

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE
EXTRACELLULAR HEMOGLOBINS OF THE BRANCHIOPOD
CRUSTACEANS LEPIDURUS BILOBATUS
AND DAPHNIA PULEX

by

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Title: STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE
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Hemoglobin (erythrocrucorin) is found in many representatives of the invertebrate phyla. Among the arthropods, however, its presence is restricted to a few insect species and several minor groups within the Subphylum Crustacea. The arthropodan hemoglobins which have been studied fall into two groups based on structural patterns: The insect hemoglobins, which appear to be monomers or dimers of 14-15,000 molecular weight subunits and the branchiopod crustacean hemoglobins which exist as large polymeric proteins. Branchiopod hemoglobins are similar to those of the insects in that they both contain one mole heme per 17-20,000 grams protein. However, subunit molecular weights assessed by methods other than heme content differ strikingly among the branchiopod species investigated. Previous studies showed that the subunits of some branchiopod hemoglobins (eg. Cyzicus) are polypeptides with molecular weights similar to insect hemoglobins ($M_r = 15,000$). In contrast, other Branchiopod hemoglobins (eg. Artemia)

have been shown to be composed of large polypeptides ($M_r = 125,000$) each polypeptide associated with up to seven heme groups.

A major question exists with respect to the subunit structure of the branchiopod hemoglobins. Are they similar to the vertebrate hemoglobins with 15-17,000 molecular weight subunits or are these subunits long polypeptide chains composed of a linear series of covalently linked heme-containing units (domains)?

The purpose of this study has been to perform a careful analysis of the structure of the extracellular hemoglobins from two representatives of the branchiopod crustaceans, the tadpole shrimp, Lepidurus bilobatus and the cladoceran Daphnia pulex. A variety of chromatographic and electrophoretic methods have been employed to characterize these pigments. Rapid purification strategies have been utilized as well as the inclusion of proteolytic blocking agents in the purification buffers in an attempt to obviate proteolysis during the purification process.

The extracellular hemoglobins of the notostracan Lepidurus and the cladoceran Daphnia exist as polymers with apparent molecular weights of 680-800,000 and 430-470,000, respectively. The purified hemoglobins both contain one mole heme per 18-20,000 gram protein. However, attempts

to dissociate the hemoglobins by harsh denaturing conditions have shown that Lepidurus and Daphnia hemoglobins are composed of subunits with molecular weights around 34,000.

Gentle proteolysis of Lepidurus and Daphnia hemoglobins with enzymes of different specificities produces 15-17,000 molecular weight polypeptide chains. These proteins, isolated by column chromatography, have a heme content similar to those of the intact pigment from which they were isolated and bind oxygen reversibly. It is proposed, therefore, that the intact 34,000 molecular weight subunits of Lepidurus and Daphnia hemoglobins consist of two linearly linked myoglobin-like oxygen binding domains.

Oxygen equilibrium studies of Lepidurus hemoglobin and its digestion products have been performed. Oxygen binding properties of intact Lepidurus hemoglobin show a low oxygen affinity with a slight Bohr effect. In contrast, the domains isolated from digestion of Lepidurus hemoglobin display a relatively high oxygen affinity and lack a Bohr effect. The native molecule seems necessary for the expression of the heterotropic interactions and relatively low oxygen affinity.

Homogeneous 15,000 molecular weight fragments of Lepidurus hemoglobin produced by proteolytic cleavage of the native molecule have been isolated by gel column chromatography and ion exchange chromatography. Two dimensional

tryptic peptide maps of Lepidurus globin are presented and suggest that the 34,000 molecular weight subunit may be made up of two domains that are dissimilar in primary structure.

This study is the first to suggest that the subunits of some branchiopod crustacean hemoglobins may be constructed of a series of covalently linked structural and functional domains. Recent work by others on related species is supportive of these conclusions.

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CHAPTER 1

INTRODUCTION

In contrast to the almost universal occurrence of hemoglobin in the vertebrates, its distribution in the invertebrate animals is phylogenetically erratic. Invertebrate hemoglobin, also called erythrocrucorin, is represented in all the major phyla and several minor phyla. Many studies of the function and structure of these invertebrate pigments have been carried out. Typically these hemoglobins can be categorized into two groups based on their size and location. (For review see Manwell, 1960, 1964; Mangum, 1976; Antonini and Chiancone, 1977; Weber, 1978; Terwilliger, 1980). The first group, the intracellular hemoglobins and myoglobins, are relatively small molecules of about 15-17,000 molecular weight or dimers and tetramers of 15-17,000 molecular weight subunits. Structural studies of several of these pigments have shown that only limited homologies in primary sequence exist between the invertebrate monomeric hemoglobin polypeptides and vertebrate hemoglobins (Imamura et al., 1972, Garlick et al., 1979). However, limited homologies in sequence do not necessarily

preclude homologies in regard to higher orders of structure. The intracellular monomeric hemoglobin from Glycera dibranchiata (Polychaete, Annelid) shows only limited sequence homology with sperm whale myoglobin yet the pigments share similar tertiary structures (Padlan and Love, 1974).

The second group, the extracellular hemoglobins, are typically large polymeric aggregates, many with molecular weights greater than one million. Structural studies of these remarkable proteins have revealed that although they show certain similarities to the vertebrate hemoglobins and low molecular weight invertebrate hemoglobins, the extracellular hemoglobins exhibit some intriguing alternatives to the more usual hemoglobin structure described above.

Annelid extracellular erythrocrucorins are polymers with molecular weights between $2.6-3.6 \times 10^6$. Some dimerization of these large molecules has been reported (Terwilliger et al., 1977a). Most annelid hemoglobins appear to be composed of twelve submultiples of approximately 250,000 molecular weight. When viewed in the electron microscope these submultiples seem to be arranged as a two-tiered hexagonal array around a central core (Roche, 1965; Terwilliger, R.C. et al., 1976). The central core appears to be empty in most of

the molecules visualized; however, two cases of a possible central submultiple occupying this space has been reported (Van Bruggen and Weber, 1974; Terwilliger et al., 1977; Garlick, 1980). Subunit molecular weights of hemoglobins from some annelid species are heterogeneous when treated with sodium dodecyl sulfate. Molecular weights have been reported between 12,000 and 37,000 (Shlom and Vinogradov, 1973; Terwilliger, R.C. et al., 1976; Vinogradov et al., 1977, 1980). The hemoglobins of other species, however, have been reported to have homogeneous subunits with respect to molecular weight ($M_r = 14-16,000$) when analyzed under denaturing conditions (Garlick and Terwilliger, 1975, 1977; Garlick and Riggs, 1978). These findings may imply that these large polymeric molecules are composed of monomeric subunits similar to vertebrate hemoglobins. However, recent amino acid sequence data on one polypeptide chain from earthworm erythrocrucorin show that this subunit shares little sequence homology with other hemoglobin chains (Garlick and Riggs, 1978; Garlick, 1980). Furthermore, the helical content of annelid extracellular hemoglobins seems to be low (40%) when compared to other hemoglobins (75%) (Harrington et al., 1973; Ascoli et al., 1976). Thus, it is difficult at present to determine how similar the tertiary structure of these proteins is to other members

of the globin family. An additional dilemma is the striking lack of stoichiometry between heme content and subunit size in these molecules. Although the smallest polypeptide chain described above for some annelid erythrocytes is 15-17,000, nearly all annelid extracellular hemoglobins studied so far contain one heme per 22-27,000 gram protein (Waxman, 1975; Terwilliger, R.C. et al., 1975a, 1976; Garlick, 1980). This discrepancy may reflect problems in purification of these molecules (Terwilliger, 1980; Garlick, 1980). However, another possible explanation is that a unique relationship may exist in the way the heme is associated with the protein moiety. For example, some polypeptide chains may not bind a heme and act as a spacer. Alternatively, one heme may be associated with more than one of the polypeptide chains.

Hemoglobins are also widespread among the molluscs. These hemoglobins, again, can be thought to fall into two general structural classes; low molecular weight intracellular hemoglobins and large polymeric extracellular hemoglobins. Monomeric and dimeric myoglobins are abundant in the radular musculature, nerve and heart tissue of many gastropod molluscs (Read, 1966) whereas dimeric and tetrameric hemoglobins occur in the ctenidia or circulating blood cells of a number of bivalve molluscs. Several

of these pigments have been studied in detail and all seem to be composed of 15-17,000 molecular weight subunits (Read, 1966; Furuta et al., 1977).

Extracellular molluscan hemoglobins have been studied in representatives from two groups of molluscs; the planorbid snails (Svedberg and Eriksson-Quensel, 1934; Figueiredo et al., 1973; Wood and Mosby, 1975; Terwilliger, N.B., et al., 1976; Terwilliger et al., 1977b) and clams of the families Carditidae (Terwilliger et al., 1978; Terwilliger and Terwilliger, 1978) and Astartidae (Terwilliger, 1980). These pigments have been shown to have quaternary and subunit structures that are unique among the hemoglobins. The hemoglobin of the planorbid snail Helisoma exists as a 1.7×10^6 molecular weight polymer (Svedberg and Eriksson-Quensel, 1934; Terwilliger, N.B. et al., 1976). As seen in the electron microscope the molecule is circular and displays a unique ten-fold symmetry about a central axis. This unusual shape is consistent with the findings that the smallest subunit molecular weight that can be obtained by harsh methods of denaturation has a molecular weight of 175,000-200,000, one tenth that of the intact molecule (Waxman, 1975; Wood and Mosby, 1975; Terwilliger, N.B. et al., 1976). This is in striking contrast to the 15-17,000 molecular weight subunits of most other hemoglobins. However,

this pigment does contain one mole heme per 18-19,000 gram protein. Terwilliger, N.B. et al. (1976) and Terwilliger, R.C. et al. (1977b) have shown that gentle proteolysis of the molecule cleaves Helisoma hemoglobin into heme-containing functional units (domains) with molecular weights of 15-17,000. These domains have one mole heme per approximately 17,000 gram protein and the isolated domains bind oxygen reversibly. It is likely that the 175,000 molecular weight polypeptide consists of 8-12 heme-containing domains. Similar patterns have been seen for hemoglobin from another planorbid snail Planorbis corneus (Wood and Gullick, 1979).

A similar subunit structure has been proposed for the extracellular hemoglobin of the clam Cardita borealis (Terwilliger et al., 1978). The molecule has an apparent molecular weight of about 12×10^6 . This unusually large molecular weight value is, however, consistent with its appearance in the electron microscope where the hemoglobin appears as an assortment of rod-shaped structures of varying lengths. Its ultrastructure is unlike any polymeric hemoglobin so far described. It resembles neither the two-tiered hexagonal array of the annelid hemoglobin nor the ten membered ring of the planorbid snail hemoglobin. The subunits of Cardita and a related species Astarte hemoglobins have been described as

relatively large polypeptide chains with molecular weights of about 290,000 as analyzed by sodium dodecyl sulfate gel electrophoresis (Waxman, 1975; Terwilliger et al., 1978; Terwilliger, 1980). However, Cardita hemoglobin, like other hemoglobins, contains one mole heme per 17-20,000 gram protein. When dissociated, this hemoglobin can be digested into 15-17,000 molecular weight oxygen binding domains similar to those seen for Helisoma hemoglobin (Terwilliger and Terwilliger, 1978).

It is not known whether the domains of Cardita or Helisoma hemoglobins are structurally alike. However, amino acid compositions of isolated mono- and polydomains of Helisoma are very similar to one another suggesting that the domains may be similar in primary sequence (Terwilliger et al., 1977b). On the other hand, Wood and Gullick (1979) have reported the possibility of intramolecular heterogeneity in the hemoglobin from Planorbis corneus.

Hemoglobins are also found in several groups of the phylum Arthropoda (Fox, 1949 ; 1957). The occurrence of hemoglobin in this group is unusual in that most arthropods utilize the copper-containing respiratory protein hemocyanin. Generally speaking, the hemoglobins appear to fall into two groups based on their structure. The first group, the insect hemoglobins, are relatively low

molecular weight molecules: monomers, dimers and possibly tetramers of 14-15,000 molecular weight subunits. These molecules are characterized by the pigments found in dipteran larvae of Chironomus (Thompson et al., 1968) and Gastrophilus (Adair, 1946; Keilin and Wang, 1946) and the adult hemipteran Buenoa (Bergstrom, 1977). The putative tetrameric hemoglobins of Buenoa and the dimeric hemoglobin of Gastrophilus are found in the tracheal cells of the animal's respiratory system. The monomeric and dimeric hemoglobins of Chironomus are unusual in that they are free in the hemolymph. As many as nine hemoglobins may be produced by Chironomus and they appear to be products of individual genetic loci (Thompson and English, 1966). Certain hemoglobins present in Chironomus have been studied in detail and resemble the typical vertebrate myoglobins and hemoglobins with respect to sequence (Buse et al., 1969) and tertiary structure (Huber et al., 1971).

The second group, the branchiopod hemoglobins, are unusual in that they form large polymeric aggregates with molecular weights ranging from 220-670,000 depending on the species from which the pigment was extracted. In an earlier study Svedberg and Eriksson-Quensel (1934) found that the molecular weight of Daphnia pulex hemoglobin is approximately 420,000. Both the conchostracan Cyzicus and the anostracan Artemia have hemoglobins with molecular

weights of 220-270,000 (Bowen et al., 1976; David et al., 1977; Moens and Kondo, 1976, 1978). Sugano and Hoshi (1971) have estimated that the molecular weights of Daphnia magna and Moina macrocopa hemoglobins are about 670,000 based on sedimentation rates. Similarly, the hemoglobin from the notostracan Triops has a molecular weight of 600,000 (Horne and Beyenbach, 1974).

The hemoglobins of the branchiopod crustaceans, like those of the insects, contain one heme per 17-20,000 gram protein. However, subunit molecular weights obtained by methods other than heme content differ strikingly among the branchiopod species investigated. David et al. (1977) reported a minimum molecular weight of 15,500 for Cyzicus (Crustacea Conchostraca) hemoglobin based on sodium dodecyl sulfate gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis of hemoglobin from the cladoceran Moina shows a major subunit with a molecular weight of 23,000 which after incubation in sodium dodecyl sulfate denaturing solution for up to 35 hours produces smaller polypeptides with molecular weights of 11,700 and 8,900 (Hoshi et al., 1976). In contrast, Moens and Kondo (1976, 1978) report that Artemia hemoglobin consists of two 125,000 dalton polypeptides and have suggested that seven heme groups are associated with this large polypeptide chain. Bowens et al. (1976), also using sodium

dodecyl sulfate gel electrophoresis, have found that Artemia hemoglobin has subunits of 13,000 and 17,000 daltons, a value that is in closer agreement with the minimum molecular weight based on heme content.

Thus, a major question exists with respect to the subunit structure of branchiopod hemoglobins. Are they similar to insect hemoglobins with 15-17,000 molecular weight subunits or are these subunits long polypeptide chains composed of a linear series of oxygen binding domains as seen in the molluscan hemoglobins described above?

Our understanding of the structure of these unusual Arthropod hemoglobins is dependent on careful analysis of the nature of the pigment's subunit. This study was undertaken to describe the structure and function of the extracellular hemoglobins of the branchiopod crustaceans Lepidurus bilobatus (order Notostraca) and Daphnia pulex (order Cladocera). One of the problems encountered in studies of crustacean hemoglobins is that it is difficult to obtain large quantities of hemoglobin without possible proteolytic contaminants in the preparation. Lepidurus (tadpole shrimp) was selected because hemoglobin from this relatively large branchiopod can be purified quickly in large quantities and proteolytic contamination is unlikely. Furthermore, no studies had been published on the

subunit structure of Notostracan hemoglobins based on denaturing experiments. Daphnia pulex (water-flea) hemoglobin has been studied as a comparison within the branchiopod crustaceans.

The results of this thesis will show that Lepidurus and Daphnia hemoglobins are large polymeric aggregates composed of subunits which appear to be two covalently linked heme-containing units. These putative domains have been isolated by gentle proteolysis and have been shown to bind oxygen reversibly. Peptide maps of Lepidurus hemoglobin are presented and suggest that the two domains are non-identical in primary sequence. Oxygen equilibrium experiments performed on Lepidurus hemoglobin are also discussed.

CHAPTER 2

MATERIALS AND METHODS

ANIMALS

Notostracan branchiopods (tadpole shrimps or shield shrimps) comprise a very small group of Euphyllopod crustaceans consisting of a single family with two genera: Triops and Lepidurus. These relatively large (0.1-3.0 gram) branchiopod crustaceans usually occur in fresh or brackish water pools in drier parts of the world where water is often of an ephemeral nature. It is generally believed that Triops occurs in pools that regularly dry up and that Lepidurus is found in more permanent waters.

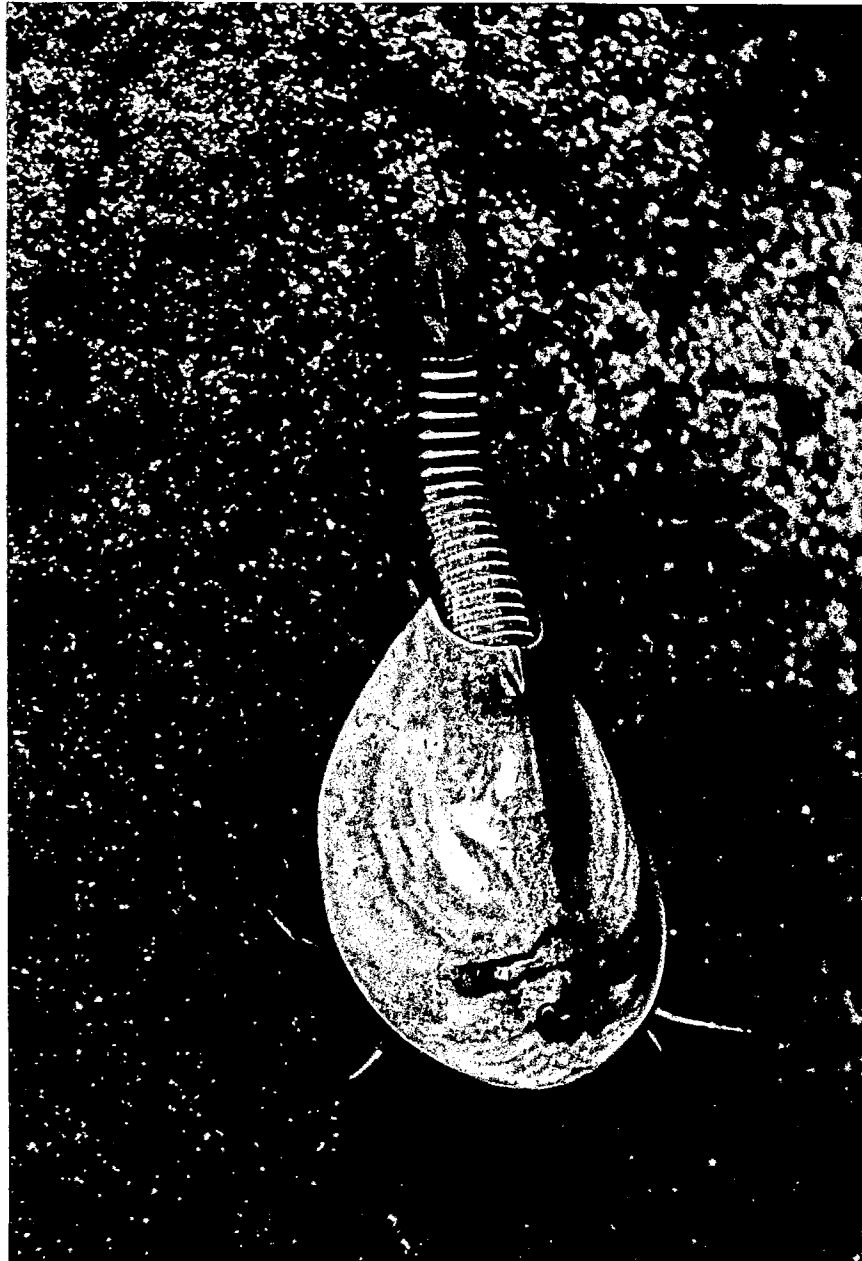
The genus Lepidurus is represented in North America by five species, four of which have been shown to occur in the Pacific Northwest. The five species of Lepidurus differ only slightly in morphology. Triops is represented by only one species in North America and four species worldwide (for review see Linder 1952; Longhurst 1955). Triops has also been recorded from Oregon although this species was never seen together with Lepidurus in any

of the pools visited during this study.

Lepidurus bilobatus (Fig. 1) is a typical tadpole shrimp. The animal possesses a large shield-like dorsal carapace covering the head and thorax and a flexible abdominal region which extends beyond the posterior margin of the carapace. This abdominal region ends at the telson which bears a supra-anal plate, not present in Triops, and two long furca. The first eleven segments (thorax) normally bear one pair of rather primitive jointed appendages each. The abdominal segments (body rings), however, bear a series of more simple, plate-like appendages with several pairs of these appendages being associated with each body ring. The appendages do not extend all the way to the telson; thus, an "apodous" region exists. The number of appendages and body segments is variable from species to species as well as within a species. This interspecific variation seems to be more prevalent in the genus Triops than in the genus Lepidurus. Lepidurus bilobatus has 33 segments, 60 pairs of appendages and 6 apodous segments (Linder, 1952).

Lepidurus has mouthparts for gnawing and chewing and is thought to be largely a predatory animal feeding on small crustaceans. It seems to be omnivorous, however, and will eat any food material it finds while foraging along the bottom of the pond. Lepidurus has been observed

Fig. 1 Lepidurus bilobatus. Approximately 2x life size.



eating some of the larger branchiopod species found in the pond. Lepidurus has also been seen to be cannibalistic when held under laboratory conditions. It is not known if this behavior exists in nature. A partial species list of animals associated with Lepidurus is presented in Table I.

Specimens of Lepidurus bilobatus brought to the laboratory and used in this study were assumed to be adult animals. Many of the animals carried eggs in brood pouches which are located on the eleventh thoracic appendage. It is not known, however, whether those animals not carrying eggs were male animals. No males have been described in this species (Longhurst, 1955). Although putatively parthenogenetic and hermaphroditic species of Lepidurus and Triops have been described (Longhurst, 1954, 1955), nothing has been published regarding the reproductive biology of Lepidurus bilobatus. There is no further information from this study to broaden our knowledge concerning this problem. Furthermore, since the question of sex in these animals is not resolved hemolymph used for this study was pooled from all animals regardless if they carried eggs or not.

Lepidurus bilobatus used in this study was collected in brackish water ponds in Lake County, Oregon during the spring and summer months of 1978 and 1979. The animals

Table I. Partial list of species associated with Lepidurus bilobatus.

Brick water hole

Crustacea

Branchinecta dissimilis

Caenestheriella setosa

Diaptomus forbesi

Cyriconcha alba

Daphnia middendorffiana

St. Patrick's water hole

Crustacea

Branchinecta dissimilis

Caenestheriella setosa

Diaptomus nevadensis

Insecta

Notonecta sp.

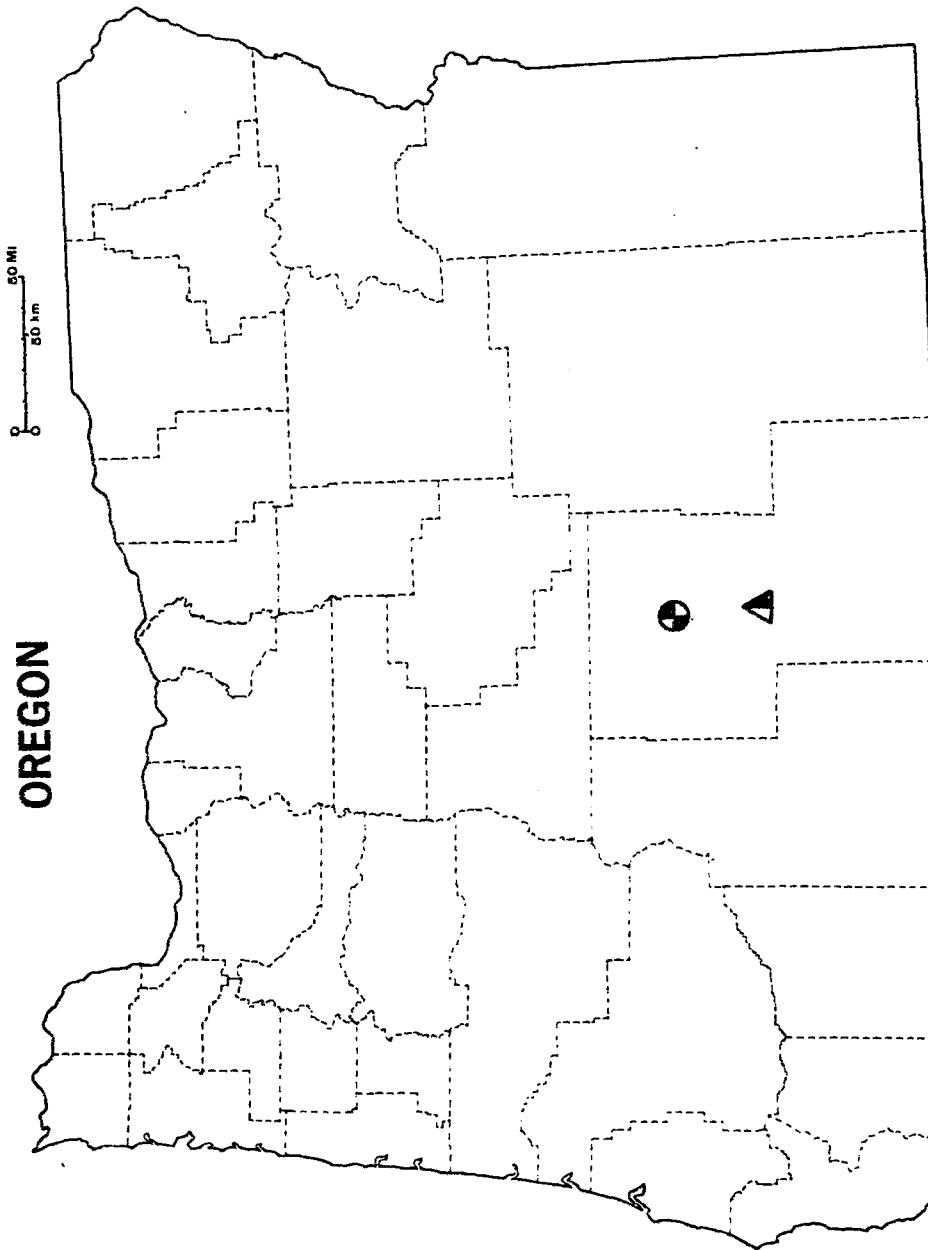
were collected from two sites (Fig. 2). The first site, Brick water hole, was sampled the first season and lies approximately 40 miles north of the city of Lakeview, Oregon. Due to the difficulty of access to this pond a second site near St. Patrick's mountain was chosen the second year. This site is approximately nine miles SSE of the town of Christmas Valley and is located with the Christmas Administration unit, Lakeview District of the U.S. Bureau of Land Management. The geographic location of Brick water hole is T29S, R20E, Sect. 32, NW NW and St. Patrick's water hole is T28S, R18E, Sect. 27, SW SW.

This region is characterized as high desert and represents the northern-most extension of the Great Basin. The ponds and lakes of this area are characteristically shallow and occupy flat basin floors between ridges or lie at the feet of small mountains. The climate is characteristically one of extreme variation. Air temperatures range from more than 35° C in the summer to less than -23° C in the winter (Phillips and Van Dengurgh, 1971). Evaporation of the ponds is high on clear, windy days but low at night due to a marked drop in evening temperatures. Daily ranges of temperature often exceed 30° C.

St. Patrick's water hole and Brick water hole have been bulldozed by the U.S. Bureau of Land Management to

Fig. 2 Location of Lepidurus bilobatus collecting sites
in Lake County, Oregon. ▲ Brick water hole.

⊕ St. Patrick's Mountain.



act as watering holes for range cattle. The pools are at least five feet deep yet the depths of the ponds vary with their age and state of repair and season. The water is extremely cloudy with suspended particulate matter at all times of the collecting season.

Certain chemical and physical parameters of the ponds have been measured. Both Brick and St. Patrick's water holes are slightly alkaline with pH ranging from 7.3-7.5 in June 1978. Conductivity was measured as 600 and 450 $\mu\text{mhos}/\text{cm}^2$ for Brick and St. Patrick's water hole, respectively. Water temperatures of the ponds vary with season. Midwater temperatures of St. Patrick's water hole range from 12-15^o C through the 1979 season. There is a slight gradient of temperature from the bottom of the pond to the surface with surface water temperatures often being equal to the air temperature. Oxygen concentration measured at St. Patrick's water hole was fairly constant between 7-9 ppm throughout the summer of 1979. Only a slight oxygen gradient from the surface to the bottom of the pond was observed.

The animals for this study were collected by dip net or seine and returned to the Oregon Institute of Marine Biology in Charleston, Oregon. Lepidurus bilobatus was carried in chilled, aerated containers in water from their natural ponds and maintained in large aquaria at the

laboratory under conditions simulating the natural environment as nearly as possible. The animals were fed live fairy shrimps (Branchinecta dissimilis) when these animals were available. Due to the lack of success at culturing the fairy shrimp in large quantities food for Lepidurus was supplemented with frozen brine shrimp (Artemia salina) obtained from Metaframe (San Francisco Bay Brand, Newmark, CA.).

The cladocerans (water-fleas) are primarily fresh water organisms. Aside from fast-flowing streams, brooks and heavily polluted waters they are abundant everywhere (Pennak, 1978). The water fleas are characteristically small animals (0.2-3.0 mm). Although the body is not clearly segmented, the animal is divided into three morphological regions: thorax, abdomen and post-abdomen. The thorax and abdomen are covered by a thin, often transparent, carapace which has a general bivalved appearance, but which is actually a single, folded piece that gapes at the ventral side. In lateral view the shell is variously shaped depending on species as well as season and developmental stage. The head is a compact structure which is not enclosed by the carapace. The most conspicuous feature of the head is a single, large compound eye. There are 5-6 pairs of lobed, leaf-like throacic legs bearing numerous hairs and setae. The abdomen is

suppressed but the post-abdomen is large and is curved forward so that the dorsal side is downward. The post-abdomen bears two terminal claws that seem to be used chiefly for cleaning debris from the thoracic legs.

The water fleas are filter feeders. Complex movements of setose thoracic legs create water currents which serve to aid in filtering food particles from the water. Algae and protozoa are assumed to be the chief food of cladocerans. However, it is also known that any sort of organic debris as well as bacteria are included in their food items.

Reproduction is parthenogenetic during the greater part of a season at which time only female animals are produced. This mode of reproduction can continue for many generations. Male animals do appear, however, under certain conditions. The exact nature of the stimulus which causes the production of male water fleas is not known. However, crowding, decreased food supply and increased water temperature seem to correlate with increased numbers of male animals. These conditions are also correlated to the production of sexual eggs. Sexual eggs are larger than parthenogenetic eggs and relatively few in number. After the sexual egg is fertilized, the walls of the brood chamber become thickened and dark, and form an ephippium which is released upon molting and

sinks to the bottom of the pond. The ephippia are capable of withstanding drying and freezing. Their production is clearly an adaptation to adverse environmental conditions.

Daphnia pulex for this study were collected in seasonal ponds near Charleston, Oregon. These ponds are most likely formed from collections of rain water in the deflation plain area behind Bastendorff beach. The animals were collected by dip net and identified according to Pennak (1978). Although the genus Daphnia has been given thorough taxonomic treatment, it is a troublesome taxon and identification is at times worrisome due to seasonal and geographical variations in head and carapace morphology. However, it is unusual to find two species of Daphnia coexisting (Pennak, 1978). If two species are found together, one of them is usually much more abundant than the other. The animals were collected in the winter and early spring months of 1978 and 1979. Water temperatures of the ponds varied according to season. Temperature of the ponds ranged from 15-20° C to near 0° C (the pond surface was frozen).

Daphnia pulex was returned to the laboratory, sorted in order to remove other contaminating animals and prepared for purification of the hemoglobin. Table II includes a partial list of species associated with Daphnia in the ponds. No attempt was made to separate Daphnia

Table II. Partial list of species associated with Daphnia pulex in Bastendorff beach ponds.

Insecta

Chironomus sp.

Chaoborus sp.

Dysticus sp.

Amphibia

Ambystoma gracile

Rana aurora

by sex or developmental stage. In addition to its presence in the hemolymph and muscle tissues (Fox, 1955, 1957), hemoglobin has been noted by Tessier (1932) to occur in the parthenogenetic eggs of Daphnia when the eggs are in the brood pouch. On the other hand, Fox (1948) has not detected hemoglobin in sexual eggs. Qualitative observations made during this study are consistent with these observations; collections made early in the season when the animals are carrying parthenogenetic eggs in their brood pouches appear to yield more hemoglobin than individuals collected later in the season which are carrying sexual eggs.

HEMOGLOBIN PURIFICATION

LEPIDURUS BILOBATUS

Before hemolymph extraction, the animals were washed thoroughly with distilled water and dried with Kimwipes. Blood was obtained by gently breaking a thoracic appendage and collecting the hemolymph in an ice cold beaker containing 0.05 M Tris-HCl, 0.1 M NaCl, 0.01 M MgCl₂ and 1 mM phenylmethylsulfonyl fluoride (PMSF), a proteolytic blocking agent, (pH 7.0 or pH 8.0). After centrifugation in a refrigerated Sorvall RC-2B at 12,000 g for 10 minutes,

the supernatant was applied to a column of Sepharose 4B or Bio Gel A5M (1.9 x 90 cm) in equilibrium with the extraction buffer without PMSF. All purification procedures were rapidly carried out in the cold (0° - 4°C). The Sepharose 4B column had been previously calibrated with Euzonus mucronata hemoglobin [$M_r = 3 \times 10^6$ (Terwilliger et al., 1977a)] , Cancer magister hemocyanin [$M_r = 9.0 \times 10^5$ (Ellerton et al., 1970)] , Artemia salina hemoglobin [$M_r = 2.5 \times 10^5$ (Moens and Kondo, 1976, 1978)] , and Callianassa californiensis hemocyanin [$M_r = 1.7 \times 10^6$ and 4.3×10^5 (Roxby et al., 1974)] . The Bio Gel A5M column had been previously calibrated with Eudistylia vancouveri chlorocruorin [$M_r = 3.1 \times 10^6$ (Terwilliger et al., 1975b)] , Cancer magister hemocyanin and Artemia salina hemoglobin. All calibrants were prepared from living organisms collected in the Charleston area except for live Artemia salina which were purchased from a Metaframe (San Francisco Bay Brand, Newark, CA) distributor in Portland, Oregon.

DAPHNIA PULEX

Daphnia pulex was concentrated in nylon netting, then washed with distilled water and rinsed with extraction buffer (0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl,

0.01 M $MgCl_2$, 1 mM PMSF). All purification procedures were rapidly performed at 0-4° C. The hemoglobin was extracted by squeezing the animals in the nylon netting and collecting the extract in a chilled beaker containing a small amount of the extraction buffer with the proteolytic blocking agent PMSF. The solution was bubbled with carbon monoxide to convert the hemoglobin to the carbonmonoxy form. The extract was then centrifuged at 16,300 g for 15 minutes at 4° C in a Sorvall RC-2B refrigerated centrifuge. The supernatant was quickly filtered through Whatman No. 1 filter paper to remove a lipid layer and brought successively to 20 and 40% saturation with ammonium sulfate. The solutions were stirred for 10 minutes at 4° C prior to centrifugation at 16,300 g for 15 minutes, and the precipitates were discarded. A greyish-red pellet was collected by bringing the solution to 60% saturation, resuspended in 0.03 M Tris-HCl (pH 8.0), 0.1 M NaCl, desalted by elution through a column of Sephadex G-75 (6.0 x 15.0 cm) in equilibrium with the Tris-HCl (pH 8.0) buffer eluted through DEAE cellulose (Sigma Chem. Co.) also in equilibrium with the Tris-HCl buffer. This step removed several contaminants and resulted in a clear red solution. The protein was then concentrated by ammonium sulfate precipitation (60% saturation), resuspended in 0.03 M Tris-HCl (pH 8.0)

and applied to a column of Sepharose 4B (2.5 x 55 cm) equilibrated in that buffer. After elution, the hemoglobin was absorbed directly to a column of DEAE cellulose (6.0 x 15.0 cm) (Sigma Chem. Co.) which had been equilibrated in the Tris-HCl buffer (pH 8.0). The column was eluted with a linear salt gradient (125 ml each chamber) between the pH 8.0 Tris-HCl buffer and the same buffer 0.15 M in NaCl.

The apparent molecular weight of purified Daphnia hemoglobin was determined by chromatography on Bio Gel A5M (1.9 x 90 cm) in equilibrium with 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.01 M MgCl₂. The column had been previously calibrated with Eudistylia vancouveri chlorocruorin, Cancer magister hemocyanin, Artemia salina hemoglobin, and bovine heart lactate dehydrogenase ($M_r = 150,000$) (Sigma Chem. Co.).

ANALYTICAL METHODS

Sedimentation velocity experiments were performed on purified Lepidurus and Daphnia carbonmonoxy hemoglobins in a Beckman Spinco Model E ultracentrifuge equipped with an automatic split-beam photoelectric scanning absorption system and an RTIC temperature control unit. Sedimentation coefficients were corrected to water at

20° C ($S_{20,w}$) as described in Svedberg and Pederson (1940). Samples of Lepidurus hemoglobin used in pH sensitivity experiments were dialyzed against the appropriate buffer for 24 hours (4° C) prior to the centrifuge run. pH measurements were made of the samples after each experiment to ensure that equilibrium had been reached. Buffer solutions were made according to standard recipes (Biochemists Handbook). The following buffers were used for the pH ranges given: Acetate-sodium hydroxide (pH 4.0 - 6.0), Potassium dihydrogen phosphate - disodium hydrogen phosphate (pH 6.0 - 7.0), Tris-HCl (pH 7.0 - 8.5), Sodium bicarbonate - sodium carbonate (8.5 - 11.0). All buffers were 0.1 ionic strength or 0.125 ionic strength when 10 mM $MgCl_2$ was added. All buffers contained 10 mM disodium EDTA or 10 mM $MgCl_2$.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out in 1.5 mm slab gels (Studier, 1973) with a discontinuous buffer system (Laemmli, 1970). Gel concentrations of 10 and 12.5% were used with a constant ratio of acrylamide to bisacrylamide of 30:0.8. Sodium dodecyl sulfate gel electrophoresis in 8 M urea was performed according to Weber and Osborn (1975). Sodium dodecyl sulfate/urea incubation buffer was prepared from freshly dissolved urea which was then passed through Amberlite MB-1 mixed bed resin and stored as frozen

aliquots until use. Freshly prepared pigment or purified, lyophilized proteins were first denatured in boiling incubation buffer, with or without 8 M urea containing 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride for 1.5 minutes at 100° C. Calibrants were bovine serum albumin ($M_r = 68,000$), ovalbumin ($M_r = 43,000$), α -chymotrypsinogen ($M_r = 25,700$), sperm whale metmyoglobin (mol. wt. 17,200) and lysozyme ($M_r = 14,300$) (Sigma Chem. Co.). The gels were stained with Coomassie Blue according to Fairbanks et al. (1971). Globin prepared by the method of Teale (1959), was denatured in boiling 6 M guanidine hydrochloride (Heico Co., extreme purity) with 2-mercaptoethanol, incubated for 2 h at 37° C and dialyzed extensively against the sodium dodecyl sulfate incubation buffer prior to analysis by sodium dodecyl sulfate gel electrophoresis. Apparent molecular weights of Lepidurus globin denatured in 6 M guanidine hydrochloride and reduced with dithiothreitol were determined on Sephacryl S-200 (1.4 x 86 cm) equilibrated with 6 M guanidine hydrochloride, 50 mM Tris-HCl (pH 7.5) and 0.5 mM dithiothreitol (Fish et al., 1969; Belew et al., 1978). Samples were dissolved in the incubation buffer and incubated at 50° C for 4 hours prior to applying the sample to the column. The column was calibrated with Blue Dextran, bovine serum albumin,

ovalbumin, α -chymotrypsinogen A, sperm whale metmyoglobin and potassium ferricyanide under identical denaturing conditions. A similar column was prepared in the absence of dithiothreitol for studies involving reduced and alkylated proteins. Alkylated proteins and calibrants were applied directly to the column after the reaction mixture was treated with excess 2-mercaptoethanol. Fractions from both columns were monitored at 280 nm.

Lyophilized globins were performic acid oxidized according to the method of Hirs (1956) and carboxymethylated with iodoacetamide according to Hirs (1967).

Isoelectric focusing of the Lepidurus cyanmethemoglobin was performed in tubes containing 5% acrylamide gel and 2% in a 3:2 mixture of pH 3.5 - 10.5 and pH 4 - 6 Ampholines (LKB Chem. Co.). The upper buffer reservoir contained 0.02 N NaOH and the lower buffer reservoir contained 0.01 M phosphoric acid. The focusing was carried out at 120 V for 10 hours (4^o C). The gels were removed and scanned at 540 and 280 nm with a Zeiss PMQ II spectrophotometer.

Gel electrophoresis was carried out on globin at low pH in the presence of 6.25 M urea (Panyim and Chalkey, 1969; Poole et al., 1974). Samples for urea gel electrophoresis were dissolved in a solution of freshly prepared 10 M urea (deionized with Amberlite MB-1) which was 5%

in acetic acid and 1% in 2-mercaptoethanol and allowed to denature at room temperature for at least 4 hours prior to electrophoresis. Pre-electrophoresis of the gels and electrophoresis of the sample were carried out at 2 ma per tube. The gels were stained with Coomassie Blue and destained with 10% acetic acid.

Regular disc gel electrophoresis was performed on carbonmonoxyhemoglobin (Davis, 1964). Amino acid analysis was carried out on purified dehemed proteins as described by Spackman et al. (1958) and tryptophan was assayed as in Edelhoeh (1967).

Absorption maxima were determined for the purified pigments using a Zeiss PMQ-II spectrophotometer. Absorption maxima of oxygenated hemoglobin samples were determined on aliquots equilibrated with air. Bubbling with oxygen did not change the absorption spectra. Deoxygenated hemoglobin samples were prepared by adding a small aliquot of sodium dithionite to a hemoglobin solution. Carbonmonoxyhemoglobin was prepared by reacting concentrated sulfuric acid with concentrated formic acid and bubbling the liberated carbon monoxide gas through the hemoglobin solution. The CO-gas was first bubbled through dilute solution of sodium hydroxide to neutralize any acid fumes that may have been present. Cyanmet-derivatives were prepared by first treating a hemoglobin

sample with a small amount of potassium ferricyanide followed by another small aliquot of potassium cyanide.

Oxygen binding of both intact Lepidurus hemoglobin and the fraction separated by gel chromatography were studied spectrophotometrically (Benesch et al., 1965) with a Zeiss PMQ II spectrophotometer equipped with a temperature controlled cell holder and tonometers purchased from Eck and Krebs Company. Samples were dialyzed in the cold versus the appropriate buffer before analysis. Absorbances were measured at 565, 555 and 540 nanometers to determine percent oxygenation. The pigment appeared to be very unstable with a tendency to form methemoglobin during binding experiments. The oxygen binding experiments, therefore, were performed in the presence of a reductase system described by Hayashi et al. (1973). Isosbestic points at 585 and 525 nm were monitored throughout the binding experiments.

The heme content of Lepidurus hemoglobin was determined by the pyridine hemochromogen method described by Swaney and Klotz (1971). Sperm whale metmyoglobin and pure hematin (Sigma Chem. Co.) were used as standards. The extinction coefficient for Lepidurus bilobatus hemoglobin was obtained from purified pigment dialyzed against distilled water. Samples of known volume and absorbance were frozen, lyophilized and dry weights determined.

Lepidurus globin was cleaved by reaction with cyanogen bromide. The lyophilized protein was dissolved in 70% formic acid (10 - 20 mgm/ml) and an amount of cyanogen bromide was added such that the ratio of cyanogen bromide to protein equaled 1:2 (mgm CNBr:mgm protein). The reaction allowed to continue at room temperature (20° C) for 24 hours at which time the reaction mixture was diluted 1:1 with distilled water, frozen and lyophilized. Products from the reaction were analyzed by sodium dodecyl sulfate slab gel electrophoresis (15% acrylamide). The gels were calibrated with α -chymotrypsinogen A, sperm whale metmyoglobin, lysozyme and cytochrome c.

Hemoglobins were digested with subtilisin (Carlsberg Type VIII, Sigma Chemical Co.) in 0.04 M sodium glycinate buffer (pH 10.5), 0.01 M in EDTA at 25° C. The reaction was stopped by the addition of excess phenylmethylsulfonyl fluoride. The concentration of enzyme used relative to hemoglobin was 1 mg enzyme per 100 mg protein. A control aliquot of the hemoglobin sample was treated in the same way without the addition of subtilisin. The digestion products were separated on a Sephacryl S-200 column (1.7 x 75 cm) in equilibrium with the pH 10.5 sodium glycinate buffer or a Sephadex G-100 column (1.6 x 80 cm) in equilibrium with 0.05 M Tris-HCl (pH 8.0), 0.1 M in NaCl. The columns had been previously calibrated with

Blue Dextran, bovine serum albumin, ovalbumin, α -chymotrypsinogen A, sperm whale metmyoglobin and potassium ferricyanide.

Two dimensional peptide mapping was performed according to Stephens (1978). Lyophilized protein samples were dissolved in 0.1 ammonium bicarbonate. TPCK-treated trypsin (Worthington) was added at a 1:100 enzyme to protein ratio and the mixture incubated at 25° C for 24, 48 or 72 hours. At the end of the digestion period the mixture was diluted with an equal volume of distilled water, frozen and lyophilized. Sperm whale apomyoglobin was treated similarly and served as a control.

The dried digest mixture was dissolved in 0.1 M ammonium bicarbonate (0.5 nanomoles/ μ l) and spotted on 10 x 10 cm, 0.1 mm thickness silica gel Chromogram sheets (Eastman Chemical Co.) [Poly(ethylene terephthalate) backed]. The small application spot (no greater than 2 mm in diameter) was applied 1.25 cm from the left edge of the sheet. Ascending chromatography was performed in an Eastman Chromogram developing apparatus (No. 13259). The solvent used consisted of chloroform-methanol-ammonium hydroxide (4:5:1, v/v). The plates were dried with hot air from a hand-held hair drier.

Electrophoresis of the plates was carried out at pH 3.5 using pyridine-acetic acid-water at a ratio of

1:10:489. Dried plates were sprayed with electrophoresis buffer until their matte finish just barely became glossy. Electrophoresis was carried out at 1000 volts using an LKB Multiphor electrophoresis system equipped with an LKB Constant Power Supply. The plates were laid horizontally on the glass plate and cooled by circulating water (8° C) through the glass plate. Platinum wire electrodes were constructed which ran the full length of the buffer troughs. The plates were connected to the buffer troughs by three layers of Whatman N. 1 chromatography paper cut to the appropriate size. A clean 10.5 x 20 cm plate of glass was placed over the electrophoresis plate in order to minimize evaporation.

Upon completion of the electrophoresis, the plates were dried with hot blown air and sprayed with the fluorogenic reagent fluorescamine (3 mgm/10 ml acetone) and stabilized by spraying the plate with 5% triethylamine in acetone. Tryptic spots were visualized by illumination with UV light and photographed with Kodak Plus-X black and white film (ASA 125) according to Stephens (1978).

CHAPTER 3

RESULTS

STUDIES OF LEPIDURUS BILOBATUS HEMOGLOBIN

Carbonmonoxy- and oxyhemoglobin of Lepidurus bilobatus each chromatograph on Sepharose 4B at pH 7.0 in the presence of 0.01 M $MgCl_2$ as a slightly asymmetric peak with an apparent molecular weight of 680,000 (Fig. 3). Chromatography on Bio Gel A5M of oxy- and carbonmonoxy-hemoglobin at pH 8.0 in the presence of 0.01 M $MgCl_2$ indicates the hemoglobin has an apparent molecular weight of 800,000 (Fig. 4). The size of the native molecule is difficult to determine due to the peculiar polymerization behaviour that the pigment displays under certain conditions of pH and ligand state. Treatment of the pigment with potassium ferricyanide and potassium cyanide for two hours at 20° C prior to chromatography on Bio Gel A5M at pH 8.0 results in a minor hemoglobin peak which elutes ahead of the main 800,000 molecular weight peak suggesting some polymerization of the cyanmethemoglobin into apparent dimers. Similarly, chromatography on Sepharose 4B of oxyhemoglobin at pH 8.0 in the

Figure 3 Sepharose 4B column chromatography of Lepidurus bilobatus oxyhemoglobin (4° C). Column buffer 0.05 M Tris-HCl (pH 7.0), 0.1 M NaCl, 0.01 M MgCl₂. Column volume 1.9 x 90 cm.

Calibrants: a, Euzonus mucronata hemoglobin; b, Cancer magister hemocyanin; c, Callianassa hemocyanin; d, Artemia salina hemoglobin. ● Absorbance at 280 nm;
▲ Absorbance at 540 nm.

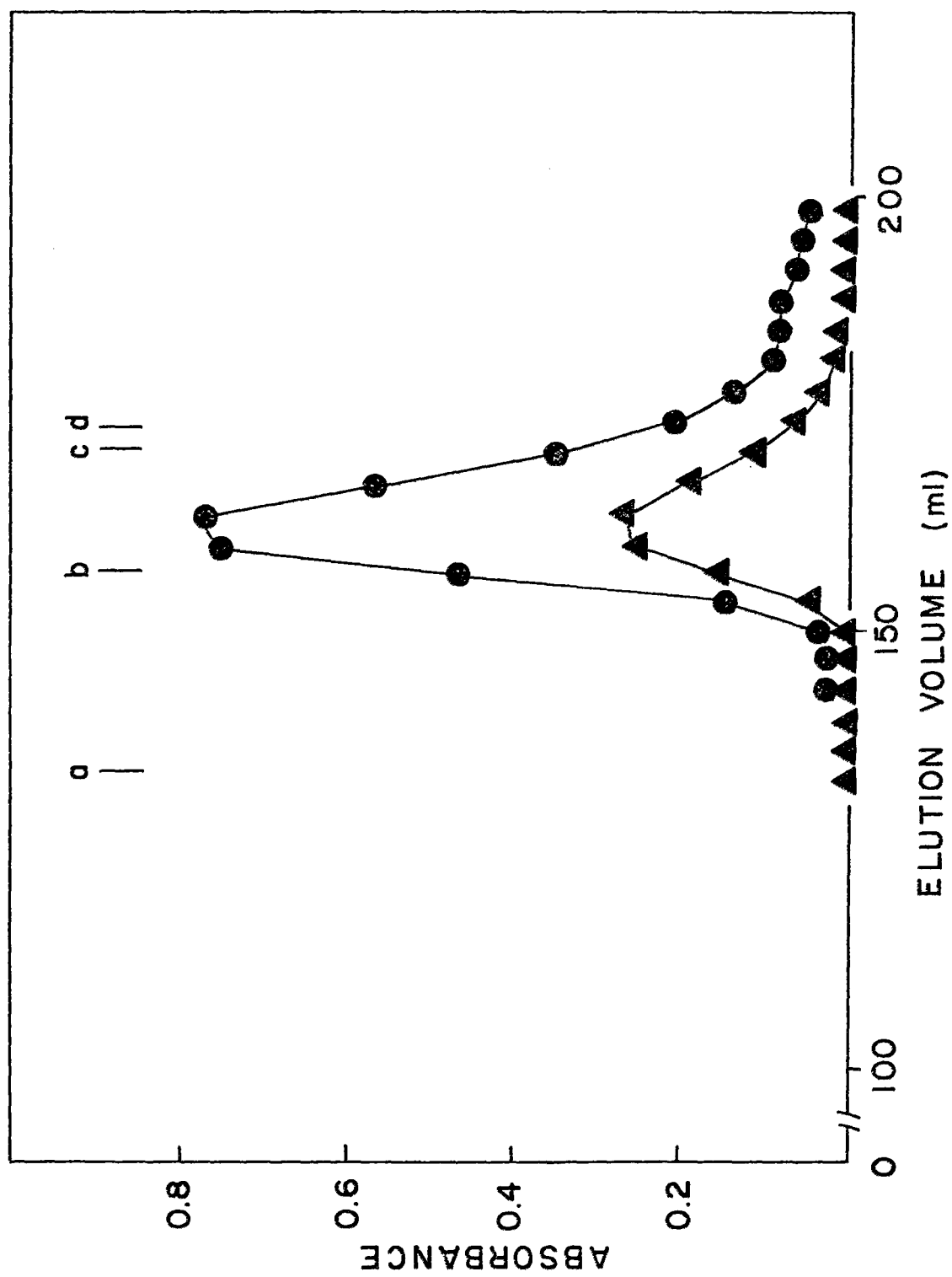
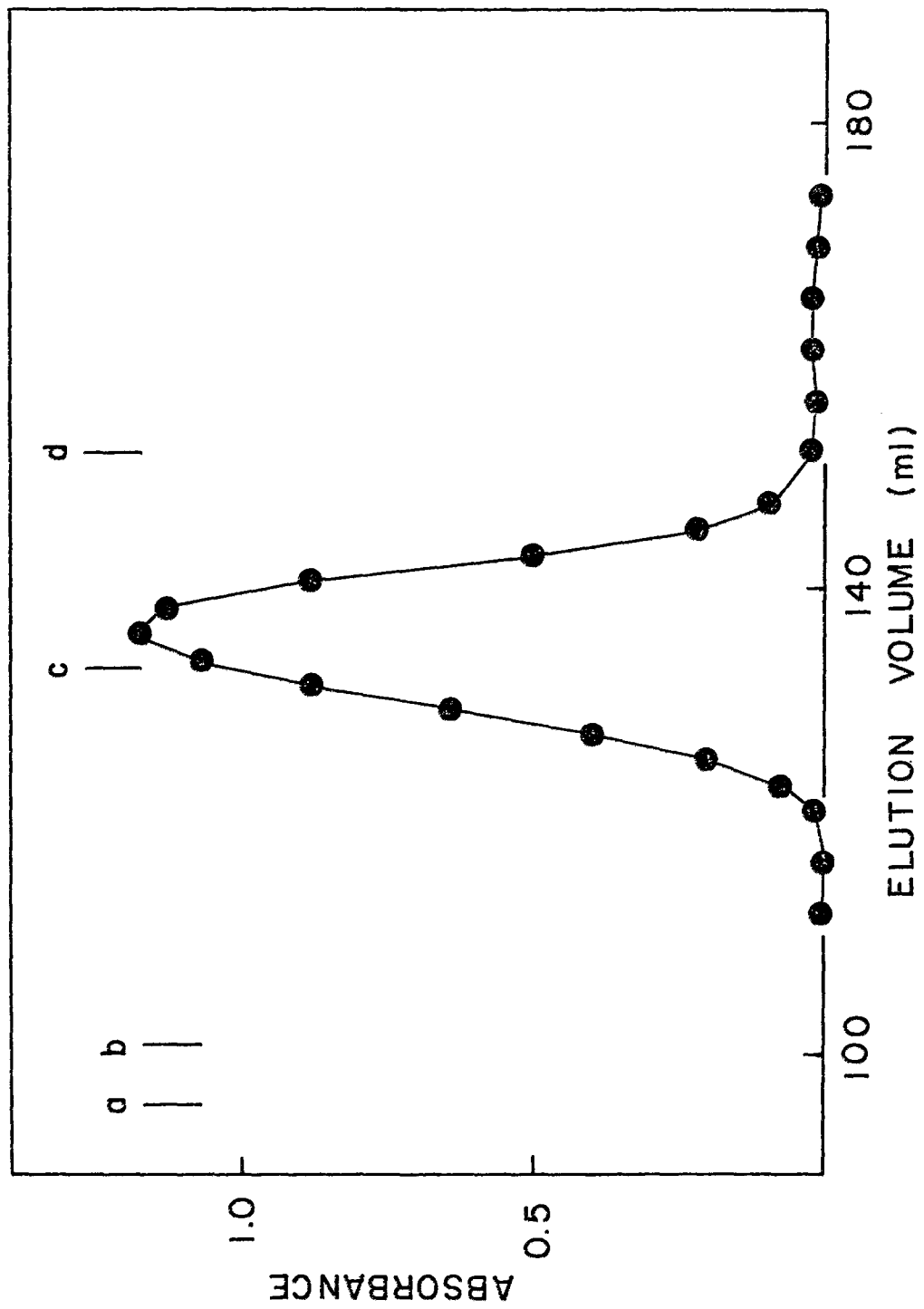


Figure 4 Bio Gel A5M column chromatography of Lepidurus bilobatus oxyhemoglobin. Calibrants: a, Blue Dextran. b, Eudistylia vancouveri chlorocruorin. c, Cancer magister 24S hemocyanin. d, Cancer magister 16S hemocyanin. ● absorbance at 540 nm.



presence of $MgCl_2$ or of methemoglobin at pH 7.0 results in the shift of the elution molecular weights of approximately 1.2×10^6 and 1.8×10^6 , respectively, indicating the polymerization of the protein to apparent dimers and trimers, respectively. Chromatography on Sepharose 4B at pH 7.0 in the presence of 0.01 $MgCl_2$ of a dilute sample of oxyhemoglobin results in a main peak of about 680,000 molecular weight with a shoulder indicating the presence of some lower molecular weight material (Fig. 5).

Lepidurus carbonmonoxyhemoglobin (0.2 mg/ml) has a sedimentation coefficient ($S_{20,w}$) of 18.2 at pH 8.0 in the presence of 0.01 M $MgCl_2$. The effect of pH on the sedimentation behaviour of the hemoglobin in the presence of $MgCl_2$ is shown in Fig. 6. The molecule has an average sedimentation value of 18.7S over the pH range of 6.6 - 9.0. Above pH 9.5 the sedimentation coefficient decreases slightly to around 17.1S. Heterogeneous sedimentation boundaries are noted in nearly all experiments. Small amounts of slower sedimenting material are present. The amount of this low molecular weight material is variable but usually represents less than 10% of the total heme protein.

The pigment appears to be stable over a wide range of pH in the presence of EDTA (Fig. 7). The sedimentation coefficient is approximately 18.2S between pH 4.7 - 8.8.

Figure 5 Association-dissociation behavior of Lepidurus bilobatus oxyhemoglobin on Sepharose 4B. \circ absorbance 540 nm. \blacktriangle , absorbance 415 nm. Column buffer 0.05 M Tris-HCl (pH 7.0), 0.1 M NaCl, 0.01 M $MgCl_2$. Column volume 1.9 x 85 cm.

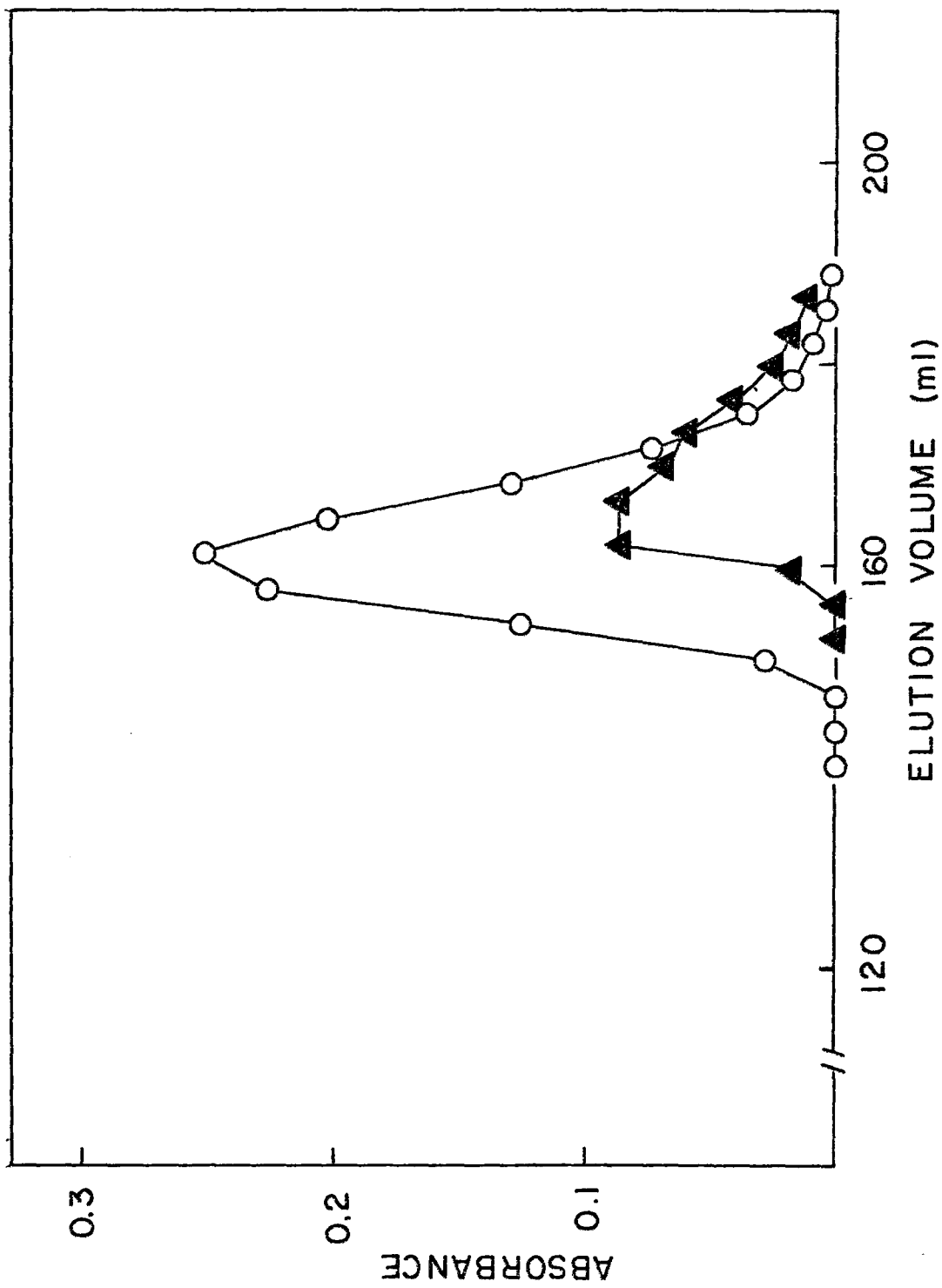


Figure 6 The effect of pH on the sedimentation of Lepidurus bilobatus carbonmonoxyhemoglobin in the presence of 0.01 M $MgCl_2$.

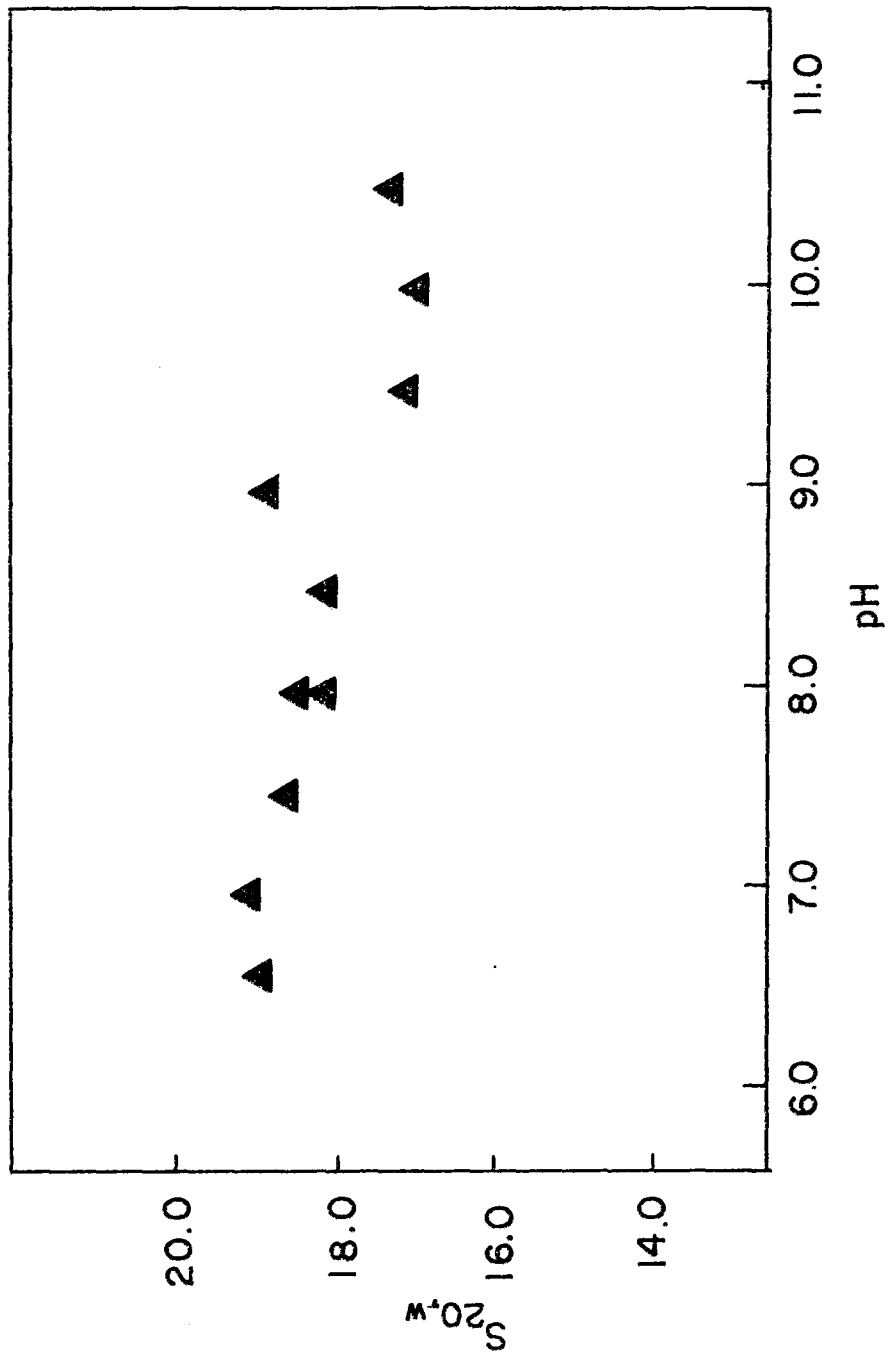
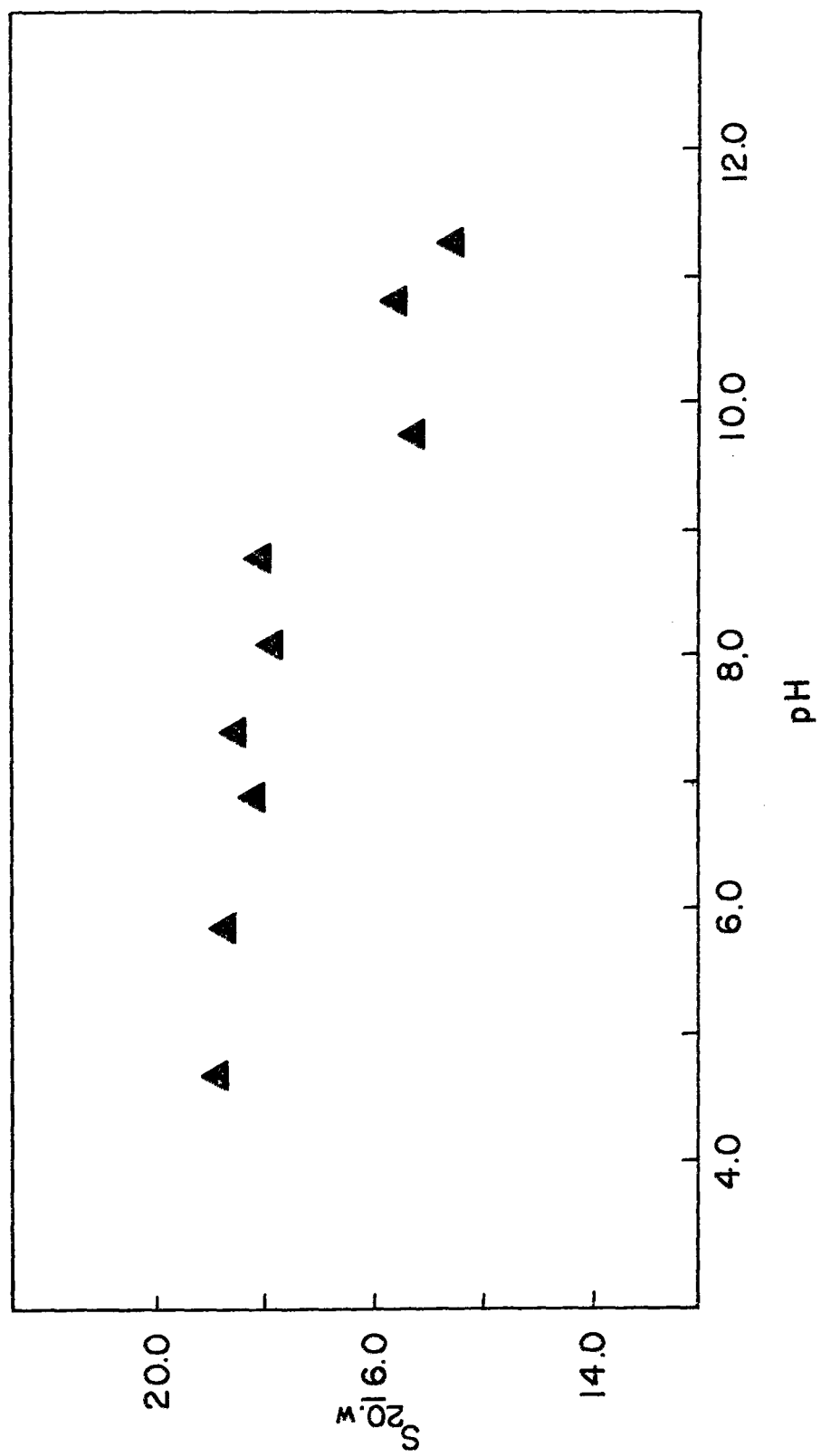


Figure 7 The effect of pH on the sedimentation coefficient of Lepidurus bilobatus carbonmonoxyhemoglobin in the presence of 0.01 M EDTA.



Above pH 9.0 the sedimentation coefficient decreases to around 15.5S. Single boundaries are obtained between pH 5.9 and 8.8. The sedimentation boundaries are heterogeneous, however, for experiments performed at pH 4.7 and above pH 9.0 suggesting the presence of some low molecular weight material.

Isoelectric focusing of the purified cyanmethemoglobin shows that the pigment is homogeneous with a pI equal to 4.9 (Fig. 8). Gel scans at 280 and 540 nm of the isoelectric focused sample indicates that the protein purified by column chromatography is free of contaminants. The amino acid composition of the purified hemoglobin is presented in Table III. There is one mole heme per 18,400 grams protein based on an absorption coefficient for Lepidurus hemoglobin of $\epsilon_{\text{mg/ml}}^{280} = 1.31$.

In order to identify the smallest polypeptide subunit of this polymeric hemoglobin the protein was analysed under a variety of denaturing conditions. 1. Sodium dodecyl sulfate gel electrophoresis of hemoglobin or globin in the presence of 2-mercaptoethanol shows one major staining band corresponding to a molecular weight of $33,500 \pm 3,300$ (S.D.) (9 trials) and two minor bands of molecular weight $61,900 \pm 2,000$ (S.D.) and 64,000 (S.D.) (9 trials) (Fig. 9a). 2. Gel electrophoresis (5% acrylamide) was carried out in tubes with sodium

Figure 8 Spectrophotometric scan of isoelectric focused Lepidurus bilobatus cyanmethemoglobin at 280 nm. Gel concentration 5% acrylamide.

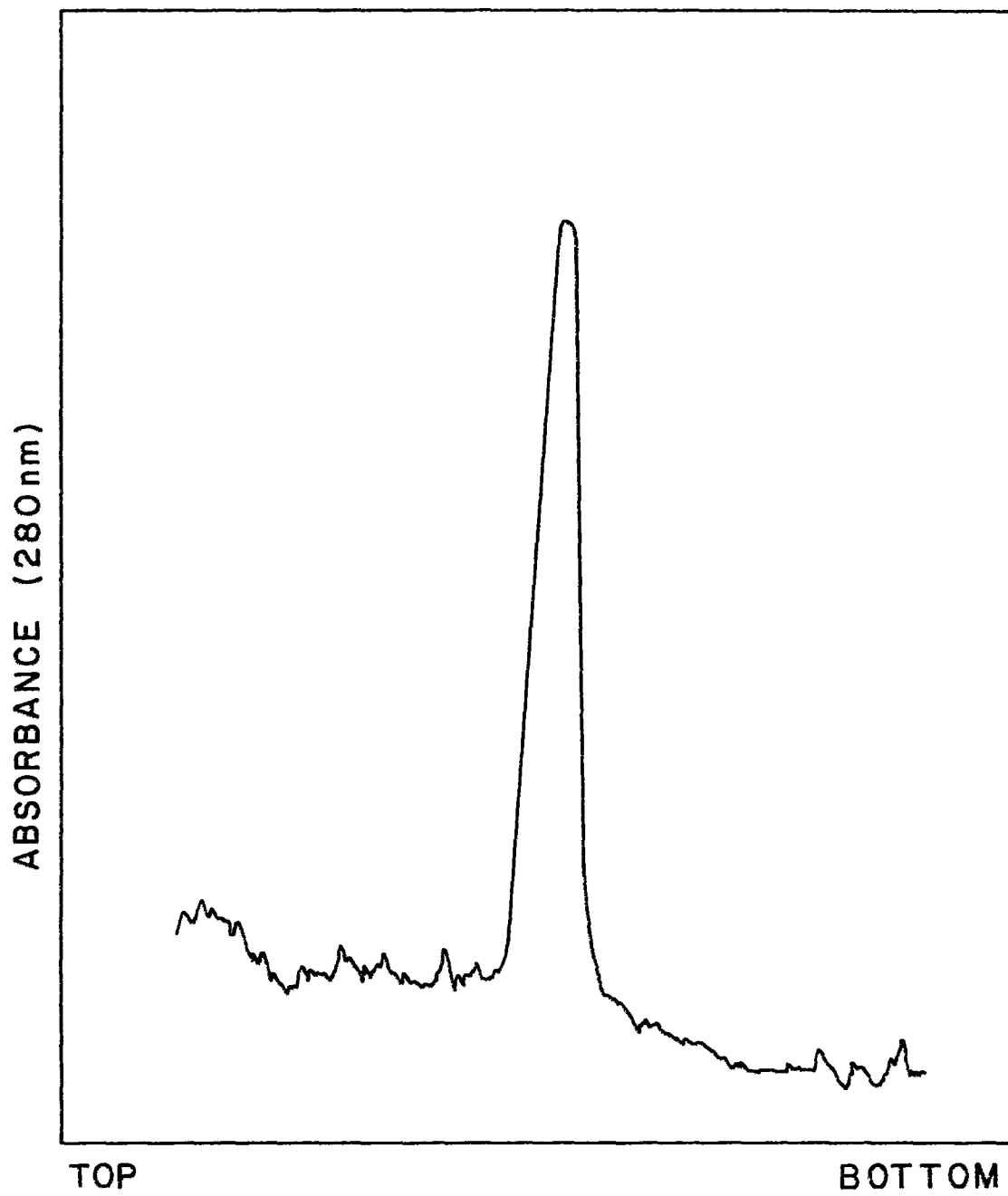


Table III. Amino acid composition of Lepidurus bilobatus hemoglobin.

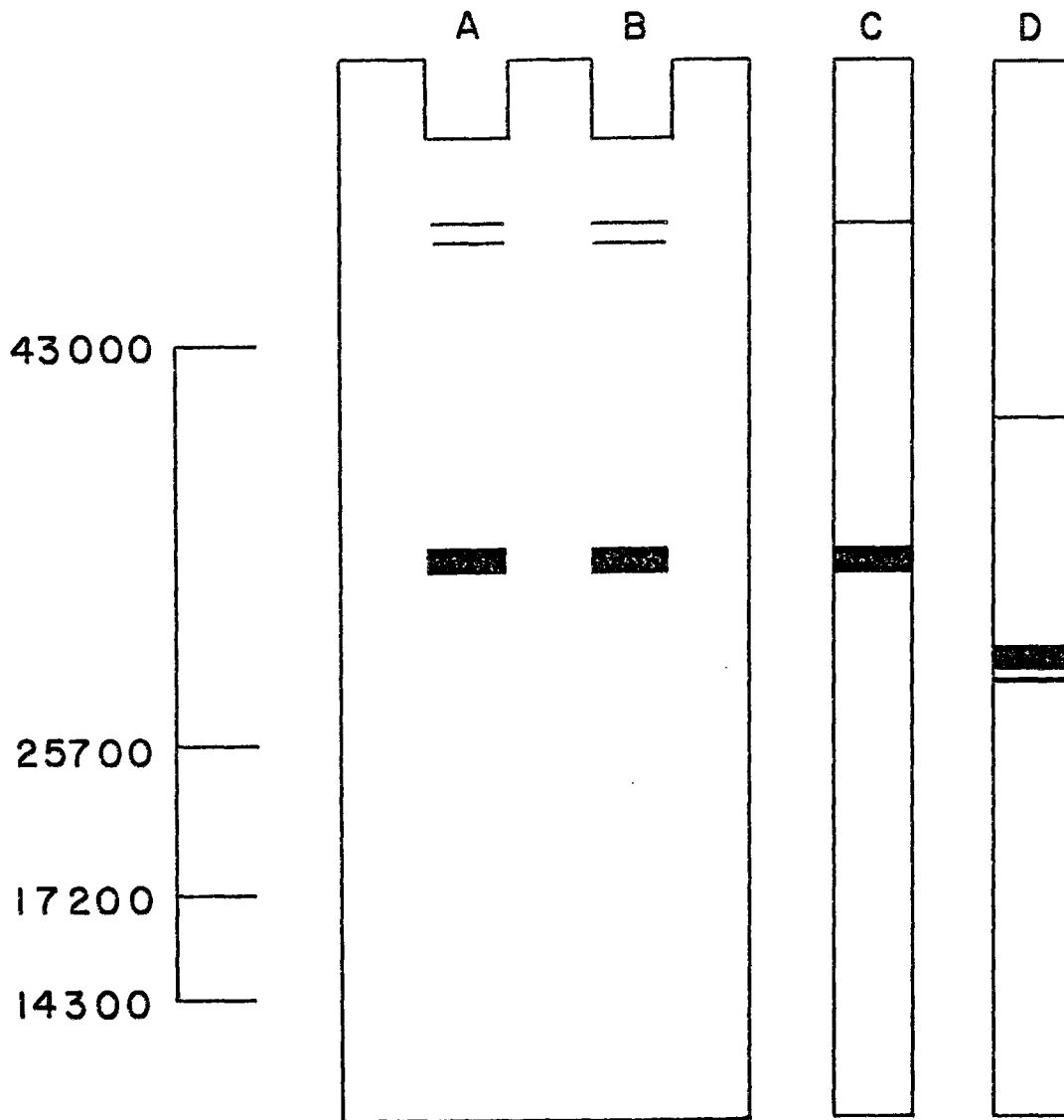
Amino Acid	Intact Hb Mole %	Digest Fraction Mole %
Lys	3.5	5.8
His	2.4	2.3
Arg	4.8	4.1
Thr**	9.4	10.6
Ser**	7.1	7.0
Glu	9.7	12.4
Pro	2.7	2.3
Gly	8.4	6.3
Ala	11.4	12.2
½ Cys*	1.3	-
Val***	8.0	9.5
Met	0.9	0.8
Ileu***	3.5	3.1
Leu	10.4	14.4
Tyr	2.3	1.0
Phe	5.4	4.4
Trp	0.9	-

* Determined as cysteic acid as in Hirs (1956)

** Extrapolated to zero time hydrolysis

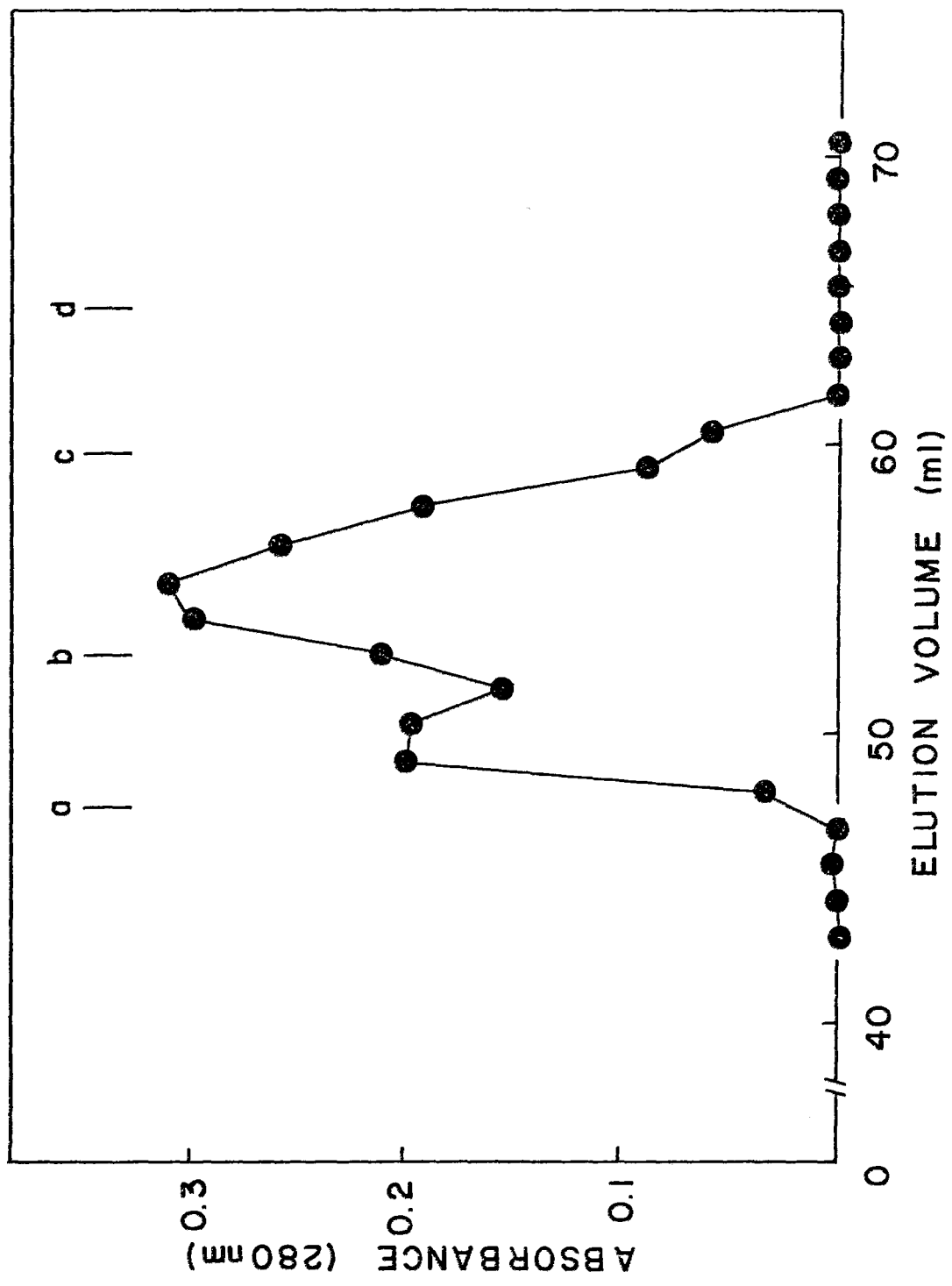
*** Highest value after 72 h hydrolysis

Figure 9 Electrophoretic patterns of Lepidurus bilobatus hemoglobin. A and B: Sodium dodecyl sulfate slab gel electrophoresis. Gel concentration 12.5% acrylamide. A. Purified Lepidurus hemoglobin; B. Lepidurus globin previously denatured in 6 M guanidine hydrochloride. C. Sodium dodecyl sulfate tube gel electrophoresis in 8 M urea of Lepidurus hemoglobin. Gel concentration 5% acrylamide. D. Urea gel electrophoresis of Lepidurus globin. Gel concentration 5% acrylamide.



dodecyl sulfate, 2-mercaptoethanol and 8 M urea. Electrophoresis of cyanmethemoglobin, globin or lyophilized hemoglobin under these conditions also gives one major band of 33,000 molecular weight and a trace of the 66,000 molecular weight material (Fig. 9c). 3. Performic acid oxidation and carboxymethylation of the protein prior to electrophoresis in sodium dodecyl sulfate or sodium dodecyl sulfate with urea give the same electrophoretic patterns as shown in Fig. 9a. 4. Globin, previously denatured in hot (100° C) 6 M guanidine hydrochloride with 2-mercaptoethanol and subsequently dialyzed against the sodium dodecyl sulfate incubation buffer, also electrophoreses as one major band with a molecular weight of 34,000 and two minor bands of 62 and 64,000 molecular weight (Fig. 9b). 5. Lepidurus globin was denatured in 6 M guanidine hydrochloride, reduced with dithiothreitol and chromatographed on a 1.4 x 86 cm column of Sephacryl S-200. The protein elutes as two fractions with apparent molecular weights of 34,000 and 66,000 (Fig. 10). Analysis of the peaks by sodium dodecyl sulfate slab gel electrophoresis indicates that the low molecular weight fraction is essentially all 34,000 molecular weight protein. The high molecular weight fraction is mostly 66,000 molecular weight protein with some 34,000 molecular weight material. No protein smaller

Figure 10 Sephacryl S-200 column chromatography of reduced Lepidurus bilobatus globin. Column buffer 0.05 M Tris-HCl (pH 7.5), 6 M guanidine hydrochloride, 0.5 mM dithiothreitol. Column volume 1.4 x 86 cm. Flow rate 5.6 ml/hour. Calibrants: a, bovine serum albumin. b, ovalbumin. c, α -chymotrypsinogen. d, sperm whale myoglobin.



than 34,000 molecular weight was detected in either fraction. Identical results are obtained for alkylated globin chromatographed on the same column in the absence of reducing agent.

Tube gel electrophoresis of globin in 6.25 M urea at acid pH shows that the pigment consists of one major and two minor polypeptides (Fig. 9d).

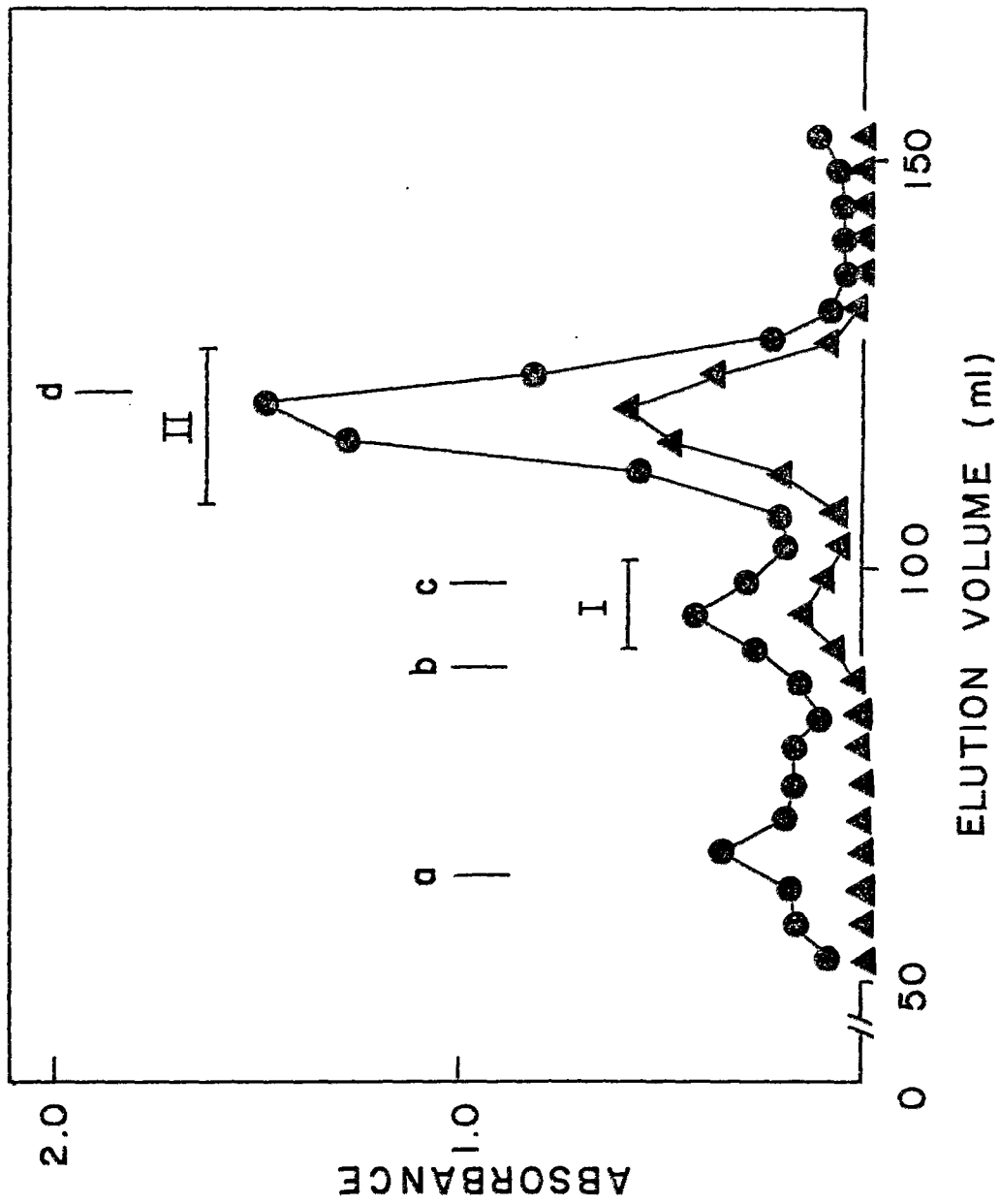
The protein was treated with enzymes in an attempt to explore the nature of the subunit structure. The results of the digestion experiments were analyzed by sodium dodecyl sulfate slab gel electrophoresis. Lepidurus hemoglobin which has been dialyzed against 0.04 M sodium glycinate buffer (pH 10.5), 0.01 M EDTA, has an identical electrophoretic pattern as native hemoglobin after treatment with sodium dodecyl sulfate incubation buffer in the presence of 2-mercaptoethanol. However, digestion of the oxyhemoglobin with subtilisin at pH 10.5 in 0.04 M sodium glycinate buffer, 0.01 M EDTA results in the production of polypeptides with molecular weights of 14 - 16,500 plus some minor bands between 20 - 30,000 molecular weight when analyzed by sodium dodecyl sulfate gel electrophoresis.

The relative amounts of the cleavage products are dependent on the enzyme to hemoglobin ratio used during the digestion. Enzyme to hemoglobin ratios (w/w) of 1/1000, 1/500, 1/250, 1/100, and 1/50 were used in the

digestion experiments. Heterogeneity with respect to molecular weight of the products was seen in all cases. The amount of lower molecular weight material produced is greater with a higher enzyme concentration or a longer digestion time. Prolonged digestion results in further breakdown of the hemoglobin with concomitant poor detection of protein.

The 14 - 16,000 molecular weight digestion products have been isolated by gel column chromatography. When Lepidurus hemoglobin is dialyzed against 0.04 M sodium glycinate buffer (pH 10.5), 0.01 M EDTA, and chromatographed on a column of Sephacryl S-200 equilibrated in the same buffer, the hemoglobin elutes in the void volume. These results are consistent with the sedimentation coefficient (15.5S) determined at high pH in the presence of EDTA. Sephacryl S-200 chromatography of hemoglobin digested with subtilisin for 2.5 hours at 25° C in the pH 10.5 sodium glycinate buffer at a ratio of 1:100 (gm enzyme: gm protein) elutes as two heme containing fractions (Fig. 11). These fractions (I and II) elute with apparent molecular weights of 40 - 50,000 and 17 - 22,000, respectively. No material is present in the void volume under these conditions. The ratio of protein to heme (absorbance at 280 nm/540 nm) in Fractions I and II is similar to that of the intact hemoglobin suggesting that the two fractions

Figure 11 Chromatography on Sephacryl S-200 of a 2.5 hr subtilisin digest of Lepidurus bilobatus hemoglobin (1 mg enzyme to 100 mg protein, 25° C). Fractions collected indicated by area under the bars. Column buffer 0.04 M sodium glycinate (pH 10.5) 0.01 M EDTA. Column volume 1.7 x 75 cm. Calibrants: a, Blue Dextran; b, bovine serum albumin; c, ovalbumin; d, sperm whale myoglobin. ● Absorbance at 280 nm; ▲ Absorbance at 540 nm.



contain about one mole heme per 18,000 gram protein.

Sodium dodecyl sulfate gel electrophoresis in 12.5% polyacrylamide gels was performed on the Sephacryl S-200 fractions identified in Fig. 12. Fraction I has one major staining band with a molecular weight of $29,400 \pm 400$ (S.D.) (25 trials) and a trace of 34,000 molecular weight material. Fraction II shows only two bands with molecular weights of $16,600 \pm 250$ (S.D.) (24 trials) and $14,850 \pm 100$ (S.D.) (23 trials). The amino acid content of Fraction II is compared with native hemoglobin in Table III. Polyacrylamide gel electrophoresis in 7.5% gels at pH 8.9 shows that Fraction I has one major band and one minor band. Fraction II has two major bands and 4 - 6 minor bands (Fig. 13).

Digestion experiments with subtilisin have been performed under a variety of conditions with consistent results. Results similar to those described above are obtained for hemoglobin digested with subtilisin at pH 8.0 in the presence of $MgCl_2$. There is, however, a slight increase in heterogeneity of the 14 - 16,000 molecular weight material when the protein is digested at pH 8.0 and analyzed by sodium dodecyl sulfate gel electrophoresis. Under both conditions, digestion of the hemoglobin with subtilisin can be characterized by a repeatable sequence of events as analyzed by sodium dodecyl sulfate slab gel

Figure 12 Sodium dodecyl sulfate slab gel electrophoresis patterns of products formed during subtilisin digestion of Lepidurus bilobatus hemoglobin. Gel concentration 12.5% acrylamide. A. control; B. whole digest; C. Fraction I; D. Fraction II.

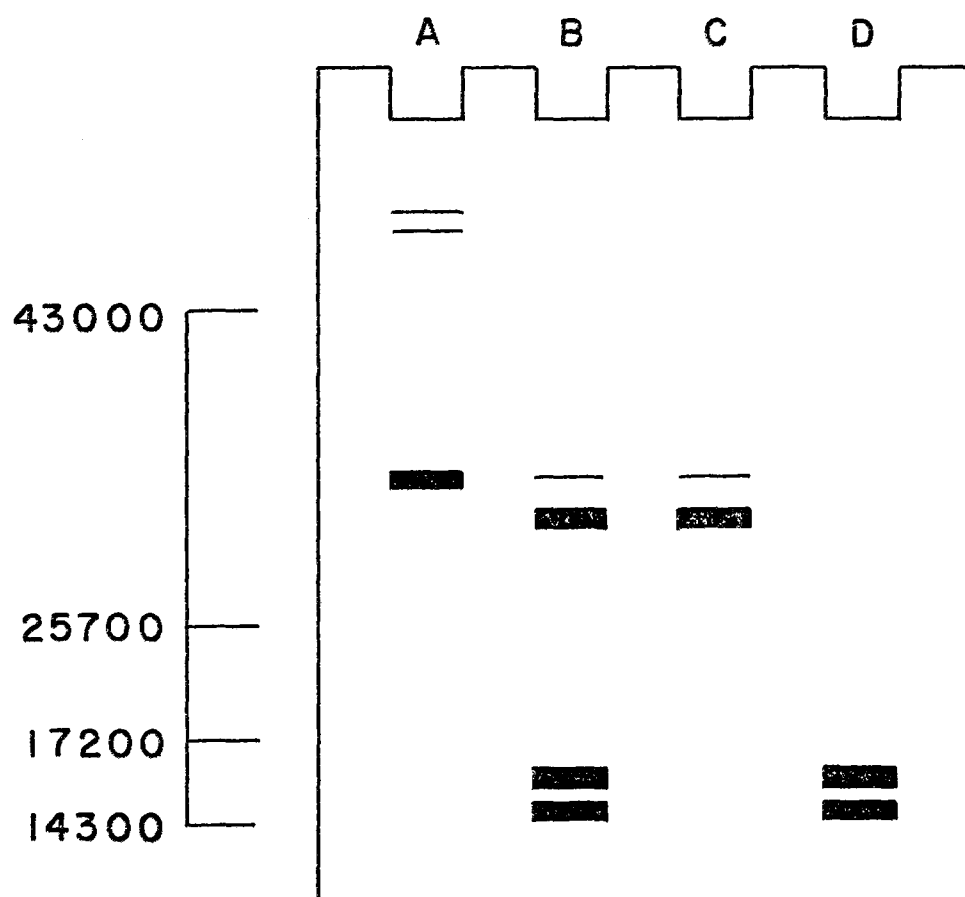


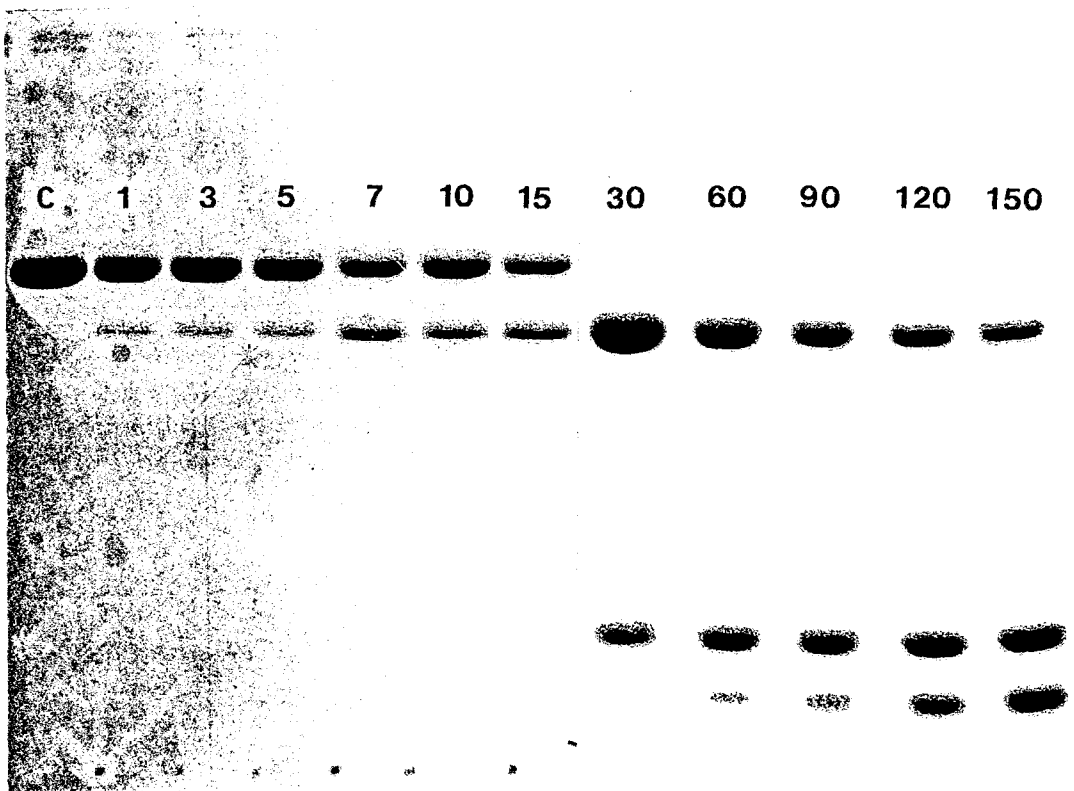
Figure 13 Disc gel electrophoresis of Fractions I and II from Sephacryl S-200 chromatography of Lepidurus hemoglobin digestion material. A. Fraction I. B. Leading edge of Fraction II. C. Trailing edge of Fraction II. D. Leading and trailing edge of Fraction II combined. 7.5% acrylamide. pH 8.9.



electrophoresis (Fig. 14). Within several minutes one sees the appearance of a 30,000 molecular weight material. This 30,000 molecular weight material disappears over time. Concurrently, two major bands are produced with molecular weights around 14 - 16,000. Time course digestion experiments show that these two bands change in relative abundance to one another when samples are incubated with constant ratios of enzyme to hemoglobin. Similar results are seen when the enzyme ratio is varied and digestion time held constant. Sodium dodecyl sulfate slab gel analysis of relatively early samples taken during timed digestions detect more 16,500 molecular weight material than 14,000 molecular weight material. In later samples the reverse is true; there is more 14,000 and less 16,000 molecular weight material. There is also less total stained protein after long incubation periods suggesting that the protein is being digested further under these conditions. No stained material is detected at the dye front.

An attempt was made to isolate a purified heme-containing digestion fragment. Sephacryl S-200 column chromatography of the cleavage products obtained from a one hour subtilisin digest (1 gm enzyme: 100 gm protein) of Lepidurus hemoglobin (pH 10.5, 0.01 M EDTA) resolves a single major heme-containing peak with an apparent

Figure 14 Sodium dodecyl sulfate slab gel electrophoresis of time course analysis of Lepidurus bilobatus hemoglobin digested with subtilisin. Numbers represent digestion time in minutes. c, 2.5 hour control hemoglobin.



molecular weight of 18 - 23,000. Sodium dodecyl sulfate gel electrophoresis of fractions along the elution curve show that the peak is made up of primarily 14 - 16,000 molecular weight material with a trace of 30,000 molecular weight material. The amount of 30,000 molecular weight material in this peak varies slightly from digest to digest. Increased amounts of this material seem to correlate with the higher molecular weights (23,000) determined for the mixture by gel column chromatography. The amount of protein recovered in the 18 - 23,000 peak represents approximately 25 - 33% of the total protein applied to the column. This suggests that a large portion of the material is digested into smaller peptide fragments which either elute in the salt volume or adhere to the column matrix. Although absorbance at 280 nm is detected in the salt peak it does not account for the missing protein. Absorbance is still detected after many column volumes of buffer have been washed through the column. This absorbance may indicate the slow elution of hydrophobic fragments. A brown material is also present on the column and can only be removed by extensive washing of the column. Although this material is unidentified it may represent hydrophobic heme groups that have been released from the polypeptide chains during the digestion. No protein is detected by sodium dodecyl sulfate slab gel

electrophoresis of the material from the salt peak, wash volume or the brown material.

Three heme-containing peaks are resolved when the 18,000 molecular weight peak from the subtilisin digestion is passed through a column of DEAE Sephadex A-50 (Sigma Chem. Co.) (1.0 x 15 cm) equilibrated in 0.05 M Tris HCl (pH 8.0), 0.05 M NaCl (Fig. 15). The relative heights of the peaks varies from one experiment to the next but the position of elution peaks is unchanged.

Sodium dodecyl sulfate slab gel analysis of the three peaks is shown in Fig. 16. The electrophoretic pattern of DEAE Fraction A is heterogeneous with respect to molecular weight with bands corresponding to 15,000, 17,000 and 30,000. In one experiment, however, Fraction A was homogeneous with a molecular weight of 16,500. DEAE Fractions B and C are both homogeneous and have apparent molecular weights of $15,150 \pm 40$ (S.D.) (3 trials) and $15,400 \pm 70$ (S.D.) (4 trials), respectively. Co-electrophoresis of Fractions B and C results in a single band with an apparent molecular weight of 15,300. Samples of Fractions A, B, and C were analyzed by tube gel electrophoresis in 6.25 M urea at pH 2.2 in the presence of reducing agent. DEAE Fraction A is heterogeneous and shows one major band with 3 - 4 minor bands. Fractions B and C each electrophorese as single major

Figure 15 DEAE Sephadex A50 chromatography of a 1 hour subtilisin digestion of Lepidurus bilobatus hemoglobin (enzyme:hemoglobin ratio, 1:100). Column buffer 0.05 M Tris-HCl (pH 8.0), 0.05 M NaCl. Fractions collected indicated by black bars. ● absorbance 415 nm.

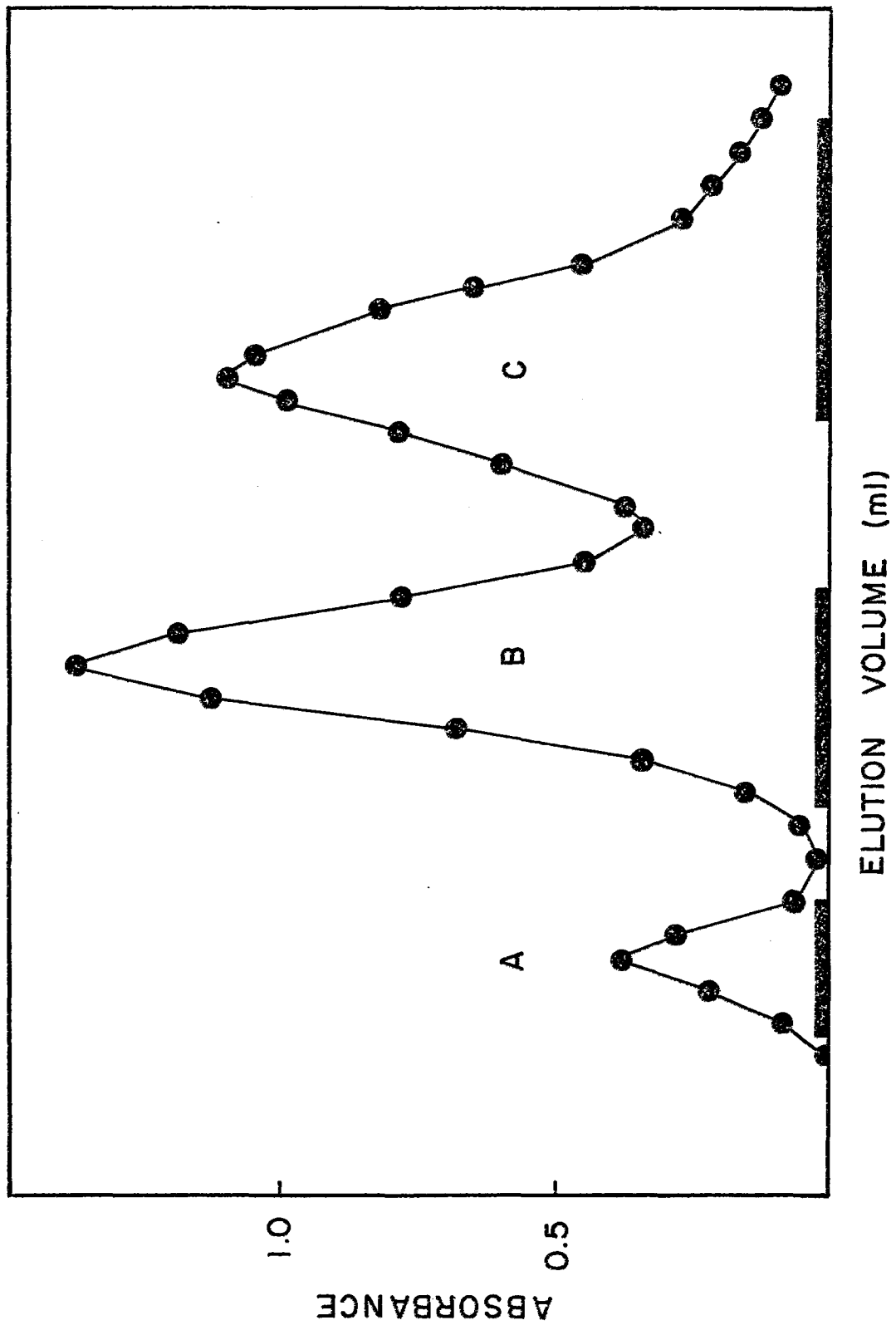
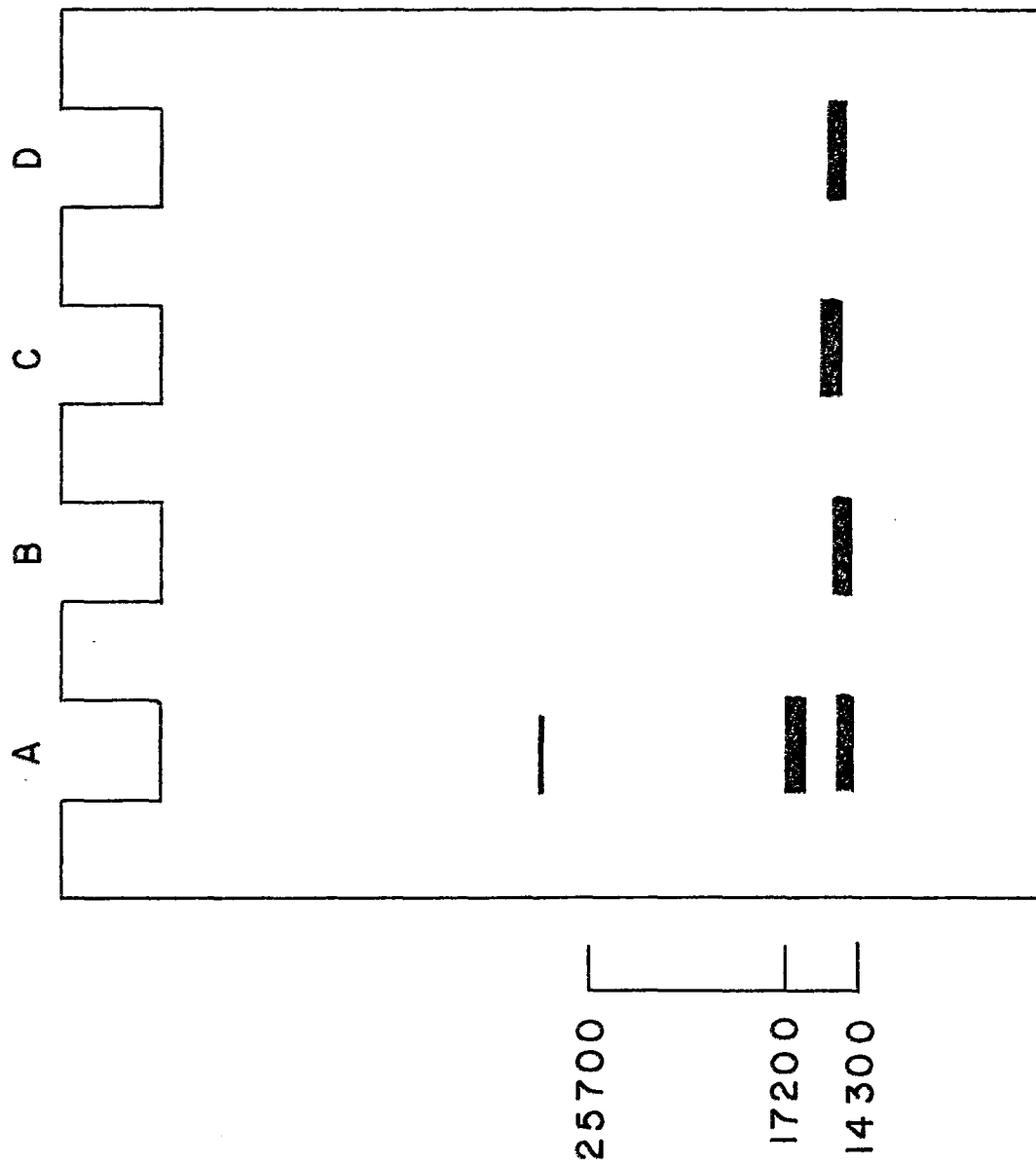


Figure 16 Sodium dodecyl sulfate slab gel electrophoresis of DEAE Sephadex Fractions A, B, and C. A. Fraction A. B. Fraction B. C. Fraction C. D. Fractions B and C. 12.5% acrylamide.



bands with slight heterogeneity being detected in overloaded gels (Fig. 17). Electrophoresis of Fractions B and C on the same gel show that the two major proteins are very similar in mobility and can only be separated under these conditions if the electrophoresis is allowed to continue after the tracker dye has run out of the gel.

Sodium dodecyl sulfate gel electrophoresis (15% acrylamide) of cyanogen bromide cleaved Lepidurus globin resolves two major bands and three minor bands (Fig. 18). The major bands have molecular weights of 20,100 and approximately 12,600. The minor bands have molecular weights of approximately 29,600, 26,700, and 24,000. A very faint fuzzy stained trace of material is present near the dye front. Control hemoglobin which had not been treated with cyanogen bromide had a molecular weight of approximately 29,000 under these conditions.

Tryptic peptide patterns were obtained for alkylated whole Lepidurus globin and purified 34,000 molecular weight material isolated by column chromatography in 6 M guanidine hydrochloride according to the method of Stephens (1978) as described in Materials and Methods. The peptide map of whole alkylated Lepidurus globin is presented in Fig. 19. Fluorescent staining of the tryptic peptides of Lepidurus hemoglobin reveals 34 - 36 intensely staining invariable spots and 8 - 10 lighter staining

Figure 17 Urea gel electrophoresis of Fractions B and C from DEAE Sephadex A50 chromatography of subtilisin cleaved Lepidurus bilobatus hemoglobin. A. DEAE Fraction B. B. DEAE Fraction C. C. DEAE Fractions B and C. 5% acrylamide. pH 2.2.

DEAE
B



DEAE
C



DEAE
B + C



Figure 18 Sodium dodecyl sulfate slab gel electrophoresis of Lepidurus bilobatus hemoglobin cleaved with cyanogen bromide. 15% acrylamide.

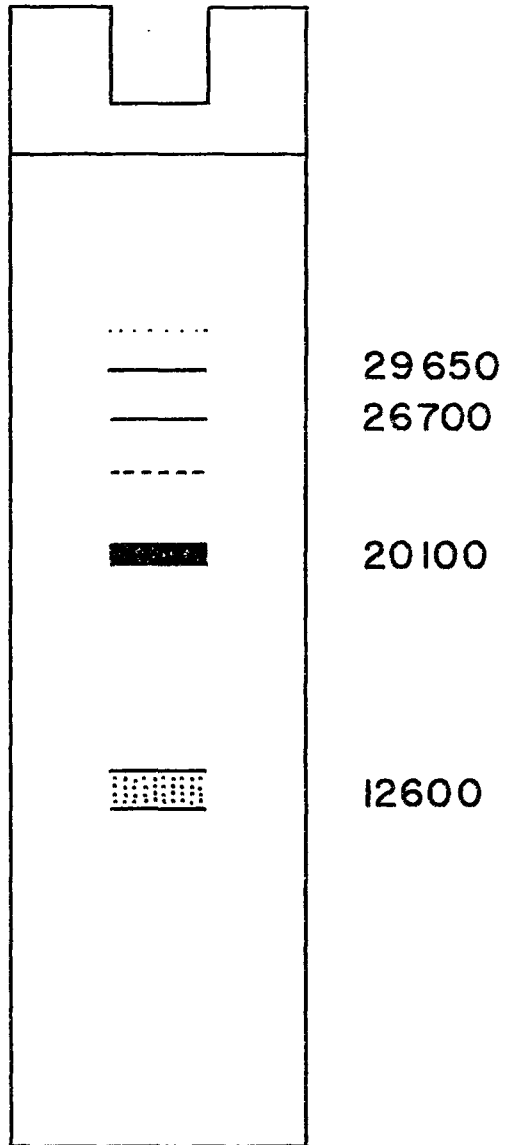


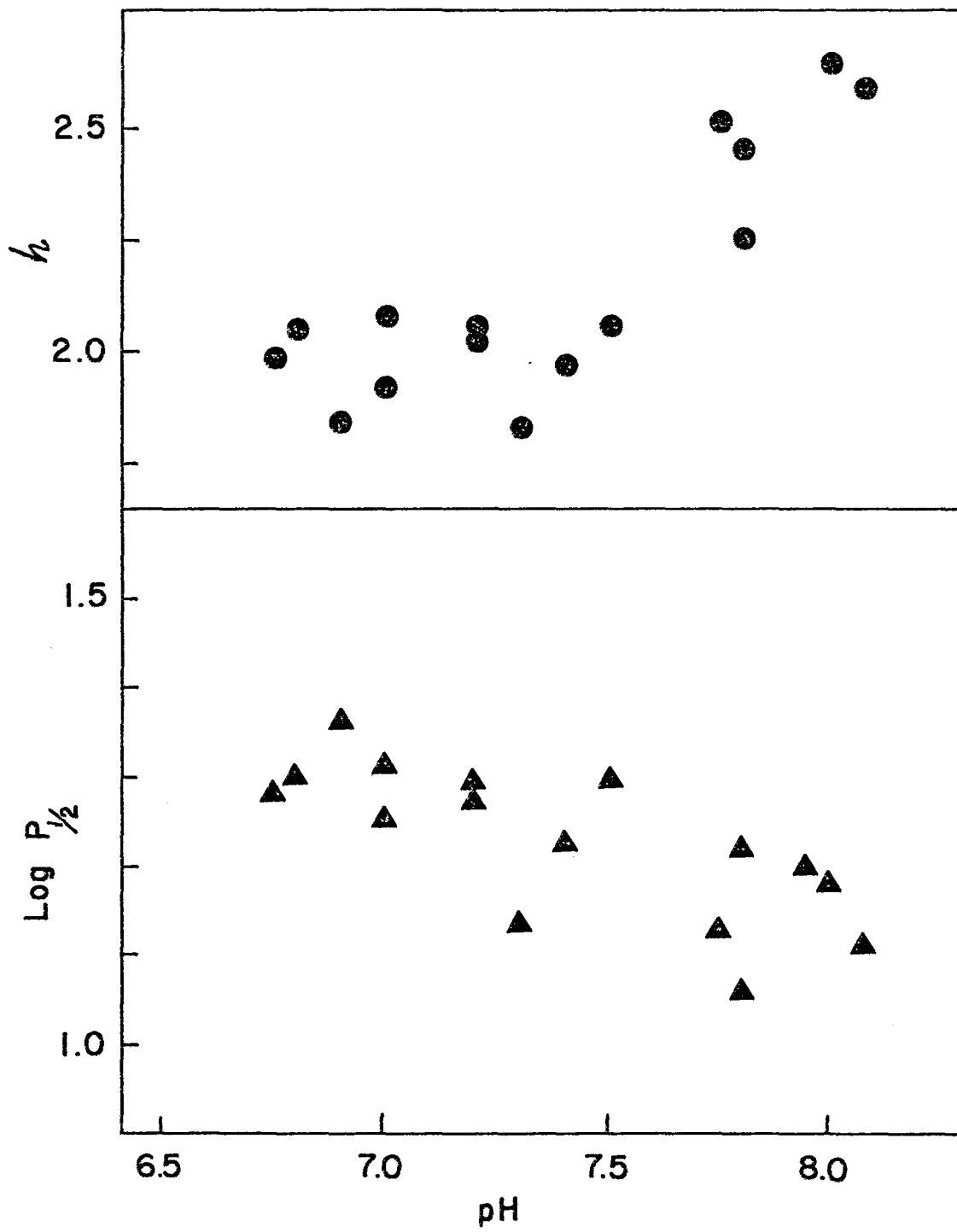
Figure 19 Tryptic map of whole Lepidurus bilobatus
hemoglobin. Enzyme : protein ratio 1:100. 24 hours,
25° C. Procedure as described in Materials and Methods.



spots. These results are reproducible under a variety of conditions including longer digestion times and increased enzyme concentrations. Simultaneous digestion of sperm whale myoglobin apoprotein under identical conditions gives the number of spots (24) predicted for this protein by its lysine and arginine content. Trypsin at the concentration used in this experiment was also digested and mapped. No spots were detected. The number and pattern of peptides detected for the purified 34,000 molecular weight subunit are identical or nearly identical to those obtained for the intact molecule. Preliminary maps of tryptic digests of the 62 - 64,000 molecular weight material also reveal a peptide pattern very similar to both the intact molecule and the purified 34,000 molecular weight subunits.

Lepidurus hemoglobin has a relatively low oxygen affinity, $P_{1/2} = 20$ mm Hg (20° C). It exhibits significant cooperativity. The Hill coefficient, h , a measure of this cooperativity is equal to 2.0 under the conditions tested (pH 7.2, 20° C). The pigment shows a slight Bohr effect; ϕ , defined as $\frac{\log P_{1/2}}{\log \text{pH}}$, is equal to - 0.13 between pH 6.75 and 8.0 (Fig. 20). The value for h is unchanged over the pH range 6.75 - 7.6; above pH 7.6 the Hill coefficient, h , increases to around 2.5. Intact Lepidurus hemoglobin has a heat of oxygenation as

Figure 20 The effect of pH on oxygen binding of Lepidurus bilobatus hemoglobin. All experiments performed in 0.05 M Tris-HCl buffer, 0.1 M NaCl, 0.01 M MgCl₂ at 20° C. Each point represents the mean of 3 or 4 experiments.



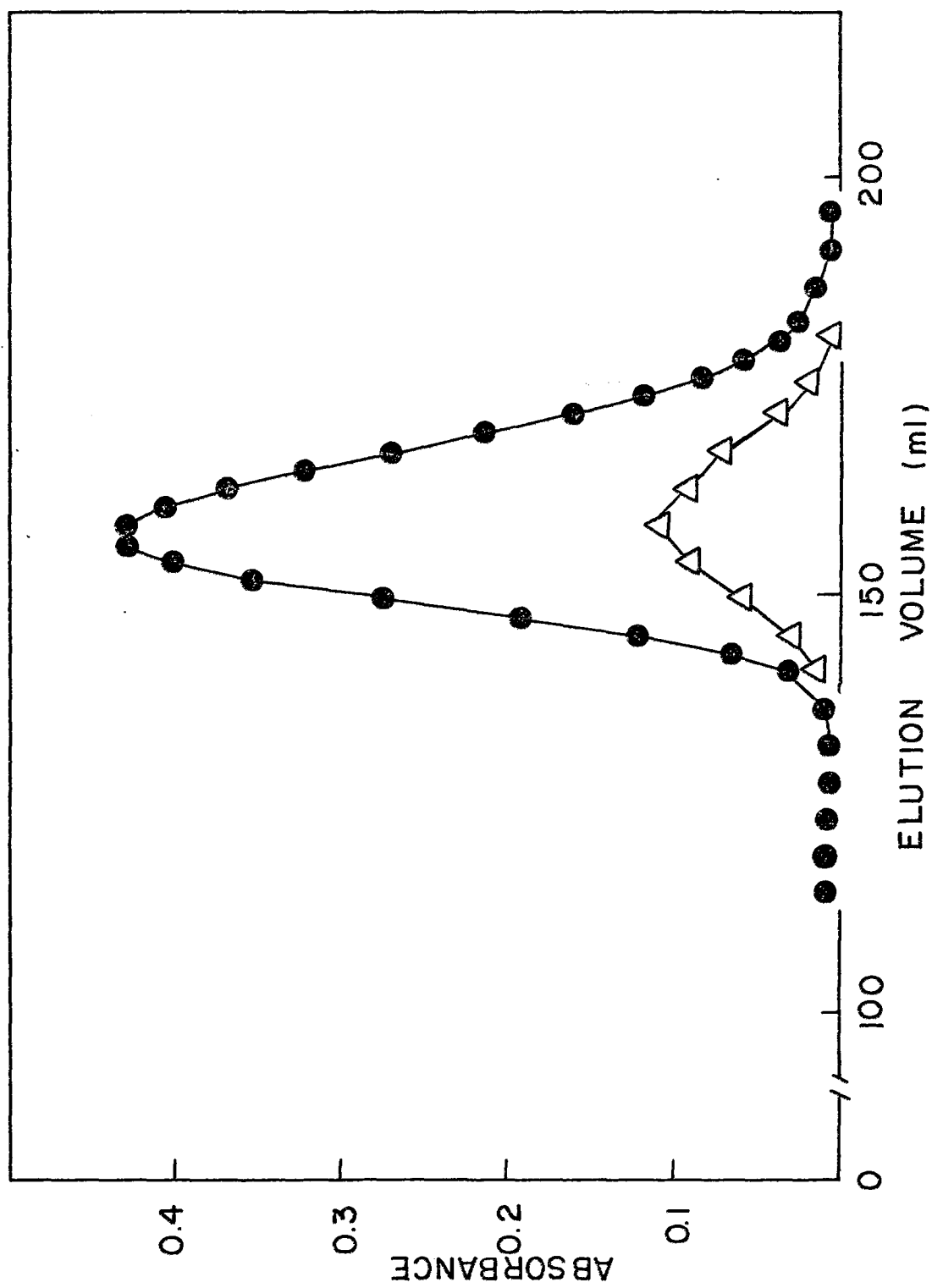
calculated by the van't Hoff equation of -7.4 Kcal/mole over the temperature range 10° to 25° C in 0.05 M Tris HCl (pH 7.2), 0.1 M NaCl, 0.01 M $MgCl_2$. This value includes the heat of solution of oxygen.

Oxygen binding studies of Sephacryl S-200 Fraction II (Fig. 11) obtained by subtilisin digestion were performed at pH 7.1, 7.3, and 8.0 in the presence of the reductase system of Hayashi et al. (1974) (Table IV). The digested material has a high oxygen affinity, $P_{1/2} = 2.4$ mm Hg (24° C), which is insensitive to hydrogen ion concentration over the pH range from 7.1 to 8.0. The Hill coefficient, h , is variable and sometimes high. Certain experiments have shown $H = 1.7$ and others $h = 3.3$. In order to test for the possible polymerization of the digestion material in the deoxy state, the digestion material was chromatographed on a column of Sephadex G-100 (1.2×35 cm) in the presence of sodium dithionite. No shift in the elution pattern was detected (Fig. 21).

Table IV. Oxygen Equilibria of Intact Lepidurus Hemoglobin and Digestion Products

<u>Sample</u>	<u>pH</u>	<u>P_{1/2}</u>	<u>h</u>
Intact	7.0	19.3	1.9
	7.3	13.6	1.8
	8.0	14.8	2.6
Fraction II	7.1	2.2	3.0
	7.3	2.5	2.8
	8.0	2.4	3.3
	8.0	2.6	1.7

Figure 21 Sephadex G-100 column chromatography of 18,000 molecular weight material from Lepidurus bilobatus hemoglobin cleaved with subtilisin. ● oxyhemoglobin. △ deoxyhemoglobin. All absorbances at 540 nm.



STUDIES OF DAPHNIA PULEX HEMOGLOBIN

Chromatography of purified Daphnia hemoglobin on Bio Gel A5M at pH 8.0 in the presence of 0.01 M $MgCl_2$ shows one symmetrical peak with an apparent molecular weight of 430,000 (Fig. 22). Similar results ($M_r = 415-470,000$) are obtained on Sepharose 4B at pH 7.0 with $MgCl_2$. Daphnia carbonmonoxyhemoglobin elutes behind Lepidurus bilobatus hemoglobin yet ahead of Artemia salina hemoglobin when these pigments are chromatographed on a Bio Gel A5M column under identical conditions (Fig. 23).

Sedimentation velocity experiments performed on Daphnia carbonmonoxyhemoglobin show that the pigment sediments as a single boundary with $S_{20,w} = 16.9$ at pH 7.0 and $S_{20,w} = 17.4$ at pH 8.0 in 0.05 M Tris HCl, 0.1 M NaCl, 0.01 M $MgCl_2$.

Electrophoresis of Daphnia hemoglobin under a variety of denaturing conditions is shown in Fig. 24. The subunit is heterogeneous with respect to molecular weight under all conditions tested. Sodium dodecyl sulfate gel electrophoresis of the pigment on 10% acrylamide slab gels shows 4 major bands with molecular weights between 33 - 37,000 (Fig. 24a). The major bands have molecular weights of $33,700 \pm 300$ (S.D.), $35,100 \pm 200$ (S.D.)

Figure 22 Bio Gel A5M column chromatography of Daphnia pulex hemoglobin (4° C). Column buffer 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.01 M MgCl₂. Column volume 1.9 x 90 cm. Calibrants: a, Eudistylia vancouveri chlorocruorin. b, Cancer magister 24S hemocyanin. c, C. magister 16S hemocyanin. d, lactate dehydrogenase. ● OD_{540nm}.

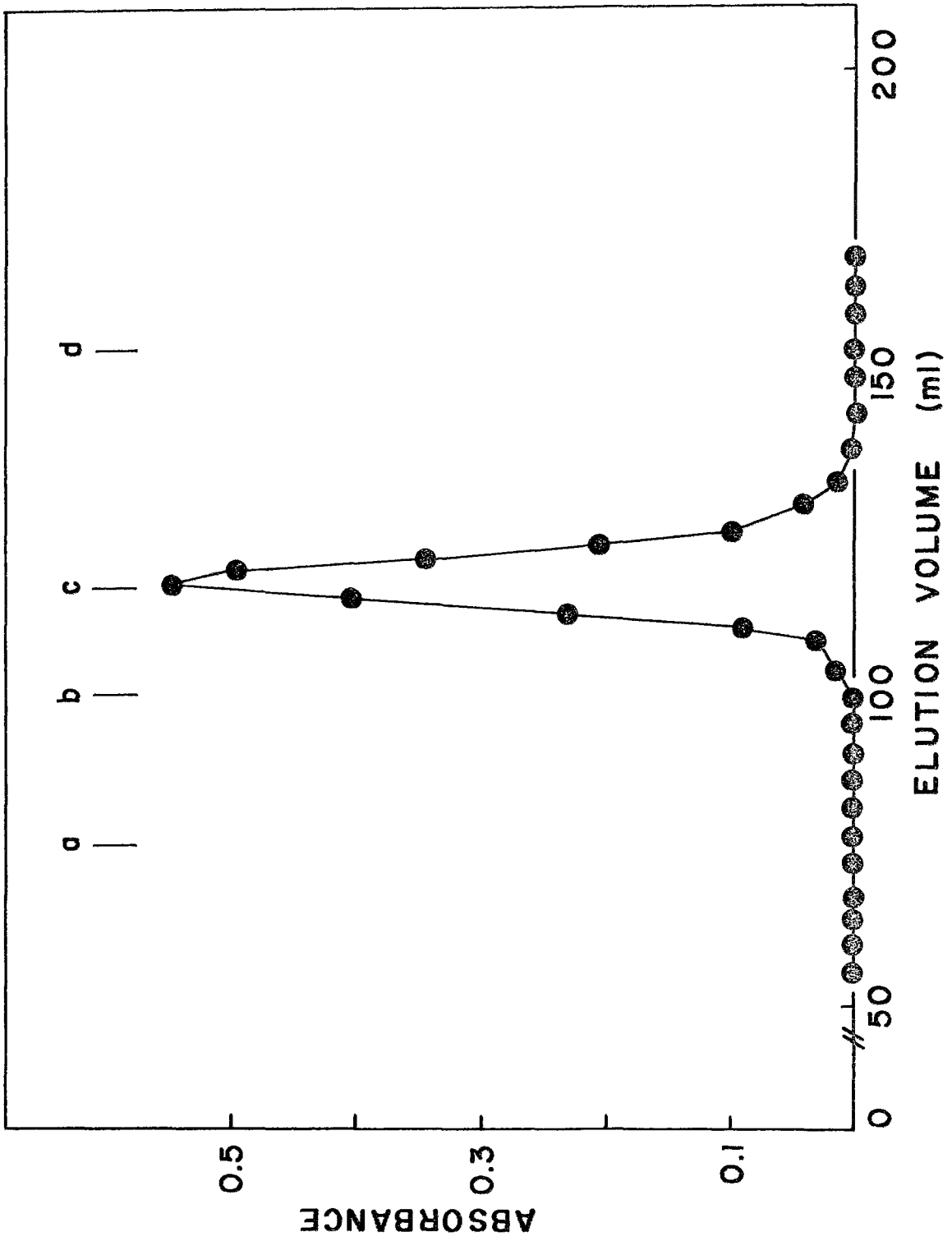


Figure 23 Bio Gel ASM column chromatography of Lepidurus bilobatus hemoglobin, Daphnia pulex hemoglobin and Artemia salina hemoglobin. Column buffer 0.05 M Tris-HCl (pH 8.0), 0.1 M NaCl, 0.01 M MgCl₂. Calibrants: a, Eudistylia vancouveri chlorocruorin. b, Cancer magister 24S hemocyanin. c, Cancer magister 16S hemocyanin. Column volume 1.9 x 90 cm.

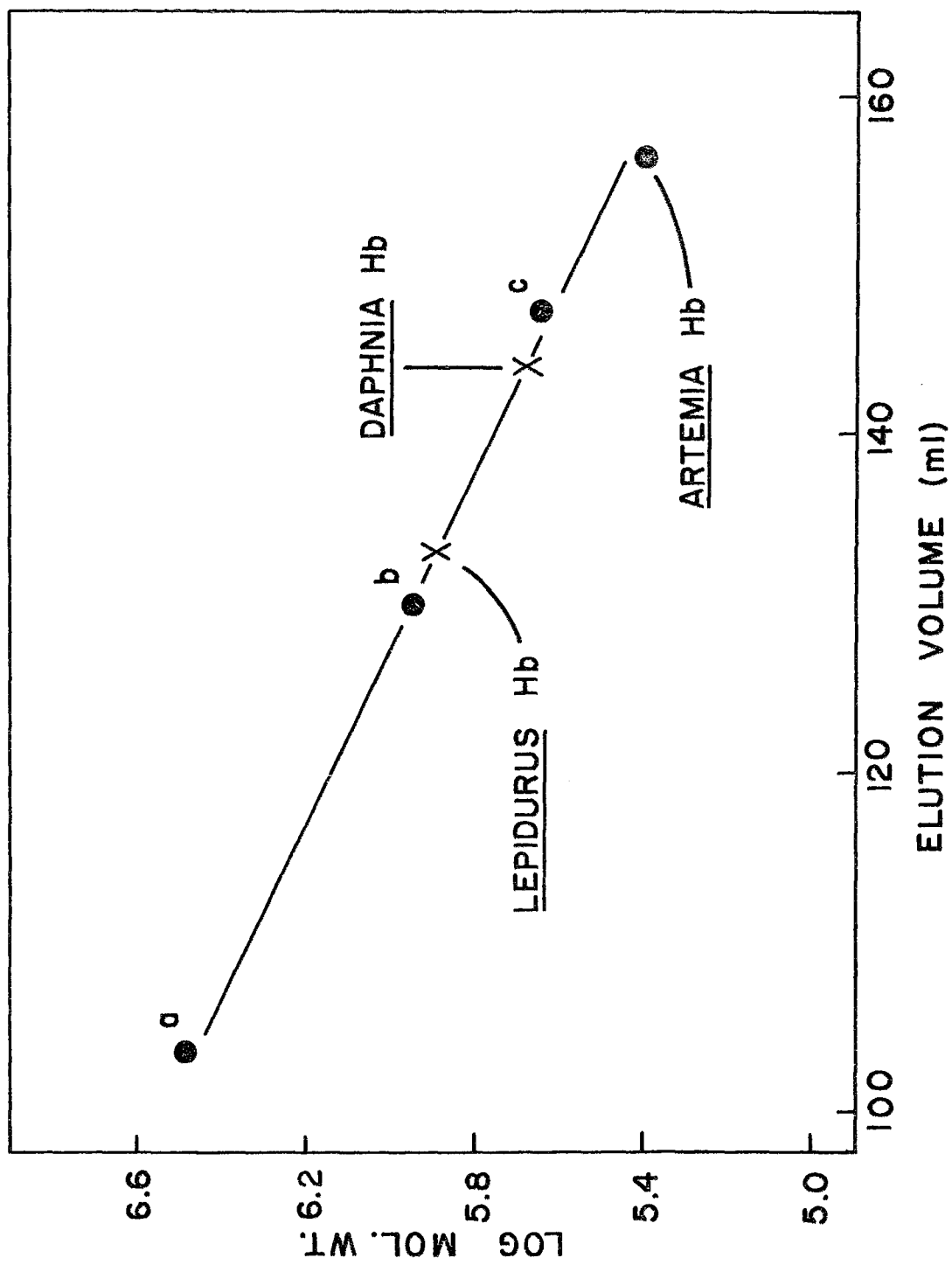
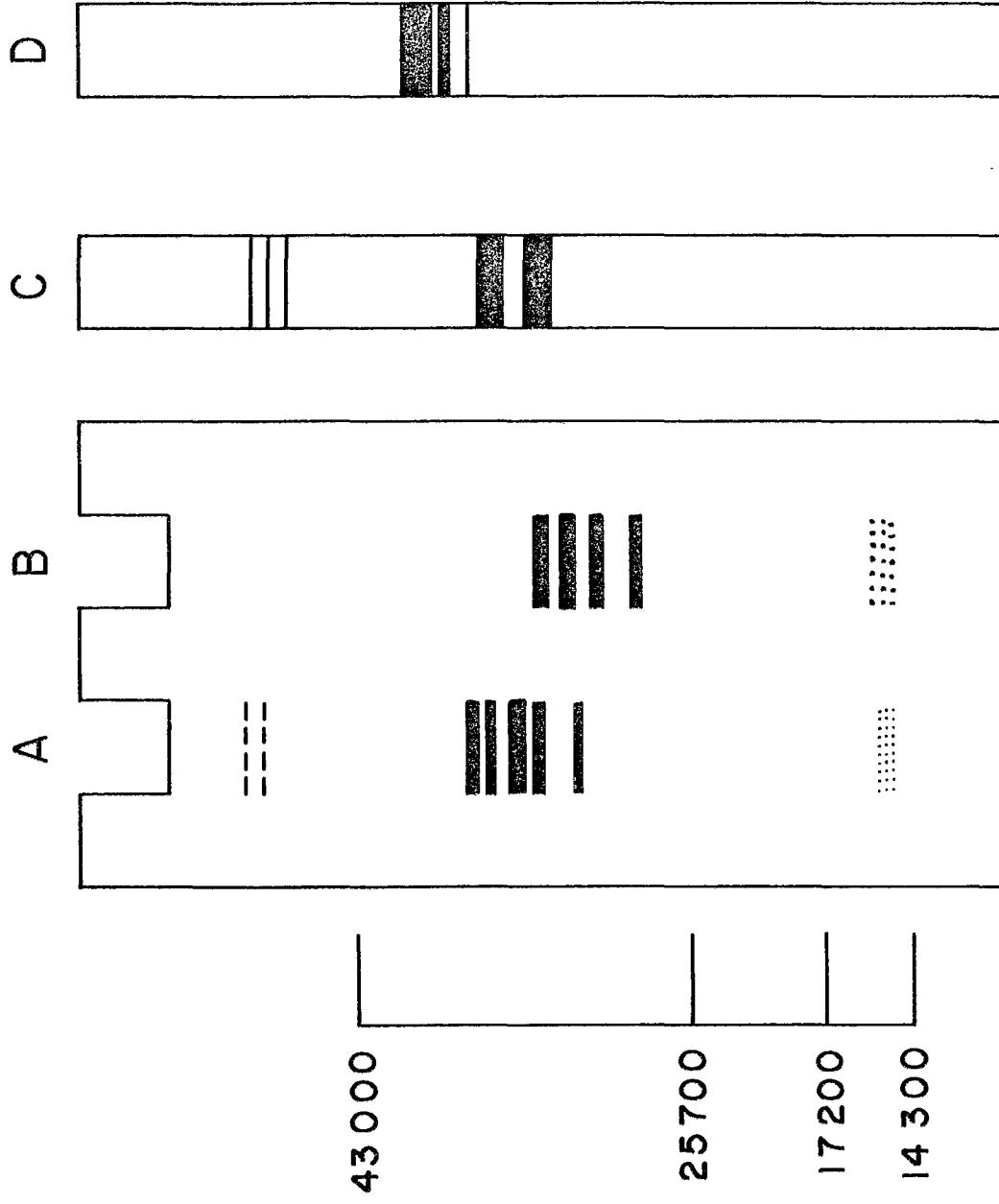


Figure 24 Electrophoretic patterns of Daphnia pulex hemoglobin. A. Sodium dodecyl sulfate slab gel electrophoresis. Gel concentration 12.5% acrylamide. B. Sodium dodecyl sulfate slab gel electrophoresis of hemoglobin previously denatured in boiling 6 M guanidine hydrochloride. Gel concentration 12.5%. C. Sodium dodecyl sulfate tube gel electrophoresis in 8 M urea. Gel concentration 5%. D. Tube gel electrophoresis of Daphnia hemoglobin in 6.25 M urea. Gel concentration 5%. pH 2.2.



35,500 \pm 400 (S.D.), and 37,400 \pm 200 (S.D.) (4 trials). One preparation showed an additional band with an apparent molecular weight of 31,400 \pm 100 (S.D.) (4 trials). Small amounts of higher molecular weight proteins ranging from 53 - 86,000 also are detected. The number of these higher molecular weight bands varies from one preparation to the next: however, they are present in all preparations as a small amount of the total protein. Sodium dodecyl sulfate gel electrophoresis of the same samples on a 12.5% acrylamide slab also shows a lightly stained diffuse band of 16,000 molecular weight material. However, most of the protein in all the above experiments is in the 31 - 37,000 molecular weight range. Identical results are obtained when analyzing lyophilized globin which had been oxidized with performic acid prior to treatment with sodium dodecyl sulfate. Bands corresponding to the 31 - 37,000 and 53 - 86,000 molecular weight proteins are also detected if unpurified pigment is immediately treated with boiling sodium dodecyl sulfate incubation buffer. Sodium dodecyl sulfate gel electrophoresis in the presence of 8 M urea of native hemoglobin or performic acid oxidized globin shows two prominent major staining bands with molecular weights of 33,000 and 36,000 (Fig. 24c). Some very faint bands with apparent molecular weights ranging from 47,000 - 80,000 are also present.

No material is detected with molecular weights smaller than 33,000 under these conditions.

Lyophilized Daphnia hemoglobin, previously denatured in boiling 6 M guanidine hydrochloride with reducing agent and dialyzed against sodium dodecyl sulfate incubation buffer, electrophoreses on 12.5% acrylamide slab gels as four major bands with molecular weights of 28,000, 30,700, 32,000, and 33,400 (Fig. 24b). Electrophoresis of protein in overloaded gels also shows 3 minor bands between 53,000 and 58,500 molecular weight as well as a blurred trace of 15,500 molecular weight material. Most of the protein is found in the 29 - 33,000 molecular weight range.

Samples from two separate hemoglobin preparations were analyzed by gel electrophoresis in 6.25 M urea at pH 2.2. The pigment had been incubated in 10 M urea 5% in acetic acid, 0.01 M in 2-mercaptoethanol overnight prior to the electrophoresis. Both samples yield three closely spaced bands (Fig. 24d).

The amino acid content of the purified hemoglobin is presented in Table V. There is one heme per 18 - 20,000 gram protein based on $\epsilon_{280}^{\text{mg/ml}} = 1.31$ determined for Lepidurus hemoglobin and $\epsilon_{540}^{\text{mM}}$ of 11.0 for cyanmet hemoglobin (Van Assendelf and Zijlstra, 1975).

Sedimentation velocity experiments on Daphnia.

Table V. Amino Acid Composition of Daphnia pulex hemoglobin.

Amino Acid	Intact Hb g/100g protein	38,000 peak g/100g protein	Digest g/100g protein
Lys	4.6	5.0	5.0
His	1.3	1.6	1.5
Arg	2.9	3.0	2.7
Asp	11.6	10.8	10.3
Thr**	6.0	4.1	4.1
Ser**	8.2	8.7	10.0
Glu	8.3	8.3	8.9
Pro	3.9	3.5	3.5
Gly	12.6	13.4	13.2
Ala	14.9	15.7	16.0
½ Cys*	0.5	0.5	0.4
Val***	7.4	6.8	6.7
Met	0.8	0.8	0.8
Ileu***	4.3	4.5	4.3
Leu	8.5	9.1	8.8
Tyr	1.1	1.0	0.7
Phe	3.1	3.3	3.1
Trp	-	-	-

* Determined as cysteic acid as in Hirs (1956).

** Extrapolation to zero time hydrolysis.

*** Highest value after 72 h hydrolysis.

hemoglobin at pH 10.5 in EDTA show the protein behaves as a 3S material. Column chromatography of this pigment on Sephacryl S-200 in the same buffer resolves a major and a minor heme-containing peak with apparent molecular weights of 38,300 and 22,300, respectively (Fig. 25). The ratio of absorbance at 280 nm to 540 nm for the carbonmonoxyhemoglobin of both peaks is the same as that found for the intact hemoglobin suggesting that both dissociation fractions contain one mole heme per 18 - 20,000 gram protein. SDS slab gel analysis of the major heme-containing peak shows that it contains from 4 - 6 polypeptides with molecular weight ranging from 31 - 37,000 with the smallest chain staining the heaviest (Fig. 26b). The minor peak contains protein that electrophoresis as a diffuse band with an apparent molecular weight of 18,000 (Fig. 26c). Amino acid analysis of the 38,000 molecular weight peak (Fig. 25) is similar to that of the intact pigment (Table V).

Limited proteolysis of the 38,000 molecular weight fraction as prepared by column chromatography at pH 10.5 in the presence of 0.01 M EDTA was carried out at 25° C using a 1:50 (g enzyme:g protein) concentration of subtilisin to hemoglobin ratio in the 0.04 M sodium glycinate buffer. The reaction was allowed to proceed for one hour at which time it was stopped by the addition of excess

Figure 25 Sephacryl S-200 column chromatography of Daphnia pulex hemoglobin. Column buffer 0.04 M sodium glycine (pH 10.5), 0.01 M EDTA. Calibrants: a, bovine serum albumin. b, ovalbumin. c, sperm whale metmyoglobin. Column volume 1.8 x 85 cm. ● OD_{415nm}, ▲ OD_{280nm}.

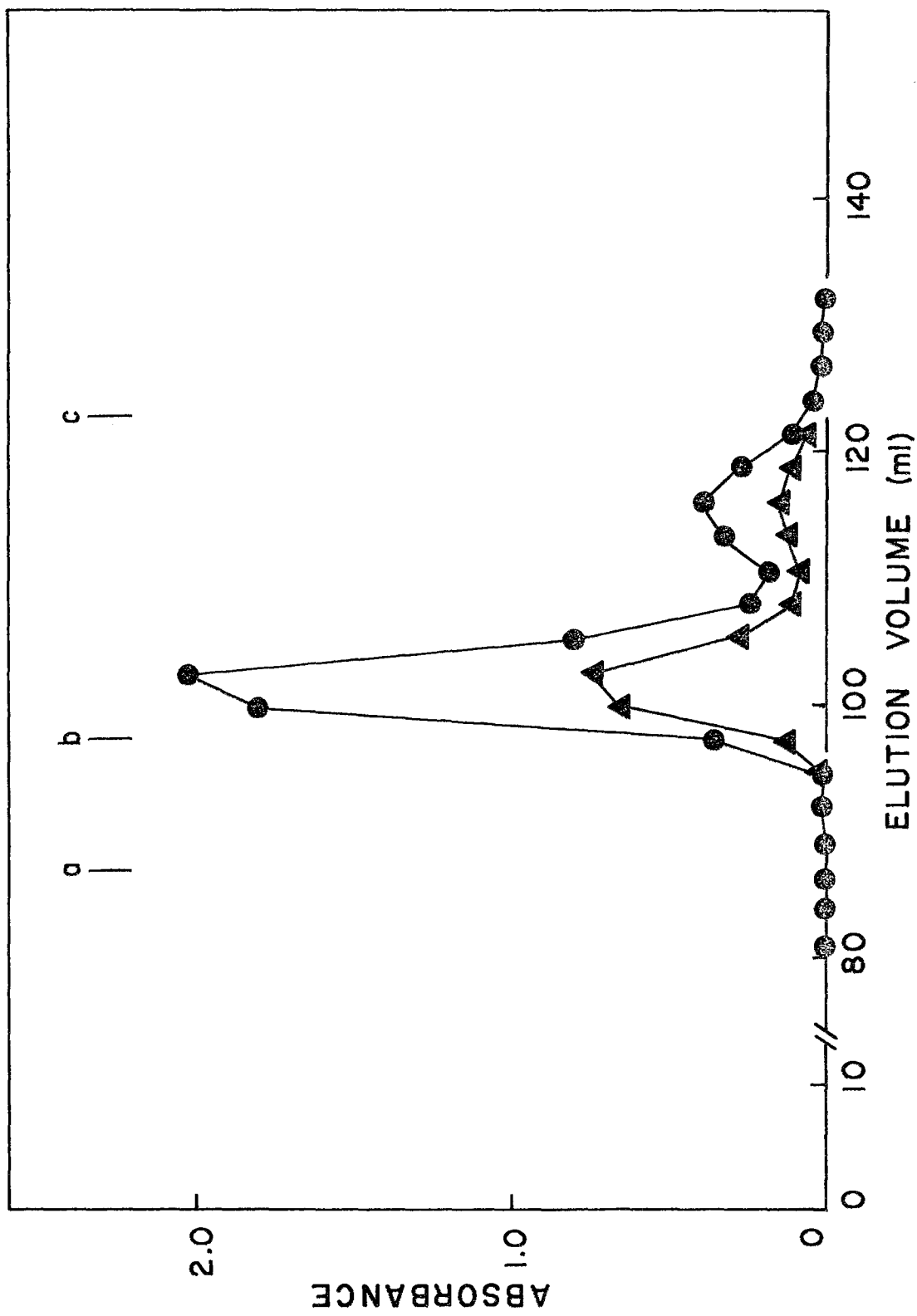
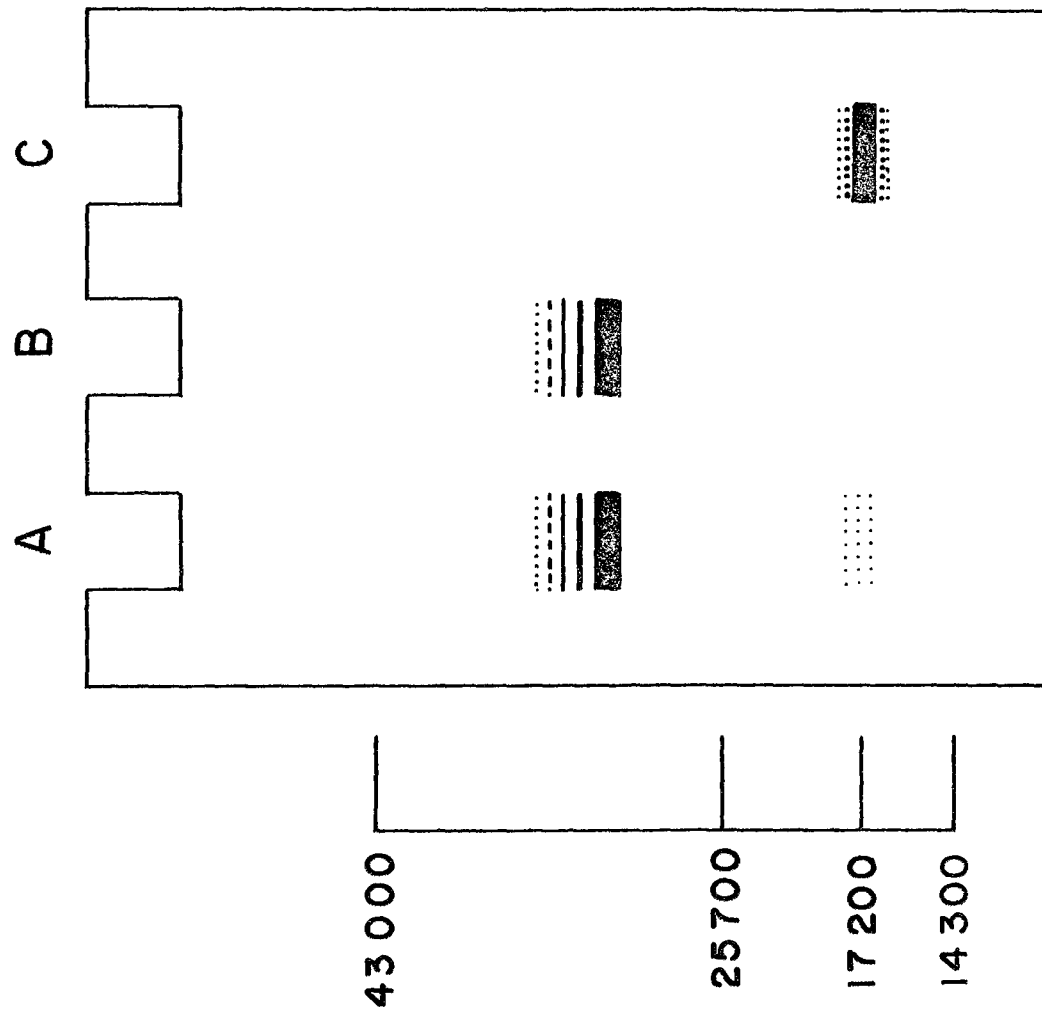


Figure 26 Sodium dodecyl sulfate slab gel electrophoretic patterns of dissociation products of Daphnia pulex hemoglobin. A. Unfractionated sample of dissociated hemoglobin. B. Sephacryl S-200 heavy fraction. C. Sephacryl S-200 light fraction. Gel concentration 12.5% acrylamide.



PMSF. This mixture was then chromatographed on a column of Sephacryl S-200 equilibrated in the pH 10.5 glycinate buffer. Hemoglobin which had been incubated at room temperature without enzyme and in the presence of PMSF served as a control.

The digestion products elute as a major and a minor heme-containing peak (Fig. 27). The minor peak has an apparent molecular weight of 38,000 corresponding to the undigested material, and the major peak elutes with an apparent molecular weight of 22,000. Chromatography of the control shows only 38,000 molecular weight material.

Samples of the digestion products and control were treated with sodium dodecyl sulfate incubation buffer for electrophoretic analysis (Fig. 28). The control shows one major staining band with molecular weight around 32,000 and minor bands between 34 - 36,000; a diffuse trace of lower molecular weight material can be detected on heavily overloaded gels. The 38,000 molecular weight peak (Fig. 27) contains several proteins between 32 - 36,000 molecular weight when analyzed by SDS slab gel (Fig. 28c). The 22,000 molecular weight fraction electrophoreses in SDS as one major band with apparent molecular weight of 15,950 and 2 minor bands of 17,000 and 14,500 (Fig. 28d). The heme to protein ratio of the 22,000 molecular weight material is similar to that of the intact

Figure 27 Sephacryl S-200 chromatography of a one hour subtilisin digest of Daphnia pulex hemoglobin (1 mgm enzyme to 100 mgm protein, 25^o C). Area under the bar represents the material digested. Open circles are hemoglobin eluted prior to digestion. Solid squares are hemoglobin eluted after digestion. Column buffer 0.04 M sodium glycine (pH 10.5), 0.01 M EDTA. Column volume 1.8 x 85 cm. All absorbances read at 540 nm.

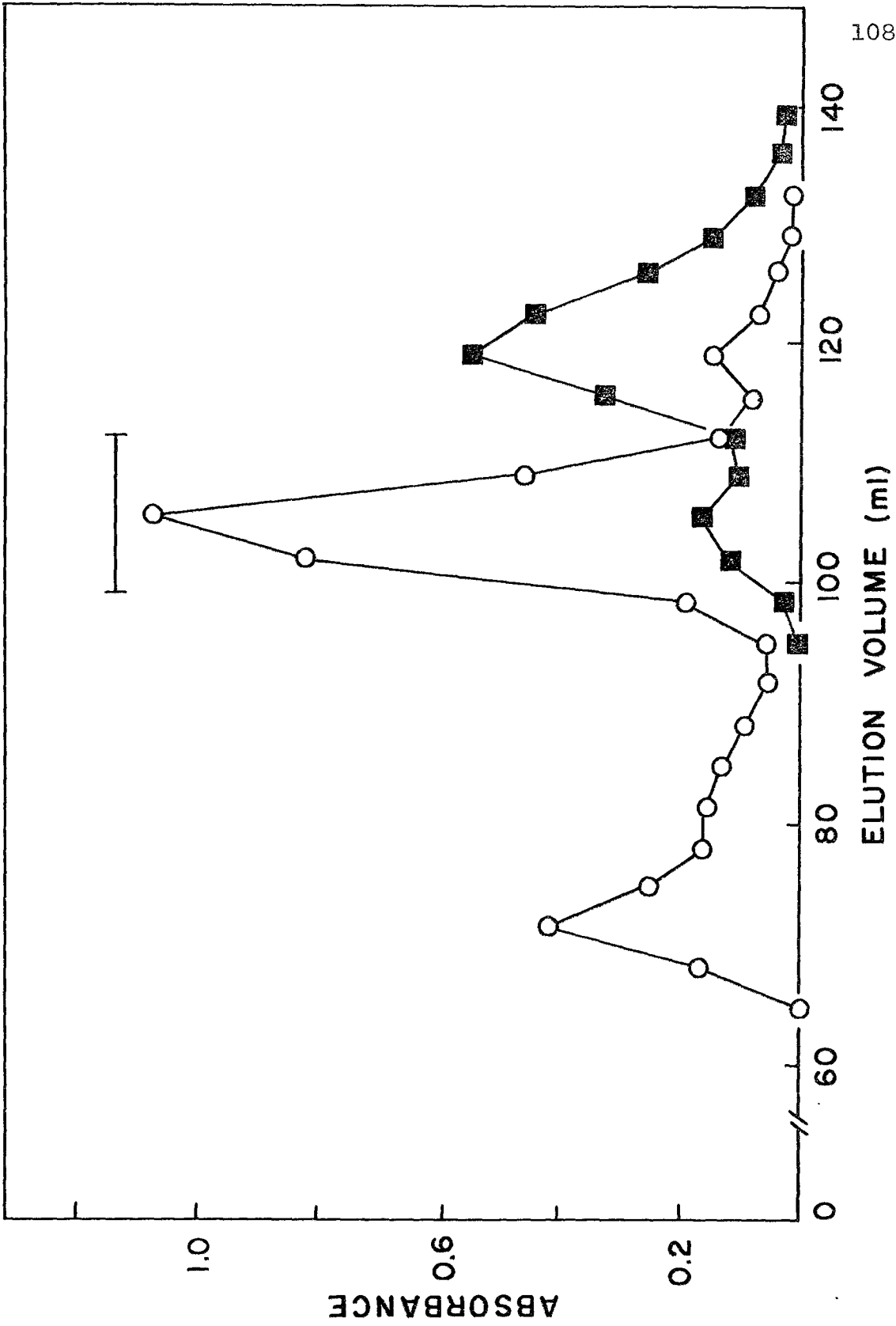
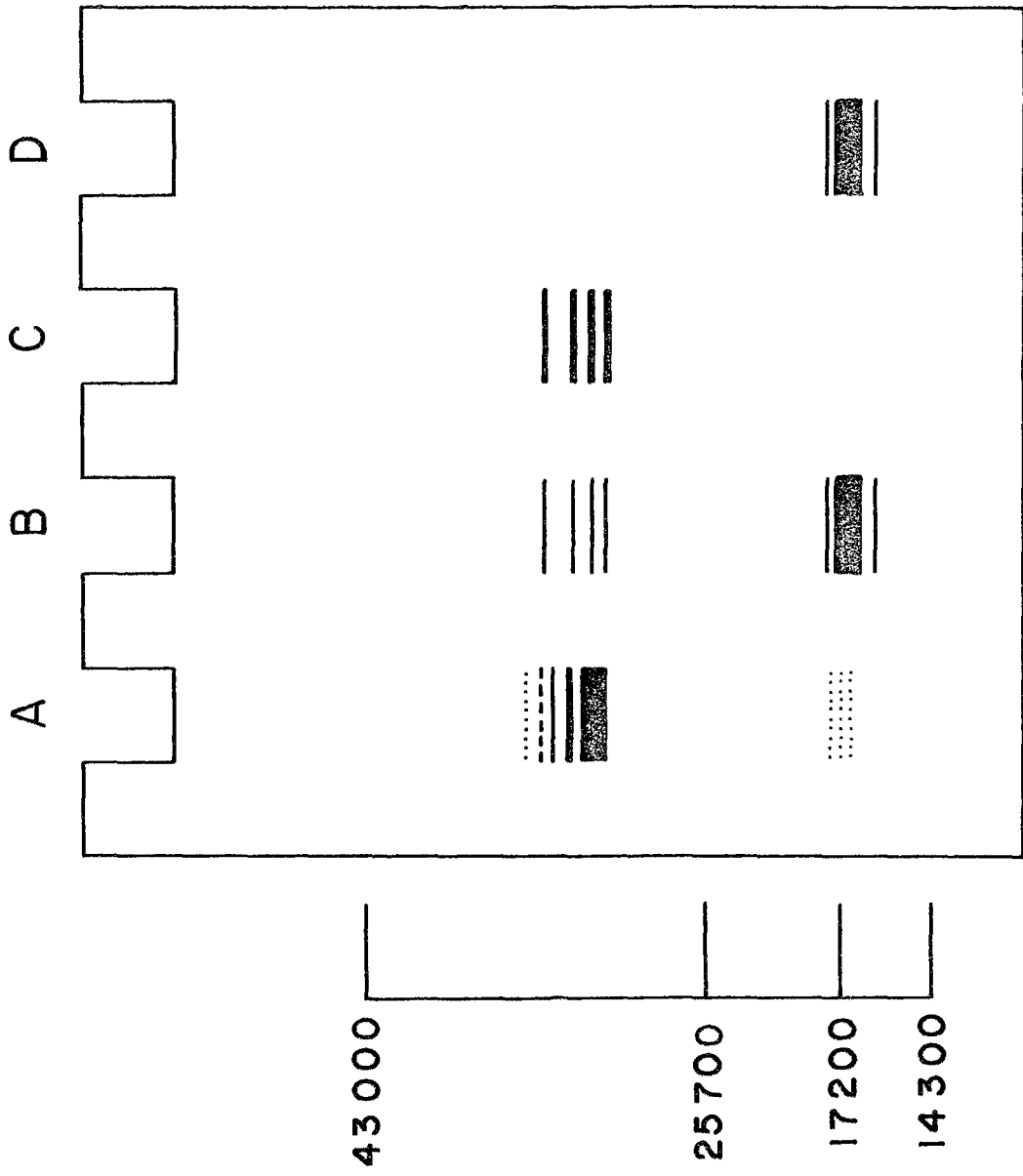


Figure 28 Sodium dodecyl sulfate slab gel electrophoresis patterns of products formed during subtilisin digestion of Daphnia pulex hemoglobin. Gel concentration 12.5% acrylamide. A. Control. B. Whole digest. C. Digest heavy fraction. D. Digest light fraction.



molecule and the 38,000 dissociation product from which it was produced. The amino acid composition of this digestion product also is similar to the intact pigment and the 3S dissociation material (Table V).

CHAPTER 4

DISCUSSION

It is concluded that Lepidurus and Daphnia extra-cellular hemoglobins are large polymeric aggregates composed of subunits which appear to be two covalently linked heme-containing units. These putative domains have been isolated by gentle proteolytic cleavage of the intact molecule. The domains isolated from Lepidurus hemoglobin have been shown to bind oxygen reversibly. Peptide maps of whole globin from Lepidurus suggest that the two domains may be non-identical in primary sequence.

Lepidurus hemoglobin exists as a high molecular weight polymer. However, the size of the native pigment has proven difficult to measure with certainty, as variations in the molecular weight are found when the hemoglobin is chromatographed under different conditions. Chromatography of oxy-hemoglobin on Sepharose 4B shows that the pigment has an apparent molecular weight of 680,000 in the presence of 0.01 M $MgCl_2$. However, when oxy-hemoglobin is applied to a column of Bio Gel A5M under identical buffer conditions, the molecule elutes with an apparent molecular weight of 800,000. This

variation is not likely to be the result of differences in hemoglobin concentration or ligand state as the samples were prepared in a similar manner. However, this discrepancy may reflect differences in the way the hemoglobin interacts with the agarose gels. As has already been reported for other gels (eg. Sephadex) some proteins exhibit abnormal behavior on agarose gels (Hjertén, 1961; Fasold et al., 1971; Andrews, 1962, 1964; Siegel and Monty, 1966; Locascio et al., 1969). Carbohydrate content, differences in shape and charge of the native molecule and uncertainties regarding the state of aggregation might account for this fact when different proteins are chromatographed. In addition, however, the same protein may exhibit different elution behaviors when chromatographed on different kinds of agarose gels (Ward and Arnot, 1965; Locascio et al., 1969). Clearly, any undetected interaction between protein and gel can lead to erroneous conclusions about the molecular weight of the protein.

The molecular weight reported above for Lepidurus hemoglobin is similar to the molecular weights reported for other notostracan hemoglobins. The range of molecular weights reported for Lepidurus hemoglobin is reflected in the range of values reported for the other species. For example, Triops hemoglobin has a molecular weight of 600,000 (Horne and Beyenbach, 1974). Yet recent studies

of the hemoglobin from Lepidurus apus lubbocki show that this pigment has a molecular weight of 798,000 (Ilan and Daniel, 1979b). The large differences in molecular weight are unexplained at present. These results may reflect unique species-specific hemoglobins. On the other hand, the results may be explained by the different methods of purification and analysis used. The molecular weight of Triops hemoglobin was determined by column chromatography and sucrose density centrifugation on material that had been previously frozen. Alternatively, L. apus lubbocki hemoglobin was analyzed by sedimentation equilibrium on freshly prepared material. Differences in the calculated partial specific volumes and the method for calculating the molecular weight of the two pigments may account for the observed discrepancy.

Variations in molecular weights reported for the notostracan hemoglobins makes it difficult at present to know the actual size of these pigments. It can be said, however, that the notostracan hemoglobins are large molecules with molecular weights between 600 - 800,000 molecular weight. It is intriguing to note, also, the similarity between the molecular weight ($M_r = 800,000$) found for Lepidurus bilobatus hemoglobin by chromatography on Bio Gel A5M and for L. apus lubbocki ($M_r = 798,000$) hemoglobin by equilibrium centrifugation. Further careful

studies of these unusual hemoglobins performed under controlled laboratory conditions in an effort to obviate differences in preparation and analysis might aid in answering this question.

Native Daphnia pulex hemoglobin has an apparent molecular weight of 430 - 470,000 (pH 7.0 - 8.0 in the presence of 0.01 M $MgCl_2$) as determined by gel chromatography on Bio Gel A5M. This value is lower than the molecular weight ($M_r = 670,000$) of native Moina macrocopa and Daphnia magna hemoglobins (Sugano and Hoshi, 1971). However, this molecular weight for D. pulex hemoglobin is in fair agreement with that reported for the same species by Svedberg and Eriksson-Quensel in 1934. Although these discrepancies between Daphnia pulex and the other cladoceran hemoglobins might be attributed to different techniques, Daphnia pulex hemoglobin can be differentiated from Lepidurus hemoglobin ($M_r = 680 - 800,000$) and Artemia salina hemoglobin ($M_r = 250,000$) when analyzed under the same buffer conditions on Bio Gel A5M. These results suggest that the hemoglobins of these different species have different quaternary structures. One obvious explanation for the behavior of Daphnia hemoglobin is that the pigment exists in a state of dissociation-association equilibrium. This alternative should be studied more closely. However, dilute samples

of Daphnia hemoglobin, chromatographed on the same Bio Gel A5M column, show the same elution pattern as the more concentrated samples suggesting that the molecule does not dissociate.

The smallest polypeptide subunit of Lepidurus hemoglobin appears to have a molecular weight of 33 - 34,000. This value is obtained using two different strategies of denaturation. The first strategy involves electrophoretic studies in which the molecule has been denatured with the detergent sodium dodecyl sulfate in the presence of reducing agents. Under these conditions a molecule will theoretically bind sodium dodecyl sulfate by hydrophobic interactions in a constant stoichiometry such that the intrinsic charge of most proteins will be masked and an approximately constant negative charge per unit mass will be obtained (Weber and Osborn, 1975). The protein-sodium dodecyl sulfate complex thus formed is thought to exist in a conformation in which the length of the complex varies uniquely with the molecular weight of the molecule. Electrophoresis of the complex in the presence of sodium dodecyl sulfate allows the polypeptide chains to have a mobility determined solely by their molecular weight. However, failure to reduce disulphide bonds will restrict the conformational freedom of the polypeptide thereby decreasing the amount of sodium

dodecyl sulfate bound and the susceptibility of disulphide linkages to reducing agent. It is important to note that the protein-sodium dodecyl sulfate complex is not a random coil but contains a high degree of order (Reynolds and Tanford, 1970a). Several proteins, including some vertebrate hemoglobins seem to show disulphide linked configurations which may be highly resistant to reducing agents (Reynolds and Tanford, 1970b); Waxman, 1975; Terwilliger et al., 1975a; Terwilliger, 1980).

In an attempt to reduce any disulphide linkages which may occur in Lepidurus hemoglobin and which may be inaccessible to reducing agent due to some unusual protein configuration, the protein was denatured in boiling 6 M guanidine hydrochloride with 2-mercaptoethanol prior to treatment with sodium dodecyl sulfate. Under these conditions the protein should be conformationally altered to random coils (Fish et al., 1970) and the reducing agent should be free to react with all disulphide linkages. These experiments as well as experiments performed on performic acid oxidized or carboxymethylated protein give results identical to those obtained with sodium dodecyl sulfate denaturation alone.

In an attempt to eliminate the possibility that the 34,000 molecular weight subunit is an artifactual product of anomalous binding of sodium dodecyl sulfate

a second denaturation strategy was employed. The protein was denatured in 6 M guanidine hydrochloride in the presence of dithiothreitol and applied to a column of Sephacryl S-200 equilibrated with 6 M guanidine hydrochloride and the reducing agent. Under these conditions the protein elutes as two fractions of 34,000 and 66,000 molecular weight. Identical results are obtained for the protein which is alkylated with iodoacetamide in 8 M guanidine hydrochloride with 2-mercaptoethanol prior to chromatography. No protein is present with a molecular weight lower than 34,000. Therefore, it is unlikely that the 34,000 molecular weight material is a dimeric aggregate of smaller polypeptides since it is resistant to breakdown under harsh conditions which are known to disrupt such a structure.

Although the major subunit of Lepidurus hemoglobin seems to have a molecular weight of 34,000, two minor components of 62 and 64,000 molecular weight were present under all of the above conditions. One question one should ask is whether this material is part of the hemoglobin or some contaminant of the purification? Because these polypeptides do not dissociate under the drastic treatment to which they were subjected, they are most likely not dimeric aggregates of the 34,000 molecular weight material. However, it seems unlikely that these minor

components are non-heme contaminants for two reasons. First, contaminating native molecules of this size ($M_r = 62 - 64,000$) would be expected to separate from the native hemoglobin during the purification by gel column chromatography. Second, it also seems unlikely that the 62 - 64,000 molecular weight materials are subunits of a polymeric contaminant that coincidentally co-chromatographs with the hemoglobin as the purified hemoglobin appears to be quite homogeneous by isoelectric focusing. Therefore, it seems more probable that the 62 - 64,000 molecular weight material may be part of the hemoglobin molecule. Further evidence that this is so will be presented below.

Attempts to determine whether the 62 - 64,000 molecular weight material is associated with a heme moiety have proven unsuccessful. The hemoglobin so far has not displayed a dissociation behavior that allows the isolation of this material unless the molecule is first denatured.

It is not possible at this time to determine what relationship the 62 - 64,000 molecular weight material has to the 34,000 molecular weight subunit or how it might be involved in the quaternary structure of the intact pigment. The possibility that 34,000 molecular weight polypeptide is a degradation product of the 62 - 64,000 material caused by the purification or that both come from a larger polypeptide is also unlikely for several reasons. First, the

hemoglobin is removed quickly from the animal in a way that does not contaminate the hemolymph with any gut contents and placed directly into ice cold buffer containing the proteolytic blocking agent phenylmethanesulfonyl fluoride. Second, purified hemoglobin left standing at room temperature for 2.5 hours without phenylmethanesulfonyl fluoride has an identical electrophoretic pattern in sodium dodecyl sulfate as control hemoglobin held at 4° C. Third, hemoglobin removed directly from the animal and placed immediately into boiling sodium dodecyl sulfate incubation buffer with reducing agent shows the predominant 34,000 molecular weight subunit with a slight trace of the 62 - 64,000 molecular weight protein.

The 62 - 64,000 molecular weight material is present in only small quantities and is heterogeneous with respect to molecular weight based on sodium dodecyl sulfate gel electrophoresis. Results of urea gel electrophoresis show that the pigment contains one major polypeptide chains, presumably corresponding to the 34,000 molecular weight subunit, and two minor polypeptides, presumably the 62,000 and 64,000 molecular weight proteins. This interpretation is consistent with urea gel electrophoresis of 34,000 and 66,000 molecular weight fractions obtained by column chromatography in 6 M guanidine hydrochloride. Although the fractions are contaminated due to the

overlapping of the elution peaks, electrophoresis of the 66,000 molecular weight fraction shows an enhancement of the minor bands seen in electrophoresis of the whole pigment. Similarly, urea gel electrophoresis of the 34,000 molecular weight fraction from the guanidine column shows smaller amounts of the minor bands. These results imply that the 62 - 64,000 molecular weight material is heterogeneous with respect to charge as well as size and that the native hemoglobin molecule is composed predominantly of one kind of major subunit ($M_r = 34,000$) which is homogeneous by both criteria.

The smallest polypeptide that can be produced from Daphnia pulex hemoglobin using the conventional methods of protein denaturation described above for Lepidurus hemoglobin has a molecular weight of 31 - 37,000. These results agree well with those of Ilan and Daniel (1979a) for the hemoglobin of Daphnia sp. ($M_r = 31,000$) yet differ from that reported for the related cladoceran Moina macrocopa ($M_r = 23,000$) (Hoshi et al., 1976). Some variation in the molecular weight of the D. pulex subunit is seen in samples treated with 6 M guanidine hydrochloride or 8 M urea prior to sodium dodecyl sulfate gel electrophoresis. This may suggest that the subunits are slightly smaller than determined by standard sodium dodecyl sulfate electrophoresis or that some spurious

proteolysis has occurred. Nonetheless, under the harsh conditions used to dissociate and denature the molecule, it is only on overloaded gels that one sees traces of material with molecular weights lower than 30,000.

Some higher molecular weight protein ($M_r = 53 - 86,000$) is also detected in sodium dodecyl sulfate slab gel electrophoresis of the intact molecule. The amount of this material varies from one preparation to the next but it is always present in small amounts. It is not known whether it is part of the hemoglobin molecule. The 53 - 86,000 molecular weight protein is not detected in sodium dodecyl sulfate slab gel electrophoresis of hemoglobin which had been previously denatured in 6 M guanidine hydrochloride nor upon column chromatography of the dissociated molecule at high pH. This may imply that the material is an aggregate of lower molecular weight polypeptides which are resistant to sodium dodecyl sulfate denaturation alone.

The subunit of Daphnia pulex hemoglobin is heterogeneous both by sodium dodecyl sulfate gel electrophoresis and gel electrophoresis in 6.25 M urea. The heterogeneity is less, but is still present when the protein is treated with 8 M urea prior to sodium dodecyl sulfate gel electrophoresis. Heterogeneity in Daphnia pulex hemoglobin is in contrast to the data reported for Daphnia sp. (Ilan

and Daniel, 1979a) but can be explained in a number of ways. The polymer could consist of more than one kind of subunit; this subunit heterogeneity could then be detected by the sensitive slab gel method. There might also be hemoglobin heterogeneity within the Daphnia population, either by sex, age or subspecies. There is also the possibility that although an attempt was made to obviate proteolysis during purification it was still present. The results of studies of Moina hemoglobin probably illustrate the kinds of problems one can expect with spurious proteolysis. Hoshi et al. (1976) observed that after denaturation of the protein for 35 hours in sodium dodecyl sulfate at 37° C, the 23,000 molecular weight subunit became 11,700 and 8,900 molecular weight polypeptides. Some low molecular weight ($M_r = 14 - 18,000$) material is seen in samples of Daphnia pulex hemoglobin run on overloaded sodium dodecyl sulfate gels as well as in protein brought to high pH. It may be that the dissociation of the native pigment at high pH makes the protein more sensitive to proteolysis.

The subunits of Lepidurus and Daphnia hemoglobins are unusual in that they appear to be twice as large as the typical subunit of vertebrate hemoglobins and myoglobins. However, the occurrence of relatively large subunits may be characteristic of all the branchiopod hemoglobins.

Ilan and Daniel (1979a, 1979b) have recently reported that the hemoglobins of Lepidurus apus lubbocki and Daphnia sp. have subunits of 34,000 and 31,000 molecular weight, respectively. Also, although previous studies of hemoglobin from the clam shrimp Cyzicus hierosolymitanus suggested a monomeric subunit ($M_r = 15,000$) (David et al., 1977), re-examination of the pigment under conditions which attempt to prevent proteolysis show that the subunit molecular weight of Cyzicus hemoglobin is about 30,000 (Ilan and Daniel, 1979a). In addition, I have found that hemoglobin subunits from two other congeneric species of tadpole shrimp, Lepidurus couesi and L. lynchi as well as the clam shrimp Caenestheriella setosa also have molecular weights around 30 - 34,000. Artemia hemoglobin is probably the most unusual of these pigments in that it is thought to be composed of two polypeptides of approximately 125,000 molecular weight, each of which contains a number of hemes per polypeptide chain (Moens and Kondo, 1976, 1978). Under certain conditions of incubation in sodium dodecyl sulfate the 125,000 molecular weight subunit of Artemia hemoglobin dissociates or is cleaved by proteolysis into two polypeptides of 80,000 and 50,000 (Moens and Kondo, 1978). There is some controversy regarding the nature of this subunit, however. Bowen et al. (1976) have shown by sodium dodecyl sulfate gel

electrophoresis that Artemia hemoglobin consists of both 13 and 17,000 molecular weight subunits. The subunit molecular weight of Artemia hemoglobin determined in our laboratory is 125,000, in agreement with Moens and Kondo (1978). Although the 13 - 17,000 molecular weight subunits found by Bowen et al. (1976) agree with the heme content of the protein, it is possible that they represent proteolytic products of the incubation method employed.

Hemoglobin from another anostracan, Streptocephalus, may also be composed of two, perhaps, three, 100 - 120,000 molecular weight polypeptide chains (Ilan and Daniel, 1979a).

It is interesting to note that the branchiopod hemoglobins studied thus far seem to fall into two classes based on their molecular structure which are also in accordance with their taxonomic groupings. The first group, the hemoglobins of the branchiopods without carapaces (Anostraca), represented by Artemia and Streptocephalus, are dimeric or possibly, trimeric molecules composed of subunits which are long polypeptide chains and which may be associated with seven heme groups each. The second group, those branchiopods with carapaces (Notostraca, Diplostraca), represented by Lepidurus, Daphnia and Cyzicus possess polymeric hemoglobins composed of a large number of subunits which are

associated with two heme groups each. Thus, the molecular characteristics of the branchiopod crustacean hemoglobins appear to be consistent with traditional crustacean systematics which is based on external morphology.

One intriguing hypothesis that can be drawn from this information is that the hemoglobins of the closely related carapaced branchiopods may be various aggregation states of a common 220 - 270,000 molecular weight submultiple. For example, the conchostracans Cyzicus and Caenestheriella have molecular weights of 220 - 280,000 (Ar and Schejter, 1971; David et al., 1977). The present study and studies by other workers have shown that Daphnia pulex and Lepidurus hemoglobins have molecular weights of 420 - 470,000 and 700 - 800,000, respectively (Svedberg and Eriksson-Quensel, 1934; Ilan and Daniel, 1979b). If one assumes that the hypothetical submultiple has a molecular weight around 250 - 260,000, dimers and trimers of this unit would have molecular weights around 500 - 520,000 and 750 - 780,000 respectively. These values are in fair agreement with the intact molecular weights estimated for Cyzicus, Daphnia pulex and Lepidurus hemoglobins. Based on subunit molecular weights reported above one can further estimate from this model that one submultiple would contain approximately 8 polypeptide chains, a dimer of submultiples would have 16, and a

trimer approximately 24 and that each intact molecule would contain 16, 32 and 48 hemes, respectively. The similarity in the size of the subunits of these pigments as well as the different apparent aggregation states of Lepidurus bilobatus hemoglobin are consistent with this idea. Amino acid composition of these molecules (Table VI) are also very similar suggesting that there may be a high degree of homology in their primary and tertiary structures.

In this regard it is interesting to ask what relationship Artemia hemoglobin has to the other branchiopod hemoglobins. The native molecular weight of this pigment is very similar to that of Cyzicus hemoglobin ($M_r = 250,000$). Yet the subunit of Artemia hemoglobin is almost four times the size of other branchiopod hemoglobin subunits which implies that this molecule has a different quaternary structure. However, similarities in amino acid composition between Artemia hemoglobin and other branchiopod hemoglobins (Table VI) suggests that these pigments are closely related. Amino acid sequence data should help explain the relationship between the branchiopod hemoglobins.

The presence of one mole heme per 18 - 20,000 grams of Lepidurus and Daphnia hemoglobins suggests that the 34,000 molecular weight subunits of these molecules are associated with two hemes per polypeptide chain. This

Table VI. Amino acid compositions of Branchiopod hemoglobins

	<u>Lepidurus</u> <u>lynchi</u>	<u>L. apus</u> ²	<u>Triops</u> ¹	<u>Cyzicus</u> ³	<u>Caenestheriella</u> <u>setosa</u>	<u>Artemia</u> ⁴ <u>Hb-II</u>
Lys	4.3	4.5	4.4	5.1	5.7	6.3
His	3.3	2.5	1.9	3.9	4.4	3.0
Arg	4.9	5.0	5.0	3.4	3.5	3.2
Asp	10.9	10.6	11.9	13.2	12.3	12.6
Thr	6.3	6.6	6.9	5.1	4.9	4.9
Ser	6.7	7.2	6.9	5.2	4.4	5.0
Glu	10.4	10.3	11.3	10.4	10.4	10.0
Pro	2.4	2.2	3.1	2.9	3.6	2.8
Gly	7.4	8.8	8.8	6.8	6.8	6.5
Ala	12.4	11.7	12.6	9.9	11.7	9.5
½ Cys	-	0.0	0.6	0.0	-	0.0
Val	8.5	7.6	6.9	9.6	9.4	9.2
Met	0.8	0.7	0.0	0.6	0.8	0.6
Ileu	4.0	3.8	3.8	3.7	3.7	3.5
Leu	12.4	11.8	10.1	12.1	12.1	11.5
Tyr	0.9	1.5	1.3	1.5	1.1	1.4
Phe	4.4	4.3	3.8	5.4	5.2	5.2
Trp	-	0.8	0.6	1.3	-	1.2

1 Horne and Beyenbach, 1974

2 Ilan and Daniel, 1979b

3 Ar and Schejter, 1970

4 Bowen et al., 1976

is in contrast to most other hemoglobins whose smallest subunit molecular weights are 15 - 17,000 and which contain one heme per subunit. One hypothesis that is consistent with this finding is that the smallest polypeptide chains of Lepidurus and Daphnia hemoglobins consist of two covalently linked 15 - 17,000 molecular weight heme-containing units. In addition, these structural units might be arranged into compact myoglobin-like tertiary structures or structural domains which are connected by more exposed regions of polypeptide.

This hypothesis is supported by the experiments in which the hemoglobins are digested with the enzyme subtilisin. Under these conditions the pigments are cleaved into polypeptides with molecular weights of approximately 17,000 as determined by sodium dodecyl sulfate gel electrophoresis. The molecular weight of these digest products are similar to those of the typical myoglobin molecule. Furthermore, the domains must form compact tertiary structures which are resistant to the digestion or are digested at a slow rate. If this were not the case, subtilisin digestion should result in major peptides which are not integral multiples of 17,000 molecular weight. The regions of polypeptide connecting the domains, on the other hand appear to be more accessible to the protease. Furthermore, the lack of greater heterogeneity of the digest fragments,

especially of Lepidurus hemoglobin, might suggest that the polypeptide linker connecting the two domains is relatively short. If it were long, non-specific cleavages might produce much greater heterogeneity with respect to molecular weight.

Digestion fragments, of these two hemoglobins have been isolated by gel column chromatography on Sephacryl S-200. In both Lepidurus and Daphnia the ratio of protein to heme in the digestion fractions is similar to that in the intact hemoglobins. This suggests that the fractions contain one heme per 18,000 gram protein. Oxygen equilibrium studies show that the digestion products of Lepidurus hemoglobin isolated by chromatography bind oxygen reversibly and are, therefore, functional as well as structural entities. However, the fragments have different oxygen binding characteristics than the intact hemoglobin. The intact pigment has a relatively low affinity for oxygen and exhibits a slight Bohr effect and significant cooperativity. In contrast, the oxygen affinity of the isolated domains is relatively high and the Bohr effect, present in the intact molecule, is lacking.

The oxygen binding characteristics displayed by the 14 - 16,000 molecular weight domains isolated from Lepidurus hemoglobin are similar in some ways to those one might expect for a myoglobin-like molecule. Human

myoglobin (Rossi-Fanelli and Antonini, 1958) and the myoglobins of some annelids (Weber and Pauptit, 1972; Garlick and Terwilliger, 1977) also show high oxygen affinities and lack the Bohr effect. Similarly, the monomeric extracellular insect hemoglobins (Wollmer et al., 1972) share these myoglobin-like characteristics. In contrast to myoglobins and monomeric hemoglobins, the putative monodomains from Lepidurus hemoglobin, at times, show an unusually high degree of cooperativity. Hill coefficients (h) range from 1.7 - 3.3. One possible explanation for this unusual cooperativity is that the monodomains undergo a ligand linked association at low oxygen tensions. This behavior has been shown in the monomeric lamprey hemoglobins (Briehl, 1963; Anderson and Gibson, 1971). However, association of the domain fraction has not been demonstrated by column chromatography of the deoxy-pigment and argues against unusual ligand-linked aggregation states. Another explanation for the cooperative behavior of this domain is that varying amounts of partially digested 30,000 molecular weight material may cause the mixture of digestion fragments to assume a state of partial association which may also be reflected in the relatively high molecular weights obtained occasionally by column chromatography for the monodomain fractions. It should be pointed out also that caution should be

taken in analyzing single tonometer determinations of oxygen equilibrium characteristics of high affinity pigments. One can conclude, however, that the native molecule seems to be necessary for the expression of its heterotropic interactions and relatively low oxygen affinity.

Unusually large subunits constructed of putative oxygen binding domains have been found in the extracellular molluscan hemoglobins of the planorbid snails Helisoma and Planorbis (Terwilliger, N.B. et al., 1976; Terwilliger et al., 1977b; Wood and Gullick, 1979). Helisoma hemoglobin is a polymeric molecule with an apparent molecular weight of 1.7×10^6 . Although the pigment contains one mole heme per 18 - 19,000 grams protein the molecule cannot be dissociated into polypeptide chains smaller than 175 - 200,000 molecular weight in 6 M guanidine hydrochloride in the presence of reducing agent or by sodium dodecyl sulfate gel electrophoresis of reduced and carboxymethylated globin. Smaller heme-containing polypeptides (domains) of approximately 15 - 17,000 molecular weight and multiples thereof can be isolated, however, by gentle proteolysis of the pigment with the protease subtilisin (Terwilliger et al., 1977b). These fragments have been shown to bind oxygen reversibly. Similar to Lepidurus domains, however, the domains from Helisoma hemoglobin have oxygen equilibrium properties

different from those of the undigested pigment. The Bohr effect, low affinity and cooperativity present in the intact molecule are lacking in the isolated mono- and polydomains of Helisoma.

A model similar to that proposed for Helisoma has also been suggested for the extracellular hemoglobins which are found in the clams Cardita and Astarte (Terwilliger et al., 1978; Terwilliger and Terwilliger, 1978; Terwilliger, 1980). The subunit of Cardita hemoglobin under denaturing conditions is a large polypeptide with a molecular weight of 290 - 300,000. Cardita hemoglobin, like that of Helisoma and most other animals, contains one mole heme per 16 - 22,000 grams protein. Digestion of dissociated Cardita hemoglobin with subtilisin results in subunits of 15 - 17,000 molecular weight and integrals of this value (Terwilliger and Terwilliger, 1978). The digestion products from this molluscan hemoglobin also contain heme and bind oxygen reversibly.

Interestingly, a similar model has been proposed for the structural organization of the subunits of the hemocyanin from the Roman snail Helix pomatia (Brouwer and Kuiper, 1972; Lontie et al., 1973; Gielens et al., 1975; Brouwer et al., 1976, 1979) and other molluscan hemocyanins (Wood, 1976; Gullick and Wood, 1978). Based on copper content, the molecular weight of the minimal

functional unit of molluscan hemocyanin is 50,000. However, the subunit of these copper-containing non-heme respiratory proteins are large polypeptides with molecular weights between 250 - 400,000 (Brouwer and Kuiper, 1973). They can be digested by gentle proteolysis with subtilisin and trypsin into copper-containing oxygen binding domains whose molecular weights are about 50,000 and multiples thereof. It is concluded from these experiments that the subunit of Helix hemocyanin consists of a series of seven or eight of these 50,000 molecular weight units arranged in a covalently linked, linear array of structural and functional domains. This interpretation is supported by electron micrographs of putative molluscan subunits which show a linear chain of seven or eight globular units ("string of pearls") linked by exposed stretches of polypeptide (Siezen and Van Bruggen, 1974). If the proposed structure for crustacean and molluscan extracellular hemoglobins is correct, the subunit of these heme-containing pigments could share a similar pattern of serially linked units with the copper-containing molluscan hemocyanins.

One question of interest regarding the multidomain molecules is how similar are the putative domains of a molecule to one another with respect to primary sequence? To begin answering this question, further structural

studies were undertaken on Lepidurus hemoglobin. Two approaches have been utilized: 1. peptide mapping of the intact hemoglobin and purified subunits and 2. digestion experiments designed to isolate the purified domains. Unfortunately it is not possible at this time to tell, conclusively, whether or not the putative domains are structurally alike. However, the results from these experiments imply that the domains may be dissimilar.

Peptide maps of Lepidurus globin containing both the major 34,000 molecular weight subunit and the two minor 62 - 64,000 molecular weight components exhibit a pattern of approximately 45 - 50 peptide spots (Fig. 19). This peptide pattern is unaltered by increasing the length of the digestion period or the ratio of enzyme to protein. Similarly, globin which has been carboxymethylated in order to obviate artifactual spots caused by spurious disulphide linkages has a nearly identical peptide pattern to that shown in Fig. 19. This large number of spots is three times the number of spots that would be predicted based on the lysine and arginine content of the intact hemoglobin per minimum molecular weight of 17,000. Theoretically, the number of peptides that would be predicted from a tryptic digest is equal to the total number of lysyl and arginyl residues present in the molecule plus one. Therefore, based on the lysine

and arginine content of Lepidurus hemoglobin, a homogeneous 34,000 molecular weight polypeptide chain composed of two identical or nearly identical, repeated sequences would have approximately 14 - 15 tryptic peptides. Similarly, a subunit made up of two non-identical, tandemly linked sequences would have approximately twice the number of peptides (30) predicted by the amino acid composition. The number of tryptic peptides experimentally detected for Lepidurus hemoglobin is larger than the number of spots predicted for a homogeneous subunit made up of two domains of similar sequence or totally dissimilar sequences. These results are presently puzzling. However, attempts to minimize the number of tryptic peptides by carefully excluding contaminating proteins have proven unsuccessful in altering the pattern. Peptide maps of purified and alkylated 34,000 molecular weight subunits from column chromatography in 6 M guanidine hydrochloride show nearly identical patterns to maps of the whole molecule as well as the 62 - 64,000 molecular weight fraction. Thus, it seems unlikely that the presence of the 62 - 64,000 molecular weight material during mapping of the intact hemoglobin can explain the large number of spots. Therefore, the presence of 45 - 50 spots from tryptic digests of Lepidurus hemoglobin suggests that the putative domains comprising the major subunit of this pigment are dissimilar

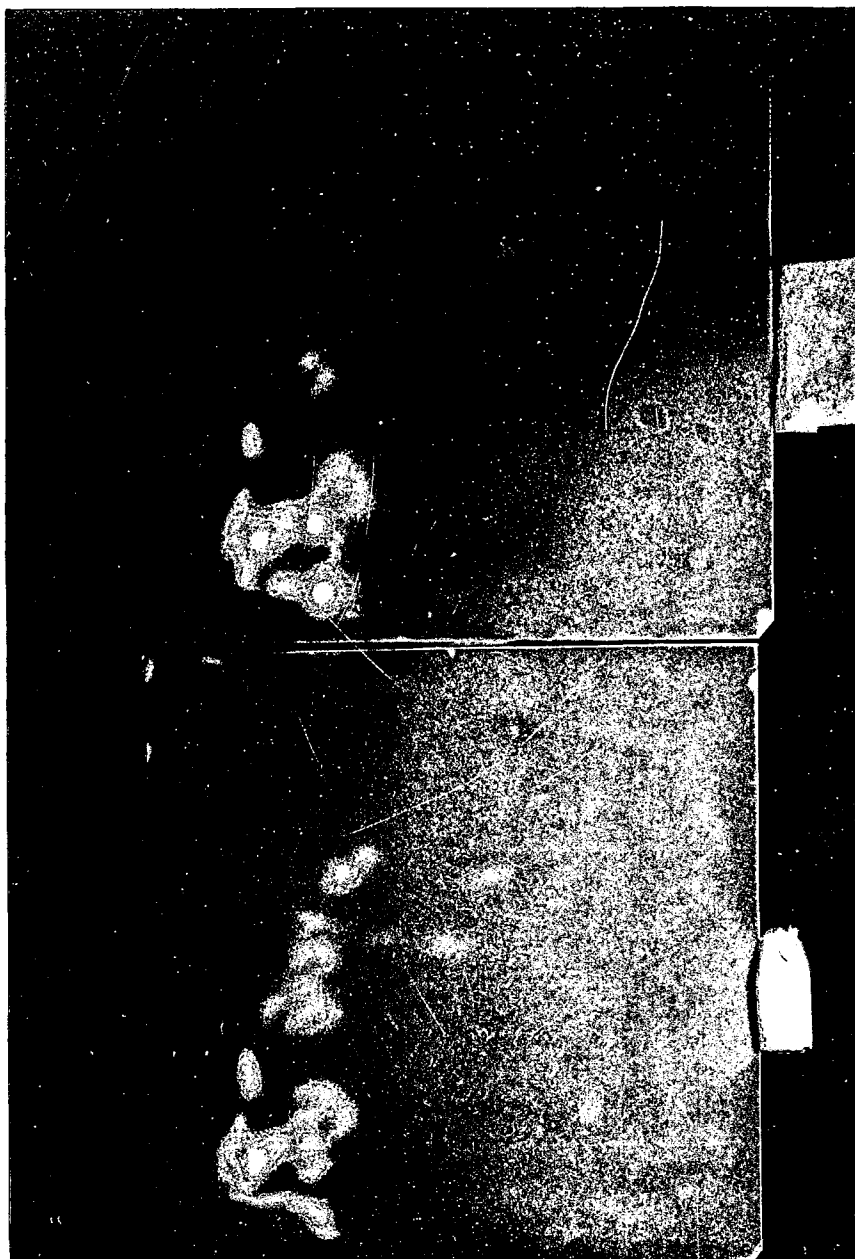
in primary sequence. Alternatively, there could be some, as yet, undetected heterogeneity of the 34,000 molecular weight subunit. Although there are arguments against this, it should be pointed out that one interpretation of the results of cyanogen bromide cleavage of Lepidurus hemoglobin may suggest the possibility of heterogeneity among the polypeptide chains. Further studies will be needed to resolve this dilemma.

In pursuing studies to further characterize the subunit structure of Lepidurus hemoglobin, an attempt has been made at isolating a purified domain. Hemoglobin which has been digested with the enzyme subtilisin has been shown to be cleaved into two heme-containing units of approximately 14 - 16,000 molecular weight as determined by sodium dodecyl sulfate gel electrophoresis and gel column chromatography. However, chromatography of the carbonmonoxide treated digestion material on DEAE Sephadex A50 results in the separation of three heme-containing fractions (Fig. 15). Analysis of the three fractions by sodium dodecyl sulfate slab gel electrophoresis and urea gel electrophoresis has shown that Fraction A from the DEAE Sephadex column is heterogeneous with respect to molecular weight and charge. However, Fractions B and C are homogeneous with respect to molecular weight and nearly homogeneous with respect to

charge. Co-electrophoresis of Fractions B and C on sodium dodecyl sulfate slab gel and urea tube gels shows that these materials have very similar electrophoretic mobilities under these conditions. These similarities are reflected in peptide maps of the individuals fragments (Fig. 29). Tryptic peptide maps of Fractions B and C exhibit similar patterns of approximately 23 - 25 spots. This large number of spots is approximately one and half times the number of spots that would be predicted based on the lysine and arginine content of the combined digestion fragments (Table III) per minimum molecular weight of 15,000. This large number of spots is puzzling. However, it seems to be consistent with the peptide maps of the whole hemoglobin. The slight heterogeneity seen in urea gels of these fractions may account for some of the spots seen on the maps. However, the small quantities of these contaminants makes this hypothesis unlikely.

The notostracan branchiopods (tadpole shrimps) live in temporary or semi-permanent brackish water ponds in arid regions of the world where water is often at a premium. Desert pools are often ephemeral in nature and, consequently, may exhibit substantial seasonal variation in their chemical and physical parameters. These parameters include salinity (conductivity) and pH as well as diurnal fluctuations in temperature and oxygen

Figure 29 Tryptic peptide maps of DEAE Sephadex Fractions B (left) and C (right). Enzyme:protein ratio, 1:100, 24 hours, 25° C. Digestion and mapping of fractions performed simultaneously and under identical conditions.



concentration (Horne, 1971). Because tadpole shrimps (and other branchiopod crustaceans) experience large variations in their environment, the physiological value of their hemoglobins and the parameters affecting hemoglobin function are of interest. Oxygen equilibrium studies have been performed on Lepidurus bilobatus hemoglobin in an attempt to help determine the physiological value of this respiratory pigment to the shrimp.

Lepidurus hemoglobin has a relatively low oxygen affinity compared to other branchiopod extracellular hemoglobins (Table VII). When tested in 0.05 M Tris-HCl buffer (pH 7.2), 0.1 M NaCl, 0.01 M MgCl₂ at 20° C the pigment has a P_{1/2} of approximately 20 mm Hg and a Hill coefficient, h, of 2.0. In contrast, another tadpole shrimp, Triops, has a P_{1/2} of 6.8 mm Hg and a Hill factor of 1.5 at pH 7.2 (23° C) (Horne and Beyenbach, 1971). Diplostracan hemoglobins also have high oxygen affinities. Daphnia magna hemoglobin has a P_{1/2} equal to 3.1 mm Hg at pH 7.7 (17° C) (Fox, 1945) and hemoglobin from the clam shrimp, Cyzicus, has a P_{1/2} equal to 0.035 mm Hg (Ar and Schejter, 1970).

The differences in oxygen affinity are striking. However, the differences in oxygen affinity of these hemoglobins from related animals may correlate with differences in their habitats and habits. Triops is

Table VII Functional Characteristics of Branchiopod Hemoglobins*

	P_{50}	h	Conditions under which P_{50} and h were measured	ϕ	ΔH	Reference
<u>Anostraca</u>						
<u>Artemia salina</u>						
Hb-I	6.5	1.81	0.1M Borate buffer (pH 8.5) 0.6M NaCl, 0.02M KCl, 25° C			Van den Branden <u>et al.</u> , 1978
Hb-II	3.99	2.0				
Hb-III	1.83	2.05				
<u>Artemia salina</u>						
Hb-I	5.34	1.62	0.1M Borate buffer (pH 8.5) 0.6M NaCl, 0.02M KCl, 25° C	-0.09 (pH 8-9)	-10.8**	D'Hondt <u>et al.</u> , 1978
Hb-II	3.7	1.92		-0.21 (pH 8-9)	-12.0**	
Hb-III	1.8	1.58		-0.03 (pH 8-9)	-5.4**	
<u>Notostraca</u>						
<u>Triops longicaudata</u>	6.8	1.5	0.1M Phosphate buffer 0.1M NaCl, 23° C	-0.23	-9.2**	Horne and Beyenbach 1971
<u>Lepidurus bilobatus</u>	20	2.0	0.05M Tris HCl (pH 7.2)	-0.13 (pH 6.8-7.6)	-7.4	Present study
<u>Lepidurus lynchi</u>	21.4	3.1	0.04 M Tris HCl (pH 7.9) 0.1M NaCl, 0.01M MgCl ₂ 21° C			

Table VII Continued. Functional Characteristics of Branchiopod Hemoglobins*

	$P_{1/2}$	h	Conditions under which $P_{1/2}$ and h were measured	ϕ	ΔH	Reference
<u>Diplostraca</u>						
<u>Cyzicus hierosolymitanus</u>	0.035	2.3	0.01M Tris maleate buffer (pH 7.2), 28 ^o C			Ar and Schejter, 1970
<u>Daphnia magna</u>	3.1 4.9		pH 7.7, 17 ^o C 17 ^o C, 1% carbon dioxide			Fox, 1945
<u>Daphnia Magna</u>	3.5		0.1M Phosphate buffer (pH 7.2), 20 ^o C	0.0		Sugano and Hoshi, 1971
<u>Ceriodaphnia</u>	0.8		pH 7.7, 17 ^o C			Fox, 1945
<u>Moina macrocopa</u>	2.1	2.7**	0.1M Phosphate buffer (pH 7.2), 20 ^o C	0.0	-16.0**	Sugano and Hoshi, 1971; Hoshi, <u>et al.</u> , 1968

* $P_{1/2}$, Half-saturation oxygen tension; h, Hill's cooperativity coefficient; ϕ Bohr factor; ΔH , overall heat of oxygenation, in Kcal/mole.

** Values calculated from data by author.

found in shallow ephemeral ponds which may experience high temperatures and very low oxygen concentrations (Horne, 1971). A pigment with a relatively high affinity for oxygen may be useful under these stressful conditions. Lepidurus may also occur in temporary ponds but there seems to be a tendency for most members of this genus to live in waters which dry out less regularly than the Triops pools (Longhurst, 1955). These differences in the size of the ponds may be reflected in the oxygen concentration of the water. Oxygen concentrations measured by the author at St. Patrick's water hole (Lake County, Oregon) show that the pools are nearly saturated with oxygen throughout the season the animals are present in the ponds. Along these lines, Ceriodaphnia generally lives in fouler water than Daphnia magna and has a hemoglobin with a higher oxygen affinity (Fox 1945). Similarly, the very high affinity of Cyzicus hemoglobin may be correlated with its habit of foraging for food in the anoxic mud at the bottom of the ponds in which it lives.

Another possible explanation for the differences in oxygen affinity of Lepidurus hemoglobin and the diplostracan hemoglobins may reflect differences in structural morphology of these two groups. Cladoceran and conchostracan gills are held within the tightly fitting carapace possibly restricting ventilation. Clam shrimp are

frequently found lying on the bottom of the pond with their carapaces closed. Lepidurus gills, on the other hand, are continually exposed to the oxygenated water. Measurement of oxygen content within the carapace of these small animals are not available. However, Fox (1948) has suggested that the oxygen tension within the carapace of Daphnia may be lower than the oxygen content of the surrounding medium.

Lepidurus hemoglobin possesses a small, normal Bohr effect over the pH range 6.8 to 7.6 with ϕ equal to -0.13. This value is slightly lower than values reported for other branchiopod hemoglobins. Artemia salina Hb-II has a normal Bohr effect over the pH range 8.0 - 9.0 with a ϕ value of -0.21 (D'Hondt et al., 1978). Triops hemoglobin displays a normal Bohr effect with ϕ equal to -0.23 to -0.31 over the pH range 6.2 - 8.1 (Horne and Beyenbach, 1971). Traditionally, the Bohr effect has been understood as an increase in deoxygenation of the pigment at the target organ as effected by the pH of the blood. The Bohr shift in the polychaete annelid Arenicola cristata ($\phi = -0.3$ to -0.4) is responsible for about 13% of the oxygen transported by the hemoglobin (Mangum, 1976). It seems unlikely that the slight Bohr effect exhibited by Lepidurus hemoglobin is responsible for a large proportion of the oxygen delivered to the tissues. This could not be known

until blood PO_2 's are measured. However, the relatively small sensitivity to pH seen in the tadpole shrimp hemoglobin may be adaptive in allowing loading of this relatively low affinity pigment if the ponds in which the animal lives become more acidic due to the breakdown of organic wastes in the water. Interestingly, most of the diplostracan branchiopod hemoglobins which have been studied lack a Bohr effect (Fox, 1945; Sugano and Hoshi, 1971). On the other hand, Daphnia magna hemoglobin has been shown to shift its binding curve to the right (indicating a lower affinity) in the presence of 1% carbon dioxide (Fox, 1945). The importance of the Bohr effect in Lepidurus hemoglobin and the apparent lack of a Bohr shift in some diplostracan hemoglobins is an intriguing question.

Hemoglobin from Lepidurus displays a decrease in oxygen affinity with a corresponding change in the shape of the equilibrium curve, as the temperature is increased. The heat of oxygenation for Lepidurus hemoglobin is -7.4 Kcal/mole over the temperature range $10^\circ - 25^\circ$ C. This relatively low sensitivity to temperature is similar to that shown by Triops hemoglobin as calculated from Horne and Beyenbach's data ($\Delta H = -9.2$ Kcal/mole) and may be useful in allowing the hemoglobin to saturate with oxygen during periods when the animal is in shallow, warm water.

The presence of hemoglobin in the blood of Lepidurus suggests a respiratory function. Functional studies of other branchiopod hemoglobins have shown them to appear capable of transporting oxygen from the body surfaces to the tissues over a range of oxygen partial pressures. Gilchrist (1954) and Hoshi and Shimada (1965) have presented direct evidence for oxygen transport in Artemia and Moina, respectively. The hemoglobin of Triops has been shown to be valuable in transporting oxygen in larger animals although the presence of hemoglobin does not seem to be necessary for survival (Horne and Beyenbach, 1971). Large animals (>1g) whose hemoglobin had been blocked with carbon monoxide showed reduced metabolic rates. Metabolic rates of smaller shrimps (0.1 - 0.2 g), however, seemed unaffected by the carbon monoxide. Horne and Beyenbach (1971) feel that, perhaps, the smaller surface to volume ratio of the larger Triops places a premium on the hemoglobin, whereas the smaller shrimps may rely more on diffusion pressures and efficient circulation. Obviously, further studies need to be performed in order to understand the function of the hemoglobin of these fascinating animals.

CHAPTER 5

SUMMARY OF FINDINGS

Lepidurus bilobatus and Daphnia pulex hemoglobins are large polymeric aggregates with apparent molecular weights of 680 - 800,000 and 430 - 470,000, respectively. Although both pigments have minimum molecular weights of 18 - 20,000 based on heme content, the smallest polypeptide chains that can be isolated under a variety of harsh denaturing conditions have molecular weights of approximately 34,000. This suggests that the subunits of Lepidurus and Daphnia hemoglobins are associated with two heme groups. Digestion experiments have shown that the subunits of Lepidurus and Daphnia hemoglobins can be cleaved into smaller polypeptides of approximately 18,000 molecular weight. The digested polypeptides have been isolated by column chromatography and have been shown to bind reversibly suggesting that they are functional as well as structural units (domains). Oxygen equilibrium experiments performed on the intact Lepidurus hemoglobin show that the pigment has a relatively low oxygen affinity and possesses a slight Bohr effect. However, oxygen binding studies performed on the isolated domains show

that this material has very high oxygen affinity and lacks a Bohr effect, similar to many monomeric myoglobins and hemoglobins. Tryptic peptide maps of Lepidurus hemoglobin suggest that the two putative domains are dissimilar in primary sequence. The presence of some 62 - 64,000 molecular weight material is detected under all conditions of denaturation tested. It is likely that this material is a part of the hemoglobin molecule. A partially purified domain has been isolated. Peptide maps of this material contain a larger number of spots than is predicted from amino acid composition.

CHAPTER 6

APPENDIX

STUDIES OF LEPIDURUS LYNCHI HEMOGLOBIN

MATERIALS AND METHODS

Lepidurus lynchi was collected from Greaser reservoir in Lake County, Oregon during the summer of 1979 and identified according to Linder (1952). The animals were transported in chilled, aerated containers to the Oregon Institute of Marine Biology where they were bled immediately. Extraction, purification, and analysis of hemoglobin were performed as previously described for Lepidurus bilobatus. All procedures were carried out in the cold (4° C) and in the presence of phenylmethylsulfonyl fluoride when appropriate.

RESULTS

Lepidurus lynchi oxy-hemoglobin elutes from Bio Gel A5M equilibrated in 0.05 M Tris HCl (pH 8.0), 0.1 M NaCl, 0.01 M MgCl₂ as a single symmetric peak with an

apparent molecular weight of 800,000. Isoelectric focused carbonmonoxy-hemoglobin shows that the pigment thus purified is homogeneous.

Sodium dodecyl sulfate slab gel electrophoresis of performic acid oxidized globin in the presence of 2-mercaptoethanol resolves one major band with a molecular weight of 34,000 and two minor bands with molecular weights of 62 and 64,000 (Fig. 30b).

Urea gel electrophoresis in tubes (5% acrylamide) shows one major band and one minor band (Fig. 30c). The major band may be a closely spaced doublet. The 280/540 ratio of purified Lepidurus lynchi oxyhemoglobin is similar to that of L. bilobatus suggesting that the molecule contains mole heme per 18,000 gram protein.

A single oxygen equilibrium experiment was performed in the presence of the reductase system (Hayashi et al., 1973) in 0.05 M Tris HCl (pH 7.9), 0.1 M NaCl, 0.01 M MgCl₂. L. lynchi hemoglobin has a P_{1/2} of approximately 21 mm Hg with significant cooperativity, h = 3, at 20° C.

The amino acid composition of the purified hemoglobin is reported in Table VI. Absorption maxima for L. lynchi hemoglobin are reported in Table VIII.

Figure 30 Electrophoretic analysis of Caenestheriella setosa and Lepidurus lynchi hemoglobins. A and B: Sodium dodecyl sulfate slab gel electrophoresis. 12.5% acrylamide. A. Caenestheriella setosa. B. Lepidurus bilobatus. C and D: Urea gel electrophoresis. 5% acrylamide. C. Lepidurus lynchi. D. Caenestheriella setosa.

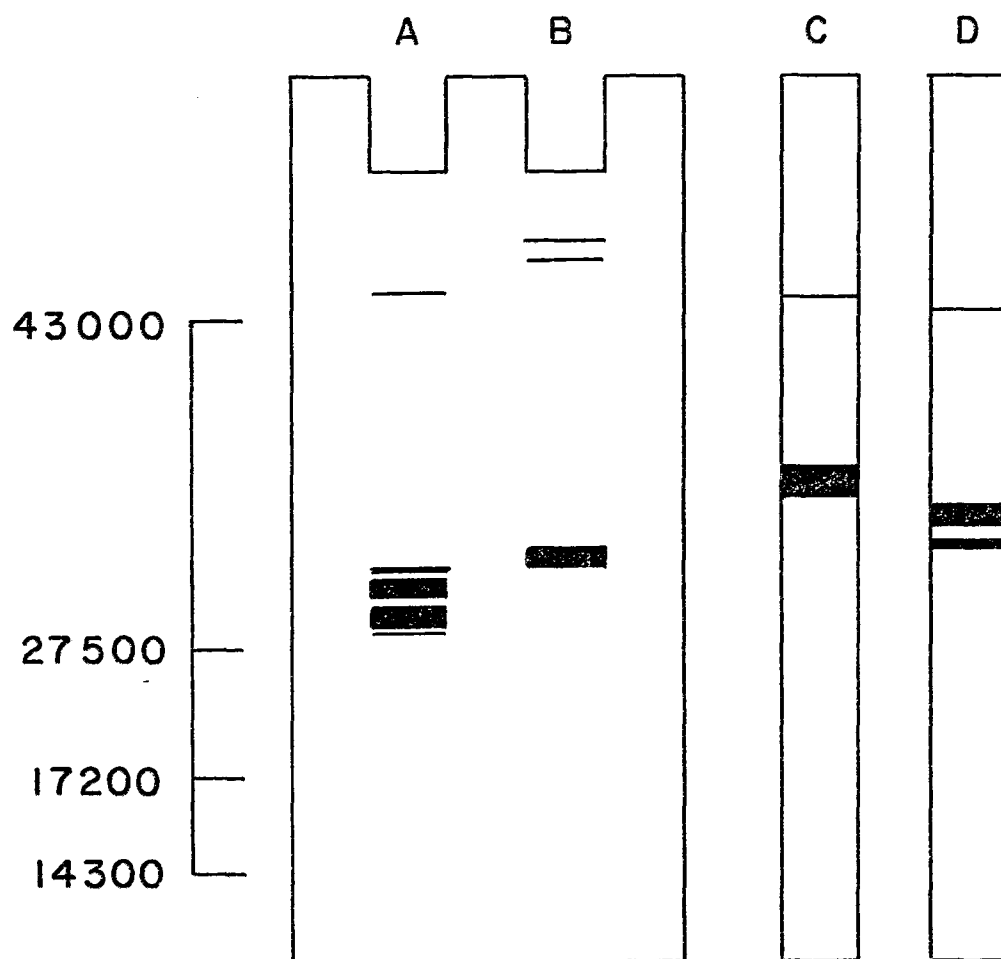


Table VIII Absorption maxima

	<u>Oxy Hb</u>			<u>Deoxy Hb</u>		<u>CO Hb</u>		<u>Cyanmet Hb</u>
<u>Lepidurus bilobatus</u>	414	542	578 560		567		540 571 557	539
<u>Lepidurus lynchi</u>	415	540	573 560	430	558	422	539 566 558	
<u>Daphnia pulex</u>							538 568	
<u>Caenestheriella setosa</u>	415	539	572 558	426	555	420	538 566 556	

STUDIES OF CAENESTHERIELLA SETOSA HEMOGLOBIN

MATERIALS AND METHODS

Caenestheriella setosa was collected at St. Patrick's water hole, Lake County, Oregon during the summer 1979 and identified according to Pennak (1978). The animals were transported to the Oregon Institute of Marine Biology as described for L. lynchi. Hemolymph was extracted from individual animals and pooled. Before hemolymph extraction, the animals were washed by allowing them to swim in a beaker of distilled water. Individual C. setosa were then dried and placed under a dissecting scope. The valves were forced open and the hemolymph was drawn into a capillary pipette. The hemolymph was immediately collected in a chilled beaker containing 0.05 M Tris HCl (pH 8.0), 0.1 M NaCl, 0.01 M MgCl₂, and 1 mM phenylmethylsulfonyl fluoride. The solution was bubbled with carbon monoxide and centrifuged at 12,000 g for 10 minutes before the supernatant was applied to a column of Bio Gel A1.5M (1 x 60 cm) in equilibrium with the extraction buffer. All analyses of C. setosa were performed as previously described for L. bilobatus.

C. setosa hemoglobin which had previously been dialyzed against 0.04 M sodium glycine buffer (pH 10.5),

0.01 M EDTA (25° C) was digested with the enzyme subtilisin and analysed by sodium dodecyl sulfate slab gel electrophoresis (15% acrylamide). Enzyme to protein ratios of 1/1000, 1/500, 1/250, 1/100, and 1/50 were used. Digestions were stopped by the addition of excess phenylmethylsulfonyl fluoride.

RESULTS

Caenestheriella setosa carbonmonoxyhemoglobin elutes from Bio Gel A1.5M at pH 8.0 in the presence of 0.01 M MgCl₂ with an elution volume similar to Artemia salina carbonmonoxyhemoglobin ($M_r = 250,000$; (Moens and Kondo, 1978) (Figure 31). Sedimentation velocity experiments performed on the carbonmonoxyhemoglobin at pH 7.0 and 8.0 in the presence of 0.01 M MgCl₂ show that the protein has a sedimentation coefficient (S) equal to 11.3 - 11.8.

A single isoelectric focusing experiment (5% acrylamide) performed for 24 hours (4° C) shows that the carbonmonoxyhemoglobin is composed of a major and a minor component. The major and minor components have pI equal to 5.2 and 4.8, respectively.

Sodium dodecyl sulfate slab gel electrophoresis (12.5% acrylamide) of the pigment in the presence of 2-mercaptoethanol shows two major bands with molecular

weights equal to 31,100 and 29,500 and three minor bands with molecular weights equal to 49,900, 32,000, and 28,400 (Figure 30a). There is no trace of lower molecular weight material. A similar pattern was obtained for unpurified hemoglobin removed from the animal and immediately placed in boiling sodium dodecyl sulfate incubation buffer with 2-mercaptoethanol. In addition, an aliquot of unpurified hemoglobin was allowed to stand at room temperature for 2 hours prior to incubation in sodium dodecyl sulfate. No change in electrophoretic pattern was detected. Electrophoresis of purified C. setosa hemoglobin in sodium dodecyl sulfate, 8 M urea, in the presence of 2-mercaptoethanol shows two major bands with molecular weights equal to 28,000 and 29,000 and a trace band with a molecular weight of 49,500.

Polyacrylamide tube gel electrophoresis (5% acrylamide) in the presence of 6.25 M urea (pH 2.2) of purified hemoglobin resolves one major, one minor, and one trace band (Figure 30d). There is one mole heme per 22,700 grams protein based on an extinction coefficient for Caenestheriella setosa hemoglobin $\epsilon_{280}^{280} = 1.87$. Absorption maxima are presented in Table VIII. The amino acid composition of the purified hemoglobin is presented in Table VI.

Sodium dodecyl sulfate slab gel electrophoresis of

a one hour digest of C. setosa carbonmonoxyhemoglobin at pH 10.5 (0.01 M EDTA) resolves two major bands with molecular weights of 14 - 16,000 and several minor bands corresponding to the intact subunits (Figure 32c). The amount of this relatively low molecular weight material is dependent on the enzyme to protein ratio used in the digestion experiment. Control hemoglobin which had been incubated for one hour without subtilisin showed an electrophoretic pattern similar to intact hemoglobin indicating that no spurious proteolysis of the molecule had occurred.

Figure 31 Bio Gel A1.5M column chromatography of Caenestheriella setosa hemoglobin. ▲ C. setosa carbonmonoxyhemoglobin. -○- Lepidurus bilobatus oxyhemoglobin. -□- Artemia salina carbonmonoxyhemoglobin. All absorbances at 540 nm. Column volume 1 x 60 cm.

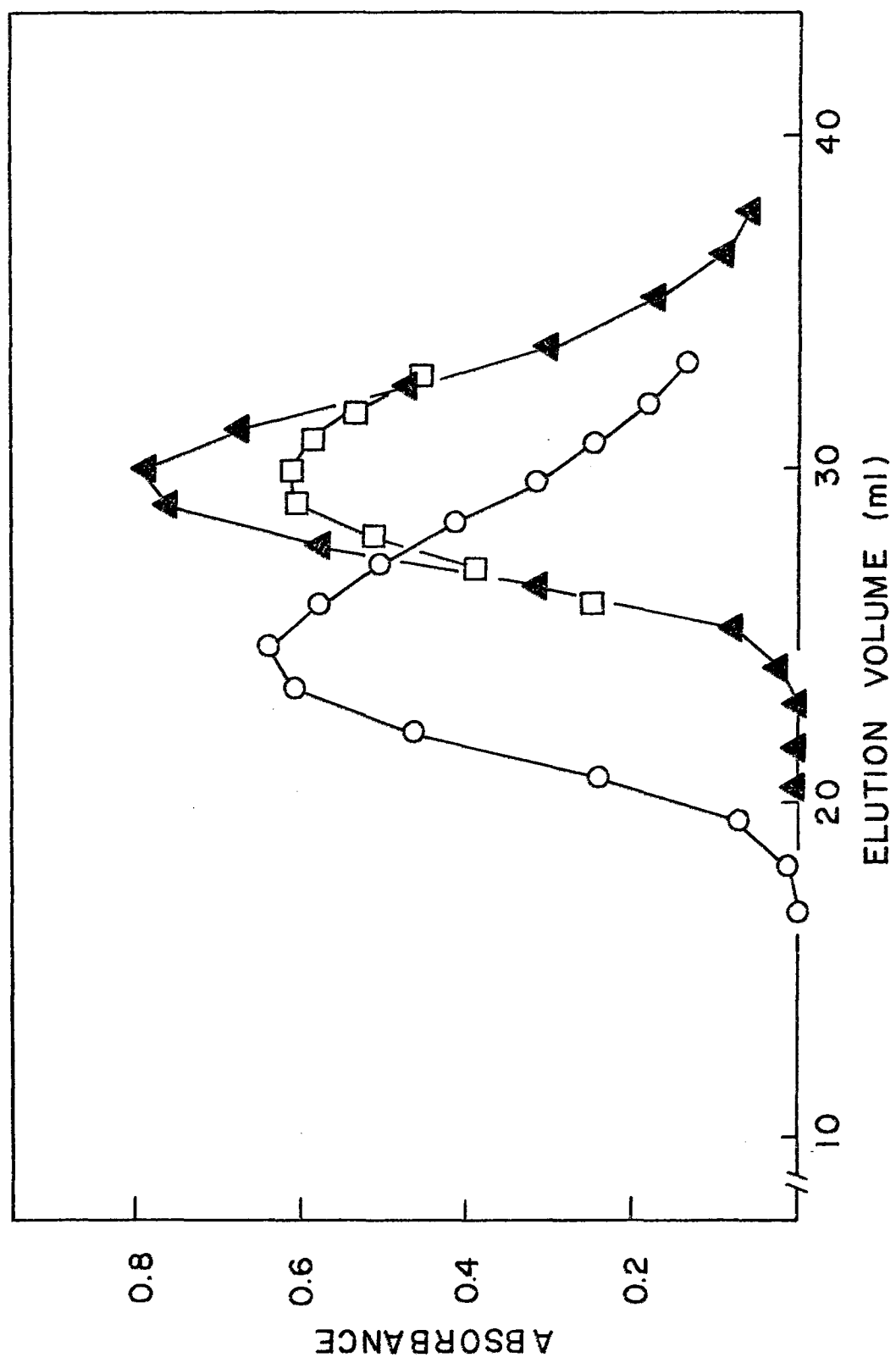
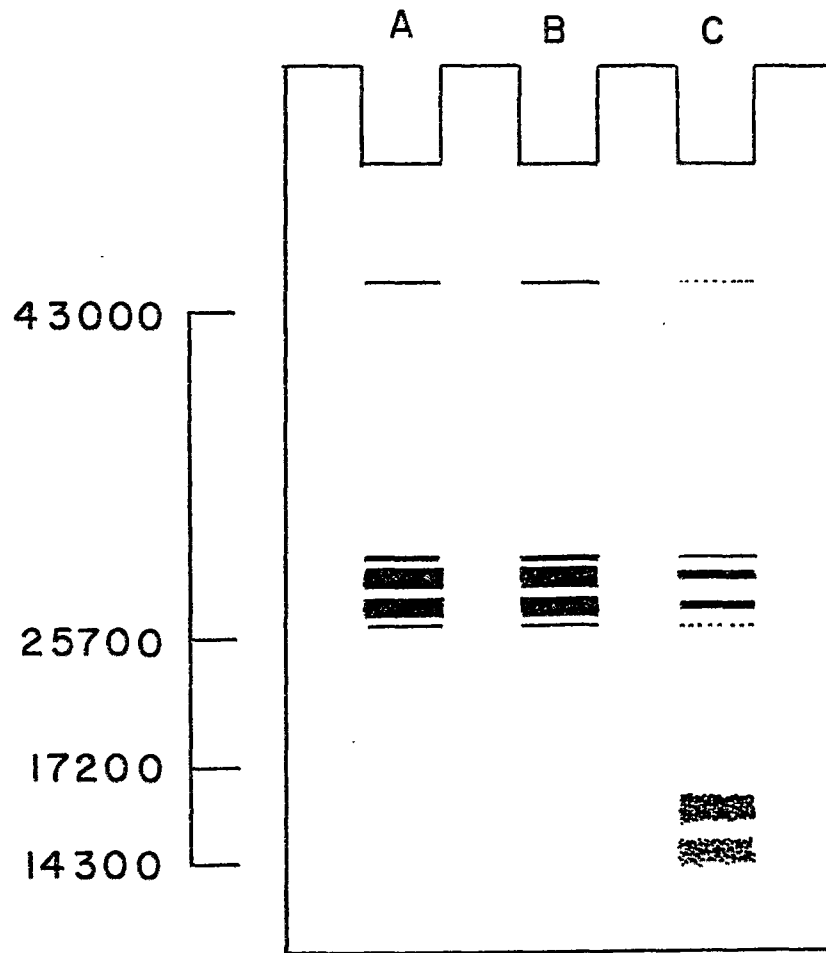


Figure 32 Sodium dodecyl sulfate slab gel electrophoresis of Caenestheriella setosa hemoglobin digested with subtilisin. A. Native hemoglobin. B. Control without subtilisin, with PMSF. C. One hour digest. Enzyme to protein ratio, 1:100 (25° C). 12.5% acrylamide.



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