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A COMPARATIVE SURVEY OF SOME HOLOTHURIAN HEMOGLOBINS

by

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A THESIS

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

December 1980

Rec'd 12/81

APPROVED: _____

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An abstract of the Thesis of

Michael Stuart Roberts for the degree of Master of Science in the Department of Biology to be taken December 1980

Title: A Comparative Survey of Some Holothurian Hemoglobins

Approved: _____

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The hemoglobins of five holothurian species have been purified and compared. Those of the species of Cucumaria were so similar that they could not be distinguished by any of the methods employed. Cucumaria curata and Cucumaria pseudocurata could only be told apart by the presence of a distinctive ossicle unique to Cucumaria curata. The apparent molecular weights of native hemoglobins from all five species range from 32,000 to 35,000 daltons. Investigation of subunit structure shows that these hemoglobins are dimeric. Cucumaria hemoglobins are heterodimers while that of Sclerodactyla is a homodimer. The subunit molecular weights range from 15,800 to 17,800. Cucumaria pseudocurata hemoglobin, like that of Cucumaria miniata, has a high affinity for oxygen. The amino acid composition of Cucumaria pseudocurata hemoglobin is also very similar to those of other species of Cucumaria. Proposed further structural studies into holothurian hemoglobins are discussed.

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ACKNOWLEDGEMENTS

I wish to thank Dr. Rolf Ingermann, Mark Silbersteen, Russell Reasoner, Eric Sweid, and many others for help in obtaining animals. I am grateful to Dr. Robert Becker for help with the amino acid analysis, and to my advisor, Dr. Robert C. terwilliger for help and many useful suggestions in the course of this investigation.

DEDICATION

I wish to dedicate this thesis to my wife, Judith Sidney Garrett Roberts, whose unfailing help and encouragement made this effort possible.

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INTRODUCTION

Hemoglobins are respiratory proteins capable of reversibly binding oxygen. They are generally in solution in a circulating aqueous medium, and may be intra- or extracellular. Their function is to transport oxygen from surface tissues to those deeper within the organism. One or more intracellular and/or extracellular hemoglobins may be present at a time. Animals may also possess myoglobin, a respiratory pigment found within tissues, whose function may be to facilitate diffusion of oxygen into tissues, (Scholander, 1960) or to store oxygen against times of hypoxia (Wittenberg, 1970). Hemoglobins and myoglobins are found in representative species of many phyla. The physical structures of vertebrate hemoglobins were the first to receive study. The tertiary structure of sperm whale myoglobin, a monomer of single polypeptide chain, was elucidated by Kendrew, (1963) with X-ray crystallography. Subsequently Perutz (1964, 1969, 1970) showed that horse hemoglobin has a tetrameric quaternary structure consisting of four polypeptide chain subunits. Each subunit has a molecular weight and tertiary structure very similar to those determined for whale myoglobin.

The structures of invertebrate hemoglobins, also termed

erythrocruorins, have been widely studied. (For review, see Terwilliger, 1980). Erythrocruorins exhibit a wide range of molecular weights. For example, monomers with molecular weights of approximately 16,000 are found in the insect Chironomus, (Huber et al., 1971) and the polychaete Glycera, (Padlan and Love, 1974) and a gigantic oligomer with a molecular weight of 12,000,000 is present in the clam Cardita, (Terwilliger et al., 1978).

In the phylum Echinodermata hemoglobins are known from a number of species of Holothuroidea. (One study has been made of the structure of an ophiuroid hemoglobin, (Hajduk and Cosgrove, 1974)). Howell (1885) was the first investigator to demonstrate, by chemical means, the presence of an iron containing protein in a holothurian, Thyonella gemmata, which was found by spectroscopy to be a hemoglobin. Using similar methods Hogben and Van der Linden (1927) found hemoglobin in coelomic cells of Cucumaria frauenfeldi, and Van der Heyde (1921) reported hemoglobin-containing cells in the water vascular system of Sclerodactyla briareus (= Thyone briareus). Crescitelli (1945) and Hetzel (1960) found intracellular hemoglobins in Cucumaria miniata and Cucumaria piperata, respectively. All the above species are thought to be rather closely related and are placed in the order Dendrochirotida. Two species of the order Molpadonia, Paracaudina chilensis (=

Caudina chilensis) and Molpadia roretzii, were studied by Kobayashi (1932) who included a summary of some earlier investigators' findings. Sorby (1876) and Anson et al., (1924) had reported that the hemoglobins from different species of animals appeared to show different absorption spectra. Kobayashi was able to differentiate between hemoglobin solutions of two closely related species of holothurians by their absorption spectra.

Svedberg (1933) developed the ultracentrifuge as a means of determining the molecular weights of proteins by sedimentation rate. Sclerodactyla briareus (= Thyone briareus) hemoglobin was found by this method to have an apparent molecular weight of 23,000 (Svedberg and Pederson, 1940). A similar species, Thyonella gemmata, was found by Manwell (1966) to possess a hemoglobin whose molecular weight by ultracentrifugation was 41,000.

Terwilliger and Read (1970) used gel permeation chromatography to determine the molecular weights of carbonmonoxyhemoglobins from Cucumaria miniata, Cucumaria piperata, and Molpadia intermedia. A range of 36,000 - 40,000 was reported, which suggests that these hemoglobins are all dimeric. This was verified experimentally with purified Cucumaria miniata hemoglobin, which was treated with a reducing agent followed by alkylation with iodoacetic acid or n-ethylmaleimide. Subsequent column chromatography

showed that monomers resulted under these conditions.

Oxidation of purified Molpadia intermedia hemoglobin with $K_3Fe(CN)_6$ converted the pigment in large part to monomers, but identical treatment of Cucumaria miniata hemoglobin resulted in high molecular weight aggregates. A molecular explanation of this may be found in the amino acid analyses of these pigments, which showed that Cucumaria hemoglobins are rich in the amino acid cysteine whereas Molpadia intermedia and Molpadia oolitica hemoglobins lack any cysteine residues (Terwilliger and Read, 1970, 1972). It was suggested that the aggregation of Cucumaria hemoglobins under oxidizing conditions results from the formation of disulfide bonds between cysteine residues of adjacent subunits. This phenomenon is probably an experimental artifact and should not be considered a mechanism stabilizing the dimeric configuration. Certainly, the stability of Molpadia hemoglobin's dimeric structure is not a function of interaction between cysteine residues (Terwilliger and Read, 1970).

Ligand linked association, and dissociation reactions have been described for holothurian hemoglobins. Bonaventura and Kitto (1973) and Terwilliger (1975) found independently that the hemoglobin of Cucumaria miniata exists as a dimer in the oxy state but becomes a putative tetramer when deoxygenated chemically. It may be that an

equilibrium state between dimeric and tetrameric configurations occurs under these conditions. Such molecular behavior may explain in part the rather high molecular weight of the hemoglobin of Thyonella gemmata proposed by Manwell (1966). Hemoglobin from Molpadia arenicola is reported to undergo conformational changes upon deoxygenation; this pigment aggregates to tetramers and higher oligomers (Bonaventura and Kitto, 1973), which is reversible upon re-oxygenation. The molecular mechanism underlying such conformational shifts is unknown.

Dissociation may also occur as a function of hemoglobin concentration. Purified Molpadia arenicola hemoglobin dissociates into monomers when the protein concentration of a hemoglobin solution is brought to less than two mg per ml (Bonaventura and Kitto, 1973). This finding seems to indicate a difference between this pigment and the hemoglobins of Molpadia intermedia and Molpadia oolitica which Terwilliger and Read (1970, 1972) found to maintain their apparent molecular weights even when very dilute.

Kitto et al., (1976) attempted to determine the primary structures (amino acid sequences) of hemoglobins from five species of holothurians. In all cases the attempts failed, and it was determined that the N-terminus of each globin chain was blocked. In the case of Molpadia arenicola globins the blocking agent was found to be an acetyl

group. Such N-terminal blockage may have functional consequences. Vertebrate hemoglobins are believed to transport carbon dioxide and hydrogen ions by ionic interactions with terminal amino graoups on subunit chains. Furthermore, conformation of vertebrate deoxy tetramers is thought to be stabilized by ionic interactions between the N-terminus of one subunit chain and the C-terminus of another, termed "salt links". A different mechanism is probably responsible for stabilizing deoxy state oligomers of holothurian hemoglobins.

Organic phosphates are known to reduce the affinity of vertebrate hemoglobins for oxygen (Benesch, R. and Benesch, R.E., 1969), and are thought to act by cross linking globin chains. The organic phosphate 2,3 diphosphoglycerate is believed to bind in part to N-terminal amino groups (Arnone, A., 1972). If these amino groups are occupied, as in the holothurian hemoglobins examined, then it might be expected that 2,3 diphosphoglycerate would not affect the oxygen affinities of such holothurian hemoglobins. This has been experimentally verified (Bonaventura et al., 1976. Another finding was that the C-terminus amino acid residue of Molpadia arenicola hemoglobin was serine, whereas in vertebrate hemoglobins the C-terminal residue is usually histidine. This may have important functional consequences, especially with respect to the

Bohr effect. The Bohr effect is a reversible equilibrium system in which the presence of CO_2 and H_3O^+ reduce the affinity of hemoglobins for oxygen. Within the organism the presence of oxygen at the respiratory surface drives off CO_2, thus providing a two-way gas exchange system. The mechanism postulated for the Bohr effect in vertebrate hemoglobins involves interactions of CO_2 and H_3O^+ with C-terminal histidine residues and N-terminal amino groups. As Molpadia arenicola hemoglobin has blocked N-terminals and non-histidine C-terminal residues, the absence of the Bohr effect might be expected, as was found to be the case (Bonaventura and Kitto, 1976). Cucumaria miniata hemoglobin has not yet been sequenced, but as it too has been found to lack a Bohr effect (Manwell, 1959) blocked N-terminal amino groups and perhaps non-histidine C-terminal residues might be predicted.

Cooperativity between subunits during oxygen binding by holoturian hemoglobins has been investigated by a number of workers. Cooperativity is said to exist when an experimentally derived value called the Hill coefficient, represented by "n", is greater than 1. In oxygen binding studies Manwell (1959) reported an "n" value of 1.4 for Cucumaria miniata hemoglobin, which is the same figure reported by Steinmeier and Parkhurst (1979) for Thyonella gemmata hemolysate. Terwilliger and Read (1972) reported

an "n" value of 1.6 for purified Molpadia oolitica hemoglobin, and the hemoglobin of Molpadia arenicola was found by Bonaventura and Kitto (1973) to have an "n" value of 1.45. These figures show that cooperativity between subunits in oxygen binding exists, but to about half the extent described for many vertebrate hemoglobins (Prosser, 1973).

Steinmeier and Parkhurst (1979) studied carbon monoxide and oxygen reaction rate of Thyonella gemmata hemolysate. Thyonella hemoglobin had a low affinity for carbon monoxide. Conformational differences exist between the oxy and carbon monoxy hemoglobins. In this dimer binding of either ligand is biphasic. It takes five times as long for both hemes to bind ligands than for just one heme to become liganded.

The structural and functional data obtained for holothurian hemoglobins to date show many differences from those of vertebrate hemoglobins, yet the holothurians represent the hemoglobin-possessing group of invertebrates most closely related to the vertebrates. In view of the commonly accepted hypothesis that vertebrate and invertebrate hemoglobins share common ancestry (Goodman and Moore, 1974) holothurian respiratory pigments are of obvious importance. Further sequencing attempts might produce useful information as to relationships between

groups of holothurians, and between holothurians and other groups.

Taxonomic relationships between holothurian species are not clear. The fossil record is quite sparse as these are soft-bodied organisms, unlike most other echinoderms. Biochemical techniques may prove useful tools in investigating relationships between groups of holothurians and have been employed by Kobayashi (1932), Manwell (1963), and Rutherford (1977).

In this thesis a number of holothurian species have been studied and compared. Structural and functional characterizations of Cucumaria curata and Cucumaria pseudocurata hemoglobins are presented for the first time. Cucumaria miniata has been reinvestigated as to subunit heterogeneity. New data was obtained for the hemoglobins of Eupentacta quinquesemita and Sclerodactyla (= Thyone) briareus. The data from these species are compared and contrasted.

MATERIALS AND METHODS

Cucumaria miniata Brandt, Cucumaria pseudocurata Deichmann, and Eupentacta quinquesemita (Selenka) were collected among intertidal rocks at Cape Arago, Oregon during low slack-water intervals of spring tides. Cucumaria curata Cowles was collected near Pescadero Point, California and shipped live. Sclerodactyla briareus (Lesueur) was obtained from the Supply Department, Woods Hole Marine Biological Laboratories, Woods Hole, Massachusetts. Animals were stored under running sea water or used immediately.

The four Pacific species were identified with the key to Holothuroidea in Light's Manual (1975). S. briareus, an Atlantic species, was identified with a key to Holothuroidea by Pawson (1977). Three factors were used in the identification process; external whole-body morphology, structure of the calcareous ring supporting the feeding tentacles, and structure of the ossicles within the body wall. The ossicles were prepared by macerating pieces of body wall with sodium hypochlorite in aqueous solution.

All five species contain hemoglobin in erythrocytes. Structural studies upon hemoglobins from C. miniata and C. piperata were done by Terwilliger and Read (1970). Sclerodactyla briareus was reported by Manwell (1966) to

contain intracellular hemoglobin. C. curata and C. pseudo-curata (which are very small species) have not been previously reported to contain respiratory pigments.

In all three species of Cucumaria erythrocytes occur freely in the perivisceral coelomic fluid and in the organs of the water vascular system. From these species the cells containing hemoglobin were collected by slicing the animals along an interambulacral plane starting from the oral end, and by draining the coelomic fluid into a chilled beaker over ice. The coelomic cavities were flushed with saline solution to remove any remaining erythrocytes.

E. quinquesemita and S. briareus contain erythrocytes only in the water vascular system. No trace of erythrocytes was found in the perivisceral coelomic fluid. The polian vesicles, tentacular ampullae and podial ampullae were sliced or punctured and the fluid contents drawn or scraped out, and collected into chilled containers on ice.

Hemolymph of all species was centrifuged in a refrigerated Sorvall RC-2B at 130 x g for ten min immediately after collection. The pelleted hemocytes were re-suspended in filtered sea water and again centrifuged at 130 x g for 10 min. This washing procedure was carried out three times.

Washed pelleted cells were shaken with 2.5 ml of distilled water over ice for fifteen minutes to effect lysis. A final centrifugation at 13,000 x g for 10 minutes

yielded a red supernatant.

Exclusion column chromatography was performed on a G-100 Sephadex dextran (Sigma Chemical Co.) column 1.1 cm in diameter with a bed height of 54 cm. The column was previously calibrated with Blue Dextran, bovine serum albumin (M_r 68,000), α chymotrypsinogen A (M_r 25,100), and sperm whale metmyoglobin (M_r 17,800). The column was equilibrated with a buffer 0.1 M in NaCl, 0.05 M in Hcl, and titrated with trihydroxyaminomethanehydrochloride (tris) to pH 7.45.

Ion exchange column chromatography was carried out upon diethylaminoethyl (DEAE) cellulose. Column volume varied with sample volume; however, a column volume of 5.5 cubic mls. was usually employed. Columns were equilibrated with 0.01 M ammonium bicarbonate. Elution was accomplished by application of a linear sodium chloride gradient between two 250 ml buffer reservoirs, the first contained 0.01 M ammonium bicarbonate and the second 0.01 M ammonium bicarbonate and 0.20 M in sodium chloride.

Hemoglobin solutions purified by DEAE-cellulose chromatography were used in polyacrylamide disc gel electrophoresis according to the method of Davis (1964). Samples were converted to the carbon monoxy state or to the cyanmet state. The reservoir buffer was modified to include 0.10 g of KCN/liter after Moss and Ingram (1968).

Globins were prepared after Teale (1959), and subjected to polyacrylamide gel electrophoresis at pH 2.2, in 6.25 M urea, according to the methods of Panyim and Chalkey (1969); and Poole et al. (1974). The urea solution was deionized by passage through a bed of MB-1 amberlite beads immediately before use. Gels were poured in tubes and preelectrophoresed two hours. Samples were run at two milliamperes per tube, for three and one half to four hours, depending on tube length.

It was subsequently found that lyophilized hemoglobin samples gave identical results with their corresponding globins in electrophoreses, and were much easier to dissolve in urea solutions. Globins and their corresponding hemoglobins were treated with 2-mercaptoethanol or performic acid prior to urea gel electrophoresis. After electrophoresis gels were stained with Coomassie Brilliant Blue R stain, (Fairbanks et al., 1971).

Globins reduced or oxidized as described above were incubated for 1.5 minutes at 100° C in a solution containing 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 1 mM phenylmethylsulfonylfluoride (PMSF), and electrophoresed on 1.5 mm slab gels (Studier, 1973) with a discontinuous buffer system, after Laemmli (1970).

Gels were of 15% acrylamide concentration. Calibrants used in determination of subunit molecular weights were

ovalbumin, chymotrypsinogen A., sperm shale metmyoglobin, and lysozyme (molecular weight 14,300) (Sigma Chemical Co.). Gels were stained in Coomassie Brilliant Blue R dissolved in isopropyl alcohol and acetic acid, according to Fairbanks et al. (1971).

Amino acid analyses were carried out according to Spackman et al. (1958). Cysteine and cystine were computed as cysteic acid according to Hirs (1956), on performic acid oxidized samples. Tryptophan was not determined.

Oxygen binding experiments upon whole hemoglobins were performed after Benesch et al. (1965). A Zeiss PMQ II spectrophotometer was used to measure optical densities and to determine the absorption maxima of intact hemoglobins in the oxy and deoxy states. Freshly prepared oxyhemoglobin was converted to carbonmonoxyhemoglobin by deoxygenating the hemoglobin with sodium hydrosulfite, followed by bubbling carbon monoxide gas through the hemoglobin solutions. The carbon monoxide was prepared by dehydrating formic acid with concentrated sulfuric acid.

RESULTS

Structural Properties

Cucumaria curata and Cucumaria pseudocurata

The amount of hemoglobin in individual specimens of these species varied. The coelomic fluid of some individuals was bright red while in others it was light pink, or had no discernible color. For six individuals of C. pseudocurata with a wet weight of approximately 50 mg, the mean hematocrit was 3.6 volume percent.

The oxyhemoglobin of C. pseudocurata chromatographs on a column of Sephadex G-100 as a single asymmetric peak (Fig. 1). The apparent molecular weight of this fraction, obtained by a plot of log molecular weight versus elution volume, is 34,500 (Fig. 2). If the hemoglobin was first deoxygenated with sodium dithionite prior to gel permeation chromatography and eluted in the presence of 0.05 M sodium dithionite, the protein had an apparent molecular weight of 51,000 (Fig. 3).

Ion exchange chromatography of C. pseudocurata hemoglobin on DEAE-cellulose in equilibrium with 0.01 M ammonium bicarbonate resulted in the pigment adhering to

the cellulose as a tight red band. The hemoglobin could be eluted from the column by application of a sodium chloride gradient (described above) as a single asymmetric peak (Fig. 4).

Purified intact hemoglobin was treated with either carbon monoxide or converted to the cyanmet state, and electrophoresed in polyacrylamide gels of different concentrations and at different pHs. Two bands were observed for carbonmonoxy hemoglobin and one band for cyanmet hemoglobin.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out on hemoglobin which had been purified by gel chromatography and ion exchange chromatography. Two bands could be detected as represented in Fig. 5. These bands exhibit approximately equal amounts of protein polypeptide subunits, based on staining intensities of the bands. The apparent molecular weights of these bands are about 15,500 and 17,500. The same bands were seen when the hemoglobin was first oxidized with performic acid prior to electrophoresis.

Electrophoresis of purified apohemoglobin was carried out at pH 2.2 in the presence of 6.25 M urea. Again, two bands were resolved (Fig. 6). The band which migrated furthest into the gel stained somewhat more heavily than the trailing band.

The amino acid composition of purified C. pesudocurata hemoglobin is presented in Table 1.

Cucumaria curata hemoglobin was taken through exactly the same purification regime as that of C. pseudocurata. The oxyhemoglobin behaved as a 32,000 molecular weight fraction on G-100 Sephadex gel chromatography (Fig. 7, Fig. 8) and appeared as a single asymmetric peak on DEAE-cellulose (Fig. 9). Purified hemoglobin electrophoresed in sodium dodecyl sulfate gel as two bands (Fig. 10). Two bands were again observed by urea gel electrophoresis. The hemoglobins of C. pseudocurata and C. curata could not be distinguished when co-electrophoresed under these conditions.

Cucumaria miniata

Purification of the hemoglobin from this species and subsequent analysis of the pigment yielded results in agreement with those previously reported (Terwilliger and Read, 1970). However, sodium dodecyl sulfate polyacrylamide gel electrophoresis showed two bands, Fig. 11. This pair of bands could also be seen when the protein was oxidized with performic acid prior to electrophoresis. Two bands were detected when purified C. miniata was electrophoresed in the presence of 6.25 M urea in

polyacrylamide gel at low pH (Fig. 12). The further migrating band stained more heavily than the trailing band. When the pigments of C. miniata, C. pseudocurata and C. curata were co-electrophoresed they could not be distinguished from one another.

Sclerodactyla

This species contains small amounts of hemoglobin in cells which appear to be confined to the water vascular system. The oxyhemoglobin chromatographed on Sephadex G-100 as a fraction with an apparent molecular weight of 34,000 (Fig. 13). DEAE ion exchange chromatography was carried out as described above. A single asymmetric peak was observed.

Electrophoresis in sodium dodecyl sulfate polyacrylamide gel showed a single band with an apparent molecular weight of 15,800 (Fig. 14). When co-electrophoresed with C. miniata hemoglobin three bands results.

Eupentacta quinquesemita

This species also contains small quantities of erythrocytes which, like those of S. briareus, occur only

in the water vascular system. E. quinquesemita oxy hemoglobin seems to be very labile, subject to rapid oxidation. Chromatography of the oxyhemoglobin on Sephadex G-100 showed two peaks (Fig. 15). The first fraction eluted with the void volume and is presumed to be aggregated methemoglobin. The second fraction showed an apparent molecular weight of 33,000 (Fig. 16). Owing to the very small quantities of hemoglobin contained in individual specimens, the extreme lability of the pigment, and the difficulty of obtaining specimens, no further studies were carried out.

Absorption Maxima

The absorption maxima for all five species studied were determined in the oxy and carbonmonoxy states. They are summarized in Table 2.

Oxygen Equilibrium Studies

Oxygen equilibrium studies were carried out on freshly prepared C. pseudocurata oxyhemoglobin at pH 7.0, 7.4, and 8.0, at 20° C. Two replicates were used at each pH value. This showed a mean P_{50} value of 7.1 mmHg, and a mean "n" value of 1.64. Both "n" and P_{50} values are

independent of pH. No Bohr effect was observed.

Morphological Studies

Living specimens were examined in terms of gross morphology, and individuals of each species were dissected. Internally the species are very similar. The only observed difference in internal structures was in the gonads of the females of C. curata and C. pseudocurata. These, in the winter months, become greatly enlarged and resemble strings of beads. The gonads of the males and those of both sexes of the other three species resemble simple tubules. The two smallest species are brooding forms, producing a small number of eggs which they protect. The other species are broadcast fertilizers. It may be that brooding in these small species is an adaptation to life in mussel beds.

Externally, the five species are readily distinguishable. C. miniata is generally of a mottled red color and can reach 20 cm in extended length. The feeding tentacles are a bright orange-red and the tube feet or pedicels are soft and retractile and are not more numerous on the undersurface of the body. (Many holothurians tend to orient themselves such that one surface of the body is always the "underside." In species exhibiting such behavior the undersurface is termed a ventor.) C. curata is black, has a dark

undersurface, black feeding tentacles, and retractile pedicels more numerous on the undersurface. C. pseudocurata can be visibly differentiated from the preceding form only by pigmentation. This species ranges from brown to white, always with a lighter undersurface, which is often red or pink. These two species rarely exceed 2 cm in length. S. briareus is blackish in color, has black feeding tentacles and, most distinctively, has pedicels scattered evenly over the entire body surface, unlike the other species whose tube feet are restricted to ambulacral bands. This species is an Atlantic form which burrows in soft sediments. E. quinquesemita is white, with pale yellow to light orange feeding tentacles. Specimens reach about 10 cm in extended length. They are very stiff and rigid when handled, and their pedicels are non-retractile.

Some individuals of each species were observed for many months in aquaria equipped with running water. About 15 female C. pseudocurata produced eggs in captivity, some of which developed into young holothurians and were returned to the capture site. E. quinquesemita often eviscerated in aquaria but survived the experience. C. miniata eviscerated less commonly but several which did died soon thereafter. Specimens of the two small species could not be induced to eviscerate despite prolonged provocation. Individuals sliced longitudinally as erythrocytes were collected

usually expanded the feeding tentacles when placed in sea water. This was not observed in the other species. Exsanguinated specimens were frozen or stored in 70% ethanol/water for purposes of species verification.

Sclerite Morphology

Sclerites, or ossicles, are microscopic calcareous structures found within the body wall of almost all holothurians. Their form and structure is of great importance in taxonomy. A species often has several major kinds of sclerites. Each such type of sclerite is represented by many individual ossicles showing variations of the type. There is a descriptive nomenclature used to describe ossicles.

Individuals of all species studied were dissolved in 25% sodium hypochlorite solution, and the sclerites washed in distilled water and photographed. C. curata and C. pseudocurata had the same sclerite types. However, C. curata had an additional, distinctive kind of sclerite apparently unique to the species (Fig. 17).

DISCUSSION

The hemoglobins of holothurians are contained in nucleated amoeboid cells termed hematocytes (Hyman, 1955). On a microscope slide the cells move about and extrude pseudopodia. Hematocytes aggregate into clumps when removed from an animal. The mechanism by which this occurs is not known, and clumping is not reduced by use of heparinized saline solution. Some individuals appeared to have clumps of hematocytes within the coelom when the specimens were opened. The clumping phenomenon may not be merely a preparative artifact. Cell clumps will enmesh foreign material within them and once formed are difficult to disperse.

A yellow substance, perhaps a lipid, is often seen as a floating contaminant of the hemolysate preparation. It is easily removed by millipore filtration or column chromatography.

The range of apparent molecular weights of the oxy hemoglobins of the five species investigated in this study are in agreement with values published for other holothurian species. The dimeric nature of these hemoglobins seems an established pattern.

When purified C. pseudocurata hemoglobin was

deoxygenated with sodium dithionite and re-chromatographed on G-100 Sephadex the apparent molecular weight was raised, indicating an aggregation state. These results are similar to those of Bonaventura and Kitto (1973) and Terwilliger (1975) who carried out the same experiment with C. miniata hemoglobin. The deoxy aggregate molecular weights of these two species, 51,000 and 55,000 daltons, respectively, lie approximately half way between the expected molecular weights of dimeric and tetrameric configurations. It may be that these experimentally derived molecular weight values represent equilibria of association and dissociation between subunits which occurs so rapidly that single asymmetric peaks are produced upon gel chromatography.

The five hemoglobins investigated appear to be homogeneous by gel permeation and ion exchange chromatography. Single asymmetric peaks were produced by ion exchange chromatography on DEAE-cellulose. However, E. quinquesemita hemoglobin may be heterogeneous as it showed a fraction in the void volume, which presumably represents oxidized pigment.

Native C. pseudocurata hemoglobin was further investigated as to homogeneity by polyacrylamide disc gel electrophoresis. Two bands were resolved when carbonmonoxyhemoglobin was electrophoresed but only one band occurred when the pigment was first converted to the cyan

met derivative. An explanation for this phenomenon may be that C. pseudocurata hemoglobin might, like that of Thyonella gemmata, have a low affinity for carbon monoxide, and part of the sample may be converting in situ to methemoglobin, oxyhemoglobin, or other molecular species. Aggregation may play a role as disc gel electrophoresis is thought to resolve proteins as a function of their molecular weights. Should part of the sample aggregate, two bands might occur. On the other hand, hemoglobins are known to form very stable bonds with the cyanide ligand, resulting in single molecular species. Thus, no apparent heterogeneity was present when the protein was electrophoresed in the cyanmet state.

Subunit heterogeneity was investigated by two electrophoretic techniques. Inclusion of urea in the resolving gel and in the incubation buffer denatures proteins and overcomes electrostatic forces between subunits. Resolution of the polypeptide chains occurs as a function of the net electrical charge of each chain. A newer technique, sodium dodecyl sulfate electrophoresis (SDS electrophoresis) denatures proteins by binding anionic groups to the protein "backbone" and resolution of subunits is a function of their molecular weights. When these electrophoretic techniques were employed, two bands were resolved from the hemoglobin of all three species of Cucumaria. These same

bands were also seen when the hemoglobins were first oxidized with performic acid, which was intended to prevent disulfide linkages from forming between sulfhydryl groups of different polypeptide chains. From these data it was included that the hemoglobins of each species of Cucumaria examined are composed of two non-identical, electrophoretically distinct subunits. Under the same conditions, the hemoglobin of S. briareus electrophoresed in both urea and SDS gels as a single component, and therefore appears to be a homodimer.

The amino acid composition of C. pseudocurata hemoglobin appears to be very similar to those obtained by Terwilliger and Read (1970) for the hemoglobins of C. miniata and C. piperata. Based on molar ratios of the amino acids present, a species diversity index was calculated according to the method of Harris and Teller (1973) comparing C. pseudocurata to C. miniata and C. piperata, similar species, and to Molpadia intermedia and Molpadia oolitica, dissimilar species. It was found that by this method C. pseudocurata is closely related to the other two Cucumariids but quite distant from the Molpadid species. Amounts of the amino acid proline were approximately equal in the three Cucumaria hemoglobins, which is significant because proline is known to interrupt the helical structure of proteins (Schellman and Schellman, 1964) and this similarity in number of

of proline residues may indicate a corresponding similarity in tertiary structure as well (Terwilliger and Read, 1970). No holothurian hemoglobin tertiary structure has been determined.

C. pseudocurata hemoglobin appears to bind oxygen in a fashion similar to that of C. miniata reported by Terwilliger and Read (1970). Because several hundred individuals of this small species are required for a hemoglobin preparation oxygen binding studies have been limited.

It has been suggested that C. curata and C. pseudocurata might be conspecific (Smith and Carlton, 1975). Rutherford (1977) addressed this problem by collecting numbers of individuals from each of thirteen sites between California and British Columbia, and compared their isozymes by starch gel electrophoresis. He found no variations between populations and concluded that only one species is valid, C. curata, which was first described. He also presented drawings of integumentary ossicles as these are important in holothurian taxonomy. The ossicles were isolated from one population. The anal and tentacular protions of each individual specimen were amputated prior to maceration, leaving a "tube."

In the course of this study the hemoglobins of C. curata and C. pseudocurata were compared in a parallel investigation. The specimens of C. curata were obtained

from a site at or very near the type locale reported by Cowles (1907) in the original description of the species. C. pseudocurata specimens were obtained from a number of sites near Cape Arago, Oregon. Ossicles were removed from entire individuals of each putative species.

Molpadid holothurians can be differentiated from Dendrochirotid by their hemoglobins, and within the latter group S. (Thyone) briareus can be distinguished from species of Cucumaria by subunit electrophoresis. However, the hemoglobins of the three species of Cucumaria studied cannot be distinguished from one another by the electrophoretic techniques used.

A difference between the ossicles of C. curata and those of C. pseudocurata was observed. Both species have distinctively shaped perforated ossicles of a general type termed a "plate." Specimens of C. curata were found to have an additional type of ossicle not observed in a C. pseudocurata ossicle preparations (Fig. 14). It resembles a type figured by Cowles in the original species description. It is possible that the extra ossicle occurs only in those portions of the holothurians which Rutherford excised. Alternatively, the extra ossicle might only be found in individuals living at the type locale, at which Rutherford probably did not sample. It is uncertain as to how much emphasis the extra ossicle data properly be given.

A comparison of isozymes from C. pseudocurata and the corresponding isozymes from other species of Cucumaria might show whether Cucumaria species are distinguishable by this method. Certainly, comparative electrophoresis of Cucumaria hemoglobins does not provide a basis for distinguishing between species. Until further data is available the question of whether C. curata and C. pseudocurata are separate species should be regarded as unsettled.

Consideration was given as to what advantage might be conferred to these holothurians by possession of hemoglobin. These small forms dwell in mussel beds, which are exposed to air for several hours a day. It was observed that the holothurians contract when the mussel beds are exposed. This thickens the integument and presumably reduces diffusion of gases across the body wall. Perhaps the function of hemoglobin in this form is one of storage, to provide oxygen to the tissues during periods of prolonged contraction. C. miniata hemoglobin binds oxygen very much like that of C. pseudocurata, yet the two species occupy very different habitats. C. miniata lives beneath large fixed rocks in burrows in which they fit very tightly. Generally, only the feeding tentacles and a short portion of the anterior of the animal are extruded. Defense against predators such as Pycnopodia helianthoides and Solaster dawsoni consist of contraction into the burrow,

where little standing water is found. It may be that the hemoglobin is an oxygen storage device for periods of long contraction. This storage function hypothesis is conjecture and requires further data before conclusions can be drawn.

The holothurian hemoglobins of this study appear to have structural and functional properties similar to those of other Dendrochirotid species. A new finding, however, is that Cucumaria miniata hemoglobin is composed of two non-identical subunits and may be considered a heterodimer.

Further structural studies of holothurian hemoglobins should include pigments from species of the order Aspidochirotida. At least one, Parastichopus californicus, is known to contain a compartmentalized hemoglobin (Prosser and Judson, 1952), of which no structural data have been published. Members of the Aspidochirotida are errant, very active holothurians, and their hemoglobins may prove to have different structural and/or functional properties than those of Dendrochirotid of Molpadid species.

It would be of interest to know at what point in development that juvenile C. pseudocurata individuals begin manufacture of hemoglobin. Such a project is feasible as development is direct in this species and the eggs give rise to individuals about 2.5 mm in length and a little less than 1 mm in diameter. Individuals could be pooled and homogenized in a tissue grinder and the homogenate

centrifuged. The supernatant could then be examined by spectroscopy at wavelengths sensitive to C. pseudocurata hemoglobin.

Further structural studies upon the hemoglobins examined in this investigation are possible on a finer scale. Enzyme digest of these pigments can be electrophoresed on SDS gels, resulting in "peptide maps." Such enzyme digestion products can also be compared by two dimensional thin layer chromatography on silica gel. These techniques may reveal subtle differences between hemoglobins not apparent by the electrophoretic methods employed in this study. Some preliminary work involving both of the above techniques has been carried out.

Additional oxygen binding studies will be necessary for a better understanding of how holothurian hemoglobins function. I suspect that echinoderms evolved the water vascular system originally as a means of facilitating exchange of gases. This structure is a unique respiratory system, which, judging by the multiplicity of echinoderm species appears to be very effective. It is therefore remarkable that some holothurian species, but not all, have found it advantageous to possess hemoglobin in addition to the water vascular system. As echinoderms must have diverged from other deuterostome stocks a very long time ago, the genetic potential for hemoglobin synthesis

must be even more ancient.

Figure 1. Chromatography of Cucumaria pseudocurata hemoglobin on Sephadex G-100. Buffer 0.05 I Tris HCl (pH 7.45) 0.01 M in NaCl. = absorbance 416 nm.

= absorbance 280 nm.

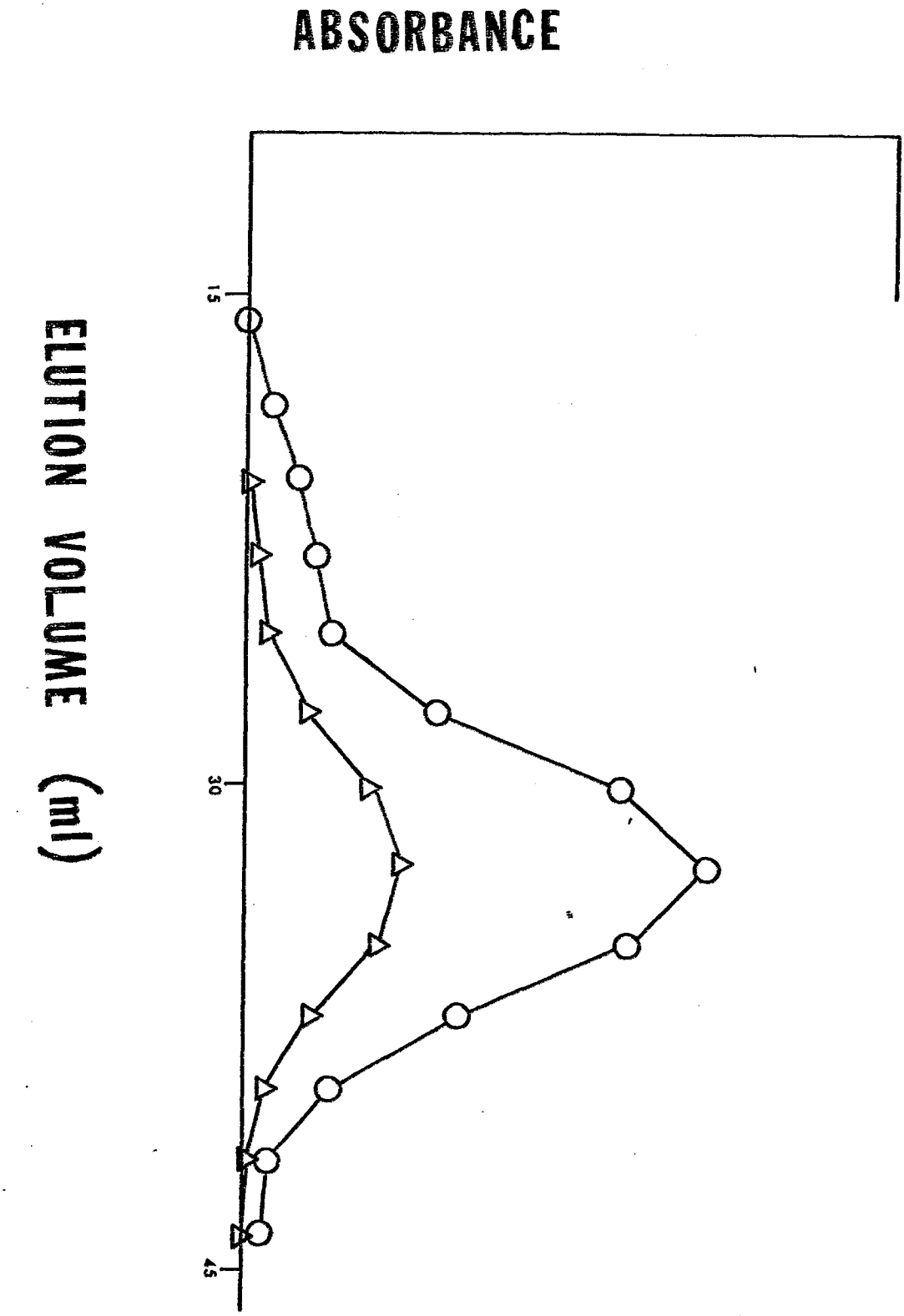
ABSORBANCE
ELUTION VOLUME (ml)
15
30
45

Figure 2. The molecular weight of Cucumaria pseudocurata hemoglobin on Sephadex G-100. Data is from Figure 1. The protein calibration markers are (a) bovine serum albumin, (b) chymotrypsinogen A, and (c) sperm whale metmyoglobin. The arrow indicates the volume at which the protein elutes from the column.

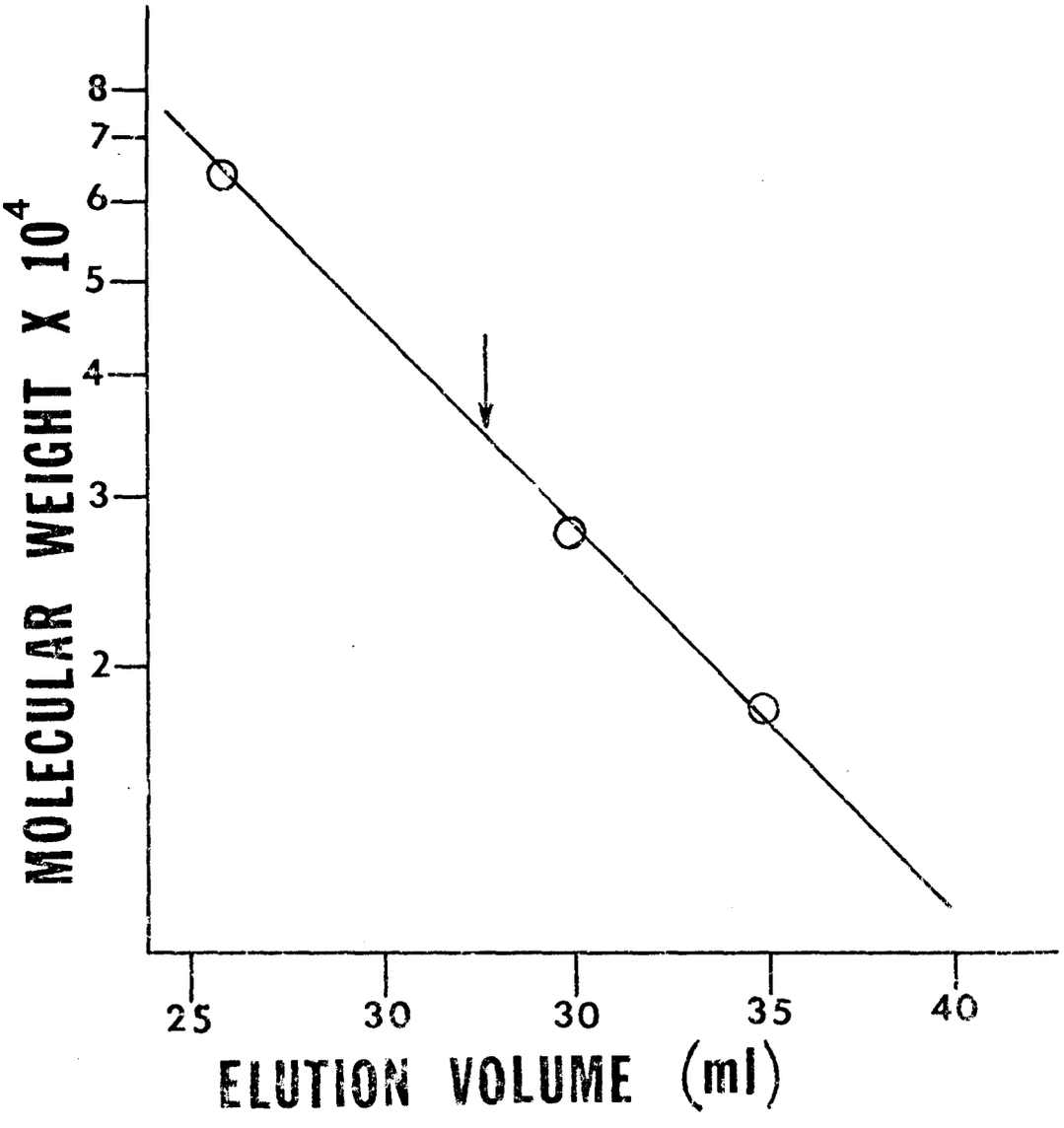
MOLECULAR WEIGHT X 10^4
8
7
6
5
4
3
2
25
30
30
35
40
ELUTION VOLUME (ml)

Figure 3. Chromatography of Cucumaria pseudocurata hemoglobin on Sephadex G-100. The solid line represents chromatography of oxyhemoglobin and the dashed line represents chromatography of deoxyhemoglobin. The dashed line curve is shorter than the other because only half as much deoxy hemoglobin was chromatographed. Buffer 0.05 I Tris HCl (pH 7.45) 0.01 M in NaCl. = absorbance 280 nm. = absorbance 416 nm. = absorbance 416 nm by deoxy hemoglobin.

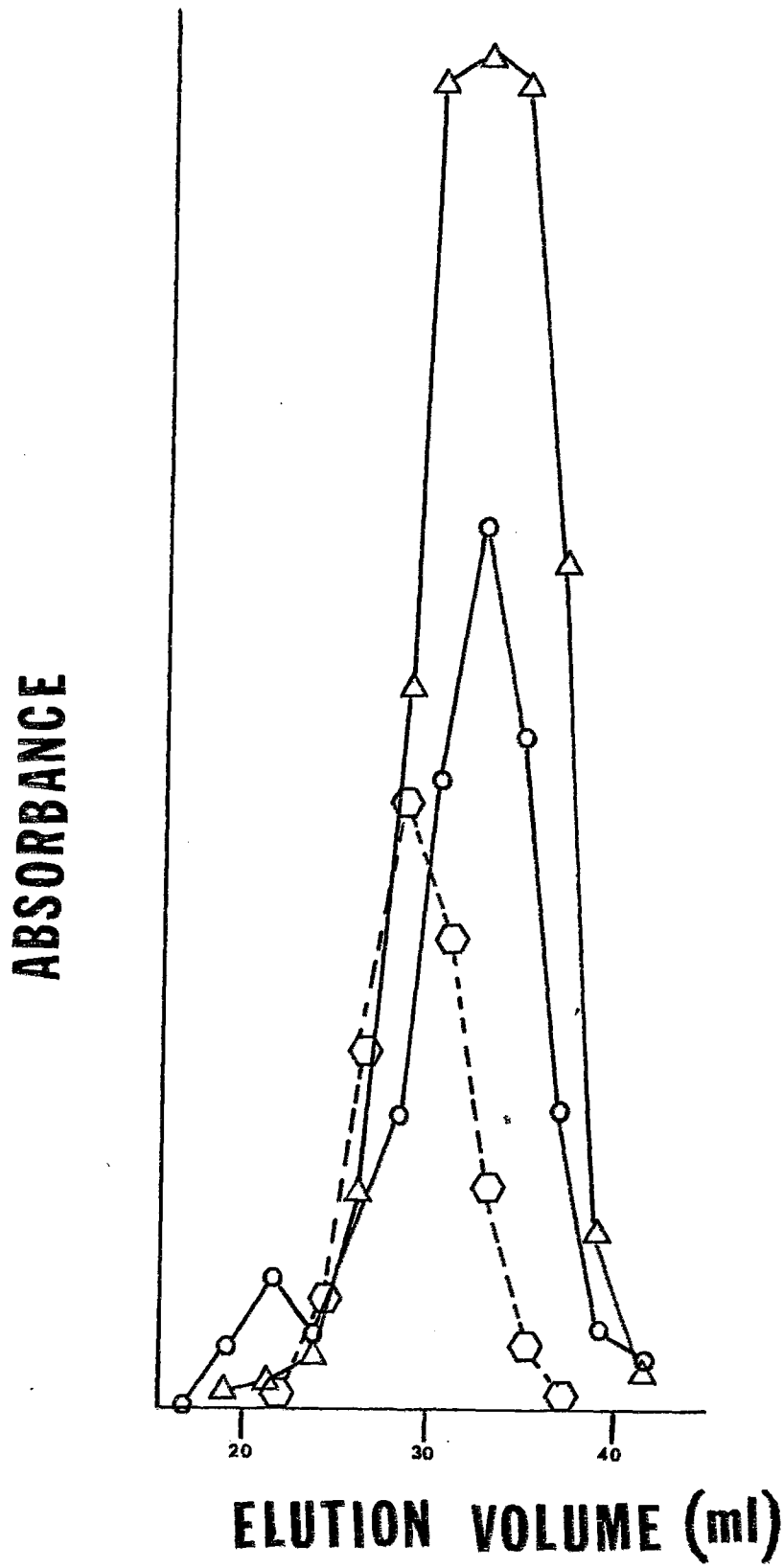
ABSORBANCE
20
30
40
ELUTION VOLUME (ml)

Figure 4. DEAE-cellulose chromatography of Cucumaria pseudocurata hemoglobin. Buffer: 0.01 M ammonium bicarbonate (pH 7.55). The pigment elutes in the presence of a 0-0.2 M NaCl linear gradient. = absorbance 416 nm.

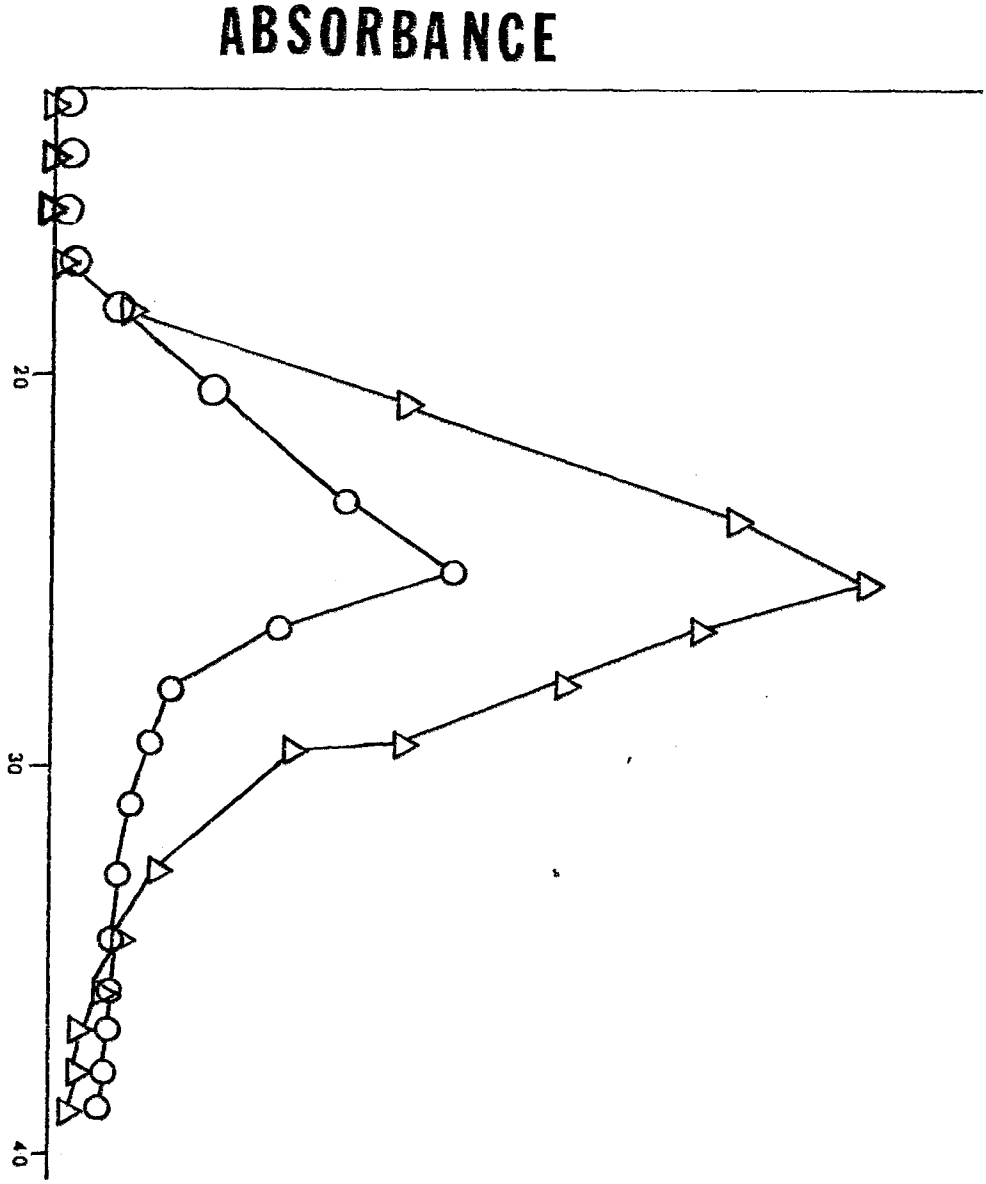
ABSORBANCE
20
30
40

Figure 5. Sodium dodecyl sulfate slab gel electrophoresis of Cucumaria pseudocurata hemoglobin. Gel concentration 15%.

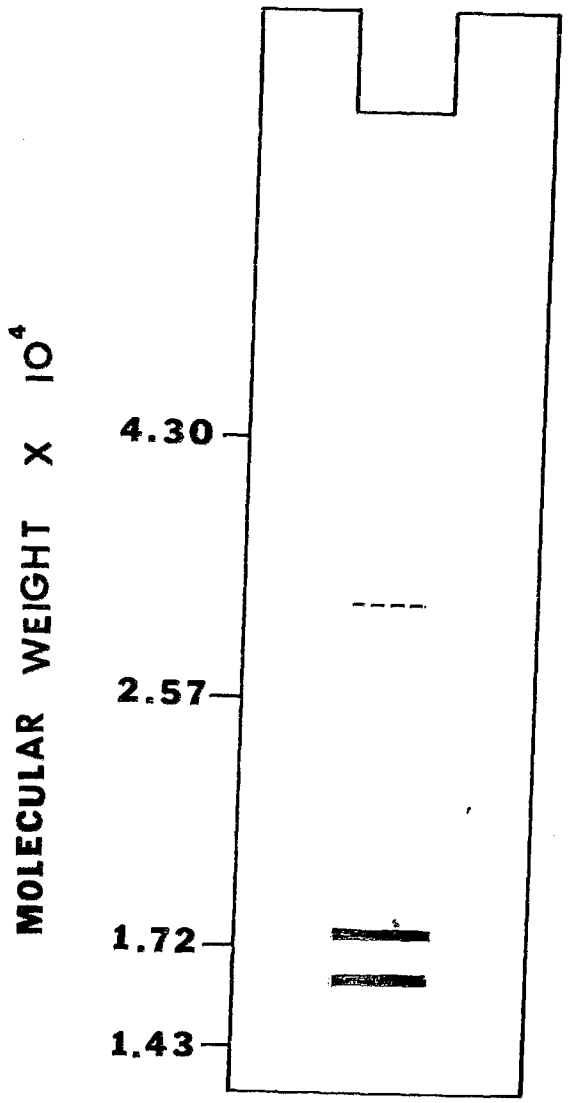
MOLECULAR WEIGHT X 10^4
4.30
2.57
1.72
1.43

Figure 6. Urea gel electrophoresis of Cucumaria pseudocurata hemoglobin. The protein was first reduced with 2-mercaptoethanol.

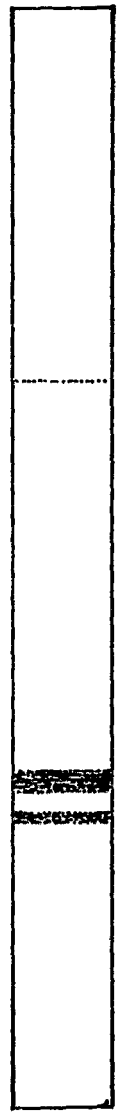

Figure 7. Chromatography of Cucumaria curata hemoglobin on G-100 Sephadex. = abosrbance 416 nm. = absorbance 280 nm.

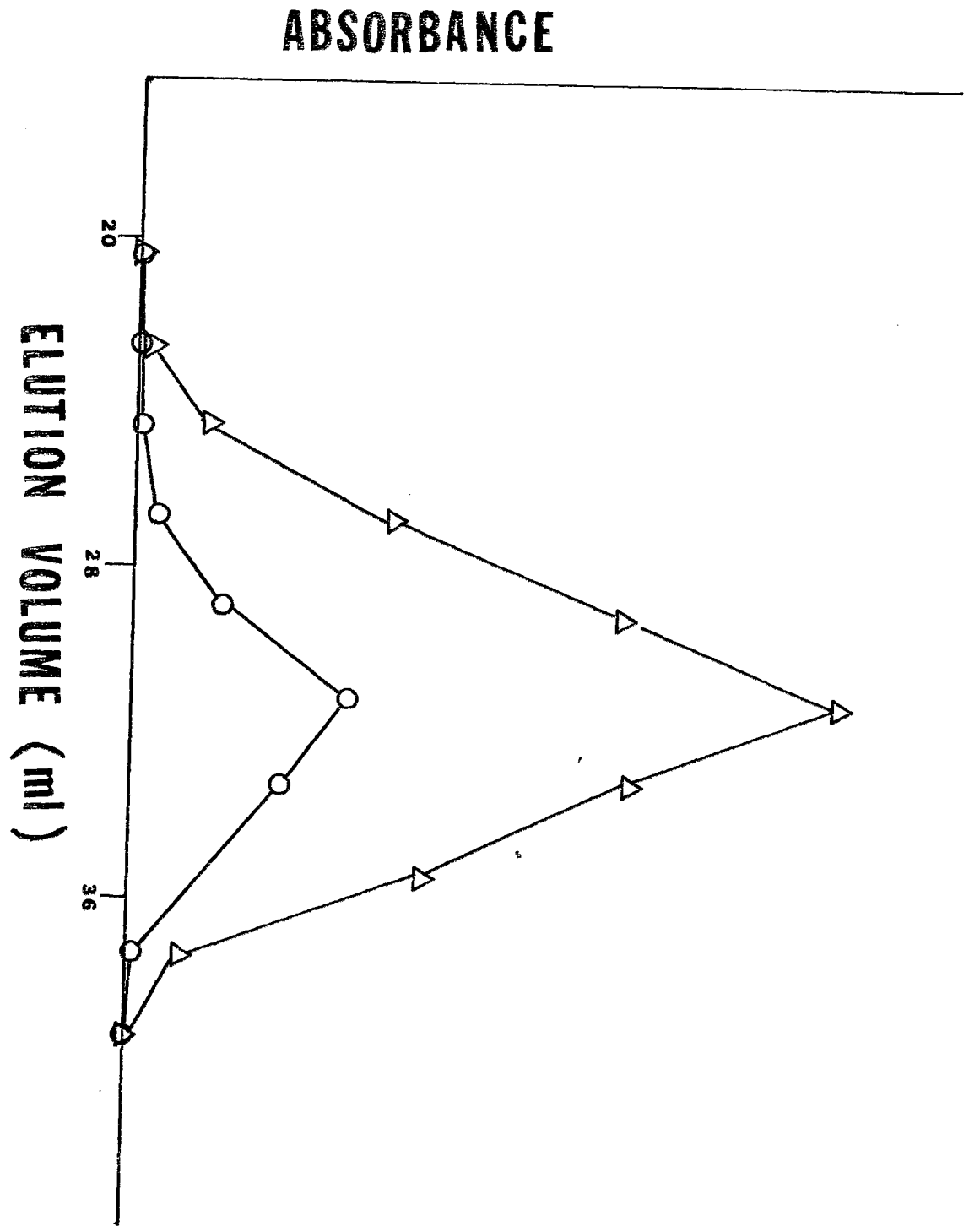
ABSORBANCE
ELUTION VOLUME (ml)
20
28
36

Figure 8. The molecular weight of Cucumaria curata hemoglobin on Sephadex G-100. Data is from Figure 7. The protein calibration markers are (a) bovine serum albumin, (b) chymotrypsinogen A, and (c) sperm whale metmyoglobin. The arrow indicates the volume at which the protein elutes from the column.

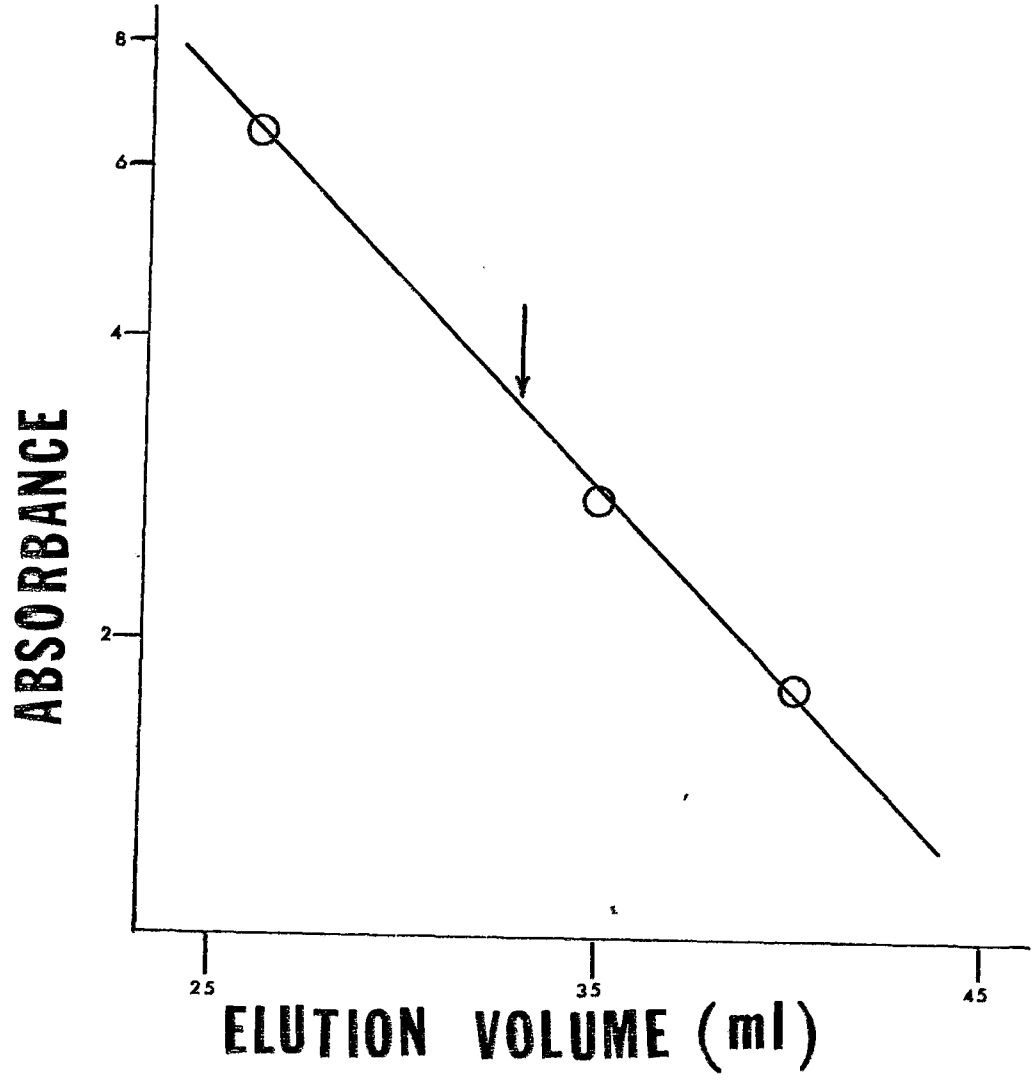
8
6
4
2
ABSORBANCE
25
35
45
ELUTION VOLUME (ml)

Figure 9. DEAE-cellulose chromatography of Cucumaria curata hemoglobin. = absorbance 416 nm. = absorbance 280 nm.

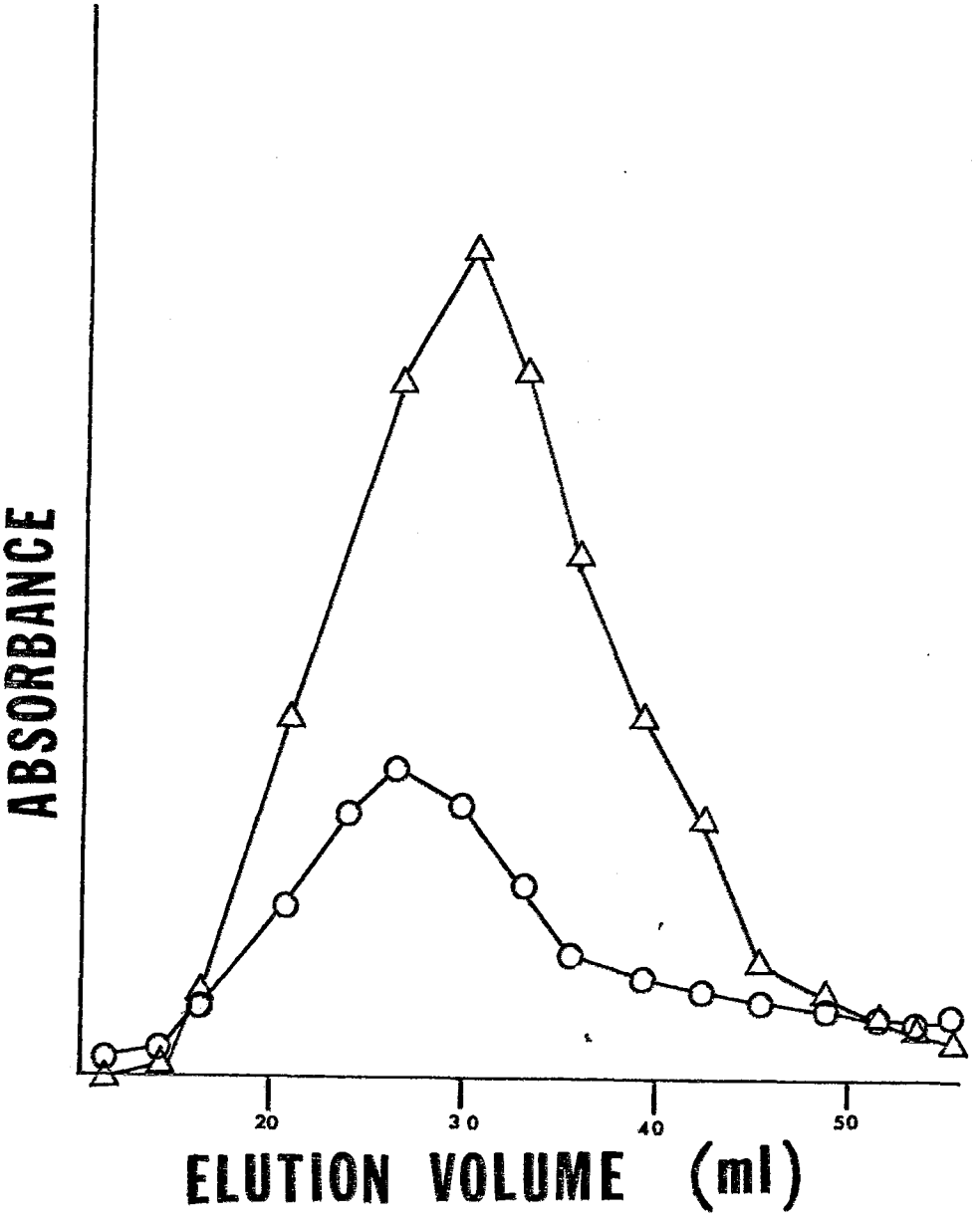
ABSORBANCE
20
30
40
50
ELUTION VOLUME (ml)

Figure 10. Sodium dodecyl sulfate slab gel electrophoresis of Cucumaria curata hemoglobin. Gel consistancy 15%.

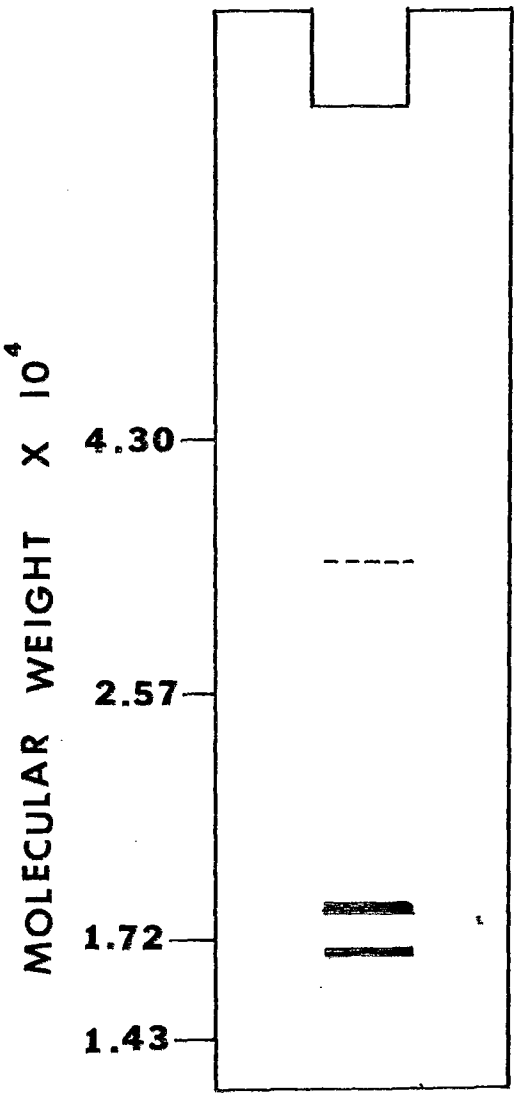
MOLECULAR WEIGHT X 10^4
4.30
2.57
1.72
1.43

Figure 11. Sodium dodecyl sulfate slab gel electrophoresis of Cucumaria miniata hemoglobin. Gel consistancy 15%.

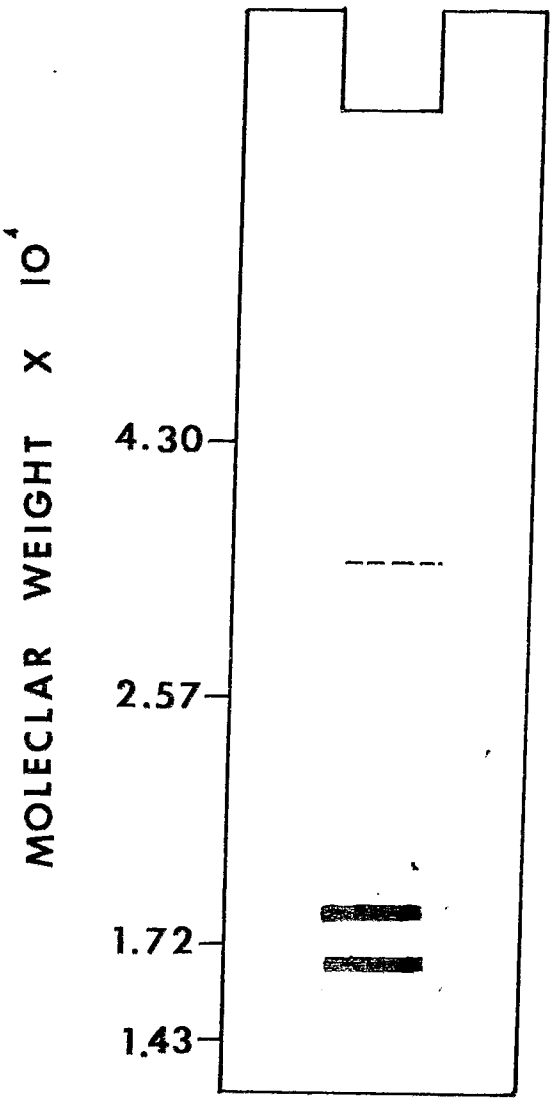
MOLECLAR WEIGHT X 10⁴
4.30
2.57
1.72
1.43

Figure 12. Urea gel electrophoresis of Cucumaria miniata hemoglobin. The protein was first reduced with 2-mercapto-ethanol.

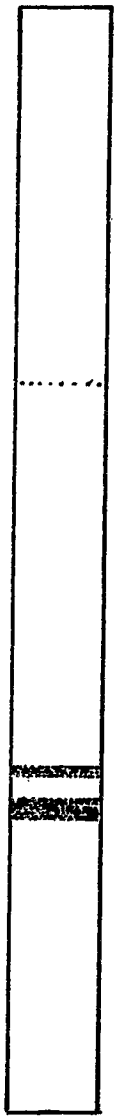

Figure 13. Chromatography of Sclerodactyla briareus hemoglobin on Sephadex G-100. = absorbance 416 nm. = absorbance 280 nm.

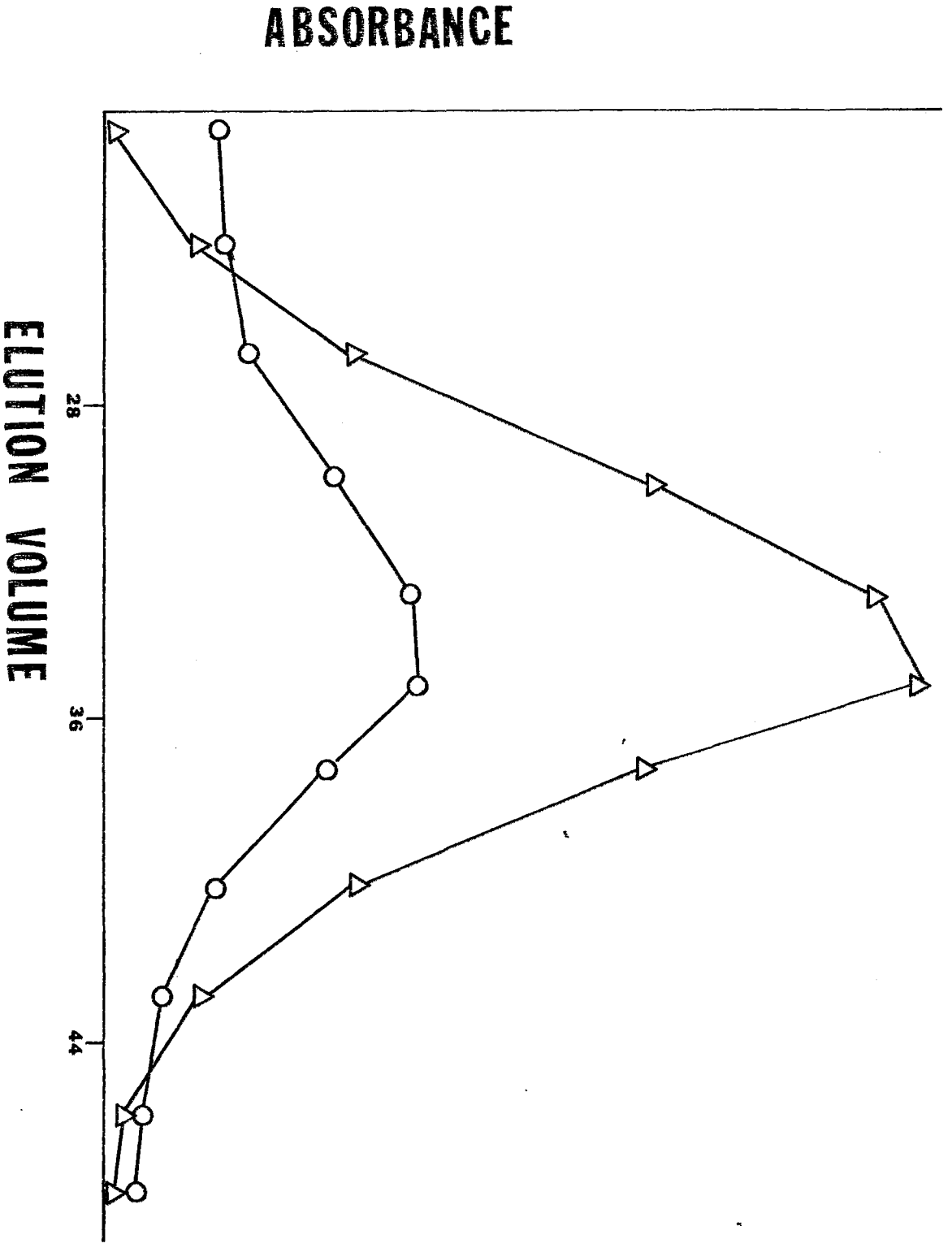
ABSORBANCE
ELUTION VOLUME
28
36
44

Figure 14. Sodium dodecyl sulfate slab gel electrophoresis of Sclerodactyla briareus hemoglobin. Gel consistancy 15%.

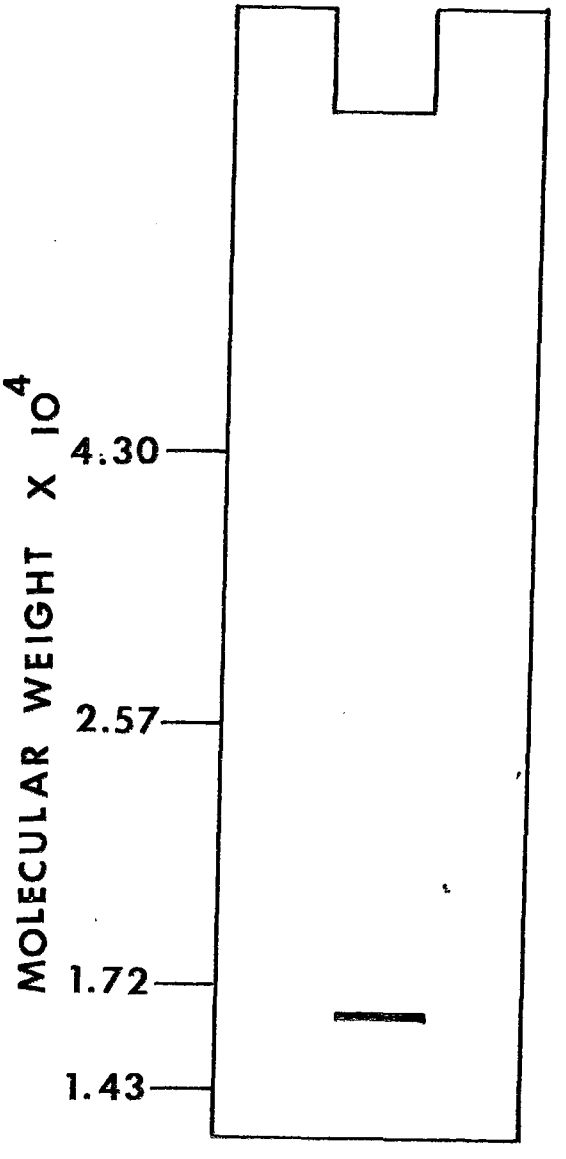
MOLECULAR WEIGHT X 10^4
4.30
2.57
1.72
1.43

Figure 15. Chromatography of Eupentacta quinquesemita hemoglobin on Sephadex G-100. Buffer: 0.05 I Tris HCl (pH 7.45) 0.01 M in NaCl. = absorbance 416 nm.

= absorbance 280 nm.

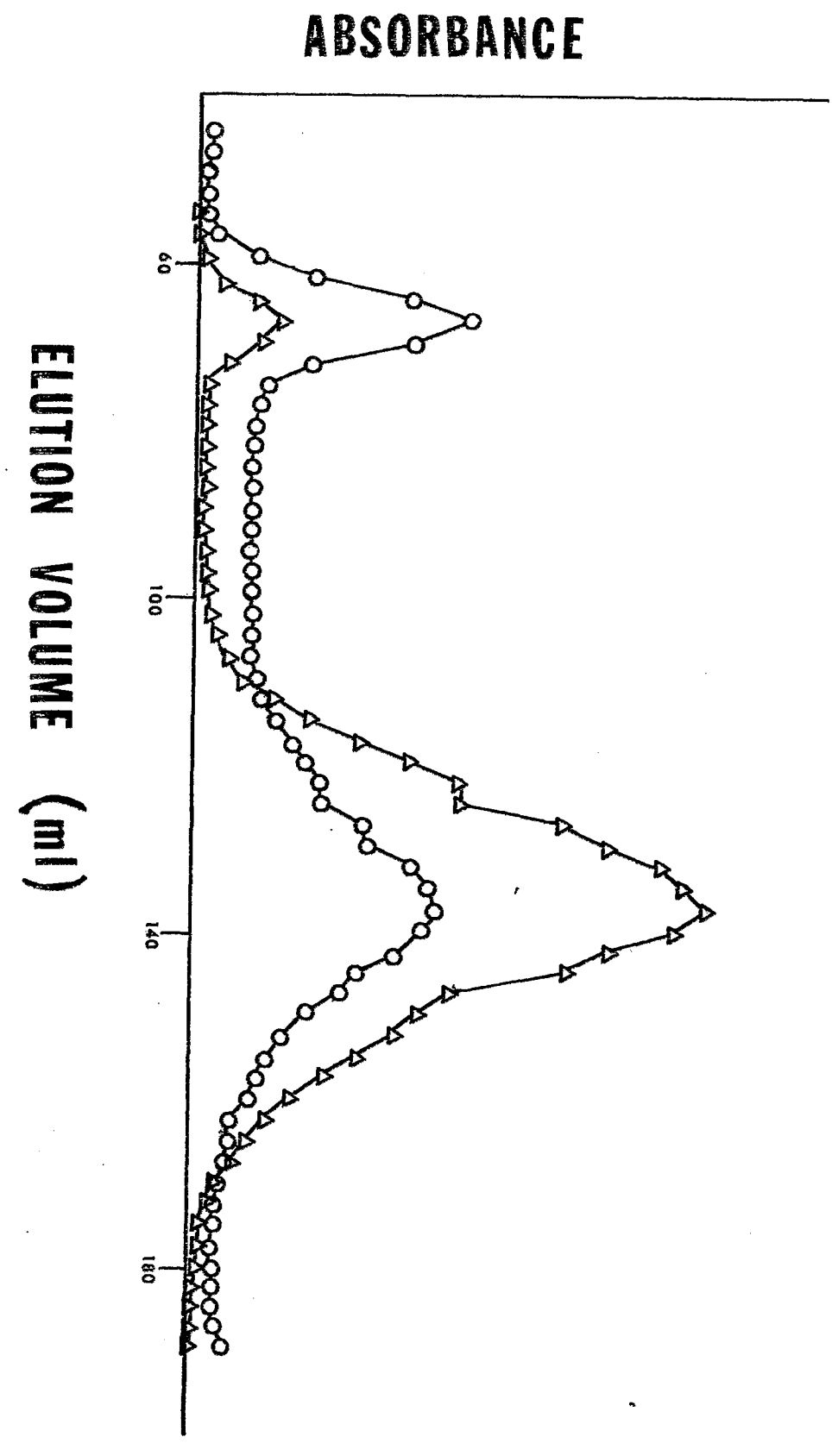
ABSORBANCE
60
100
140
180
ELUTION VOLUME (ml)

Figure 16. Molecular weight of Eupentacta quinquesemita hemoglobin on Sephadex G-100. Data is from Figure 13. Protein calibration markers are (a) bovine serum albumin (b) chymotrypsinogen A (c) sperm whale metmyoglobin. The arrow indicates the volume at which the hemoglobin elutes from the column.

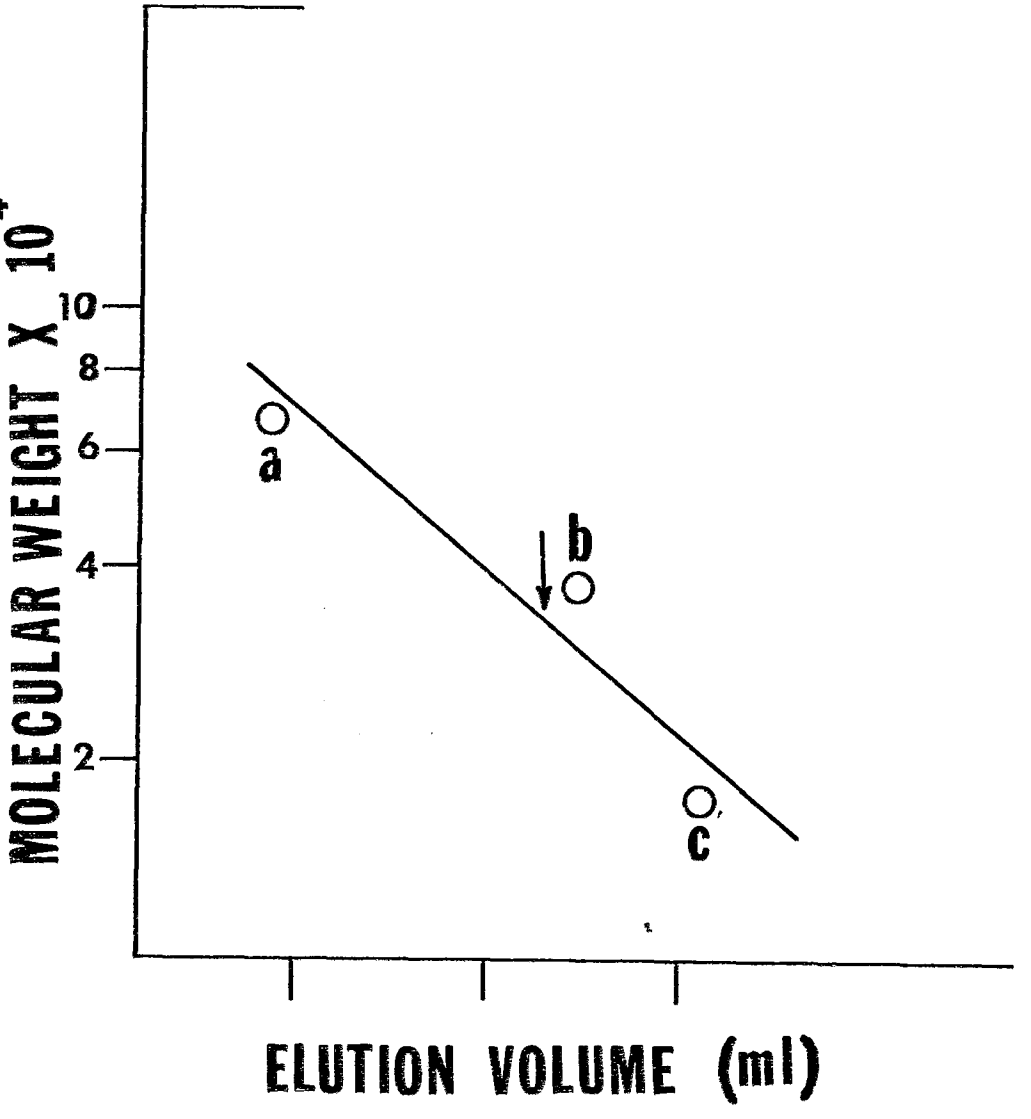
MOLECULAR WEIGHT X 10^4
10
8
6
4
2
a
b
c
ELUTION VOLUME (ml)

Figure 17. Ossicles. The upper figure is the "extra ossicle" found only in Cucumaria curata; the lower figure represents an ossicle type found in both Cucumaria curata and Cucumaria pseudocurata.

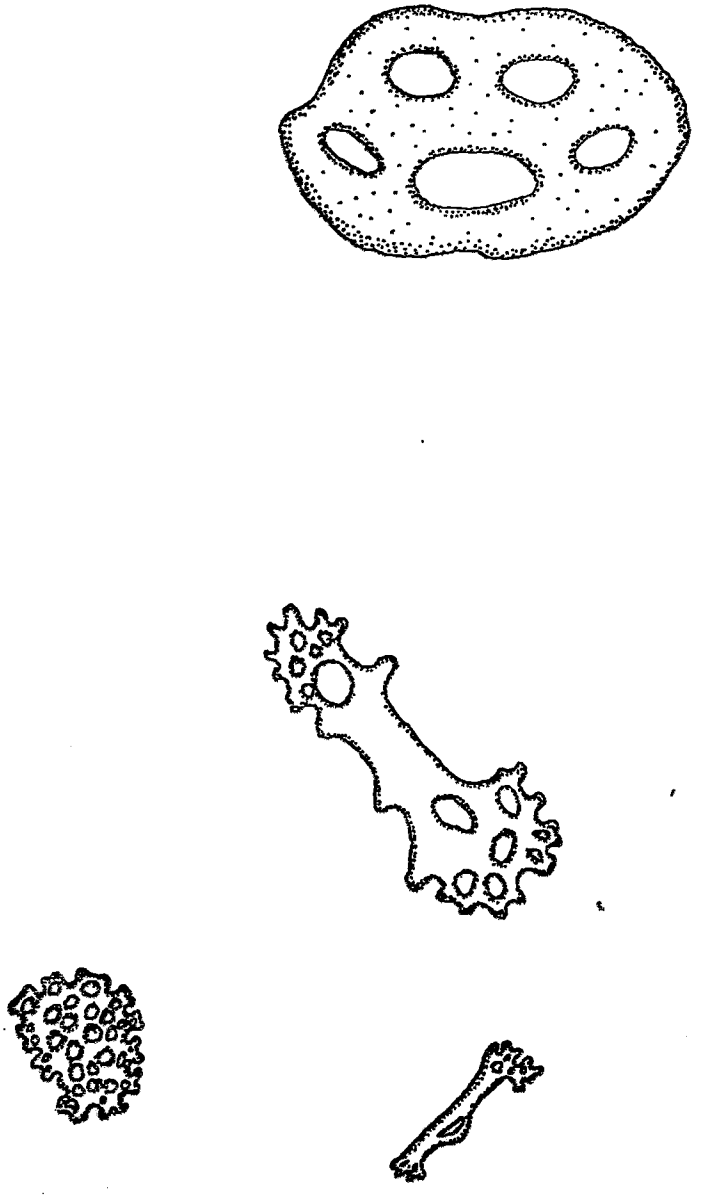

TABLE 1. The amino acid composition of *Cucumaria pseudocurata* hemoglobin. *

Lysine	0.073
Histidine	0.036
Arginine	0.064
Aspartic acid	0.111
Threonine	0.057
Serine	0.067
Glutamic acid	0.119
Proline	0.016
Glycine	0.079
Alanine	0.081
½Cysteine	0.012
Valine	0.050
Methionine	0.031
Isoleucine	0.072
Leucine	0.077
Tyrosine	0.013
Pheylalanine	0.041
Tryptophan	0.010

* The numbers represent amino acid molar ratios.

TABLE 2. Absorption maxima.

Species	Ligand	Soret	—	—
C. miniata	O_2	416	542	578
	CO	420.2	535	568
C. pseudocurata	O_2	416	541	578
	CO	422	539	572
C. curata	O_2	415.8	542	579
	CO	422	539	572
S. (Thyone) briareus	O_2	415	542	579
E. quinquesemita	O_2	416.2	544.5	579
	CO	417.9	548	565

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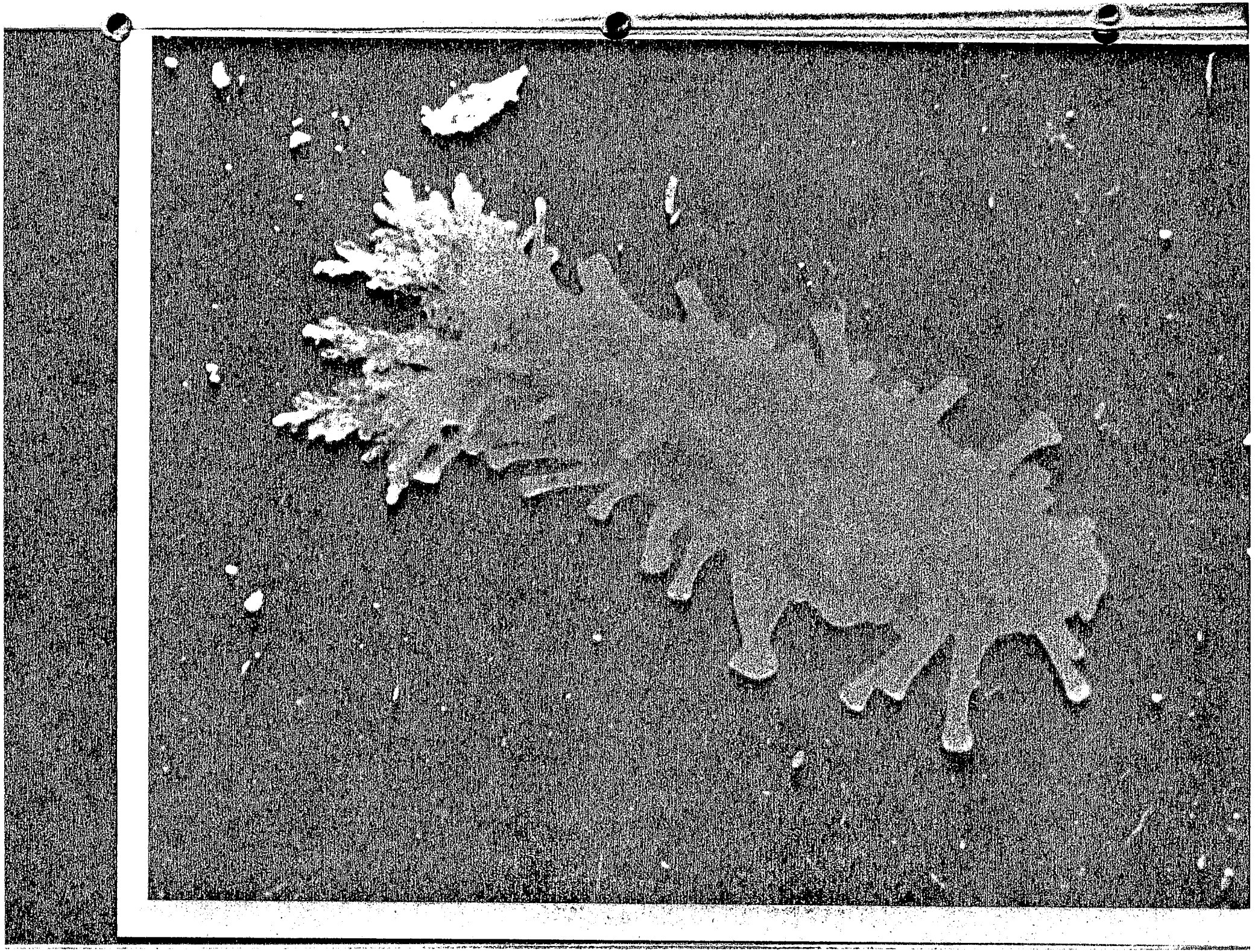

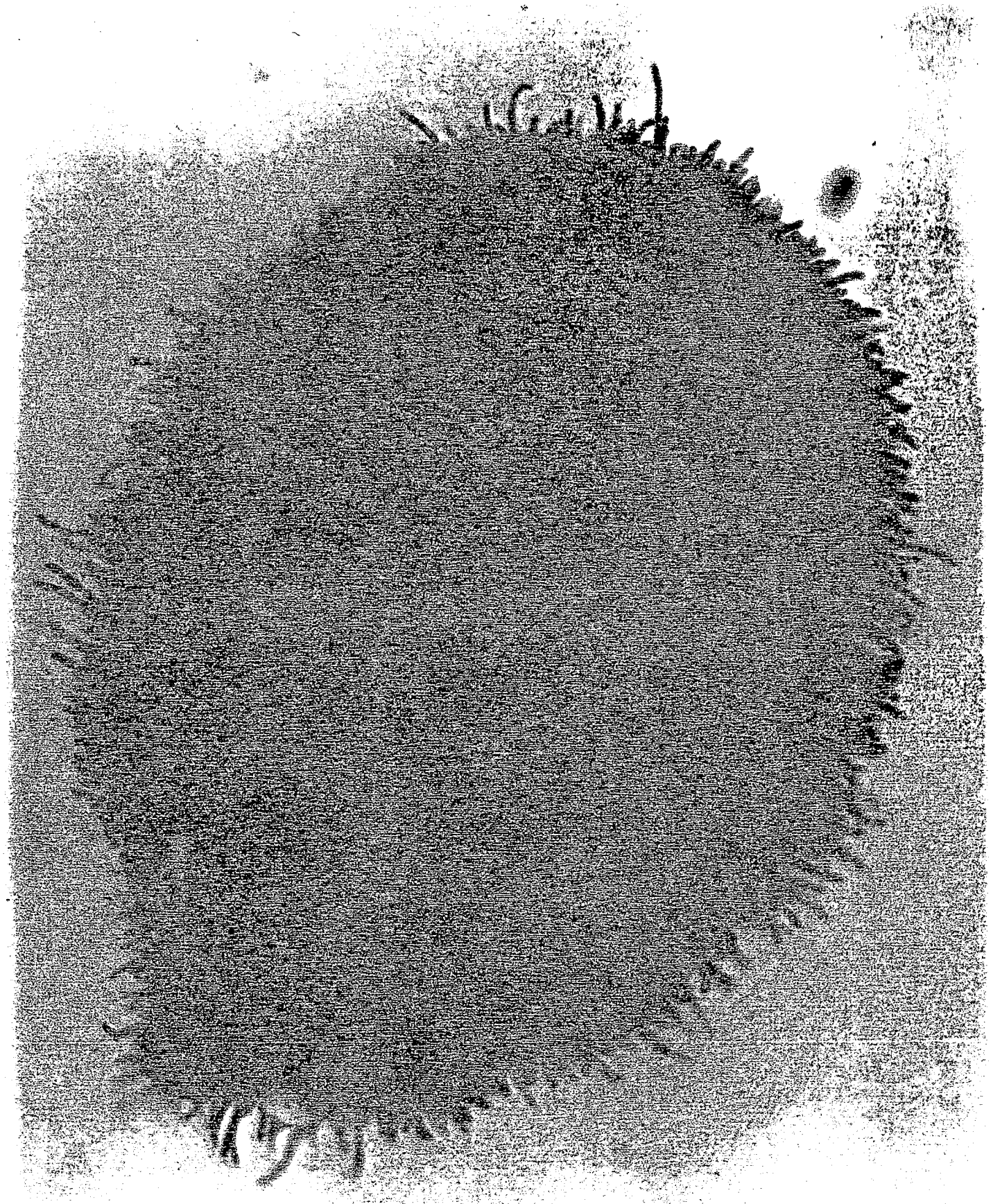


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