

SNBUNIT STRUCTURE OF CANCER PRODUCTUS  
HEMOCYANIN DURING EARLY DEVELOPMENT

by

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A THESIS

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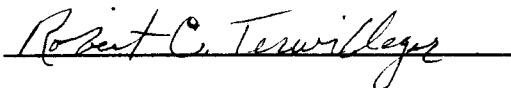
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## An Abstract of the Thesis of

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Title: SUBUNIT STRUCTURE OF CANCER PRODUCTUS HEMOCYANIN DURING EARLY  
DEVELOPMENT

Approved:



Robert C. Terwilliger

The structure of hemocyanin from Cancer productus adults, eggs, and embryos was studied. Hemocyanin from all three sources is present in two molecular weight fractions, 940,000 and 480,000, as determined by gel chromatography. Hemocyanin subunit structure was studied by using various gel electrophoresis systems. Hemocyanins from the three sources consist of four common subunits with molecular weights ranging between 81,000 and 73,000. The number and relative concentrations of subunits from egg hemocyanin are indistinguishable from those of maternal hemocyanin. Embryonic hemocyanin has two additional subunits on SDS with approximate molecular weights of 69,000 and 66,000. It can also be clearly distinguished from maternal hemocyanin by the peptide maps of hemocyanin subunits from SDS gels. Three hypotheses explaining the difference in molecular weight between adult and embryonic hemocyanin subunits are discussed: 1) difference in amino acid sequence involving neutral amino acids, 2) degree of glycosylation, and 3) proteolysis.

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## INTRODUCTION

The hemocyanin of Cancer magister, a brachyuran decapod closely related to Cancer productus, has been intensely studied over the past 20 years. Electron micrographs of purified hemocyanin show a characteristic two-hexamer structure for the 25S hemocyanin molecule and a one-hexamer structure characteristic of the 16S molecule (Van Bruggen et al., 1981; Ellerton et al., 1970). The hemocyanin dissociates into its subunits at high pH and dissociation is first observed at pH 8.9. When C. magister hemocyanin is submitted to a denaturing agent six subunits can be resolved (Larson et al., 1981). The number of subunits varies from species to species among the crustacean hemocyanins, though they always portray the typical hexameric structure in electron micrographs and often exist as multiples of hexamers (Van Holde and Miller, 1982).

An ontogenetic study of hemocyanin subunit structure was done with C. magister, Cancridae (Terwilliger and Terwilliger, 1982) and Hemigrapsus nudus, Grapsidae (Larson, 1982). C. magister megalops and first instar juvenile hemocyanins electrophorese with one unique subunit missing in the adult. In addition, one of the subunits characterizing adult hemocyanin is missing in megalops and first instar juvenile. The embryos of H. nudus differ in their hemocyanin subunit structure from the adult crabs by missing one subunit present in the adult as well as having two additional subunits the adult lacks. The investigation of Hemigrapsus zoea indicated a hemocyanin subunit pattern different from

the adult as well.

The overall purpose of this thesis is to complete the study of hemocyanin subunit structure during the larval development of brachyuran crabs with an investigation of the unfertilized eggs and embryos of C. productus, Cancridae. It would be especially interesting to follow up with O<sub>2</sub> binding studies to see whether these structurally different, yet ontogenetically related hemocyanins bind oxygen cooperatively, show a Bohr effect, and exhibit O<sub>2</sub> affinities specific for each stage. O<sub>2</sub> binding properties of juvenile C. magister hemocyanin have already been under investigation (Terwilliger et al., 1982) showing that the O<sub>2</sub> affinity of larval hemocyanin is lower than that of adult hemocyanin. To my knowledge these are the only studies on hemocyanins of decapod crustaceans focusing on the structure and function of hemocyanin during ontogeny.

A comparable ontogenetic study involving the structure and function of an oxygen carrying protein was done with chick embryo hemoglobin (Manwell et al., 1966). Studies focusing on structural aspects alone were done with the various larval hemoglobins of the insect Chironomus (Manwell, 1966; Wulker et al., 1969; Vafopoulou-Mandalos and Laufer, 1982), the three types of hemoglobin of the brine shrimp Artemia (Bowen et al., 1976; Heip et al., 1978), and the hemocyanin of the embryos, young and adult animals of the mollusc Sepia (Decleir et al., 1971).

This study first focuses on the description of adult C. productus native hemocyanin to confirm the presence of 25S and 16S molecules using C. magister hemocyanin for calibration. Hemocyanin subunit structure is studied at pH 8.9 and using denaturing agents. To demonstrate the

uniqueness of each hemocyanin subunit peptide maps are run. In the second part of the thesis the details of egg and embryo hemocyanin structures are described in comparison to the corresponding maternal hemocyanins.

## METHODS AND MATERIALS

Purification of the Hemocyanin

Animals were collected at Coos Bay, Oregon. 1.5-2 ml of hemolymph were taken with a syringe from the legjoints of adult crabs and stored for 30 min. on ice to allow clotting. The sample was then centrifuged for 12 min. at 10,000 g. The supernatant was fractionated at 4°C on a BioGel A-5M column with a buffer: 0.1 ionic strength Tris-HCl at pH 7.2 in the presence of 0.1 M NaCl, 0.05 M MgCl<sub>2</sub>, and 0.01 M CaCl<sub>2</sub>. The column was calibrated with Blue Dextran, spermwhale met myoglobin, and C. magister hemocyanin. For a description of whole hemolymph the hemolymph was centrifuged as described above and the supernatant was electrophoresed without prior fractionation on BioGel A-5M.

When ovaries were dissected from adult crabs care was taken that no neighboring maternal tissue was cut. Ovarian tissue and unfertilized eggs (mature oocytes) were teased apart and the egg mass was rinsed many times with freshly filtered seawater before it was worked on further. Fertilized eggs (embryos), 4-6 weeks old, could be separated from the pleopods by cutting the pleopod hairs. Care was taken that the pleopods were not cut. These procedures were carried out on ice. A column buffer solution containing 1.0 mM PMSF (phenylmethylsulfonyl fluoride) was added to egg and embryo preparation before homogenization. The homogenate was first fractionated on a G-100 Sephadex gel column and concentrated by vacuum dialysis prior to further fractionation on BioGel A-5M. Column buffer and temperature conditions were the same as with hemolymph

preparations.

Molecular weights of C. productus hemocyanin subunits were estimated by comparison with C. magister hemocyanin subunits on SDS (sodium dodecyl sulfate) gels. In order of decreasing size the apparent molecular weights of C. magister subunits are: 81,800  $\pm$  800, 77,200  $\pm$  700, 75,100  $\pm$  600, 71,900  $\pm$  800, 69,000  $\pm$  600, and 67,300  $\pm$  900 (Larson et al., 1981).

The description of maternal and egg hemocyanin is based on three egg/mother and two egg/no mother samples. Comparison of maternal and embryonic hemocyanins was done for two embryo/mother collections.

#### Electrophoresis

Hemocyanin samples were electrophoresed in a regular PAGE (polyacrylamide gel electrophoresis) system with the gel dimensions 11 x 15 x 1.5 cm at pH 7 and pH 8.9 as modified from Davis (1964). The pH 7 gels were predominantly 5% acrylamide concentration. The pH 8.9 regular slab gels were run in the presence or absence of 0.024 M EDTA (ethylenediaminetetraacetic acid) and were 7.5% acrylamide concentration. The marker dye was 0.05% bromophenol blue. The electrophoresis buffer systems were as follows: pH 7 gels, upper electrode buffer 0.05 M in maleate-NaOH, pH 7, and lower electrode buffer 0.05 M in HCl, pH 6.4, both titrated to the desired pH with 1 M Tris; pH 8.9 gels, upper electrode buffer 0.05 M in Tris-glycine, pH 8.9, and lower electrode buffer 0.05 M in Tris-HCl, pH 8.1. Regular gels (pH 7, pH 8.9) were run at constant current (35 ma/gel). pH 7 gels were electrophoresed for 3 h, pH 8.9 gels for 1.5 h. Staining was done with Coomassie brilliant blue R after

Fairbanks et al. (1971). Gels were destained in 10% acetic acid.

SDS slab gels were run in a discontinuous system (Laemmli, 1970). Hemocyanin samples had a protein concentration of about 1-2 mg/ml. Samples were mixed 1:1 with incubation buffer containing 2% SDS, 10% glycerin, 1 mM EDTA, 0.01% bromophenol blue, 62.5 mM Tris-HCl, 5% 2-mercaptoethanol or 50 mM DTT (dithiothreitol), and 1 mM PMSF. The samples were heated for 1.5 min. in a boiling water bath and stored at  $-20^{\circ}\text{C}$ . All buffer systems contained 1 mM EDTA. SDS slab gels, 7.5% acrylamide concentration, were electrophoresed at constant voltage (100V) for 3-6 h. The gels were stained and destained as described above.

#### Peptide Mapping

The peptide mapping procedure is essentially that described in Cleveland et al. (1977). The hemocyanin sample was first electrophoresed either in a pH 8.9 regular gel, 7.5% acrylamide concentration, or on a SDS slab gel, 7.5% acrylamide concentration. Sample wells were loaded with as much as 50  $\mu\text{g}$  of protein and electrophoresis time was increased to about 6 h. Gels were then stained for 20 min. in a solution containing 0.1% Coomassie brilliant blue R, 50% methanol, and 10% acetic acid and destained in 5% methanol and 10% acetic acid. Bands corresponding to hemocyanin subunits were cut and the slices stored in SDS stacking gel buffer at  $-20^{\circ}\text{C}$ . Peptide maps, 15% acrylamide concentration, were obtained by overlaying the slices in the sample wells with 10  $\mu\text{l}$  of a solution containing 20% glycerin and 50% stacking gel buffer and a second layer of 10  $\mu\text{l}$  of a solution consisting of 10% glycerin, 20% stacking gel buffer, and 50% enzyme solution ( 0.6  $\mu\text{g}/\mu\text{l}$  of

Staphylococcus aureus V8 proteinase - Miles Laboratories, U.K.). Gels were electrophoresed at a constant voltage (100V) until the Coomassie blue dye front reached the bottom of the 4.5 cm wide stacking gel. The voltage was then turned off for 35 min., sufficient time to allow partial digestion of the hemocyanin. Electrophoresis was resumed at 100V until the dye front reached the bottom of the separating gel. All buffer systems contained 1 mM EDTA. The gels were stained with Coomassie brilliant blue R as described above.

#### Electron Microscopy

Purified 940,000 and 480,000 molecular weight fractions were negatively stained for electron microscopy. Formvar grids were carbon-stabilized and made hydrophilic by glow discharge before a drop of the hemocyanin sample was added to the grid. The sample was blotted off after 30-60 sec, but not completely. Then a drop of 1-2% potassium phosphotungstate stain was added which was removed after 30 sec. The negatively stained hemocyanin was then observed in the Philips 300 electron microscope at 60 and 80 KV.

#### Detection of Copper on Gels

The detection of copper on gels is based on the quenching of fluorescence of bathocuproine sulfonate by copper. Protein bands containing copper can be made visible in ultraviolet light (Bruyninckx et al., 1978). To detect whether copper is present at all and to identify the subunit to which copper is bound, pH 7 and pH 8.9 regular slab gels were electrophoresed in the absence of EDTA and without denaturing

and reducing agents. After electrophoresis the gels were washed in a 16 mM ascorbate-glacial acetic acid bath for 1 min. and in a 0.28 mM bathocuproine sulfonate solution for another minute. After a 10 min. exposure to UV light, only the protein bands containing copper, as low as 1 nmol, appeared dark and were marked with India ink. The gel was then stained and destained with Coomassie blue as described above.



## RESULTS

Hemocyanin of Female and Male Adult Cancer Productus

When whole hemolymph of adult C. productus is fractionated on a BioGel A-5M column, two major peaks are resolved (Fig.1). The relative average molecular weight of peak I is 940,000 and of peak II is 480,000. The ratio of the absorbance measured at 280 nm to that at 340 nm is 6:1 for peak I. The ratio can be 10:1 to 13:1 for peak II, predominantly in the later elution fractions of peak II. The high absorbance ratios for peak II have been noted for both female and male adult crabs. While the 280/340 nm absorbance ratio for peak I is fairly constant, ratio values for peak II vary noticeably during the year.

When whole hemolymph is electrophoresed on a pH 7 regular (no SDS) slab gel, two major dark staining fractions are resolved (Fig.2, A-E). These two bands have electrophoretic mobilities similar to those seen in C. magister hemocyanin (Terwilliger and Terwilliger, 1982), corresponding to 25S and 16S molecules. In some adults, male or female, a third band, designated X, electrophoreses just behind the 16S fraction (Fig.2, D,E). Two lightly staining bands electrophorese behind the 25S fraction. The relative staining intensities of the 25S, X, and 16S bands are not the same from animal to animal (Fig.2, A-E). When the gels are stained for copper, 25S and 16S bands as well as band X react positively. No reaction could be detected in the top two bands.

When fraction I is run on a pH 7 gel it electrophoreses as 25S band

Figure 1. Chromatography of hemolymph from C. productus adult on a column of BioGel A-5M (200-400 mesh). Column volume 1.8 x 95 cm. Buffer 0.1 ionic strength Tris HCl (pH 7.2), 0.1 M in NaCl, 0.05 M in MgCl<sub>2</sub>, and 0.01 M in CaCl<sub>2</sub>. Absorbance at 280 (▲) and 340 (●) nm.

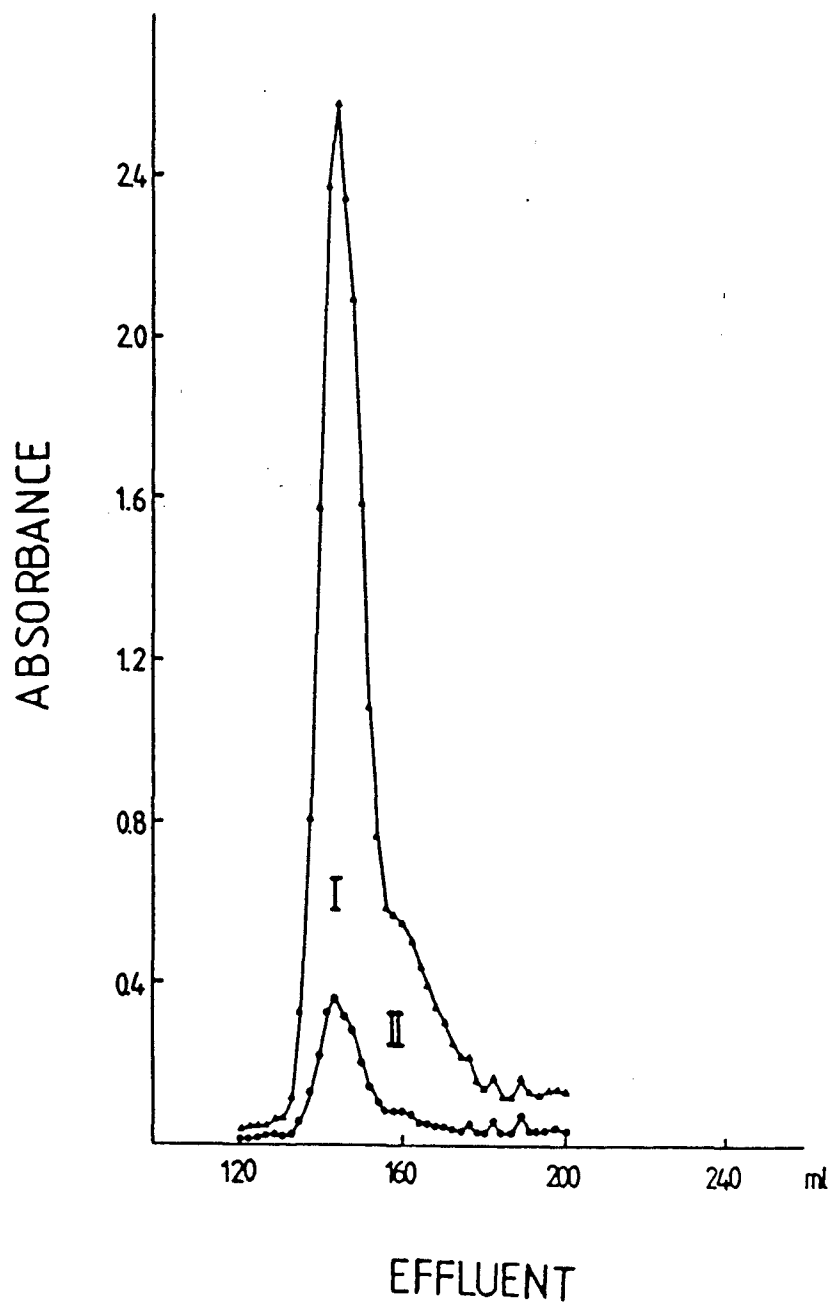
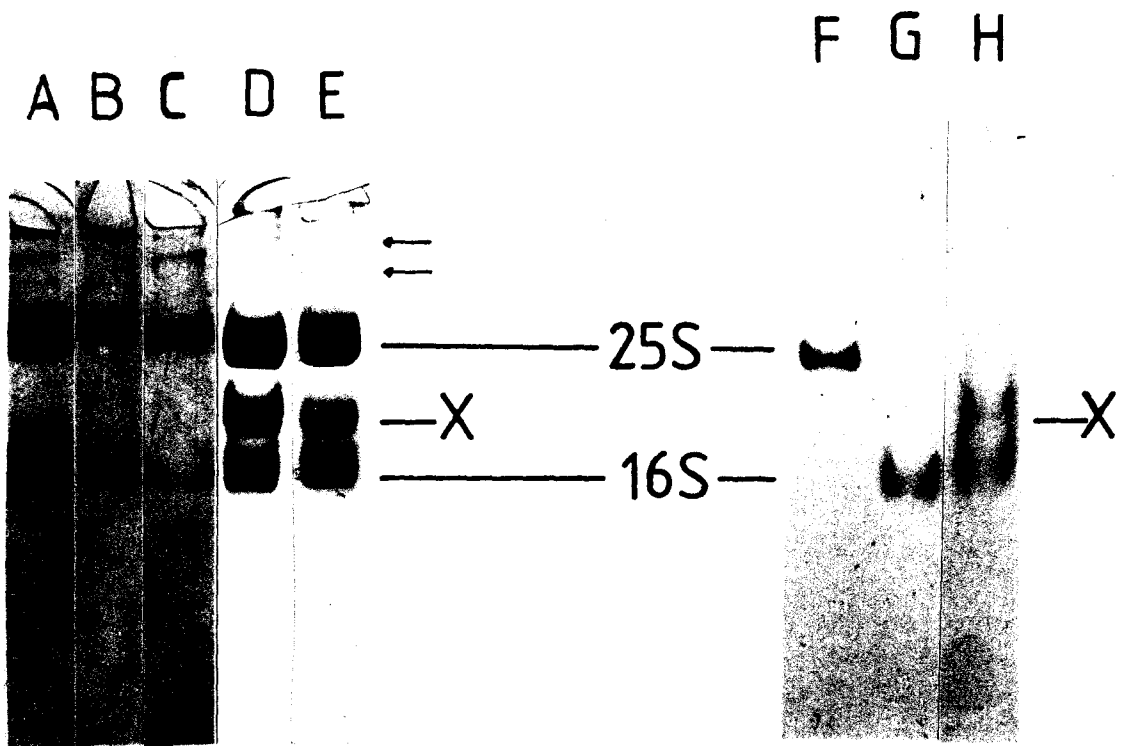


Figure 2. Regular gel electrophoresis at pH 7 on 5% acrylamide gel of whole hemolymph of adult C. productus (A-E) and on 4% acrylamide gel of purified adult 25S (F) and 16S hemo-cyanins (G,H).



and some low mobility bands behind it (Fig.2, F). Fraction II electrophoreses as a 16S band (Fig.2, G). In those animals whose hemocyanin contains band X, it is present in fraction II as a more slowly migrating band behind the 16S band (Fig.2, H). The hemocyanins of males and females were indistinguishable from one another by the above techniques.

The presence of bands in regions of the gel where hemocyanin components are usually absent led us to investigate the possibility of non-hemocyanin molecules co-chromatographing on BioGel A-5M with hemocyanin. The A-5M column results were electrophoretically scanned on pH 7 and 8.9 non-denaturing gels as well as on SDS gels (Fig.3) by sampling aliquots from fractions in the vicinity of peaks I and II. The results confirmed the presence of other molecules besides hemocyanin in peaks I and II. Therefore, a two-step purification scheme was used for further analysis of the 25S hemocyanin. After chromatography on BioGel A-5M, peak I was electrophoresed on a pH 7 gel. The band corresponding to 25S hemocyanin was then re-electrophoresed on SDS gel.

SDS slab gel electrophoresis of different samples of adult 25S hemocyanin (from A-5M) consistently shows four bands, labeled I-IV. The staining intensities of I and IV are always strong, those of II and III differ from animal to animal (Fig.4, A,B,). Two additional protein bands are observed in the high molecular weight region of the separating gel (Fig.4, A). These bands are not present in the doubly-purified 25S hemocyanin samples (Fig.4, B). The apparent molecular weights of subunits I-IV of C. productus adult 25S fraction are:  $80,600 \pm 500$ ,  $78,500 \pm 500$ ,  $77,200 \pm 300$ , and  $73,500 \pm 300$  (S.D. of five determinations).

Figure 3. Electrophoretic scan of adult fractionated hemolymph by sampling aliquots of fractions right and left of peaks I and II. Regular gel electrophoresis at pH 7 on 5% acrylamide gels (top), regular gel electrophoresis at pH 8.9 on 7.5% acrylamide gels (middle), SDS gel electrophoresis on 7.5% acrylamide gels (bottom).

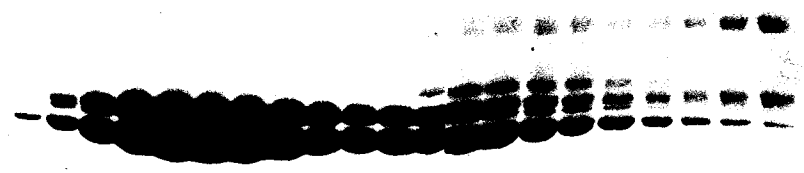
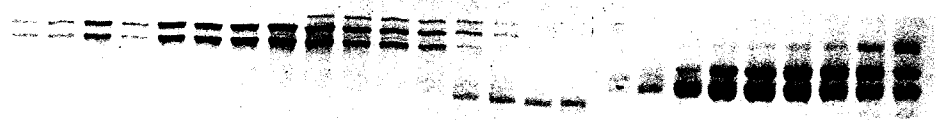
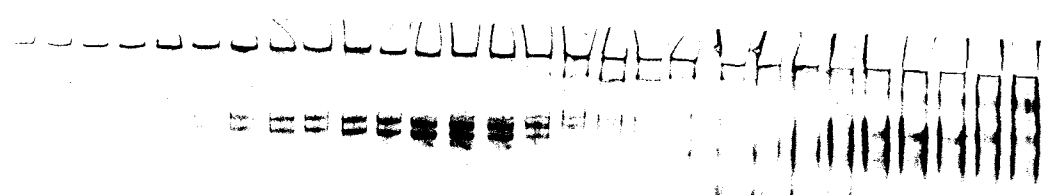
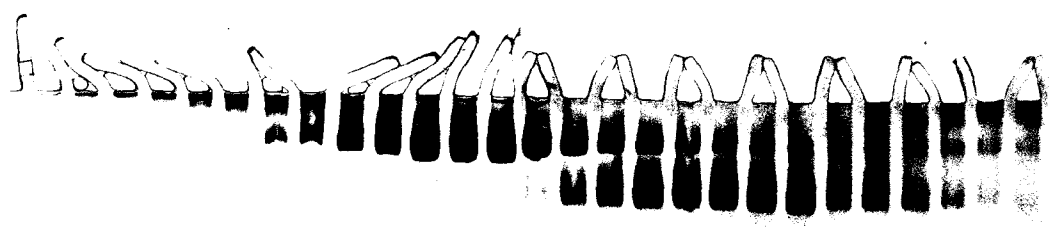
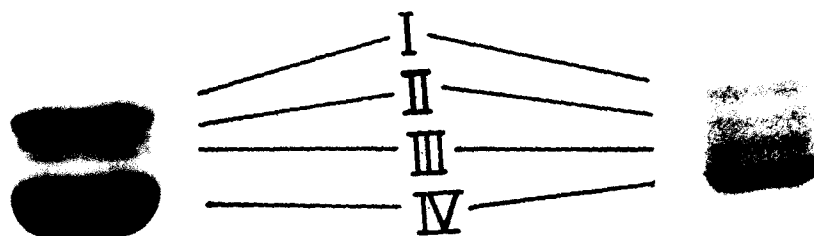




Figure 4. SDS gel electrophoresis of two adult 25S hemocyanins on 7.5% acrylamide gels (A,B). Purified 25S hemocyanin (A), doubly-purified 25S hemocyanin (B).

A

B



Different electrophoretic banding patterns are observed between hemocyanins from different animals regardless of sex when whole hemolymph (Fig.5, A-D) or 25S hemocyanin (Fig.5, E) are electrophoresed at pH 8.9. Four major bands, separated by charge, are always discernible in all animals studied: these are labeled p, q, r, and s. In many, but not all animals, bands q and r are doublets. In some animals band r electrophoreses as a triplet (Fig.5, E). All major bands (p, q, r, s) show fluorescence quenching which suggests that these bands contain copper and correspond to hemocyanin subunits. Besides the banding pattern corresponding to the hemocyanin subunits at the bottom of the pH 8.9 regular gel, other bands are observed. In the mid region several broad bands (Fig.5, arrows) show fluorescence quenching indicative of copper. Cross-referencing these mid region bands onto a SDS gel indicates that they are aggregates of subunits I-IV. In the upper region there are several bands. Cross-referencing of these upper bands onto SDS results in a trace of hemocyanin subunits and a high molecular weight non-hemocyanin component. The upper bands react positively to the quenching of fluorescence by copper.

When bands p-s from the pH 8.9 gel were individually re-electrophoresed on a SDS gel, the pattern in Fig.6 was obtained. Bands r and s consistently re-electrophoresed as shown. The relative concentrations of SDS subunits resulting from re-electrophoresis of bands p and q varied from one animal to another, and in one case, band p electrophoresed as SDS band III. Thus bands r and s are invariable while bands p and q show a variety of patterns.

Figure 5. Regular gel electrophoresis at pH 8.9 on 7.5% acrylamide gels of adult whole hemolymph (A-D) and purified adult 25S hemocyanin (E).

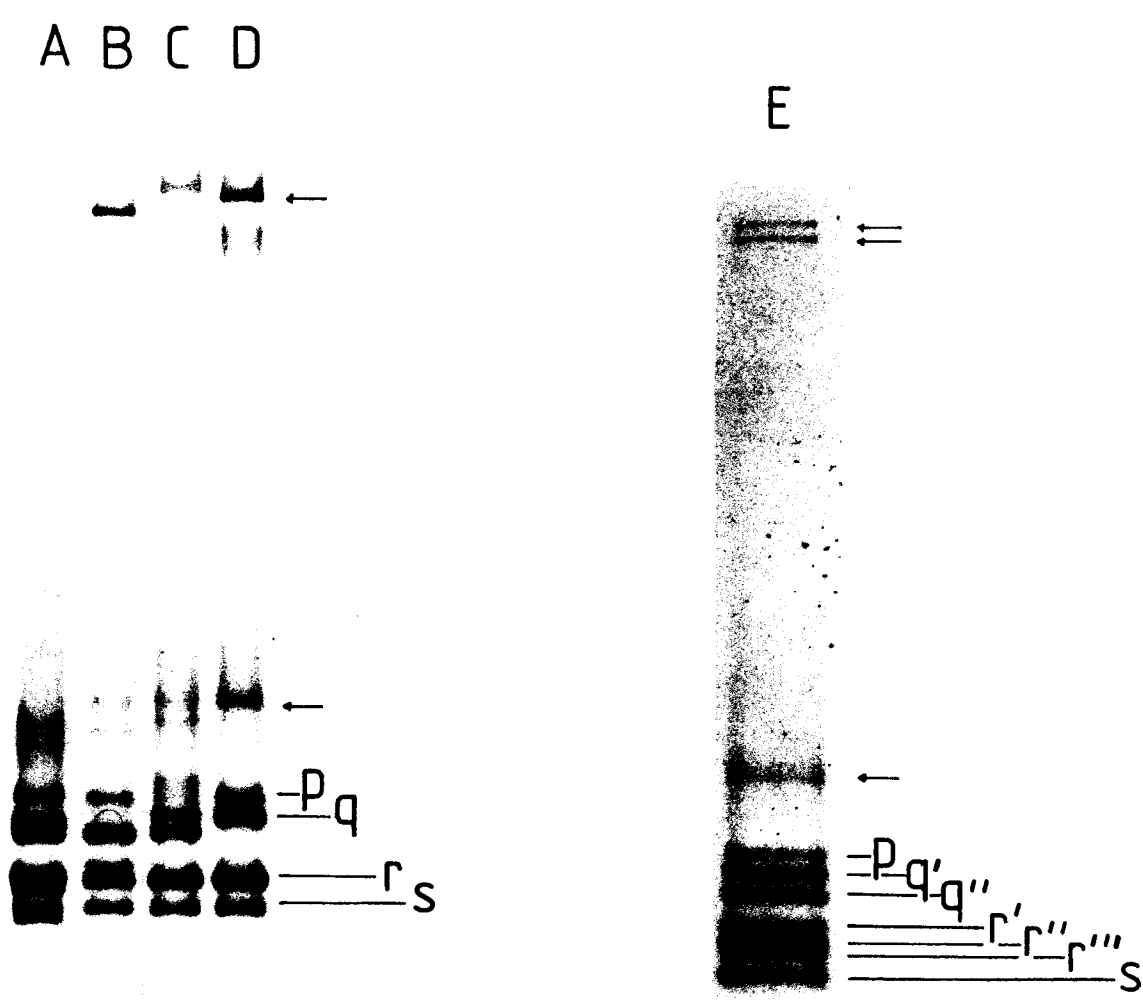
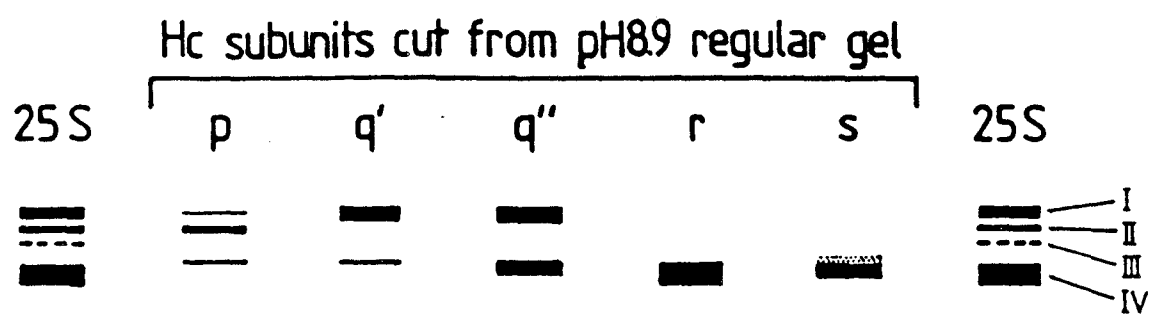


Figure 6. Two-step electrophoresis of C.productus adult 25S hemo-  
cyanin subunits from pH 8.9 regular gel to SDS gel.



### Hemocyanin of the Developmental Stages

Because adult C. productus hemocyanin from different animals gives varying banding patterns on pH 8.9 regular and SDS slab gels, it was necessary to compare hemocyanin structures of the 25S fractions from a mother animal and her unfertilized or fertilized eggs. Two developmental stages were analyzed: unfertilized eggs (referred to as "eggs") and fertilized eggs (referred to as "embryos").

#### Unfertilized Eggs

Eggs from all crabs studied contained hemocyanin. Analysis of BioGel A-5M fractions corresponding to adult fractions I and II indicated 940,000 and 480,000 molecular weight hemocyanin components respectively, as well as other proteins not present in the maternal blood (Fig.7). The 280/340 nm absorbance ratio for egg fraction I is similar to that of the adult hemocyanin. A valid ratio for egg fraction II could not be determined.

An electron micrograph of the egg sample after fractionation on a G-100 Sephadex gel column shows the typical two-hexamer and one-hexamer molecules known for other crustaceans (Van Bruggen et al., 1981; Van Holde and Miller, 1982) as well as other material (Fig.8). The 25S molecule appears as a hexagon whose corner touches the side of a square; the two opposite sides of the square appear fuzzy. The presence of predominantly two-hexamer molecules is another proof besides



Figure 7. Chromatography of hemolymph from the mother animal (top) and C. productus egg homogenate (bottom) on a column of BioGel A-5M (200-400 mesh). Column volume 1.8 x 95 cm. Buffer 0.1 ionic strength Tris HCl (pH 7.2), 0.1 M in NaCl, 0.05 M in MgCl<sub>2</sub>, and 0.01 M in CaCl<sub>2</sub>. Absorbance at 280 (▲) and 340 (●) nm.

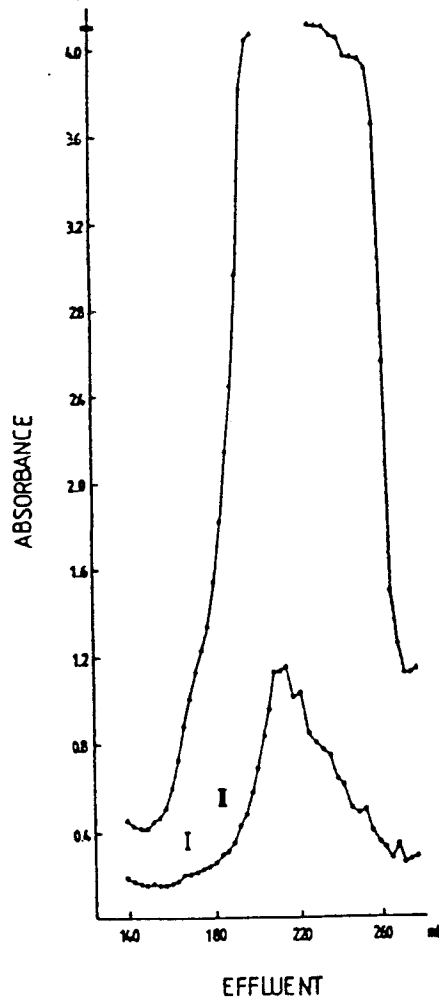
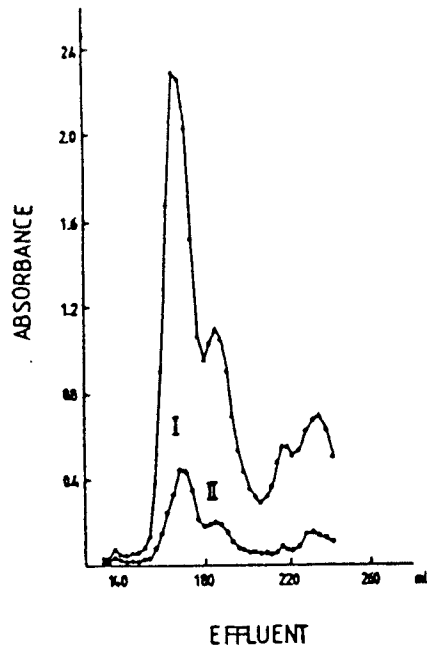
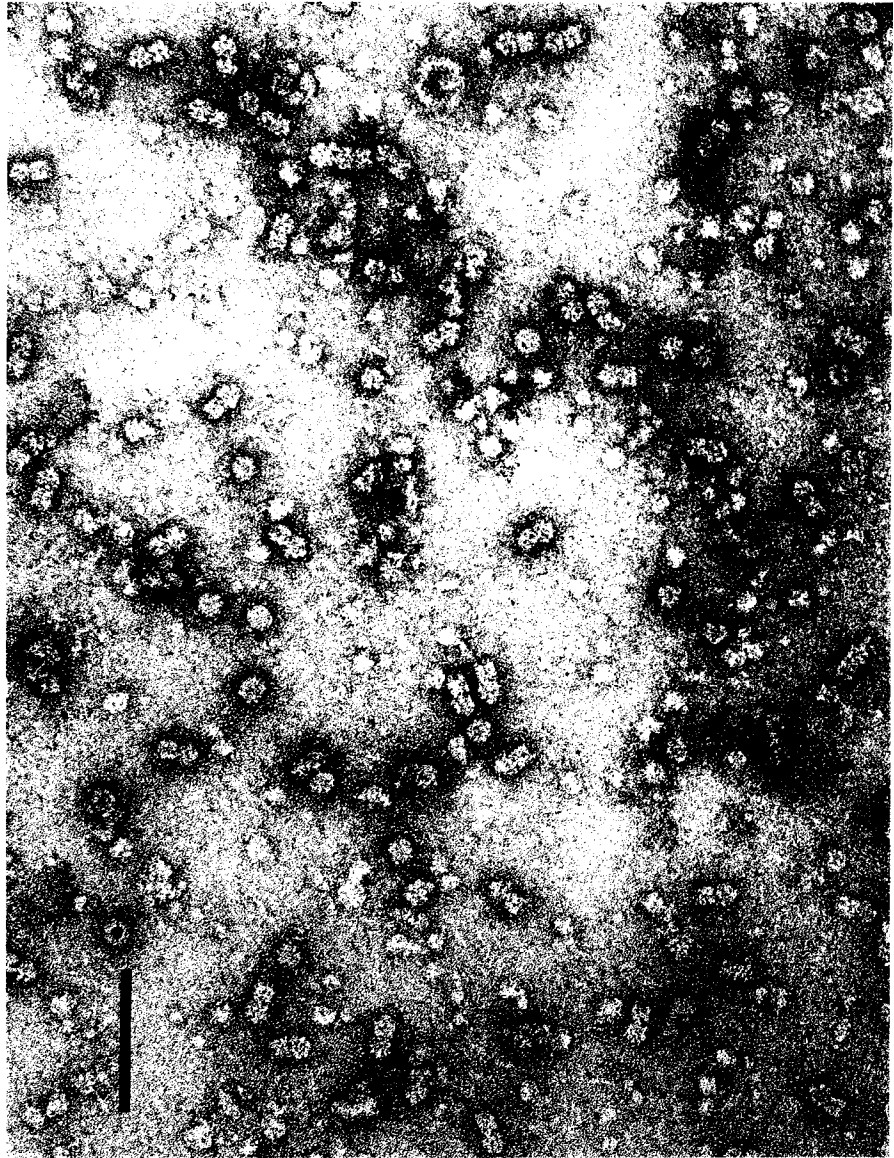


Figure 8. Electron micrograph (courtesy of Eric Schabtach) of C. productus egg hemocyanin negatively stained with potassium phosphotungstate. Magnification bar represents 100 nm.



chromatographic results that C. productus eggs contain hemocyanin.

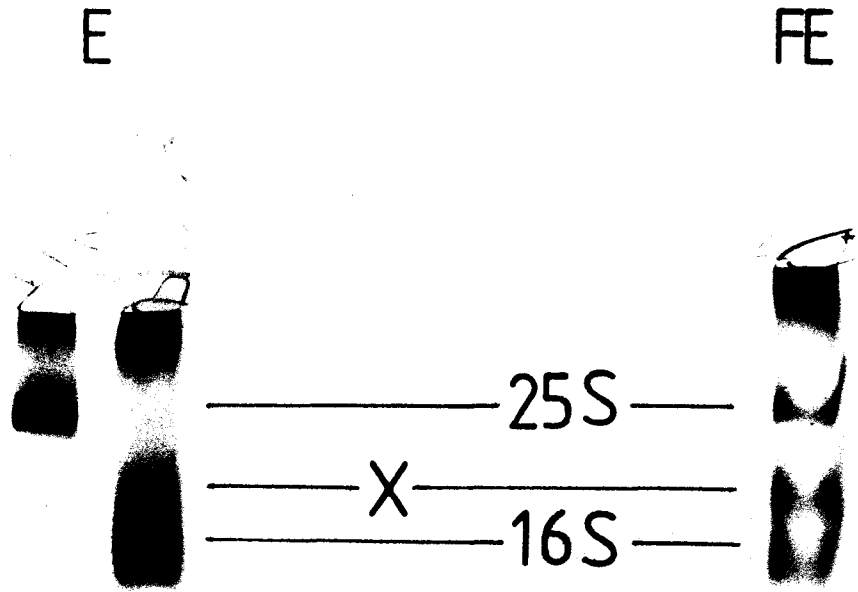
Comparison of adult and egg hemocyanin on a pH 7 regular gel is shown in Fig.9. Egg fraction I electrophoreses as 25S hemocyanin as well as some low mobility component which is not directly comparable to the low mobility bands behind the 25S band of adult hemocyanin (Fig. 9, A). Egg fraction II electrophoreses as band X and 16S hemocyanin as well as other proteins. When egg peak I is concentrated and then run on a pH 7 gel at the same loading volume as adult peak I, the 25S bands of each are indistinguishable (Fig9, B). The 25S hemocyanin fractions of egg and adult reacted positively to the quenching of fluorescence by copper.

The subunit pattern of the egg hemocyanin on a pH 8.9 gel is indistinguishable from that of the mother crab (Fig.10). Like the maternal hemocyanin, egg hemocyanin bands p, q, r, and s, plus the more slowly migrating midband, react positively for the presence of copper. The only distinction between maternal and egg hemocyanin is in the upper region of the gel. The high molecular weight non-hemocyanin components of the maternal blood, identified in the pH 8.9 gel as very slowly migrating bands, are not present in the egg sample. Instead, some slightly slower moving components are present in low concentration.

The 25S hemocyanin of the eggs and the maternal hemocyanin do not differ from each other on SDS gels either and emerge as subunits I-IV (Fig.11). Additional SDS bands present in the egg BioGel A-5M peak I samples are eliminated by further purification of the egg 25S protein on the pH 7 gel before electrophoresis on a SDS gel as was done for the

- Figure 9. Regular gel electrophoresis at pH 7 of C. productus egg, embryonic, and maternal hemocyanins on 5% acrylamide gels.
- A. Purified 25S and 16S fractions of C. productus eggs (E) and partially purified hemocyanin fraction of C. productus embryos (FE, fertilized egg) from a G-100 Sephadex gel column. Column volume 3 x 18 cm.
- B. Purified C. productus egg (E), embryonic (FE), maternal (M) 25S hemocyanins. Total protein concentration of maternal 25S hemocyanin two times that of the egg 25S sample. Total protein concentration of the embryonic 25S sample two times that of the maternal 25S sample.

A.



B.

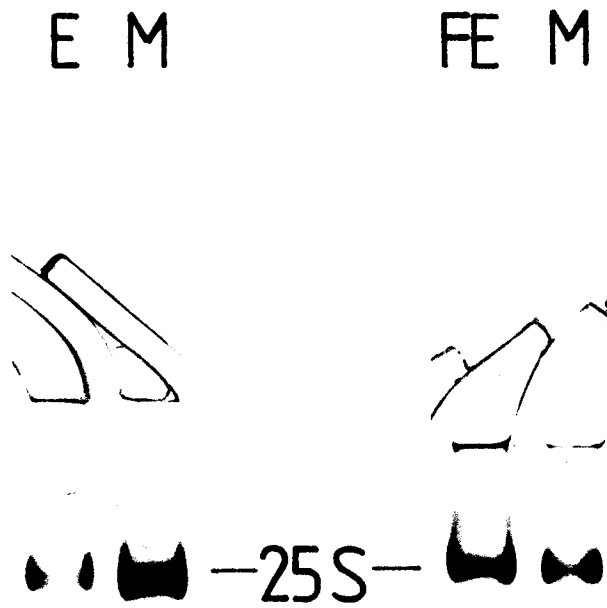



Figure 10. Regular gel electrophoresis at pH 8.9 of purified C. productus egg (E), embryonic (FE), and maternal (M) 25S hemocyanins on 7.5% acrylamide gels. Total protein concentration of egg 25S sample five times that of maternal 25S sample. Total protein concentration of the embryonic 25S sample eight times that of the corresponding maternal 25S sample.



M E



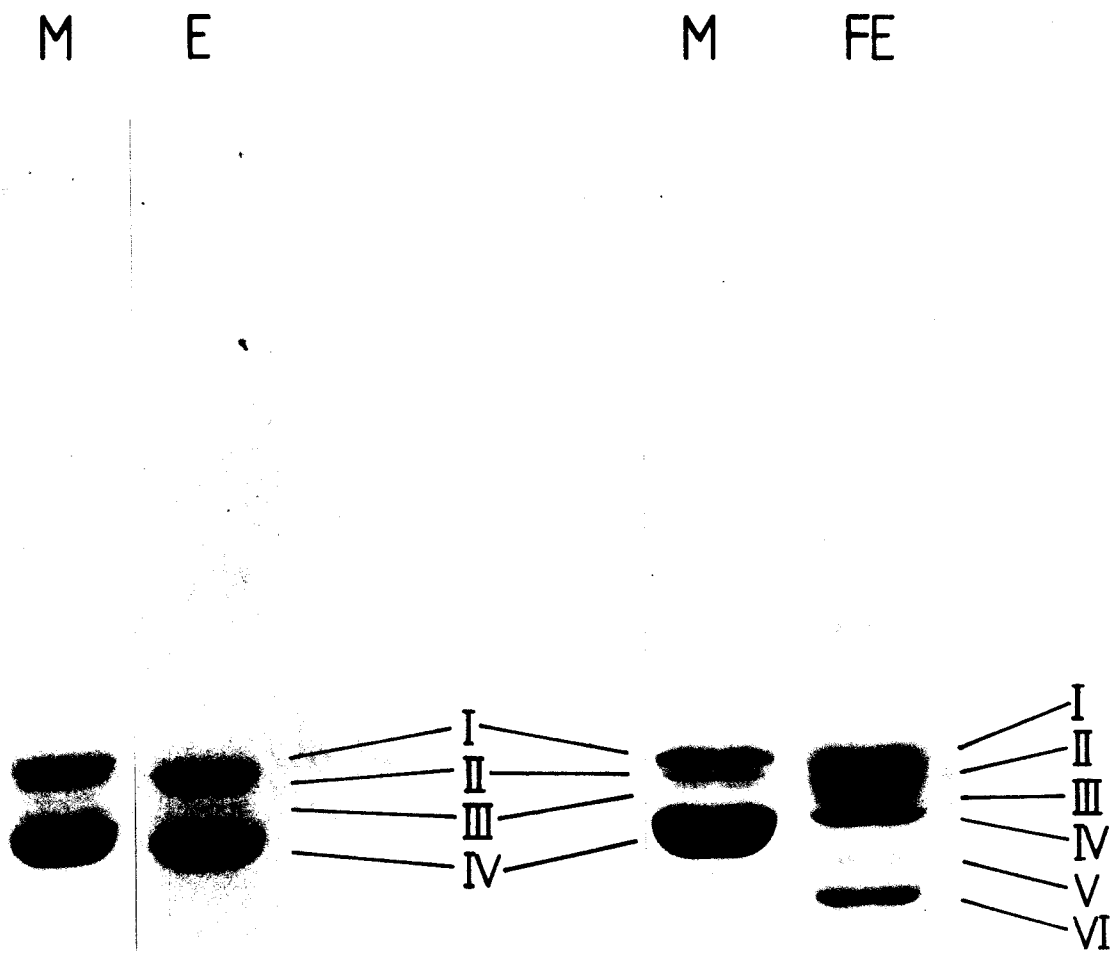
$\text{—P—q'—q''}$   
 $\text{—r—s}$

M FE



$\text{—P—q'—q''}$   
 $\text{—r'—r''—r'''—s}$

Figure 11. SDS gel electrophoresis of purified C. productus egg(E), embryonic (FE), and maternal (M) 25S hemocyanins on 7.5% acrylamide gels. Total protein concentration of egg 25S sample three times that of the corresponding maternal 25S hemocyanin sample. The total protein concentration of the embryonic and corresponding maternal 25S hemocyanins is the same.



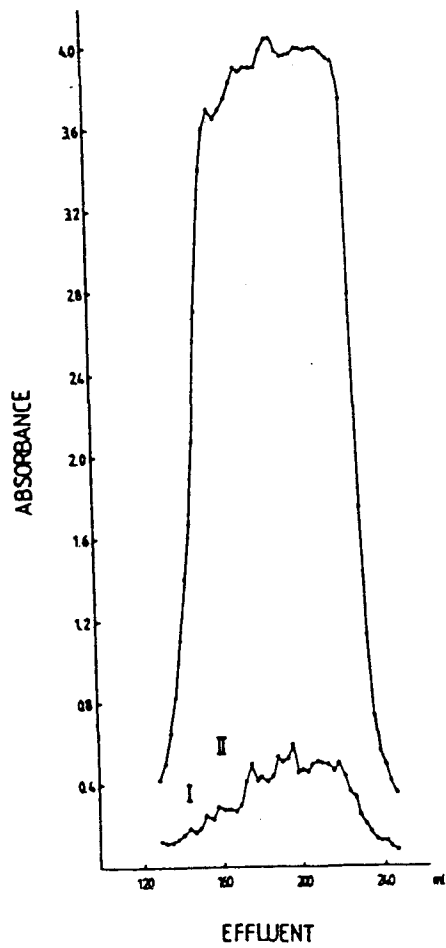
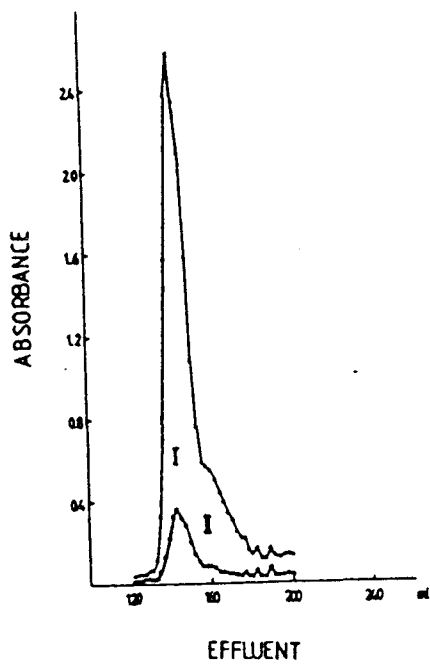
maternal hemocyanin. Cross-referencing experiments support earlier findings in that maternal and egg 25S hemocyanins cannot be differentiated from one another.

#### Fertilized Eggs (Embryos)

C. productus embryos, removed from the external surface of the mother crab after 4-6 weeks of development, contain hemocyanin. The crude embryo homogenate (after chromatography on G-100) electrophoreses on a pH 7 gel as several low mobility bands plus bands corresponding to adult 25S hemocyanin, band X, and 16S hemocyanin (Fig9, A). Loading volumes brought to about the same protein concentration of embryo peak I and adult peak I show indistinguishable 25S bands on pH 7 gel (Fig.9, B). The chromatographic pattern of an embryo homogenate on BioGel A-5M (Fig.12) is similar to that of eggs (Fig.7), except that there is a marked increase in absorbance at 280 nm in the region corresponding to adult hemocyanin peak I.

The hemocyanin banding pattern of the embryo sample on pH 8.9 non-denaturing gels (Fig.10) is indistinguishable from that of the corresponding maternal hemocyanin, and the embryo putative hemocyanin bands appear to contain copper. However, clear differences can be seen on SDS between 25S hemocyanin fractions of the mother and her embryos (Fig.11). In the embryo sample, one can distinguish subunits  $I_{FE}-IV_{FE}$ , corresponding in molecular weight to maternal  $I_M-IV_M$  plus two additional subunits,  $V_{FE}$  and  $VI_{FE}$  with molecular weights approximately  $69,700 \pm 1,800$  and  $66,000 \pm 1,500$  (S.D. of two determinations).

Figure 12. Chromatography of hemolymph from the mother animal (top) and C. productus embryo homogenate (bottom) on a column of BioGel A-5M (200-400 mesh). Column volume 1.8 x 95 cm. Buffer 0.1 ionic strength Tris HCl (pH 7.2), 0.1 M in NaCl, 0.05 M in MgCl<sub>2</sub>, and 0.01 M in CaCl<sub>2</sub>. Absorbance at 280 (▲) and 340 (●) nm.



The maternal 25S hemocyanin consists of subunits  $I_M-IV_M$ . Subunits  $II_M$  and  $III_M$  exhibit apparent reversed intensities compared to the pattern of embryo peak I. However, after reelectrophoresing the embryo 25S band from a pH 7 gel onto SDS, subunits  $II_{FE}-III_{FE}$  appear indistinguishable from  $II_M-III_M$ . Other hemolymph components can be excluded by this double purification step of the embryonic 25S hemocyanin, leaving  $I_M-IV_M$  and  $I_{FE}-VI_{FE}$  as SDS 25S hemocyanin subunits. The results of a cross-referencing experiment involving maternal and embryonic hemocyanins from the same animal are presented (Fig.13).

#### Peptide Mapping - Adult, Egg, and Embryo

Subunits  $I_M-IV_M$  give distinct peptide maps (Fig.14, 15). Peptide maps of subunits p, q, r, and s of adult 25S hemocyanin are distinct from each other. Those of  $q'$  and  $q''$  cannot be distinguished nor can the peptide maps of  $r'$  and  $r''$ .

An additional proof that the 25S hemocyanin molecules of mother and egg are the same is that the peptide maps of pH 8.9 hemocyanin subunits and SDS hemocyanin subunits (Fig.14) are indistinguishable. Similarly, peptide maps of p, q, r, and s of maternal and embryonic 25S hemocyanin cannot be distinguished. However, peptide maps of maternal and embryonic SDS hemocyanin subunits are not identical (Fig.15). Peptide maps of SDS hemocyanin subunits  $I_{FE}-VI_{FE}$  are distinct from each other. On comparison of peptide maps  $I_M$ ,  $I_{FE}$ , and  $V_{FE}$  one can see that they share some bands. Similarly,  $IV_{FE}$  and  $VI_{FE}$  are comparable, but not the same. Peptide maps of embryonic SDS hemocyanin subunits prepared via the double purification

Figure 13. Two-step electrophoresis of embryonic (top) and maternal (bottom) 25S hemocyanin subunits from pH 8.9 regular gel to SDS gel.



Hc subunits cut from pH8.9 regular gel

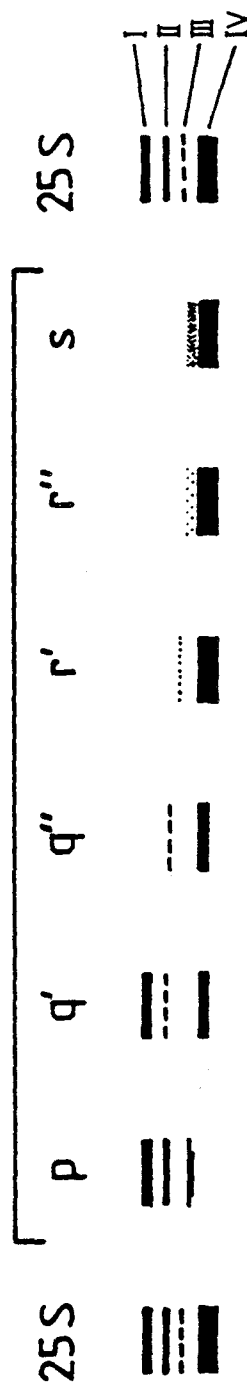
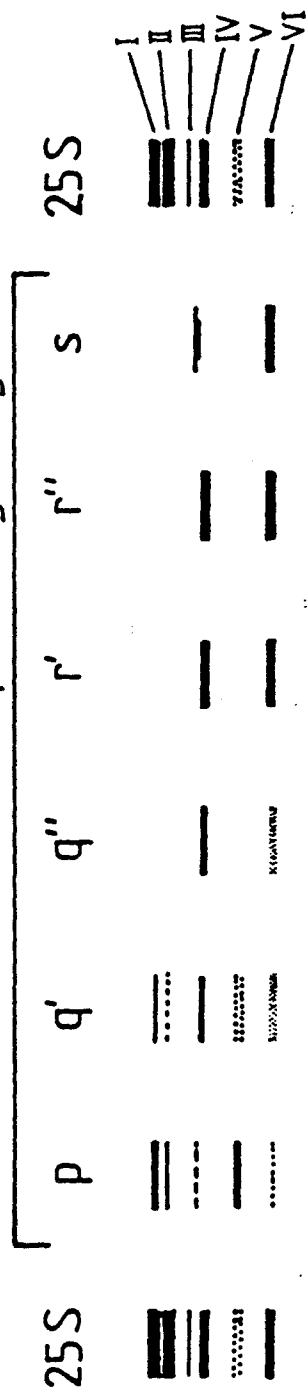


Figure 14. Peptide maps on 15% acrylamide gels of egg (E) and maternal (M) 25S hemocyanin subunits. Slices are cut from a 7.5% SDS gel, transferred to a 15% SDS gel, and then treated with 0.6  $\mu\text{g}/\mu\text{l}$  Staphylococcus aureus V8 protease as described in methods.

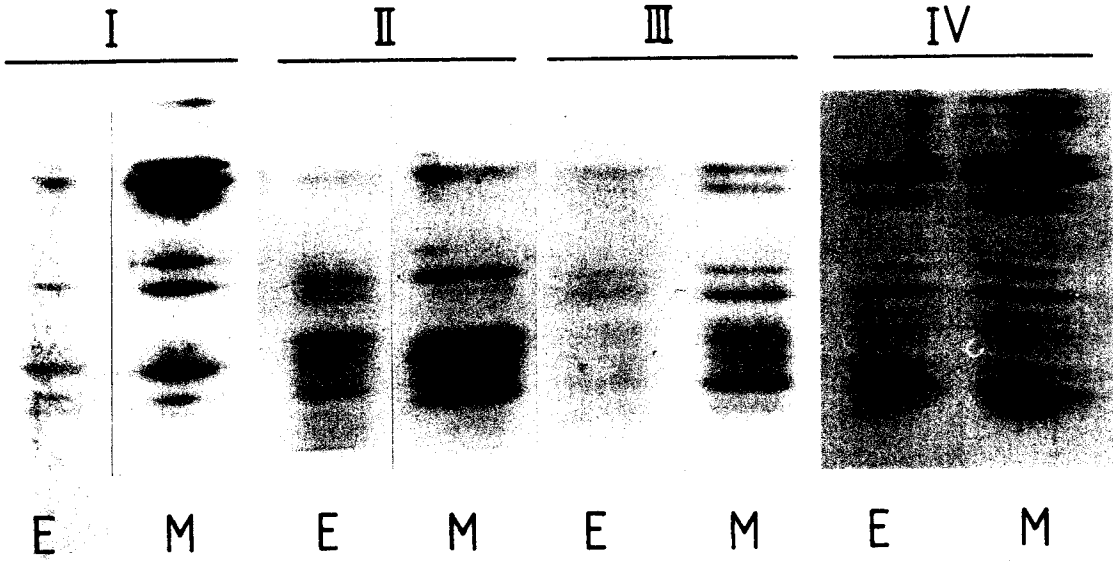
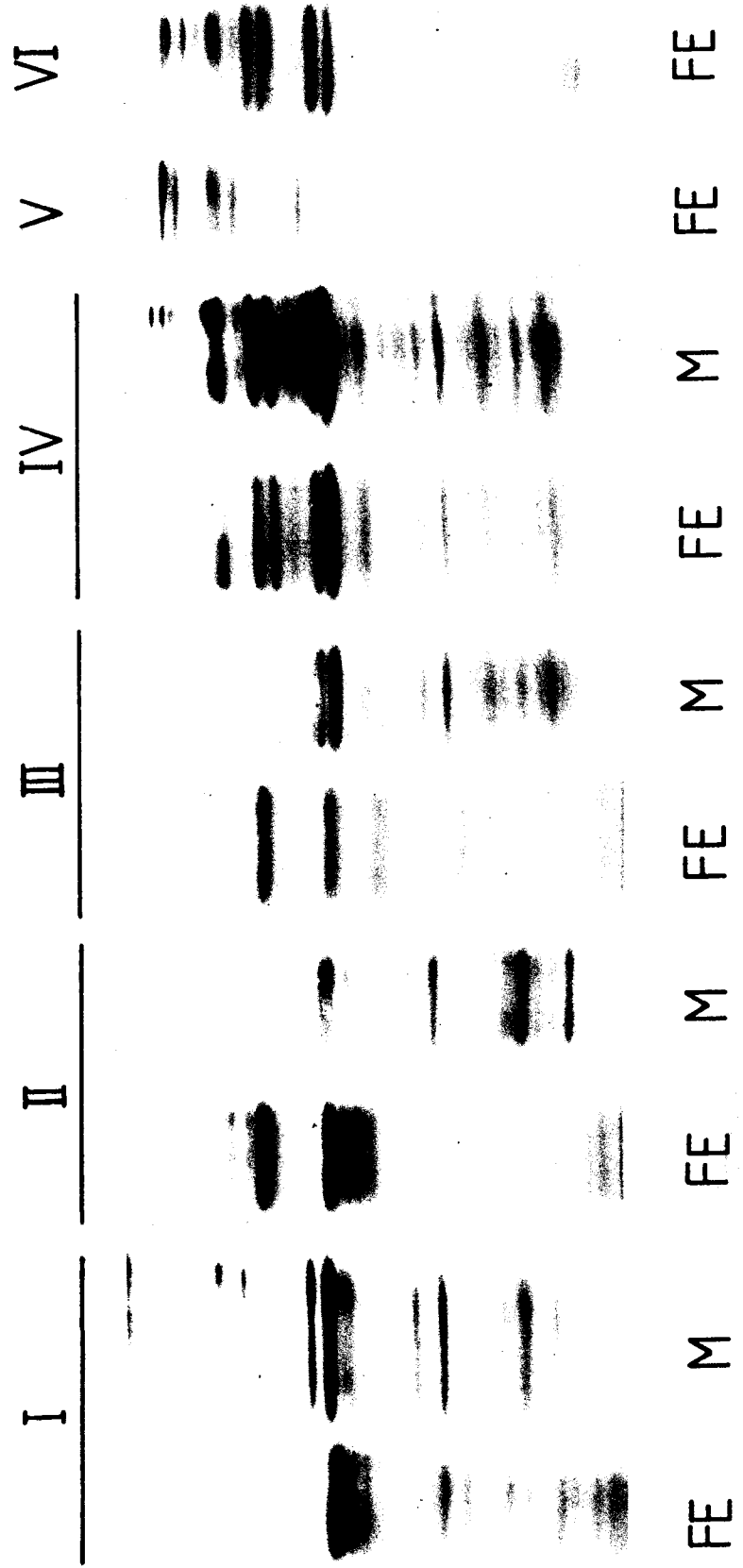


Figure 15. Peptide maps on 15% acrylamide gels of embryonic (FE) and maternal (M) 25S hemocyanin subunits. Slices are cut from a 7.5% SDS gel, transferred to a 15% SDS gel, and then treated with 6  $\mu\text{g}/\mu\text{l}$  Staphylococcus aureus V8 protease as described in methods.



procedure are less complex though still distinguishable from peptide maps of maternal SDS hemocyanin subunits. It was observed that peptide maps of pH 8.9 hemocyanin subunits are always resolved better and have a simpler banding pattern than those of SDS hemocyanin subunits.

## DISCUSSION

Adult C. productus hemocyanin is a typical crustacean hemocyanin consisting of 25S (940,000 dal) and 16S (480,000 dal) molecules. The 25S hemocyanin molecule has a unique subunit pattern as seen on peptide maps. An as yet unidentified protein elutes with peak II and electrophoreses just behind the 16S band at pH7. Not all animals investigated have band X. Its occurrence is independent of sex.

The subunit structure of hemocyanin at pH 8.9 is very complex among the adult C. productus population at Coos Bay, Oregon. Varying banding patterns involving subunits p, q, r, and s are observed. Each subunit has been seen to electrophorese as a doublet (Fig.5). Subunits p and s exhibit low intensity while subunits q and r have strong intensities. A sex specific difference underlying the banding pattern on a pH 8.9 regular gel could not be detected. A polymorphic hemocyanin structure that has been well described is that of Uca pugilator (Fielder et al., 1971; Sullivan et al., 1983). Analyses of the dialyzed stripped hemocyanin samples (at pH 8.9) by regular disc gel electrophoreses revealed complex polymorphic patterns. No correlation was observed, however, between either the sex or the size of the individual and its hemocyanin pattern. Another example for the occurrence of polymorphic hemocyanin is Callinectes sapidus (Horn and Kerr, 1969). An explanation for the polymorphism might be changes in the hemocyanin structure during the molting cycle as is suggested in the papers by Kaim-Malka et al. (1983)

on Idotea balthica, Ceccaldi and Martin (1969) on Carcinus maenas, and Ceccaldi (1970) on Penaeus kerathurus.

Egg and embryo 25S hemocyanins were compared to the 25S hemocyanins of their corresponding mothers because of polymorphic banding patterns among the adult C. productus. The approximate molecular weight of 25S hemocyanin of mother, egg, and embryo is 940,000, and 480,000 for the 16 S hemocyanin as determined on BioGel A-5M.

The electron micrograph of the egg sample shows two-hexamer and one-hexamer molecules as they have been described for other crustaceans. This result provides additional evidence that the egg contains hemocyanin, and also includes C. productus in the list of crustacean species whose 25S molecule is a dodecamer and whose 16S molecule consists of only one hexamer.

Maternal and egg 25S hemocyanins are indistinguishable using the various PAGE systems described in methods. This result reflects what has been shown for Daphnia (Dresel, 1948). In this species hemoglobin passes from the maternal blood into the eggs, while still in the ovary, a few hours before the eggs are laid. Maternal and embryonic 25S hemocyanins are indistinguishable at pH 7 and pH 8.9 as well as on peptide maps of hemocyanin subunits from pH 8.9 regular gels. Yet they appear to be different according to results obtained from SDS gels, cross-referencing experiments, and SDS peptide maps. The possibility of a hypothetical non-hemocyanin protein that is separated from the hemocyanin subunits on a pH 8.9 gel is suggested by the results of cross-referencing experiments as well as peptide maps of hemocyanin subunits from pH 8.9 and SDS gels. Low mobility bands electrophorese on cross-referencing the 25S hemocyanin



band from a pH 7 gel onto a pH 8.9 gel. However, low mobility bands are missing when cross-referencing 25S hemocyanin subunits from a pH 8.9 gel onto SDS. Similarly, the resolution of peptide maps from pH 8.9 hemocyanin subunits is better than when subunits obtained from a SDS gel are run on a peptide map. In addition, the peptide maps of pH 8.9 hemocyanin subunits are less complex for both, adult and embryo, than those of SDS hemocyanin subunits.

Three hypotheses can account for the difference in the number of hemocyanin subunits on SDS between mother and embryo. First, it can be assumed that the polypeptide chains of the maternal hemocyanin have an amino acid sequence similar to that of embryonic polypeptide chains with the exception of a stretch of neutral amino acids. This sequence might not influence the hemocyanin subunit pattern on a pH 8.9 gel, but a difference in molecular weight concerning the shorter embryonic chains lacking the neutral sequence could be detected on SDS. Second, the difference could be due to a difference in the degree of glycosylation of maternal and embryonic hemocyanin subunits. This difference would be detected on SDS alone. Assuming precursor molecules of secretory proteins to have a higher molecular weight than the adult form of the protein this hypothesis appears less likely, unless the hemocyanin is newly synthesized in the embryo. Thirdly, proteolysis is considered. It is possible that the embryo preparation has a larger amount of proteases present than the egg preparation. As was shown in a study on the barnacle Tetraclita squamosa rufotincta (Gilboa-Garber et al., 1983) the number of proteolytic enzymes is increased when fully developed larvae are ready to hatch. Similarly, one might imagine the interference of

feeding enzymes present in larger amounts in the embryo. Presumably, at an early point in development the embryo synthesizes its own m-RNA. If this is true the de novo synthesis of hatching and feeding enzymes is a possibility as much as the de novo synthesis of subunits  $V_{FE}$  and  $VI_{FE}$ .

To show that  $V_{FE}$  and  $VI_{FE}$  are indeed newly synthesized hemocyanin subunits characteristic of the embryonic stage, m-RNA extracted from the embryo homogenate and that collected from the tissue synthesizing hemocyanin in the adult crab assumed to be the hepatopancreas, is translated in a cell free system and the outcome is compared. The experiment should give unequivocal results concerning the size of maternal and embryonic hemocyanin polypeptide chains.

An experiment that is designed to test the possibility of proteolysis involves labeling of 25S maternal hemocyanin with an isotope and mixing it with the embryo homogenate. After an incubation time long enough to allow proteolysis to occur the sample is fractionated, electrophoresed, and autoradiographed. If labeled subunits  $V_M$  and  $VI_M$  are observed it can be assumed that proteases must be present in the embryo homogenate that induce the cleavage of the polypeptides designated  $V_{FE}$  and  $VI_{FE}$ .

If  $V_{FE}$  and  $VI_{FE}$  are due to proteolysis then it is possible that the number of hemocyanin subunits on SDS will be the same for mother and embryo. Similarly, it is possible that one or several of the subunits  $I_{FE}$ - $IV_{FE}$  are a result of proteolysis. Aforementioned experiments will clarify this point.

## SUMMARY

Adult C. productus hemocyanin has been shown to be a typical crustacean hemocyanin. It exists as 25S and 16S molecules dissolved in the hemolymph. When it is submitted to a denaturing agent four unique subunits can be resolved. Among the C. productus population at Coos Bay, Oregon, polymorphic banding patterns were observed when hemocyanin subunit structure was studied at pH 8.9. The possibility of varying hemocyanin structures during the molt needs to be investigated. The eggs and embryos of C. productus contain hemocyanin. Egg and maternal hemocyanins are indistinguishable. When embryonic 25S hemocyanin is denatured six unique subunits can be resolved. The two additional subunits have lower molecular weights than any of the subunits of adult hemocyanin. Studying stage specific hemocyanin structures as well as the oxygen binding properties of these hemocyanins might ultimately provide a model for understanding more complex systems in addition to a better understanding of the evolution of oxygen carrying proteins among the invertebrates.

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