CHARACTERIZATION AND SUBUNIT STRUCTURE OF THE HEMOCYANIN OF A POLYPLACOPHORAN, *KATHERINA TUNICATA* (WOOD)

by

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Hemocyanin of the chiton, Katherina tunicata, is present in the native state as a 60 S approximately 4×10^6 molecular weight protein. Essentially no work has been published on the structure of polyplacophoran hemocyanin. The dissociation behavior of this molecule is characterized with respect to pH and presence or absence of divalent cations, and compared to the published work on other molluscan hemocyanins. The subunit of this 60S hemocyanin has a molecular weight of approximately $3 - 4 \times 10^5$ (11S). Dissection of the subunit has been carried out through the use of the enzymes subtilisin and trypsin. The digestion experiments have resulted in the isolation and purification of a seemingly pure 56,000 dalton fragment. This corresponds to a minimal functional unit, or "domain" of the hemocyanin molecule. A 90,000

molecular weight fragment has also been isolated. This fragment is apparently different from the 56,000 dalton fragment.

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INTRODUCTION

Oxygen transport pigments are of four major types: hemoglobins, hemerythrins, chlorocruorins, and hemocyanins. Hemocyanins are second to hemoglobin in frequency of occurrence among the animal phyla (Prosser, 1973). Hemocyanins are extracellular proteins with respiratory capabilities and are found dissolved in the hemolymph of members of the phyla Arthropoda and Mollusca. The characteristic blue color of the oxygenated protein is due to the copper associated with this protein. One oxygen molecule is bound per two copper atoms. Native, or whole, hemocyanin is defined in this thesis as the conformational state of the moleculr that is predominant in the hemolymph of the animal. This conformational state is determined by ultracentrifugation of hemolymph or by column chromatography in conditions similar in pH and ion concentration to the hemolymph. Native hemocyanin ranges in molecular weight from 4×10^5 to 9×10^6. The quaternary structure of these macromolecules is sensitive to pH, ionic strength, divalent cation concentration, and degree of oxygenation: it will dissociate or associate in response to solution conditions.

Among the arthropods, hemocyanin has been found in members of the classes Crustacea, Arachnida, and Merostomata. No hemocyanin has been reported for the

classes Progoneata (millipedes), Chilopoda (centipedes), Insecta, or in the "subclass" Entomostraca of the Crustacea (Van Holde and Van Bruggen, 1971). The Arthropod hemocyanins are composed of 75,000 to 80,000 dalton subunits, each binding one oxygen molecule. Formic acid oxidation experiments indicate that these subunits are possibly composed of two identical 37,000 dalton polypeptide chains, each containing one copper atom, or from three 25,000 dalton chains in which two contain copper and one lacks it (Ellerton et al., 1970). Other investigators believe there is one 75,000 dalton polypeptide chain that comprises the subunit (Loher and Mason, 1973; Carpenter and Van Holde, 1973). The 75,000 molecular weight subunits form 6, 12, 24, or 48 oxygen binding sites. Heterogeneity of the arthropodan hemocyanin subunits has been widely reported (Murray and Jeffrey, 1974; Sullivan et al., 1974; Sugita and Sekiguchi, 1975; Sullivan et al., 1976; Hamlin and Fish, 1977; Miller et al., 1977; Van Den Berg et al., 1977; Markl et al., 1979a, 1979b). Possible subunit homogeneity has infrequently been found to exist as well (Terwilliger et al., 1979). Therefore, arthropod hemocyanin is composed of 75,000 molecular weight subunits, each containing a single oxygen binding site, and which aggregate to form the intact molecule.

In the phylum Mollusca, hemocyanins are found among the classes Gastropoda, Cephalopoda, and Polyplaco-

phora. None has been found to date in the Monoplacophora, Scaphopoda, or Bivalvia. The molecular weight of native molluscan hemocyanin ranges from 4×10^6 (60S) in the Cephalopoda and Polyplacophora to 9×10^6 (100S) in the Gastropoda.

Most molluscan hemocyanin research has centered on the 9×10^6 molecular weight, 100S, gastropod molecule. As outlined in Figure 1, this 100S molecule reversibly dissociates to one-half (60S), one-tenth (20S), and one-twentieth (11S) of the whole 100S molecule in response to pH, ionic strength, divalent cation concentration, and state of oxygenation (Eriksson-Quensel and Svedberg, 1936; Konings et al., 1969; DePhillips et al., 1970; Bois D'Enghien et al., 1971; Elliott et al., 1972; Siezen and Van Bruggen, 1974; Siezen and van Driel, 1974; Quitter et al., 1978).

The 100S native hemocyanin appears in the electron microscope to be a six-tiered cylinder with tenfold rotational symmetry (Figure 1A) (Van Bruggen et al., 1962a; Fernandez-Moran et al., 1966; Van Bruggen, 1968; Mellema and Klug, 1972). End-on views of this cylinder show a five-fold "collar" structure within the central cavity (Van Bruggen, 1968; Mellema and Klug, 1972). The "collar" is believed to limit the length of the cylinder since its removal leads to the formation of tubular polymers of indefinite length (van Breeman et al., 1975; Wood and

Mosby, 1977; Gullick et al., 1979; van Breeman et al., 1979; van Breeman et al, 1979).

One-half molecules (60S) are formed by dissociation along the perpendicular axis of the cylinder (Figure 1B). End-on views of one-half molecules show some ends containing the "collar", while the others lack this structure, suggesting the "collar" is present only at one end of the gastropod 60S molecule. The presence of the "collar" is also inferred from the asymmetrical staining apparent in electron micrographs of side views of the one-half cylinder. One end of the cylinder - where the "collar" would be present - shows greater intensity of staining than the other end.

The one-tenth molecules arise by five-fold splitting of the one-half molecule (Figure 1C). Each one-tenth contains a piece of the "collar", and appear as an "arc + blob" in electron micrographs (Siezen and Van Bruggen, 1974). They exist in two forms - "Compact" 1/10 at relatively high ionic strength and slightly alkaline pH, and "Loose" 1/10 at low ionic strength and/or higher pH (Siezen and Van Bruggen, 1974).

One-twentieth molecules form by a lengthwise splitting of the one-tenth molecule (Figure 1D). They appear as a flexible structure of seven or eight spherical units, arranged linearly, as beads on a chain. Each bead or spherical unit is proposed to have a molecular

weight of approximately 50,000 daltons. Based on copper to protein ratios of one Cu per 24,000 daltons protein (Ghiretti, 1962), each of these spherical units corresponds to a minimal functional unit containing one oxygen binding site. Isolation of these functional units, or "domains", is possible only by the breaking of covalent bonds. Limited proteolytic cleavage has been used as a tool in the separation of "domains" (Brouwer and Kuiper, 1973; Lontie et al., 1973; Bannister et al., 1975; Gielens et al., 1975; Brouwer et al., 1976; Bonaventura et al., 1977). 70% formic acid also will dissociate the subunit into 50,000 and 25,000 molecular weight units (Dijk et al., 1970).

In summary, the native gastropod hemocyanin molecule is composed of twenty 11S subunits arranged in a cylinder to form the 100S molecule. The 11S subunit contains 7 - 9 oxygen binding sites. Each binding site is proposed to have a molecular weight of approximately 50,000.

Cephalopod hemocyanin exists as a 50 - 60S molecule in its native state (Eriksson-Quensel and Svedberg, 1936; Van Holde and Cohen, 1965; Salvato et al., 1979). Electron microscopy shows that this molecule closely resembles the one-half molecules of gastropod hemocyanin. It consists of a three layered cylindrical structure (Van Bruggen et al., 1962b). End-on views show the circle to contain a five-fold inner ring. The 60S

cephalopod molecule does not show the asymmetrical staining patterns as does the 60S gastropod structure. It has been suggested that this lack of asymmetry prevents dimerization to a gastropod-like 100S molecule (Van Holde and Van Bruggen, 1971). Dissociation of cephalopod hemocyanin occurs to 20S and 11S subunits, strongly resembling the dissociation products of gastropod hemocyanin (Van Holde and Cohen, 1965; DePhillips et al., 1969; Salvato et al., 1979). The fractional nomenclature of hemocyanin dissociation products becomes cumbersome for the different classes of molluscs. A gastropod whole (100S) molecule dissociates to one-half (60S), one-tenth (20S), and one-twentieth (11S) molecules. A cephalopod whole (60S) molecule dissociates to one-fifth (20S) and one-tenth (11S) molecules.

Polyplacophoran hemocyanin, like the cephalopod molecule, exists as a 60S molecule (Svedberg and Pederson, 1940). The minimum subunit size of the hemocyanin of the chiton, Stenoplax conspicua, as assayed by SDS gel electrophoresis, is comparable to Loligo, Octopus, and Busycon hemocyanin (Waxman, 1975). No further structural information on polyplacophoran hemocyanin has been published.

The purpose of this work is first to characterize the dissociation behavior of a chiton (Katherina tunicata) hemocyanin with respect to pH and presence or absence of divalent cations. A second purpose is to study the subunit

structure of this protein. A complex and intriguing system is suggested by the large polypeptide chain size (11S and approximately 350,000 daltons) and number of oxygen binding sites (7 - 9). In this study, attempts have been made to dissect the polypeptide chain in order to identify and isolate a minimal functional "domain" or "domains". A comparison of the above data with similar studies of other molluscan hemocyanins should be interesting in that among the classes of Mollusca there apparently is great constancy of subunit size but differences in the size of the native molecule. Numerous questions arise through such a comparison. What factor(s) determine the differences and similarities in structure among these classes? What correlation could be drawn between the structural architecture of hemocyanin and molluscan phylogeny? How do the structures correlate with the animal's oxygen needs as well as the parameters of its environment? The information gathered through the structural studies of Katherina tunicata hemocyanin presented here can be added to the general body of hemocyanin knowledge, allowing one to speculate as to some of the answers to these questions.

MATERIALS AND METHODS

Katherina tunicata (Wood) was collected in the vicinity of Cape Arago, Charleston, Oregon. Animals were kept in tanks with running seawater until use.

The animals were bled by slitting the pallial groove and allowing the hemolymph to drip into an ice-cooled beaker containing a few drops of a protease inhibitor, phenylmethanesulfonyl flouride (PMSF) (Sigma Chem. Co.) made up 6 mg/ml in 95% ethanol. From one to six animals were used for each preparation. Hemolymph was centrifuged at 12,000 x g for 10 minutes at 4^{o} C to remove particulate matter. If necessary, the blood was concentrated on sucrose before application to a column of Sepharose 4-B equilibrated with 0.05 M Tris-HCl buffer pH 7.4, 0.01 M $MgCl_2$, and 0.02 M NaCl. All chromatography was carried out at 4^{o} C.

Protein concentration of purified hemocyanin was approximated from the absorbance at 345 nm using $E^{1\%}_{1cm}$ = 2.79, as determined for Loligo pealei hemocyanin (Nickerson and Van Holde, 1971).

Buffer solutions were made according to standard recipes. The following were used for the pH ranges given: Potassium dihydrogen phosphate - disodium hydrogen phosphate (pH 6.0 - 7.0), Tris - hydrochloric acid (pH 7.0 - 8.5), Sodium bicarbonate - sodium carbonate (pH 8.5 -

11.0). All buffers were 0.1 ionic strength or 0.125 ionic strength when 0.01 M $MgCl_2$ was added. All buffers contained either 0.01 M disodium EDTA or 0.01 M $MgCl_2$.

Sedimentation velocity studies were performed with a Spinco Model E analytical ultracentrifuge equipped with an RTIC unit and scanning aptics. All runs were performed near 20° C. Sedimentation coefficients were calculated by the method of least squares from the plot of ln r vs s and corrected to the viscosity and density of water at standard conditions (Svedberg and Pederson, 1940).

Regular 5% or 7.5% disc gel electrophoresis was carried out at pH 8.9 according to Ornstein (1964) and Davis (1964) with the addition of 0.01 M EDTA to all buffers.

SDS gel electrophoresis was performed on 7.5% acrylamide gels with a ratio of acrylamide to bis-acrylamide of 37:1 (Weber and Osborne, 1975). Tube gels and slab gels were done according to methods described by Laemmli (1970), Studier (1973), and Ames (1974). Before electrophoresis, boiling incubation buffer, containing 0.01 M PMSF was added to the protein; this mixture was boiled for 2 minutes. Calibrants were phosphorylase A, bovine serum albumin, and ovalbumin (Sigma Chem. Co.). Gels were stained with Comassie Blue (Fairbanks et al., 1971).

Digestion experiments were carried out on samples dialyzed against 0.05 M Tris-HCl buffer plus 0.01 M EDTA,

pH 8.5, unless otherwise noted. Under these conditions, the hemocyanin is present as a 17 - 18S molecule. The starting material could be further dissociated at a higher pH, but experimental results at pH 9.6 (EDTA) were no different than those at pH 8.5. As the higher pH is considered a harsh environment for optimal enzyme activity, pH 8.5 was selected.

The hemocyanin solution was incubated at 30° C with subtilisin (Sigma Chem. Co.) or trypsin (Worthington Biochemicals) at an enzyme to hemocyanin ratio of 1:1000 (w/w). Samples were taken at specified intervals in time course digestions and analysed by SDS gel electrophoresis or at one hour for the preparation of individual fragments. Subtilisin digestion was stopped by the addition of 1/20 volume of PMSF (6 mg/ml in 95% ethanol); tryptic digestions were halted by 0.2% (w/w) soybean trypsin inhibitor (Calbiochem) according to Brouwer et al., (1979).

The one hour digestion material from subtilisin or trypsin digests was chromatographed on a 1.7 x 90 cm column of Sephadex G-200 (Sigma Chem. Co.) which was equilibrated with the digestion buffer. The column was calibrated with blue dextran, bovine serum albumin, ovalbumin, and α chymotrypsinogen A.

Ion exchange chromatography was performed using a 0.6 x 28 cm column of Sephadex DEAE A-50 (Sigma Chem. Co.)

equilibrated with the digestion buffer and eluted with a 0.05 - 0.5 M linear gradient of NaCl.

RESULTS

Dissociation Studies

The purification elution profile of Katherina tunicata hemocyanin on a column of Sepharose 4-B equilibrated with Tris-HCl buffer, pH 7.4 plus 0.01 M $MgCl_2$ is shown in Figure 2. The first peak is whole 60S hemocyanin. This value is obtained through calibration of the 4-B column and is corroborated by sedimentation equilibrium runs (R. C. Terwilliger, personal communication) and sedimentation velocity experiments. The second peak is a yellow pigmented substance. This yellow pigment is not found in hemolymph of other classes of the Mollusca and is worthy of further study. Hemocyanin from the first peak is used for the following experiments.

Dissociation behavior of the whole molecule in response to pH in the presence and absence of divalent cations is illustrated in Figures 3 and 4. In the presence of divalent cations (0.01 M $MgCl_2$) (Figure 3), the 60S molecule is intact between pH 6.5 and 8.5. Above pH 8.5, the molecule dissociates to the one-fifth structure. There is a gradual decrease in $S_{20,w}$ as the pH increases from 8.5 to 11; the molecule has dissociated to the one-tenth (11S) structure at pH 11.

At pH 7, in the absence of divalent cations and in the presence of 0.01 M EDTA, the hemocyanin exists pri-

marily as the whole molecule (Figure 4). With an increase in alkalinity, dissociation of the 60S to one-fifth molecules (18 - 19S) occurs; above pH 8 no 60S material is present. There is again a gradual decrease in $S_{20,w}$ values with an increase in pH, consistent with a dissociation from one-fifth to one-tenth molecules.

Regular disc gel electrophoresis at pH 8.9 and 10.5 is shown in Figure 5. One major band is present at pH 8.9 (Figure 5A). At pH 10.5, one major band and one lightly staining band is present (Figure 5B).

Subunit Structure

Katherina tunicata hemocyanin is present as a 17S molecule in pH 8.5 buffer with 0.01 M EDTA, which are the conditions under which all digestion experiments were carried out. This 17S material was digested with the enzyme subtilisin. An SDS gel electrophoresis analysis of the course of digestion over time is shown in Figure 6. Within 30 seconds, the digestion is in progress, yielding the 350,000 molecular weight subunit plus an approximately 250,000 and 100,000 dalton fragments (Figure 6B). As the time course proceeds, the 350,000 molecular weight subunit disappears and the appearance of 250,000, two molecular weight bands that are between 120,000 and 200,000 , 94,000, 90,000, 56,000 (present as a doublet), 51,000, 47,000, 44,000, and 42,000 dalton

products are evident (Figure 6C-H). After one hour (Figure 6I), only the 94,000, 90,000, 56,000 doublet, and the 42,000 to 51,000 molecular weight products are present. After two and one-half hours the 94,000 dalton product has disappeared; the 90,000, 56,000 doublet, and 42,000 - 51,000 molecular weight products remain (Figure 6J).

A one hour digestion of the hemocyanin subunit by subtilisin was chosen for separation of proteolytic fragments. At this time all larger molecular weight material (250,000 - 350,000 daltons) had been digested and very little had been degraded to very small pieces, since not much material was present at the dye interface in SDS gel electrophoresis. Chromatography of a one hour digest on Sephadex G-200 is shown in Figure 7. The digest elutes as two peaks. The first peak (Fraction I) chromatographs with an apparent molecular weight of 81,000 and has an $S_{20,w}$ value of 6S. Fraction I is heterogeneous when analysed by SDS slab gel electrophoresis, appearing as 94,000, 90,000, a faint 74,000, and 56,000 (doublet) dalton bands (Figure 8B). On pH 8.9 disc gel electrophoresis, this 81,000 molecular weight peak shows two heavily staining bands and one lightly staining band (Figure 9A). The second peak (Fraction II) chromatographs with an apparent molecular weight of 41,000 and exhibits an $S_{20,w}$ value of 4S. On SDS slab gels, this peak

electrophoreses as four bands with molecular weights of 51,000, 47,000, 44,000, and 42,000 (Figure 8C). Regular pH 8.9 disc gel electrophoresis shows six bands - two heavily staining and four lesser staining bands (Figure 9B).

Anion exchange chromatography on DEAE Sephadex of the 81,000 dalton Sephadex G-200 peak (Fraction I), resolved two major fractions (Figure 10). The first DEAE peak electrophoreses as a single band with a molecular weight of 90,000 by SDS slab gel electrophoresis (Figure 11B). Disc gel electrophoresis at pH 8.9 also shows a single band (Figure 12B). This first DEAE peak shows a slight trailing shoulder. SDS electrophoresis of this shoulder gives two bands - a 90,000 and a very faint 74,000 molecular weight band (Figure 11C). This faint 74,000 dalton band corresponds to the faint band seen in the SDS analysis of the Sephadex G-200 Fraction I (Figure 11A). The second DEAE peak is present as a 56,000 dalton double band according to SDS (Figure 11D). A single band is present on pH 8.9 regular disc gels as shown in Figure 12C.

Redigestion with subtilisin of the fragments isolated by DEAE chromatography resulted in no further digestion after two hours of incubation. These fragments, therefore, are apparently resistant to further cleavage by subtilisin under these experimental conditions.

In order to approach the dissection of the polypeptide chain in a slightly different manner, and to compare the resulting fragments, a different enzyme, trypsin, was employed. The hemocyanin preparation and the buffer conditions for digestion were the same as that for the subtilisin digestions. An analysis of the progress of a trypsin digestion over time by SDS gel electrophoresis is shown in Figure 13. After five minutes (Figure 13C), the main identifiable bands are the 350,000 dalton subunit, a 250,000, and a 56,000 dalton doublet, with many molecular weight bands between 74,000 and 250,000. Over the remainder of the one hour time course, the 350,000 dalton subunit nearly disappears, the presence of the 250,000 dalton band reaches a point of apparent stability, and the staining intensity of the 56,000 molecular weight doublet increases, as do many of the 74,000 to 250,000 molecular weight bands (Figure 13 D-H).

The application of a one hour tryptic digest of Katherina hemocyanin to a Sephadex G-200 column, resolved two peaks, as shown in Figure 14. The first peak chromatographs with an apparent molecular weight of 250,000 and is 11S in the ultracentrifuge. This peak shows great heterogeneity in SDS, with fourteen bands ranging in molecular weight from 43,000 to 350,000 (Figure 15B). It should be noted that there is no 90,000 or 56,000 dalton bands. Disc gel electrophoresis shows one major

and six minor bands (Figure 16A). The second peak elutes at a molecular weight of 98,000, has an $S_{20,w}$ value of 6S, and by SDS gel electrophoresis shows a faint band corresponding to a molecular weight of 90,000 and a heavily staining 56,000 dalton doublet (Figure 15C). Disc gel electrophoresis gives two major and four minor bands (Figure 16B).

Redigestion of the Sephadex G-200 250,000 dalton peak with trypsin led to no further proteolysis after incubation for one hour. Digesting this peak with subtilisin for one hour yielded, as analysed by SDS gel electrophoresis, products formed in a one hour subtilisin digest which has been described above - a 94,000, a major 90,000, and four 42,000 to 51,000 molecular weight bands, with the addition of some less than 40,000 molecular weight products (Figure 17). There is a noticable absence of any 56,000 dalton material.

DISCUSSION

Katherina tunicata native hemocyanin exists as a 60S, approximately 4 x 10^6 molecular weight protein. In this respect, chiton hemocyanin is much like the whole hemocyanin of the cephalopods, and is in contrast to the 100S native gastropod molecule. Electron microscopy of whole Katherina tunicata hemocyanin corroborates chromatographic, sedimentation velocity, and sedimentation equilibrium data. A three tiered cylinder very similar to the 60S cephalopod hemocyanin molecule is seen in these electron micrographs (Terwilliger, personal communication). No 100S material has ever been found for Katherina hemocyanin. Under the conditions examined in this study, there is no tendency for the molecule to dimerize.

Divalent cations are normally present in the hemolymph of molluscs. The tendency for divalent cations such as $CaCl_2$ or $MgCl_2$ to stabilize native hemocyanin under conditions of ionic strength and pH that generally lead to dissociation of the molecule has been reported for cephalopods and gastropods. The behavior of Katherina hemocyanin in the presence or absence of $MgCl_2$ over the pH range 6 - 11 will be compared to the reported behavior of other molluscan hemocyanins.

Katherina hemocyanin dialyzed versus buffer solutions containing 0.01 M $MgCl_2$ result in stabilization of the

native hemocyanin molecule from pH 6 to pH 8.5. At pH 9, the molecule has dissociated to primarily one-fifth (20S) molecules. With a further increase in pH, dissociation results in the formation of one-tenth (11S) molecules. A gradual decrease in $S_{20,w}$ values is seen in the dissociation between the 20S and 11S molecules. Under the same conditions described for Katherina, the hemocyanin of the cephalopod, Loligo pealei, is stable as a 60S molecule to pH 10, at which point a breakdown directly to the one-tenth (11S) molecule occurs, with only trace amounts of the one-fifth molecule present (Van Holde and Cohen, 1965). In the presence of 0.01 M $CaCl_2$, the whole 100S molecule of the gastropod Helix pomatia is also stable up to pH 10 (Konings, 1969). In the presence of $CaCl_2$, the Helix 100S hemocyanin molecule dissociates directly to the 20S, with no 60S intermediate (Siezen and van Driel, 1974). Whole Murex trunculus hemocyanin is also stable to pH 10 in buffers containing 0.01 M $MgCl_2$ although one-half molecules are present in the dissociation process (Wood and Mosby, 1973). The stabilizing effect of Ca^{2+} and Mg^{2+} has also been reported to occur for Helix pomatia hemocyanin in the presence of Sr^{2+} and Ba^{2+}, although to a lesser extent (Vannoppen-Ver Eecke and Lontie, 1973). Therefore, Katherina hemocyanin appears to dissociate at a lower pH (pH 9) than Loligo pealei, Helix pomatia, and Murex trunculus hemo-

cyanin (pH 10) in the presence of $MgCl_2$.

In the absence of $MgCl_2$ and in the presence of 0.01 M EDTA, Katherina hemocyanin is a whole 60S molecule at pH 7. Increase in alkalinity above this pH vlaue results in dissociation to the 20S one-fifth molecule and gradually to the 11S one-tenth molecule at pH 11. This dissociation is very similar to that reported for the cephalopod Loligo pealei in the absence of divalent cations (Van Holde and Cohen, 1965). Loligo is present as a 60S molecule until approximately pH 7.5, at which point dissociation occurs to the one-fifth molecule. Further increase in pH leads to a gradual decrease in $S_{20,w}$ value from the 19S one-fifth to the 11S one-tenth molecule. Hemocyanin of the gastropod, Helix pomatia, in the absence of divalent cations, dissociates from the whole 100S molecule to the 60S one-half and 20S one-tenth near pH 8. At pH 10 the molecule has further dissociated and exists primarily as an 11S one-twentieth component (Siezen and van Driel, 1974b). Murex trunculus hemocyanin, in buffers containing 0.01 M EDTA, exhibited total dissociation of the molecule to one-twentieths at pH 8.2 (Wood and Peacocke, 1973). Hemocyanins of the gastropods Buccinum undatum and Neptunea antigua, in buffers containing EDTA, dissociate to the 60S and 20S from the 100S whole molecule around pH 8.0 - 8.5 (Wood, 1973). The hemocyanin of Colus gracilus, another gastropod, dissoci-

ates at about pH 7.5 in the presence of EDTA (Wood, 1973). These three gastropods were investigated only over the pH range 7.5 - 9.2, and there is little data regarding the 11S one-twentieth molecule formation. In the cases of Loligo and Helix hemocyanins, the presence of EDTA to chelate any divalent cations in the hemocyanin solutions would shift the pH at which dissociation of the blood occurs to a lower value.

In comparing the behavior in the presence and absence of divalent cations of the three classes of molluscs - a polyplacophoran, cephalopod, and gastropods, it is apparent that the hemocyanin from each group dissociates in response to alkaline conditions and the intact molecule is stabilized by divalent cations. There are variations between classes and between species within a class as to the extent of stabilization by cations and the degree of dissociation exhibited.

An interesting behavior of Katherina hemocyanin is that it exhibits a progressive decrease in $S_{20,w}$ value in dissociating from one-fifth (20S) to one-tenth (11S) molecules. This same behavior is reported for Loligo pealei hemocyanin (Van Holde and Cohen, 1965). It is suggested that the intermediate values obtained represent equilibrium or near equilibrium mixtures of 20S and 11S molecules. Broad boundaries and continual decrease of sedimentation coefficients over the pH range is behavior

expected for a monomer-dimer equilibrium (Gilbert and Gilbert, 1973). Another possibility is that the one-fifth and one-tenth molecules exhibit a change in conformation and therefore a change in the sedimentation values, similar to the "Compact" and "Loose" one-tenth and "Fast" and "Slow" one-twentieth molecules described for Helix pomatia hemocyanin (Siezen and van Driel, 1974). The homo- or heterogeneity of the system at particular pH values could be approached through sedimentation equilibrium studies.

The pH dissociation of Katherina hemocyanin does not appear totally reversible. This statement is cautious since attempts at reassociation were quite limited. Hemocyanin at pH 10.5, EDTA (13S) or pH 9.0 EDTA (17S) was dialyzed against 0.05 M Tris-HCl buffer at pH 7.4 plus 0.01 M $MgCl_2$. There was a shift of the sedimentation value to 21S, which would correspond to the one-fifth molecule. Reassociation to the whole 60S molecule was not observed.

Dissociation of the hemocyanin of the cephalopod, Octopus vulgaris, is reversible in 3M urea but not under pH dissociating conditions. Lowering of the pH of a hemocyanin solution which had been dissociated at high results in a high molecular weight polydisperse system (Salvato et al., 1979). Total reversibility of pH dissociated molecules in gastropods has been reported in

Helix pomatia and Murex fulvascens although reassociation to the whole molecule from hemocyanin subunits was not possible for Murex trunculus (Konings et al., 1969; Wood and Peacocke, 1973; Brouwer et al., 1978). Some additional reassociation conditions should be attempted for polyplacophoran hemocyanin before declaring reassociation irreversible. One alternative is stepwise buffer changes. That is, lower the pH in one step and introduce divalent cations in the next. Experimenting with different ionic strength reassociation buffers may be effective, since among the gastropods, differences between species in dissociation behavior in response to ionic strength have been reported (Hoebeke and Elliott, 1971; Siezen and van Driel, 1974b). Possibly, as Salvato et al., (1979) reports for Octopus hemocyanin, dissociation in 3 M urea may be reversible for Katherina hemocyanin.

At pH 10.5, Katherina tunicata hemocyanin exists as a 13S molecule. Regular disc gel electrophoresis at this pH gives one major band and one closely preceeding very faint band. This apparently near homogeneous system suggests that a more pure system than has been reported for gastropod hemocyanins (Brouwer et al., 1978; Brouwer et al., 1979) may exist for Katherina hemocyanin. This is important in that if all polypeptide subunit chains were alike, interpretation of digestion experiments

may be simplified.

Dissection through the use of proteolytic enzymes has been useful in attempts to understand the structural nature of macromolecules. Cleavage of a molecule by proteases will preferentially occur in exposed stretches of peptide chain rather than the compact folded regions (Brouwer, 1975). Examples of molecules that have been studied in this manner are serum albumin, immunoglobulins, fibrinogen, myosin, paramyosin, and collagen (Mihalyi, 1972). The "string of pearls" configuration proposed as a model for the subunit structure of molluscan hemocyanin suggests that this method would be quite useful in the isolation of minimal functional units or "domains". Proteolytic cleavage has been employed for the study of gastropod hemocyanin structure and results seem to agree with the proposed one-twentieth structures of Siezen and Van Bruggen (1974a) and Van Breeman et al., (1977). The use of enzymes has been applied to the study of the subunit structure of the hemocyanin of the polyplacophoran, Katherina tunicata.

Digestion of dissociated (17S) Katherina hemocyanin with subtilisin leads to the formation of 94,000, 90,000, 56,000 (doublet), and four 42,000 to 51,000 molecular weight fragments. The 94,000, 90,000, and 56,000 dalton doublet are separated from the remainder of the digestion

mixture by column chromatography. The 90,000 and 56,000 dalton fragments are isolated in pure form by anion exchange chromatography. The 56,000 dalton piece would correspond to a minimal functional unit, or "domain", of the string of pearls structure described for the polypeptide chain of Helix pomatia by Siezen and Van Bruggen (1974). The molecular weight of this fragment is determined as 56,000 by SDS gel electrophoresis, although it is present as an 81,000 molecular weight component by G-200 chromatography. Dimerization of the 56,000 dalton fragment appears to occur in non-SDS conditions. The SDS migration of the 56,000 dalton component as two closely running bands may indicate some heterogeneity, although regular disc gels show a single homogeneous species. The presence of an 80,000 molecular weight fragment by Sephadex G-150 chromatography that is 100,000 to 120,000 daltons by sedimentation equilibrium, and which is present as a 65,000 molecular weight doublet in SDS gel electrophoresis has been reported for the gastropod, Lymnaea stagnalis, after digestion with trypsin or plasmin (Gullick et al., 1979). These authors find this 65,000 dalton piece to be the "collar" fragment of the hemocyanin molecule, which they have separated after digestion and subsequent polymerization of the remainder of the molecule. An SDS doublet is also found in the analysis of an otherwise pure 50,000 molecular

weight tryptic "domain" of Helix pomatia hemocyanin (Brouwer et al., 1979). This double band behavior is as yet unexplained.

The 90,000 molecular weight fragment (by SDS gel electrophoresis, gel chromatography, and sedimentation velocity) does not appear to be a precursor to the 56,000 dalton fragment. Redigestion of the 90,000 dalton material with subtilisin for up to two hours gave no further digestion; no trace of a 56,000 molecular weight product was seen. The 56,000 dalton doublet and the 90,000 molecular weight fragment absorb at 345 nm, which suggests the presence of copper. It is necessary to perform oxygen binding experiments on these fragments to determine their functional nature.

Chromatography of a one hour trypsin digestion of the dissociated (17S) hemocyanin molecule of Katherina tunicata resolves two peaks. One has an apparent molecular weight of 250,000 and the other elutes at 98,000 daltons. Most of the 98,000 dalton peak contained a 56,000 dalton doublet as resolved by SDS gel electrophoresis. This material is very much like the fragment isolated from the subtilisin digest. They are similar in chromatographic behavior and SDS banding patterns. Although caution must be exercised in comparing digestion products generated by different enzymes, the supposition that enzymatic cleavage will occur selectively

in exposed stretches of polypeptide chain rather than in compact regions allows such a comparison.

The redigestion of the G-200 peak corresponding to a 250,000 molecular weight product provides further comparison. Re-exposure of this peak to trypsin for up to one hour gave no further digestion. When incubated with subtilisin, the 250,000 dalton material is digested to a 90,000 molecular weight fragment plus approximately six fragments less than 50,000 daltons. There is no 56,000 molecular weight material present.

The isolation of two fragments that are apparently non-identical indicates microheterogeneity - heterogeneity among the domains in the polypeptide chain. From the structure seen by electron microscopy, this is reasonable. The gastropod one-half molecules are asymmetric - showing "collar" material at one end of the cylinder. Cephalopods do not show this asymmetry, but there is central "collar" material within the circular end-on views. Electron micrographs of Katherina hemocyanin also show this central material.

The role of the "collar" in other molluscan hemocyanins has been approached through studies that apparently clip off the "collar" material by proteolytic cleavage. When this happens, the molecule undergoes an infinite end to end polymerization which can be detected

in the ultracentrifuge and by electron microscopy (van Breeman et al., 1975; Gullick et al., 1979). In order to compare these reported results for gastropod hemocyanin with the polyplacophoran molecule, an attempt at tubular polymer formation was made. A two hour digestion with trypsin at an enzyme to hemocyanin ratio of 1:20 was performed at 30^{0} C on material equilibrated with 0.1 M Tris-HCl buffer, 0.025 M $CaCl_2$, pH 8.0 (Wood and Mosby, 1977; Gullick et al., 1979). The digestion was stopped as described in Materials and Methods. Turbidity was monitored over the course of digestion at 345 nm in order to detect large scale aggregation. No increase in turbidity was found - there was a slight initial decrease in the first five minutes followed by little or no change throughout the remainder of the experiment. Sedimentation velocity analysis of the two hour digest showed a major boundary of 57S plus one or two slowly sedimenting peaks whose $S_{20,w}$ values were undeterminable. Katherina tunicata hemocyanin, therefore, does not form tubular polymers under conditions in which such structures are observed for the gastropod molecule.

Speculation as to the structure of polyplacophoran hemocyanin subunits and their arrangement within the whole molecule based on this quite limited data is a questionable venture, but some ideas will be presented here. Again, reference must be made to the proposed

model for gastropod hemocyanin (See Figure 1). According to van Breeman et al., (1977), a gastropod polypeptide chain consisting of eight globules is suggested: six domains belonging to the wall of the cylinder and two domains forming part of the "collar". The side-by-side arrangement of two subunits within the one-tenth molecule of the gastropod is schematically drawn in Figure 18A. Five of these structures would come together in a one-half molecule cylinder - all "collar" pieces at one end. Finally, two asymmetrical one-half molecules connect into the whole cylinder, now with a "collar" at each end.

The polyplacophoran whole structure is equivalent in size to a one-half gastropod molecule. However, it must be structurally different as this molecule does not form the 100S polymer. Structural differences have been illustrated between cephalopod and gastropod hemocyanins. When mixtures of dissociated Loligo pealei and Helix pomatia hemocyanin were placed in reassociating conditions, each species reassociated specifically (Van Bruggen and Fernandez-Moran, 1966). If a "collar" exists in the whole 60S molecule of the chiton, Katherina tunicata, which electron microscopy does suggest, it must be situated symmetrically so as to be present at both ends of the molecule and prevent further polymerization and cause stabilization of the whole 60S struc-

ture. If eight "domains" are assumed for Katherina hemocyanin subunits, the two arrangements shown in Figure 18 B and C are possible. This would place five "collar" pieces at each end. This would correlate with electron micrographs which show all end-on views to contain an apparent five-fold structure within the cylinder. The presence of only seven spherical units could be arranged within the model shown in Figure 18C as well.

All structural models with this "collar" structure suggest heterogeneity among "domains" in the subunit. Such heterogeneity is also suggested here by the proteolysis studies for Katherina tunicata hemocyanin. Although largely speculation, it is possible that the 56,000 molecular weight fragments are part of a "collar" and the 90,000 molecular weight material is a "di-domain" which comprises the body of the three-tiered cylinder. Evidence against this speculation are the results of the digestion experiment to remove the 56,000 dalton material and induce polymerization. Under the conditions reported, no polymerization occurred. Nevertheless, the structure of a "collar" in the polyplacophoran molecule may be such that removal does not result in a molecule polarized so as to encourage end-on aggregation.

This work on the structure of Katherina tunicata hemocyanin is significant in that an apparently pure 56,000 molecular weight component has been isolated.

Also, an apparently different fragment with a molecular weight of 90,000, which is about twice the value of the 56,000 fragment, has been purified. What is the actual role of these fragments in the whole molecular structure of this hemocyanin? What is the relationship between these fragments - are they truly different, or clipped by enzymes in such a way as to appear different? Amino acid analysis of the fragments may give an indication. Are these the only components of the subunit, or are there other "domains" that are quickly degraded to low molecular weight fragments? In interpreting digestion experiments, it would be of great interest to be able to follow the actual process - to know what the starting material is and how each of the fragments arise. An approach to this would be to stop a digestion experiment early - before too many pieces are generated - separate the fragments and then continue the digestion course on the isolated pieces, monitoring the formation of cleavage products. This and other approaches need to be taken to decipher the subunit architecture of such macromolecular structures as hemocyanins.

SUMMARY

Katherina tunicata native hemocyanin exists as a 60S molecule with an approximate molecular weight of 4×10^6. Dissociation behavior of this molecule in the presence of $MgCl_2$, and in the absence of $MgCl_2$ and presence of EDTA is described and compared to other classes of Mollusca. In the presence of $MgCl_2$, *Katherina* hemocyanin dissociates at a lower pH (pH 9) than the hemocyanin of the cephalopod, *Loligo pealei*, and of the gastropods, *Helix pomatia* and *Murex trunculus* (pH 10). In the absence of $MgCl_2$ and presence of EDTA, *Katherina* hemocyanin dissociation is very similar to the dissociation of the hemocyanin of *Loligo pealei*. Both of these hemocyanins dissociate just on the alkaline side of pH 7.0. The gastropod hemocyanins cited in this thesis dissociate in the absence of divalent cations at a higher pH than the cephalopod or polyplacophoran hemocyanin, between pH 7.5 and 8.5.

In order to dissect the subunit of *Katherina tunicata* hemocyanin, digestion experiments were performed. A one hour subtilisin digestion of the subunit of *Katherina* hemocyanin yielded two peaks by Sephadex G-200 chromatography. Fraction I contained a 56,000 dalton doublet and a 90,000 molecular weight fragment by SDS gel electrophoresis analysis. Fraction II con-

tained many molecular weight pieces between 43,000 and 51,000. The two Fraction I components can be separated by DEAE anion exchange chromatography and each appear rather pure by SDS and regular disc gel electrophoresis.

A one hour trypsin digestion gives two peaks by Sephadex G-200 chromatography. Fraction I elutes with an apparent molecular weight of 250,000 and is heterogeneous by SDS and regular disc gel analysis. Fraction II contains primarily a 56,000 dalton doublet by SDS analysis. Redigestion of Fraction I by subtilisin yields the same SDS pattern found for a subtilisin digest of the subunit of Katherina hemocyanin, with the absence of any 56,000 dalton material.

It therefore appears on the basis of this digestion data, that the subunit of Katherina tunicata hemocyanin is heterogeneous. That is, the subunit is possibly composed of more than one type of functional unit, or oxygen binding site.

Figure 1. Schematic diagram of the dissociation process of the 100S gastropod hemocyanin molecule. The 100S molecule (A) dissociates successively to one-half (60S) (B), one-tenth (20S) (C), and one-twentieth (11S) (D) molecules. (From Mellema and Klug, 1972; Siezen and Van Bruggen, 1974; Van Bruggen et al., 1977).

FIGURE 1

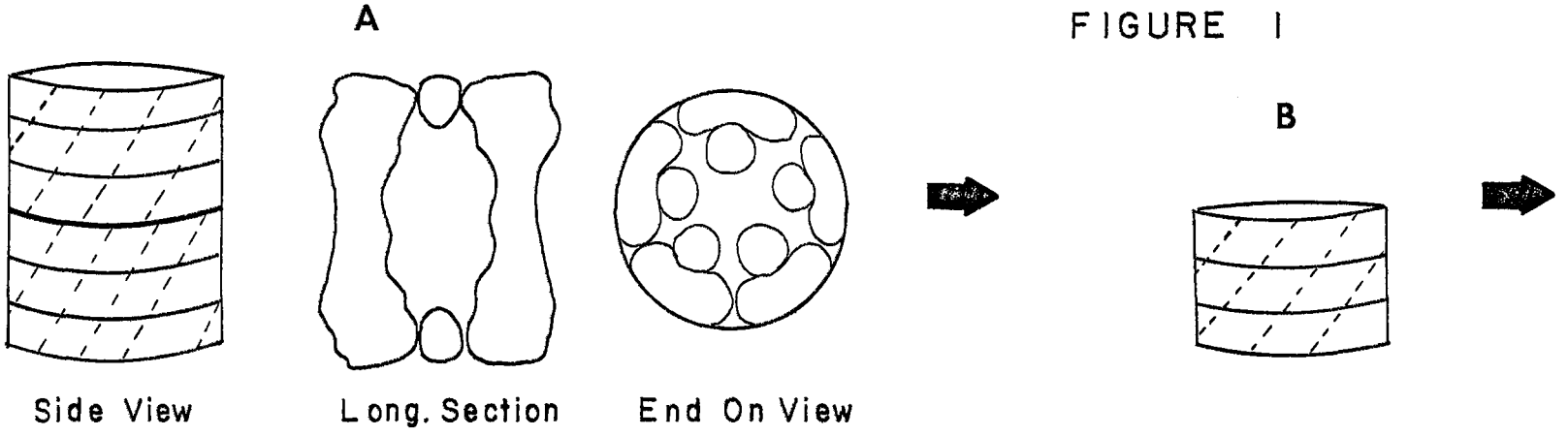

A
B
Side View
Long. Section
End On View
100S WHOLE MOLECULE
60S ONE-HALF
C
collar
wall
D
20S ONE-TENTH
11S ONE-TWENTIETH

Figure 2. Chromatography of Katherina tunicata hemocyanin on Sepharose 4-B. Buffer = 0.05 M Tris-HCl, 0.02 M NaCl, 0.01 M $MgCl_2$, pH 7.4. (A) Limulus polyphemus hemocyanin, MW= approximately 3×10^6, (B) Cancer magister hemocyanin, MW= 9.4×10^5, (C) Thyroglobulin, MW= 6.7×10^5.

FIGURE 2

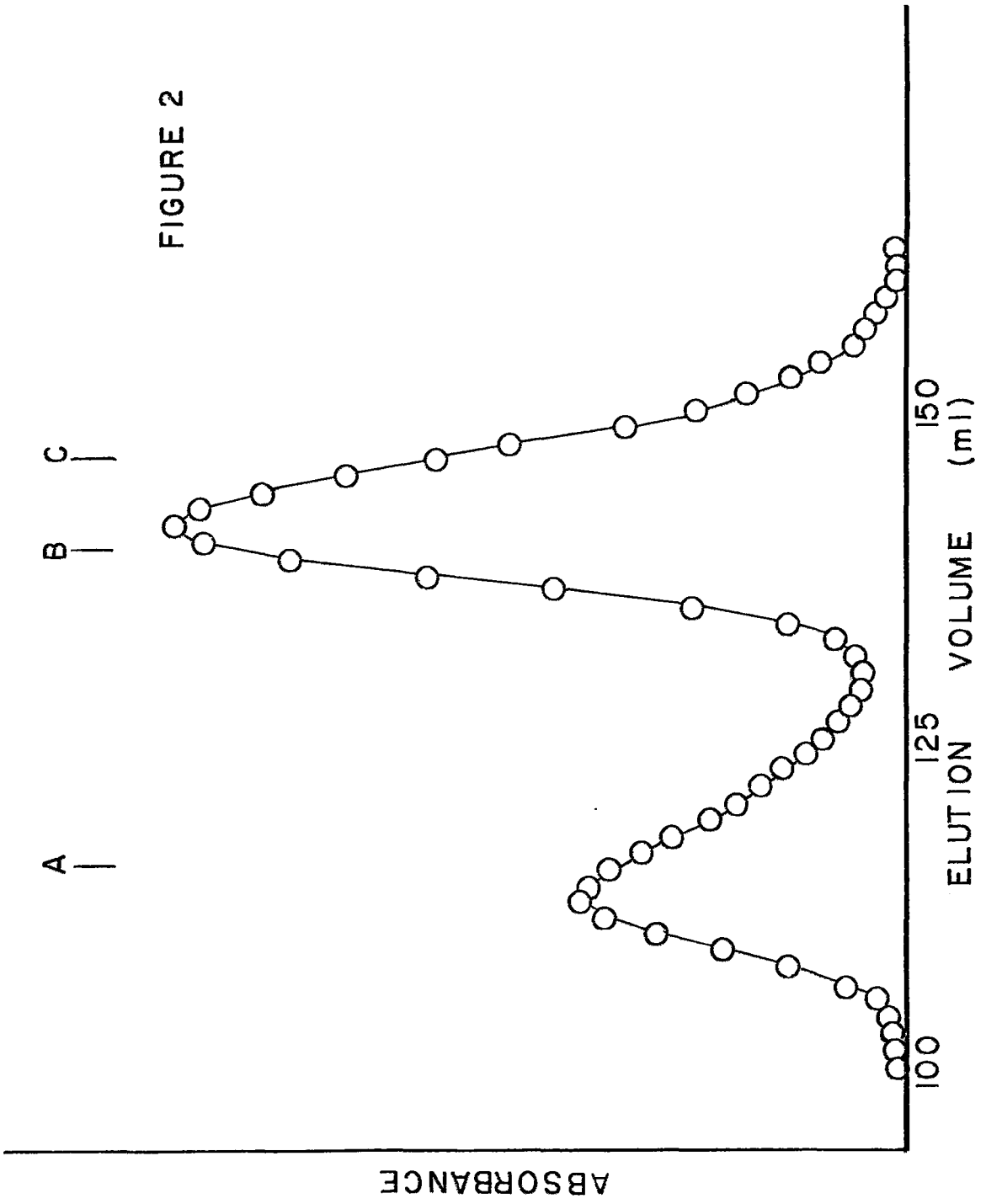


Figure 3. Diagram of sedimentation coefficient ($S_{20,w}$) of Katherina tunicata hemocyanin versus pH in the presence of 0.01 M $MgCl_2$. The solid circles represent the sedimenting boundary present in the greatest amount.

FIGURE 3

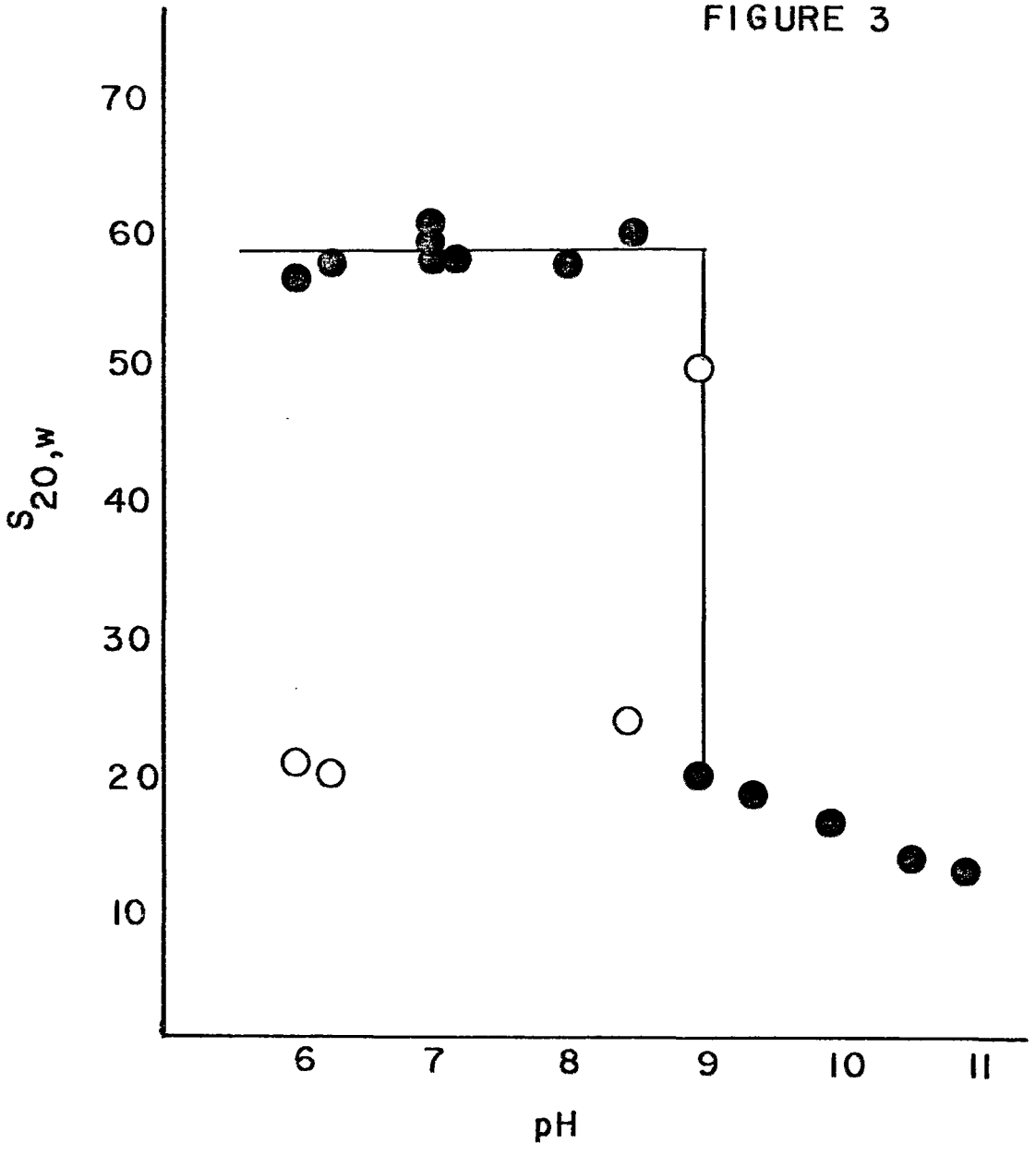


Figure 4. Diagram of sedimentation coefficient ($S_{20,w}$) of Katherina tunicata hemocyanin versus pH in the absence of $MgCl_2$ and in the presence of 0.01 M EDTA. The solid circles represent the sedimenting boundary present in the greatest amount.

FIGURE 4

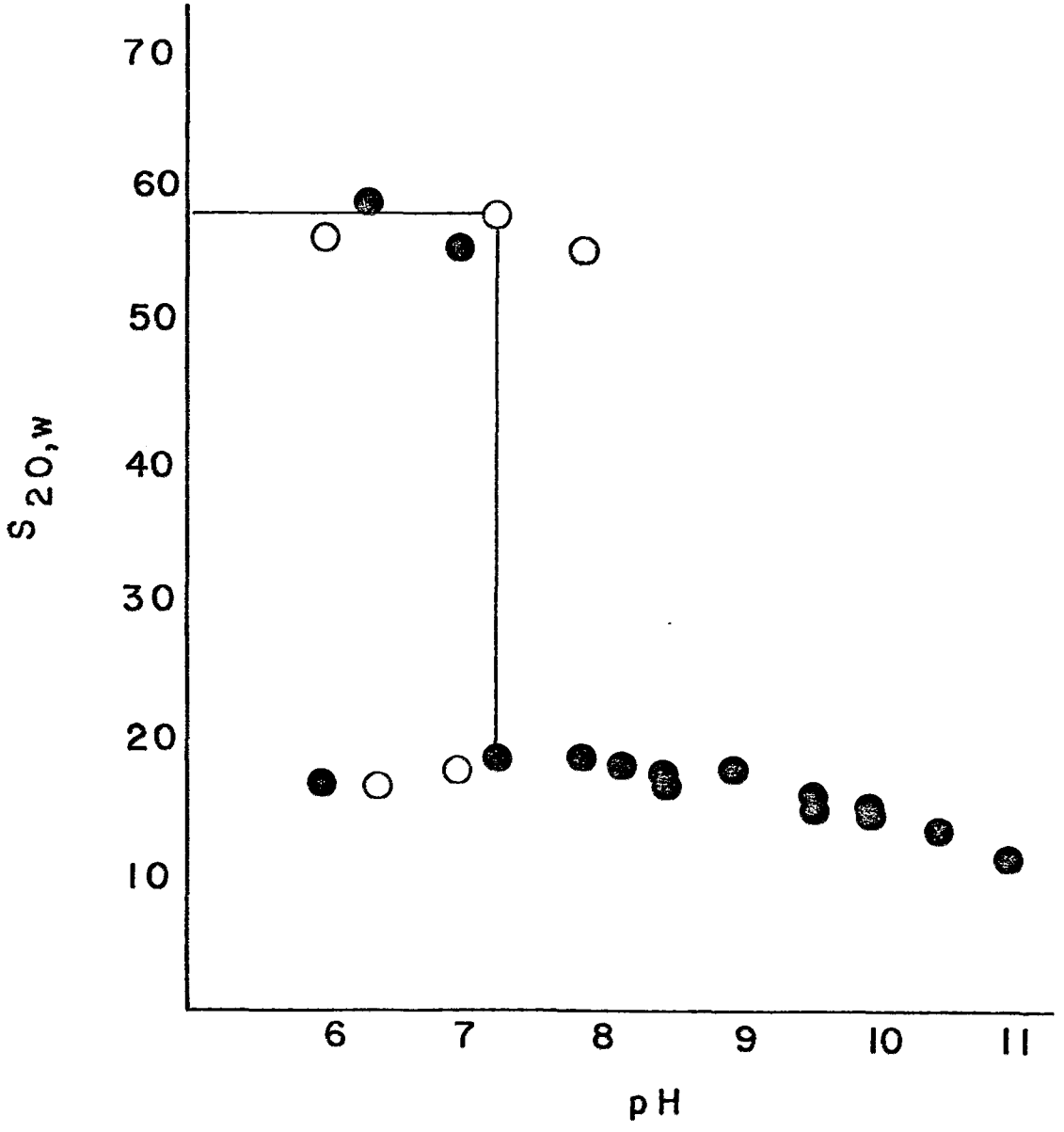


Figure 5. Regular disc gel electrophoresis of Katherina tunicata hemocyanin. (A) pH 8.9, 7.5% acrylamide, (B) pH 10.5, 5% acrylamide.

FIGURE 5

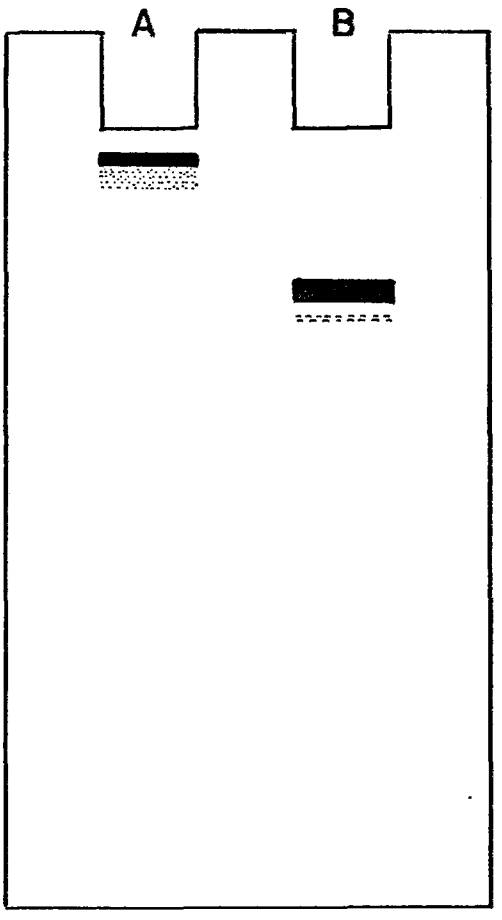


Figure 6. SDS slab gel electrophoresis time course analysis of Katherina tunicata hemocyanin (pH 8.5, 17S) digested with subtilisin. (A) pH 8.5 hemocyanin of K. tunicata, (B) 30 second digestion, (C) 1 minute, (D) 3 minutes, (E) 5 minutes, (F) 10 minutes, (G) 15 minutes, (H) 30 minutes, (I) 1 hour, (J) 2½ hours.

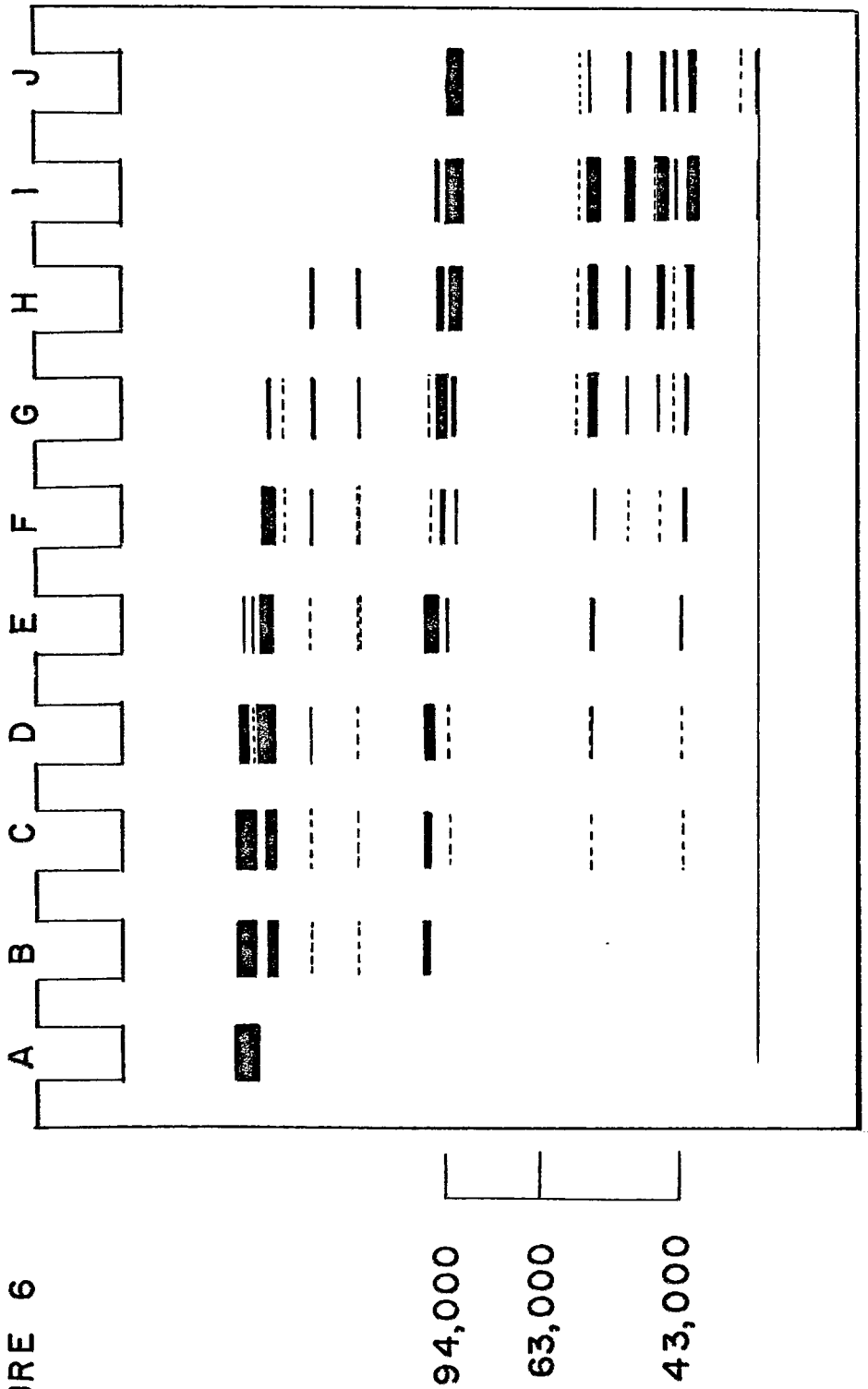


FIGURE 6

Figure 7. Chromatography of a one hour subtilisin digestion of Katherina tunicata hemocyanin on Sephadex G-200. △ = absorbance at 280 nm, ○ = absorbance at 345nm, (A) Bovine serum albumin, (B) ovalbumin, (C) α chymotrypsinogen A.

FIGURE 7

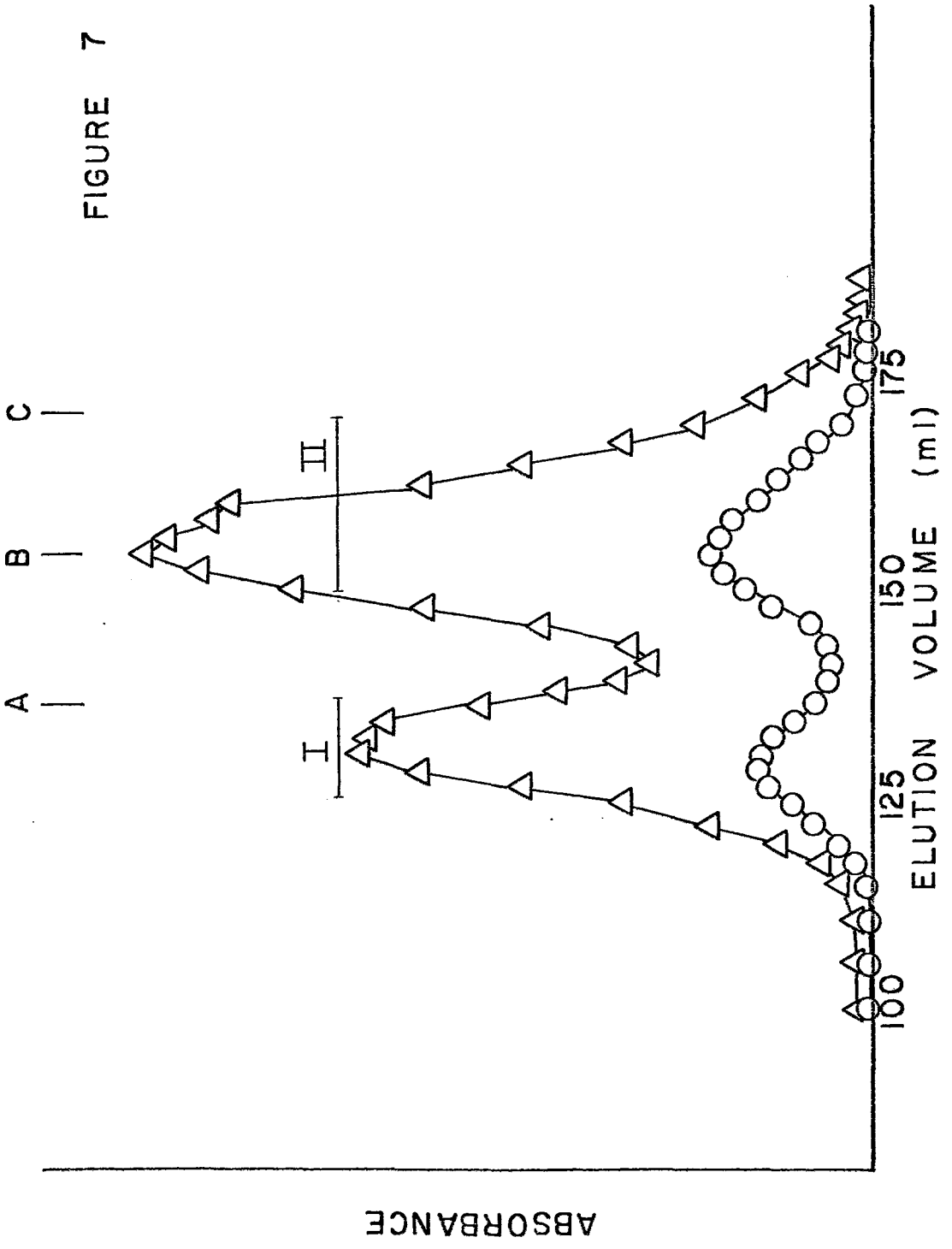


Figure 8. SDS slab gel electrophoresis of the products isolated by Sephadex G-200 chromatography of a one hour subtilisin digest of Katherina tunicata hemocyanin. (A) One hour subtilisin digest before application to Sephadex G-200, (B) subtilisin G-200 Fraction I, (C) subtilisin G-200 Fraction II, (D) leading edge of subtilisin G-200 Fraction I.

FIGURE 8

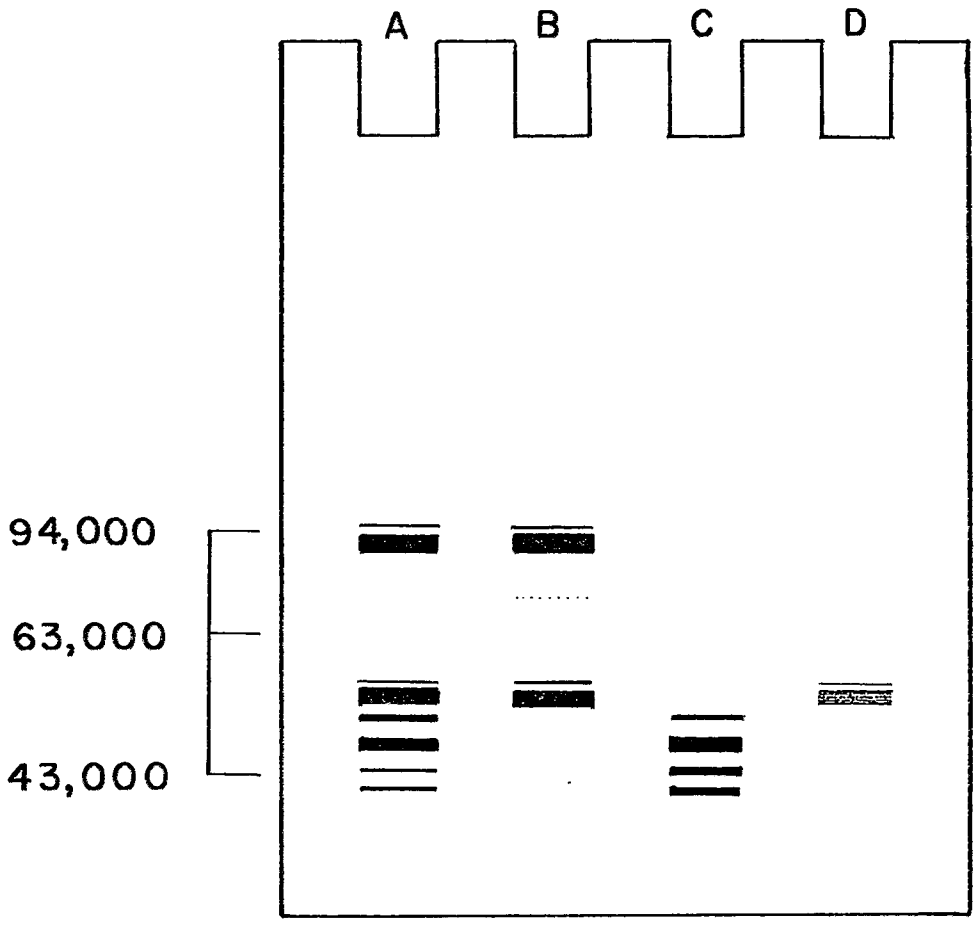


Figure 9. Regular pH 8.9 disc gel electrophoresis of the products isolated by Sephadex G-200 chromatography of a one hour subtilisin digest of Katherina tunicata hemocyanin. 7.5% acrylamide. (A) Subtilisin G-200 Fraction I, (B) subtilisin G-200 Fraction II.

FIGURE 9

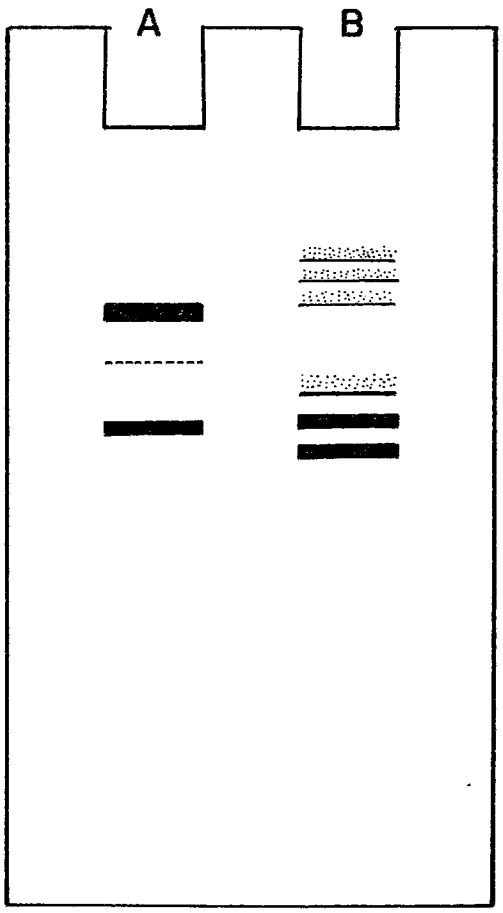


Figure 10. DEAE ion exchange chromatography of Katherina tunicata hemocyanin subtilisin G-200 Fraction I.

△ = absorbance at 280 nm, ○ = absorbance at 345 nm.

FIGURE 10

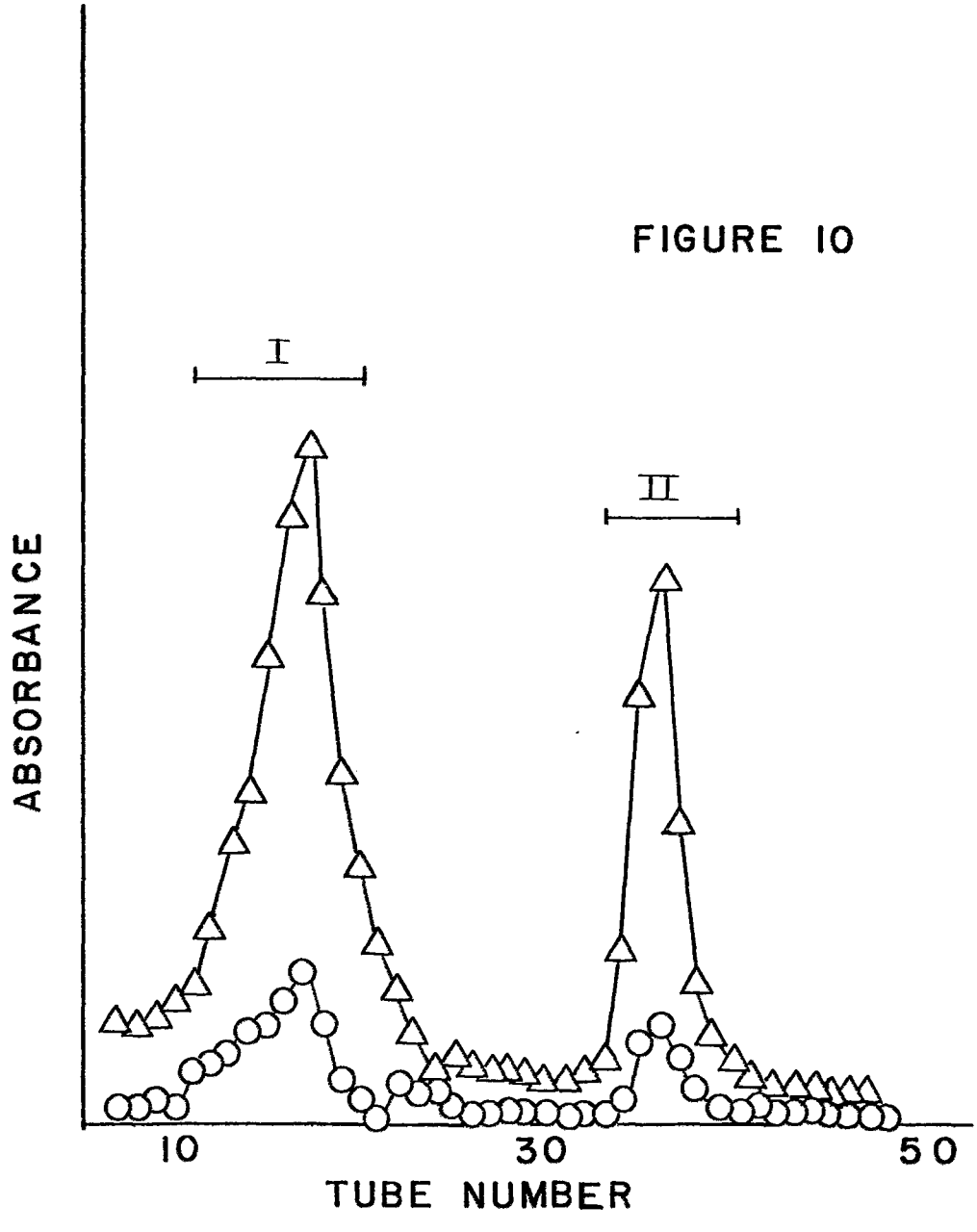


Figure 11. SDS slab gel electrophoresis of the fragments of Katherina tunicata hemocyanin isolated by DEAE ion exchange chromatography of the subtilisin G-200 Fraction I. (A) Subtilisin G-200 Fraction I, (B) DEAE Fraction I, (C) DEAE trailing shoulder of Fraction I, (D) DEAE Fraction II.

FIGURE II

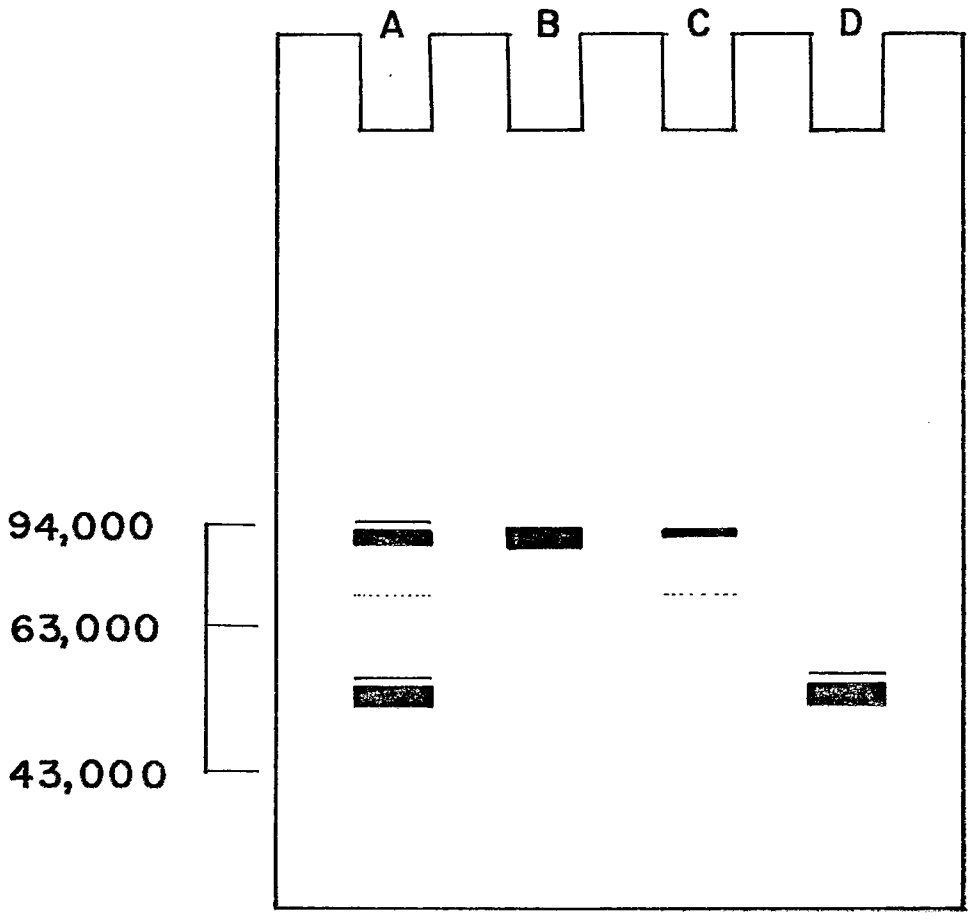


Figure 12. Regular pH 8.9 disc gel electrophoresis of the fragments of Katherina tunicata hemocyanin isolated by DEAE ion exchange chromatography of the subtilisin G-200 Fraction I. 7.5% acrylamide. (A) Subtilisin G-200 Fraction I, (B) DEAE Fraction I, (C) DEAE Fraction II, (D) DEAE Fractions I + II.

FIGURE 12

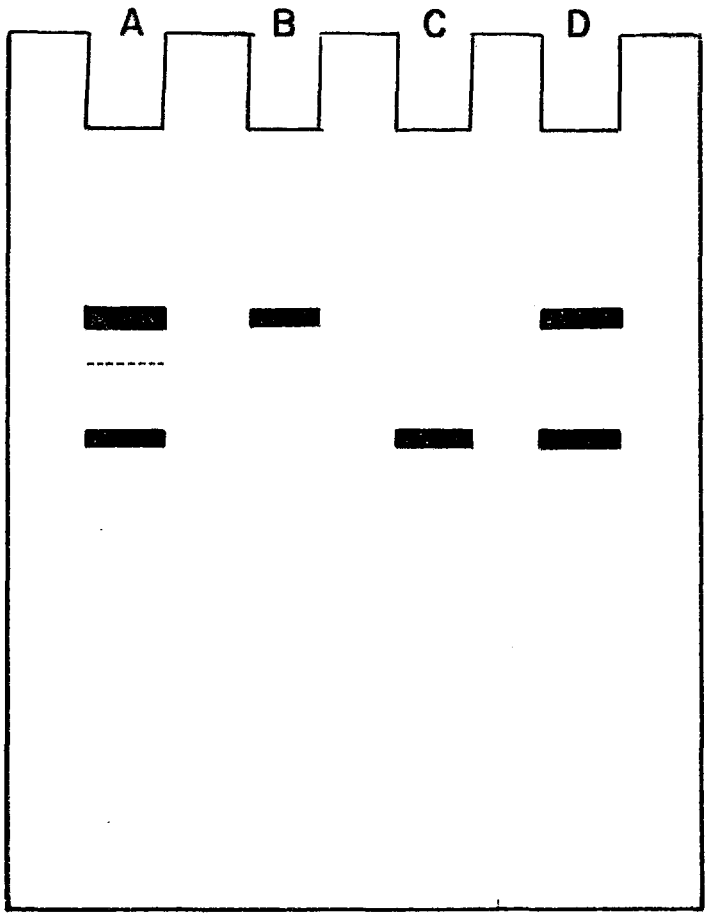


Figure 13. SDS slab gel electrophoresis time course analysis of Katherina tunicata hemocyanin (pH 8.5, 17S) digested with trypsin. (A) pH 8.5 hemocyanin of K. tunicata, (B) 1 minute, (C) 5 minutes, (D) 10 minutes, (E) 15 minutes, (F) 30 minutes, (G) 45 minutes, (H) one hour.

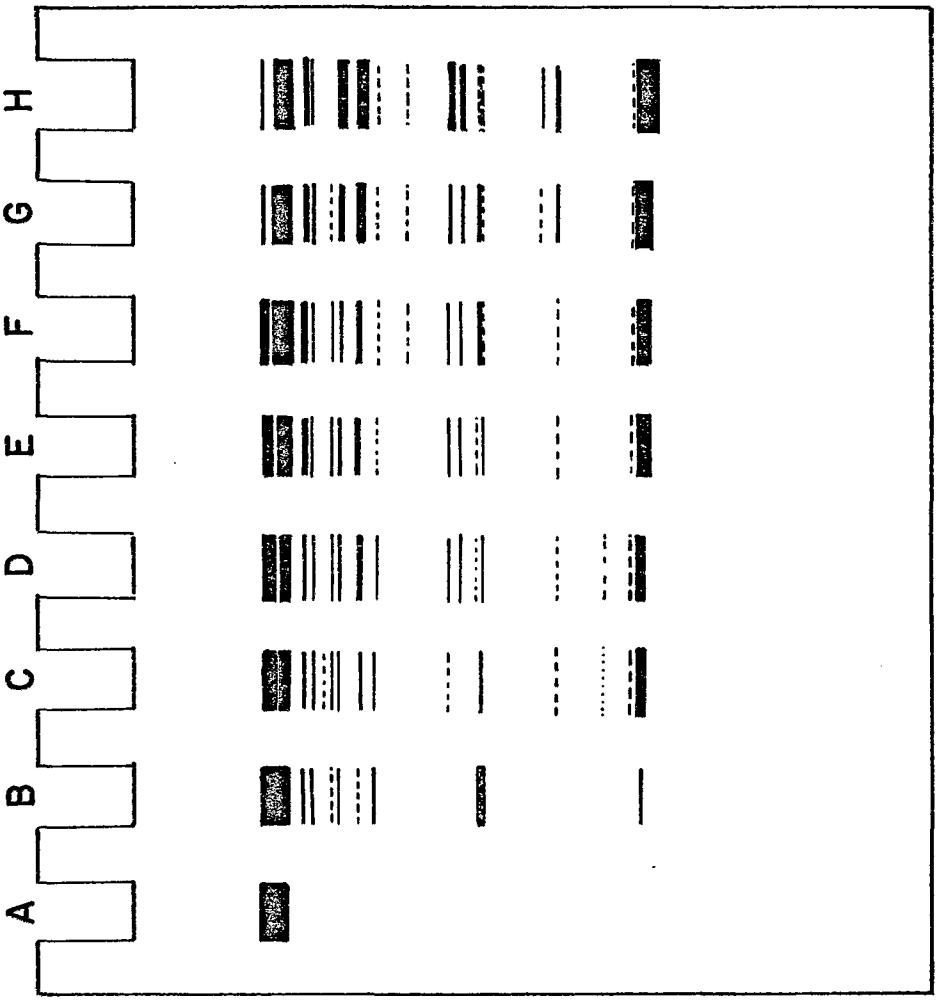


FIGURE 13

Figure 14. Chromatography of a one hour tryptic digestion of Katherina tunicata hemocyanin on Sephadex G-200. △ = absorbance at 280 nm, ○ = absorbance at 345 nm. (A) Blue dextran, (B) bovine serum albumin, (C) ovalbumin, (D) ∝ chymotrypsinogen A.

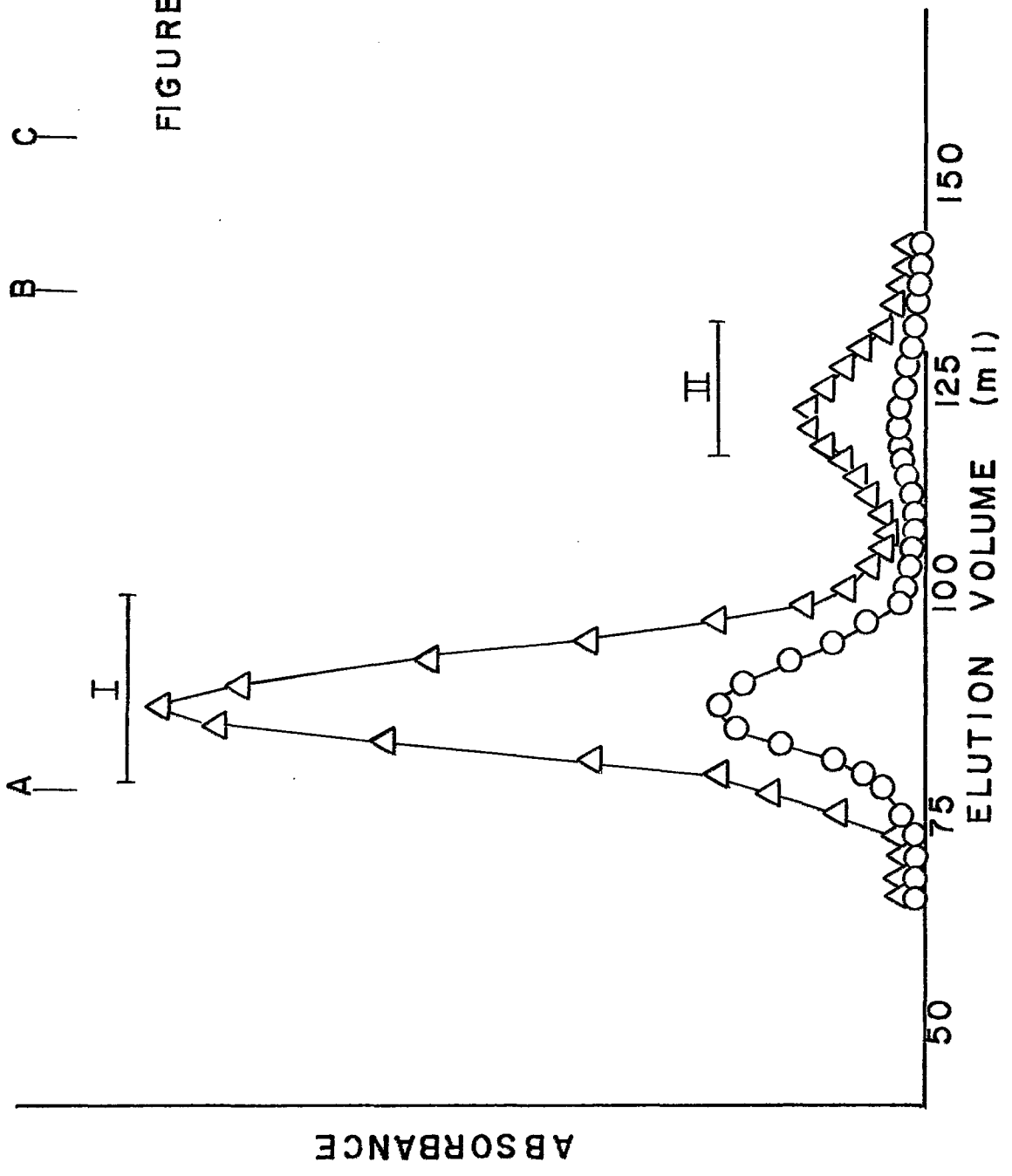


FIGURE 14

Figure 15. SDS Slab gel electrophoresis of the products isolated by Sephadex G-200 chromatography of a one hour tryptic digest of Katherina tunicata hemocyanin. (A) one hour trypsin digest befor application to Sephadex G-200, (B) trypsin G-200 Fraction I, (C) trypsin G-200 Fraction II.

FIGURE 15

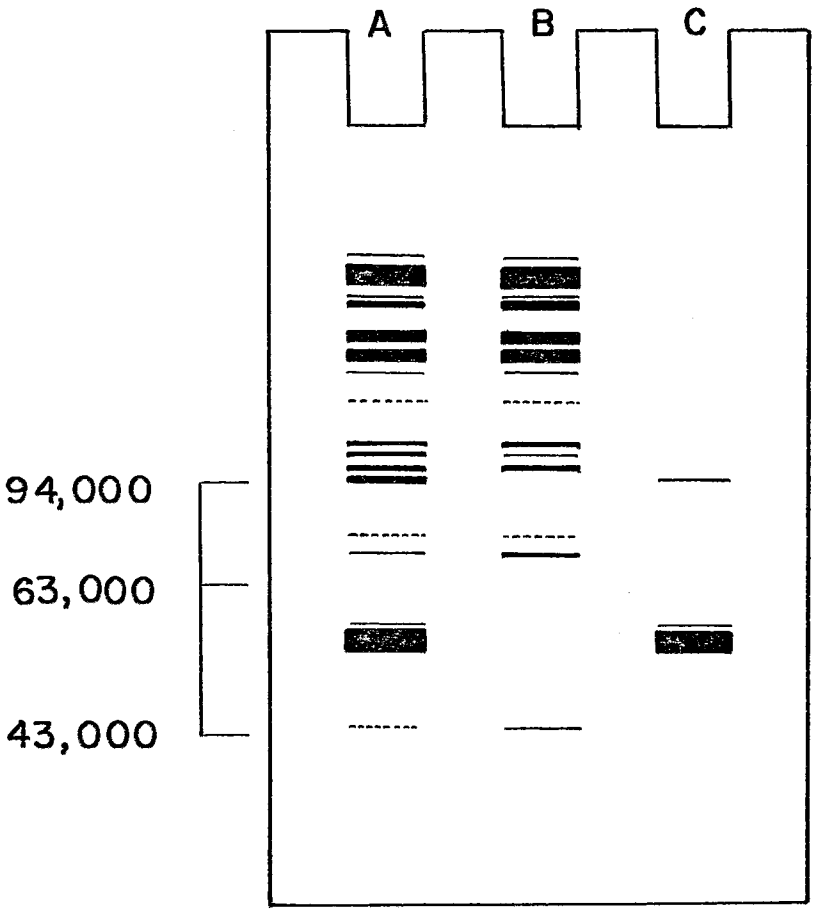


Figure 16. Regular pH 8.9 disc gel electrophoresis of the products isolated by Sephadex G-200 chromatography of a one hour tryptic digest of Katherina tunicata hemocyanin. 7.5% acrylamide. (A) Trypsin G-200 Fraction I, (B) Trypsin G-200 Fraction II.

FIGURE 16

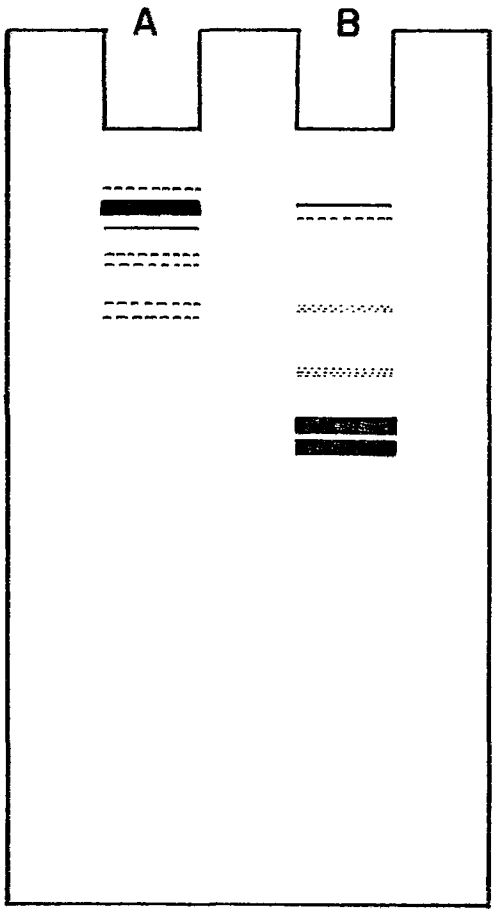


Figure 17. SDS slab gel electrophoresis of redigestion of Katherina tunicata hemocyanin trypsin G-200 Fraction I. (A) Trypsin G-200 Fraction I, (B) redigestion of trypsin G-200 Fraction I with subtilisin for one hour, (C) one hour subtilisin digestion of Katherina tunicata hemocyanin.

FIGURE 17

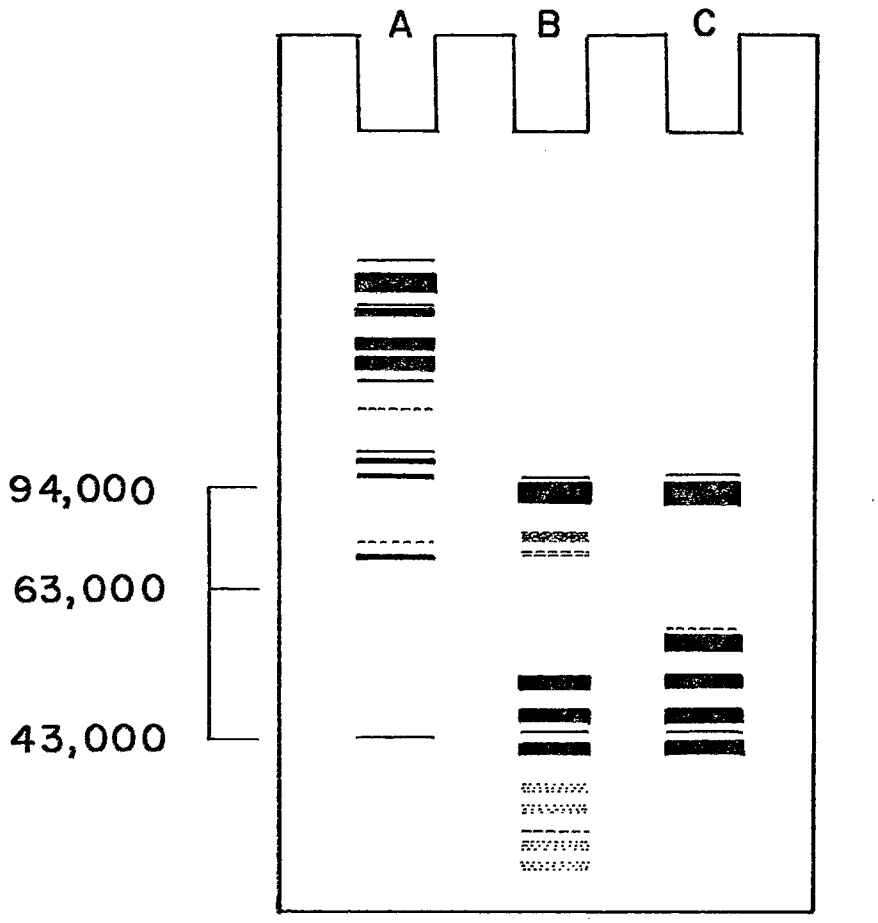
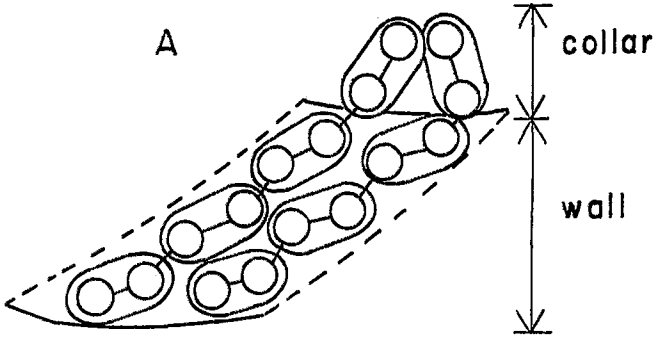
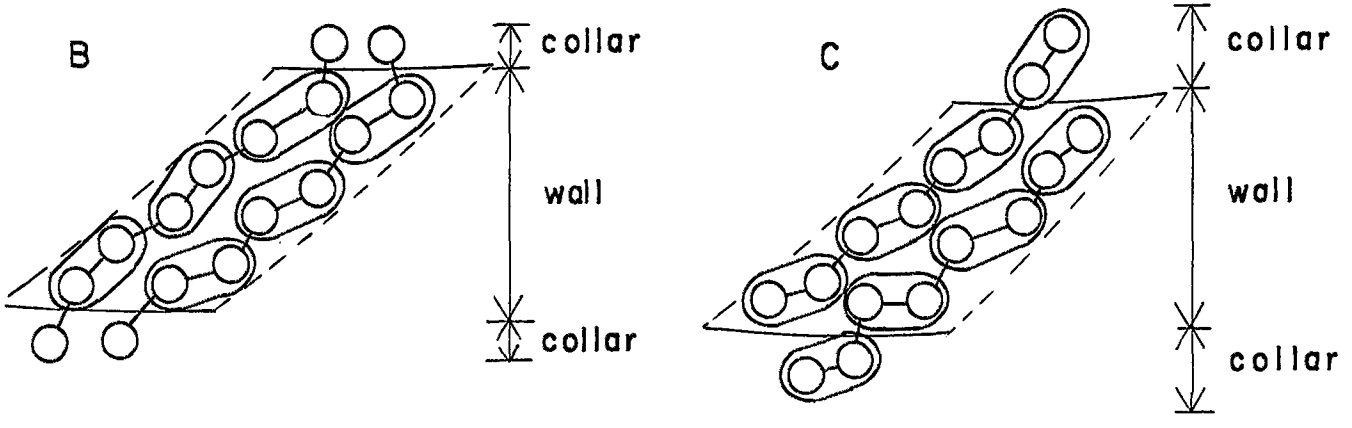


Figure 18. Suggestions for the arrangement of the one-tenth (11S) molecules of Katherina tunicata hemocyanin within the one-fifth (20S) molecule. (A) Model of the one-tenth (20S) gastropod hemocyanin molecule, according to Van Bruggen et al (1977), (B and C) models of K. tunicata one-fifth (20S) molecule.

FIGURE 18



Proposed model of gastropod
20S hemocyanin molecule (Van Bruggen et al, 1977)



Possible models of polyplacophoran 20S hemocyanin molecule

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