The Structure of Arthropod and Mollusc Hemocyanins*

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SUMMARY

The hemocyanins from molluscs and from arthropods differ in the size of their polypeptide chains. A variety of physical techniques including sodium dodecyl sulfate polyacrylamide gel electrophoresis and column chromatography in sodium dodecyl sulfate and guanidine HCl indicate that the polypeptide chain of mollusc hemocyanin has a molecular weight of 290,000. These results were corroborated by quantitative end group analyses. Several experiments designed to count the number of tryptophan and methionine-containing peptides in the hemocyanin from the whelk Busycon canaliculatum indicate that sequence homology within the polypeptide chain of the mollusc hemocyanins accounts for their large size. Digestion of the native protein with subtilisin produces a 50,000-dalton fragment in high yield which corresponds to one binding site for oxygen.

On the other hand, the polypeptide chain molecular weight of lobster hemocyanin is 76,000 to 78,000 and this seems to be a general property of all arthropod hemocyanins. The pigment from lobster consists of two very similar polypeptide chains which are not present in equal amount. Analysis of the cysteine-containing and of the tryptophan-containing tryptic peptides confirms the value of the molecular weight. However, separation of fragments which contain methionine indicates that there is sequence homology within the polypeptide chain of this protein. It is concluded that the molluscs and arthropod hemocyanins have little structural similarity.

The second largest class of invertebrate respiratory pigments, the hemocyanins, are of interest because of their occurrence in two very diverse phyla: the arthropods and the molluscs. While copper-containing proteins are widespread in nature, functioning both as enzymes and as components of electron transfer systems, it is remarkable that their role as oxygen carriers is unique to the members of these phyla.

The hemocyanins are large molecules, with masses of 1 to 9 \( \times 10^4 \) daltons, which contain many copper atoms and many \( O_2 \) binding sites. A central question concerning these molecules is the size of the polypeptide chains which compose them. In this paper evidence is presented to answer this question for the mollusc and the arthropod pigments. The results show that arthropod hemocyanin is made of small polypeptide chains (75,000 daltons) each of which can bind one \( O_2 \) molecule, while the mollusc hemocyanins are composed of giant polypeptide chains (300,000 daltons) each with six \( O_2 \) binding sites.

MATERIALS AND METHODS

The sources of all reagents and their preparation have been described (1). 

Chemical Modifications—Performic acid oxidation and reduction and alklylation with iodoacetamide were carried out as previously described (1). Cleavage with CNBr was performed essentially as described by Bucci et al. (2), and the extent of reaction was estimated by the disappearance of methionine after acid hydrolysis as well as by the recovery of homoserine and homoserine lactone.

Periodate oxidation of the sugar moieties of the hemocyanins was performed exactly as described by Komatsu et al. (3). After stopping the reaction with polyethylene glycol, the protein was prepared for Na dodecyl-SO\(_4\) polyacrylamide gel electrophoresis as described below.

Protein modification by sertaxylation was performed as described by Klotz (4), and acetylation experiments were carried out according to Bucci et al. (5). The disruptive effects of these reagents were determined by Na dodecyl-SO\(_4\) polyacrylamide gel electrophoresis.

Modification of protein sulfhydryl groups with ethyleneimine after reduction was performed as described by Cole (6), except that 6 \( n \) guanidine HCl was used to denature the protein.

The methionine residues of Homarus hemocyanin were labeled with iodomethyl-l-\(^{14}\)C]acetamide (5 to 12 mCi/mmol) (New England Nuclear) by a modification of the method of Wilkinson (7) as discussed by Platt et al. (8). Modified proteins were dialyzed against 1% ammonium bicarbonate to remove excess reagents before digestion with trypsin.

Following two-dimensional paper electrophoresis and chromatography, autoradiographs were prepared with Kodak RP Royal X-Omat x-ray film.

Chemical Analyses—Amino sugars were quantitated on the short column (12 cm) of a Beckman model 120C amino acid analyzer using the pH 4.25 citrate buffer of the long column. Hydrolyses were carried out for several times between 4 and 20 hours in 3 \( n \) HCl with \( \beta \)-alanine as an internal standard. In this system, \( \beta \)-alanine elutes at 30 min, glucosamine at 44 min, and galactosamine at 49 min.

Neutral sugars were assayed by the phenol-sulfuric acid method of Dubois et al. (9) using mannose as standard. By scaling the volumes used by a factor of 5, 1 \( \mu \)g of sugar gives an absorbance of 0.1 at 489 nm. Sialic acid was determined by the colorimetric assay of Warren (10).

Isolation of the neutral sugars from Busycon hemocyanin was performed as described by Siro (14) following hydrolysis of 10 mg of protein in 1 \( n \) H\(_2\)SO\(_4\) for 8 hours at 110°C. The sugars were then converted to their O-trimethylsilyl derivatives as described by Reinhold (15) and were analyzed on a Perkin-Elmer 990 chromatograph.
Amino acid analyses were performed as previously described (1). Half-cystine and methionine were determined as cysteic acid and methionine sulfoxide following performic acid oxidation. The value for half-cystine was corroborated by determining carboxymethylcysteine following reduction and alkylation with iodoacetamide. Mercaptoacetic acid (0.1%) was included in these hydrolysates to protect this derivative from oxidation.

Tryptophan was determined by the method of Edelhoch (11) in 0.1 guanidine HCl, or by amino acid analysis following hydrolysis in 6 M guanidine HCl or by amino acid analysis following hydrolysis in 3 N p-toluenesulfonic acid (12).

Determination of homoserine in hydrolysates of CNBr-reacted material was performed as described by Tang and Hartley (13). The NH$_2$-terminal amino acid of the various proteins under study was identified as the dansyl derivative on polyamide plates as described by Weiner et al. (16). These results were checked by the cyanate method of Stark and Smyth (17), which was also used to obtain a quantitative estimate of the NH$_2$-terminal residue. To account more carefully for losses incurred in transfers when using this method, carbamyl norleucine was included in the cyclization step. This derivative was prepared as described by Stark and Smyth (17) for the amino acid leucine and purified from any under-derivatized material on Aminex AG 50W-X2 in H$_2$O. Quantitation of the derivative in a stock solution was obtained after hydrolysis in 6 M HCl for 100 hours, which converted it back to the free amino acid.

The COOH-terminal amino acid(s) was determined by enzymatic digestion of the denatured protein with carboxypeptidase B. Digestions were carried out in 0.1 Na dodecylSO$_4$, 0.2 m N-ethylmorpholine acetate at pH 8.0 to 8.5; the substrate to enzyme ratio was generally kept at 500:1 (w/v). The substrate was prepared by dialyzing in Na dodecylSO$_4$-dialysis buffer. Digestions were carried out in 5 to 10 ml containing $\beta$-alanine as an internal standard. Aliquots (0.5 to 1.0 ml) were then removed at various times and pipetted into conical centrifuge tubes containing 0.2 ml of 1 N HCl to stop the reaction. The precipitated protein was centrifuged down and the supernatant was applied directly to the cation exchanger.

Polyacrylamide Gel Electrophoresis—Na dodecylSO$_4$ polyacrylamide gel electrophoresis was carried out as described by Weber et al. (18) with the borate-acetate buffer, pH 8.5, of Davies and Stark (19). Gel electrophoresis at pH 4.5 in 5 m urea was performed in 0.07 M b-alanine-acetic acid buffer as described by Epper (20). Methods of Isolation and Identification of Peptides—Separation of peptides generally included a combination of gel filtration and ion exchange chromatography. Purity was assessed at each step by spotting the peptides on pre-wetted cellulose thin layer plates and subjecting them to electrophoresis at pH 6.0 to 6.5 (0.5% pyridine with glacial acetic acid), 1000 volts for 40 min, or at pH 3.5 (5% pyridine, 0.5% acetic acid), 1000 volts for 60 min, in a Savant flat-bed electrophoresis apparatus. Paper electrophoresis was carried out at 2000 to 3000 volts on Whatman No. 3MM paper in tanks (Savant) containing Varsol as coolant. Three buffer systems were used: pH 6.0 (10% pyridine, 1% acetic acid); pH 5.7 (pH 6.0 buffer titrated with glacial acetic acid); pH 4.9 (8% glacial acetic acid, 2% formic acid). Peptides were detected with ninhydrin-cadmium reagent (21) or with aniline-xylene reagent (22). Tryptophan-containing peptides were located with Ehrlich’s reagent (23), and glycopeptides were located by the silver nitrate reagent of Trevelyan et al. (24).

Enzymatic Digestions—Digestions with trypsin were done in 1% NH$_4$HCO$_3$, 1 m urea for 12 to 18 hours at room temperature with a trypsin to protein ratio of 1:100. An estimate of the completeness of the reaction was obtained by treating an aliquot of the digested protein with carboxypeptidase B for 2 hours and analysing for the lysine and arginine released per mol of protein. Digestion of native Buscon hemocyanin was carried out as described by Lontie et al. (25) for Helix pomatia hemocyanin, except that the reaction mixture was buffered with 0.1% Tris-HCl, pH 8.2. After 6 hours the reaction was stopped by the addition of phenylmethane sulfonyl fluoride, and the mixture was filtered on Sephadex G-200 in the same buffer. Digestion of denatured hemocyanin with pronase was done by suspending salt-free, reduced, and alkylated protein in 1% ammonium bicarbonate and adding 1% (w/v) pronase. An equal amount was added after 8 hours and the digestion continued for a total of 18 to 20 hours. The lyophilized digestion mixture was completely dissolved in 0.2 m ammonium bicarbonate with a few drops of ammonia. A drop of toluene was included as a preservative during the digestion.

Carboxypeptidase Y was the gift of Dr. J. Manning of Rockefeller University, and was used as described by Hayashi et al. (26); the ratio of substrate to enzyme was 200:1, and a blank consisting of enzyme alone was also run.

Purification Techniques—A brief description of the purification procedures which were used for each pigment is given below. It should be noted that the protease inhibitors phenylmethane sulfonyl fluoride and sodium tetrathionate, both at 1 mM, were added to freshly drawn blood to prevent proteolytic cleavage by enzymes present in the blood or in tissue contaminants. Buffer A, which was used in all gel filtration steps, consisted of 0.1 m NaH$_2$PO$_4$, 0.1 m KCl, and 1 mM EDTA, pH 7.0.

Buscon canaliculatum—Specimens of this gastropod mollusc were obtained from Woods Hole Oceanographic Center, Woods Hole, Mass., and were bled by foot puncture. The hemolymph was centrifuged for 10 min at 8000 x g to remove particulate matter, and then was concentrated in a Beckman-Spinco model L centrifuge (80,000 x g, 2 hours). Final purification was achieved by gel filtration on Sepharose 4B and a trace of hemocyanin (200 to 500 mg) could be obtained from a single animal.

Loligo pealei—Hemolymph from the cephalopod mollusc Loligo was obtained by heart puncture at Woods Hole and shipped on ice to Cambridge. The purification scheme was the same as for Buscon. Approximately 50 mg could be obtained from an individual animal.

Octopus vulgaris and Octopus dofleini—Hemocyanin from the cephalopod mollusc O. vulgaris from the Zoological Station at Naples was generously provided by Dr. M. Brunori. After purification by preparative ultrafiltration, the material was freeze-dried in the presence of sucrose as described by Ghiratti (27) and shipped as powder to Cambridge. The mixture was dissolved in Buffer A and dialyzed for 24 hours before final purification on Sepharose 4B.

Unpurified hemolymph from O. dofleini from the North Pacific was collected by Dr. B. Shapiro and shipped as the lyophilised powder. This material was dissolved in Buffer A, centrifuged to remove insoluble matter, and purified by gel filtration.

Arthropods—The decapod crustaceans Libinia emarginata and Cancer irroratus, and the merostome Limulus polyphemus were obtained from Woods Hole, and were bled by removing a leg. The hemolymph was allowed to clot on ice for 30 min in the presence of the protease inhibitors phenylmethane sulfonyl fluoride and sodium tetrathionate (1 mM) and the coagulated material was then centrifuged at low speed. The supernatant, which contained the hemocyanin, was concentrated by high speed centrifugation and further purified on Sepharose 4B.

The lobster Homarus americanus was purchased from local fish stores and bled by heart puncture. This method was chosen to obtain the maximum yield from each animal (75 to 200 mg per 1 pound animal), but the actual purification was identical to that employed for the other crustaceans.

RESULTS

Polypeptide Chain Sizes—The molecular weights of the polypeptide chains of the arthropod and mollusc hemocyanins were estimated by gel filtration and by gel electrophoresis in denaturing solvent. The results of these experiments are shown in Figs. 1 to 3 for the arthropod proteins, and in Figs. 4 and 5 for the mollusc pigments; no chemical modification altered these patterns. The results are clear; the polypeptides of mollusc hemocyanins have molecular weights of 2.9 to 3.0 x 10$^5$, while those of arthropod hemocyanins have masses of 75,000 to 80,000 daltons. Several of the arthropod hemocyanins consisted of two polypeptides, appearing as two closely spaced bands after gel electrophoresis in

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slices, the mass ratio of the larger to the smaller polypeptide was 1.5 to 1.0. Cross-linking experiments done on native lobster hemocyanin was estimated by amino acid analysis of gel fractionation of peptides obtained from alkylated, but not reduced, hemocyanin. Its purification was carried out independently on this material.

The considerable overlap on the Aminex column shows up quite clearly on DEAE-Sephadex chromatography (Fig. 7) where several of the radioactive peptides appear in more than one fraction. Thus, I_a and I_b are identical, as are II_a and II_b, III_a and IV_a, and III_b and IV_b. From the amino acid composi-
FIG. 3. Determination of molecular weights by gel filtration in Na dodecyl-SO₄ and guanidine HCl. Samples of purified hemocyanin were denatured by boiling in 5% Na dodecyl-SO₄, 0.1% 2-mercaptoethanol, 0.1 M Tris-HCl, pH 8.0, and incubated at 37° overnight. The sample was applied to a column of Sepharose 4B (90 × 2.5 cm) in 0.2% Na dodecyl-SO₄, 0.04 M Tris sulfate, pH 8.0. The logarithm of the molecular weight of each standard is plotted as a function of the logarithm of its distribution coefficient, $K_d$. The flow rate was 5 ml/hour; 2.5-ml fractions were collected. For gel filtration in guanidine HCl, samples were prepared by boiling in 6 M guanidine HCl, 0.1% 2-mercaptoethanol, and incubated overnight at 37°. The denatured protein was applied to a Sepharose 4B column (1.5 × 45 cm) in this solvent and eluted at 2.5 g/hour; 1.2 to 1.3 g fractions were collected. The standards, which were prepared as described above, included myosin (220,000 daltons), muscle phosphorylase (92,000 daltons), bovine serum albumin (68,000 daltons), and bovine erythrocyte carbonic anhydrase (30,000 daltons). (A) denotes the calibration curve for the Na dodecyl-SO₄ column; (B) designates that for the guanidine column.

FIG. 4. Na dodecyl-SO₄ column chromatography of Octopus vulgaris hemocyanin. Eight to ten milligrams of reduced and Na dodecyl-SO₄-denatured protein were applied to a column of Sepharose 4B in 0.2% Na dodecyl-SO₄. The chromatographic conditions are exactly as described in Fig. 3. The component at 390 ml contained no amino acids after acid hydrolysis, and is probably an artifact due to light scattering by the Na dodecyl-SO₄ micelle.

FIG. 5. Na dodecyl-SO₄ polyacrylamide gel electrophoresis of mollusc hemocyanins. Na dodecyl-SO₄ polyacrylamide gels (5% acrylamide, 0.15% methylene bisacrylamide) of the purified mollusc hemocyanins examined in this study were run for 5 hours in borate-acetate buffer, pH 8.5. From left to right: Busycon hemocyanin which had been incubated for 1 week in 70% formic acid as described by Dijk et al. (28); Busycon hemocyanin; Loligo hemocyanin; Stenoplax hemocyanin; Octopus vulgaris hemocyanin; O. dactylini hemocyanin. All samples were reduced and then denatured by boiling in Na dodecyl-SO₄ as described by Weber et al. (18).
Amino acid composition and quantitation of NH$_2$-terminal peptide of pronase digest of lobster hemocyanin

In this experiment 43.6 nmol of digested protein were applied to a column of Dowex 50W-X2 (0.9 × 20 cm). The composition is based on a 24-hour acid hydrolysate, and analyses of controls containing only pronase or hemocyanin which had been treated in the same way as the digest gave negligible amounts of amino acids.

| Amino acid       | Nmoles in flow-through | Mols/mol protein |
|------------------|------------------------|------------------|
| Aspartic acid    | 39.2                   | 0.90             |
| Threonine        | 10.5                   | 0.24             |
| Serine           | 10.5                   | 0.24             |
| Glutamic acid    | 46.2                   | 1.06             |
| Glycine          | 10.6                   | 0.24             |
| Alanine          | 8.5                    | 0.20             |
| Valine           | 12.1                   | 0.28             |
| Isoleucine       | 6.0                    | 0.14             |
| Leucine          | 9.3                    | 0.21             |
| Phenylalanine    | 5.1                    | 0.12             |

(B) Electrophoretic properties of the peptide isolated from flow-through of Dowex 50W-X2 following chromatography of pronase digest of lobster hemocyanin

Migration distances (in cm) which are defined with respect to the origin, are negative toward the anode and positive toward the cathode.

| Isolated peptide | pH 2.0 | pH 3.47 | pH 6.5 |
|------------------|--------|---------|--------|
| Unknown          | -1.1   | -12.95  | -14.1  |
| Aspartic acid    | 5.8    | -7.0    |        |
| Arginine         | 9.6    |         |        |
| PCA              | 13.6   | -7.7    |        |
| PCA-Alanine      | -5.3   |         | -8.3   |
| N-acetyl aspartic acid | -    |         |        |

* PCA, pyrorridone carboxylic acid.

Tryptic digest of reduced and alkylated protein on DEAE-cellulose, followed by electrophoresis on Whatman No. 1 paper of the fractions that absorbed light at 280 nm (Fig. 8). Tryptophan peptides were detected by Ehrlich’s reagent, and were found only in the first two ultraviolet-absorbing fractions obtained from the DEAE-cellulose column.

When the material which remained at the origin in Fraction II was subjected to electrophoresis at pH 8.9 (0.1 M ammonium bicarbonate titrated with ammonia), none of the tryptophan-positive material moved. However, chromatography in 1-butanol-pyridine-acetic acid-water resolved two more peptides and left no material at the origin (Fig. 8). Thus, a total of 7 tryptophan peptides could be detected of the 10 or 11 expected from the amino acid composition.

Two approaches were taken toward analyzing the methionine-containing peptides of lobster hemocyanin. The first involved cleavage of the reduced and alkylated protein with CNBr as described by Waxdal et al. (2). Amino acid analysis showed that greater than 95% of the methionine had been derivatized. When a sample of CNBr-cleaved protein was taken up in 10% Na dodecyl-SO$_4$ and the fragments were separated on Na dodecyl-SO$_4$ polyacrylamide gels, only four strongly staining bands were seen (Fig. 9).

Because the experiments with CNBr could account for only half of the expected number of cleavage products (unless there are several Met-Met sequences), a second approach to determine the number of unique methionine-containing peptides was tried. This entailed the specific alkylation of methionine residues at pH 3.5 to form the carboxymethylsulfonium derivative with iodo[1-14C]acetamide. Although no attempt was made to separate the peptides, it was noted that all of them were retarded on a column of P-2, and all are therefore smaller than 10 to 15 residues.

When this material was separated by two-dimensional electrophoresis-chromatography, only 4 spots were detected by autoradiography (Fig. 10). The radioactive areas were then cut out from the paper and placed in scintillation vials, and eluted into 0.5 ml of 1 M acetic acid at 37° before counting. The distribution of counts (Peptide 1, 14,722 cpm; Peptide 2, 6,850 cpm; Peptide 3, 12,122 cpm; Peptide 4, 6,943 cpm) shows that one peptide contains approximately twice the number of counts as any other one. This confirms the conclusion, deduced from the isolation of free homoserine in the CNBr digest, that a Met-Met sequence exists in the protein. However, the number of methionine peptides is only half the expected number.

Busycon Hemocyanin—Fractionation of a tryptic digest of Busycon hemocyanin on DEAE-cellulose and paper electrophoresis of the ultraviolet-absorbing peaks resolved 10 tryptophan-positive regions (Fig. 11) which could not be further resolved by chromatography. The cleavage of reduced and alkylated protein with CNBr was carried out as described by Waxdal et al. (2). Following lyophilization, the residue was extracted with water and the soluble
TABLE III

(A) Determination of sulphydryl content of lobster hemocyanin

Alkylation in 6 M guanidine HCl in the presence and absence of reducing agent, performic acid oxidation, and spectrophotometric titration of sulphydryl groups are described elsewhere (1). Bovine ribonuclease, which was used as a control, contains four disulfide bonds and no free cysteine (31).

| Derivative                     | Moles/75,000 g | No. of determinations |
|-------------------------------|----------------|-----------------------|
| Cysteic acid                  | 4.7 ± 0.2      | 3                     |
| Cm-cysteine (with reduction)  | 4.8 ± 0.4      | 5                     |
| Cm-cysteine (no reduction)    | 0.5 ± 0.1      | 3                     |
| Cm-cysteine (native, no reduction) | 0.4 ± 0.1     | 2                     |
| Titration with dithiopyridine (in guanidine HCl) | 0.3 ± 0.1     | 1                     |
| RNase-cysteic acid            | 7.8 ± 0.1      | 1                     |
| Cm-cysteine                   | 7.7 ± 0.1      | 1                     |
| Cm-cysteine (no reduction)    | 0.1 ± 0.1      | 1                     |

(B) Susceptibility to reduction of sulphydryl groups of native lobster hemocyanin

Freshly purified lobster hemocyanin (1 mg/ml) in 0.1 M Tris-HCl, pH 8.0, was incubated with dithiothreitol at room temperature. Aliquots (0.2 ml) were withdrawn and pipetted into 0.5 ml of 0.1 M iodoacetamide in the same buffer. The reaction was stopped after 30 min by the addition of 0.1 ml of 1 M acetic acid, and the solution was dialyzed against 0.2 M acetic acid overnight at 4°C. The samples were then hydrolyzed in 6 N HCl and 0.1% mercaptoacetic acid, and analyzed for Cm-cysteine; the same analysis was used for determining total protein; DTT, dithiothreitol.

| Reduction time | DTT concentration | Alkylation time | Moles Cm-cysteine/mol protein |
|----------------|-------------------|----------------|-----------------------------|
| min            | (mg/ml)           | min            |                             |
| 0              | None              | 20             | 0.36                        |
| 20             | 0.01 M            | 20             | 1.01                        |
| 40             | 0.01 M            | 20             | 1.07                        |
| 60             | 0.01 M            | 20             | 1.11                        |

peptides were submitted to paper electrophoresis at pH 1.9. It was possible to identify one ninhydrin-positive spot in this fraction. A portion of the insoluble fraction was taken up in 10% Na dodecyl- SO4 and when the CNBr fragments were separated on Na dodecyl- SO4 polyacrylamide gels, only five strongly staining bands were seen (Fig. 12). A second aliquot was dissolved in 8 M urea and subjected to electrophoresis on polyacrylamide gels at pH 4.5 (Fig. 12). Eight to eleven bands could be identified in this system. According to these results, at best, only 1/6 of the expected number of peptides was obtained, indicating that the molecule is made up of repeating polypeptide fragments.

Additional evidence for this conclusion was obtained by limited proteolysis of Busycon hemocyanin. A tryptic digest of lobster hemocyanin (30 mg) in which the sulphydryl groups had been reduced and alkylated with iodo[1-14C]acetamide was applied to a column of Aminex AG 50W-X2 (0.9 × 15 cm) at room temperature which had been equilibrated with 0.1 M pyridine-acetate, pH 3.1. After 100 ml of starting buffer had been pumped through the column, a linear gradient was begun which consisted of 200 ml of the starting buffer and 200 ml of 2.0 M pyridine-acetate, pH 3.0. The flow rate was 18 ml/hour and 2.5-ml fractions were collected. The fractions designated I to IV were taken for further purification. The chromatogram was analyzed by the ninhydrin method; 100 μl were taken from alternate fractions for alkaline hydrolysis.

![Fig. 6. Fractionation of the sulphydryl-containing tryptic peptides of lobster hemocyanin. A tryptic digest of lobster hemocyanin (30 mg) in which the sulphydryl groups had been reduced and alkylated with iodo[1-14C]acetamide was applied to a column of Aminex AG 50W-X2 (0.9 × 15 cm) at room temperature which had been equilibrated with 0.1 M pyridine-acetate, pH 3.1. After 100 ml of starting buffer had been pumped through the column, a linear gradient was begun which consisted of 200 ml of the starting buffer and 200 ml of 2.0 M pyridine-acetate, pH 3.0. The flow rate was 18 ml/hour and 2.5-ml fractions were collected. The fractions designated I to IV were taken for further purification. The chromatogram was analyzed by the ninhydrin method; 100 μl were taken from alternate fractions for alkaline hydrolysis.](http://www.jbc.org/)

Also, the main fraction from the DEAE-cellulose column produced a major peak of material which contained only the 50,000-dalton protein (Fig. 15).

Only tyrosine and histidine were detected as N terminus terminal residues by the dansyl technique. The amino acid composition of this component is not significantly different from intact hemo-
TABLE IV
Amino acid compositions of sulfhydryl-containing tryptic peptides of lobster hemocyanin

Hydrolyses were in 6 N HCl and 0.1% mercaptoacetic acid for 24 hours; no corrections have been made for the destruction of any amino acids; residues which were present in less than 1/10 mol per mol of peptide were omitted. Peptide III, was stained for tryptophan using Ehrlich's reagent.

| Amino acid       | I  | IIa | IIb | IIIa | IVa |
|------------------|----|-----|-----|------|-----|
| Lysine           | 0.2| 1.0 | 1.3 | 0.9  | 4.2 |
| Histidine        | 1.1|     |     |      |     |
| Arginine         | 0.8| 0.8 | 1.0 | 0.2  | 2.0 |
| Aspartic acid    | 0.2| 1.0 | 0.2 | 2.0  | 4.0 |
| Threonine        | 1.1|     |     |      |     |
| Serine           | 0.9| 1.0 | 1.2 | 0.9  | 1.05|
| Glutamic acid    | 1.0| 1.05|     | 1.1  | 3.0 |
| Proline          | 1.1| 1.2 | 1.2 | 1.2  | 2.3 |
| Glycine          | 1.0| 1.0 |     | 2.0  | 2.0 |
| Alanine          |    |     |     |      |     |
| Valine           | 1.1| 1.1 | 1.4 |      | 2.15|
| Isoleucine       | 0.86| 2.1 | 1.4 |      | 2.3 |
| Leucine          |    |     |     | 1.0  | 0.95|
| Phenylalanine    |    |     |     |      |     |
| Tryptophan       | 1.0| 1.0 | 0.7 | 0.8  | 1.0 |
| Cm-cysteine      |    |     |     |      | 0.95|
| Total residues   | 7  | 7   | 12  | 7    | 11  |

Fig. 8. Electrophoretic chromatographic analysis of Fractions I to IV. Electrophoresis of aliquots of Fractions I-IV was carried out at pH 5.7, 2500 volts, for 1 hour on Whatman No. 1 paper. When the peptides were stained for tryptophan with Ehrlich's reagent, only Fractions I and II gave positive results (top). Chromatography in 1-butanol-pyridine-acetic acid-water of the material which remained at the origin after electrophoresis of Fraction II resolved two more tryptophan-positive peptides (bottom).

Fig. 9 (left). Na dodecyl- SO₄ polyacrylamide gel electrophoresis of lobster hemocyanin CNBr fragments. After treatment with CNBr and lyophilization to remove excess reagent and formic acid, a sample of reduced and alkylated hemocyanin was dissolved in 5% Na dodecyl- SO₄ and subjected to electrophoresis on 14-cm Na dodecyl- SO₄ polyacrylamide gels (15% acrylamide, 0.405% methylene bisacrylamide) for 21 hours in borate-acetate buffer, pH 8.5. The anode is at the bottom. The 4 major bands have approximate molecular weights of 20,000, 10,000, 7,000, and 4,000.

Fig. 10 (right). Two-dimensional map of the methionine-containing tryptic peptides of lobster hemocyanin. After lyophilization of the radioactive fraction obtained by gel filtration, the methionine-containing tryptic peptides which had been alkylated with iodo[1-¹³C]acetamide were applied to a sheet of Whatman No. 1 paper (22 X 37 cm). Electrophoresis was carried out in Varsol-cooled tanks at pH 5.7 (1 hour, 2500 volts), and was followed by descending chromatography in 1-butanol-pyridine-acetic acid-water for 16 hours. The radioactive peptides were detected by autoradiography for 48 hours.

Fig. 11. Fractionation of the tryptophan tryptic peptides of Busycon hemocyanin. Reduced and alkylated hemocyanin (25 to 30 mg) was digested with trypsin and the product was applied to a column of DEAE-cellulose (25 ml) in 10 mM ammonium bicarbonate, pH 8.4. After 150 ml of starting buffer had been pumped through the column, a linear gradient was begun which consisted of 125 ml each of starting buffer and 0.5 mM ammonium bicarbonate, pH 7.9. The flow rate was 12 ml/hour and 2.0- to 2.5-ml fractions were collected. Every fourth tube was lyophilized and the contents dissolved in 200 µl of buffer; each sample was then applied to a sheet of Whatman No. 1 paper and electrophoresis was carried out at pH 5.7 for 1 hour at 3000 volts. The maps were then stained with Ehrlich's reagent to locate the tryptophan peptides.
FIG. 12. Fractionation of the CNBr fragments of *Busycon* hemocyanin. *Left,* 8 M urea polyacrylamide gel electrophoresis at pH 4.5 of the water-insoluble CNBr fragments was performed on 8-cm gels (10% acrylamide, 0.3% methylene bisacrylamide) for 2.5 hours at 4° as described by Eipper (20). The cathode is on the bottom. *Right,* Na dodecyl-SO₄ polyacrylamide gel electrophoresis of the water-insoluble CNBr fragments was performed on 9-cm gels (12% acrylamide, 0.3% methylene bisacrylamide) for 12 hours in borate-acetate buffer, pH 8.5. The anode is at the bottom. The five major bands had approximate molecular weights of 19,000, 13,000, 10,500, 8,700, and 7,500.

that all of it might be attached to one amino acid, and it was decided to perform a tryptic cleavage of reduced and alkylated protein, and then to purify this peptide by conventional means. This was done in 3 steps: gel filtration on G-25, in which case the peptide eluted ahead of most of the other tryptic peptides, and then on G-50; this material was then further purified on DEAE-cellulose, and gave one ninhydrin-positive fluorescent spot upon paper electrophoresis.

It was clear from the amino acid composition (Table V) that the peptide was not completely cleaved by trypsin; this is not unusual if either lysine or arginine is near the site of carbohydrate attachment.

The huge size of this peptide, however, made it difficult to resolve the question of how many sites of attachment for carbohydrate there are in lobster hemocyanin. Thus, in a similar experiment, the carbohydrate-containing fraction from a separation on G-50 of the tryptic peptides was pooled and digested with Pronase for 24 hours. This material was then further purified on DEAE-cellulose, and gave one ninhydrin-positive fluorescent spot upon paper electrophoresis.

Attempts to fractionate a tryptic digest of *Busycon* hemocyanin in order to isolate the glycopeptides were unsuccessful. After several steps of gel filtration and ion exchange chromatography two impure peptides could be resolved (data not shown).

The presence, however, of mannose and glucosamine was particularly interesting because several workers (32, 33) have used the intact hemocyanin molecule as an electron-dense marker for the lectin concanavalin A when the latter was bound to the membranes of animal cells. This application suggested, based on the specificity of concanavalin A for α-D-glucosamine and α-D-mannose (34), that it should be possible to purify one or more of the glycopeptides by affinity chromatography on a column of Sepharose-bound concanavalin A. When this was done, the material that eluted with 0.1 M α-methylmannoside gave only one highly fluorescent spot upon electrophoresis which stained with both ninhydrin and silver nitrate (Fig. 16). This does not eliminate the possibility that this spot may include more than one electrophoretically similar peptide, although the amino acid composition of this fraction is very simple (Table V). The yield of the glycopeptide indicates that there is one glycopeptide per 50,000 g of protein, or carbohydrate chains per polypeptide chain.
FIG. 14. Na dodecyl-SO₄ polyacrylamide gel electrophoresis of samples taken from fractionation on DEAE-cellulose of the 50,000-dalton material. Na dodecyl-SO₄ gel electrophoresis of DEAE-cellulose column fractions was performed as described in Fig. 1. The numbers on the figure correspond to the fraction numbers shown in Fig. 13B. The standard contains myoglobin, carbonic anhydrase, glutamate dehydrogenase, and bovine serum albumin.

Busycon and lobster hemocyanin indicates that they may be class II glycopeptides (35) with the structure:

\[
\begin{align*}
    &A - \text{Asn} - B - (\text{Ser or Thr}) - \\
    &N - \text{Acetylglucosamine} \\
    &\text{(mannose)}_n
\end{align*}
\]

where \(A\) and \(B\) represent any amino acid.

**Discussion**

The experiments described here were designed to determine the subunit structure of the arthropod and the mollusc hemocyanins. The results show that the polypeptide chain molecular weights for arthropod and mollusc hemocyanins are 75,000 and 290,000, respectively. These values were obtained by gel filtration, gel electrophoresis, and by quantitation of the NH₂- and COOH-terminal amino acids. Values obtained by other investigations (28, 35-39) appear to be incorrect.

In the hemocyanins, 1 \(O_2\) molecule is bound by 2 copper atoms. There are 2 copper atoms per 75,000 daltons of arthropod hemocyanin and per 50,000 daltons of mollusc hemocyanin. Accordingly, one polypeptide chain of arthropod hemocyanin binds 1 \(O_2\) molecule, while one polypeptide chain of mollusc hemocyanin binds six \(O_2\) molecules.

Both polypeptides appear to have some degree of sequence homology. The peptide fractionation experiments of Pickett et al. (39) and Nardi et al. (40) on lobster hemocyanin have shown that only the number of tryptic peptides which could account for 35,000 daltons of protein could be detected. This result is concordant with the isolation of the small number of cyanogen bromide peptides reported here and suggests that the 75,000-dalton polypeptide contains regions with sequence homology. On the other hand, attempts to fractionate the CNBr fragments and the tryptophan-containing tryptic peptides of *Busycon* hemocyanin suggest that the minimum chemical molecular weight is 50,000. The only way to reconcile the values ob-

**TABLE V**

| Amino acid | Lobster hemocyanin | *Busycon* hemocyanin |
|------------|--------------------|----------------------|
|            | Tryptic peptide    | Promase peptide      |
| Lysine     | 1.0                | 0.6                  |
| Histidine  | 3.8                | 1.0                  |
| Arginine   | 1.0                | 1.0                  |
| Aspartic acid | 12.4   | 2.0                  | 0.75               |
| Threonine  | 3.9                | 0.9                  |
| Serine     | 4.8                | 1.0                  |
| Glutamic acid | 14.0   | 0.8                  | 0.9                |
| Proline    | 2.0                | 1.0                  |
| Glycine    | 5.2                | 0.1                  | 1.0                |
| Alanine    | 5.1                | 1.0                  |
| Valine     | 4.0                | 0.9                  |
| Methionine | 0.8                |                      |
| Isoleucine | 2.3                |                      |
| Leucine    | 8.1                |                      | 0.75               |
| Tyrosine   | 8.1                |                      |
| Phenylalanine | 6.8   |                      |
| Tryptophan |                    |                      |
| Mannose    | 3.5                | 3-4                  |
| Glucosamine| 0.4                | 0.4                  |

**Total number of residues**

|             | Lobster hemocyanin | *Busycon* hemocyanin |
|-------------|--------------------|----------------------|
|             | 81                 | 5                    | 10                  |

**FIG. 15.** Na dodecyl-SO₄ polyacrylamide gel electrophoresis of the final purification of the 50,000-dalton component by gel filtration. Na dodecyl-SO₄ gel electrophoresis of the final purification step on Sephadex G-100 in 5 M urea (Fig. 13C) was performed as described in Fig. 1.
It is clear from column chromatography and Na dodecyl-
results of limited proteolysis of the native hemocyanin with sub-
polypeptide chain of 290,000 daltons.

In this experiment, the tryptic peptides that eluted in the void
volume of a Sephadex G-25 column were applied to a 3.5-ml column
of Sepharose-bound concanavalin A at 4°C, and were allowed to
equilibrate with the column for 30 min before elution was begun
with 0.1 M ammonium bicarbonate, pH 7.9. At Fraction 14 the
buffer was changed to one that contained 0.1 M α-methyl manno-
side. The flow rate was 36 ml/hour and 3-ml fractions were col-
lected.

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Fig. 16. Purification of a glycopeptide of Busycon hemocyanin.
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