STRUCTURE AND SUBUNIT COMPOSITION OF THE HEMOCYANIN FROM THE PURPLE SHORE CRAB HEMIGRAPSUS NUDUS

by
KRISTIN LARSON

A THESIS

Presented to the Department of Biology
and the Graduate School of the University of Oregon
in partial fulfillment of the requirements
for the degree of
Master of Science

March 1982

An Abstract of the Thesis of

Kristin Larson for the degree of Master of Science in the Department of Biology to be taken March 1982

Title: STRUCTURE AND SUBUNIT COMPOSITION OF THE HEMOCYANIN FROM THE PURPLE SHORE CRAB HEMIGRAPSUS NUDUS

Approved: _____
Robert C. Terwilliger

Hemocyanins serve as oxygen carriers for diverse numbers of molluscs and arthropods. Arthropod hemocyanins are large, polymeric copper-containing proteins with molecular weights ranging from 4.5×10^4 to 9×10^6. These giant respiratory proteins consist of subunits with molecular weights of 70,000-80,000 and an $s_{20,w} = 5$. Amongst various groups of arthropods, these 5S subunits are found arranged in aggregates of six (ca. 16S hexamer), twelve (ca. 25S 2-hexamer), twenty-four (ca. 39S 4-hexamer) and forty-eight (ca. 60S 8-hexamer). The state of aggregation appears to be characteristic for each species and may be specific to different taxonomic groups. Among the decapod crustaceans 25S and 16S hemocyanins

predominate.

Many crustacean hemocyanins are characterized by striking subunit heterogeneity. It has been suggested that the degree of subunit heterogeneity and state of aggregation are somehow related. In fact, there is now evidence that the subunits play specific roles in the hemocyanin assembly prosesses. It appears that some subunits are involved in assuring the correct assembly of the hexamer configuration while others may act as hexamer-hexamer linkers in the formation of the larger aggregates.

This thesis presents a detailed description of the structure of the hemocyanin of Hemigrapsus nudus (Dana), the purple shore crab. The hemocyanin of H. nudus exists in the hemolymph as two different multi-subunit polymers, 15% of which are thought to be 16S hexamers and 85% of which are thought to be 25S 2-hexamers. These polymers are composed of heterogeneous subunits. The 25S hemocyanin of the adult crab can be differentiated from the 16S hemocyanin based on identity and numbers of subunits as determined by polyacrylamide gel electrophoresis and peptide mapping.

Different stages of development of Hemigrapsus nudus, the early eggs, the late eggs and the zoeae, also appear to contain hemocyanin whose quaternary structure corresponds

to that of the adult. However, the putative egg and zoea hemocyanins differ from one another as well as from the adult, based on analysis of subunit structure. The significance of these differences in subunit compositions are discussed with respect to the quaternary structure of the hemocyanin as well as possible functional implications for the animal.

VITA

NAME OF AUTHOR: Kristin Larson

DATE OF BIRTH: January 8, 1958

UNDERGRADUATE AND GRADUATE SCHOOLS ATTENDED:

Lewis and Clark College
Pacific University
University of Oregon

DEGREES AWARDED:

Bachelor of Science, 1980, Lewis and Clark College

AREAS OF SPECIAL INTEREST:

Biochemistry
Respiratory Physiology

PROFESSIONAL EXPERIENCE:

Research Assistant, Organic Chemistry Synthesis Division, University of California, Lawrence National Laboratory, Livermore, California, Summer 1976

Research Assistant, Nuclear Chemistry Division, University of California, Lawrence National Laboratory, Livermore, California, Summer 1977

Teaching Assistant, Department of Chemistry, Lewis and Clark College, Portland, Oregon, 1978

Research Assistant, Organic Chemistry Division, University of California, Lawrence National Laboratory, Livermore, California, Summer 1979

AWARDS AND HONORS:

Recipient - Sigma Xi Grant for research. Awarded June 1981

PUBLICATIONS:

Bissell, E.R., Larson, K., Croudace, M.C. (1982) Some 7-Substituted-4-Trifluor-Methyl-Courmarins. Journal of Chemistry and Engineering Data (in press)

PAPERS PRESENTED:

Western Society of Naturalists, Santa Barbara, California, December 1981

ACKNOWLEDGEMENTS

This thesis is dedicated to my parents, Paul and Diana, who have given me the freedom and support to pursue my educational interests in a rich and varied manner. I also wish to thank Robert Terwilliger, my thesis advisor, for his keen guidance throughout this project. His good humor and enthusiasm will long remain a bright spot for me. I am also grateful to Nora Terwilliger whose answers to my manifold questions retained enough mystery to keep my curiosity piqued.

This research was supported in part by a grant from the Sigma Xi Society and NSF Grant PCM 80-1255-1.

TABLE OF CONTENTS

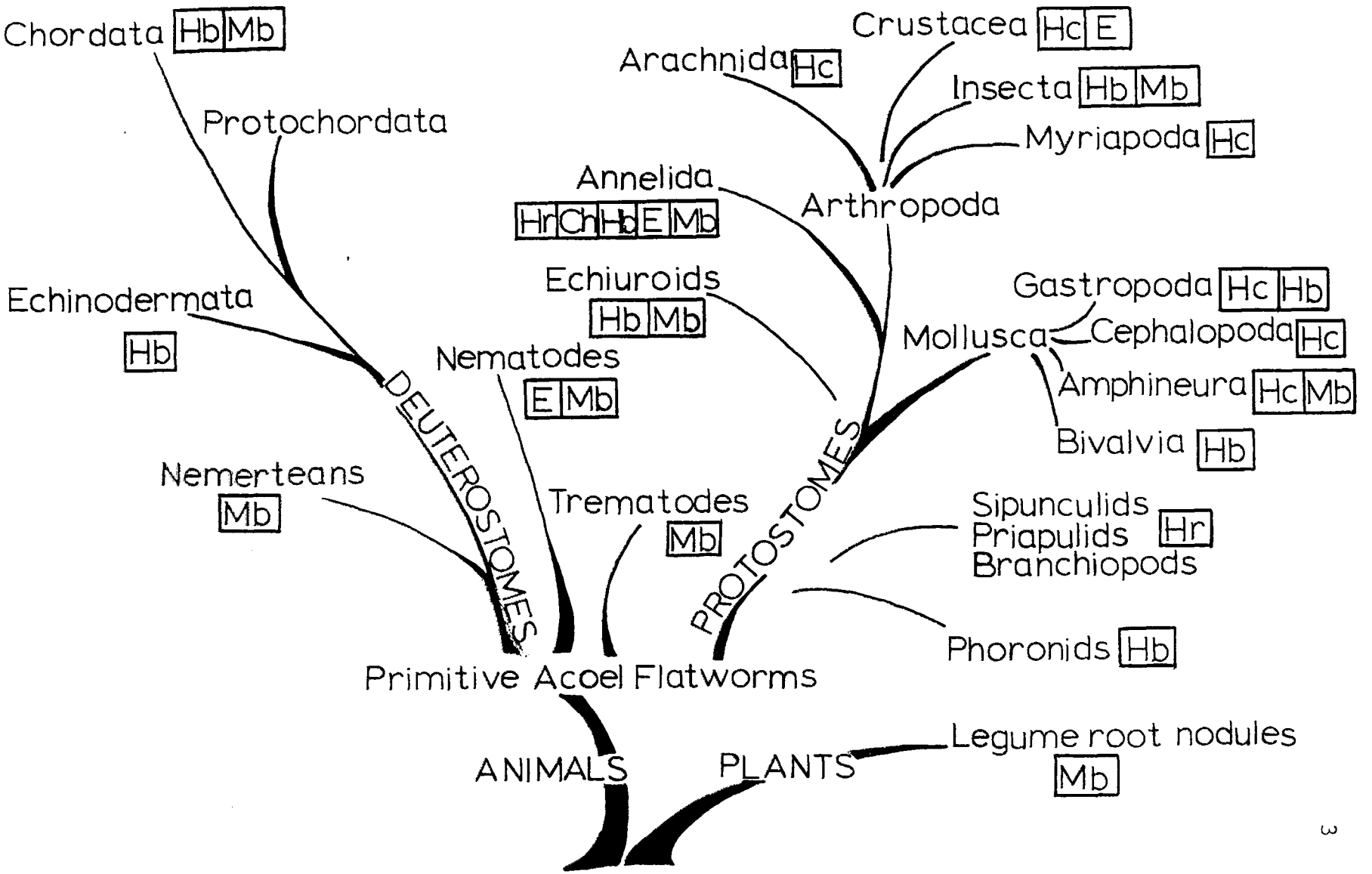
LIST OF FIGURES

INTRODUCTION

Hemocyanin is a copper-containing respiratory protein which has been studied extensively (for reviews see: Van Holde and Van Bruggen, 1971; Lontie and Witters, 1973; Bonaventura et al., 1977; Antonini and Chiancone, 1977; Bannister, 1977; Wood, 1980; Bonaventura and Bonaventura, 1980; Mangum, 1980; Lamy and Lamy, 1981; Mangum, 1981; Van Holde and Miller, in press). These high molecular weight, polymeric proteins are found freely dissolved in the hemolymph of diverse numbers of molluscs and arthropods (Fig. 1). In the phylum Mollusca, hemocyanins are found among the classes Gastropoda, Cephalopoda and Polyplacophora. To date, none has been found in the Monoplacophora, the Scaphopoda or in the Bivalvia. Among the Arthropoda, hemocyanin appears in the classes Crustacea, Arachnida (spiders and scorpions) and Merostomata (horseshoe crabs). No hemocyanin has been reported for the Progoneata (millipedes), Chilopoda (centipedes) or Insecta, with the possible exception of Scutigera longicornis (Chilopoda: Myriapoda) (Rajulu, 1969).

The name hemocyanin is somewhat misleading since the molecule does not have heme as its prosthetic group.

Figure 1. Phylogenetic distribution of respiratory proteins (modified from van Holde and Miller, in press). Mb = myoglobin, Hb = hemoglobin, E = erthrocruorin, Ch = chlorocruorin, Hr = hemerythrin, Hc = hemocyanin.


Chordata Hb Mb
Protochordata
Echinodermata Hb
Nemerteans Mb
Arachnida Hc
Crustacea Hc E
Insecta Hb Mb
Myriapoda Hc
Annelida Hr Ch Hb E Mb
Arthropoda
Echiuroids Hb Mb
Nematodes E Mb
Gastropoda Hc Hb
Mollusca
Cephalopoda Hc
Amphineura Hc Mb
Bivalvia Hb
DEUTEROSTOMES
PROTOSTOMES
Trematodes Mb
Sipunculids
Priapulids Hr
Branchiopods
Phoronids Hb
Primitive Acoel Flatworms
ANIMALS
PLANTS
Legume root nodules Mb

Rather, it contains two copper atoms at the active site which combine reversibly with an oxygen molecule. Hemocyanin is named for the bluish color it displays upon oxygenation (Fredericq, 1878) which reflects a change in the oxidation state of the copper atoms:

$$\underset{\text{deoxyhemocyanin}}{\underset{\text{clear}}{\text{Cu (I) Cu (I)}}} + O_2 \rightleftharpoons \underset{\text{oxyhemocyanin}}{\underset{\text{blue}}{\text{Cu (II) } O_2^{-2} \text{ Cu (II)}}}$$

The mechanism for oxygen binding was first proposed by Orgel (1958) and later confirmed using resonance spectroscopy (Loehr et al., 1974; Freedman et al., 1976).

Although the hemocyanins of arthropods and molluscs appear homologous in terms of amino acid content (Ghiretti-Magaldi et al., 1975), they differ radically at all other structural levels. This is readily apparent when one compares the quaternary structures of these hemocyanins. In arthropod hemocyanins there are two copper atoms per approximately 70,000 daltons of protein. This corresponds to the molecular weight of the minimum polypeptide chain or functional subunit. The subunits, which are observed in vitro only, are obtained by raising the pH, lowering the divalent cation concentration or by lowering the ionic strength of the medium. The subunits are known to have a sedimentation coefficient of about 5S (Eriksson-Quensel and Svedberg, 1936; Van Holde and Van Bruggen, 1971).

Conversely, there are approximately 50,000 daltons of protein per two copper atoms in molluscan hemocyanins and this does not represent a minimal functioning subunit, rather it is known as an oxygen binding domain (Lontie, et al., 1973; Brouwer, et al., 1976). These domains are linked together like a "string of pearls" to form a large subunit with as many as eight oxygen binding sites and $S^{o}_{20,w}$ = 11 (Brouwer and Kuiper, 1973; Waxman, 1975). The molluscan hemocyanin subunits, as found in the native proteins, are aggregated into giant cylindrical molecules with molecular weights up to 9,000,000. These polymers are composed of 20 subunits. In arthropods, the native hemocyanins generally occur as six subunit aggregates known as hexamers ($S^{o}_{20,w}$ = 16) and/or multiples thereof: 2-hexamers (25S), 4-hexamers (39S) and 8-hexamers (60S) (Van Holde and Miller, in press) (Fig. 2).

Markl (1979b) has suggested that the state of aggregation of arthropod hemocyanin appears to be characteristic for each species and may be specific to different taxonomic groups (Fig. 3). A number of exceptions occur, however. For instance, the 60S molecule is found only among the primitive horseshoe crabs and the 39S molecule appears exclusively among some of the Thalassinid shrimps and the more ancient Chelicerates. Most of the other taxonomic groups possess either the 25S 2-hexamer or 16S

Figure 2. Structural characteristics of the arthropod hemocyanins.

ARTHROPOD HEMOCYANINS

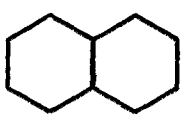
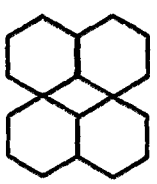
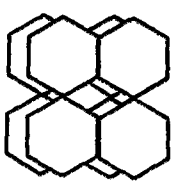
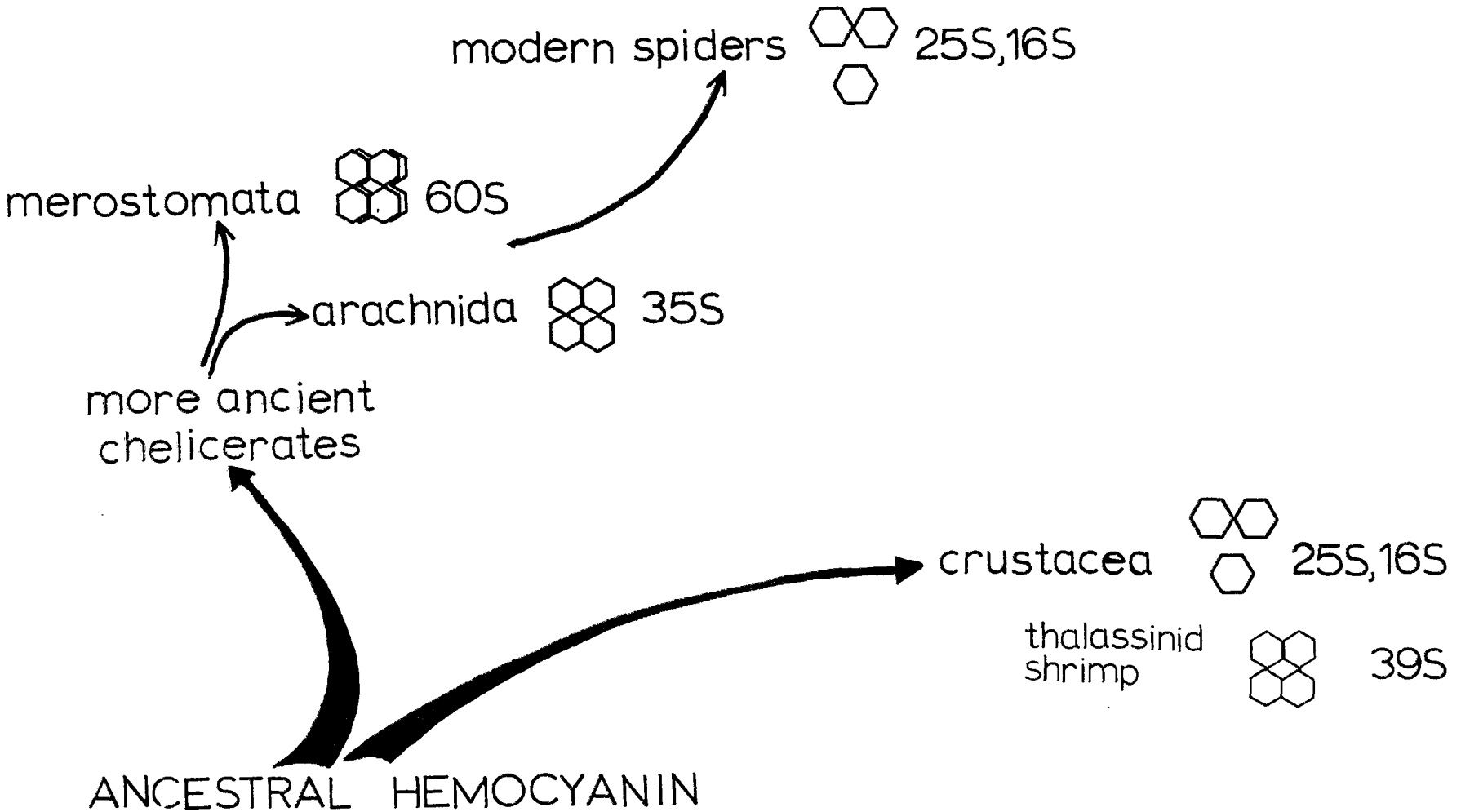
proposed arrangement	M.W.	$S_{20,w}^{\circ}$	name
	450,000	16S	HEXAMER
	900,000	25S	2-HEXAMER
	1,800,000	39S	4-HEXAMER
	3,600,000	60S	8-HEXAMER

Figure 3. Distribution of various hemocyanin polymer types within the phylum Arthropoda (modified from Markl and Kempter, 1981a). ⬡ = 16S hexamer hemocyanin.


modern spiders
25S,16S
merostomata
60S
arachnida
35S
more ancient
chelicerates
crustacea
25S,16S
thalassinid
shrimp
39S
ANCESTRAL HEMOCYANIN

single hexamer hemocyanin molecule or both.

As was noted, arthropod hemocyanins exist, *in vivo*, as a small number of discreet molecular classes denoted by sedimentation coefficients 16S, 25S, 39S, and 60S. It is generally accepted that these molecular populations are not in equilibrium; however, a few scattered exceptions occur (cf. Arisaka and van Holde, 1979). The large aggregates observed in arthropod hemocyanins can be dissociated to individual polypeptide chains (subunits) by raising the pH and/or removing divalent cations with a chelating agent or lowering the ionic strength. In other words, both H^+ and Ca^{++} (Mg^{++}) have stabilizing effects on the large hemocyanin aggregates.

Formerly, it was assumed that the subunits obtained by dissociation of the hemocyanin from a given organism were all the same and represented the products of a single gene. However, with the advent of high resolution probes it has become clear that hemocyanins are composed of more than one type of polypeptide chain or subunit. This subunit heterogeneity is particularly striking in those hemocyanins which form the larger aggregates. For example the 60S molecule of *Limulus* is composed of 8-15 electrophoretically distinct subunits (Markl *et al.*, 1979a; Brenowitz *et al.*, 1981). Yet heterogeneity is also observed in the smaller aggregates: the hemocyanin of

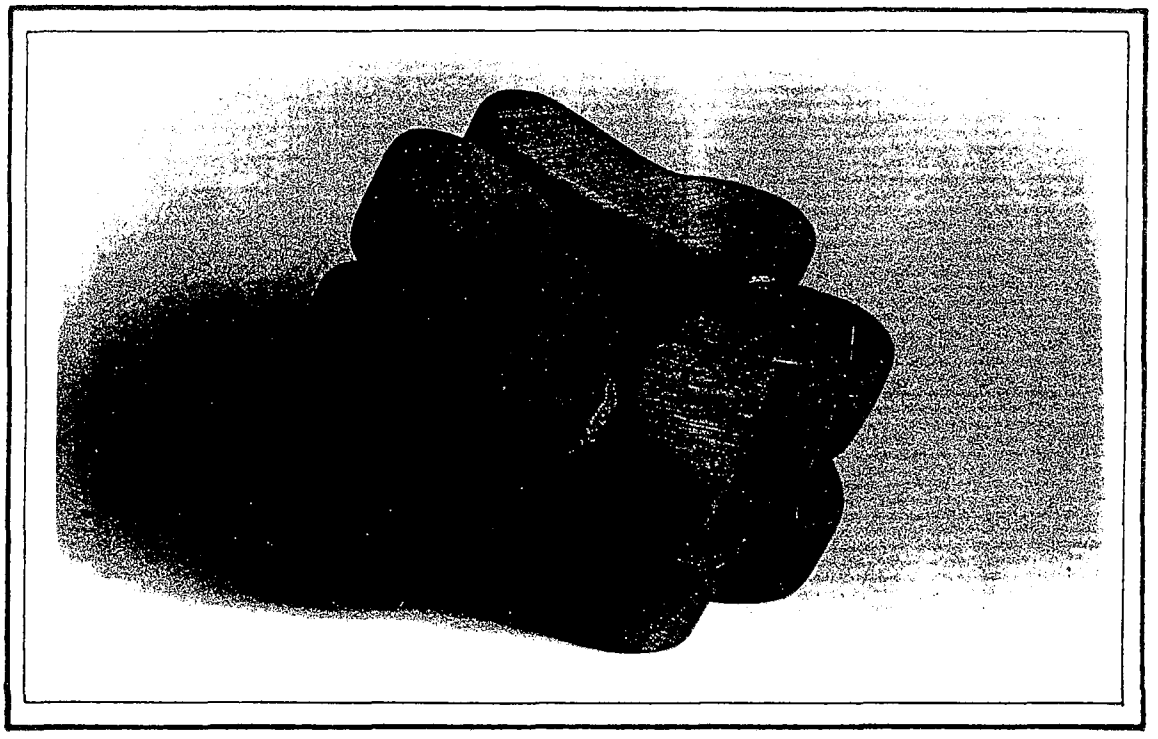
Palinurus which exists, in vivo, as a hexamer contains 6 different subunit types (Markl et al., 1979b).

It is difficult to make any generalization with respect to the role played by these heterogeneous subunits in arthropod hemocyanins, although the heterogeneity does appear to have both functional (Sullivan et al., 1974) and structural (Lamy et al., 1981a) implications. One trend that has become increasingly apparent among hemocyanins in the decapod crustaceans is that in those species where both 16S and 25S molecules are present in the native hemocyanin, the 25S molecule contains more subunit types than the 16S molecule. Evidence now suggests that these additional subunits are involved in linking the two hexamers together (Fig. 4 and 5). In some instances, the interhexameric bond is a disulfide bridge (Jeffrey et al., 1978; Markl et al., 1981), in others the "linker bond" shows a marked sensitivity to calcium (Morimoto and Kegeles, 1971; Terwilliger, in press), while in still others, it is contended that polar and ionic interactions are primarily responsible for the stability of the double hexamer conformation (Herskovits et al., 1981b).

The recent studies of Markl and Kempter (1981a, 1981b) have added yet another dimension to our understanding of subunit heterogeneity. Using immunochemical techniques they have been able to divide the diverse numbers of

Figure 4. Models of arthropod hemocyanin structures.
A) 16S single-hexamer hemocyanin molecule,
B) 25S 2-hexamer hemocyanin molecule.

A



B

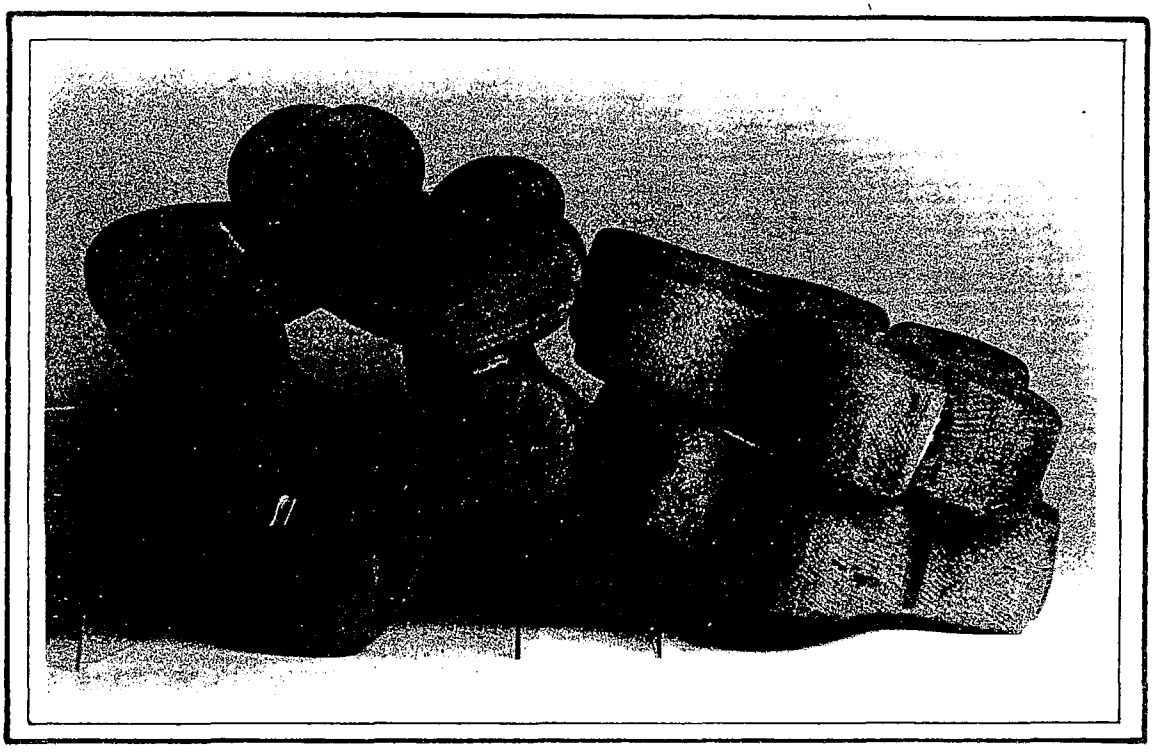
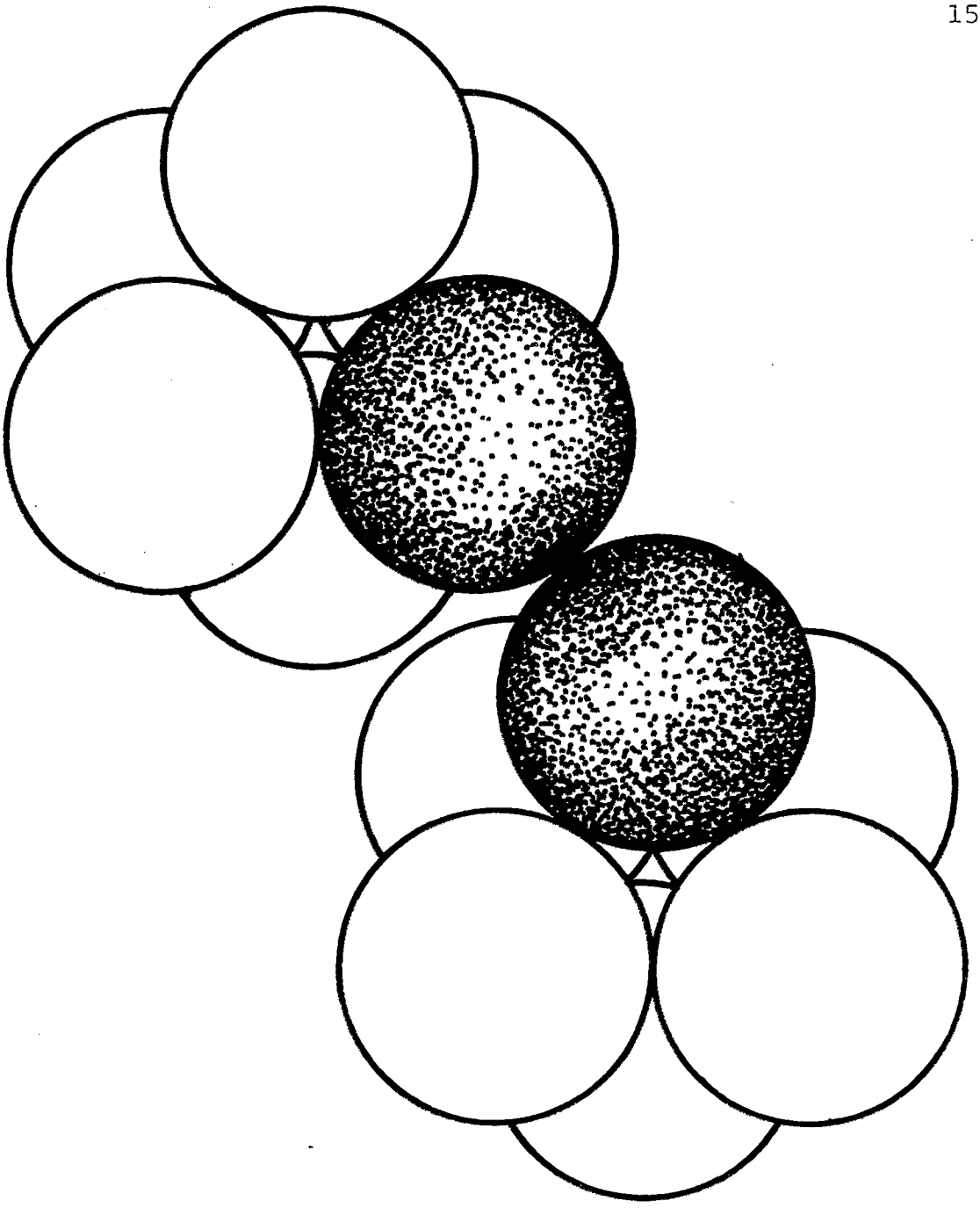


Figure 5. A schematic model of a 2-hexamer molecule held together by two dimerized "linker" subunits (shaded).



subunit types (from six different Crustacean species) into two classes based on conformation. One class is called "variable subunits" which show great specificity in their immunological reactions with anti-serum. It is suggested that these subunits, in their specificity, play an important role in assuring the correct assembly of the hexamers. The second class is called "conservative subunits" because their primary structure appears to have been well conserved during evolution. They are nonspecific in their immunological reactions and will cross-react with the anti-sera of other subunits. Markl and Kempter suggest that the conservative subunits are involved in the formation of the two-hexameric state and could possibly act as linkers.

With this study, Markl and Kempter have attempted to analyze the implications of subunit heterogeneity in terms of evolution. Another approach would be to trace the emergence of heterogeneity in the hemocyanins of developing animals. In general, there is a paucity of information regarding ontogenesis of the hemocyanins. To date only two such studies have been published. The first, carried out on the hemocyanins of a mollusc, Sepia officinalis, throughout its development, remains inconclusive with respect to the precise nature of the respiratory proteins present (Decleir & Richard, 1970; Decleir et al., 1971).

The other study focuses on the differentiation of hemocyanin subunit compositions during the ontogency of Cancer magister (Terwilliger and Terwilliger, 1980; in press). The megalops and first instar juveniles of this species are shown to contain hemocyanins which differ in a number of ways from that of the adult crabs. Perhaps the ontological changes observed in hemocyanins reflect the changing needs of the organism as it metamorphoses and moves into new environments.

Heterogeneity in subunits has been observed in the hemocyanins of many, if not all arthropods. In fact, there is no known case of a native hemocyanin containing less than two subunit types. The purpose of this study is to document subunit heterogeneity in the hemocyanin from a family of crabs hither-to-now undescribed. Hemigrapsus nudus (Dana), the subject for this study, is one of four members of the family Grapsidae found on the Pacific Coast (Fig. 6). Unlike most of the brachyurans previously described, H. nudus is an inhabitant of the mid to high intertidal zone and as such may contain a hemocyanin which in some way reflects adaptation to the respiratory rigors imposed by this habitat. Data are presented here on the quaternary structure of the hemocyanin of H. nudus, in addition to general factors which influence its state of aggregation. Also included is a detailed characterization

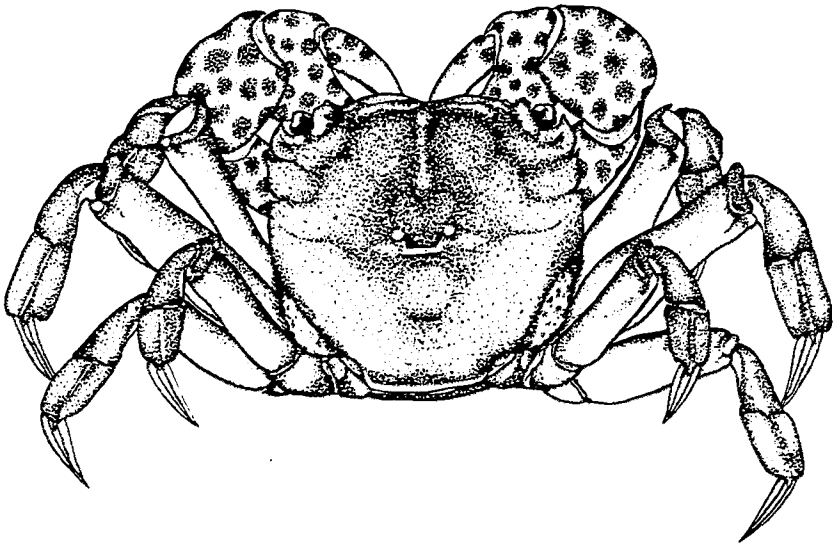


Figure 6. Hemigrapsus nudus (Dana) the purple shore crab (Scale drawing by Maggie Rudy).

of the subunit composition of the protein. This description of the hemocyanin of adult Hemigrapsus nudus constitutes the first part of the thesis.

The second part presents data regarding the hemocyanins of Hemigrapsus nudus at different developmental stages. As noted, only one other study has focused on the ontogenic expression of hemocyanin within a species of Arthropods (Terwilliger and Terwilliger, 1980; in press). Preliminary findings in that study indicate that the hemocyanin of Cancer magister undergoes ontogenic changes. Accordingly, I have characterized the hemocyanin of the eggs and zoea of H. nudus in an attempt to answer the following questions:

- Are the hemocyanins of the different developmental stages of Hemigrapsus nudus different from that of the adult crabs?
- Can the emergence of subunit heterogeneity and extent of aggregation be traced through the developing crab?
- Is it possible to determine at which ontogenic stage the hemocyanin genome is accelerated?

The information presented here on the hemocyanins of Hemigrapsus nudus, both adult and larval, contributes to our understanding of this remarkable protein and provides a strong data base for further comparative studies.

METHODS AND MATERIALS

Purification

Adult Hemigrapsus nudus (Dana) were collected from the mid-intertidal zone at North Cove, Cape Arago, Oregon. Intermoult male crabs ranging in size from 3.5 to 6.0 cm were used exclusively for this study of adult hemocyanins in an effort to eliminate possible variation due to sex, age, or moult cycle.

Fresh, post-branchial hemolymph was obtained by puncture at the basal joint of the third walking leg. The 1-4 millilitres of hemolymph obtained from each animal was pooled in the presence of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor and allowed to clot on ice for 15 minutes.

Following centrifugation in a refrigerated Sorvall RC-2B at 12,000 x g for 10 minutes, the supernatant was immediately applied to a 1.8 x 110 cm column of BioGel A-5M (200-400 mesh) which was equilibrated to 0.05 ionic strength Tris-HCl, pH 7.5 buffer that was 0.1 M in NaCl and 10 mM in $MgCl_2$ and $CaCl_2$. The column had been previously calibrated with fresh Cancer magister hemocyanin

M_r = 940,000 and 450,000 (Ellerton et al., 1970). All chromatography was performed at 4° C. The fractions thus obtained were analyzed for absorption maxima at both 280 and 340 nm on a Zeiss PMQ II spectrophotometer. Flushing the samples with oxygen did not change the absorbance reading at 340 nm. Hemocyanin samples which were used in subsequent experiments were purified in this manner.

Electrophoresis

High resolution polyacrylamide gel electrophoresis was used to analyze the subunits of purified hemocyanin. For this study, 7.5% slab gels (13 x 11 x 0.0015 cm) of constant acrylamide : bisacrylamide (37:1 ratio) were employed.

Regular Gel Electrophoresis

Regular gel electrophoresis in the absence of denaturants was performed according to Davis (1964). Purified hemocyanin samples of known concentration were dialyzed versus the appropriate buffer (upper electrode buffer) and electrophoresed into 7.5% polyacrylamide gels. A current of 35 mA was applied for 1-5 hrs. depending on the pH and buffer system utilized. Calibrants included

the whole and purified 25S, 16S and 5S components of Cancer magister hemocyanin (Ellerton et al., 1970; Larson et al., 1981) and the whole blood of Callinassa californica (M_r = 1.7 x 10^6 and 4.3 x 10^5, Roxby et al., 1974).

Electrophoresis carried out at pH 7.0 and pH 7.4 utilized a 0.05 M Tris-HCl/Tris-maleate buffer system both in the presence and absence of EDTA. At pH 8.9 where crustacean hemocyanins usually dissociate into 5S subunits, electrophoresis was carried out in a 0.05 M Tris-glycine/ Tris-HCl buffer system. Gels were stained for protein in Coomassie brilliant blue R according to Fairbanks et al. (1971) and destained in 10% acetic acid. Protein banding patterns resolved by this method shall be referred to as "native" protein patterns.

SDS Gel Electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis followed the discontinuous system described by Laemmli (1970). Purified hemocyanin samples of known concentration were denatured in boiling incubation buffer containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerin, 10 mM Tris-HCl (pH 6.3) and 0.01% bromphenol blue for 2 minutes at 100° C. These samples were electrophoresed into 7.5% slab gels with 3 cm stacking gels and run at

100 v for approximately 4 hours. The gels were stained in Coomassie brilliant blue R (Fairbanks et al., 1971) and destained in 10% acetic acid. Gel calibrants included phosphorylase A (M_r = 93,000), bovine serum albumin (M_r = 68,000) and ovalbumin (M_r = 43,000) (Sigma Chemicals).

Cross-referencing of Polyacrylamide Gel Protein Patterns

In order to determine the true number of different polypeptide chains present in the hemocyanin of Hemigrapsus nudus, isolated protein bands from one gel system were re-electrophoresed into a different system. Most frequently cross-referencing was done between "native", pH 8.9, gel protein bands and the SDS gel system. Following electrophoresis in the first system, gels were removed from the electrophoretic apparatus; stained and destained rapidly, according to Cleveland et al. (1977). The protein band of interest was carefully excised and left to soak in 0.5 ml of SDS incubation buffer at room temperature for 30 minutes and then overnight at -20° C. After thawing, the protein in the slice was electrophoresed into an SDS gel as described previously and stained per usual.

The method of cross-referencing protein patterns

was also employed between the regular gel systems. In this case it is not possible to stain for protein because, unlike the SDS system, there is no detergent present in the regular gel incubation buffer to solublize the protein after it is fixed. Rather a template of the protein banding pattern was used to locate band positions in the first unstained gel. Gel slices were removed from the first system and incubated overnight at 4° C in 0.5 ml of the upper electrode buffer appropriate to the second gel system. Electrophoresis and staining techniques were then carried out as described previously.

Peptide Maps

To confirm the unique nature of the subunit protein chains present in the hemocyanin of H. nudus, peptide maps of individual subunits were made. The procedure involves the partial digestion of the proteins by a protease in the presence of SDS (Cleveland et al., 1977). Protein bands were cut from both SDS and pH 8.9 regular gels and soaked for 30 minutes in SDS stacking gel buffer at room temperature. Gel slices were then transferred to a 15% SDS slab gel with a 4-5 cm stacking gel. The wells of the slab gel, containing the excised slices, were overlaid with 4 mg Staphlococcus aureus V8 protease

(Worthington). Electrophoresis was then initiated at 100 v and allowed to proceed until the tracking dye met the stacking gel/resolving gel interface. Digestion took place in the stacking gel for 45 minutes. Electrophoresis was then resumed at 100 v for 3 hours until the dye front reached the bottom of the gel. Gels were stained in Coomassie brilliant blue R (Fairbanks et al., 1971) and and destained in 10% acetic acid.

Copper Staining

Since hemocyanin is a copper-containing protein, its presence in polyacrylamide gels can be detected by a test specific to this metal. The test is based on the quenching of fluorescence of bathocuproine sulfonate by Cu^{+} (Bruyninckx et al., 1978). Regular gels at pH 7.0 and 8.9 were pre-electrophoresed in the gel buffer appropriate to the pH system used, excluding tetramethyl-ethylenediamine (TEMED) and ethylenediaminetetraacetic acid (EDTA) and diluted 1:4, for 4 hours to remove free radicals present in the gel. The gels were then loaded and run as previously described, in the absence of EDTA. Immediately following electrophoresis, gels were soaked for 1 minute in 16 mM ascorbate in glacial acetic acid and then for 1 minute in 0.28 mM aqueous bathocuproine sulfonate solution.

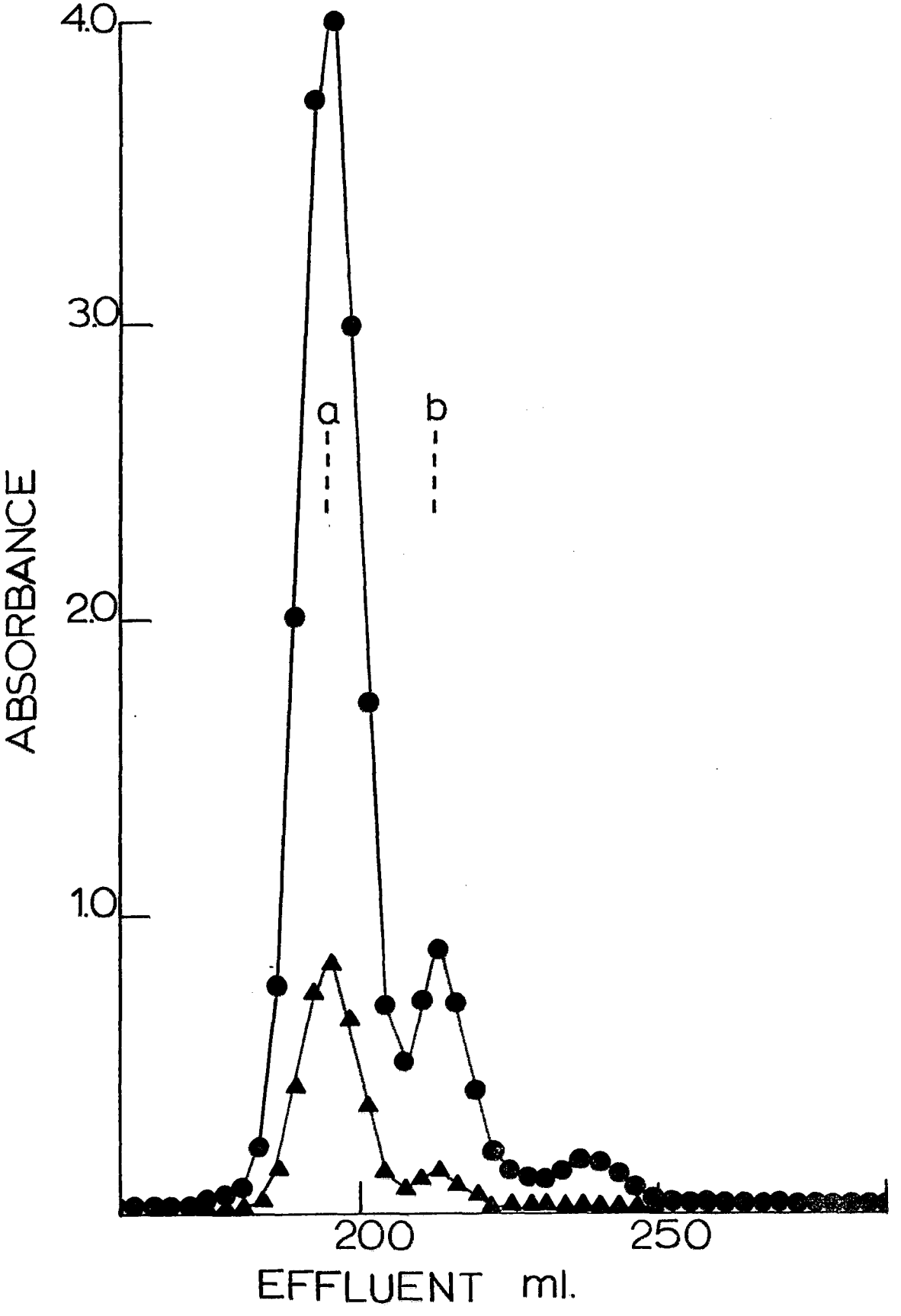
The staining pattern on the gel was observed by UV light illumination. Copper, where present, was indicated by dark bands against a light field of fluorescence and marked with pin holes and India ink. After staining for copper the gels were stained for protein with Coomassie brilliant blue R (Fairbanks et al., 1971) and destained in 10% acetic acid.

RESULTS

The hemocyanin of adult Hemigrapsus nudus chromatographs on BioGel A-5M at pH 7.5 in the presence of divalent cations as 2 peaks (Fig. 7). The material from the first peak has an apparent molecular weight of 940,000. This fraction comprises approximately 85% of the total protein present. The second, minor peak has an apparent molecular weight of 450,000 and makes up the remaining 15%. It is assumed by analogy with numerous other crustacean hemocyanins that peak I is 25S 2-hexamer hemocyanin and peak II is predominantly 16S single hexamer hemocyanin. If fractions containing either the putative 25S or 16S material are concentrated and re-chromatographed on the same column they elute as a single peak corresponding to peak I or II, respectively.

Absorption maxima are read at both 280 nm and 340 nm. The ratio of these two absorption maxima does not vary significantly for hemocyanin from the phylum Arthropoda and can therefore give important information regarding protein purity and degree of oxygenation of the hemocyanin. Purified samples of 25S hemocyanin from H. nudus which

Figure 7. Elution profile of hemolymph from Hemigrapsus nudus on a column of BioGel A-5M (200-400 mesh). Column volume = 1.8 x 110 cm. Column buffer was 0.05 ionic strength Tris-HCl (pH 7.5), 0.1M in NaCl, 10 mM in $MgCl_2$ and 10 mM in $CaCl_2$. Calibration proteins: a) purified 25S hemocyanin from Cancer magister, b) purified 16S C. magister hemocyanin. Absorbances read at 280 nm (●) and 340 nm (▲).


4.0
3.0
2.0
1.0
ABSORBANCE
a
b
200
250
EFFLUENT ml.

have been scrubbed with oxygen give a 280 nm/340 nm ratio of about 4.57. The ratio for 16S material is typically near 6.85.

The pattern resulting from electrophoresis of purified hemocyanin at either pH 7.0 or pH 7.4 is depicted in Figure 8. Under these conditions of pH and ionic strength, the 25S 2-hexamer molecule remains intact and electrophoreses as a single slow-moving band which stains strongly for copper. Under the same conditions, the 16S fraction runs as two distinct protein bands, a lower band and a central band in between the other two bands. The lower protein band from the 16S fraction stains very strongly for copper. It is concluded that the lower band is 16S single-hexamer hemocyanin by reference to the hemocyanin of Cancer magister and the central or "mid-band" protein remains, as yet, unidentified.

At pH 8.9 both the 25S and 16S aggregates dissociate into 5S subunits which can be resolved on regular disc gels at this pH (Fig. 9). The purified 25S fraction is resolved as 4 protein bands labled A to D. The purified 16S fraction yields a similar pattern with two important exceptions: A slow-moving band, A' is present only in this fraction, and band D present in the 25S fraction cannot be detected. All protein bands resolved on regular gels at pH 8.9, with the exception of A' which was present

Figure 8. Regular gel electrophoresis at pH 7.0 on 7.5% acrylamide gel of H. nudus hemocyanin. a - b) purified 25S hemocyanin from major peak (Fig. 7), c - d) purified 16S fraction: mid-band protein (MB) and 16S hemocyanin, from minor peak (Fig. 7), e - f) whole hemolymph of H. nudus.

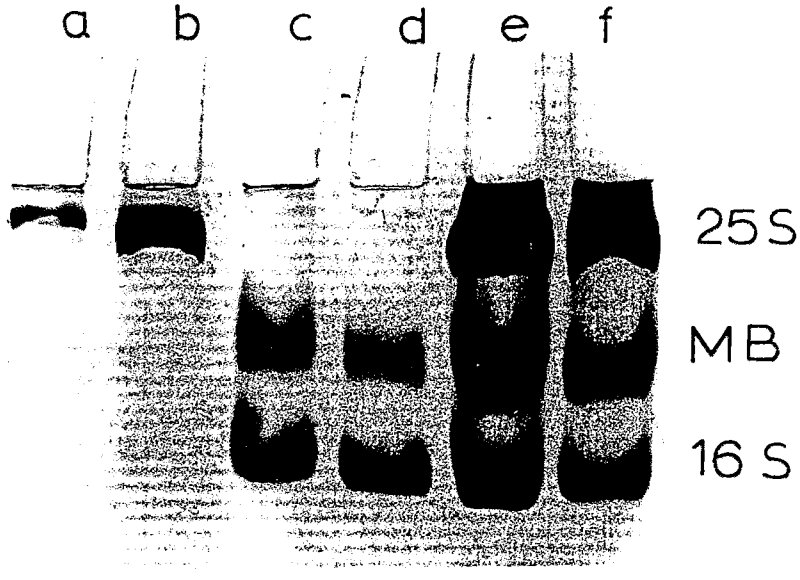
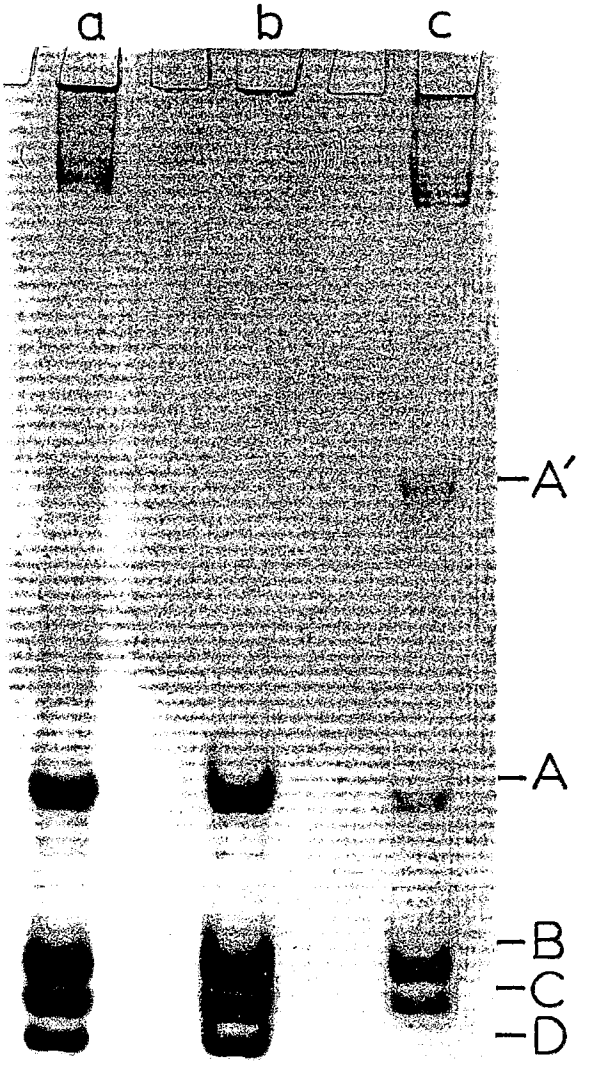

a
b
c
d
e
f
25 S
MB
16 S

Figure 9. Regular gel electrophoresis at pH 8.9 on 7.5% acrylamide gels of H. nudus hemocyanin. a) whole hemolymph, b) purified 25S hemocyanin, c) purified 16S fraction. Protein components are labeled A' - D in order of increasing mobilities.


a
b
c
A′
A
B
C
D

in low concentrations, stained for copper.

Purified samples of 25S and 16S hemocyanin were denatured with SDS in the presence of 2-mercaptoethanol and electrophoresed on SDS slab gels (Fig. 10). Five protein bands of different staining intensity were resolved with molecular weights ranging from 76,200 to 82,300. The bands are labled 1-5 in order of descending size. Both 25S and 16S give indistinguishable banding patterns in this system.

The results from cross-referencing experiments between native gels at pH 8.9 and SDS gel electrophoresis are shown in Figures 11 and 12. The important points gleaned from these experiments are as follows: 1) Native protein band B (pH 8.9) electrophoreses as 2 protein bands, 3 and 5, of differing molecular weight in the SDS system and, 2) Native band A' (pH 8.9), unique to the 16S fraction, and native band D (pH 8.9) found only in the 25S fraction, co-electrophoreses to the same position, band 2, in SDS. From cross-referencing experiments between regular gel systems it was found that the mid-band protein of the 16S fraction observed at pH 7.0 and 7.5 electrophoreses to position A' on a pH 8.9 regular gel.

Further confirmation for the unique character of the bands resolved in both native gels at pH 8.9 and those observed in SDS was provided by peptide maps (Fig. 13 and

Figure 10. SDS gel electrophoresis of H. nudus hemocyanin on 7.5% acrylamide gels. a) purified 16S fraction, b) purified 25S hemocyanin, c) whole Cancer magister hemocyanin. Protein components are labeled 1 - 5 in order of decreasing apparent molecular weights which range from 76,200-82,300.

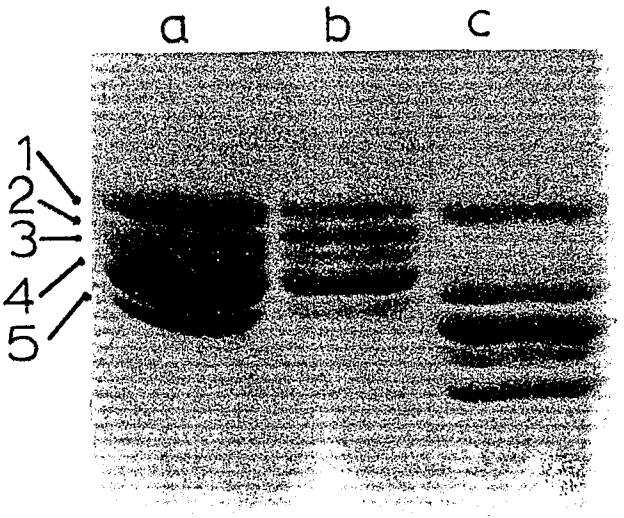

a
b
c
1
2
3
4
5

Figure 11. Results of a cross-referencing experiment. Individual bands were cut from a regular gel at pH 8.9 and run in the SDS gel system as described in Methods. The wells, labeled A' - D, contain the corresponding protein band cut from the pH 8.9 regular gel (Fig. 9). Bands b_1 and b_2 are the upper and lower sections of band B run separately. Band X is the interspace cut from between bands C and D. The normal SDS banding pattern of 25S hemocyanin is shown in wells indicated by arrows and labeled 1 - 5 as in Fig. 10.

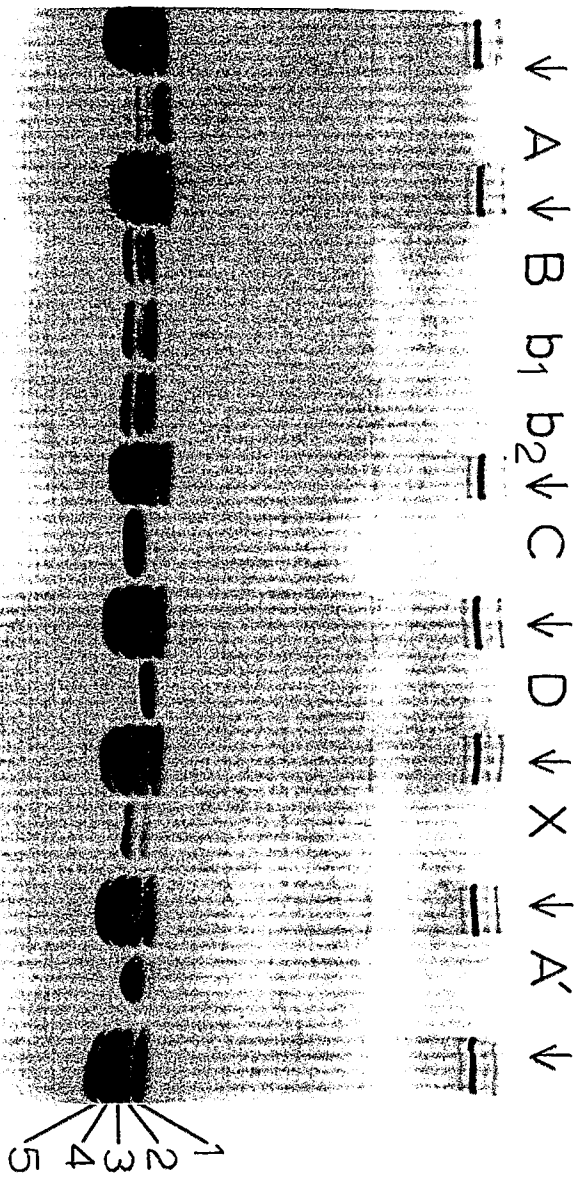
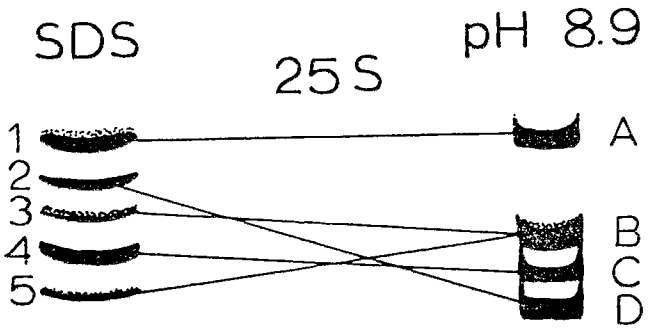
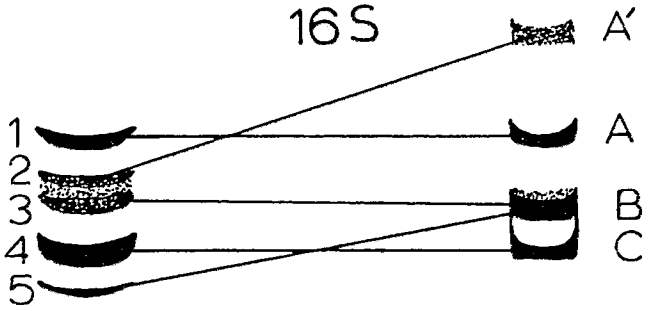

A
B
b1
b2
C
D
X
A'
1
2
3
4
5

Figure 12. Proposed correspondance of protein bands obtained by regular gel electrophoresis at pH 8.9 with the bands obtained by SDS gel electrophoresis as suggested by cross-referencing experiments (Fig. 11). A) 25S hemocyanin, B) 16S fraction, C) result of cross-referencing experiment where slices containing the proteins from bands D (25S only) and A' (16S only) were electrophoresed into the same well in SDS. Note that both D and A' run to the same position (2) in SDS.

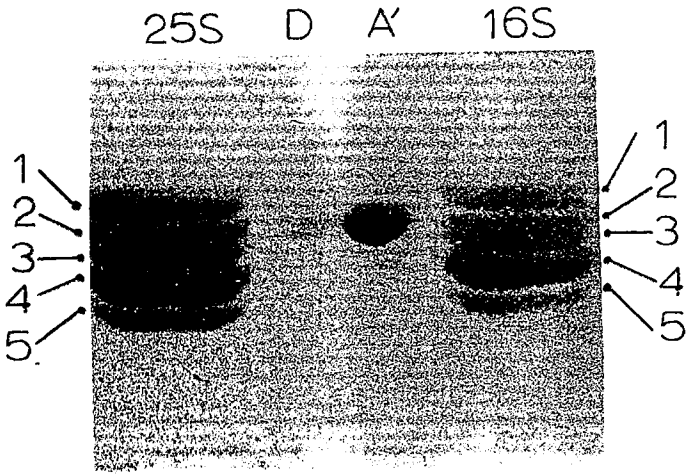
A



B



C



14). It was found that a given polypeptide chain will give the same peptide map whether it is cut from regular gels or from SDS gels. In the case of Hemigrapsus nudus hemocyanin six distinct polypeptides exist. Of these, five are certainly hemocyanin as judged by copper content and apparent molecular weights. The exact identity of the sixth protein, A' on pH 8.9 regular gels and mid-band on pH 7.0 and 7.5 regular gels, remains unresolved.

The experiments just described were also performed on whole hemolymph from both male and female crabs, purified hemocyanin from the female crabs (with and without eggs), on a freshly moulted individual and several other individual crabs as well. The results of these experiments did not differ from those just described for pooled intermoult male hemocyanin.

Figure 13. Peptide map on 15% acrylamide gel of the five protein components resolved on regular gels at pH 8.9 (Fig. 9). Each band, labeled A' - D, was cut from either the 25S or 16S fraction and treated with Staphylococcus aureus V8 protease as described in Methods. Bands b_1 and b_2 are the upper and lower sections of band B, run separately.

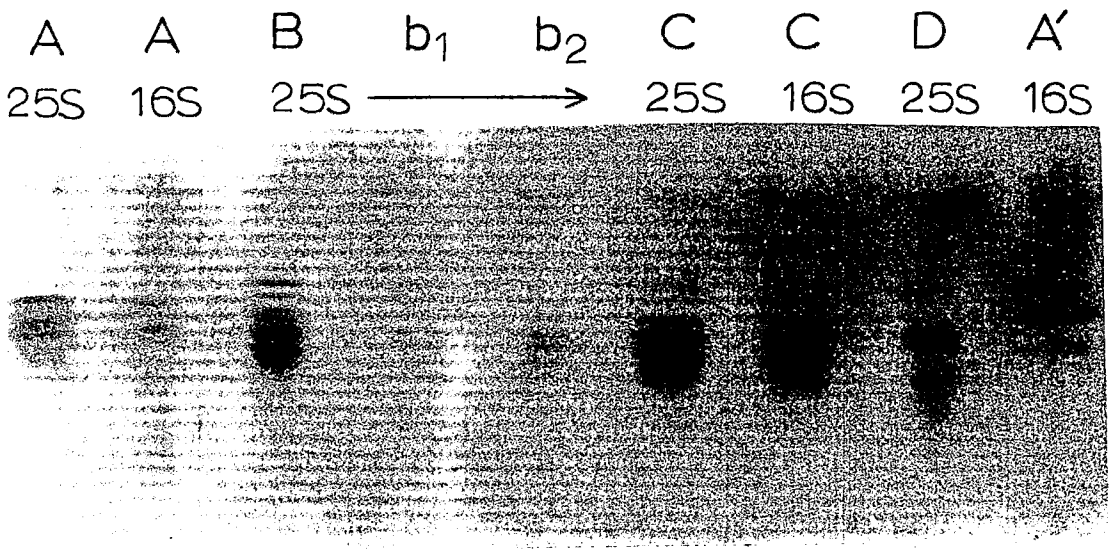
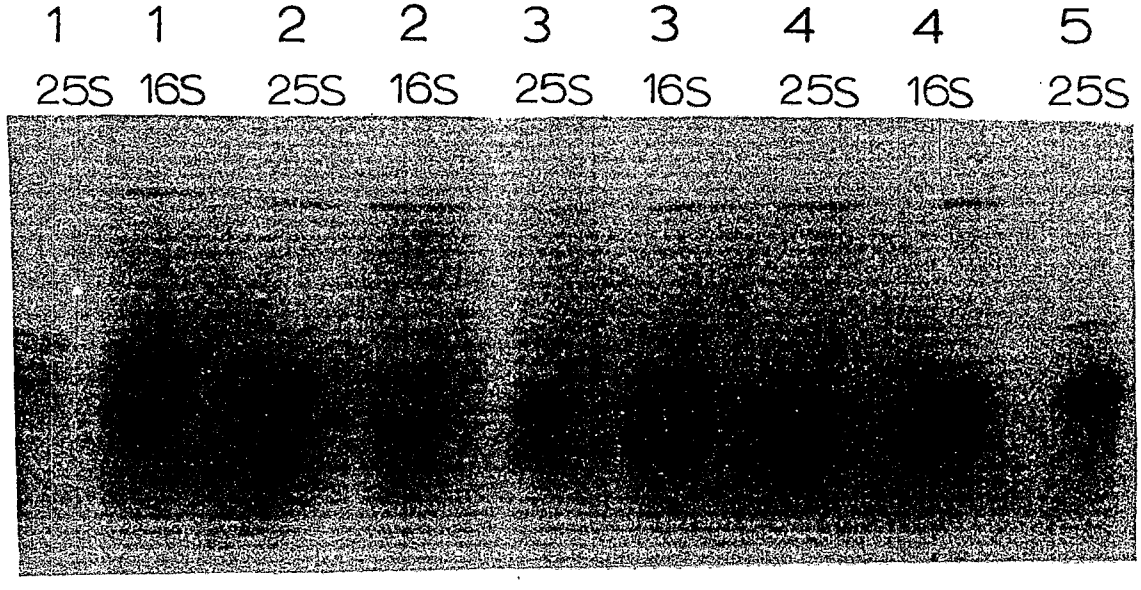

A
25S
A
16S
B
25S
b1
b2
C
25S
C
16S
D
25S
A'
16S

Figure 14. Peptide map on 15% acrylamide gel of the five protein components resolved on SDS gels (Fig. 10). Each band, labeled 1 - 5, was cut from either the 25S or 16S fraction and treated with Staphylococcus aureus V8 protease as described in Methods.


1 1 2 2 3 3 4 4 5
25S 16S 25S 16S 25S 16S 25S 16S 25S

DISCUSSION

Quaternary Structure

The hemocyanin of Hemigrapsus nudus exists, in vivo, as two distinct polymeric aggregates which can be separated by exclusion chromatography. The major component of this blood is a 25S 2-hexamer molecule as judged by its apparent molecular weight (940,000), copper-staining on polyacrylamide gels and its 280 nm:340 nm absorbance ratio. The average value observed in H. nudus hemocyanin for this ratio is 4.57 which is well within the limits of 4 to 5 ascribed for pure hemocyanin (Markl, 1979b).

The second component, a 16S single-hexamer hemocyanin, comprises up to 15% of the total protein present. However, due to the presence of an additional unknown protein, the exact quantity of 16S hemocyanin in this fraction has proven difficult to measure with certainty. That an additional protein is present is evidenced by the disproportionately high 280 nm:340 nm ratio of 6.85. Additionally, when purified 16S samples are resolved on a regular gel at either pH 7.0 or 7.5 two bands of approximately equal

protein staining intensity are observed. Based on relative mobilities of these two proteins in this electrophoretic system in comparison to purified 16S hemocyanins of other organisms, it is concluded that the lower-most band is 16S hemocyanin and the mid-band is the unknown protein. This view is supported by copper-staining and cross-referencing experiments. Efforts to separate the unknown protein from the 16S hemocyanin fraction have been unsuccessful.

The two hemocyanin species observed in Hemigrapsus nudus (25S 2-hexamers and 16S hexamers) do not represent a rapidly equilibrating system; rather they behave as a mixture of discrete aggregates. This is shown by the failure of purified hemocyanin solutions, either 25S or 16S, to produce increased products of dissociation or reassociation upon dilution. The lack of apparent equilibrium or deviance from the mass action law among the hemocyanins is attributed to the effects of heterogeneity at the subunit level (Herskovitz et al., 1981a, 1981b).

Subunit Composition

The 25S and 16S aggregates of Hemigrapsus nudus hemocyanin can be dissociated to six distinct subunits whose apparent molecular weight in SDS range from 76,200 to 82,300. The presence of dissociation products with

apparently differing molecular weights suggests that the hemocyanin of Hemigrapsus nudus is composed of a heterogeneous mixture of polypeptide chains. Yet, it can be argued that the SDS gel method is not reliable for absolute molecular weight determinations. Therefore, other lines of evidence in support of the existence of microheterogeneity in H. nudus hemocyanin are proffered here and will be the subject of the remainder of this discussion.

Using two different electrophoretic procedures it is possible to dissociate the hemocyanin of Hemigrapsus nudus into two different protein banding patterns. The first pattern, obtained by regular gel electrophoresis at pH 8.9, arises mainly as a result of charge differences among the putative subunits. In this system each of the purified aggregates, 25S and 16S, produces 4 major protein bands. One of the bands (A') has a markedly retarded mobility in this system. The other banding pattern, from SDS gel electrophoresis, is the result of differences in apparent molecular weights. In SDS both the 25S and 16S molecules give identical patterns consisting of five bands each. The fact that the SDS gel system resolves one band more than observed in the regular gel system indicates that two polypeptides are present which have similar charge characteristics but different molecular weights in SDS. This idea was confirmed by cross-referencing experiments;

band B of the regular gel system migrates as bands 3 and 5 in SDS.

The cross-referencing experiments proved very instrumental in the determination of subunit numbers since it is unlikely that any one component was concealed in both systems. The most revealing information came from experiments where proteins were cut from regular gels at pH 8.9 and analyzed in the SDS system. Four of the subunits obtained from regular gels were homogeneous in both systems and all ran as single bands in SDS. Only band B, which appeared homogeneous on regular gels, was shown to be heterogeneous in SDS. On the other hand, band 2 of the SDS system, which appeared homogeneous, was actually composed of two proteins separable by regular gel electrophoresis at pH 8.9. It is interesting to note that of the two proteins which migrate to position 2 on SDS, one is observed only in the 25S fraction (D) and the other (A') is unique to the 16S fraction such that only when unpurified hemocyanin was run in the SDS gel system, did band 2 really represent a heterogeneous mixture of A' and D. Furthermore, on regular gels, at pH 8.9, band A' migrates as the slowest band; far behind the other subunits, and band D migrates as the fastest band. In other words, two seemingly different proteins with vastly different mobilities in the regular gel system have the

same approximate molecular weights as determined by SDS electrophoresis in the presence of reducing agents.

It was originally hypothesized that band D existed as a single subunit only when it was a part of the 25S 2-hexamer aggregate but dimerized when associated with the 16S fraction and thus ran as a slower band, A'. Upon exposure to the disulfide-bridge reducing agent, 2-mercaptoethanol, present in the SDS system, the dimer would presumably dissociate and run as a single band to position 2 and become indistinguishable from its single chain counterpart; D. Experiments with and without 2-mercaptoethanol did not offer proof for this hypothesis. Additionally it was found by cross-referencing gel slices between pH 7.0 and 8.9 regular gels that band A' was also the contaminating, mid-band protein observed on pH 7.0 (and 7.5) regular gels, and as such not likely to be hemocyanin at all.

The cross-referencing experiments between regular and SDS gels argue strongly in support of heterogeneity at the subunit-polypeptide level. Not only are multiple protein bands observed in the SDS system, where proteolysis and/or anomolous protein-SDS interactions might account for the observed heterogeneity, but multiple protein bands are also detected on regular gels. Moreover, each band isolated by the regular gel system corresponded neatly

to a protein band resolved by SDS gel electrophoresis.

The most unequivocal evidence for polypeptide heterogeneity came from peptide maps obtained by limited proteolysis of individual protein bands. Each of the subunits isolated by SDS gel electrophoresis produced a unique map. In particular, SDS band 1 produced the same characteristic map regardless of whether it was cut from a 25S fraction or a 16S fraction. This was also true for SDS bands 3, 4 and 5. However, the map obtained from SDS band 2 of 25S hemocyanin differed markedly from the map obtained from SDS band 2 cut from a 16S fraction; confirming the findings of the cross-referencing experiments proposed in Figure 12.

In addition, peptide maps obtained from subunits isolated on regular gels at pH 8.9 correlated closely with those maps obtained from SDS and thus further verified cross-referencing findings. For instance, the map of band A (reg. gel) closely resembled the map of band 1 (SDS); band B (reg. gel) which was suspected to be a doublet of SDS bands 3 and 5 gave a map that was a composite of the maps from 3 and 5. Finally, a map of band A' (reg. gel, 16S only) correlated with the map of 16S band 2 (SDS), while a map of band D (reg. gel, 25S) matched the map of 25S band 2 (SDS).

In light of these findings it is possible to conclude

that the single hexamer, 16S molecules have at least 4 individual subunits which are also present in the 25S linked hexamers. Based on relative staining intensities, it is proposed that subunits 1 and 4 (SDS) occur twice in the 16S molecule making up the six subunits of the 16S hexamer. Furthermore, the 25S 2-hexamer population appears to have a subunit associated with it that is not present in the 16S fraction. Although no evidence exists at present, it is possible that this subunit is necessary for the assembly of subunits to a linked 25S 2-hexamer configuration. In the words of Markl and Kempter (1981a; 1981b), the protein isolated as band D on regular gels at pH 8.9 may be a "conservative subunit" and as such, act as a hexamer-hexamer link. Such a system has been described in a number of arthropods, the simplest known is that of the marine isopod Ligia pallassi (Terwilliger, in press). The 16S hemocyanin of this organism is composed of six subunits of a single type, M_1, and the 25S 2-hexamer hemocyanin has 2 subunit types, M_1 and M_2, in an apparent ratio of 5:1. Since a dissociated 2-hexamer aggregate is incompetent to reassociate in the absence of M_2 subunits it is suggested that the M_2 subunit acts a linker in this system.

Finally, it is concluded that genetic or sexual polymorphisms can not account for the observed

heterogeneity since identical results were obtained from individual crabs of both sexes.

Summary

The native hemocyanin of adult Hemigrapsus nudus exists as two non-equilibrating populations of multi-subunit aggregates: 25S 2-hexamers and 16S hexamers. The 2-hexamer molecule has a molecular weight of about 940,000 and comprises 85% of the protein present in purified hemocyanin. The 16S fraction includes the single hexamer hemocyanin molecule which has a molecular weight of about 450,000 and makes up most of the remaining 15% of the protein. Also present in the fraction which elutes at 16S is an additional protein of unknown identity.

Dialysis against an alkaline buffer, pH 8.9, in the absence of divalent cations results in the dissociation of the 25S and 16S aggregates to 5S subunits. The subunit mixture was resolved by regular polyacrylamide gel electrophoresis and gave characteristic patterns of four protein bands each. The resulting patterns from the 25S and 16S molecules differed with respect to a slow-moving band present only in the 16S fraction and a fast-moving band found only in the 25S fraction.

Denaturation of the 25S and 16S molecules in the

presence of SDS and 2-mercaptoethanol yielded a pattern of five protein bands with apparent molecular weights ranging from 76,200 to 82,300. The banding patterns produced by the 25S and 16S molecules are indistinguishable from each other.

Through a series of cross-referencing experiments, where isolated protein bands from the regular gel system at pH 8.9 were analyzed in the SDS gel system, it was possible to correlate the two patterns produced by these electrophoretic systems. The results showed that all but one of the bands resolved by regular gel electrophoresis corresponded to distinct and separate bands in the SDS system; the other band resolved as two bands in SDS.

In other cross-referencing experiments it was found that the unidentified contaminant present in the 16S fraction represented the slow-moving band observed on regular gels at pH 8.9.

Peptide maps provided proof for the correlations determined by cross-referencing experiments and lent strong support to the idea that each band separated by SDS represented a unique polypeptide chain.

It is concluded that the hemocyanin of adult Hemigrapsus nudus is composed of five unique subunits, four of which occur in both the 25S and 16S aggregates. The fifth subunit, observed only in the 25S fractions, is thought

to act as a "linker molecule" and in this capacity aids in the formation of the 25S 2-hexamer configuration.

METHODS AND MATERIALS

Ovigerous Hemigrapsus nudus (Dana) were collected off Fossil Point, Coos Bay, Oregon. These animals were kept in tanks of running seawater until needed.

Egg Hemocyanin

Eggs, both early and late in development, were obtained by removing the berried pleopods and extracting the egg masses from them. Following removal, the eggs were soaked in filtered seawater in the cold for 1-2 hours. Eggs were crushed in a tissue grinder to a fine homogenate in the presence of a small amount of phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor. The extract was then centrifuged at 12,000 x g for 15 minutes at 4^{o} C in a Sorvall RC-2B refrigerated centrifuge. The supernatant was quickly passed through Whatman No. 1 filter paper to remove the lipid layer and centrifuged again at 12,000 x g for 10 minutes. The supernatant, which was brownish-orange, was immediately applied to a column of Sephadex G-75 (1.8 x 32 cm) in equilibrium with a Tris-HCl buffer, pH 7.5,

that was 0.1 M in NaCl, 10 mM in $MgCl_2$ and 10 mM in $CaCl_2$. The void volume peak, which was a clear orange solution and should contain hemocyanin if present was collected and concentrated briefly on sucrose at 4° C and then applied to a BioGel A-5M column (1.8 x 110 cm) in equilibrium with the same buffer. Further analyses were performed as previously described for adult Hemigrapsus nudus.

Zoea Hemocyanin

The zoea of Hemigrapsus nudus were collected following their release from ovigerous crabs held in running seawater tanks. The zoea were placed in nylon netting and rinsed with filtered seawater. Body fluid was extracted by squeezing the animals in the nylon netting and collecting the filtrate in a chilled beaker containing a few drops of PMSF. The crushed animals were rinsed several times with small quantitites of filtered seawater which were also added to the contents of the chilled beaker. The combined extract was then centrifuged at 12,000 x g for 15 minutes at 4° C in a Sorvall RC-2B refrigerated centrifuge. A clear orange supernatant was removed and concentrated briefly on sucrose at 4° C before application to a BioGel A-5M column (1.8 x 110 cm) in equilibrium with a Tris-HCl buffer, pH 7.5 that was 0.1 M in NaCl,

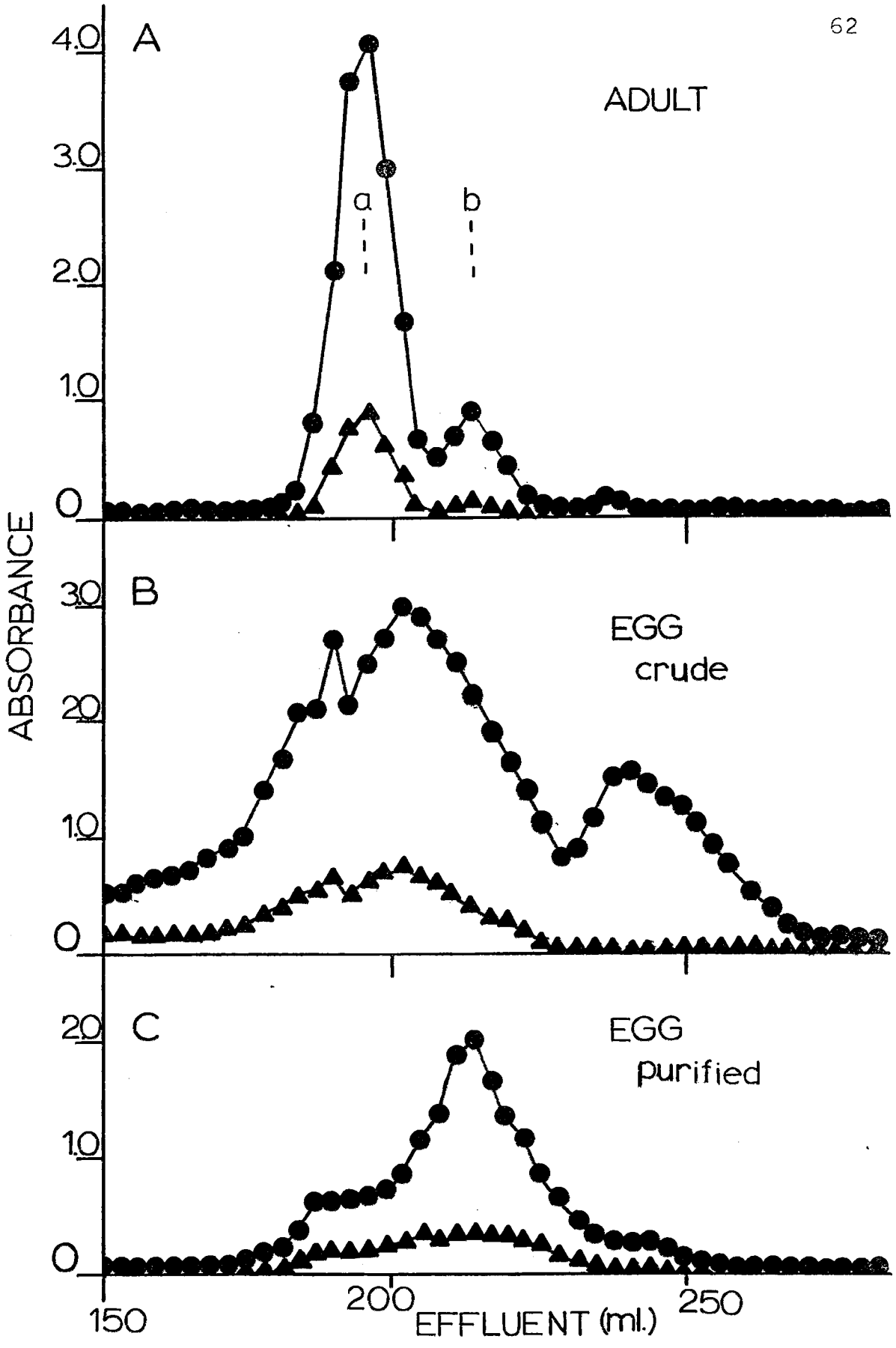
10 mM in $MgCl_2$ and 10 mM $CaCl_2$. All subsequent analyses were performed as previously described for adult Hemigrapsus nudus.

RESULTS

Egg Hemocyanin

The crude homogenate obtained from the eggs of Hemigrapsus nudus chromatographs as several protein peaks (Fig. 15b). Rechromatography of samples with elution volumes similar to those of adult hemocyanin yielded a single peak with a shoulder on the leading edge (Fig. 15c). The material from the main peak has an apparent molecular weight of 450,000 daltons as judged by its elution volume. The shoulder corresponds to an apparent molecular weight of 940,000. It is assumed by analogy with adult Hemigrapsus nudus hemocyanin and numerous other crustacean species that the main peak is composed primarily of 16S single hexamer hemocyanin and the shoulder is 25S 2-hexamer hemocyanin. Ratios of absorption maxima read at both 280 nm and 340 nm give values of about 6 for the 25S shoulder and about 13 for the main 16S peak. These values are high in contrast to those obtained from adult 25S and 16S hemocyanins (4.57 and 6.85, respectively), suggesting the presence of additional proteins. Since experiments with eggs both early and late in development

Figure 15. Chromatography of hemolymph from H. nudus adult (A), crude egg homogenate (B) and purified egg (C) on a column of BioGel A-5M (200-400 mesh). Column volume = 1.8 x 110 cm. Column buffer was 0.05 ionic strength Tris-HCl (pH 7.5), 0.1 M in NaCl, 10 mM in $MgCl_2$ and 10 mM in $CaCl_2$. Calibration markers: a) purified 25S hemocyanin from Cancer magister and b) purified 16S C. magister hemocyanin. Absorbances read at 280 nm. (●) and 340 nm. (▲).


A
ADULT
a
b
4.0
3.0
2.0
1.0
0
B
EGG
crude
3.0
2.0
1.0
0
C
EGG
purified
2.0
1.0
0
ABSORBANCE
150
200
250
EFFLUENT (ml.)

gave the same results, they will not be distinguished from each other in this report.

Electrophoretic analysis of purified egg hemocyanin on regular gels at pH 7.0 yielded patterns similar to the adult hemocyanin (Fig. 16); 25S material resolves as a single slow moving protein band which stains strongly for copper, and the 16S fraction runs as two distinct protein bands. The lower band stains strongly for copper and faint copper staining was detected for the mid-band protein. It is concluded by reference to adult 16S hemocyanin that the lower band, resolved in the egg 16S fraction, is 16S hemocyanin. The egg hemocyanin as resolved by this system differs from that of the adult in two ways. First the so-called mid-band protein is present in much higher relative concentration in the egg hemocyanin than in the adult hemocyanin. Secondly there appears to be considerably more 16S hemocyanin relative to 25S hemocyanin in the egg preparations in comparison to the adult hemocyanins.

At pH 8.9 both the 25S and 16S egg hemocyanins dissociate into 5S subunits which can be resolved on regular disc gels (Fig. 17). The purified 25S material is resolved into three protein bands which apparently correspond to bands B, C and D of the adult 25S hemocyanin. The purified 16S material resolves as two protein bands

Figure 16. Regular gel electrophoresis at pH 7.0 on 7.5% acrylamide gel of adult and egg hemocyanins from H. nudus. a) purified egg 25S hemocyanin, b - e) samples of egg 16S fraction taken at different points along the major peak shown in Figure 15 C, f) whole adult hemolymph.

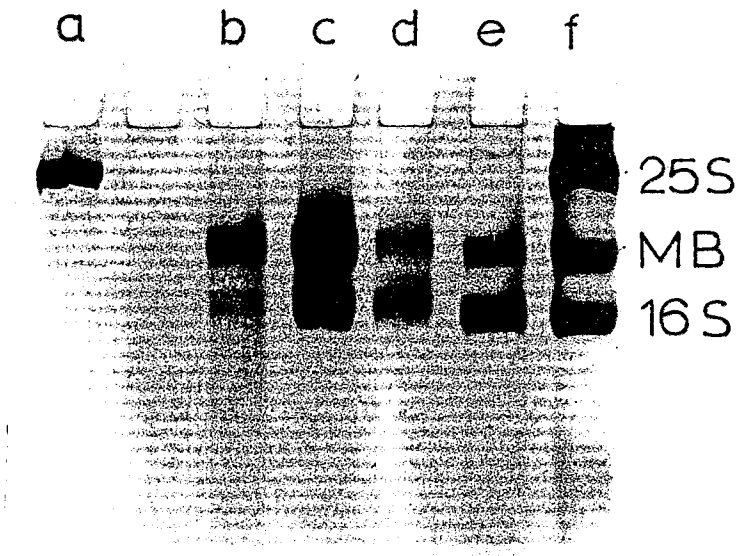
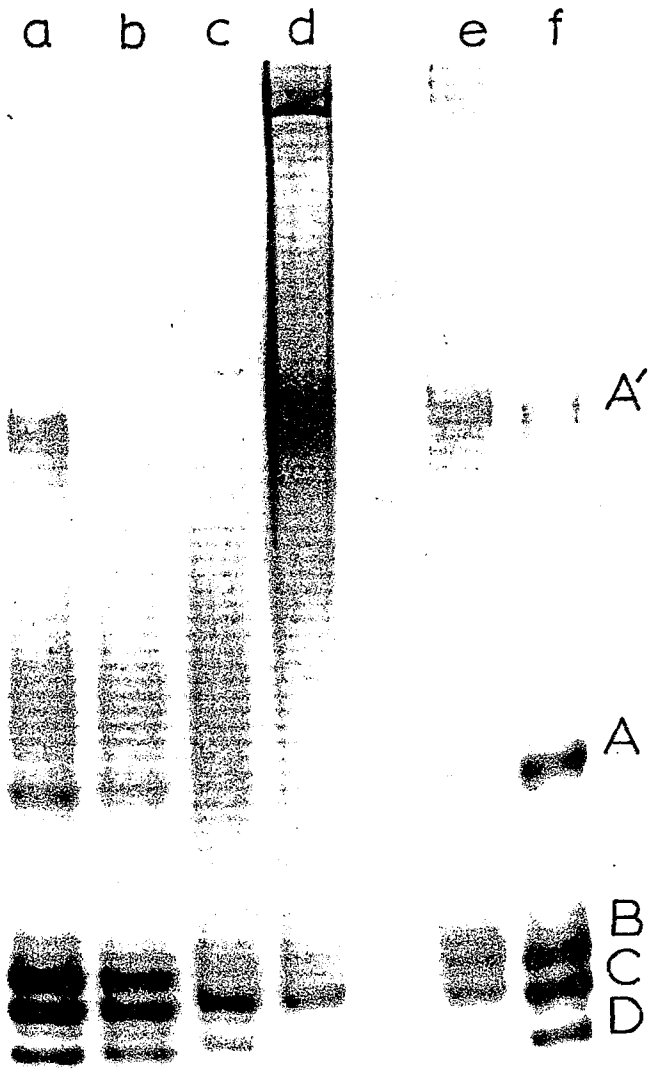

a
b
c
d
e
f
25S
MB
16S

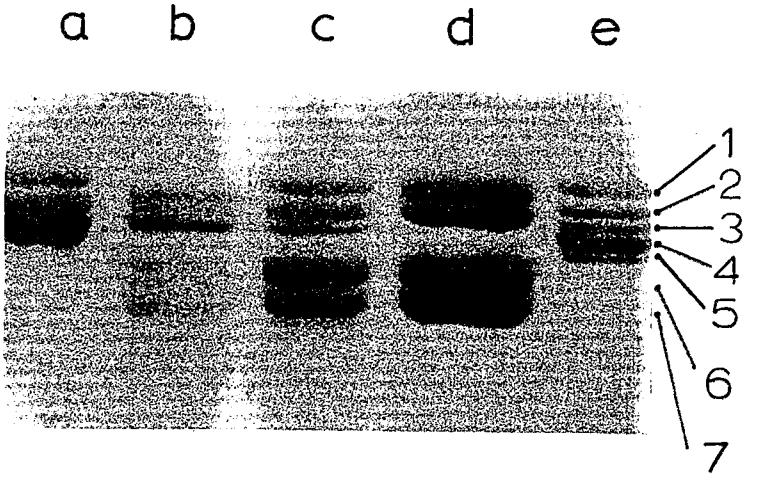
Figure 17. Regular gel electrophoresis at pH 8.9 on 7.5% acrylamide gel of adult and egg hemocyanins from H. nudus. a) whole adult hemolymph, b) purified adult 25S, c) purified egg 25S hemocyanin, d) purified egg 16S fraction, e) purified adult 16S fraction, f) whole adult hemolymph.


a
b
c
d
e
f
A′
A
B
C
D

which correspond to adult bands B and C. High molecular weight material is also present in the egg 16S fraction including a band at position A'. Despite basic similarities between the egg and adult hemocyanins as resolved in this system two important differences occur: adult band A can not be detected in either of the egg 25S or 16S fractions and, although adult band B is observed in the egg fractions it is present in much lower relative concentrations.

Purified samples of egg 25S and 16S hemocyanin were denatured with SDS in the presence of 2-mercaptoethanol and electrophoresed on SDS slab gels (Fig. 18). The purified 25S egg hemocyanin resolves as 3-4 protein bands in this system which correspond to adult SDS bands 2, 3, 4 and trace quantities of 5. Adult band 1 is not detected in egg 25S hemocyanin. Also present in the 25S egg fraction were faster moving bands. These protein bands might be the result of proteolytic degradation or are due to slight contamination by the mid-band protein. The purified egg 16S hemocyanin resolves as 2 protein bands in SDS which correspond to adult SDS bands 3 and 4. Adult bands 1 and 5 are not detectable in the purified 16S hemocyanin. When the mid-band protein is isolated and run in the SDS system it resolves as four protein bands two of which have molecular weights which correspond to

Figure 18. SDS gel electrophoresis on 7.5% acrylamide gel of adult and egg hemocyanins from H. nudus. a) whole adult hemolymph, b) purified egg 25S hemocyanin (with slight contamination from mid-band protein), c) purified egg 16S fraction, d) mid-band protein (cut from pH 7.0 regular gel and run in SDS system), e) purified adult 25S hemocyanin. The adult bands are labeled 1 - 5 in order of decreasing molecular weights. Bands 6 and 7 have molecular weights of 74,300 and 69,900, respectively.


a
b
c
d
e
1
2
3
4
5
6
7

adult bands 1 and 2, the other two have a higher mobility than the adult subunits and are labled 6 and 7. The molecular weight values for bands 6 and 7 as determined by SDS gel electrophoresis are about 74,300 and 69,900, respectively.

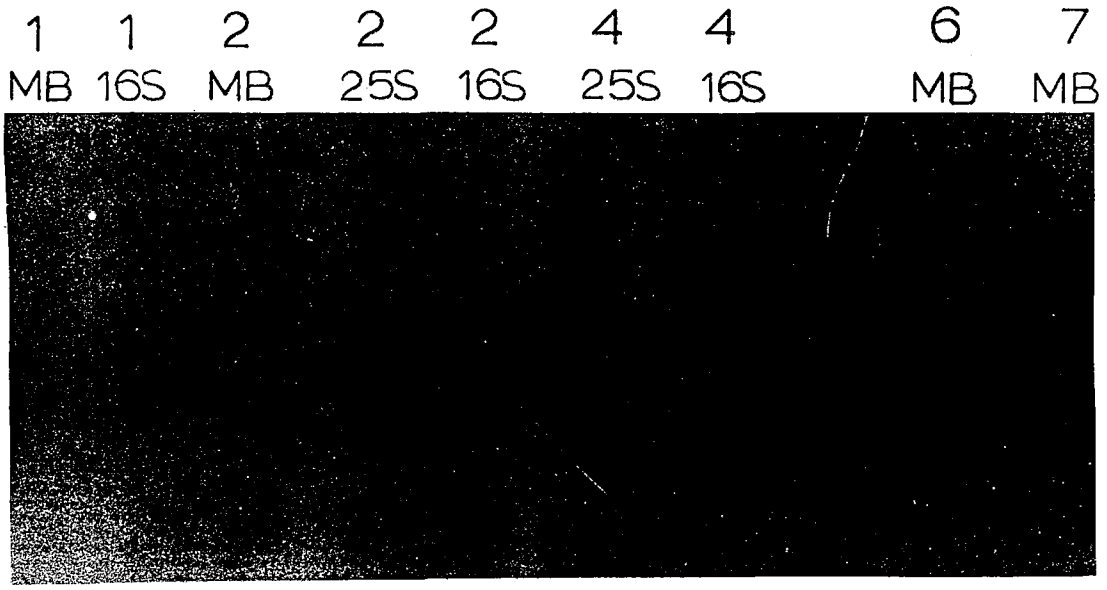
Through cross-referencing experiments it was possible to correlate the adult hemocyanin protein bands as resolved on regular gels (pH 8.9) with the 5 protein bands observed in the SDS system. A similar cross comparison was carried out on egg hemocyanins. Egg band B of pH 8.9 regular gels electrophoresed to position 3 in SDS. Band C electrophoresed to SDS position 4 and band D (25S only) electrophoresed to position 2. The mid-band protein ran to 4 positions; 1, 2, 6 and 7. The results from the cross-referencing experiments with egg hemocyanins parallel those obtained from similar experiments on adult hemocyanins with 3 important exceptions. First, egg band B resolves as only one major band in SDS (band 3), this is in contrast to adult band B which resolves as two major protein bands in SDS (3 and 5). Secondly, it was found that egg mid-band protein resolves as four bands on SDS (1, 2, 6 and 7) while adult mid-band runs to SDS position 2 only. A third difference which will be discussed later is the conspicuous absence of band A from both 25S and 16S egg hemocyanin and corresponding absence of subunit 1 in the

SDS samples.

To confirm the relationship between subunits observed in both SDS and on regular gels at pH 8.9, and to establish the true relationship between adult and egg hemocyanins peptide maps were made of individual egg proteins. Each of the egg subunits isolated by SDS gel electrophoresis produced a unique peptide map which corresponded closely to the maps obtained from adult subunit bands (Fig. 19). For instance, the map of egg band 4 from either the 25S or 16S fractions matched the peptide map of adult band 4. In adult hemocyanin it was shown that the map of 25S band 2 differed from the map of 16S band 2; the adult 25S band 2 is a hemocyanin subunit and the 16S band 2 represents the mid-band protein. The same situation is also true of the egg hemocyanins. The peptide map of band 2 from the egg 25S fraction is different from that of the egg 16S band 2. The map of egg 25S band 2 resembles the maps of both adult bands 2 and 3. This can probably be explained by cross-contamination between these neighboring bands. The map of the egg 16S band 2 corresponds exactly to the map of adult 16S band 2.

Additionally, the maps of egg bands 1 and 6 which are also known to be components of the egg mid-band protein produce peptide maps which are indistinguishable from the map of adult 16S band 2. In other words, three of the

Figure 19. Peptide map on 15% acrylamide gel of the seven protein components of purified egg homogenate resolved on SDS gels (Fig. 18). The bands, labeled 1 - 7, were cut from either the 25S, 16S or mid-band fractions and treated with Staphylococcus aureus V8 protease as described in Methods.


1
MB
1
16S
2
MB
2
25S
2
16S
4
25S
4
16S
6
MB
7
MB

components of the egg mid-band protein are the same as the adult mid-band protein. The fact that a single protein type runs as three different bands in SDS can probably be attributed to heightened sensitivity of this egg protein to proteolysis not observed in the adult protein. Another possibility is that the mid-band protein components have been chemically modified in such a way as to give them slightly different mobilities in the SDS system. Egg band 7, the fourth component of the mid-band protein, produces a unique map and appears to be a protein that is not present in the adult hemocyanin. An analysis of egg band 3 is not included here since it was not possible to obtain this subunit in high enough concentrations for analysis by peptide maps.

Zoea Hemolymph

The results of experiments with the zoea are less conclusive than those with egg hemocyanins. This is due to the difficulty in obtaining sufficient quantity of zoea hemolymph for a complete analysis. The results presented here are tentative.

The hemolymph of the zoea of Hemigrapsus nudus chromatographs on BioGel A-5M at pH 7.5 in the presence of divalent cations, as a complicated curve not unlike

that of the crude egg homogenate shown in Figure 15b. Rechromatography of samples with elution volumes similar to those of adult hemocyanin yields a small peak of very low concentration.

On regular gels at pH 7.0, the purified hemolymph from the zoea resolves as two protein bands. The upper band corresponds closely to adult 25S hemocyanin. The second band which is considerably wider than the first runs slightly behind the adult 16S hemocyanin band. Both of the zoea protein bands resolved in this system stain for copper.

Due to the low concentration of zoea hemolymph available it was not possible to resolve this protein on regular gels at pH 8.9. Instead the protein was lyophilized and run in the SDS electrophoresis system in the presence of 2-mercaptoethanol (Fig. 20). A single strongly staining protein band accompanied by several faint bands was resolved by this system. The major band has an apparent molecular weight in SDS of 77,900 and will be referred to as band 3.

Peptide maps were made of zoea protein bands (Fig. 21). Each of the peptide maps produced from zoea hemolymph proteins are the same. Furthermore they do not bear an immediate resemblance to any of the adult hemocyanin peptide maps.

At this point it is impossible to draw any conclusions regarding the existence of hemocyanin in the zoea of Hemigrapsus nudus. It can be stated, however, that the hemolymph of this organism possesses at least one copper-containing protein which has an apparent molecular weight similar to that of the adult hemocyanin. Also, this zoea protein is composed of at least one subunit whose molecular weight in SDS is within the range expected for hemocyanin subunits. Since this study of zoea is not complete, further discussion of this aspect of my research will not be made in this thesis.

Figure 20. SDS gel electrophoresis on 7.5% acrylamide gel of adult and putative zoea hemocyanins from H. nudus. a) purified adult 25S hemocyanin, b - c) purified zoea hemolymph, d) purified adult 16S hemocyanin, e) whole hemolymph from Cancer magister. The adult protein bands are labeled 1 - 5 in order of decreasing molecular weights. The major zoea protein band as an apparent molecular weight of 77,900.

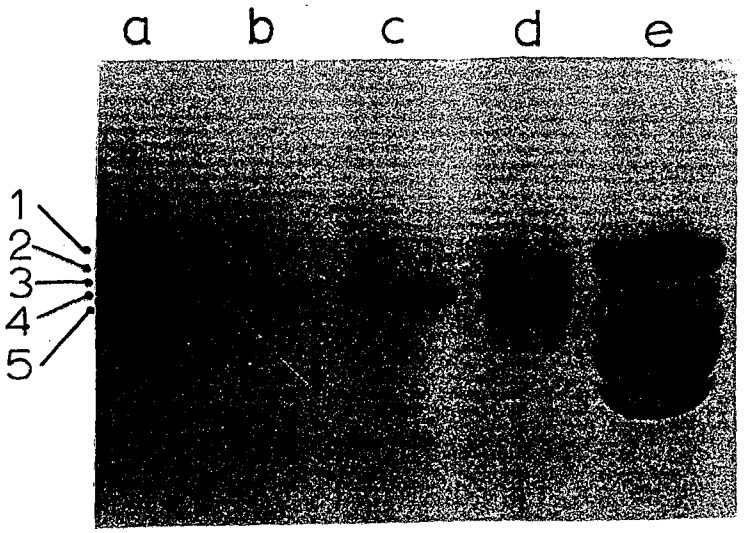
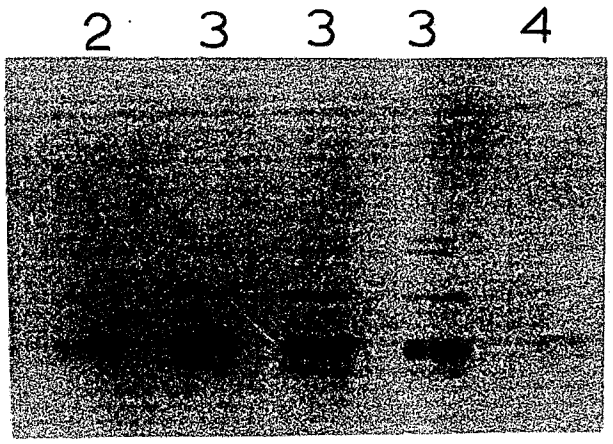

a
b
c
d
e
1
2
3
4
5

Figure 21. Peptide map on 15% acrylamide gel of the major and minor protein components of zoea hemolymph resolved on SDS gels (Fig. 20). The bands, labeled 2 - 4 were cut from the SDS gel and treated with Staphylococcus aureus V8 protease as described in Methods. Three different concentrations of band 3 were analyzed.


2
3
3
3
4

DISCUSSION

The results of this experiment shed light on an area of hemocyanin research that has long remained unexplored. Two significant facts have emerged. First, the eggs and possibly the zoea of Hemigrapsus nudus contain hemocyanin. The existence of hemocyanin in either of these developmental stages in arthropods has not been reported. It is suspected however, that hemocyanin exists in the zoea of Cancer magister (N.B. Terwilliger, personal communication). Confirmation for the existence of 25S and 16S hemocyanin in the eggs and zoea of Hemigrapsus nudus awaits electron microscopy, however, the structural studies argue strongly for this conclusion. This is particularly striking in light of the structural similarities shared by the eggs, zoea and adult hemocyanins. Putative hemocyanins from the adult and egg stages elute as two protein peaks by molecular exclusion chromatography and apparently correspond to the 25S 2-hexamer and 16S hexamer hemocyanins reported for other crustacean hemocyanins (Markl, 1979b). Regular gel electrophoresis at pH 7.0 and copper-staining experiments further corroborate this finding. An additional protein which elutes with the adult 16S fraction is also present

in the egg 16S fraction and electrophoreses midway between the 25S and 16S hemocyanins on regular gel at pH 7.0. This protein has complicated the interpretation of the data: Is it an apohemocyanin? Is it a hemocyanin subunit with differing copper content? Is it a non-respiratory protein associated with hemocyanin? The problem is even more acute in the egg hemocyanins where the protein dissociates and runs as 4 separate protein bands in SDS as opposed to the single band observed for adult mid-band protein. The solution to this problem awaits successful separation of 16S hemocyanin from this additional unknown protein.

The second outstanding fact to emerge from this study is that the putative hemocyanins contained in the eggs and zoea of Hemigrapsus nudus differ from those of the adult. This conclusion is based on a comparative study made between the developmental and adult hemocyanin subunit compositions.

In the first part of this thesis it was demonstrated that the hemocyanin molecules of adult H. nudus were composed of 5 subunit types and that the 16S hexamer lacked a subunit that was present in the 25S 2-hexamer. Different subunit types also exist in the egg hemocyanins, but their numbers and relative concentrations differ from those of the adult. For instance, a most obvious

difference is the absence of band A in the egg preparations. In adult hemocyanins, band A is a relatively strong staining protein band which electrophoreses to position 1 in SDS. The fact that purified 16S and 25S egg hemocyanins, when cut from pH 7.0 regular gels (excluding mid-band) are also missing band 1, demonstrates further that this particular subunit is not present in the egg hemocyanins. Another difference observed between the adult and egg hemocyanins is found in band B. If this protein band is cut from an adult sample and run in SDS it separates into two distinct subunits, 3 and 5. Conversely band B of egg hemocyanin runs to position 3 only. In other words egg band B represents a single unique subunit (instead of two); this fact probably accounts for the lowered relative staining intensity of band B observed in the egg preparations. Finally, as mentioned, adult mid-band protein, present in 16S samples only, electrophoreses to position 2 in SDS. Mid-band protein from egg 16S electrophoreses as 4 bands in SDS. Not only does it resolve at position 2 but to a position roughly equivalent to hemocyanin position 1 and two new positions, 6 and 7. Since it is unlikely that mid-band protein represents a hemocyanin, it will not be considered as a contributor to subunit heterogeneity in the egg hemocyanins.

In the first part of this thesis it was demonstrated

that the 25S 2- hexamer molecule of the adult hemocyanin contained a subunit that was not present in the 16S single hexamer fraction. The possible role of this subunit as a hexamer-hexamer linker was discussed. The same phenomenon is also observed in the egg hemocyanins. That is, band D is present in the 25S hemocyanin of the eggs and missing from the egg 16S fraction. But the two molecular populations are the same in every other respect.

This finding ties in nicely with the ideas of Markl and Kempter (1981a, 1981b) regarding variable and conservative subunits. As defined, variable subunits have specific conformations and as such probably specific roles in hexamer assembly. In the case of Hemigrapsus nudus, perhaps the subunits which appear and disappear throughout ontogeny are the "variable subunits". These are the components whose specific functions come and go as the functional demands of the animal are altered. Conversely, the conservative subunits are non-specific in their conformation, they vary little from crustacean species to species. This suggests that they have not differentiated much during evolution. Markl and Kempter suggest that these conservative subunits are involved in the formation of double hexamers. Perhaps the subunit represented as band D in both the egg and adult 25S hemocyanins of Hemigrapsus nudus is a "conservative subunit". If this

is true, then this study shows that not only has the subunit been conserved through evolution but throughout ontogeny as well.

In summary, the hemocyanin observed in the eggs of Hemigrapsus nudus represents a simplified version of the adult hemocyanin. It appears that the egg 16S hexamer is composed of two different subunit types and the 25S 2-hexamer consists of three subunit types; the third possibly acting as a hexamer-hexamer link. The functional and physiological implications of this simplification are not known. Perhaps it reflects the less demanding needs of a developing embryo for oxygen. Or maybe the expression of only a portion of the hemocyanin genome has been accelerated at the egg stage and is awaiting some physiological or environmental stimulus to complete its expression.

The answer to these questions and a cascade of others have only recently been addressed. But judging from what little has been learned, the study of ontogenic changes in hemocyanins promises to be an exciting new area of research.

BIBLIOGRAPHY

Antonini, E. and E. Chiancone (1977). Assembly of multi-subunit respiratory proteins. Ann. Rev. Biophys. Bioeng. 6, 239-271.

Arisaka, F. and K.E. van Holde (1979). Allosteric properties and the association equilibria of hemocyanin from Callianassa californiensis. J. Mol. Biol. 134, 41-73.

Bannister, J.V. (Ed.) (1977). Structure and Function of Haemocyanin. Berlin: Springer-Verlag. 295 pp.

Bonaventura, J., C. Bonaventura, and B. Sullivan (1977). Non-heme oxygen transport proteins. In "Oxygen and Physiological Function (Jobsis, F., ed). pp. 177-220, Professional Information Library, Dallas, Texas.

Bonaventura, J. and C. Bonaventura (1980a). Hemocyanins - Relationships in their structure, function and assembly. Am. Zool. 20, 7-17.

Brenowitz, M., C. Bonaventura and J. Bonaventura, and E. Gianazza (1981). Subunit composition of a high molecular weight oligomer: Limulus polyphemus hemocyanin. Arch. Biochem. Biophys. (In press).

Brouwer, M. and H.A. Kuiper (1973). Molecular weight analysis of Helix pomatia α-hemocyanin in guanidine hydrochloride, urea, and sodium dodecyl sulfate. Eur. J. Biochem. 35, 428-435.

Brouwer, M., M. Wolters and E.F.J. van Bruggen (1976). Proteolytic fragmentation of Helix pomatia α-hemocyanin: Structural domains in the polypeptide chain. Biochemistry 15, 2618-2623.

Bruyninckx, W.J., S. Gutteridge and H.S. Mason (1978). Detection of copper on polyacrylamide gels. Analyt. Biochem. 89, 174-177.

Cleveland, D.W., S.G. Fischer, M.W. Kirschner and U.K. Laemmli (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252, 1102-1106.

Davis, B.J. (1964) Disc gel electrophoresis - II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. 121, 404-427.

Decleir, W. and A. Richard (1970). A study of the blood proteins in Sepia officinalis L. with special reference to embryonic hemocyanin. Comp. Biochem. Physiol. 34, 203-211.

Decleir, W., J. Lemaire, and A. Richard (1971). The differentiation of blood proteins during ontogeny in Sepia officinalis L. Comp. Biochem. Physiol. 40B, 923-930.

Ellerton, H.D., D.E. Carpenter and K.E. van Holde (1970). Physical studies of hemocyanins. V. Characterization and subunit structure of the hemocyanin of Cancer magister. Biochemistry 9, 2225-2232.

Eriksson-Quensel, I.-B. and T. Svedberg (1936). The molecular weights and pH stability regions of the hemocyanins. Biol. Bull. 71, 498-547.

Fairbanks, G., T.L. Steck, and D.F.H. Wallach (1971). Electrophoretic analysis of the human erythrocyte membrane. Biochemistry 10, 2606-2616.

Fredericq, M.L. (1878). Sur l'hémocyanine, substance nouvelle du sang de Poulpe (Octopus vulgaris). C.R. Acad. Sci. (Paris) 87, 996-998.

Freedman, T.B., J.S. Loehr and T.M. Loehr (1976). A resonance Raman study of the copper protein hemocyanin. New evidence for the structure of the oxygen-binding site. J. Am. Chem. Soc. 98, 2809-2815.

Ghiretti-Magaldi, A., G. Tamino and B. Salvato (1975). The monophyletic origin of hemocyanins on the basis of the amino acid composition. Structural implications. Boll. Zool. 42, 167-179.

Herskovits, T.T., L.J. Erhunmwunsee, R.C. San George and A. Herp (1981a). Subunit structure and dissociation of Callinectes sapidus hemocyanin. Biochim. Biophys. Acta 667, 44-58.

Herskovits, T.T., R.C. San George and L.J. Erhunmwunsee (1981b). Light scattering investigation of the subunit dissociation of Homarus americanus hemocyanin. Effects of salts and ureas. Biochemistry 20, 2580-2587.

Jeffrey, P.D., D.C. Shaw and G.B. Treacy (1978). Hemocyanin from the Australian freshwater crayfish Cherax destructor. Characterization of a dimeric subunit and its involvement in the formation of the 25S component. Biochemistry 17, 3078-3084.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227, 680-685.

Lamy, J., M.C. Bijholt, P.-Y. Sizaret, J. Lamy and E.F.J. van Bruggen (1981a). Quaternary structure of scorpion (Androctonus australis) hemocyanin; Localization of subunits with immunological methods and electron microscopy. Biochemistry 20, 1849-1856.

Larson, B.A., N.B. Terwilliger and R.C. Terwilliger (1981). Subunit heterogeneity of Cancer magister hemocyanin. Biochim. Biophys. Acta (In press).

Loehr, J.S., T.B. Freedman and T.M. Loehr (1974). Oxygen binding to hemocyanin: A resonance Raman spectroscopic study. Biochem. Biophys. Res. Commun. 56, 510-515.

Lontie, R. and R. Witters (1973). Hemocyanin. In: Inorganic Biochemistry. G.L. Eichorn, Ed. Amsterdam: Elsevier. pp. 344-358.

Lontie, R., M. deLey, H. Robberecht and R. Witters (1973). Isolation of small functional subunits of Helix pomatia haemocyanin after subtilisin treatment. Nature New Biol. 242, 180-182.

Mangum, C.P. (1980). Respiratory function of the hemocyanins. Amer. Zool. 20, 19-38.

Mangum, C.P. (1981a). Oxygen transport in the blood. In: Biology of Crustacea Vol. II. L.H. Mantel, Ed. New York: Academic Press. (In press).

Markl, J., A. Markl, W. Schartau and B. Linzen (1979a). Subunit heterogeneity in arthropod hemocyanins. I. Chelicerata. J. Comp. Physiol. 130, 283-292.

Markl, J., A. Hofer, G. Bauer, A. Markl, B. Kempter, M. Brenzinger and B. Linzen (1979b). Subunit heterogeneity in arthropod hemocyanins. II. Crustacea. J. Comp. Physiol. 133, 167-175.

Markl, J., H. Decker, W. Stocker, A. Savel, B. Linzen, W.G. Schutter and E.F.J. van Bruggen (1981). On the role of dimeric subunits in the quaternary structure of arthropod hemocyanins. Hoppe-Seylers Z. Physiol. Chem. 362, 185-188.

Markl, J. and B. Kempter (1981a). Subunit heterogeneity in arthropod hemocyanins. In: Invertebrate Oxygen Binding Proteins: Structure, Active Site, and Function. J. Lamy and J. Lamy, Eds. New York: Marcel Dekker. pp. 125-137.

Markl, J. and B. Kempter (1981b). Subunit heterogeneity in crustacean hemocyanins as deduced by two-dimensional immunoelectrophoresis. J. Comp. Physiol. 141, 495-502.

Morimoto, K. and G. Kegeles (1971). Subunit interactions of lobster hemocyanin. I. Ultracentrifuge studies. Arch. Biochem. Biophys. 142, 247-257.

Orgel, L.E. (1958). Enzyme-metal-substrate complexes as co-ordination compounds. Biochem. Soc. Symp. 15, 8-20.

Rajulu, G.S. (1969). Presence of haemocyanin in the blood of a centipede Scutigera longicornis (Chilopoda: Myriapoda). Curr. Sci. 38, 168-169.

Roxby, R., K.I. Miller, D. Blair and K.E. van Holde (1974). Subunits and association equilibria of Callianassa californiensis hemocyanin. Biochemistry 13, 1662-1668.

Sullivan, B., J. Bonaventura and C. Bonaventura (1974). Functional differences in the multiple hemocyanins of the horseshoe crab, Limulus polyphemus. Proc. Natl. Acad. Sci. USA 71, 2558-2562.

Terwilliger, N.B. and R.C. Terwilliger (1980). Developmental changes in Cancer magister hemocyanin. (Abstract). Amer. Zool. 20, 907.

Terwilliger, N. (1982). Effect of subunit composition on quaternary structure of isopod (Ligia pallasii) hemocyanin. Biochemistry (In press).

Terwilliger, N.B. and R.C. Terwilliger (1982). Changes in the subunit structure of Cancer magister hemocyanin during larval development. J. Exp. Zool. (In press).

van Holde, K.E. and E.F.J. van Bruggen (1971). The Hemocyanins. In: Biological Macromolecules Vol 5, Pt A. S.N. Timasheff and G.D. Fasman, Eds. New York: Marcel Dekker. pp. 1-55.

van Holde, K.E. and K.I. Miller (1982). The Hemocyanins. (In press).

Waxman, L. (1975). The structure of arthropod and mollusc hemocyanins. J. Biol. Chem. 250, 3796-3801.

Wood, E.J. (1980). The oxygen transport and storage proteins of invertebrates. Essays in Biochemistry 16, 1-47.

Lilah L. Chambers