemocyanin. Figure 8 contrasts the Hill plots of oxygen binding by the Sepharose 4B purified hemocyanin (24S and 16S) with those of the hexameric forms (16S) and the monomeric forms (5S with a trace of 16S) of Ligia hemocyanin. The hexamers obtained by chromatography at high pH and low ionic strength (fraction I of Figure 4) have approximately the same oxygen affinity and high degree of cooperative interactions as the unchromatographed hexamers that were prepared by dialysis against EDTA-containing buffer at pH 9.0. In contrast, hexamers prepared by reassociation of chromatographically isolated monomers (fraction II of Figure 4) show somewhat reduced cooperativity and a lower oxygen affinity. The 5S subunits of Ligia hemocyanin, prepared by chromatography at high pH and low ionic strength, show little or no cooperativity in oxygen binding. At high pH in EDTA-containing buffers the monomers have a much lower oxygen affinity than do the 16S

Table I. Effect of pH and Cofactors on O<sub>2</sub> Binding Characteristics of Purified Ligia Hemocyanin at 20°C.

molecular species	treatment of hemocyanin	cofactor	pH	log p <sub>1/2</sub>	n <sub>H</sub>	k <sup>a</sup> (s <sup>-1</sup> )
dodecamers and hexamers	Sepharose 4B	Mg <sup>2+</sup>	7.1	1.44	3.6	400
		EDTA	7.1	1.53	2.9	> 400
		Mg <sup>2+</sup>	8.0	0.95	4.0	350
		EDTA	8.0	1.15	3.4	
		Mg <sup>2+</sup>	9.2	0.93	4.7	170
hexamers	Sepharose 4B	EDTA	9.0	1.02	3.2	325

<sup>a</sup>The estimates of oxygen dissociation rates (k) are averages from multiple experiments. These rates represent the final stages of oxygen dissociation since much of the reaction was lost in the instrumental dead-time.

Figure 8. Hill plots of oxygen binding to various aggregation states of Ligia exotica hemocyanin. Temperature, 20°C; oxygen pressure is expressed in mmHg. Purified hemocyanin dialysed versus 0.1 ionic strength Tris-HCl, pH 9.2, made 10 mM in MgCl<sub>2</sub> and 0.1 M in NaCl (○); fraction I from Sephacryl S-200, pH 9.3 (□); fraction II from Sephacryl S-200, pH 9.5 (△).

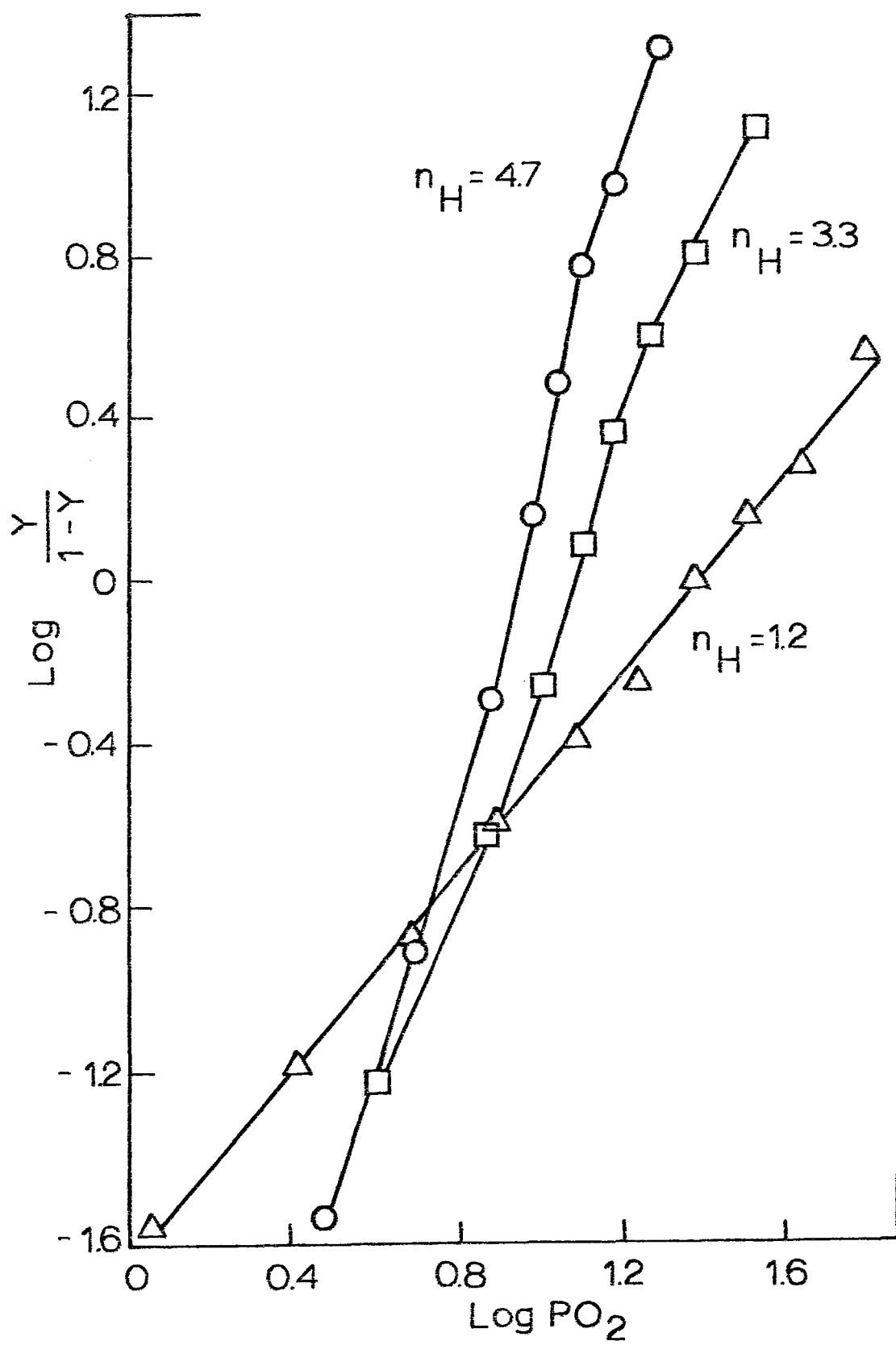


Table II. Oxygen Binding Characteristics of Dissociated and Reassociated Ligia Hemocyanin at 20°C.

molecular species	treatment of hemocyanin <sup>a</sup>	cofactor	pH	log $p_{\frac{1}{2}}$	$n_H$	$k^b$ (s <sup>-1</sup> )
hexamer (from Table I)	Sepharose 4B	EDTA	9.0	1.02	3.2	325
hexamer	fraction 1	EDTA	9.3	1.07	3.3	
hexamer	a. reassociated from monomers of fraction II	EDTA	8.0	1.28	2.1	>400
	b. reassociated from monomers of fraction II	EDTA + NaCl	8.0			100
monomer	a. fraction II	EDTA	9.5	1.37	1.2	340
	b. fraction II	EDTA + NaCl	9.5			120

<sup>a</sup>Fraction I (hexamers) and fraction II (monomers) are the two major zones from a Sephacryl column.

<sup>b</sup>The estimates of oxygen dissociation rates ( $k$ ) are averages from multiple experiments. These rates represent the final stages of oxygen dissociation since much of the reaction was lost in the instrumental dead-time.



hexamers.

### Kinetics of Oxygen Dissociation

Rapid-mixing methods were used to study the kinetics of oxygen dissociation from the various Ligia hemocyanin preparations. The reaction was extremely fast under all conditions examined. Although much of the reaction was lost in the dead-time of the rapid-mixing apparatus, the observed portion shows pH-dependent changes that parallel the pH dependence seen in oxygen equilibrium studies. The observed portion of the oxygen dissociation process from the 24S-16S mixture at pH 7.0 in magnesium occurs at a rate of approximately  $400 \text{ s}^{-1}$ . The high reaction rate results in a large degree of uncertainty in this estimate. The observed rate is considerably slower at pH 9.0, approximately  $170 \text{ s}^{-1}$ . Although more of the reaction can then be observed, it was not possible to determine if more than a single exponential process was involved. Kinetic data on the hexamer-dodecamer and hexamer preparations of Ligia hemocyanin are presented in Table I. The apparent oxygen dissociation rates are increased in the presence of EDTA, which is consistent with the EDTA-induced decrease in oxygen affinity observed in oxygen equilibria determinations. Addition of NaCl to either the fraction II monomer or the hexamer reassembled from fraction II monomers results in a marked decrease in

the rate of oxygen dissociation. This suggests that there may be an increased oxygen affinity in the presence of NaCl. These kinetic aspects of the oxygen binding properties of Ligia hemocyanin are summarized in Table II.

## DISCUSSION

Ligia hemocyanin exhibits many of the structural and functional characteristics common to crustacean hemocyanins. On the structural level, the data from analytical ultracentrifugation, column chromatography and electron microscopy indicate that Ligia hemocyanin exists in the hemolymph as 24S dodecamers and 16S hexamers with molecular weights of about 900,000 and 450,000 respectively. A similar pattern of aggregation states is found in many other crustacean hemocyanins (Ellerton et al., 1970; Van Holde and Van Bruggen, 1971; Murray and Jeffrey, 1974; Kuiper et al., 1975; Hamlin and Fish, 1977). Ligia hemocyanin is set apart from most crustacean hemocyanins, however, in that very high pH is required to dissociate it into monomers, and the isolated monomers appear to be of a single electrophoretic type. A recent study on the electrophoretic patterns of hemocyanin from six different species of isopods (Oniscoidea) was suggestive of charge heterogeneity both within and between species (Sevilla, 1977). However, since the electrophoresis which indicated heterogeneity was performed at pH 9.1, the variations in banding patterns might well reflect intermediate association phenomena rather than heterogeneous populations of monomers.

Although many crustacean hemocyanins dissociate into their monomeric subunits at high pH and low ionic strength in the presence of EDTA, this is not always the case. Electrophoresis at a pH lower than that required for complete dissociation into monomers may result in alterations in the banding pattern due to association phenomena during the electrophoresis. The existence of "resistant" hexameric forms thus makes it hazardous to make judgments on homogeneity or heterogeneity based on electrophoresis alone.

Are the electrophoretically homogeneous subunits of Ligia hemocyanin identical by other criteria? Molecular weight estimates for both denatured Ligia hemocyanin and denatured Fraction I are suggestive of some degree of heterogeneity at the subunit level. These preparations show one major and two minor bands when electrophoresed on high resolution 7.5% SDS slab gels. A doublet pattern on SDS gels has been reported for Cancer magister (Carpenter and Van Holde, 1973; Loehr and Mason, 1973) and Callinectes sapidus (Hamlin and Fish, 1977) hemocyanins and three bands within the same 70-85,000 molecular weight range have been found for Cherax destructor (Murray and Jeffrey, 1974) and Panulirus (Kuiper et al., 1975) hemocyanins. The multiple bands seen in these decapod hemocyanins, especially apparent on high resolution SDS slab gels, have been cited as evidence for multiple polypeptide chains. More recently,

six different bands have been reported for Cancer magister hemocyanin (Larson et al., 1981), and a survey of subunit heterogeneity in crustacean hemocyanins indicates a range of 2-7 polypeptide chains (Markl et al., 1979a). It is of interest that when Ligia hemocyanin monomers (Fraction II) are electrophoresed on SDS slab gels, a single sharp band appears with a calculated molecular weight of 79,500. The same results are obtained with the 5S material isolated by chromatography at pH 9.5 (50% of the total protein) or at pH 10.5 (70% of the total protein). I cannot, at present, distinguish between a number of possible explanations for the differences in banding patterns between the isolated monomeric and hexameric forms of Ligia hemocyanin. The minor bands of lower molecular weight in the hexameric fraction could be either real or artifactual. The interpretation that these minor bands represent structurally distinct monomers that are preferentially held in hexameric aggregates cannot be excluded. However, this possibility would imply that the hexameric forms made up of diverse subunits show the same electrophoretic mobility as hexamers containing only identical subunits. As pointed out in the results, no charge heterogeneity has been observed under three different conditions of pH in either 16S or 5S material.

There are several ways minor bands could appear that do not involve intrinsic subunit heterogeneity. If

proteolysis, enzymatic removal of carbohydrate bound to the hemocyanin, or carbohydrate contaminants are invoked, one must presume that the chromatographic separation of hexamers and monomers keeps the contaminating factor with the hexameric fraction. There is no evidence to either support or refute the possibility of a nonuniform binding of carbohydrate which results in heterogeneous behavior on SDS gels. Extensive precautions were taken to eliminate proteolysis during protein purification and in the incubation with SDS. Furthermore, it is likely that a proteolytic or glycolytic contaminant would have been removed by the initial purification process. Alternatively, the differences in SDS patterns of hexamers (Fraction I) and monomers (Fraction II) may reflect conformational heterogeneity of the hexameric population. Fraction I hexamers of a particularly "tight" conformation may impose constraints upon some of the subunits so that they denature differently in SDS-mercaptoethanol solutions. A likely result might be an unequal binding of SDS to the subunits, which would give rise to spurious molecular weight heterogeneity. I will return to this aspect of the discussion in Part II, Ligia pallasii hemocyanin.

The ratio of the 24S and 16S components of Ligia hemocyanin appears to be independent of protein concentration at pH 8.0,  $Mg^{2+}$ , 20°C, since dilute hemocyanin rechroma-

tographed on the same Sepharose 4B column gives an elution pattern similar to the more concentrated pigment. Furthermore, at pH 9.5 or 10.5 in EDTA, the 16S hexamers and the 5S monomers can be separated from one another by chromatography and remain as separate populations under these conditions. The presence of these unique populations of components under specific solvent conditions suggests heterogeneity. The results may be explained if the aggregated forms exist in distinct conformational states that have different pKs for dissociation. Microheterogeneity within purified protein solutions has been suggested previously to describe the pH dependent dissociation transitions in other hemocyanins (Di Giamberardino, 1967; Konings et al., 1969; Siezen and Van Driel, 1973).

The oxygen binding properties of Ligia hemocyanin, as summarized in Tables I and II, are clearly dependent on pH and divalent cation concentration. The aggregated forms show a high degree of cooperative interaction and a strong positive Bohr effect. Both the cooperativity and the oxygen affinity of the purified hemocyanin are increased in the presence of  $Mg^{2+}$ . This allosteric effect of divalent cations has been shown to occur in many other crustacean hemocyanins such as Callinassa (Miller and Van Holde, 1974) and Homarus (Pickett et al., 1966), although in Panulirus

(Kuiper et al., 1975) and Cupiennius (Loewe and Linzen, 1975) hemocyanins the effect of  $Mg^{2+}$  is to lower the oxygen affinity.

The cooperative binding behavior of both dissociation-resistance and non-resistant hexamers in the absence of  $Mg^{2+}$  contrasts sharply with the nearly non-cooperative binding curves shown by the 17S forms of Callianassa hemocyanin in the absence of  $Mg^{2+}$  (Miller and Van Holde, 1974), and the hexameric form of Cupiennius hemocyanin (Loewe and Linzen, 1975). It is relevant that when purified Ligia monomers (Fraction II) are brought to lower pH, the hexamers which form exhibit cooperative interactions in oxygen binding. The Hill coefficient,  $n_H = 2.1$ , is somewhat lower than that of the native hexamers,  $n_H = 3.2$ , but it is clear that cooperativity is maintained. This is important because the reassembled hexamers are known to be composed of subunits that are homogeneous with respect to charge and molecular weight.

Ligia monomers (Fraction II) show almost no cooperativity in oxygen binding. The fact that  $n_H = 1.2$  instead of 1.0 at pH 9.5 is probably due to the presence of a small percentage of hexamers in the preparation. Fraction II, composed largely of 5S molecules, clearly has a lower affinity than either 16S or 16S-24S preparations. Low affinity monomers have also been reported in Callianassa



hemocyanin (Miller and Van Holde, 1974) and Panulirus hemocyanin (Kuiper et al., 1975) although most arthropod hemocyanin monomers show the reverse phenomenon. It is clear that the aggregation of subunits can introduce constraints which either raise or lower the oxygen affinity of the binding sites.

The oxygen affinity of whole Ligia hemocyanin is relatively low, as one would expect for a fast-moving animal living at the air-water interface of the high intertidal where, presumably, there is no problem in obtaining oxygen. As determined by oxygen equilibrium experiments and oxygen dissociation kinetics, the process of oxygen binding is sensitive to changes in pH,  $MgCl_2$  and NaCl. With steeply changing oxygen binding curves, very slight changes in the  $pO_2$  could significantly facilitate unloading of the oxygen at the tissues. Since  $H^+$  lowers the oxygen affinity and both  $Mg^{2+}$  and NaCl increase the affinity, perhaps these factors participate in "enantiostasis" or the regulation of oxygen binding as described by Mangum and Towle (1977) for Callinectes. It has been reported that the chloride ion is an allosteric regulator of Limulus hemocyanin function although its effect in that system is to lower the oxygen affinity rather than to raise it (Brouwer et al., 1977). An allosteric effect of NaCl similar to that observed for Ligia hemocyanin has been observed in experiments with Callinectes

hemocyanin (Brouwer, M., Bonaventura, J. and Bonaventura, C. unpublished observations) and with Penaeus hemocyanin (Brouwer et al., 1978).

The apparent lack of equilibrium between populations of hexamers and dodecamers, the pH dependence of the hexamer-monomer equilibria, and the minor bands seen in SDS banding patterns for the purified hemocyanin suggest caution in concluding that Ligia hemocyanin is entirely composed of homogeneous subunits. However, it is clear that at least 70% of the hexamers are composed of a homogeneous pool of polypeptide chains. Since the formation or stability of these homohexamers is pH dependent, and the hexamers are capable of binding oxygen cooperatively, Ligia exotica hemocyanin presents us with an intriguing model for a prototype of a high molecular weight respiratory protein with highly developed homo- and heterotropic interactions in the absence of conspicuous subunit heterogeneity.

PART II: LIGIA PALLASII HEMOCYANIN

## MATERIALS AND METHODS

Ligia pallasii (Brandt) were collected at night from the sea cliffs near the mouth of Coos Bay, Oregon. The animals were identified according to Richardson (1905). To avoid possible complications due to sex differences, molt cycle or laboratory storage, only adult male intermolt isopods ranging in size from 3-5 cm in length were used, and they were bled within a few days of collection. The hemolymph was obtained by inserting a capillary pipette between the fourth and fifth thoracic segments. About 100  $\mu$ l of hemolymph could be obtained from one large male. Hemolymph from 50-75 animals was mixed with a small volume of ice-cold 0.05 ionic strength Tris-HCl buffer, pH 7.5, that was made 0.1 M in NaCl, 10 mM in MgCl<sub>2</sub> and 10 mM in CaCl<sub>2</sub>. Phenylmethylsulfonyl fluoride (1 mM) was included in the buffer to inhibit proteolysis. After centrifugation of the hemolymph at 12,000 g for 10 min, the supernatant was immediately applied to a 1.8 x 100 cm column of BioGel A-5M (200-400 mesh) at 4°C equilibrated with the same buffer minus phenylmethylsulfonyl fluoride. The column was calibrated with

Eudistylia vancouveri chlorocruorin ( $M_r = 2.8 \times 10^6$ ) (Terwilliger et al., 1975), Cancer magister 25S ( $M_r = 940,000$ ) and 16S ( $M_r = 450,000$ ) hemocyanin (Ellerton et al., 1970), and lactate dehydrogenase ( $M_r = 150,000$ ) (Sigma Chemicals).

### Electrophoresis

#### SDS Gel Electrophoresis

The SDS electrophoresis procedure followed is essentially the discontinuous system described by Laemmli (1970). Hemocyanin samples were diluted to 2 mg hemocyanin/ml column buffer, mixed 1:1 with SDS incubation buffer to yield final concentrations of 2% SDS, 5% 2-mercaptoethanol, 10% glycerin, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 62.5 mM Tris-HCl (pH 6.8), and 0.01% bromophenol blue, and heated in a boiling water bath for 1.5 min. Some samples were also prepared in the absence of 2-mercaptoethanol. The samples were stored at  $-20^{\circ}\text{C}$ . The electrophoresis, stacking gel, and separating gel buffers were 1 mM in EDTA. 7.5% acrylamide slab gels were electrophoresed at 100 V for about 3 h and stained with Coomassie brilliant blue R (Fairbanks, 1971). 5% gels were used for molecular weight determinations larger than 100,000. Calibration markers included

$\beta$ -galactosidase ( $M_r = 130,000$ ), phosphorylase A ( $M_r = 93,000$ ), bovine serum albumin ( $M_r = 68,000$ ), ovalbumin ( $M_r = 43,000$ ) and  $\alpha$ -chymotrypsinogen A ( $M_r = 25,000$ ) (Sigma Chemicals).

### Urea Gel Electrophoresis

Urea tube gel electrophoresis was performed at pH 2.3 in the presence of 6.25 M urea (Panyim and Chalky, 1969; Poole et al., 1974). The urea solutions were deionized with Amberlite MB-1 resin immediately prior to use. Hemocyanin samples were dissolved in a solution of 10 M urea (deionized), 5% glacial acetic acid, and 1% 2-mercaptoethanol at a concentration of approximately 15  $\mu$ g protein/0.2 ml solution. Samples were incubated at room temperature for at least 4 h. Pre-electrophoresis of the gels and electrophoresis of the samples were carried out at 2 mA per tube. Gels were stained with Coomassie brilliant blue R and destained with 10% acetic acid. Hemocyanin was also incubated in 6 M guanidine hydrochloride, 2% 2-mercaptoethanol at pH 7.5 in a boiling water bath for 1 min followed by 4 h at 22°C. It was then dialyzed against the urea/acetic acid/mercaptoethanol incubation buffer before analysis by urea gel electrophoresis.

## Regular Gel Electrophoresis

Regular gel electrophoresis in the absence of detergent or reducing agent was modified for slab gels from the procedure of Davis (1964). Hemocyanin samples, 20% in glycerol, were electrophoresed on either 5% or 7.5% polyacrylamide slab gels. The gels were pre-electrophoresed in the appropriate gel buffer for at least 2 hours and samples run under the same conditions. Calibrants included 25S, 16S, and 5S components of Cancer magister hemocyanin (Ellerton et al., 1970; Terwilliger and Terwilliger, 1980) and bovine serum albumin. Electrophoresis at pH 7.0 and 7.5 was carried out in a 0.05 M Tris-HCl/Tris-maleate buffer system. For dissociation experiments, the upper and lower buffers and the gel buffer were made 10 mM in  $\text{CaCl}_2$  or  $\text{MgCl}_2$ . Electrophoresis at pH 8.9 was carried out in a 0.05 M Tris-glycine/Tris-HCl buffer system. At pH 10.0 and 10.5 the electrophoresis buffer system was 0.05 M sodium bicarbonate/sodium glycinate. Unless otherwise noted, 10 mM EDTA was included in electrophoresis buffers at pH 8.9, 10.0 and 10.5. The gels were stained with Coomassie brilliant blue R and destained with 10% acetic acid. Hemocyanin was also incubated in reducing agent prior to regular gel electrophoresis at pH 7.0 in the presence of  $\text{CaCl}_2$ . Samples were made 10 mM in dithiothreitol (a 40:1 excess of dithiothreitol to

cysteine residues) or 1% in 2-mercaptoethanol and incubated at 37°C for 1.5 h before electrophoresis.

### Two-Stage Electrophoresis

In order to confirm the subunit identity of protein bands, samples which had been electrophoresed in one gel system were often re-electrophoresed in a different system. In these experiments the gels were briefly stained and destained according to Cleveland et al., (1977) after the first electrophoresis. Individual bands were then carefully cut out with a razor blade. Each slice was then soaked for 15-30 min with occasional swirling in 2 ml of the SDS incubation buffer and stored at -20°C. The slices were thawed and electrophoresed in the SDS gel system. Electrophoresis and staining were exactly as described previously.

### Gel Scans

Destained gels were scanned at 550 nm with a Zeiss PMQ II spectrophotometer. Relative concentrations of protein bands were determined by calculating the area under the peaks of the scan.

### Peptide Mapping

The procedures for obtaining peptide maps of protein bands isolated by gel electrophoresis are described by Cleveland et al., (1977). In order to have enough protein to visualize the peptide maps and maintain sufficient band separation, preparative 7.5% SDS slab gels were loaded with 30-50  $\mu\text{g}$  of hemocyanin and were electrophoresed for 5.5-6 h. The gels were briefly stained and destained after which the protein bands were cut out, soaked in SDS stacking gel buffer for 15-30 min and stored at  $-20^{\circ}\text{C}$  if necessary. The gel slices were then transferred to 15% acrylamide SDS slab gels with 4-5 cm long stacking gels. Wells of the slab gel were then overlaid with Staphylococcus aureus V8 protease (0.3 - 0.6  $\mu\text{g}$  enzyme) (Worthington Biochemical Corp.). Electrophoresis was started at 100 V and the current was turned off when the Coomassie blue stain reached the separating gel interface. Digestion was allowed to proceed for 30 min directly in the stacking gel. Electrophoresis was then resumed at 100 V and allowed to proceed until the Coomassie blue stain reached the bottom of the gel (3.5 h). The same peptide mapping procedure was also followed with protein bands obtained from urea gel electrophoresis.



### Amino Acid Analysis

The amino acid composition of purified  $M_1$  hemocyanin was determined according to Spackman et al., (1958). Cysteine and cystine were determined as cysteic acid on performic acid-oxidized hemocyanin as described by Hirs (1967).

### Dissociation Experiments

Dissociation at high pH was carried out by dialyzing the purified hemocyanin fractions overnight at 4° against either 0.05 M sodium glycinate buffer, pH 10.0 or 0.04 M sodium glycinate buffer, pH 10.5, 10 mM in EDTA. The samples were then chromatographed on a 1.8 x 97 cm BioGel A-1.5 M column equilibrated against the same buffer. Hemocyanin was also dialyzed extensively first against 0.05 ionic strength Tris-HCl buffer, pH 7.5, 10 mM in EDTA and then against 0.05 ionic strength Tris-HCl buffer, pH 7.5, made 4 M in urea. The samples were chromatographed on a 0.8 x 76 cm BioGel A-1.5 M column equilibrated against the urea buffer.

### Detection of Copper on Gels

The localization of copper on polyacrylamide gels based on the quenching of fluorescence of bathocuproine sulfonate was performed as in Bruyninckx et al., (1978). Hemocyanin samples were electrophoresed on both pH 7.5 (5% acrylamide) and pH 8.9 (7.5% acrylamide) regular slab gels in the absence of denaturants, reducing agents, and EDTA. Phosphorylase A, bovine serum albumin and ovalbumin were electrophoresed as controls. The gels were analyzed for copper as soon as they were removed from the electrophoresis apparatus. Photographs were made under UV illumination using a Minolta 35 mm camera with a UV filter, Kodak Plus-x film, ASA 125 and 20-60 sec exposures at f 5.6. The bands which appeared dark under UV illumination were marked with India ink and the gel then stained with Coomassie brilliant blue R according to Fairbanks et al., (1971).

### Oxygen Binding

Oxygen equilibrium studies were carried out by the tonometric method of Benesch et al., (1965) using a Zeiss PMQ II spectrophotometer. After equilibration to ambient  $pO_2$ , the tonometers were flushed with oxygen to insure maximum saturation. Values for the percent saturation with

oxygen were determined at 340 nm.

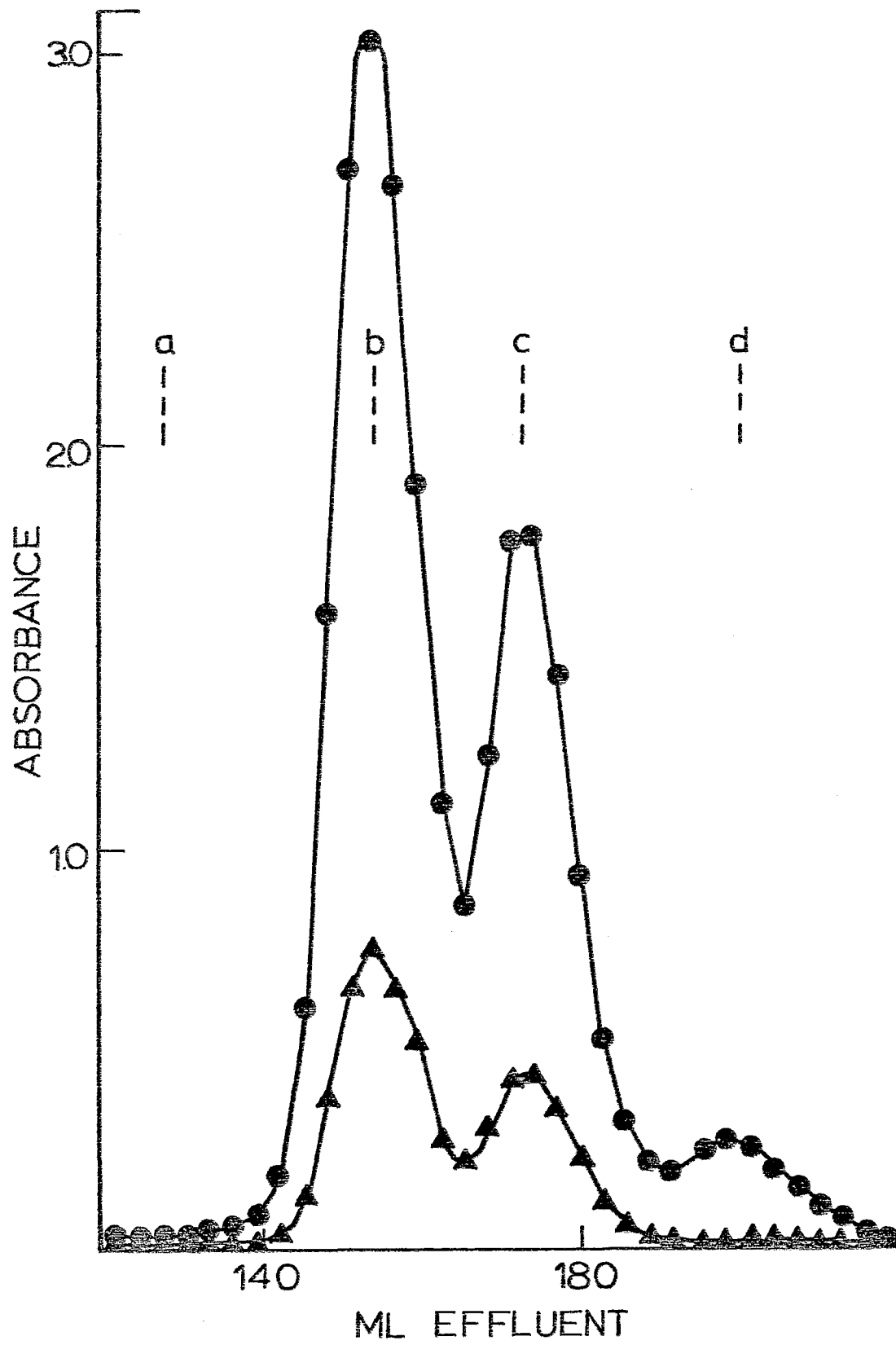
## RESULTS

Quaternary Structure

The hemocyanin of L. pallasii can be separated on a BioGel A5M column into two peaks (Figure 9). The first peak has an apparent molecular weight of approximately 900,000 and corresponds to a two-hexamer or 24S fraction. It will be designated 24S hemocyanin. The second peak has an apparent molecular weight of 450,000 and corresponds to a hexamer or 16S fraction. The 24S material comprises about 60% and the 16S material makes up about 40% of the L. pallasii hemocyanin at pH 7.5 in the presence of 10 mM MgCl<sub>2</sub>, 10 mM CaCl<sub>2</sub> and 0.1 M NaCl. A similar chromatography pattern is observed when a 0.05 ionic strength Tris-HCl buffer, pH 7.0, 10 mM in MgCl<sub>2</sub> and 0.1 M in NaCl is used. When a sample of either the 24S or the 16S material about six times more dilute than the original is rechromatographed on the same BioGel column, each sample elutes as a single peak in the same position as before. The absorbance ratios at 280 nm/340 nm for both the 24S and the 16S hemocyanin peaks are about 4.1.

A trailing peak of yellow material with an apparent molecular weight of about 150,000 is also present in Ligia

Figure 9. Elution pattern of Ligia pallasii hemocyanin chromatographed on BioGel A-5M (200-400 mesh), pH 7.5, 0.05 ionic strength Tris-HCl, 10 mM in MgCl<sub>2</sub>, 10 mM in CaCl<sub>2</sub> and 0.1 M in NaCl. Column volume 1.8 x 100<sup>2</sup>cm. Calibration proteins: (a) Eudistylia chlorocruorin, (b) 25S and (c) 16S Cancer magister hemocyanin, (d) lactate dehydrogenase. Absorbance at 280 (●) and 340 (▲) nm.



hemolymph (Figure 9). Its 280 nm/340 nm absorbance ratio is about 9.8. Analysis by electrophoresis indicated it was not hemocyanin, and it was not included as a hemocyanin component in the following experiments.

#### Subunit Composition

The purified 16S and 24S fractions each electrophorese on a 7.5% polyacrylamide SDS slab gel in the presence of 2-mercaptoethanol as a single component with an apparent molecular weight of approximately  $80,000 \pm 1500$  (S.D. of six determinations) (Figure 10B, C). The molecular weight was determined by a plot of log molecular weight versus mobility. The six polypeptide chains of Cancer magister hemocyanin, which range in molecular weight from 81,800 to 67,300 (Larson et al., 1981) can easily be resolved on this same gel system (Figure 10A). In the presence of SDS but in the absence of 2-mercaptoethanol, the 16S fraction electrophoreses as a single band corresponding to a molecular weight of 80,000 (Figure 10D) whereas the 24S fraction can be resolved into two bands with apparent molecular weights of 80,000 and 82,000 (Figure 10E). These two putative monomer bands will be referred to as  $M_1$  and  $M_2$  respectively. The staining intensity of  $M_2$  is about five times less than that of  $M_1$  as determined by measuring the area under the

Figure 10. SDS gel electrophoresis of Ligia pallasii hemocyanin on 7.5% slab gel. A-C, samples incubated in buffer containing 5% 2-mercaptoethanol. (A) control, Cancer magister 25S hemocyanin, (B) Ligia 16S, (C) 24S. D-F, 2-mercaptoethanol omitted from incubation buffer. (D) Ligia 16S, (E) 24S, (F) artificial hexamer from BioGel A-1.5M fractionation.



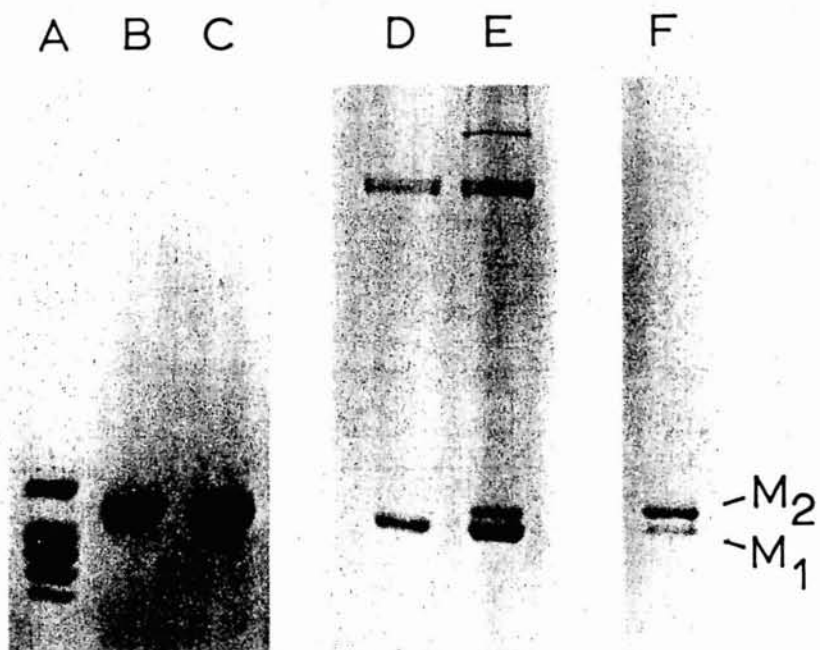


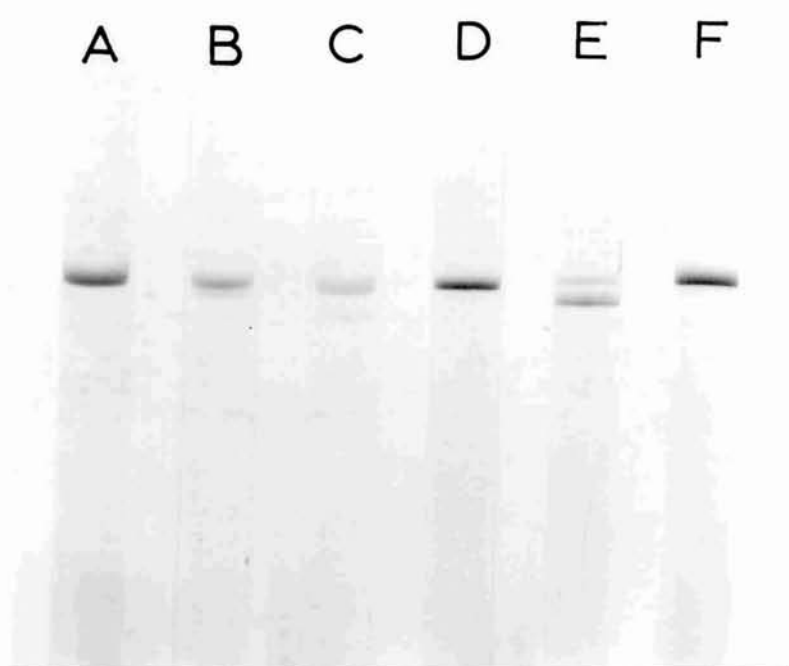
Figure 11. Diagram of SDS gel electrophoresis of Ligia pallasii hemocyanin. A-E, samples incubated in buffer containing 5% 2-mercaptoethanol; A'-E', 2-mercaptoethanol omitted from buffer. (A) 16S, (B) 24S, (C) monomer fraction from 16S sample on BioGel A-1.5M, (D) artificial hexamer fraction from 24S sample on BioGel A-1.5M, (E) monomer fraction from 24S sample on BioGel A-1.5M. Arrow indicates position of dye front.



peaks of stained gels scanned at 550 nm. The 80,000 molecular weight monomer of the 16S fraction is electrophoretically identical to  $M_1$  obtained from the 24S fraction. In the absence of 2-mercaptoethanol several bands corresponding to higher molecular weight components are also present; the 16S fraction shows one band ( $M_r = 140,000$ ) (Figure 10D) and the 24S fraction two bands ( $M_r = 140,000$  and 157,000) (Figure 10E) as determined on a 5% polyacrylamide SDS slab gel. When the various bands obtained by SDS gel electrophoresis in the absence of 2-mercaptoethanol of 16S and 24S hemocyanin are excised from the gel and re-electrophoresed in SDS with 2-mercaptoethanol, they all electrophorese as single bands with a molecular weight of 80,000 as in Figure 10B, C.

Urea tube gel electrophoresis of the hemocyanin fractions at pH 2.3 in the presence of 2-mercaptoethanol gives the following results. The 16S fraction contains only one component (Figure 12A). The 24S fraction is composed of 2 closely migrating bands (Figure 12B). The upper, more slowly migrating band is present in approximately five times higher concentration than is the lower, faster migrating band. Urea gels run on hemocyanin fractions prepared without reducing agent give similar results except that the lower band in the 24S fraction migrates slightly faster and therefore is separated farther from the upper band (Figure

Figure 12. Urea gel electrophoresis at pH 2.3 of Ligia pallasii hemocyanin. Samples incubated with 2-mercaptoethanol except where noted. (A) 16S, (B) 24S, (C) 24S minus mercaptoethanol, (D) monomer fraction from 16S sample on BioGel A-1.5M, pH 10, (E) hexamer fraction from 24S sample on BioGel A-1.5M, pH 10, (F) monomer fraction from 24S sample on BioGel A-1.5M, pH 10.



12C). The single band of the 16S fraction co-electrophoreses with the upper band of the 24S fraction. Both of these bands are equivalent to the  $M_1$  band identified by SDS gel electrophoresis, while the lower band of the 24S fraction is equivalent to  $M_2$  from SDS. These correspondences were confirmed by cutting out the bands from the urea gels and re-electrophoresing them in SDS in both the presence and absence of reducing agent. Further evidence of the equivalencies of the urea gel bands and the SDS gel bands is provided by the fact that the relative concentrations as determined by gel scans of  $M_1$  and  $M_2$  in the 24S fraction are similar in urea gels and SDS gels. Because of the possibility that incubation of the samples in urea with 2-mercaptoethanol at low pH might not result in complete cleavage of disulfide bonds or unfolding of the polypeptide chain, hemocyanin fractions were also incubated in 6 M guanidine hydrochloride, 2% 2-mercaptoethanol at pH 7.5 and then dialyzed against 10 M urea, 2% 2-mercaptoethanol at pH 2.3. Subsequent analysis of the fractions by urea gel electrophoresis showed the same results as described above.

Further characterization of  $M_1$  subunits in the 16S fraction and  $M_1$  and  $M_2$  subunits in the 24S fraction was carried out by analysis of the subunits according to the modified peptide mapping technique of Cleveland et al., (1977). The  $M_1$  and  $M_2$  subunits were separated by SDS gel electro-

phoresis in the absence of reducing agents, and peptide mapping was carried out as described in Methods. Whole 16S and 24S protein in SDS incubation buffer containing 2-mercaptoethanol were run as controls. SDS band  $M_1$  from the 24S fraction (Figure 13C) and SDS band  $M_1$  from the 16S fraction (Figure 13E) show indistinguishable peptide patterns after limited digestion by S. aureus V8 protease. The control 16S protein (Figure 13A, F) shows the same digestion pattern as band  $M_1$ . The peptide pattern of band  $M_2$  from the 24S fraction (Figure 13D) is clearly discernable from that of band  $M_1$ . The control 24S hemocyanin peptide pattern (Figure 13B) is a composite of the peptides from both  $M_1$  and  $M_2$ . The peptide mapping procedure was also carried out on  $M_1$  and  $M_2$  bands obtained by urea gel electrophoresis. The results were indistinguishable from the peptide patterns of bands obtained by SDS gel electrophoresis.

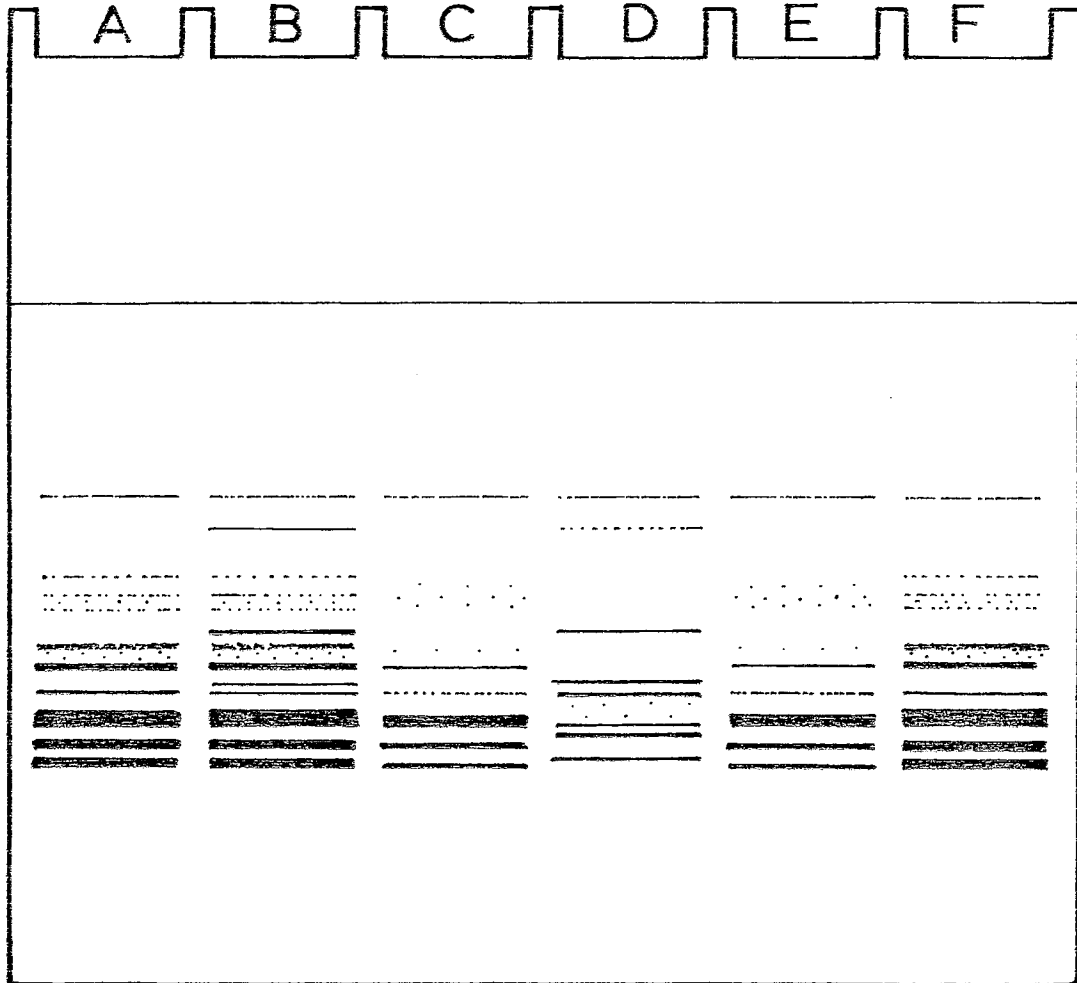
The experiments described above were carried out on samples of hemolymph which had been pooled from a large number of animals. In order to determine whether the  $M_1$  and  $M_2$  subunit pattern seen in the pooled 24S fraction was simply a reflection of polymorphism in the population, hemolymph samples were removed from individual animals and run separately on SDS gels in the absence of reducing agent. Hemolymph from animals of different sizes and sexes and females in both ovigerous and non-ovigerous stages of their repro-



Figure 13a. Modified peptide maps on 15% gel of Ligia pallasii hemocyanin subunits. Each band was cut from an SDS gel (run in the absence of 2-mercaptoethanol) and treated with 0.6  $\mu$ g Staphylococcus aureus V8 protease as described in Methods. (A) 16S fraction, (B) 24S fraction, (C)  $M_1$  from 24S fraction, (D)  $M_2$  from 24S fraction, (E)  $M_1$  from 16S fraction, (F) 16S fraction<sup>1</sup>.



Figure 13b. Diagram of Figure 13a.



ductive cycle all showed the pattern of a major  $M_1$  and a minor  $M_2$  band on SDS gels.

The amino acid composition of the  $M_1$  subunits from the 16S fraction is shown in Table III. Purified  $M_2$  subunits were not available in adequate concentration for an accurate amino acid analysis.

#### Dissociation and Reassembly of *L. pallasii* Hemocyanin

##### $Ca^{2+}$ Sensitive Reversible Dissociation to Hexamers

Whole unfractionated hemolymph electrophoreses as two major components on a regular 5% polyacrylamide slab gel at both pH 7.0 and 7.5 in the presence of  $CaCl_2$  (Figure 14B). The upper band corresponds to a two-hexamer aggregate (24S fraction) and the middle band, a hexamer (16S fraction), based on comparisons with electrophoresis of 25S and 16S components of *Cancer magister* hemocyanin (Carpenter and Van Holde, 1973; Terwilliger and Terwilliger, 1980). The lowest more diffusely staining band is presumed to be a non-hemocyanin protein as discussed below. This electrophoretic pattern is identical to that observed by fractionation of whole hemolymph by column chromatography at neutral pH in the presence of  $CaCl_2$  and/or 0.1 M NaCl (Figure 9). However, when hemolymph is electrophoresed in the absence of divalent

Table III: Amino Acid Compositions of M<sub>1</sub> Subunits of Ligia pallasii Hemocyanin and Whole Hemocyanin of Ligia italica.

Amino acid	<u>Ligia pallasii</u>	<u>Ligia italica</u>
	M <sub>1</sub> Subunits	whole hemocyanin <sup>a</sup>
	Residues/100 residues	Residues/100 residues
Lys	5.2	4.9
His	6.8	6.4
Arg	4.8	5.2
Asp	13.4	12.7
Thr	5.7	5.5
Ser	5.4	5.5
Glu	10.6	10.6
Pro	5.5	4.1
Gly	7.3	6.2
Ala	6.5	6.8 <sup>c</sup>
Cys	0.65 <sup>b</sup>	---
Val	8.0	6.8
Met	2.2	2.1
Ile	3.9	4.4
Leu	7.8	8.4
Tyr	3.8	3.5
Phe	4.9 <sup>c</sup>	6.2 <sup>c</sup>
Trp	---	---

<sup>a</sup> from Sevilla, 1978.

<sup>b</sup> determined as cysteic acid.

<sup>c</sup> not measured.

Figure 14a. Regular slab gel electrophoresis, pH 7.0, on 5% acrylamide gels of Ligia pallasii 16S and 24S fractions in the presence and absence of  $\text{Ca}^{2+}$ .

A-D: gel buffers 10 mM in  $\text{CaCl}_2$ .

- (A) Cancer magister hemocyanin,
- (B) unfractionated Ligia hemolymph,
- (C) Ligia 24S fraction,
- (D) Ligia 16S fraction.

A'-D', E': no calcium added. A'-D', same as above. (E') Ligia 24S fraction plus 16S fraction.

C'', D'', F: gel buffers 10 mM in  $\text{CaCl}_2$ .

- (C'') Ligia 24S fraction, (D'') Ligia 16S fraction, (F) Ligia 24S fraction which had been dissociated to 16S by dialysis against  $\text{Ca}^{2+}$ -free buffer prior to reassembly in this electrophoresis.

The S values reflect accepted terminology for aggregation states of crustacean hemocyanin rather than specifically determined  $S_{20,w}$  values.

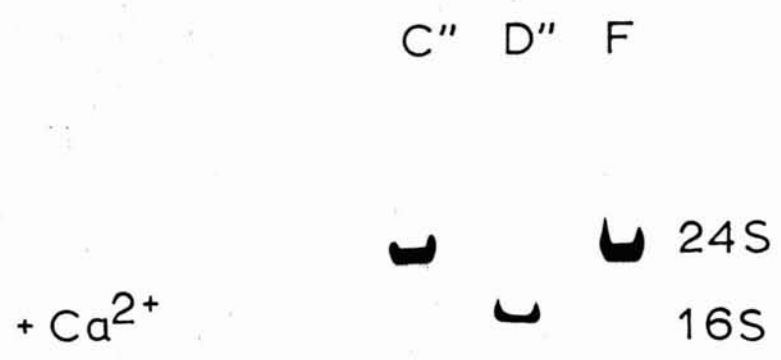
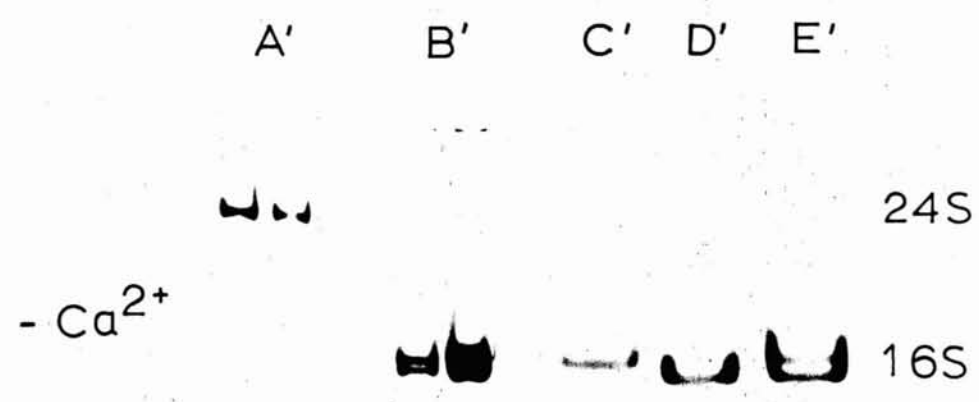
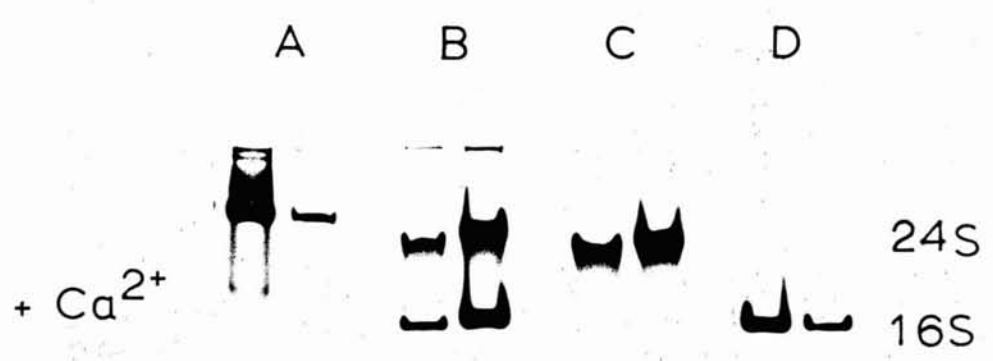
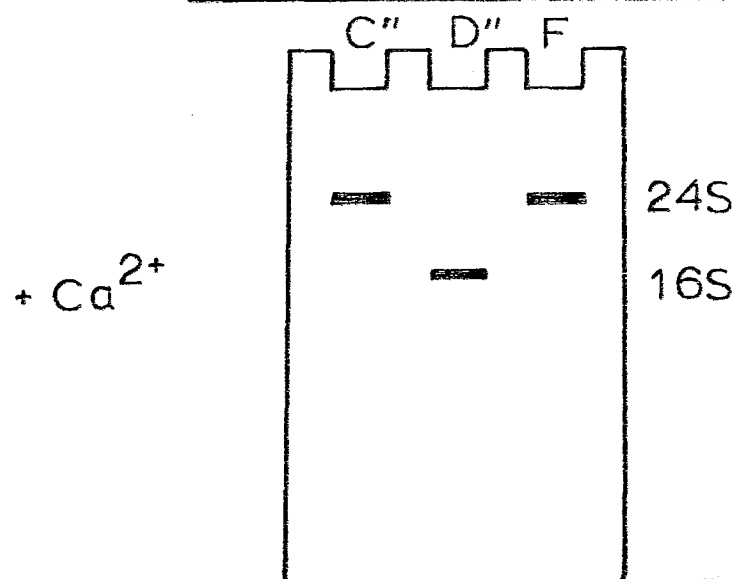
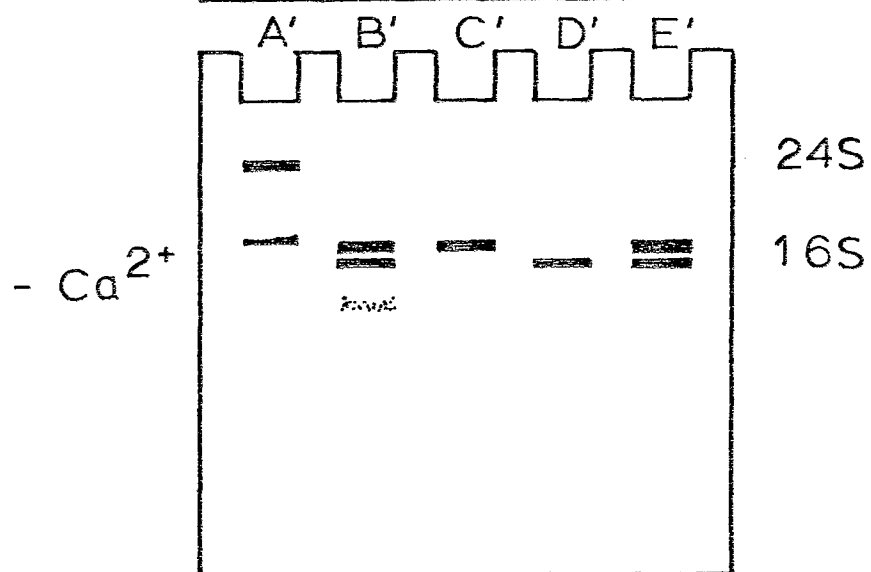
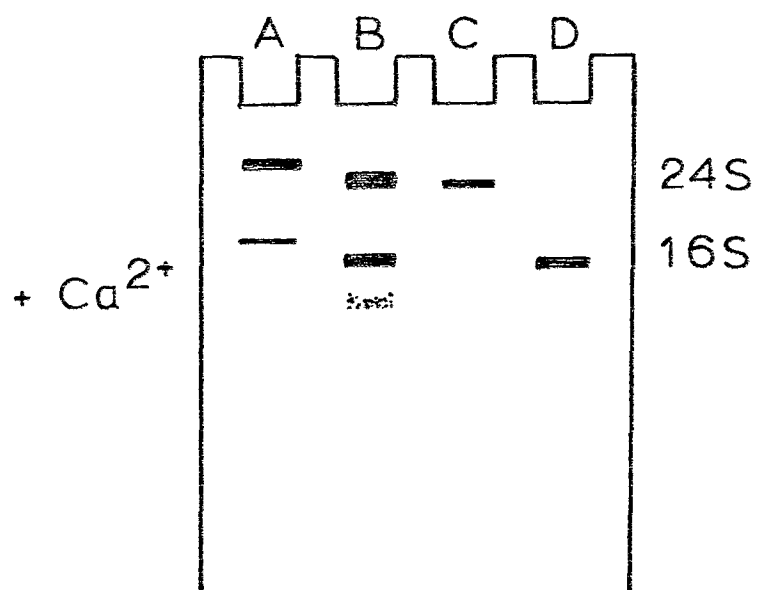




Figure 14b. Diagram of Figure 14a.



cations or when an equimolar concentration of  $MgCl_2$  is substituted for  $CaCl_2$ , the 24S band is missing. Instead, two distinct hexamer bands are present (Figure 14B'). The identities of the two hexamer bands were investigated by reelectrophoresing each band on an SDS gel in the absence of reducing agent. The upper hexamer band (Figure 14B') consists of both  $M_1$  and  $M_2$  subunits in a ratio of approximately 5:1 while the lower hexamer band is composed solely of  $M_1$  subunits.

Similar experiments were carried out on purified 24S and 16S fractions (see Figure 9). The 24S fraction electrophoreses as a single band corresponding to intact 24S molecules at pH 7.0-7.5 in the presence of  $Ca^{2+}$  (Figure 14C). In the absence of  $Ca^{2+}$ , the 24S polymer electrophoreses as a single band corresponding to a hexamer (Figure 14C'). The purified 16S fraction electrophoreses as a single band corresponding to a hexamer of slightly faster mobility than the dissociated 24S material regardless of whether or not  $Ca^{2+}$  is present between pH 7.0-7.5 (Figure 14D, D'). Co-electrophoresis of 24S and 16S samples in the absence of  $Ca^{2+}$  resolves both hexamer bands (Figure 14E). Thus the 24S molecules can be dissociated into hexamers simply by removing  $Ca^{2+}$ . Furthermore, based on electrophoresis of whole hemolymph and purified fractions, the upper hexamer band is derived from the dissociation of the

native 24S molecules; the lower hexamer band corresponds to the native 16S molecules. The diffusely staining band seen in electrophoresis of whole hemolymph and described as a non-hemocyanin protein was not observed in the purified fractions.

The  $\text{Ca}^{2+}$  sensitive dissociation of Ligia 24S hemocyanin into hexamers is a reversible process. L. pallasii 24S polymers were dialyzed overnight against the pH 7.0 Tris-maleate electrophoresis buffer made 10 mM in EDTA to insure removal of divalent cations and dissociation into hexamers. Next, EDTA was removed by dialysis against the same buffer minus EDTA. The samples were then electrophoresed on a pH 7.0 regular gel in the presence of  $\text{Ca}^{2+}$ . The 24S molecules which had been dissociated to hexamers now electrophoresed as two-hexamer molecules (Figure 14F).

Reversible dissociation of the 24S fraction into hexamers was also analyzed by column chromatography. Purified 24S hemocyanin was dialyzed against 0.05 M Tris-HCl, pH 9.0, 10 mM in EDTA, 0.1 M in NaCl, conditions which have been shown to cause L. exotica hemocyanin to completely dissociate to hexamers. The hemocyanin was then redialyzed against the pH 7.5 Tris/ $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$  buffer and rechromatographed on a BioGel A-5M column equilibrated against the same  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$  buffer. All of the hemocyanin chromatographed as a 24S fraction indicating that reassociation had occurred.

Are the two hexamers of a Ligia 24S hemocyanin aggregate linked by a disulfide-bonded dimer? A disulfide linkage has been suggested for other hemocyanins (see Markl et al., 1981). The complete dissociation of Ligia 24S hemocyanin into hexamers merely by removing  $\text{Ca}^{2+}$  at neutral pH argues against the idea that the Ligia 24S aggregate is formed by a disulfide linked dimer. However, to further investigate the presence or absence of an intermolecular disulfide bond, 16S and 24S samples were incubated in dithiothreitol or 2-mercaptoethanol. The samples were then electrophoresed on a regular gel at pH 7.0 in the presence of  $\text{Ca}^{2+}$  (conditions which normally maintain the 24S configuration). After incubation in dithiothreitol, most of the two-hexamer 24S molecules remained intact; less than 20% of the protein dissociated into a diffuse band corresponding to hexamers (Figure 15B). Electrophoresis after incubation in 2-mercaptoethanol resulted in a broad smear of stainable material on the gel ranging from what would correspond to 24S molecules down to 16S molecules.

#### Dissociation to Monomers at Alkaline pH

Dissociation of the native hexamers and two-hexamers into monomers was studied by dialyzing the two purified fractions against high pH, low ionic strength buffer as

Figure 15. Regular slab gel electrophoresis, pH 7.0, 10 mM in  $\text{CaCl}_2$ , on 5% acrylamide gel of L. pallasii hemocyanin incubated in 10 mM dithiothreitol for 1.5 hours at 37°C prior to electrophoresis. (A) control C. magister hemolymph, dithiothreitol, (B) L. pallasii 24S fraction, dithiothreitol, (C) L. pallasii 16S fraction, dithiothreitol, (D) L. pallasii 24S and 16S fractions, dithiothreitol, E-H: no dithiothreitol. (E) C. magister hemolymph, (F) L. pallasii 24S fraction, (G) L. pallasii 16S fraction, (H) L. pallasii 24S and 16S fractions.



|—A—|—B—|—C—|—D—|EFGH

described in Methods, Part II. Each fraction was then chromatographed on a BioGel A-1.5 M column equilibrated against the same buffer. The 16S hexamers dissociate completely into monomers ( $M_r = 70,000$ ) under these conditions (Figure 16A). The 24S two-hexamers dissociate into two components. About 80% of the protein is dissociated into monomers ( $M_r = 70,000$ ); the remaining 20% chromatographs as putative hexamers (Figure 16B).

SDS gel electrophoresis of the BioGel A-1.5 M chromatography fractions shows that both the monomeric fraction derived from native 16S hexamers (Figure 11C, C') and the monomeric fraction from the native 24S two-hexamers (Figure 11E, E') are composed solely of  $M_1$  subunits. The putative hexamer component derived from the native 24S fraction contains all of the  $M_2$  subunits plus some  $M_1$  subunits (Figure 10F, 11D, 11D'). The  $M_2$  subunits are present in a ratio of four  $M_2$  to two  $M_1$  subunits, compared to the one  $M_2$ , five  $M_1$  ratio found in the native 24S molecules. The same results are found when the BioGel A-1.5 M fractions are analyzed by urea gel electrophoresis (Figure 12 D-F).

Electrophoretic analyses on regular gels in the absence of denaturants or reducing agents of these high pH dissociation products are shown in Figure 17. The monomeric dissociation product of the native 16S hemocyanin electrophoreses as a single band corresponding to a 5S subunit at



Figure 16. Elution patterns for L. pallasii purified 16S and 24S hemocyanin chromatographed on BioGel A-1.5 M after dialysis vs. 0.05 M sodium glycinate buffer, pH 10.0. Column volume 1.8 x 97 cm; column buffer same as dialysate. Calibration proteins: (a) phosphorylase A; (b) bovine serum albumin; (c) ovalbumin. A, 16S fraction; B, 24S fraction. Absorbance at 280 ● and 340 ▲ nm.

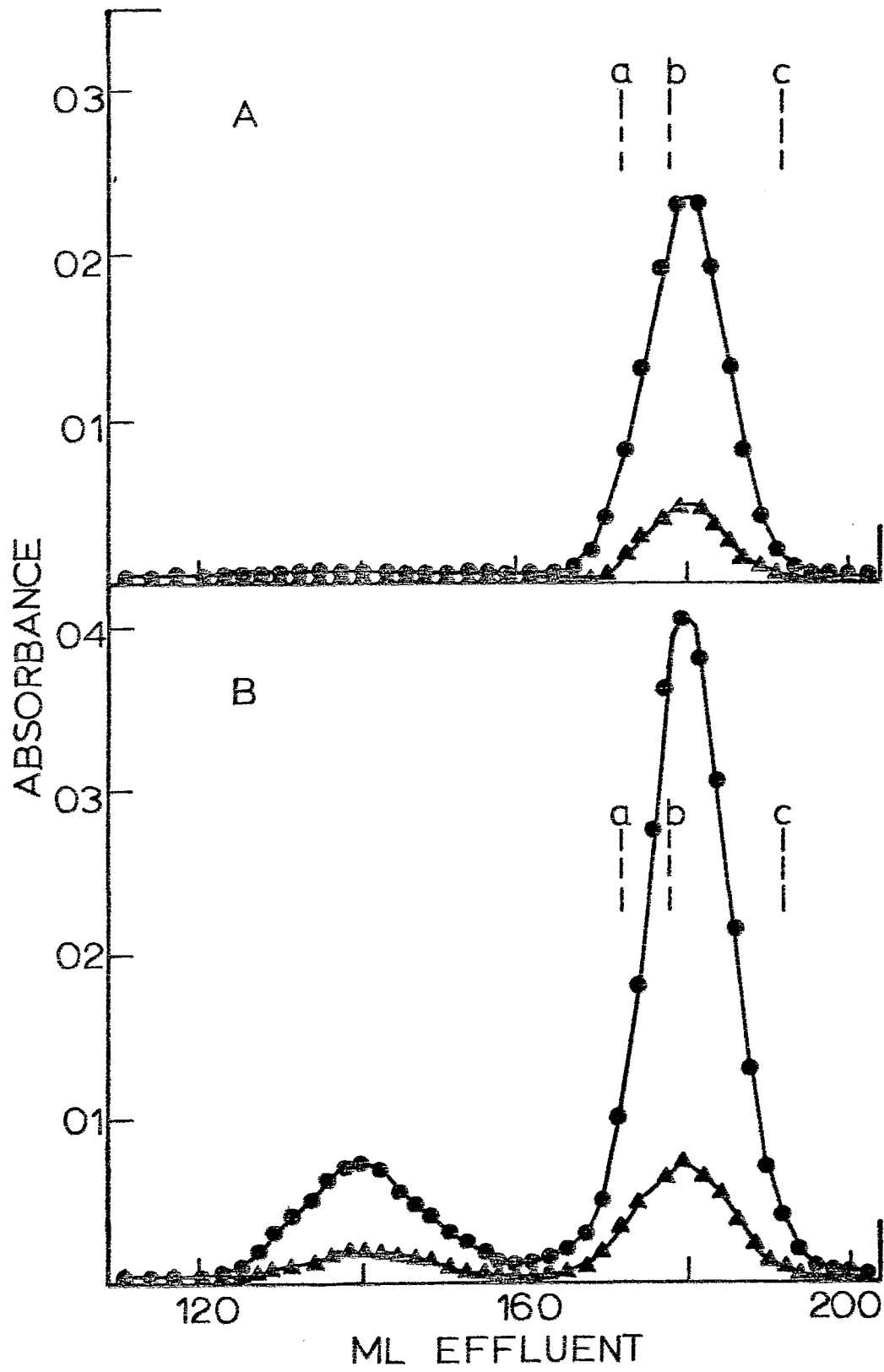


Figure 17a. Regular gel electrophoresis of L. pallasii hemocyanin. I, pH 7.5 gel, 5% acrylamide; II, pH 8.9 gel, 10 mM EDTA, 7.5% acrylamide; III, pH 10.5 gel, 10 mM EDTA, 7.5% acrylamide. (A) Cancer magister hemocyanin, (B) Ligia 24S, (C) Ligia 16S, (D) monomers from 16S fraction, BioGel A-1.5M, pH 10.0, (E) artificial hexamers from 24S fraction, BioGel A-1.5M, pH 10.0. A'-E', B''-E'', same as above.

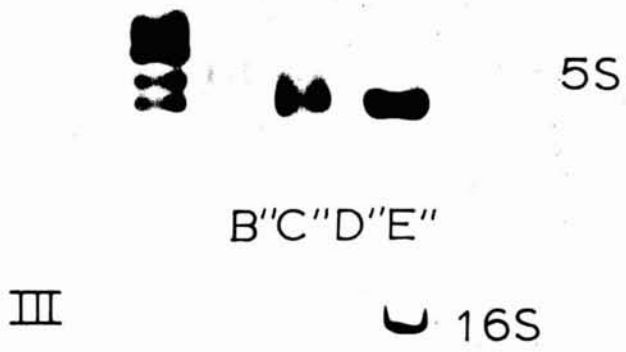
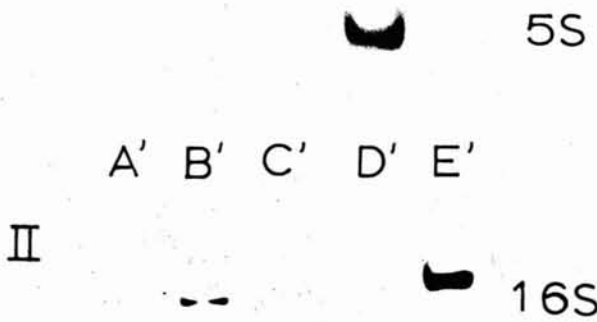
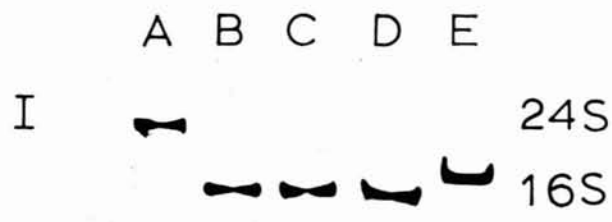
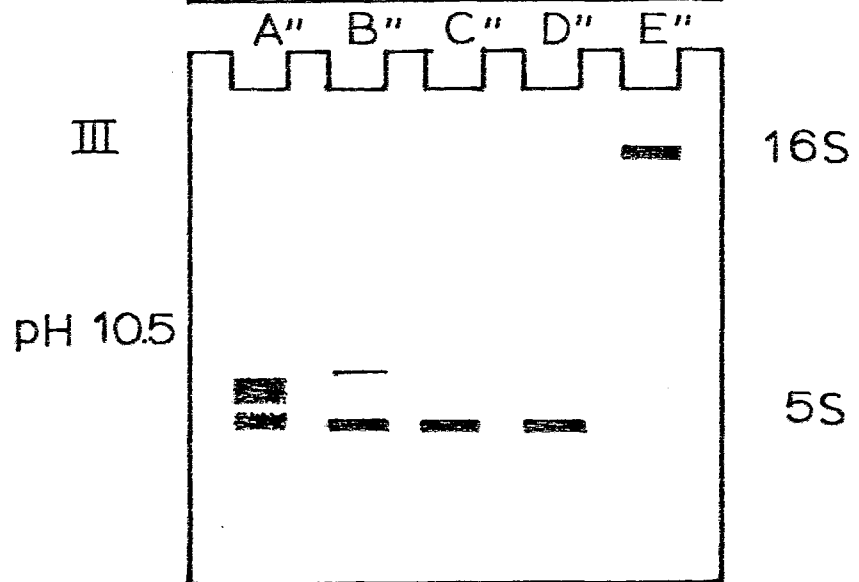
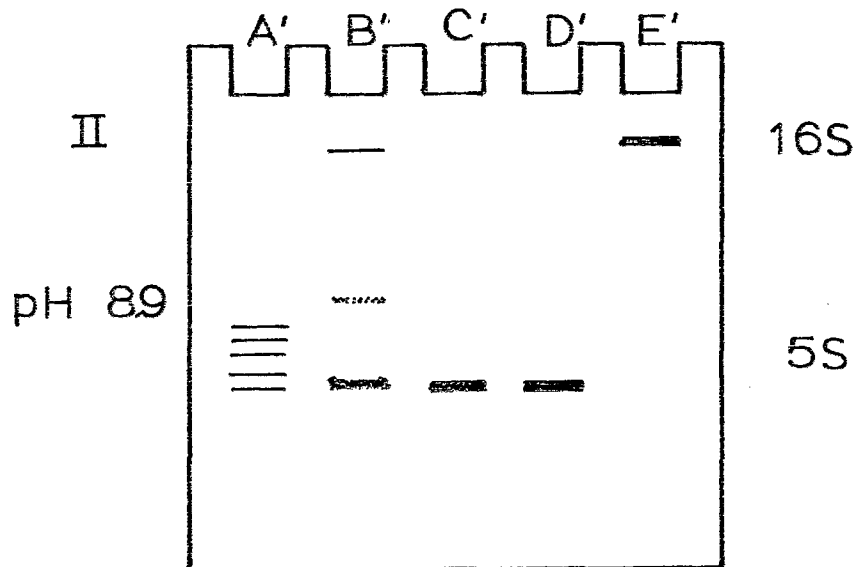
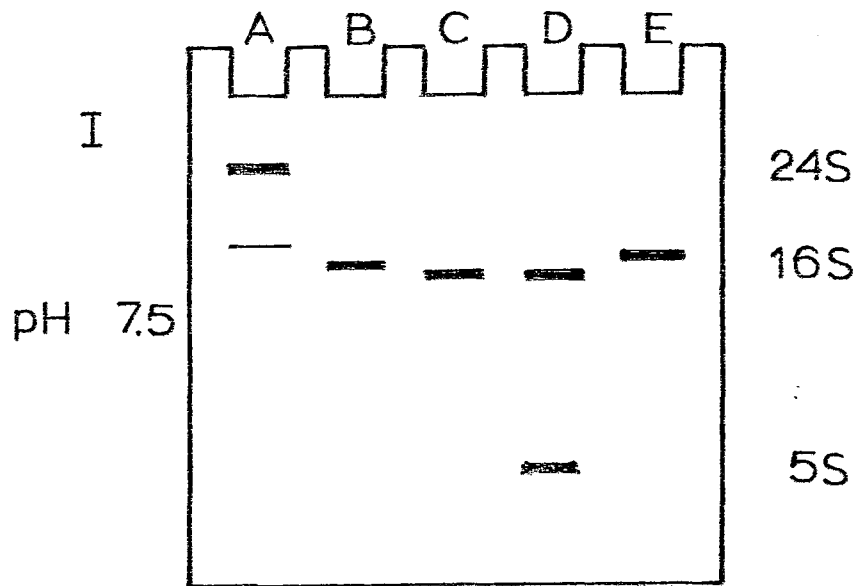


Figure 17b. Diagram of Figure 17a.



pH 8.9 (Figure 17 D'), 10.0 or 10.5 (Figure 17 D'') and shows partial reassociation to a 16S hexamer when electrophoresed at pH 7.5 (Figure 17D). The monomeric dissociation product of the 24S fraction behaves in the same manner. In contrast, the putative hexamer dissociation product of the 24S fraction electrophoreses as a single hexamer component under all conditions studied (Figure 17E-E''). Its mobility at pH 7.5 in the absence of  $\text{Ca}^{2+}$  is slower than either the hexamer band of a dissociated 24S molecule or that of a native undissociated 16S molecule. Even at high pH, the putative hexamer does not dissociate to monomer.

Is this putative hexamer component with the high  $M_2$  subunit ratio a hexamer which was part of the original 24S polymer and which is especially resistant to further dissociation into monomer, or is it a reassembly of dissociated subunits into an artificial hexamer? The following experiment indicates that it is an artificial hexamer. When the 24S fraction is dissociated into subunits at high pH and they are allowed to remain in proximity with one another during the dialysis, the resulting putative hexamer component electrophoreses as a hexamer not only at pH 7.5 but also at pH 10.5 (Figure 17 E-E''). However, when the native 24S hemocyanin at pH 7.5 is electrophoresed directly into a pH 10.5 gel without prior dialysis, no hexamer band is observed; all the protein electrophoreses as two low molecular

weight bands (Figure 17 B"). Thus the 24S aggregate remains intact until it comes in contact with the upper gel buffer at which time it presumably begins to dissociate. Reassociation of the subunits into an artificial hexamer cannot occur because the subunits are physically separated from one another in the gel.

Dissociation of the 24S fraction into monomers was attempted by dialysis against 4 M urea, pH 7.5, as described in Methods, in order to obtain a sufficient quantity of non-denatured  $M_2$  subunits for reassembly and oxygen equilibria experiments. After dialysis, the samples were chromatographed on a BioGel A-1.5 M column equilibrated against the same urea buffer. Analysis of the results indicated a series of protein fractions with a broad range of molecular weights, suggesting partial dissociation, reaggregation or denaturation had occurred. This technique was not pursued further.

#### Reassembly of Monomers

The dissociation of the native 16S molecules to  $M_1$  monomers is a reversible process. When the monomers obtained by dialysis at high pH in EDTA are dialyzed back to neutral pH in the presence or absence of divalent cations, they reassemble to hexamers as determined by column chroma-



tography. No higher molecular weight aggregates are observed. When the native 24S fraction is dialyzed against high pH in EDTA and then returned to neutral pH in the presence of divalent cations, complete reassembly to hexamers also is observed. However, the dissociated 24S fraction, which is a mixture of artificial  $M_2:M_1$  hexamers and  $M_1$  monomers, does not reassemble into two-hexamer aggregates under these conditions.

#### Detection of Copper on Gels

When the 16S and 24S samples electrophoresed on a pH 7.5 regular slab gel in the absence of EDTA were treated to detect copper, positive fluorescence quenching of both 16S and 24S components occurred (Figure 18 B-E). Thus copper is present in both the 16S and 24S fractions. Ovalbumin, run as a copper-free protein control, showed no quenching. A similar experiment run at pH 8.9 in the absence of EDTA showed copper was present in the  $M_1$  subunits of the 16S fraction (see Figure 17C'). The 24S fraction electrophoreses at pH 8.9 in the absence of EDTA as a diffuse area of dissociated protein and therefore it was not possible to differentiate between the presence of copper in subunits  $M_1$  and  $M_2$ .

Figure 18. Detection of copper in Ligia pallasii hemocyanin on regular slab gel, pH 7.5, 5% acrylamide. A-E: samples photographed under UV illumination. Dark bands represent quenching of fluorescence due to presence of copper. (A) Cancer magister hemolymph, (B) (C) Ligia 24S fraction, (D)(E) Ligia 16S fraction. A'-E': same samples after gel had been stained with Coomassie blue.

A B C D E A' B' C' D' E'



### Functional Properties

Oxygen equilibrium studies on the purified 24S and 16S fractions indicate that both polymers have similar oxygen affinities and show a strong positive Bohr effect over the pH range of 7.0-8.35. This pH range was chosen since the pH of hemolymph from several isopod species appears to be about 7.5-7.7 at 20°C (Sevilla and Lagarrigue, 1979b). The oxygen equilibrium curves of the 24S (Figure 19A) and 16S (Figure 19B) fractions are shown. The relationship between oxygen affinity and pH for both 24S and 16S hemocyanin is shown in Figure 20. The 24S and 16S fractions show a relatively high degree of cooperativity as indicated in Figure 21. The 24S fraction shows a greater cooperativity than the 16S fraction at all pH's examined, with  $n_H = 3.6-5.3$  for the 24S and  $n_H = 2.7-3.6$  for the 16S fraction. The cooperativity of the 24S fraction is more responsive to changes in pH than is that of the 16S fraction.

Aliquots of 24S and 16S fractions were dialysed against 0.05 ionic strength Tris-HCl, pH 9.0, 10 mM in EDTA, 0.1 M in NaCl, (conditions which cause L. exotica 24S molecules to dissociate to hexamer) and re-dialyzed back to the standard pH 7.5/Ca<sup>2+</sup>/Mg<sup>2+</sup> buffer in order to observe the effect of dissociation and reassociation on the oxygen binding properties. The oxygen binding curves of both the 24S and

Figure 19. Oxygen binding by L. pallasii hemocyanin, 20°C. A, 24S fraction; B. 16S fraction.

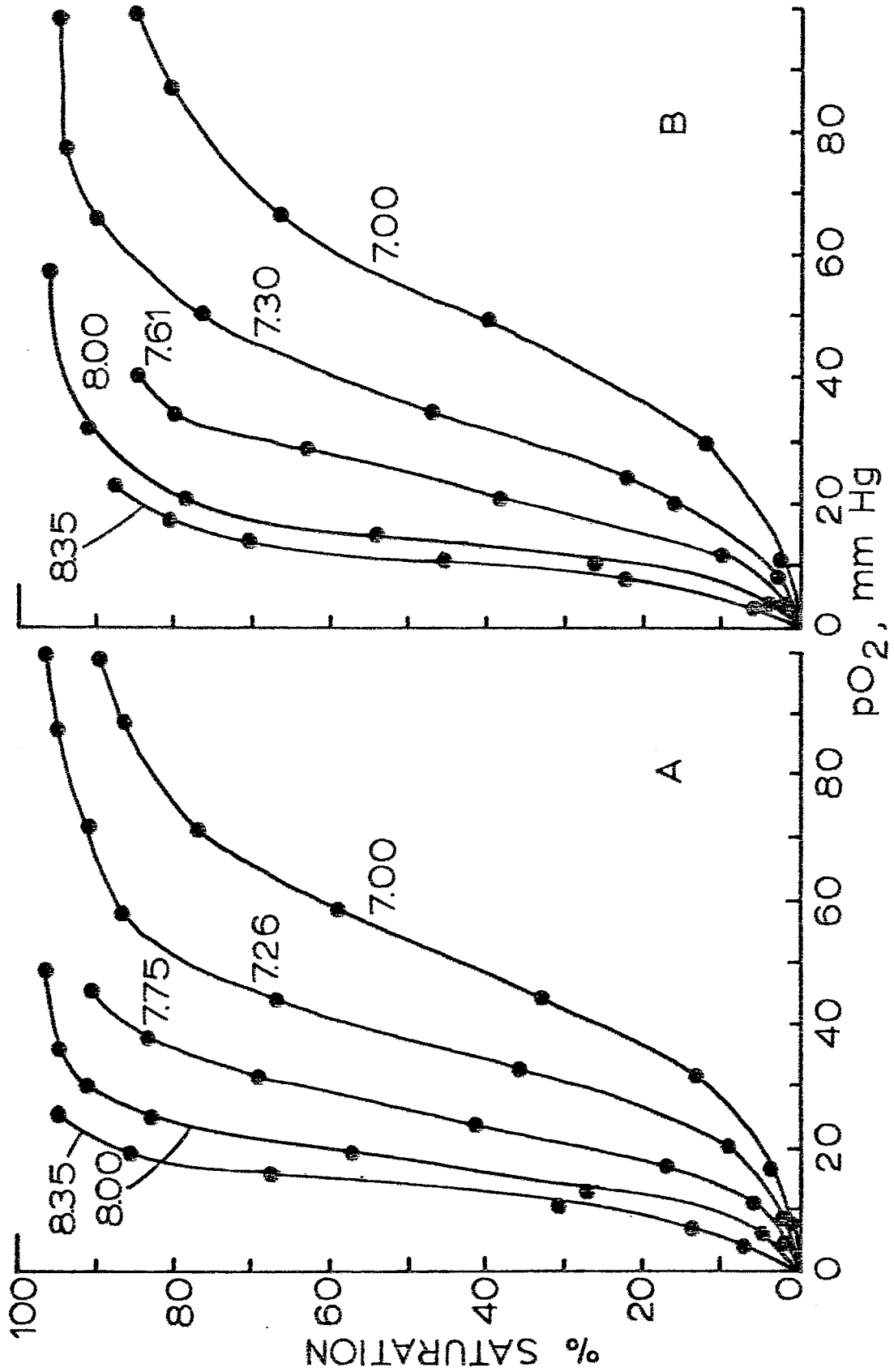


Figure 20.  $\log P_{1/2}$  as a function of pH for L. pallasii  
16S ▲ and 24S ● hemocyanin. Temperature,  
20°C.

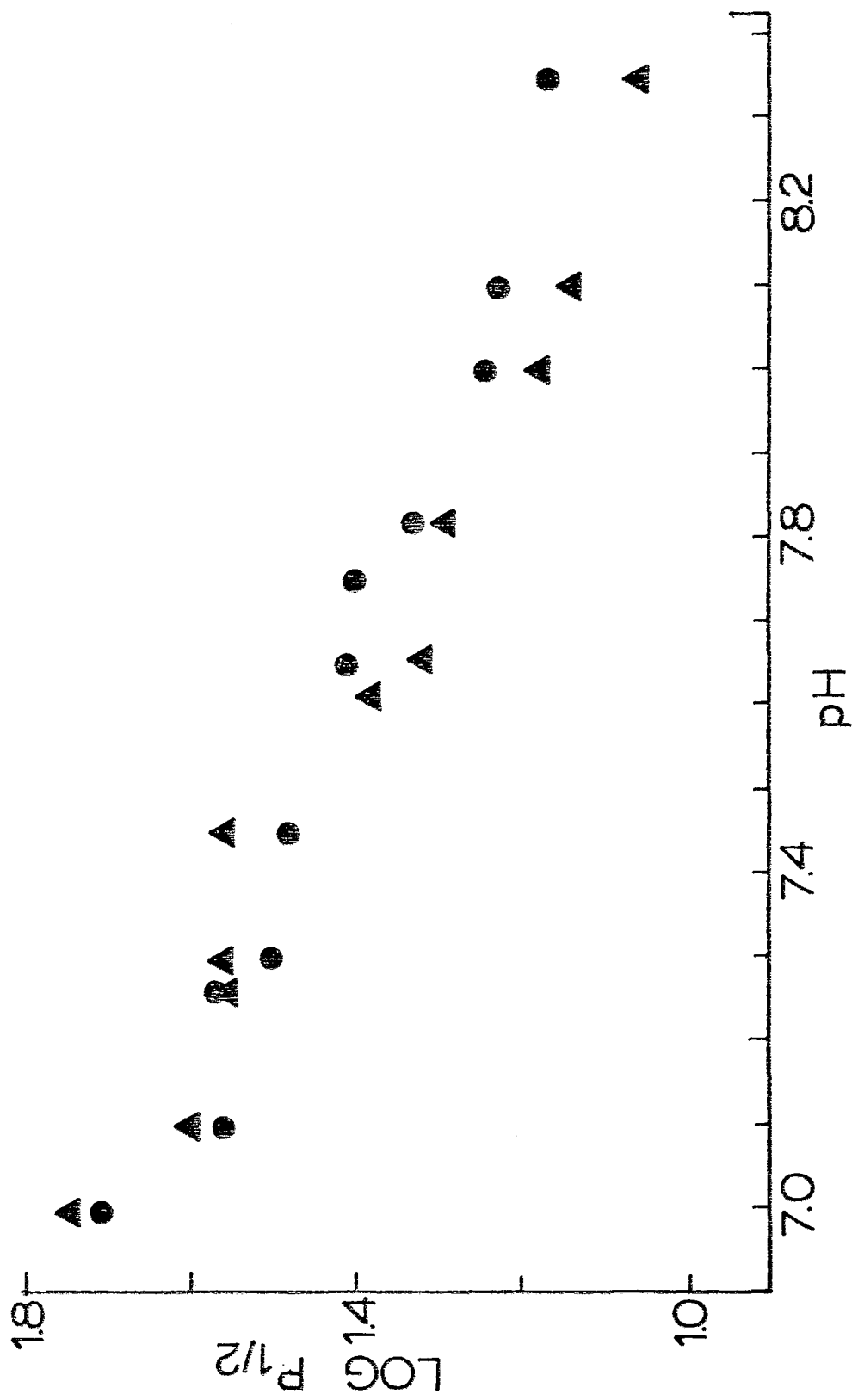
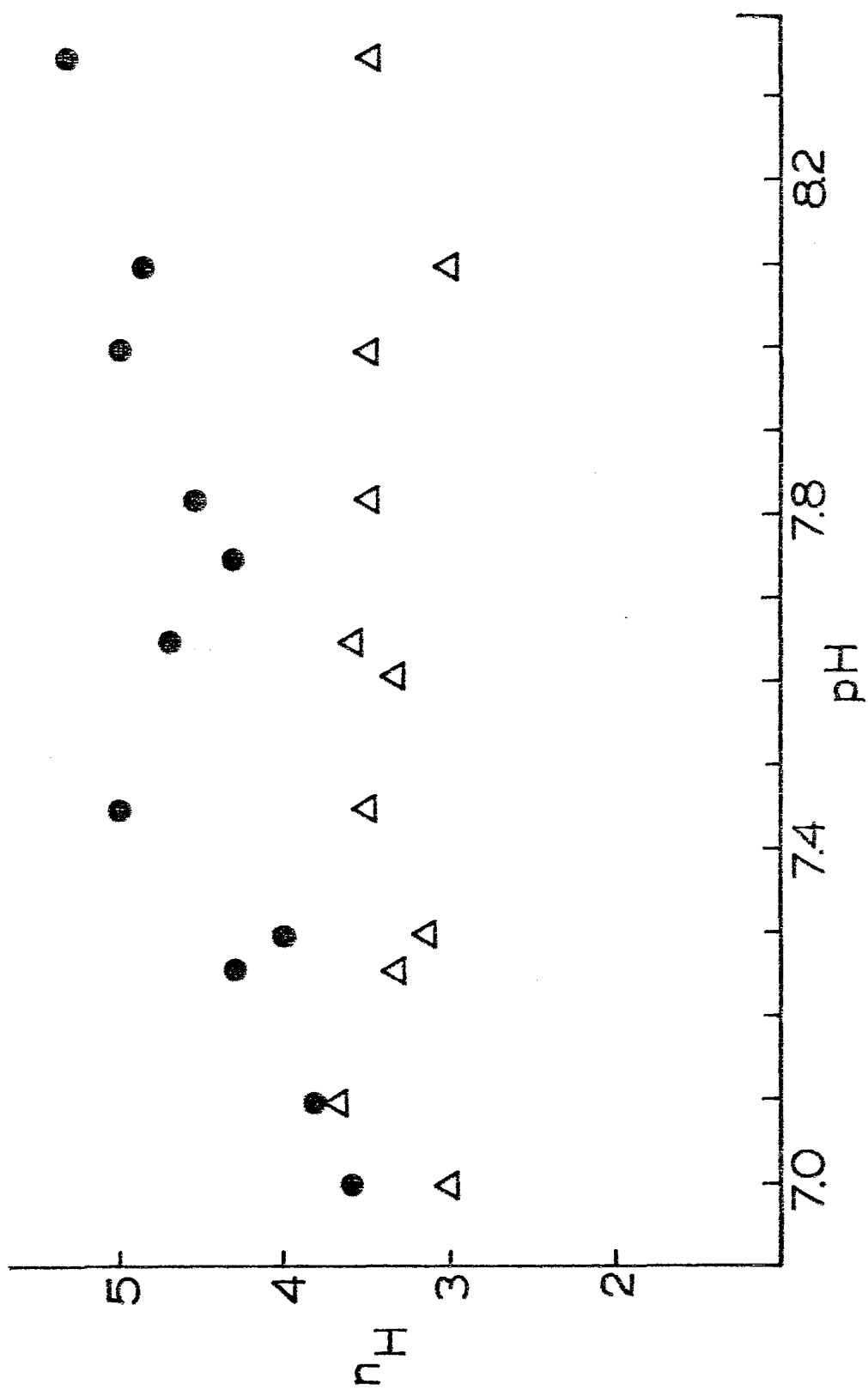




Figure 21. Hill coefficient ( $n_H$ ) as a function of pH for L. pallasii 16S<sup>H</sup>  $\Delta$  and 24S  $\bullet$  hemocyanin. Temperature, 20°C.



16S fractions were the same as prior to the dialysis steps.

The chloride concentration of the hemolymph of Ligia oceanica, a closely related species, is 596 mEq/L (Parry, 1953). Since the pH series of oxygen binding curves of L. pallasii hemocyanin was generated at a chloride concentration of 170 mEq/L, the effect of chloride ions on the oxygen binding properties was assayed at a more nearly physiological concentration, 600 mEq/L (0.5 M in NaCl). The oxygen affinities remained constant; the cooperativities increased from  $n_H = 3.5$  to 4.4 for the 16S fraction and from  $n_H = 4.5$  to 5.65 for the 24S fraction at pH 7.5.

## DISCUSSION

Quaternary Structure

Both Ligia pallasii and Ligia exotica hemocyanins clearly consist of two populations of polymers, 16S hexamers and 24S two-hexamers. Approximately the same proportion of 16S versus 24S polymers appears to be present in the hemolymph of individual isopods of both species throughout the year. The 16S component has a molecular weight of about 450,000 and appears in electron micrographs as a single hexamer; the 24S component has a molecular weight of about 900,000 and appears as two squares or a hexagon and square side by side. Both the 16S and 24S components contain copper as determined by the copper staining as well as by the 340 nm absorbance. Furthermore, the ratio of absorbance at 280 nm/340 nm is similar to that reported for other arthropodan hemocyanins. Thus the quaternary structures of the two aggregation states of Ligia hemocyanin are similar to those described for other crustacean hemocyanins (Van Holde and Van Bruggen, 1971). However, hemocyanin of the isopod Ligia is unusual in that appreciable amounts of both 16S and 24S aggregates are present whereas in most other crustaceans, either the 16S or 24S aggregate predominates. Only

a few crustaceans of the infraorder Astacidea, such as Astacus fluviatilis (Eriksson-Quensel and Svedberg, 1936), Homarus americanus (Pickett et al., 1966) and Cherax destructor (Murray and Jeffrey, 1974) have been shown to contain roughly equal concentrations of both 16S and 24S forms. Even among the isopod hemocyanins that have been studied, the structure of Ligia hemocyanin appears noteworthy since only 16S components have been reported in the hemolymph of other isopod species (Berthet et al., 1964; Van Holde and Brenowitz, 1980).

#### Subunit Composition

The molecular weights of L. pallasii hemocyanin subunits, 80-82,000, are similar to those of other arthropodan hemocyanins. The amino acid composition of the  $M_1$  subunits from the 16S fraction resembles those of other hemocyanins as well (Van Holde and Van Bruggen, 1971). It shows a relatively close correspondence to the amino acid composition of the whole hemocyanin of L. italica (Sevilla, 1978), with major differences noted only for valine and phenylalanine.

A unique feature of L. pallasii hemocyanin is that it is composed of only two subunits,  $M_1$  and  $M_2$ , in contrast to the more extensive subunit heterogeneity usually observed in other crustacean hemocyanins. The conclusion that  $M_1$

and  $M_2$  are different subunits is first based upon their different electrophoretic behavior under three different conditions: 1. SDS gel electrophoresis in the absence of reducing agent, 2. regular gel electrophoresis at pHs 8.9, 10.0 and 10.5, and 3. urea gel electrophoresis in the presence or absence of reducing agent. The fact that  $M_1$  and  $M_2$  can be resolved on SDS gels only in the absence of reducing agent suggests that  $M_1$  and  $M_2$  are two distinct polypeptide chains which are very similar in molecular weight but whose conformations differ in the native state. When the disulfide bonds are reduced, both subunits then migrate on SDS with indistinguishable apparent molecular weights. The results of limited proteolysis by V-8 protease of subunits  $M_1$  and  $M_2$  as well as of the 16S and 24S fractions substantiate the conclusion that  $M_1$  and  $M_2$  are two separate subunit populations. Since samples prepared by two different methods of denaturation, SDS and urea/2-mercaptoethanol, gave the same results, it is not likely that the difference in peptide maps between  $M_1$  and  $M_2$  is due to conformational differences alone. The peptide mapping technique further establishes that the  $M_1$  subunit of the 24S molecule is indistinguishable from the  $M_1$  subunit of the 16S molecule. It is concluded that  $M_1$  and  $M_2$  are two distinct subunits.

The subunit heterogeneity observed does not appear to be due to genetic polymorphism because the same subunit pattern was observed in blood samples from individual animals of different sizes, sexes and stages of reproductive cycle. The possibility that one of these subunits is an artifact produced by proteolytic degradation during the experimental procedures is unlikely for the following reasons. The serine protease inhibitor, phenylmethylsulfonyl fluoride, was included in the extraction buffer to minimize the possibility of proteolysis. All purification steps were carried out in the cold, and the samples were stored in the cold. Furthermore extraction and purification were done as quickly as possible and samples were not used for longer than two weeks. The two subunits are present in the same concentration in fresh blood and in blood which has been stored for several weeks. Furthermore, the two subunits are found when analyzed in the presence or absence of SDS. Therefore proteolysis as a result of unfolding in SDS is an unlikely explanation for the existence of the two subunits. While a natural degradation or aging of the protein within the animal cannot be ruled out, the existence of two subunit types does not appear to be an artifact generated in the laboratory.

The two monomers,  $M_1$  and  $M_2$ , are distributed differently between the 16S and 24S fractions. The data indicate

that the  $M_1$  subunit is found in both 16S and 24S fractions while the  $M_2$  subunit appears only in the 24S fraction. Therefore the 16S component is a homogeneous hexamer composed of only  $M_1$  subunits. The 24S component is heterogeneous and contains both  $M_1$  and  $M_2$  subunits in a ratio of 5:1 respectively. The differences in subunit composition provide an explanation for the co-existence of both components in apparently non-equilibrium conditions in the hemolymph.

#### Dissociation and Reassembly

The 24S two-hexamer aggregate of L. pallasii hemocyanin dissociates to hexamer upon the removal of  $Ca^{2+}$  at neutral pH, as demonstrated by regular gel electrophoresis at pH 7.0. The dissociation is easily reversible upon the addition of  $Ca^{2+}$ . However, an equimolar concentration of  $Mg^{2+}$  instead of  $Ca^{2+}$  is not sufficient to either maintain or reassociate the two-hexamer component. Therefore the reversible dissociation of two-hexamer to hexamer is a calcium-sensitive process. The two-hexamer component can be maintained in the absence of  $Ca^{2+}$  by a moderately high (0.15) ionic strength buffer, based on the results of BioGel A-5M chromatography at pH 7.0. It is interesting that neither the native 16S hexamers nor the hexamers resulting from cleavage of the 24S molecule when  $Ca^{2+}$  is removed dissoci-



ate further to 5S monomers. It appears that the interhexamer affinities in the 24S aggregate are more easily disrupted than are the intrahexamer bonds.

Are the hexamers of L. pallasii 24S hemocyanin linked by a disulfide-bonded dimer? Several arthropodan hemocyanins have been reported to contain a disulfide-linked dimer which is responsible for linking hexamers together into larger aggregates (Jeffrey et al., 1978; Markl, 1980; Pilz et al., 1980; Markl et al., 1981). Dissociation of these systems into hexamers requires use of a reducing agent. Some of the arthropodan hemocyanins reported to have a disulfide-linked dimer dissociate sequentially at alkaline pH, first into a heptamer (a seven-subunit aggregate consisting of a hexamer and the other half of the disulfide-linked dimer) as well as five monomers and eventually into a 7S dimer and ten 5S monomers (Markl, 1980; Pilz, 1980; Markl et al., 1981). The dissociation of L. pallasii 24S hemocyanin into hexamers merely by removing  $\text{Ca}^{2+}$  at neutral pH argues, however, against the possibility that the 24S two-hexamer of Ligia is formed by a disulfide-linked dimer. In order to further substantiate this conclusion, I tried one of the experiments often described in the literature which is used to identify such disulfide-linked dimers. Purified 24S hemocyanin was electrophoresed at pH 7.0 in the presence of  $\text{Ca}^{2+}$  (conditions which normally maintain the 24S

configuration) after the hemocyanin had been reacted with either dithiothreitol or 2-mercaptoethanol. The results show partial dissociation of the two-hexamers into hexamers. Does this prove the existence of a disulfide-linked dimer? I do not think it does. It merely points up the potential weakness of this type of experiment in producing ambiguous results. It also suggests that reports of disulfide-linked dimers based on this method may not always be accurate. The  $M_2$  subunit of L. pallasii hemocyanin would be a likely candidate for a disulfide-linked dimer, since it is present in the two-hexamer molecules but not in the one-hexamer. In addition, it has been shown to be sensitive to reducing agents, as evidenced by its behavior on both SDS and urea gels in the presence and absence of 2-mercaptoethanol. However, reducing agents do not differentiate between intermolecular disulfide bonds and intramolecular disulfide bonds. If intramolecular bonds are cleaved, the conformation and therefore the subunit binding sites of the protein could be altered. Superoxide dismutase is one example of a protein whose dimeric structure is dependent upon the integrity of intramolecular disulfide bonds (Abernethy et al., 1974). Ligia 24S hemocyanin may be another. Partial dissociation into hexamers is just as likely to be due to a change in conformation of the  $M_2$  subunits as to cleavage of a disulfide bond between two subunits. The other

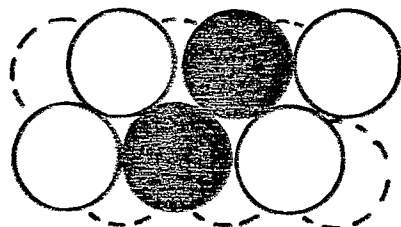
evidence, the direct dissociation of 24S hemocyanin into hexamers simply by removing  $\text{Ca}^{2+}$  at neutral pH, clearly demonstrates that the two hexamers of the Ligia 24S hemocyanin aggregate are not linked by a disulfide-bonded dimer.

SDS gels run in the absence of reducing agent reveal a small amount of high molecular weight material which subsequently re-electrophorese in the presence of reducing agents as typical Ligia monomers. The 24S fraction contains two extra bands ( $M_r = 140,000$  and  $157,000$ ) in the absence of mercaptoethanol; the 16S fraction, one ( $M_r = 140,000$ ). These high molecular weight bands most likely correspond to partially denatured or reaggregated  $M_1$  and  $M_2$  subunits rather than "native" disulfide-linked dimeric assemblages since most of the protein electrophoreses as monomeric  $M_1$  and  $M_2$ . The formation of spurious high molecular weight aggregates during molecular weight determinations in the absence of reducing agents has been documented (Poole et al., 1974).

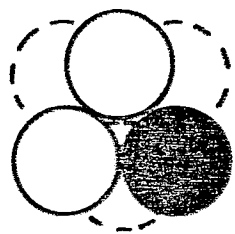
The 24S hemocyanin aggregate appears to be made up of two identical hexamers, each consisting of five  $M_1$  and one  $M_2$  subunits, as illustrated in Figure 22. Another feasible arrangement of subunits, based on the 5:1 ratio of  $M_1$  and  $M_2$  subunits present in the 24S fraction, would be two separate populations of 24S molecules, about 85% aggregates composed of  $M_1$  subunits and 15% composed of  $M_2$  subunits.

Figure 22. Proposed subunit distribution in Ligia pallasii  
24S and 16S hemocyanin. ○, M<sub>1</sub> subunits; ●, M<sub>2</sub> subunits.

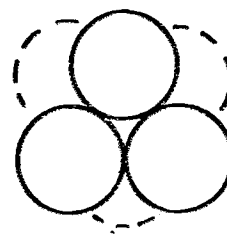
native 2-hexamer



native hexamer



$5M_1:1M_2$



$6M_1$

Intermediate between these two alternatives is the possibility of 24S aggregates formed from hexamers containing varying amounts of  $M_2$  subunits. Hexamers containing various ratios of two major subunit types have been described for the hemocyanin of Cherax destructor, where up to four hexamer bands have been resolved by gel electrophoresis (Jeffrey et al., 1976). The first alternative, a 24S two-hexamer composed of two identical hexamers, is the most likely pattern in L. pallasi hemocyanin, based on the following reasons. First, the hexamer population resulting from the dissociation of the 24S fraction electrophoreses as a single component on regular gel electrophoresis at neutral pH in the absence of  $Ca^{2+}$ . Furthermore, this hexamer band is clearly resolvable from the band corresponding to the native  $M_1$  hexamer. It can also be distinguished from the proposed artificial hexamers ( $2 M_1 : 4 M_2$ ) which migrate as a third hexamer band in this electrophoresis system. If the 24S fraction were composed of hexamers containing variable ratios of  $M_1$  and  $M_2$  subunits, it is expected that they too could be resolved on this gel system. Similarly, two separate populations of homogeneous  $M_1$  and homogeneous  $M_2$  24S aggregates could also be resolved at the hexamer level. Instead, only one band is present. Therefore it is likely that the 24S hemocyanin is composed of two identical hexamers.

Although the exact position of the  $M_2$  subunits within the 24S molecule is unknown, their proposed distribution, one to each of the two hexamers, and their absence in the native 16S aggregate implicates them as the subunits responsible for forming interhexameric bonds, an  $M_2 - M_2$  contact. At the same time, they are obviously capable of associating with  $M_1$  subunits within hexamers of the 24S aggregate. The different dissociation properties of the hexamers versus two-hexamers suggests the following. Perhaps the  $M_1$  subunit has one type of binding site which allows it to form stable hexamers at neutral pH in the presence or absence of divalent cations. The  $M_2$  subunit may have two types of binding sites, a relatively stable  $M_1 - M_2$  which permits the incorporation of one  $M_2$  subunit into a hexamer, and a  $Ca^{2+}$  sensitive  $M_2 - M_2$  site which results in the polymerization of two  $5M_1:1M_2$  hexamers into a 24S polymer.

If polymerization of monomers into hexamers were based on random assortment, one would expect to find a range of distribution of  $M_2$  subunits in the hexamers which form the 24S molecules. Instead there is a constant distribution of five  $M_1$  subunits and one  $M_2$  subunit. One explanation for the assembly of only this kind of  $M_1:M_2$  hexamer could be that the native conformation of the  $M_2$  subunit is slightly larger than that of the  $M_1$  subunit. This idea is consistent with the results of SDS electrophoresis of  $M_2$  in

the presence and absence of reducing agents. Perhaps six  $M_1$  subunits can form a stable hexamer. One  $M_2$  subunit might be able to be incorporated with 5  $M_1$  subunits into a hexamer although its presence would cause a certain amount of strain on the hexamer. However, the incorporation of more than one  $M_2$  subunit would create too much distortion and the resulting hexamer would be unstable. A slightly different sequence of events might occur if the two  $M_2$  subunits initially were to form a very stable dimer. One  $M_2$  subunit could then be incorporated into an aggregate of 5  $M_1$  subunits; the other  $M_2$  would also cause the association of five  $M_1$  subunits around itself, resulting in a two-hexamer aggregate. Either model could explain the observed association of  $M_1$  and  $M_2$  into hexamers and two-hexamers and the absence of any other kinds of hexamers.

Is dissociation of Ligia hemocyanin to its constituent 5S polypeptide chains and reassembly of 5S chains to the 16S and 24S levels of aggregation possible? The initial results of alkaline pH dialysis and column chromatography of pooled 24S and 16S L. exotica hemocyanin suggested the presence of a putative "dissociation-resistant" hexamer population which was stable even at pH 10.5 in the absence of divalent cations. Almost all other arthropodan hemocyanins which have been studied could be completely dissociated to monomers by exposure to pH between 9-10 in the absence of



divalent cations, with the exception of Penaeus setiferus hemocyanin. The 16S hemocyanin of the shrimp Penaeus exists as hexamer even at pH 10.0 in the presence of EDTA. Complete dissociation into polypeptide chains does not occur until pH 10.8 at which point the protein loses its oxygen binding capacity (Brouwer et al., 1978). Like Penaeus, L. exotica hemocyanin appeared resistant to dissociation. Investigation of this phenomenon might provide some information about the assembly of both the 16S and the 24S components. In order to better understand the process of dissociation and reassembly in isopod hemocyanin, the 16S and 24S components of L. pallasii hemocyanin were separated from one another prior to exposure to high pH. The dissociated 5S monomers of the 16S fraction readily reassembled to hexamers upon dialysis at neutral pH; the 24S fraction dissociated at high pH also reassembled - but only to the level of hexamer. Subsequent analysis of the high pH dissociated components by column chromatography revealed that the 16S fraction completely dissociates to monomer; the 24S fraction dissociates to monomer plus about 20% hexamer. SDS and urea gel electrophoresis indicate that all of the  $M_2$  subunits are in the high pH hexamer fraction in a ratio of two  $M_1$  to four  $M_2$  subunits, compared to the five  $M_1$ :one  $M_2$  ratio in the native 24S component. The high pH hexamer fraction seems to be a reassembly of dissociated subunits

into an artificial hexamer. This is demonstrated by the absence of any hexamer band when native 24S hemocyanin at pH 7.5 is electrophoresed directly into the pH 10.5 gel system without prior dialysis against the high pH buffer. Apparently, when the subunits electrophorese into the gel, they are immediately physically separated from one another as they dissociate from the native 24S component. If this occurs, they are not able to reform into artificial hexamers. However, when the 24S component is dissociated at high pH and the subunits are allowed to remain in proximity with one another, the  $M_2$  subunits reaggregate, along with some  $M_1$  subunits. A likely explanation is that the  $M_2$  subunits undergo a change in charge distribution or conformation at high pH which alters their affinity for one another. The conformational change is then maintained upon aggregation into the artificial hexamer such that the artificial hexamer is stable at all pHs, unlike the native hexamer.

The formation of these artificial hexamers helps explain the inability of the Ligia dissociated 24S component to reassemble to the two-hexamer aggregate. Since the  $M_2$  subunits are all incorporated into the stable artificial hexamers, they are not available to participate in the formation of the 24S two-hexamers. However, reassembly of the  $M_1$  monomer pool proceeds easily to the level of the  $M_1$  homo-hexamer. It is evident that a  $Ca^{2+}$  sensitive reversible

dissociation of the native two-hexamer (five  $M_1$ :one  $M_2$ ) to hexamer (five  $M_1$ :one  $M_2$ ) and a pH-dependent reversible dissociation of the native hexamer ( $M_1$ ) to monomer ( $M_1$ ) are possible. Reversible dissociation of the native two hexamer to monomer ( $M_1$  and  $M_2$ ) is more complex and will require conditions which prevent the formation of stable artificial hexamers. The difficulties reported in the literature by numerous researchers of reassembling other crustacean hemocyanin subunits to a polymerization level beyond the hexamer (for example, Morimoto and Kegeles, 1971; Jeffrey, 1979) compared to the relative ease of reassembly of chelicerate polymers may well be related to the tendency of certain crustacean hemocyanin subunits to form artificial aggregates at high pH, as illustrated by Ligia hemocyanin. Studies are underway now to obtain pure populations of L. pallasii native  $M_1$  and  $M_2$  subunits and recombine them in the appropriate stoichiometry and sequence so as to form 24S two-hexamers.

The data presented on L. exotica hemocyanin in Part I of this thesis may be better understood in light of the analysis of L. pallasii 16S versus 24S hemocyanin. The apparent heterogeneity of whole L. exotica hemocyanin as determined by SDS gel electrophoresis (Figure 7A) versus the homogeneity of the high pH dissociated monomeric fraction (fraction II, Figure 7D, E) is probably attributable

to a 24S fraction composed of several types of subunits and a 16S fraction composed of homogeneous subunits. The "dissociation resistant" hexamer of L. exotica is more likely a reaggregated artificial hexamer, as in L. pallasii.

The  $\text{Ca}^{2+}$  sensitive reversible dissociation of Ligia 24S hemocyanins into hexamers at neutral pH has also been reported in the 25S hemocyanin of the lobster Homarus americanus (Morimoto and Kegeles, 1971; Markl et al., 1981). The 24S hemocyanin of the brachyuran crab, Cancer pagurus, dissociates into hexamers at alkaline pH (Markl et al., 1979a). Other arthropodan hemocyanin aggregates appear to be linked by stronger non-covalently bonded dimers which are stable at neutral pH in the absence of  $\text{Ca}^{+2}$  but are sensitive to high concentrations of EDTA, alkaline pH and 4M urea (Eurypelma californicum, Schneider et al., 1977; Androctonus australis, Lamy et al., 1977; Lamy et al., 1979a; and Limulus polyphemus, Lamy et al., 1979b). At the other end of the spectrum are those multiple hexamer hemocyanins already mentioned which appear to be linked by disulfide bonded dimers. Thus there appear to be various kinds of interhexamer bonds within arthropodan hemocyanins. The results on L. pallasii hemocyanin present a detailed description of the structure and assembly properties of a hemocyanin with a non-covalently linked, easily disruptible two-hexamer molecule.

### Functional Studies

The oxygen binding properties of the 24S and 16S fractions of L. pallasii hemocyanin were studied in an attempt to correlate both the subunit composition and the quaternary structure of a molecule with its functional properties. The results indicate that both types of polymers have highly developed homo- and heterotropic interactions. Both fractions are relatively low affinity respiratory proteins and show Bohr effects of comparable magnitude. In addition, they respond similarly to low and physiological chloride concentrations. The one observed difference between L. pallasii 24S and 16S fractions under the conditions examined is that the 24S fraction, containing  $M_2$  subunits as well as  $M_1$  subunits, consistently shows a greater cooperativity than the 16S fraction, which contains only  $M_1$  subunits. The increase in NaCl to physiological levels appear to cause a comparable increase in cooperativity in both 24S and 16S polymers suggesting that the  $M_1$  subunit, present in both fractions, is responsible for the observed chloride effect.

The oxygen equilibrium curves of the major components as well as the unfractionated hemocyanin of Cherax destructor have been reported (Jeffrey and Treacy, 1980). Their

results indicate that the 25S and 17S components have almost identical oxygen affinities and cooperativities at pH 7.8 in the presence of  $\text{Ca}^{2+}$ . This is similar to the results shown for oxygen affinities of L. pallasii 24S and 16S fractions. Binding studies on reconstituted hexamers of the two major monomer populations of Cherax hemocyanin, which normally make up hexamers of varying monomer proportions in the animal, indicate that the two monomers contribute differently to the oxygen binding properties of the native mixed hexamers. They did not report on the functional properties of a third subunit,  $M_3'$ , which is a dimeric assemblage present in the 25S aggregate and would be analogous to L. pallasii  $M_2$  subunits.

Thus in both Ligia and Cherax hemocyanins the functional significance of two aggregation states co-occurring in the hemolymph is uncertain. However, the difference in cooperativities of Ligia 24S and 16S may be great enough to expand the functional versatility of the isopod hemocyanin. It could be that a highly specific allosteric cofactor, perhaps lactate (Truchot, 1980), is an important modulator of crustacean hemocyanin oxygen affinity as suggested by Mangum (1981) and others. Such a co-factor might exert differential effects on the 24S or 16S molecule. If that is found to be true, the isopod would have a system analogous to the specific effect of organic phosphates such as

2, 3-DPG on the oxygen binding properties of vertebrate hemoglobins.

The homo- and heterotropic behavior of the 16S hexamers of Ligia is noteworthy because the hexamers have been shown to be a homogeneous assemblage of  $M_1$  subunits. The lack of observable difference in  $P_{50}$ 's and Bohr effect between the 16S and 24S components suggests that the  $M_2$  subunits participate primarily in a structural role in the 24S polymers. The presence of the  $M_2$  subunits appears to allow the formation of the two-hexamer aggregate and therefore indirectly at least provides for an increased cooperativity of the two-hexamer versus the hexamer.

Does the  $Ca^{2+}$  sensitive dissociation of the 24S molecule occur in vivo and therefore have physiological ramifications? Miller (1980) has shown that a  $Mg^{2+}$  dependent reversible 39S-16S dissociation occurs in the hemocyanin of the ghost shrimp, Callinassa californiensis. Callinassa is an osmoconformer (Thompson and Pritchard, 1969), and under physiological conditions of low salinity, dissociation of the hemocyanin can occur. L. pallasii, on the other hand, is reported to be a good osmoregulator at salinities less than 100‰ (Wilson, 1970). Whether L. pallasii is capable of specifically regulating  $Ca^{2+}$  at a level sufficient to prevent a 24S-16S dissociation is not known. A closely related species, L. oceanica, does

maintain high levels of  $\text{Ca}^{2+}$  in the hemolymph (Parry, 1953). Therefore it is likely that dissociation of L. pallasii 24S hemocyanin does not occur in vivo. Instead, the isopod has two discrete populations of oxygen binding molecules, 16S and 24S.

Ligia hemocyanin is a much simpler system than other crustacean hemocyanins with respect to the amount of subunit heterogeneity, yet polymerization to the level of two-hexamer aggregates can still occur. The 24S molecule, which is composed of two unique subunit populations, is more complex than the 16S molecule, which is a homo-hexamer. The differences in subunit composition help explain the co-occurrences of both polymeric states in the hemolymph of Ligia. Subunits  $M_1$  and  $M_2$  play different roles in the assembly of the polymers;  $M_1$  is involved in formation of the hexamer while  $M_2$  appears to be required for aggregation to the two-hexamer state. The integrity of the hexamer is much less sensitive to low ionic strength and  $\text{Ca}^{2+}$  concentrations than is that of the two-hexamer 24S molecule, a fact which probably reflects differences in subunit binding between the  $M_1$  and  $M_2$  subunits.

Both the 16S and 24S polymers have highly developed homo- and heterotropic interactions. The oxygen binding affinities and the Bohr effects of the 16S and 24S fractions are similar to one another. Thus the 16S hexamer



composed of apparently identical  $M_1$  subunits is as functionally versatile as the heterogeneous  $M_1:M_2$  24S two-hexamer. The 24S molecule does show a consistently higher cooperativity. This suggests that the  $M_2$  subunit, while primarily involved in a structural role, also enhances the functional properties of the 24S molecule.

Charge or size heterogeneity of subunits in itself does not provide evidence of different roles in the assembly and function of multiple-hexamer aggregates. For example, the hemocyanin of the shrimp Penaeus setiferus, like the hemocyanin of Ligia, apparently contains two populations of subunits, present in a ratio of 1 to 2.6, with estimated molecular weights of 82,000 and 77,000 (Brouwer et al., 1978). Yet Penaeus hemocyanin can polymerize only to the 16S level. It is reasonable to suppose that only those amino acid substitutions which affect the conformation of the polypeptide chain and/or alter that portion of the chain specifically involved in intermolecular associations will have an effect on assembly. Similarly, only those substitutions which affect the homo- or heterotropic oxygen binding sites will affect the functional properties of the aggregate. The differences between subunits  $M_1$  and  $M_2$  in Ligia hemocyanin appear to be both structurally, and to a lesser degree, functionally significant.

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