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STRUCTURAL STUDIES ON THE PHASCOLOSOMA AGASSIZII HEMERYTHRINS

by

Frederick Anthony Liberatore
B.A., Framingham State College, 1970

A THESIS

Submitted to the University of New Hampshire
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This thesis has been examined and approved.

Gerald L. Klippenstein
Thesis director, Gerald L. Klippenstein
Asso. Prof. of Biochemistry

James A. Stewart
James A. Stewart, Asso. Prof. of Biochemistry

Arthur E. Teeri
Arthur E. Teeri, Prof. of Biochemistry

Gloria G. Lyle
Gloria G. Lyle, Asso. Prof. of Chemistry

Edward Tillinghast
Edward Tillinghast, Asso. Prof. of Zoology
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ABBREVIATIONS

Tris - tris(hydroxymethyl)aminomethane
TPCK - L-(1-tosylamido-2 phenyl) ethyl chloromethyl ketone
DFP - diisopropylphosphofluoridate
Dansyl chloride - 1-dimethylaminoaphthalene-5-sulfonyl chloride
SDS - sodium dodecylsulfate
PTC - phenylthiocarbamyl
PTH - phenylthiohydantoin
DEAE - diethylaminoethyl
TLC - thin layer chromatography
Lys - lysine
His - histidine
Arg - arginine
Asp - aspartate
Thr - threonine
Ser - serine
Glu - glutamate
Pro - proline
Gly - glycine
Ala - alanine
Cys - cysteine
Val - valine
Met - methionine
Ile - isoleucine
Leu - leucine
Tyr - tyrosine
Phe - phenylalanine
Trp - tryptophan
Asn - asparagine
Gln - glutamine
Asx - aspartate or asparagine
Glx - glutamate or glutamine

Peptide Nomenclature

A-TT = hemerythrin A tryptic peptide
TT = a pooled hemerythrin tryptic peptide
G = a pooled hemerythrin chymotryptic peptide
A,B,N = acidic, basic, and neutral
e1 = electrophoresis involved in the peptide's purification
Ch = chromatographically purified peptide
S = Dowex-50 fractionation involved in the peptide's isolation
ABSTRACT

STRUCTURAL STUDIES ON THE PHASCOLOSUMA AGASSIZII HEMERYTHRINS

by

FREDERICK ANTHONY LIBERATORE

Hemerythrin is the nonheme respiratory protein found in erythrocytes of marine worms in the phylum Sipunculoidea. The coelomic hemerythrin of Phascolosoma agassizii, the major subject of this thesis, is shown to have a trimeric quaternary structure, unusual subunit interactions, and great heterogeneity.

The coelomic hemerythrin gave an apparent molecular weight of 32,000 on Sephadex gel chromatography and an $s_{20, w}^2$ of 3.65 S in sedimentation velocity experiments. A combination of the sedimentation coefficient and the Stoke's radius determined by gel chromatography permitted the calculation of a molecular weight of 40,600. A subunit molecular weight of 12,800 was obtained by SDS-polyacrylamide gel electrophoresis. Iron analyses indicated the hemerythrin contained 0.885% Fe. Assuming 2 Fe/subunit, a minimum subunit molecular weight of 12,600 was calculated. Cross-linking experiments of native protein with dimethyl suberimidate yielded three bands on SDS-polyacrylamide gel electrophoresis and the molecular weights of these species were 13,000, 26,500, and 40,500. These results indicate that this hemerythrin has a trimeric quaternary structure.

Nine bands which stained for protein and iron were observed in
disc gel electrophoresis of the native hemerythrin. Electrophoresis in 8 M urea showed three major and one minor band. Individual native hemerythrin bands isolated by preparative electrophoresis gave one, two, or three bands when reelectrophoresed on 8 M urea gels. A model is proposed for the subunit composition of the native trimeric protein which accounts for the nine bands observed in disc gel electrophoresis.

However, by using the higher resolving power of gel isoelectric focusing with wide range ampholytes, pH 3-10, more than 20 bands were observed for native hemerythrin and five major subunit bands were found when the focusing was on gels 8 M in urea. The electrophoretic and isoelectric focusing subunit types were correlated with one another. On a mathematical basis, a trimer of five subunit types came closest to explaining the 20+ bands observed during isoelectric focusing of the native hemerythrin.

The uv-visible and circular dichroic spectra of P. agassizii hemerythrin closely matched those of G. gouldii coelomic hemerythrin.

Although the subunits of P. agassizii coelomic hemerythrin can be separated by preparative electrophoresis in 8 M urea, the more successful method utilizes DEAE-cellulose chromatography in 8 M urea. No method has been found which produces a native monomer.

Enzymatic digestions of pooled hemerythrin result in a large number of low yield peptides. The termini and a central area yielded enough peptide material for sequencing and it is postulated that these regions are essential for the tertiary structure of the protein and binding of the iron. These regions also show a strong homology with the primary structures of Golfingia gouldii coelomic hemerythrin and Dendrostomum pyroides myohemerythrin.
A myohemerythrin has been isolated from the retractor muscles of *P. agassizii*. It has an $s_{20,w}$ of 1.87 S, a molecular weight of 13,800 on SDS-polyacrylamide gel electrophoresis, and a uv-visible spectrum nearly identical to that of the fully characterized myohemerythrin from *Dendrostomum pyroides*.

Finally, a minute vascular system occurs in *P. agassizii*. 
INTRODUCTION

Hemerythrin is the nonheme iron-containing respiratory protein found in erythrocytes of worms in the marine phylum Sipunculoidea (Manwell, 1960a). Hemerythrin has also been reported in two species of the phylum Priapulodea; Priapulus caudatus and Halicryptus spinulosus; in one species of the phylum Brachiopoda, Lingula unguis; and in one species of the phylum Annelida, Magelona papillicornis (Ghiretti, 1962).

Hemerythrins are known to occur in more than one tissue within the same animal. In addition to coelomic hemerythrin, a vascular hemerythrin has been observed in several sipunculid species and a muscle hemerythrin has also been detected (Manwell, 1960b).

The coelomic hemerythrin of the sipunculid Golfingia gouldii is a well characterized oligomeric protein with a molecular weight of about 100,000 consisting of eight subunits of molecular weight 13,500 (Klotz and Keresztes-Nagy, 1963). A similar hemerythrin has been obtained from the coeloms of Dendrostomum pyroides (Ferrell and Kitto, 1970) and Sipunculus nudus (Bates et al., 1968). The vascular hemerythrin of D. pyroides (Klippenstein et al., 1972) and the hemerythrin from the brachiopod L. unguis (Joshi and Sullivan, 1973) are also octameric proteins with subunit molecular weights of about 13,500. Thus, hemerythrins from species in different phyla have an octameric quaternary structure; however, it will be shown in this thesis that another quaternary structure is found in the coelomic hemerythrin from the sipunculid Phascolosoma agassizii.

Dissociation of octameric hemerythrins has been accomplished by
use of a wide variety of physical conditions and chemical modifications. The physical conditions have included high concentrations of urea, guanidine hydrochloride, and mercaptoethanol (Joshi and Sullivan, 1973) and the detergent sodium dodecyl sulfate (Klotz and Keresztes-Nagy, 1963). All of these are considered denaturing agents.

Milder conditions which promote dissociation in octameric hemerythrins have included changes in ionic strength (Rao and Keresztes-Nagy, 1973), the binding of certain anions to the iron site (Keresztes-Nagy, 1963), and the binding of specific anions to a site distinct from the iron site (Rao and Keresztes-Nagy, 1973).

An equilibrium between octameric and monomeric hemerythrin was detected initially by the appearance of intermediate electrophoretic species when succinylated and non-succinylated octamer were mixed and allowed to hybridize (Keresztes-Nagy et al., 1965). Ultracentrifugation has allowed the direct observation of this equilibrium and has shown it to be enhanced by the presence of iron binding anions such as thiocyanate (Klapper et al., 1966). Equilibrium centrifugation did not reveal any intermediate molecular weight species of hemerythrin so it appears that the dissociation phenomena represents an all or none situation (Langerman and Klotz, 1969).

The coelomic hemerythrins of G. gouldii and D. pyroides contain two iron atoms per subunit (Klotz and Keresztes-Nagy, 1963; Ferrell and Kitto, 1970). Simple chemical tests suggested that Fe(II) is present in G. gouldii coelomic deoxyhemerythrin and Fe(III) in both the oxyhemerythrin and methemerythrin ligand forms (Klotz and Klotz, 1955). Also, the oxyhemerythrin gave a positive peroxide test while the deoxyhemerythrin did not. This led to the speculation that the oxygen bound
by this hemerythrin was reduced to the peroxide form during the binding process (Klotz and Klotz, 1966; Klotz et al., 1957).

Chemical modification techniques have been used to probe the active center of hemerythrin and the nature of the interactions between the subunits. In *G. gouldii* hemerythrin, the active center or iron binding site has been studied by chemical modification of the amino acids considered to be potential iron ligands. These were considered by Rill and Klotz (1970) to include eleven lysines, six glutamic acids, eleven aspartic acids, seven histidines, five tyrosines, one methionine, one cysteine, and the amino- and carboxyl-terminal residues. Functional groups which could be modified without destroying the active center were considered not to be involved in iron binding, while those which when modified were accompanied by destruction of the iron binding site were considered to be "probable" iron ligands. Amino acid side chains whose modification resulted in dissociation of oligomeric hemerythrin were concluded to be on or near the subunit interfaces.

Native subunits have been produced by the chemical modification of the sulfhydryl group in the coelomic hemerythrin of *G. gouldii* (Klotz and Keresztes-Nagy, 1962) and *D. pyroides* (Ferrell and Kitto, 1970). This also appeared to be true for *D. pyroides* vascular hemerythrin (Klippenstein et al., 1972). Although both the sulfhydryl groups of *D. pyroides* myohemerythrin reacted with sodium p-hydroxymercuribenzoate, the molecular weight of this hemerythrin remained unchanged (Klippenstein et al., 1972). Myohemerythrin was concluded to be a monomeric hemerythrin lacking a quaternary structure. The two cysteines in the myohemerythrin primary structure (Cote, 1973) are located well away from the single cysteine in the primary structure of *G. gouldii* coelomic
hemerythrin (Klippenstein et al., 1968).

After reacting all the carboxyl groups in G. gouldii hemerythrin with glycine methyl ester, the active site remained intact. However, reacting as few as 8 of the 18 carboxyl groups present produced monomer. Since some octamer had more groups reacted than some of the monomer present, it was concluded that electrostatic repulsion was not the cause of monomer production. Carboxyl groups were concluded not to be involved in the binding of iron in hemerythrin, although one carboxyl was suspected of being involved in subunit interactions (Klippenstein, 1972b).

Modification of all the lysines of G. gouldii oxyhemerythrin with trinitrobenzenesulfonic acid seemed to eliminate these side chains as potential iron ligands, since iron loss did not occur (Fan and York, 1969). Similar results have been reported using methyl acetimidate, succinic anhydride, or maleic anhydride to block all the lysines in metazidehemerythrin monomer. In addition, the N-terminal glycine was blocked simultaneously. A lack of significant change in the uv-visible (300-600 nm) and circular dichroic (300-550 nm) spectra indicated that neither the N-terminus nor the lysines were involved in binding the iron (Morrissey, 1971). Succinylation of metazidehemerythrin octamer resulted in total conversion to monomer when 9 amino groups were modified (Garbett et al., 1971a); however, the monomer produced was probably due to the blocking of the cysteine which also reacts with succinic anhydride (Keresztes-Nagy and Klotz, 1965).

With part of the amino groups of hemerythrin monomer blocked, up to 70% of the methionine could be selectively modified with iodoacetamide at pH 5.5 without iron loss, although some precipitation did
occur. Methionine was not involved in binding the iron of \textit{G. gouldii} coelomic hemerythrin but appeared to play a role in the maintenance of tertiary structure (Morrissey, 1971). Since there is only one methionine in the primary structure of \textit{G. gouldii} coelomic hemerythrin (Klippenstein et al., 1968), and it appears to be essential in the maintenance of tertiary structure (Morrissey, 1971), the preservation of methionine at this position, 62, would be expected in other hemerythrins of different amino acid compositions. Methionine is preserved at position 62 in the monomeric myohemerythrin of \textit{D. pyroide}\textsubscript{s} (Cote, 1973). This suggests that methionine can be expected at position 62 in the primary structure of \textit{P. agassizii} coelomic hemerythrin.

Two histidines were readily blocked in oxyhemerythrin octamer with 1-diazo-H-tetrazole yielding bis-diazo histidine residues, and a third reacted more slowly, while in monomer all three reacted readily with this reagent. All the histidines in apohemerythrin reacted readily with this reagent. Four histidines were concluded to be involved in binding the iron in \textit{G. gouldii} hemerythrin, and a fifth to be located near the subunit binding site (Fan and York, 1969). The reaction of histidine with iodoacetamide in partially amino modified \textit{G. gouldii} metazidehemerythrin monomer has shown that 1.2 histidines reacted before destruction of the active site began. An additional 0.7 histidines had already been modified during the prior succinylation or maleylation of the amino groups (Morrissey, 1971). Sequence studies indicated that histidines 34, 82, and either 73 or 77 had been partially modified with iodoacetamide. The histidine at position 82 is replaced in a variant hemerythrin by asparagine (Klippenstein, 1972a) while the other histidines remain. Thus, histidine 82 can be eliminated as an
iron ligand on the basis of these findings as well. Histidine 77 was apparently susceptible to succinylation during the initial amino group modification and this reactivity was thought to eliminate it as an iron ligand (Morrissey, 1971).

The nitration of the tyrosines of *G. gouldii* metazidehemerythrin with tetranitromethane implicated some of the tyrosines in binding of the iron (Rill and Klotz, 1970). Oxygenhemerythrin nitrated with tetranitromethane at 0° for 18 hrs. had three tyrosines nitrated and two un-modified. Heat denatured hemerythrin had all five tyrosines nitrated under the same conditions. Peptide studies indicated that tyrosines 18, 70, and probably 67 had been nitrated in the oxyhemerythrin (York and Fan, 1971). Similar results were reported for tyrosines 18 and 70 (Rill and Klotz, 1971), while tyrosines 8, 109, and 67 were considered to be potential iron ligands. Removal of the mecuriphenylsulfonate group from the cysteine of native nitrated monomer allowed some reaggregation of the subunits; however, the sedimentation value was lower than that expected for octamer. The nitration of tyrosines 18 and 70 had altered the quaternary structure, or hindered reassociation, or both. Either or both of these tyrosines were concluded to be located in the subunit binding site (Rill and Klotz, 1971).

Octameric *G. gouldii* oxyhemerythrin with 95% of the lysines modified with ethyl acetimidate was reported to remain in the oxygenated octameric form (Fan and York, 1972). Thus, it would seem that the lysines are probably not involved in subunit interactions.

Amidinated oxyhemerythrin octamer (*G. gouldii*) had three tyrosines which could be readily O-acetylated with N-acetyl imidazole resulting in the formation of metchloride monomer. Two tyrosines were
concluded to be involved as iron ligands while at least one of the three others was believed to be involved in subunit interactions (Fan and York, 1972). The status of the sulfhydryl group was not examined in the monomer produced by this modification of the tyrosines. About 0.5 tyrosine residues were available for modification with N-acetyl imidazole in metchloride- and metazidehemerythrin octamer (G. gouldii). The same reaction in 3 M urea resulted in the modification of three tyrosines and the formation of monomer. About 0.25 of the cysteine sulfhydryl groups remained unmodified (Forand, 1973). While tyrosines 18, 67, and 70 are probably masked by subunit interactions, their direct involvement in these interactions has yet to be proven. Two tyrosines, 8 and 109, are probably involved as ligands of iron.

A comparison of the acid-base titration curves of G. gouldii metazidehemerythrin octamer and monomer has shown several of the lysines and aspartic or glutamic acid residues to be more easily titrated in the monomer than in the octamer. This suggested that these groups were involved in intersubunit salt bridges (Morrissey, 1971) or they may simply be masked by subunit interactions.

Based on the evidence available, it appears that the two irons in the active center of G. gouldii coelomic hemerythrin are bound to tyrosines 8 and 109, and to histidines 25, 54, 73, and 101. These residues are preserved in the primary structure of D. pyroides myohemerythrin which differs greatly in overall amino acid composition and sequence from G. gouldii coelomic hemerythrin. However, tyrosine 67 and histidine 77 are preserved in the primary structure of D. pyroides myohemerythrin (Cote, 1973). A nonhomologous substitution for these residues in another hemerythrin, such as P. agassizii coelomic hemery-
thrin, would eliminate them as potential iron ligands with certainty.

Cysteine has been implicated in subunit interactions or else is very close to the subunit binding site in two coelomic and one vascular hemerythrin (G. gouldii coelomic and D. pyroides both types). At least one carboxyl and possibly one or more tyrosines and lysines appear to be involved in the subunit interactions of G. gouldii coelomic hemerythrin.

The uv-visible spectra of G. gouldii oxyhemerythrin and various methemerythrin ligand forms are shown in Fig. 1 (Keresztes-Nagy and Klotz, 1965). The visible-near visible absorption band (350-500 nm) of these ligand forms was assigned to a charge transfer from the ligand to the iron (Fe(II)). Another strong absorption band around 330 nm was assigned to the oxo bridge between the two irons on the basis of the spectrum of a known oxo bridged model compound (Keresztes-Nagy and Klotz, 1965).

Studies on the active site of hemerythrin by Mössbauer spectros-copy and magnetic susceptibility measurements have allowed a number of conclusions to be drawn concerning the structure of the active center of G. gouldii hemerythrin.

Initial Mössbauer studies showed that the irons of hemerythrin were near enough to interact with one another. The iron in deoxyhemerythrin was found to be in the Fe(II) high spin oxidation state. Only one iron environment was present with both nitrogen and oxygen ligands suspected in deoxyhemerythrin. Methemerythrin was concluded to have antiferromagnetically coupled irons which were in the Fe(III) high spin oxidation state (Okamura et al., 1969; Garbett et al., 1971b).

A further extensive Mössbauer study on model compounds, and the
Figure 1: The absorption spectra of various *G. gouldii* coelomic hemery-thrin ligand forms from 300-600 nm (Keresztes-Nagy and Klotz, 1965).
various ligand forms of hemerythrin, resulted in the proposal of the active site complexes shown in Fig. 2 (Garbett et al., 1969). The iron in methemerythrin and deoxyhemerythrin was found to be in equivalent states; however, the states were different for the two classes of protein. Oxyhemerythrin had antiferromagnetically coupled iron in two nonequivalent states (Fig. 3a, Garbett et al., 1969; York and Bearden, 1970).

Absorption spectra and Mössbauer data from oxyhemerythrin, and model compounds, provided evidence for an oxo bridge between the two iron atoms at the active site. Circular dichroic spectra of various methemerythrin ligand forms have revealed two classes of spectra. Some ligands coordinate via a bridging structure utilizing one molecule per active site while other ligands coordinate in a non bridging manner, one per iron (see Fig. 2, Garbett et al., 1969).

The $\text{O}_2^2$ peroxide type electronic state in oxyhemerythrin has been verified by laser resonance Raman spectroscopy. Bands were observed at 844 and 500 cm$^{-1}$ which were assigned to the 0-0 and Fe-0 stretching modes. These bands were not present in deoxyhemerythrin. When $^{18}\text{O}_2$ was substituted for $^{16}\text{O}_2$, the position of the bands shifted which confirmed their identification. The wavenumber of the 0-0 stretch was nearly identical with that of a known peroxide compound. One type of $\text{O}_2$ species appeared to be present (Dunn et al., 1973).

Room temperature magnetic susceptibility determinations on deoxyhemerythrin have shown four unpaired electrons to be present. This, along with Mössbauer evidence, conclusively demonstrated that the iron in deoxyhemerythrin was in the Fe(II) oxidation state (York and Bearden, 1970). The use of an ultrasensitive magnetometer to study metaquo- and
Figure 2: Proposed hemerythrin active site complexes and an oxygenation mechanism based on Mössbauer spectroscopy and circular dichroic studies (Garbett et al., 1969).
Iron (II) Oxygen Intermediate

Deoxyhemerythrin

Oxyhemerythrin

Ligands

Methemerythrin

\[ X = \text{OH}^-, \text{F}^-, \text{OH}_2^- \]

\[ X = \text{Cl}^-, \text{Br}^-, \text{F}^- \]

\[ \text{NCS}^-, \text{NNN}^-, \text{CN}^- \]

\[ \text{NCO}^- \]
Figure 3a: The active site of coelomic *G. gouldii* oxyhemerythrin which reflects the antiferromagnetically coupled iron in the two non-equivalent states observed with Mössbauer spectroscopy (Garbett *et al.*, 1969).

Figure 3b: Proposed active site complexes of *G. gouldii* coelomic hemerythrin consistent with both Mössbauer spectroscopy and magnetic susceptibility data (Dawson *et al.*, 1972).
oxyhemerythrin revealed the iron to be in the Fe(III) high spin oxidation state and antiferromagnetically coupled. The coupling constant of metaquohemerythrin was in the range for a known oxo bridged model compound. Thus, the structure proposed previously (Fig. 2) was considered to be consistent with the magnetic susceptibility data. However, the coupling constant for oxyhemerythrin was considered to be too large for a double bridged structure. Several structures were proposed which would be consistent with both the magnetic susceptibility and Mössbauer data (Fig. 3b, Dawson et al., 1972).

An octahedral iron configuration has been assumed in the active center of hemerythrin. The equivalent state of the iron in the deoxy- and methemerythrin ligand forms suggested an even division of the potential ligands. Thus, each iron would be bound by one tyrosine and two histidines, with an additional position occupied by an oxo bridge, and another by the binding of oxygen or some other ligand anion(s) (see Figs. 2 and 3). Five of the six coordination sites per iron can be accounted for with the available data. The sixth site of each iron is probably filled with a water molecule.

Circular dichroic spectra have been obtained for hemerythrins from a number of sipunculid species: G. gouldii (Darnall et al., 1969; Garbett et al., 1969), S. nudus (Bossa et al., 1970), and D. pyroides (Klippenstein et al., 1972). The most important finding from these studies was the strong similarity of the spectra below 250 nm. All of the spectra were typical of a protein containing a high content of α-helix. From the absorption value at 222 nm, the approximate percentage of α-helix can be computed (G. gouldii, 75%; S. nudus, 71%; D. pyroides; coelomic, 69%; vascular, 66%; and myohemerythrin, 69%).
There exists in *G. gouldii* coelomic hemerythrin a non-ligand anion binding site which binds perchlorate strongly, nitrate moderately, and phosphate weakly. Anions bound at this site slow the binding of other anions at the active site and make the cysteine sulfhydryl more difficult to chemically modify (Darnall et al., 1968). It has been noted that the pair of arginines next to the cysteine in the primary structure of *G. gouldii* hemerythrin would be a logical location for the non-ligand anion binding site. Furthermore, this site was postulated to function physiologically by binding bicarbonate ion and aiding in the removal of carbon dioxide from the coelomic cavity (Garbett et al., 1971a). Chloride ion has also been found to bind at a non-ligand anion binding site in *G. gouldii* hemerythrin. The binding of this anion enhanced the conversion of octamer to monomer (Rao and Keresztes-Nagy, 1973).

Most hemerythrins show little cooperativity between subunits in oxygen binding, including *P. agassizii* coelomic hemerythrin (Manwell, 1960c), with the value of the Hill coefficient being near 1 and a Bohr effect being absent (Manwell, 1958, 1960b; Bates et al., 1968; Ferrell and Kitto, 1970, DePhillips, 1971). Interestingly enough, the hemerythrin of the brachiopod *L. unguis* has shown a distinct Bohr effect, and at an alkaline pH (7.6) exhibited a definite cooperativity among the subunits in oxygen binding (Manwell, 1960c), while that of the annelid *M. papillicornis* did not show any cooperativity among the subunits in oxygen binding (Wells and Dales, 1974). The oxygen affinity of hemerythrins differs between species with the prototype hemerythrin of *G. gouldii* showing a P$_{50}$ $O_2$ = 4 mm Hg at 25° (Manwell, 1963), while *P. agassizii* has a P$_{50}$ $O_2$ of 10 mm Hg under similar conditions (Manwell,
1958). Intraspecies hemerythrins have also shown differing oxygen affinities which have been linked to the ecology and respiratory physiology of the worm (Manwell, 1960b).

The coelomic hemerythrin of *P. agassizii* appears to be a normal hemerythrin in terms of its Hill coefficient and lack of a Bohr effect. It has a somewhat low oxygen affinity (Manwell, 1958; 1960c). This hemerythrin will be shown to possess a quaternary structure unknown among hemerythrins and rare in other proteins. The subunit interactions of this hemerythrin are of a novel type, again, unknown among the presently characterized hemerythrins. This hemerythrin displays great heterogeneity and has resisted all attempts to produce native subunits. The unusual hemerythrin of the brachiopod *L. unguis* also displays a great deal of heterogeneity. It can be inferred from the data given that this hemerythrin has also resisted all attempts to produce native subunits (Joshi and Sullivan, 1973).

There are many questions concerning hemerythrin that remain to be answered. Among these are the following: which amino acids are involved in binding the iron? Are regions of amino acid homology, among hemerythrins of vastly differing amino acid compositions, essential for the tertiary structure and maintenance of the active site? The characterization of the unusual hemerythrin of *P. agassizii*, the subject of this thesis, will aid in providing answers to these questions.
MATERIALS AND METHODS

Purification of Hemerythrin

Sipunculid worms of the species P. agassizii were purchased from Peninsula Marine Biologica1s, Sand City, California. All purification steps were carried out at 5°. The worms were cut along their length and the rapidly clotting coelomic fluid collected in 3.5% NaCl. The resultant mixture was centrifuged at 480 x g for 15 min. and the supernatant discarded. The packed red cells were washed with 3.5% NaCl and the centrifugation repeated. Care was taken not to break up the clots. Attempts to do so resulted in premature red cell lysis and a subsequent loss of hemerythrin. The erythrocytes were lysed by the addition of 2 vol. of 0.4% NaCl followed by overnight stirring (Subramanian et al., 1968). The lysate was centrifuged at 31,000 x g for 1 hr. and the pellet discarded. The supernatant was filtered through Whatman no. 1 filter paper to remove a lipid layer and dialyzed overnight against 0.1 M NaN₃ to produce metazidehemerythrin (Keresztes-Nagy and Klotz, 1965). Dialysis against a buffer solution containing no iron-binding anions, such as 0.1 M sodium phosphate, pH 7.2, allowed the preservation of the oxyhemerythrin ligand form. The dialyzed supernatant was made 50% saturated with (NH₄)₂SO₄ by addition of the solid salt, centrifuged at 12,000 x g for 30 min., and the pellet discarded. The supernatant was made 100% saturated with (NH₄)₂SO₄, again by addition of the solid salt, and centrifuged as before. The recovered hemerythrin pellet was dissolved in 5 vol. of 0.1 M NaN₃ and dialyzed against 0.1 M NaN₃. The final purification step consisted of gel filtration on a 2.5 x 90 cm
Sephadex G-75 column (Pharmacia Fine Chemicals) using a flow rate of 20 ml/hr., with 0.1 M NaCl as the eluting solvent. Fractions of 3 ml were collected.

The coelomic hemerythrin of individual worms was examined by bleeding the worms into separate funnels containing pads of glass wool. The coelomic fluid was collected in screw capped vials containing 1-2 ml of 3.5% NaCl. The solutions were centrifuged at 750 x g and the supernatant discarded. The cells were washed two additional times in this manner. Then, 1 ml of 0.4% NaCl was added with lysis taking place at 4°, with constant stirring, overnight. Centrifugation at 27,000 x g for 1 hr. followed. The recovered supernatants of oxyhemerythrin were characterized by their disc gel electrophoretic patterns, pooled by class, dialyzed against 0.1 M NaN₃, concentrated by pressure filtration, and finally, the classes were purified by gel filtration on a Sephadex G-75 column, 2.5 x 60 cm, using a 20 ml/hr. flow rate of 0.1 M sodium phosphate buffer, pH 7.2, collecting 3 ml fractions. Disc gels were then run on samples of each purified hemerythrin class.

**Purification of Myohemerythrin**

The myohemerythrin of *P. agassizii* was isolated basically following the procedure of Klippenstein *et al.* (1972). The muscles of about 100 large worms were excised, washed with 3.5% NaCl, immersed in 0.1 M NaN₃, and stored frozen until processed. All isolation procedures were performed at about 4°. The muscles were thoroughly minced with a tissue homogenizer in 18.0 ml of 0.1 M Tris-HCl, pH 8.0, and centrifuged at 750 x g for 20 min. The supernatant was made 0.1 M NaN₃ and 5 x 10⁻³ M dithioerythritol. This solution was centrifuged at 16,000 g for 2 hrs.
and the supernatant recovered. Solid \((\text{NH}_4)_2\text{SO}_4\) was added to 40% saturation, the solution adjusted to pH 7.0, and then centrifuged at 12,000 x g for 20 min. The recovered supernatant was made 80% saturated with solid \((\text{NH}_4)_2\text{SO}_4\) and the above steps repeated. The supernatant was then made 100% saturated with \((\text{NH}_4)_2\text{SO}_4\), again by addition of the solid salt, and centrifuged at 12,000 x g for 30 min. The recovered precipitated myohemerythrin was dissolved in 4.0 ml of 0.1 M \(\text{NaN}_3\) and dialyzed against the same solution at 4°.

Standard 7% acrylamide disc gels and SDS-polyacrylamide molecular weight gels (Weber and Osborn, 1969) were run on samples of this solution. After dialysis against 0.5 M NaCl and concentration by pressure filtration to about 3 mg/ml, a sedimentation coefficient was determined by the method described in the following section.

**Native Oligomeric Molecular Weight Determinations**

The molecular weight of native coelomic hemerythrin was determined by Sephadex G-100 gel filtration (Whitaker, 1963) on a 2.5 x 90 cm column. Throughout this study, an ascending flow rate of 12 ml/hr. of 0.1 M sodium phosphate buffer, pH 7.2, was used and 3 ml fractions collected. The standards were bovine serum albumin and ovalbumin (both from Sigma), \(\beta\)-lactoglobulin (Mann), sperm whale myoglobin (Calbiochem), and *G. gouldii* metazidehemerythrin monomer. All samples pumped onto the column were of a 3 ml vol. and were 0.1 M in \(\text{NaN}_3\) which served as a low molecular weight marker of the column volume. Proteins were detected by their absorbance at 280 nm and \(\text{NaN}_3\) at 254 nm. Elution volumes of the proteins divided by the elution volume of sodium azide were plotted versus the logarithm of the molecular weights of the proteins.
The $s_{20,w}^2$ of the native coelomic hemerythrin was determined in a Beckman model E analytical ultracentrifuge (AN-D rotor) using Schlieren optics. Hemerythrin concentrations of 4.4-17.6 mg/ml in 0.5 M NaCl (adjusted to pH 7.0 with NaOH) were used in these experiments. The runs were performed at 52,640 rpm and 20°. A single sector cell was used for the coelomic hemerythrin runs and a capillary type double sector synthetic boundary cell for the myohemerythrin run.

**Denatured Subunit Molecular Weight Determinations**

SDS-polyacrylamide gel electrophoresis was done according to the method of Weber and Osborn (1969) with slight modifications. The 10 cm gels were prerun for 30 min. prior to sample loading, the trough buffer was diluted 1:3, and the gels run at 5°. Standards consisted of ovalbumin, pepsin (Worthington), β-lactoglobulin, and C. gouldii coelomic hemerythrin.

Iron analyses were performed according to the method of Rill and Klotz (1970). A standard hemerythrin solution was dialyzed against deionized distilled water and the approximate hemerythrin concentration determined by measurement of the absorption of the solution at 280, 326, and 445 nm. One ml of this solution, 2.5 ml of 0.25% o-phenanthroline (w/v) in 0.5% H$_2$SO$_4$ (v/v), and 0.5 ml of 10% hydroxylamine HCl were measured into graduated centrifuge tubes and these placed in a boiling water bath for 30 min. The volume of each tube was made up to 5 ml with deionized distilled water and the tubes centrifuged at 750 x g for 15 min. The absorption of the supernatant at 510 nm was compared with that of a blank. The iron content was then determined from a standard curve. Aliquots of the standard hemerythrin solution were hydrolyzed and
analyzed for their total amino acid content in order to obtain an accurate hemerythrin concentration.

**Amino Acid Analysis, Tryptophan Determinations, and Corrections for Serine and Threonine Degradation**

Amino acid analyses throughout this study were performed on a Beckman model 120C amino acid analyzer (Spackman et al., 1958). Hydrolysis took place in 2 ml of constant-boiling HCl, 5.7 M, for 24 hr. at 108° in evacuated glass tubes. A drop of 0.5 M hydrazine was added to each sample prior to hydrolysis to prevent tyrosine destruction (Sanger and Thompson, 1963).

Tryptophan was determined spectrophotometrically according to the following method (Beaven and Holiday, 1952). A dilute solution of hemerythrin was dialyzed against 0.1 M NaOH overnight, centrifuged at 27,000 x g for 1 hr., and the absorption measured at 280 and 294.4 nm against a blank of 0.1 M NaOH. Also, absorption values were recorded at 10 nm intervals between 320 and 360 nm. These latter values were plotted versus the appropriate wavelengths and a line extrapolated to 280 nm. The resulting values for light scattering were subtracted from the measured values at 280 and 294.4 nm. Substitution of the corrected absorption values into the following equation allowed the determination of the tyrosine to tryptophan molar ratio where D equaled the corrected absorption value at the specific wavelength.

\[
\frac{M_{\text{Tyr}}}{M_{\text{Trp}}} = \frac{0.529D_{294.4} - 0.263D_{280.0}}{0.263D_{280.0} - 0.170D_{294.4}}
\]

The number of residues of tryptophan was calculated after a determination of the number of residues of tyrosine by amino acid analysis.
To correct for the degradation of serine and threonine, identical samples were hydrolyzed for 24, 48, and 72 hr. and analyzed. The logarithms of the serine and threonine concentrations were plotted versus the corresponding hydrolysis times and a line extrapolated to zero hydrolysis time.

Absorption Measurements

Individual absorption measurements for the iron analyses, tryptophan determinations, Amido Schwartz stain quantitations, and the extinction coefficients were taken on a Beckman DU-2 spectrophotometer. Absorption spectra were recorded on a Hitachi Perkin-Elmer 124 or a Cary 15 double beam spectrophotometer. The flow from the 8 M urea DEAE-cellulose column was monitored continuously utilizing a flow cell and a Gilford spectrophotometer, model 222-A. The circular dichroism spectrum of \textit{P. agassizii} hemerythrin from 200-250 nm was recorded on a Cary model 60 Spectropolarimeter. A metazidehemerythrin solution containing 0.535 mg/ml in 0.1 M NaCl, pH 7.0, was used for these measurements in a quartz cell of 0.05 cm pathlength.

Cross-linking with Dimethyl Suberimidate

Native, pooled \textit{P. agassizii} hemerythrin was cross-linked with dimethyl suberimidate (Pierce lot 2223-4) at a protein concentration of 3.35 mg/ml and either 3.35 or 6.7 mg/ml of the cross-linking reagent (Brown \textit{et al.}, 1973). The reaction was carried out in a 0.2 M triethanolamine-HCl buffer, pH 8.5, at 4° with constant stirring for 24 or 48 hr. Any precipitated protein was removed by centrifugation. The 24 hr. reaction mixtures were dialyzed against 0.01 M sodium phosphate
buffer, pH 7.0, containing 1% SDS (w/v) and 1% B-mercaptoethanol (v/v), and the 48 hr. reaction mixtures were mixed 1:1 with the same buffer. After incubation at 37° for 2 hr., SDS-polyacrylamide gel electrophoresis was performed.

**Analytical and Preparative Disc Gel Electrophoresis**

Analytical disc gel electrophoresis on 7% acrylamide gels was according to Davis (1964). Samples mixed with 40% sucrose were layered directly on the stacking gel and the reservoir buffer layered over the sample. The gels were run at 3.5 mA/gel at 4°. Some gels were stained for iron.

The iron specific stain (Canalco) was made up just prior to use from three stock solutions. The stain solutions, 2.4 M sodium acetate buffer, pH 4.75, 10% w/v hydroquinone in absolute ethanol, and 0.25% w/v 2,4 dinitroso-1,3-napthalenediol in absolute ethanol, were mixed 20:1:1 just prior to use. Pale green bands appearing in 30-45 min. indicated the presence of iron. Brown false bands appearing after several hours were due to the presence of protein.

Preparative disc gel electrophoresis was performed in a Canalco PD2/320 apparatus. A 6 cm separating gel, 10% in acrylamide (Canalco Prep-Cryl) and 0.03% N,N'-methylenebisacrylamide, a 1 cm stacking gel 3.5% in acrylamide and 0.0625% N,N'-methylenebisacrylamide, and a 0.0375 M Tris-HCl elution buffer, pH 8.8-9.0, were used in these experiments. (The formulae for the stock solutions used to prepare these gels can be found in the Prep-Disc Instruction Manual available from the Canal Industrial Corporation.)

Hemerythrin samples, 30-50 mg, were prepared by overnight
dialysis against deionized distilled water. A drop of 0.05% bromphenol blue and solid sucrose (20-30% w/v) were added to the sample for a total volume of 10-20 ml. This solution was layered directly on the stacking gel and electrode buffer layered over it. The column was cooled to about 4° throughout the electrophoresis run and the fractions were collected at this temperature. An initial constant current of 10 mA was applied to the column until the hemerythrin had entered the stacking gel and then a current of 20-25 mA was maintained for the remainder of the run. A 60 ml/hr. flow rate of elution buffer was used in the initial experiment collecting 2 ml fractions. Thereafter, the flow rate was varied to optimize the separation of the visibly eluting hemerythrin bands. The 60 ml/hr. flow rate was lowered to 30 ml/hr. during the elution of band 1. Bands 2-8 were collected at 30 ml/hr. with a flow rate of 120 ml/hr. between bands to minimize cross-contamination. Between bands 8 and 9 a flow rate of 60 ml/hr. was maintained, with band 9 being collected at 15 ml/hr. The bromphenol blue was eluted in 4-5.5 hr. and the final hemerythrin band in 14-16 hr. Peaks were located by their absorption at 280 nm.

Peak fractions were assayed for homogeneity on analytical gels identical in composition and length to those used for the preparative disc electrophoresis. The same peak fractions were also assayed on 7% acrylamide gels, 8 M in urea. These samples were prepared by an overnight dialysis against a 0.025 M sodium phosphate buffer, pH 7.2, at 4° and then mixed with solid urea until 8 M, and finally incubated for 2 hr. at 20-25°. Loading of samples on the gels and running conditions were as previously described. The gels were stained with 0.5% (w/v) Amido Schwartz (Color Index no. 20470) in 7% acetic acid and destained electro-
phoretically or by diffusion in 7% acetic acid. The three major bands in the 8 M urea gels were cut out, homogenized, and eluted with 0.1 M NaOH at 37° with constant agitation. The final volume was adjusted to 1.5 ml/band regardless of the size of the gel slice. The eluted stain was quantitated by its absorbance at 620 nm.

Preparative electrophoresis was also used for the initial subunit separations. The conditions for these runs were as previously described with the following exceptions. The separating gel was 7.5% in acrylamide and both the separating and stacking gels were 8 M in urea. The sample solution, about 80 mg of hemerythrin, was deionized by passing it through a column of Amberlite (Mallinckrodt) MB-1 ion exchange resin. A drop of 0.05% bromphenol blue and solid urea were added to the sample to make a total volume of 10 ml which was 8 M in urea. This solution was incubated for 2 hr. at 37° prior to electrophoresis. The flow rate was a constant 60 ml/hr. collecting 2 ml fractions at room temperature. Total protein recovery was estimated spectrophotometrically at 280 nm. All of the fractions representing subunit type D, 55-70, the leading edge of type C, 82-94, and the trailing edges of subunit types B, 120-150, and A, 187-248, were pooled and aliquots were run on analytical gels, 8 M in urea, to assay the purity of the pooled fractions. Additional aliquots were hydrolyzed and analyzed for amino acid composition.

Isoelectric Focusing on Acrylamide Gels

Gel isoelectric focusing was based on the method of Wrigley (1968). The gels contained 7% acrylamide, 0.16% N,N'-methylenebisacrylamide, and 2 to 4% ampholytes (LKB Produkter AB, Bromma, Sweden) of the
desired pH range. Samples were mixed with the gel solution prior to photopolymerization which was catalyzed by 0.0006% riboflavin. In some experiments, the gels contained 8 M urea. The catholyte consisted of 0.02 M NaOH and the anolyte 0.01 M H₃PO₄. The lower buffer chamber was cooled by a flow of tap water and the gels run at 1 mA/gel until 400 V had been reached. Then, a constant 400 V was applied to the gels for about 6 hr. Washing in 5% (w/v) trichloroacetic acid fixed the protein bands in the gels and removed the ampholytes prior to staining with 0.5% Amido Schwartz in 7% acetic acid (w/v) for 1 to 2 hr. One gel from each experiment was not placed in 5% trichloroacetic acid but was cut into 5 mm slices and each of these placed in deionized distilled water and allowed to stand overnight at room temperature. A plot of the pH of the resultant solutions, versus distance down the gel of the slices, revealed the pH gradient formed during electrofocusing. An estimate could then be obtained of the isoelectric pH of the protein band in the stained gels.

Subunit Separation

A pooled P. agassizii coelomic hemerythrin sample, 19 µmoles (240 mg), was made 8 M in urea, by addition of the solid, 0.01 M in Tris-HCl, pH 8.0, by the addition of 0.1 M Tris-HCl buffer, pH 8.0, and incubated 6 hr. at 25°. The 52 ml of solution was then applied to a 2.5 x 54 cm column of DEAE-cellulose (Whatman, DE-52) which had been equilibrated with an 8 M urea 0.01 M Tris-HCl buffer, pH 8.0. This buffer was made by adding solid Tris and concentrated hydrochloric acid to a solution of 8 M urea, which had just been deionized on a column of Amberlite MB-1 ion exchange resin removing any cyanates present. The flow rate
was 252 ml/hr. initially, but was slowed to 126 ml/hr. when column pressure increased. The subunit types were eluted with a stepwise gradient consisting of 500 ml each of 0.0044 M, 0.0366 M, and 0.0662 M NaCl in the Tris-HCl-8 M urea buffer just described. The flow was monitored continuously at 280 nm and fractions collected when peaks appeared. After collection, the peak fractions were immediately dialyzed exhaustively against deionized distilled water at 4°C. Lyophilization to dryness followed this dialysis. The purity of the subunit types isolated was assayed by disc gel electrophoresis on 7% acrylamide gels, 8 M in urea, as previously described.

Chymotryptic Digestion

A 15 μmole (190 mg) sample of P. agassizii pooled coelomic hemerythrin was made 6 M with solid guanidine hydrochloride and incubated overnight at 37°C. The 26 ml of solution was then cooled to 20°C, made 0.1 M HCl, and allowed to stand 1 hr. Ethanol was then added to make a 50% solution, and this was allowed to stand an additional hr. The resultant solution was exhaustively dialyzed against deionized distilled water at room temperature. The 51 ml solution of denatured hemerythrin was made 0.1 M NH₄HCO₃ by addition of the solid salt, adjusted to pH 8.0 with NH₄OH, and digested with α-chymotrypsin. The α-chymotrypsin (Worthington lot CDI 2DB, 69 μ/mg) was added at 0 and 3 hr. digestion time at 1% (w/w) per addition. The digestion proceeded for a total of 6 hr. with constant agitation at 37°C. The digest was then frozen and lyophilized to dryness.
**Tryptic Digestion**

The lyophilized hemerythrin of subunit type A was dissolved in 10 ml of 0.1 M NH₄HCO₃, pH 8.0, and 1% (w/w) of TPCK treated trypsin (Worthington lot 34C624, 189.9 μ/mg) added to the hemerythrin solution at 0 and 3 hr. digestion time. The digestion took place at 37° with constant agitation and was terminated at 6 hr. by freezing followed by lyophilization to dryness. Part of the lyophilized material was insoluble upon resuspension in 0.1 M NH₄HCO₃, pH 8.0. The entire mixture was centrifuged at 30,000 x g for 1 hr. at 4° and the supernatant lyophilized. The precipitate was resuspended in 10 ml of 0.1 M NH₄HCO₃, pH 8.0, and an additional 1% (w/w) of TPCK treated trypsin added. The digestion proceeded for 6 hr. at 37° with constant agitation, followed by freezing and lyophilization to dryness.

**Peptide Gel Filtration**

The resultant lyophilized peptide mixtures from each of the digests described were dissolved in 1 ml of 0.1 M NH₄HCO₃ buffer, pH 8.0, aliquots taken for amino acid analysis and peptide mapping, with the remainder being run through a 1.5 x 90 cm Sephadex G-15 column. The flow rate was 5-7 ml/hr. of 0.1 M NH₄HCO₃, pH 8.0, collecting 1.5 ml fractions. Peptide peaks were located by their absorption at 254 nm or 220 nm when very weak, and then pooled, frozen, and lyophilized to dryness.

**Dowex-50 Chromatography**

The more complex peptide mixtures from the G-15 Sephadex column were separated by Dowex-50 cation exchange chromatography following the
methodology of Schroeder (1967). Throughout the chromatographic separations, the Dowex-50 x 2 columns (0.9 x 60 cm) were heated to 38° by a circulating water bath. The gradient consisted of 300 ml of pyridine acetate buffer, about pH 3.1, (32.25 ml pyridine and 557 ml glacial acetic acid diluted to 2 liters) placed in the mixing chamber, and 600 ml of pyridine acetate buffer, about pH 5.0, (332.5 ml pyridine and 286.5 ml glacial acetic acid diluted to 2 liters) in the reservoir chamber. Samples were dissolved in 2 ml of deionized distilled water and adjusted to pH 2.0 with HCl prior to loading onto the column. The column was pumped at 18 ml/hr. and 1.5 ml fractions collected.

The fractions were assayed by the alkaline hydrolysis-ninhydrin method of Hirs (1967). A 0.1 ml aliquot of every other fraction was placed in a test tube and evaporated to dryness in a 108° oven. Then, 0.15 ml of 13.5 M NaOH was added to each tube and the tubes autoclaved at 121° for 20 min. at about 20 psi resulting in alkaline hydrolysis of any peptide present. On cooling, 0.25 ml of glacial acetic acid and 0.5 ml of ninhydrin solution (Spackman et al., 1958) were added per tube, and the tubes heated for 15 min. in a boiling water bath. The peptide peaks were detected visually. The peptide fractions of each peak were pooled and lyophilized to dryness.

**Peptide Mapping**

Peptide maps (Ingram, 1958) were prepared for each of the digests, peptide fractions from the Sephadex G-15 columns, and for each of the peptide(s) isolated by Dowex-50 chromatography.

Lyophilized samples containing about 0.2 μmoles of the peptide(s) were dissolved in 0.05 ml of a pyridine-acetic acid-water buffer (133:
4.6:1,862 v/v) (Michl, 1951), pH 6.4. The sample was spotted on a sheet of Whatman 3MM paper, previously moistened with the same buffer, and electrophoresed at 55 V/cm (3,000 V and 50 mA/sheet) for 75 min. After drying for several hours, the sheets were chromatographed in a descending second dimension with n-butanol-acetic acid-water (40:6:15 v/v) for 16 hr.

Following several hours of drying, the peptide maps were examined with a uv light before dipping in the various reagents available for peptide visualization. Tryptophan-containing peptides were often located by this uv light examination.

Peptide visualization was achieved by dipping the sheets in 0.1% ninhydrin dissolved in n-butanol-acetone (7:3 v/v). Purple spots, appearing in several hours, indicated the presence of peptides. The appearance of the spots could be hastened by warming the paper in a 60° oven. Following the ninhydrin treatment, tryptophan containing peptides were visualized by dipping the sheets in Ehrlich's reagent (Smith, 1953a). This consisted of 1 g of p-dimethylaminobenzaldehyde per 90 ml of acetone followed by the addition of 10 ml of concentrated HCl. The solid was mixed with the acetone in the dipping tray, the HCl added, and the sheets dipped immediately. Purple spots, appearing 5-10 min. after dipping, were indicative of tryptophan containing peptides. Histidine and tyrosine containing peptides were located with the Pauly reagent (Smith, 1960). A solution containing 10 ml of 4.5% NaNO₂ (w/v) and 100 ml of 0.9% sulfanilic acid (w/v) in 0.12 M HCl was prepared and allowed to stand about 2 min. Then, 110 ml of 10% (w/w) Na₂CO₃ solution was added and the sheets dipped. The immediate appearance of red spots indicated histidine and brown indicated tyrosine.
Arginine peptides were located with the Sakaguchi reagent (Smith, 1953b). The maps were first dipped in a 0.1% (w/v) solution of 8-hydroxyquinoline in acetone. After drying a few minutes, the maps were dipped in a 0.5 M NaOH solution containing 2 ml of bromine per liter. A red color indicated the presence of arginine. Sheets which had not previously been dipped in these reagents could be dipped in the Toennis-Kolb (1951) reagent which detects cysteine and methionine. The dipping solution consisted of 0.4 ml of 2.0 M HCl, 0.25 ml of 1.0 M KI, 4.0 ml of 0.002 M K$_2$PtCl$_6$, and 76 ml acetone. Bleached spots against a light pink background, appearing after about 30 min., were indicative of reducing sulfur.

**Peptide Purifications**

Most of the peptide fractions isolated by Dowex-50 chromatography still represented peptide mixtures. Also, some of the G-15 Sephadex fractions were simple enough to be purified as described below.

Further peptide purification was performed by preparative paper electrophoresis, preparative paper chromatography, or preparative peptide mapping. All were done under the conditions previously described for peptide mapping. Strips of sample were used when only electrophoresis or chromatography was performed. Peptides were located by inspection with an uv light where possible, by dipping guide strips in ninhydrin, and in the case of preparative peptide maps by dipping the whole map in 0.01% ninhydrin in n-butanol-acetone (7:3 v/v). When appropriate, the specific staining reagents were used on the guide strips only.

Peptides were recovered from the paper strips by elution with 1% pyridine-water (v/v) utilizing descending chromatography in a closed
chamber. Peptide spots from the preparative maps were cut into fine pieces and eluted in test tubes with several washes of 1% pyridine. The recovered peptide solutions were lyophilized to dryness.

**Dansyl-Edman Sequencing Procedure**

Dansylation was done according to the method of Gray (1967a). A lyophilized 5 nanomole peptide sample in a small tube was dissolved in 10-15 μl of fresh 0.2 M Na₂CO₃ and lyophilized to dryness. Then, 10-15 μl of deionized distilled water and a similar volume of 2.5 mg/ml dansyl chloride in acetone was added, the solution was mixed, and then incubated at 37° for 1 hr. The sample was lyophilized to dryness and 0.05 ml of constant boiling HCl added to the tube. The glass tube was drawn out, sealed, and placed in a 108° oven for 16-18 hr. After cooling, the tube was opened and placed in a vacuum desiccator until the contents were dry.

Dansyl amino acids were identified by ascending two-dimensional chromatography on 5 x 5 cm two sided polyamide plates (Cheng-Chin) according to the system of Hartley (1970). The standard dansyl amino acid mixture (Weiner et al., 1972) contained 0.025 nanomoles of each amino acid per 0.1 μl. The standard was spotted on one side of the plate and the sample, dissolved in 10 μl of 50% redistilled pyridine, was spotted on the other side of the plate at exactly the same position. After drying with hot air, chromatography was performed in one direction with solvent I and in a perpendicular direction with solvents II, III, and IV if necessary.

Solvent I  1.5% formic acid in water
Solvent II benzene-acetic acid 9:1 (v/v)
Solvent III ethyl acetate-acetic acid-methanol 20:1:1 (v/v)

Solvent IV 0.05 M Na₃PO₄ in 25% aqueous ethanol

Solvent I was discarded after use, while solvents II-IV were used repeatedly. The plates were dried between chromatographic steps with hot air and examined under a UV light to identify the unknown dansyl amino acid by its cochromatography with one of the dansyl amino acids in the standard mixture.

The Edman degradation was used to remove sequentially N-terminal residues from peptides between dansylation steps and was performed according to the method of Gray (1967b). About 0.1 μmole of lyophilized peptide was dissolved in 200 μl of 50% aqueous redistilled pyridine in a screw capped culture vial and 100 μl of 5% phenylisothiocyanate in redistilled pyridine added. The tube was flushed with nitrogen, capped, and placed in water bath for 1 hr. at 45°.

Following coupling, the excess reagent, water, pyridine, and reaction side products were removed by evacuating the tube while warming it in a 60° water bath. If the contents of the tube remained oily after this drying procedure, they were redissolved in 50 μl of ethanol and dried again.

Cleavage of the PTC-peptide was done by adding 200 μl of anhydrous trifluoroacetic acid, flushing with nitrogen, capping, and incubating in a water bath at 45° for 30 min. When glutamine was the suspected N-terminal residue, cleavage was for 1 hr. at 25°. This prevented cyclization (Konigsberg, 1962). The trifluoroacetic acid was removed by drying in a vacuum desiccator, 150 μl water added, and this extracted three times with 1.5 ml portions of ethyl acetate. The ex-
tract, containing diphenylthiourea, was discarded. A 3 μl aliquot of the water containing the peptide was saved for dansylation and the remaining N-1 peptide frozen and lyophilized to dryness prior to the next cycle of Edman degradation.

**Sequencing by Direct Identification of PTH Amino Acids**

Sequencing by the direct identification of PTH amino acids was according to the method of Peterson et al., (1972). A sample containing 0.1-0.2 μmole of peptide in a capped culture tube was dissolved in 100 μl of coupling buffer, evacuated briefly, and refilled with nitrogen. The coupling buffer consisted of 0.4 M dimethyl allylamine in 1-propanol-water (3:2 v/v), titrated to pH 9.5 with trifluoroacetic acid, and was stored at 4° under nitrogen. Then, 5 μl of phenylisothiocyanate (stored at 4° under nitrogen) was added to the tube under a nitrogen barrier, the tube capped, mixed till cloudy, and incubated at 50° for 20 min.

The coupled peptide in solution was extracted with 500 μl of benzene and the extract discarded. (The benzene was added under a nitrogen barrier, the tube capped, mixed, centrifuged, and the benzene removed under a stream of nitrogen.) The coupled peptide was dried by flushing the tube with nitrogen while warming it in a 50° water bath. The drying was completed by evacuation still maintaining the 50° temperature with the water bath.

Cleavage of the coupled peptide involved the addition under nitrogen of 100 μl of anhydrous trifluoroacetic acid and incubation at 50° for 7 min. The trifluoroacetic acid was removed by blowing to dryness with nitrogen. The PTC amino acid was extracted with three 0.5 ml portions of peroxide free ether, 1 x 10^{-4}M dithioerythritol. The extracts were pooled and dried with a stream of nitrogen. The remaining N-1 pep-
tide was dried with nitrogen prior to another cycle through the Edman degradation procedure.

The recovered PTC derivative was converted to the PTH form by adding 0.3 ml of 1.0 M HCl, flushing the tube with nitrogen, capping, and incubating at 80° for 10 min. Extraction with 1.0 ml of ethyl acetate and then two 0.5 ml portions followed. The extracts were pooled and dried with a stream of nitrogen. The aqueous phase was saved as it could contain histidine, arginine, or cysteine (Wharton and McCarthy, 1972). When the derivative was suspected of being PTC serine or threonine, the conversion to the PTH form was by a modified procedure (Edman, 1970). A 24 hr. room temperature conversion utilizing 0.3 ml of 0.5 M HCl was used, followed by extraction with ethyl acetate and drying of the extract as previously described.

The PTH amino acids were identified by TLC chromatography (Jeppson and Sjoquist, 1966) on 20 x 20 cm silica gel plates, which contained a fluorescent indicator (Eastman Chromogram Sheets 6060) and were activated by heating 30 min. in a 108° oven prior to use. The PTH unknowns were dissolved in 5-10 µl of dichloroethane, spotted on the plates, and dried with a stream of nitrogen. The standards solutions contained 10 µmoles/ml of each PTH amino acid in 90% acetic acid. The following four standard PTH amino acid solutions were used. The standards are listed in order of increasing chromatographic mobility.

Solution I  Asn, Asp, Tyr, Trp, Met, and Leu
Solution II  His, Ser, Gly, Phe, and Ile
Solution III Arg, Gln, Thr, Lys, Ala, and Val
Solution IV CySO₃H, Glu, Phenylthiourea, and Pro

About 2 µl of the appropriate standard was spotted next to the unknown
and dried with nitrogen. The chromatographic solvent system consisted of heptane, propionic acid, and dichloroethane 58:17:25 (v/v). The chromatographic chamber was lined with solvent saturated Whatman 3MM paper and the TLC plates equilibrated 30 min. in the chamber prior to running. When the solvent front approached the top of the plates, they were removed from the chamber, the fronts marked, and the plates blown dry with cool air. The dark spots of the PTH amino acids were plainly visible against the fluorescent background of the plates upon examination with long wave uv light. The spots were circled lightly with a pencil and the plates then sprayed with 0.5% ninhydrin in n-butanol, blown dry with cool air, and heated in a 108° oven for 10-15 min. (Inagami and Murakami, 1971). Color reactions for the following PTH amino acids were observed:

- Gly and Thr: orange
- Ser, Asp, carboxymethyl-Cys, Ala: purple
- Asp: brownish purple
- Asn: bright yellow
- Cysteic acid: very faint pink
- Gln: dark green
- Glu: grayish green
- Met, Met-sulfone: brownish yellow
- Pro: very faint bluish purple
- Tyr: light yellow

The color reactions and mobilities of the PTH amino acids served in most cases to identify them. However, some PTH amino acids did not give a characteristic color with ninhydrin and had similar mobilities. The PTH derivatives of valine, leucine and isoleucine were in this category.
PTH valine was clearly resolved from a combined leucine-isoleucine spot by thin layer chromatography on polyamide sheets which had a fluorescent background (Summers et al., 1973).

The standard solution contained 2 μmoles per ml of each PTH amino acid in methanol. About 0.5 μl was spotted on one side of a 5 x 5 cm two-sided polyamide sheet, and 2 μl of the unknown at the same position on the other side, again in methanol. Chromatography began after the chamber had equilibrated for 15 min. The solvent for chromatography in the first dimension consisted of toluene, n-pentane, and acetic acid (60:30:35 v/v) containing 250 mg/liter of 2-(4'-t-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxidiazole which formed the fluorescent background and was insoluble in the solvent system used for the second dimension. When finished in one dimension, the chromatogram was blown dry with hot air and run in a second dimension in a preequilibrated chamber containing 35% aqueous acetic acid, and again, blown dry with hot air when finished. Examination under short wave uv light revealed dark PTH amino acid spots against a fluorescent yellow background. Unknowns were identified by comparing their mobilities with those of the standards seen simultaneously on the other side of the plates.

Carboxypeptidase Digests

The C-terminal residues of most chymotryptic and tryptic peptides were determined, or verified if already known by carboxypeptidase digestion (Ambler, 1967). To 0.1 mg of lyophilized chymotryptic peptide (about 0.1 μmole) was added 25 μl of 0.05 M sodium borate buffer, pH 8.5, containing 0.005 mg of DFP treated carboxypeptidase A, with digestion taking place at room temperature. Aliquots of about 6 μl were withdrawn
at various time points and added to 2 ml of the standard amino acid analysis buffer and analyzed on the automatic amino acid analyzer. If lysine or histidine was suspected to be near the C-terminus, the digest was performed in a 0.06 M sodium borate buffer, pH 9.2 (Davie et al., 1959); alternatively, 0.0025 mg of DFP treated carboxypeptidase A, and a similar amount of DFP treated carboxypeptidase B, were used with the standard 0.05 M sodium borate buffer, pH 8.5. If glutamic or aspartic acid was a probable residue in the C-terminal area, the digest was performed at pH 5.1 in a 0.05 M sodium acetate buffer (Green and Stahman, 1952; Titani et al., 1962). The digestion of tryptic peptides was performed in 25 μl of 0.05 M sodium borate buffer, pH 8.5, which contained 0.0025 mg of DFP treated carboxypeptidase A and 0.0025 mg of DFP treated carboxypeptidase B (Ambler, 1972). Otherwise, the digestion of tryptic peptides and analysis of the results was identical to that of the typical chymotryptic peptide.
RESULTS

Hemerythrin Purification

Disc gel patterns of *P. agassizii* coelomic hemerythrin purified by either the method described, or by an older technique (Klippenstein, 1972a), were essentially the same. However, the new procedure gave higher yields and more effectively removed a reddish high molecular weight impurity (Fig. 4). The fact that the protein isolated from *P. agassizii* coelomic fluid was a hemerythrin was confirmed by the observation that its uv-visible metazide-spectrum and that of *G. gouldii* metazidehemerythrin were nearly identical (Fig. 5).

Native Oligomeric Molecular Weight

The molecular weight of the native hemerythrin as determined by Sephadex G-100 gel chromatography was 32,200±800 (Fig. 6). The molecular weight did not change at higher concentrations of either oxy- or metazide-hemerythrin during gel filtration on a calibrated G-75 column (Fig. 7). A calculated molecular weight (about 32,000) was obtained at a hemerythrin concentration of about 11 mg/ml. Because of the different sample volumes in these experiments, the leading boundaries of the hemerythrin and standard peaks were used to establish mobilities.

An $s_{20}$ of 3.30±.08 S was obtained from sedimentation velocity experiments (Fig. 8). Correction of this value to the standard condition, water at 20°, gave an $s_{20}^{0,\text{w}}$ of 3.65±.09 S. The sedimentation profile was a single symmetrical peak which ruled out the presence of a higher molecular weight hemerythrin oligomer at concentrations between
Figure 4: Elution pattern of *P. agassizii* coelomic hemerythrin after gel filtration on a Sephadex G-75 column, 2.5 x 90 cm. Flow rate of the 0.1 M NaCl eluting solvent was 20 ml/hr collecting 3 ml fractions.
Figure 5: The spectrum of *P. agassizii* coelomic metazidehemerythrin (---) and *G. gouldii* coelomic metazidehemerythrin (---).
Figure 6: Calibration curve of Sephadex G-100 column (2.5 x 90 cm) utilizing a 12 ml/hr flow rate of 0.1 M sodium phosphate buffer, pH 7.2, collection 3 ml fractions. Standards are G. gouldii hemerythrin monomer (O), myoglobin (●), β-lactoglobulin (□), ovalbumin (■), and bovine serum albumin (▲). Arrow indicates the elution position of P. agassizii hemerythrin. Each point represents the mean of at least three determinations. The line is a least squares fit to the points.
Figure 7: Typical elution of *P. agassizii* coelomic oxy- or metazide-hemerythrin in high concentrations (about 10 mg/ml) from a Sephadex G-75 column, 2.5 x 90 cm, with a flow rate of 20 ml/hr collecting 3 ml fractions. The standards were the same as those used in Fig. 6. Arrow indicates the elution position of *P. agassizii* coelomic hemerythrin.
Figure 8: The dependence of the sedimentation coefficient of *P. agasizii* coelomic metazidehemerythrin on protein concentration. The value of the sedimentation coefficient at zero protein concentration was obtained by extrapolation of this line to zero protein concentration. The line is a least squares fit to the points.
4.4 and 17.6 mg/ml (Fig. 9).

Replotting Fig. 6, using the logarithm of the Stoke's radii of bovine serum albumin, ovalbumin, and myoglobin gave a Stoke's radius of 26.2 Å for P. agassizii hemerythrin (Fig. 10). Calculation of a molecular weight based on the equation \( M = \frac{6\pi \eta N_a s}{(1-\psi p)} \) (Andrews, 1970), where \( a \) is the Stoke's radius, \( \eta \) the viscosity and \( p \) the density of the medium, \( s \) the sedimentation coefficient, \( N \) the Avogadro number, and \( \psi \) the partial specific volume of the molecule, gave a molecular weight of 40,600. The value of \( \psi \), 0.73, was determined from the amino acid composition of the protein (Schachman, 1957) as obtained during the study of its iron content.

**Subunit Molecular Weight**

The subunit molecular weight obtained from SDS-polyacrylamide gels was 12,800±100 (Fig. 11). The iron content, found to be 0.885±0.005%, allowed the calculation of a minimum subunit molecular weight of 12,600±100 based on 2 Fe/subunit (Klotz and Keresztes-Nagy, 1963).

**Absorption Measurements**

The extinction coefficient at 280 nm of pooled coelomic metazide-hemerythrin was calculated directly from the absorbance of a standard hemerythrin solution, the concentration of which had been determined by amino acid analysis. Based on a subunit molecular weight of 12,600, the extinction coefficient at 280 nm was 30,600 M\(^{-1}\) cm\(^{-1}\).

The circular dichroic spectrum (200-250 nm) of P. agassizii coelomic metazidehemerythrin is shown in Fig. 12 and closely matches that of G. gouldii coelomic hemerythrin (Darnall et al., 1969). The P.
Figure 9: Sedimentation pattern of *P. agassizii* pooled coelomic metazoid-hemerythrin in 0.5 M NaCl, pH 7.0. Photographed during a run at a speed of 52,640 rpm. Temperature 20°, protein concentration 17.6 mg/ml. Migration is from left to right.
Figure 10: A plot of the mobilities of selected standards from the Sephadex G-100 molecular weight study versus the logarithm of their Stoke's radii. The Stoke's radius of *P. agassizii* coelomic metazidehemerythrin was estimated based on its mobility during gel filtration (arrow marks this). The standards were myoglobin (●), ovalbumin (■), and bovine serum albumin (▲). The line is the least squares fit to the points.
Figure 11: Calibration curve of SDS-polyacrylamide gel electrophoresis.

Mobility determined as distance of protein from origin divided by the distance of dye front from origin. Standards are G. gouldii hemerythrin monomer (○), β-lactoglobulin (□), pepsin (▲), and ovalbumin (■). Arrow indicates the relative mobility of P. agassizii coelomic hemerythrin. Each point represents the mean of at least three determinations. The line is a least squares fit to the points.
log Molecular Weight

Rf

0 0.2 0.4 0.6 0.8 1.0
Figure 12: The circular dichroic spectrum of *P. agassizii* coelomic metazidehemerythrin (---) and *G. gouldii* coelomic hemerythrin (----) (Darnall et al., 1969) from about 200-250 nm.
agassizii circular dichroism curve was characteristic of a protein containing a high percentage of α-helical structure with absorption maxima at about 210 and 222 nm. The data were plotted as molecular ellipticity \( \theta \times 10^{-3} \text{deg. cm}^2 \text{decimole}^{-1} \) versus wavelength in nm. Molecular ellipticity was calculated from the equation \( \theta = \frac{(\theta)}{10.1c} \) where \( \theta \) is the observed absorption, \( l \) is pathlength in cm (0.05 cm for reported data), and \( c \) is the concentration of protein in g per cc. For the data plotted, \( c \) was converted to residues of amino acids per cc on the basis of 113 residues per mole of hemerythrin.

The percentage of α-helix in P. agassizii hemerythrin was estimated by dividing the absorption at 222 nm by -30,600 deg. cm/decimole which has been determined as the absorption of a purely α-helical molecule (Holzworth and Doty, 1965). Based on this calculation, P. agassizii coelomic metazidehemerythrin had about 82% α-helical structure.

**Cross-linking with Dimethyl Suberimide**

After cross-linking native P. agassizii hemerythrin with dimethyl suberimide, three species were obtained on SDS-polyacrylamide gels, corresponding to molecular weights of 13,000, 26,500, and 40,500 (Figs. 13 and 14). In gels heavily loaded with protein, traces of higher aggregates were detected, these appearing as broad smears near the tops of the gels.

**Analytical and Preparative Disc Gel Electrophoresis**

A total of nine protein bands were visible on analytical disc gels after electrophoresis of native oxy- or metazidehemerythrin (Fig.
Figure 13: SDS-polyacrylamide gels of cross-linked *P. agassizii* coelomic hemerythrin. Hemerythrin was cross-linked with 3.35 mg/ml of dimethyl suberimidate for 24 hr (gel 1) or 48 hr (gel 2) or with 6.7 mg/ml of reagent for 24 hr (gel 3) or 48 hr (gel 4). Gel 5 is uncross-linked hemerythrin.
Figure 14: Calibration curve of the SDS-polyacrylamide gels shown in Fig. 13. The cross-linked *P. agassizii* hemerythrin species had mobilities (arrows) which indicated molecular weights of 13,000, 26,500 and 40,500. The standards were identical to those used in Fig. 11 and the mobilities determined for proteins in the same manner.
log Molecular Weight
17), the same bands stained readily for iron. Similarly, nine orange-yellow metazidehemerythrin bands were plainly visible during preparative disc gel electrophoresis (Fig. 15). These bands were partially separated by preparative electrophoresis utilizing a steady elution buffer flow rate (Fig. 16), and fully separated when the flow rate was increased between the visibly eluting hemerythrin bands. Analytical disc gel electrophoresis of the peak fractions from a preparative electrophoresis run, which utilized a variable flow rate, confirmed that the hemerythrin bands had been separated from one another (Fig. 17). The same fractions when run in 8 M urea gels were resolved into one, two, or three subunit types per isolated native hemerythrin band (Fig. 18).

The fractions from preparative disc electrophoresis were characterized as follows. Each fraction was electrophoresed on 8 M urea gels. The mobilities of the bands observed were compared to the mobilities of bands from electrophoresis, in 8 M urea, of unfractionated hemerythrin (Table I). Unfractionated hemerythrin had three major subunit types (designated A, B, and C) and a minor subunit type (D). The preparative electrophoresis fractions had one, two, or three bands on these gels. These could be clearly identified by their mobilities as the subunit types observed in the unfractionated hemerythrin. Results of the quantitation of the subunit bands observed in fractions from preparative disc electrophoresis that are run on analytical disc electrophoresis gels, 8 M in urea, are shown in Table II. These results lead to the proposal that the native protein is a trimer and that the multiple proteins are due to the presence of 3 variant proteins. These results led to the trimer compositions shown in Table II, column 3.
Figure 15: The visible, unstained bands of pooled native *P. agassizii* coelomic metazidehemerythrin during preparative disc gel electrophoresis. This is a reverse print from a positive film image.
Figure 16: The elution pattern of the hemerythrin bands from preparative electrophoresis utilizing a steady 60 ml/hr flow rate of elution buffer collecting 2 ml fractions.
Figure 17: Disc gel electrophoresis of pooled hemerythrin (gel P₁) and fractions 1-9 obtained from preparative disc gel electrophoresis of the pooled hemerythrin which utilized the variable flow rate described in Materials and Methods. Composition of gels is identical to those used in preparative disc electrophoresis.
Figure 18: Disc gel electrophoresis in 8 M urea of pooled coelomic *P. agassizii* hemerythrin (gels $P_1$ and $P_2$) and fractions 1-9 from preparative disc electrophoresis.
Table I

Number and Mobilities of Subunits in Proteins

Isolated by Preparative Disc Gel Electrophoresis

<table>
<thead>
<tr>
<th>Band Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Bands in 8 M Urea Gels</th>
<th>Mobilities of Bands&lt;sup&gt;b&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>.28</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
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<td>---</td>
</tr>
<tr>
<td>Pooled Hemerythrin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>.28</td>
</tr>
</tbody>
</table>

<sup>a</sup>Band numbers designate order of elution from preparative disc electrophoresis gels.

<sup>b</sup>Band mobilities computed as migration distance of the proteins divided by the migration distance of the dye front (Bromphenol Blue).

<sup>c</sup>Pooled hemerythrin mobilities are the mean values from six gels.
### TABLE II

Subunit Quantitation and a Proposed Trimer Model

<table>
<thead>
<tr>
<th>Band Number</th>
<th>Quantity of Subunit Type (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Proposed Composition</th>
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<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
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<td>(17</td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td></td>
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<td>(56</td>
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<tr>
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<tr>
<td>---</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>

<sup>a</sup>Subunit quantities reported are the mean values from a minimum of four gels.

<sup>b</sup>These subunit values are obtained from gels destained by diffusion while all other values are from electrophoretically destained gels.
The preparative electrophoresis of pooled *P. agassizii* hemerythrin, on a gel 8 M in urea, resulted in the elution profile shown in Fig. 19. Total protein recovery was estimated at about 50%. The peaks of subunit types A and D were well separated from the incompletely resolved peaks of subunit types B and C. Each of the peaks, when pooled, had primarily one band when electrophoresed on 10% acrylamide gels, 8 M in urea (Fig. 20).

**Amino Acid Composition**

The amino acid composition of pooled *P. agassizii* coelomic hemerythrin, and that of the subunit types recovered from the DEAE-cellulose column, 8 M in urea, and the preparative electrophoresis in 8 M urea, are shown in Table III.

**Isoelectric Focusing**

The pattern of native pooled coelomic *P. agassizii* hemerythrin on isoelectric focusing gels (Fig. 21) was more complex than that observed after disc gel electrophoresis. Over twenty protein bands were visible. The isoelectric points of the bands ranged from 5.36 to 7.92 ± 1.47 based on the regression line from the pH gradient shown in Fig. 22.

Similar gels, but 8 M in urea, were run on pooled hemerythrin and each of the peak fractions separated by preparative disc gel electrophoresis in order to determine subunit composition by this method. The pooled hemerythrin had five bands and the preparative electrophoresis fractions two to five bands (Fig. 23). The subunit isoelectric points on the pooled hemerythrin gel were calculated as 1, 6.81; 2, 6.53; 3, 6.40; 4, 6.22; and 5, 6.12 all ±1.63 based on the pH gradient shown in
Figure 19: The elution pattern from preparative disc electrophoresis of pooled coelomic *P. agassizii* hemerythrin. Gels were 7.5% in acrylamide and 8 M in urea. Flow rate of eluting buffer was 60 ml/hr and 2 ml fractions were collected.
Figure 20: Disc gel electrophoresis patterns of pooled peak fractions obtained from preparative electrophoresis, in 8 M urea, of pooled coelomic *P. agassizii* hemerythin. The gels were 10% acrylamide and 8 M in urea.
### Table III

The Amino Acid Composition of Pooled Coelomic

*P. agassizii* Hemerythrin and the Isolated Subunit Types

<table>
<thead>
<tr>
<th></th>
<th>Pooled&lt;sup&gt;a&lt;/sup&gt;</th>
<th>A&lt;sup&gt;b&lt;/sup&gt;</th>
<th>A&lt;sup&gt;c&lt;/sup&gt;</th>
<th>B&lt;sup&gt;b&lt;/sup&gt;</th>
<th>B&lt;sup&gt;c&lt;/sup&gt;</th>
<th>C&lt;sup&gt;c&lt;/sup&gt;</th>
<th>D&lt;sup&gt;c&lt;/sup&gt;</th>
<th>G. gouldii&lt;sup&gt;f&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Lys</td>
<td>9.5</td>
<td>10.4</td>
<td>9.4</td>
<td>8.7</td>
<td>9.1</td>
<td>8.6</td>
<td>7.0</td>
<td>11</td>
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<tr>
<td>His</td>
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<td>4.6</td>
<td>3.4</td>
<td>4.7</td>
<td>4.6</td>
<td>3.8</td>
<td>7</td>
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<tr>
<td>Arg</td>
<td>.7</td>
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<td>0.9</td>
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<td>1.0</td>
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<td>3</td>
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<tr>
<td>Asp</td>
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<td>8.5</td>
<td>12.7</td>
<td>11.6</td>
<td>11.9</td>
<td>13.7</td>
<td>17</td>
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<tr>
<td>Thr</td>
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<td>4.0</td>
<td>4.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.8</td>
<td>4.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>Ser</td>
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<td>4.8</td>
<td>5.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.2</td>
<td>7.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.2&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>14.0</td>
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<tr>
<td>Cys</td>
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<td>---</td>
<td>---</td>
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<td>---</td>
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<tr>
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<td>4</td>
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<td>1.1</td>
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<tr>
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<td>3.6</td>
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<td>3.1</td>
<td>4.7</td>
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<tr>
<td>Leu</td>
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<td>7.1</td>
<td>6.9</td>
<td>6.9</td>
<td>6.7</td>
<td>5.2</td>
<td>8</td>
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<tr>
<td>Tyr</td>
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<td>7.1</td>
<td>7.0</td>
<td>6.0</td>
<td>6.6</td>
<td>6.2</td>
<td>7.1</td>
<td>5</td>
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<tr>
<td>Phe</td>
<td>8.7</td>
<td>8.7</td>
<td>8.3</td>
<td>7.3</td>
<td>9.5</td>
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<tr>
<td>Trp</td>
<td>2.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.3&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Average of 5 analyses. Values calculated on the basis of 110 residues/mole.

<sup>b</sup>Subunit types isolated by DEAE-cellulose chromatography. Values calcu-
Table III (Continued)

lated from a single analysis on the basis of 110 residues per mole.

\( ^{c} \text{Subunit types isolated by preparative electrophoresis.} \)

\( ^{c} \text{Values calculated from a single analysis on the basis of 12,600 g per mole of hemerythrin monomer.} \)

\( ^{d} \text{Corrected for degradation due to hydrolysis.} \)

\( ^{e} \text{All tryptophan values calculated by using pooled hemerythrin tyr to trp molar ratio of 2.70:1.} \)

\( ^{f} \text{From amino acid sequence (Klippenstein et al., 1968).} \)
Figure 21: Isoelectric focusing pattern of native pooled *P. agassizii* coelomic metazidehemerythrin on a 7% acrylamide gel, 2% in ampholytes of the pH 3-10 range.
Figure 22: The pH gradient of a typical isoelectric focusing gel, 2% in ampholytes of the pH 3-10 range. The line is the least squares fit to the points between and including the solid points.
The graph illustrates the relationship between pH and distance. It shows a linear trend with pH increasing as the distance increases.
Figure 23: The gel isoelectric focusing pattern of pooled coelomic hemerythrin and preparative electrophoresis bands 1-9. The gels contained 7% acrylamide, 8 M urea, and 4% of the pH 3-10 ampholytes. Major bands 1-5 labeled from - to +.
The five subunit types were correlated with the three electrophoresis subunit types in the following manner. Hemythrin trimers, fractionated by preparative electrophoresis and of known subunit compositions, were then run on isoelectric focusing gels, 8 M in urea (Fig. 23). Preparative electrophoresis band 9, which contains trimers of only one electrophoretic subunit type, A, had isoelectric focusing subunit types 1 and 2. Preparative electrophoresis band 8, consisting of trimer type A₂B, had isoelectric focusing subunit types 1, 2, and 3. The new type, 3, must represent electrophoretic subunit type B. Also, isoelectric subunit types 1 and 2 must represent electrophoretic subunit type A. Preparative electrophoresis band 7, consisting of trimer type A₂C, lacked isoelectric focusing subunit type 3, but contained 1, 2, 4, and 5. The new isoelectric focusing subunit types, 4 and 5, must be equivalent to electrophoretic subunit type C.

The other trimers from preparative electrophoresis bands 6-2 had gel isoelectric focusing band patterns consistent with the identification of electrophoretic subunit type A as isoelectric focusing subunit types 1 and 2; B equivalent to 3; and C to 4 and 5 (see Table IV).

The narrow range ampholytes, pH 5-8, were also used in 8 M urea gels to fractionate pooled hemythrin and peak fractions 1-9 from preparative electrophoresis. On these gels (Fig. 25), pooled hemythrin had the five bands seen previously in the 8 M urea pH 3-10 isoelectric focusing gels. The isoelectric points for the major bands in the pooled hemythrin gel were 1, 6.40; 2, 6.06; 3, 5.94; 4, 5.75; and 5, 5.64 all ±0.58 based on the pH gradient shown in Fig. 26. In addition, the pooled gel had at least six minor bands.
Figure 24: The pH gradient of a typical isoelectric focusing gel, 4% in ampholytes of the pH 3-10 range, and 8 M in urea. The line is the least squares fit to the points between and including the solid points.
TABLE IV

Correlations of Subunit Bands from Electrophoresis and Isoelectric Focusing

<table>
<thead>
<tr>
<th>Preparative Electrophoresis Band Number</th>
<th>Trimer Composition on 8 M Urea Electrophoresis Gels</th>
<th>Trimer Subunit Composition on 8 M Urea Isoelectric Focusing Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>A₃</td>
<td>1, 2 -- -- --</td>
</tr>
<tr>
<td>8</td>
<td>A₂B</td>
<td>1, 2, 3 -- --</td>
</tr>
<tr>
<td>7</td>
<td>A₂C</td>
<td>1, 2, -- 4, 5</td>
</tr>
<tr>
<td>6</td>
<td>AB₂</td>
<td>1, 2, 3, -- --</td>
</tr>
<tr>
<td>5</td>
<td>ABC</td>
<td>1, 2, 3, 4, 5</td>
</tr>
<tr>
<td>4</td>
<td>AC₂+B₃</td>
<td>1, 2, 3, 4, 5</td>
</tr>
<tr>
<td>3</td>
<td>B₂C</td>
<td>-- -- 3, 4, 5</td>
</tr>
<tr>
<td>2</td>
<td>BC₂</td>
<td>-- -- 3, 4, 5</td>
</tr>
<tr>
<td>1</td>
<td>D*</td>
<td>Two Bands*</td>
</tr>
</tbody>
</table>

*Hemerythrin D electrophoresis very rapidly on disc gels. It has two subunit bands of very low pI on 8 M urea isoelectric focusing gels. It does not fall into general P. agassizii coelomic hemerythrin pattern and is present in trace quantities.
Figure 25: The gel isoelectric focusing band pattern of pooled coelomic *P. agassizii* hemerythrin and preparative electrophoresis bands 1-9. The gels contained 7% acrylamide, 8 M urea, and 4% of the pH 5-8 ampholytes. Major bands 1-5 labeled from - to +.
Figure 26: The pH gradient of a typical isoelectric focusing gel, 4% in ampholytes of the pH 5-8 range, and 8 M in urea. The line is the least squares fit to the points between and including the solid points.
A general pattern of band distribution was observed for the preparative electrophoresis peak fractions in both the pH 3-10 and pH 5-8 isoelectric focusing gels which were 8 M in urea. The more rapidly a native hemerythrin band eluted during preparative gel electrophoresis, the larger the amount of subunit types of a low isoelectric point which were present in the 8 M urea electrofocusing gels.

**Variant Electrophoretic Patterns**

Since the gel electrophoresis pattern of native coelomic *P. agassizii* hemerythrin was very complex, and a variant has been characterized in the coelomic hemerythrin of *G. gouldii* (Klippenstein, 1972a), it was considered probable that variant hemerythrins occurred in *P. agassizii*. About half of the 100 individually examined *P. agassizii* worms had coelomic hemerythrin electrophoretic band patterns which differed from those observed when using hemerythrin pooled from many animals. All the individual variant hemerythrin patterns fell into one of five recognizable classes (Fig. 27). The following percentages represent the number of individuals assigned to each class: #1, 12%; #2, 12%; #3, 8%; #4, 12%; and #5, 4%. The remaining individually examined worms had band patterns indistinguishable from the pooled hemerythrin electrophoretic band pattern.

**Hybridization**

Hemerythrin trimers, isolated via preparative electrophoresis and shown to be homogeneous (Fig. 17), had many additional bands when electrophoresed again after storage for 7-10 days at 4° (Fig. 28). A number of the new bands were recognized as previously observed electro-
Figure 27: Band patterns of native *P. agassizii* hemerythrin variant classes 1-5 on 7% acrylamide disc gels.
Figure 28: Disc gel band patterns of hemerythrin bands 1-9 from preparative electrophoresis of native pooled coelomic P. agassizii metazidehemerythrin. The hemerythrin was stored 7-10 days at 4° prior to this electrophoresis. The gels were identical in composition to those used for the preparative electrophoresis.
phoretic species of trimeric hemerythrin. However, not all the new bands could be identified. The unidentified bands probably represented trimers, composed at least in part, of the minor subunit variant types observed in the 8 M urea isoelectric focusing gels.

Chymotryptic Digest, Peptide Separation, and Purification

A peptide map of the chymotryptic digest of pooled P. agassizii hemerythrin is shown in Fig. 29. The digest was fractionated by gel filtration on a Sephadex G-15 column and seven fractions were obtained (Fig. 30). Peptide maps were prepared for each of these fractions and used as guides for further purification steps.

The μmoles of amino acids in the Sephadex fractions were as follows: G-1, 185.2; G-2, 662.4; G-3, 254.8; G-4, 30.2; G-5, 7.5; G-6, 8.5; and G-7, 5.2. Assuming 113 residues per mole of hemerythrin, the 15 μmoles used for the digest contained 1695 μmoles of amino acids. The Sephadex fractions, G-1 to G-7, contained 1153.8 μmoles of amino acids so the total recovery was 68%.

Sephadex fraction G-1 was a complex peptide mixture and was further fractionated by cation exchange chromatography on a column of Dowex-50 which yielded fractions S-1 through S-12. Of these, only S-11 contained a pure peptide. Preparative electrophoresis was performed on fraction S-1 but the yield was too low for further characterization. All of the remaining fractions were of sufficient complexity to require preparative electrophoresis-chromatography.

A peptide map of fraction G-2 revealed a complex mixture of peptides which was subjected to Dowex-50 chromatography yielding fractions S-1 through S-12. Of these, only S-12 appeared to be a pure pep-
Figure 29: Peptide map of the chymotryptic peptides of the *P. agassizii* pooled coelomic hemerythrin digest. Electrophoresis for 1 1/4 hr at 55 V/cm using a pH 6.4 pyridine-acetate buffer system. Descending chromatography for 16 hr with an n-butanol-acetic acid-water system followed the electrophoresis. Ninhydrin-positive spots are shown.
Figure 30: Elution pattern of the chymotryptic peptides of pooled *P. agassizii* hemerythrin separated by gel filtration on a Sephadex G-15 column, 1.5 x 90 cm, with a flow rate of 5-7 ml/hr of 0.1 M NH₄HCO₃, pH 8.0, collecting 1.5 ml fractions. The fractions were pooled as follows: G-1, tubes 33-41; G-2, tubes 42-52; G-3, tubes 53-66; G-4, tubes 67-79; G-5, tubes 80-88; G-6, tubes 89-97; and G-7, tubes 98-111.
tide. Preparative electrophoresis was performed on fractions S-1, S-8, and S-9. All of the remaining fractions were further fractionated by preparative electrophoresis-chromatography.

Sephadex fraction G-3 had a large number of basic peptides and appeared to be an excellent candidate for Dowex-50 chromatography. Due to a failure of the fraction collector, only the final two peptide fractions were recovered, S-1 and S-2. These both required further purification by preparative electrophoresis.

The peptides of fraction G-4 were separated by preparative chromatography while fraction G-5 was of such low yield and of such complexity that it was not further characterized.

Peptide fractions G-6 and G-7 were both purified by preparative chromatography and each consisted essentially of a dipeptide.

The composition, sequence, and major contamination of the characterized chymotryptic peptides are shown in Table V. The yields, chromatographic properties, and color reactions of these peptides are shown in Table VI.

Subunit Separation

A DEAE-cellulose column, which utilized a stepwise gradient of NaCl in a buffer which was 8 M in urea, was used to prepare purified subunit types for sequence analysis (Fig. 31). The first two peaks represented breakthrough peaks and had band patterns on 7% acrylamide gels, 8 M in urea, which were nearly identical to pooled hemerythrin. The three peaks labeled A, B, and C had the band patterns shown in Fig. 32. The mobilities of the bands in these gels, when compared to pooled hemerythrin, served to identify the hemerythrin subunit type (Table VII).
<table>
<thead>
<tr>
<th>Peptide</th>
<th>Composition</th>
<th>Contamination Maximum (%)</th>
<th>Proposed Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1 S-2 el-A-1</td>
<td>Asp 1.83 Thr 0.92 Ser 1.85 Glu 1.13 Gly 1.36 Ala 3.95 Tyr (1)¹(28% Leu)</td>
<td>x-x-Ser-Asp-Gly-Gln-(Asp, Thr Ser, Ala, Ala, Ala, Ala, Tyr)</td>
<td></td>
</tr>
<tr>
<td>G-1 S-2 el-A-2</td>
<td>Asp 2.16 Thr 0.93 Ser 2.09 Glu 1.73 Gly 2.32 Ala 3.02 Val 1.06 Leu 0.93 Tyr 0.80 (48% Pro)¹</td>
<td>x-x-Asp-Gln-Gly-Gln-x-Thr- (Asp, Ser, Ser, Gly, Ala, Ala, Ala, Val, Leu, Tyr)</td>
<td></td>
</tr>
<tr>
<td>G-1 S-7 el-A-1</td>
<td>Lys 1.08 His 1.03 Asp 3.09 Glu 2.87 Leu 2.01 Phe 0.90 (12% Gly)</td>
<td>Glu-Asp-Leu-Asp-Asp-x-His-(Glu Lys)-Gln-(Leu,Phe)</td>
<td></td>
</tr>
<tr>
<td>G-1 S-7 el-N-2</td>
<td>Lys 1.05 Ser 0.98 Glu 1.09 Gly 2.07 Ala 2.06 Val 1.57 Leu 1.04 Tyr 0.88 (31% Asp)</td>
<td>x-x-Glu-x-x-Ala-x-Gly-(Lys, Ser Gly, Ala, Val, Val, Leu, Tyr)</td>
<td></td>
</tr>
<tr>
<td>G-1 S-8 el-N-1</td>
<td>Lys 1.08 Asp 1.44 Thr 0.57 Ser 0.80 Glu 1.16 Gly 0.97 Ala 0.98 Tyr (1)³ (28% Leu)</td>
<td>x-Gly-x-Glu-(Lys, Asp, Ser, Ala, 1/2 Asp, 1/2 Thr, Tyr)</td>
<td></td>
</tr>
<tr>
<td>G-1 S-11</td>
<td>Lys 1.12 His 1.67 Asp 1.17 Glu 2.19 Gly 1.09 Ala 1.94 Phe 0.81 (20% Leu)</td>
<td>Gly-Ala-His-Lys-Ala-Glu-Gln-Gln-His-Asp-Phe</td>
<td></td>
</tr>
<tr>
<td>G-2 S-2 el-A-2</td>
<td>Asp 0.33 Thr 1.23 Ser 0.88 Glu 0.42 Gly 1.01 Ala 1.15 Trp (1)² (12% Leu)</td>
<td>x-x-Gly-1/2 Glu-(1/2 Asp, Thr, Ser, Ala, Trp)</td>
<td></td>
</tr>
<tr>
<td>G-2 S-2 el-A-3</td>
<td>Asp 1.09 Ser 1.88 Phe 1.05 (48% Glu)³</td>
<td>Asp-(Ser, Ser, Phe)</td>
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<tr>
<td>G-2 S-2 el-A-4</td>
<td>Asp 0.95 Ser 0.86 Ala 1.12 Phe 1.07 (25% Glu)</td>
<td>Asp-Ala-Ser-Phe</td>
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<tr>
<td>G-2 S-2 el-A-6</td>
<td>Glu 3.10 Ala 1.96 Met 0.97 Leu 0.90 Phe 1.08 (10% Gly)</td>
<td>Leu-Met-Glu-Glu-x-Ala-(Glu, Ala, Phe)</td>
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<tr>
<td>Protein</td>
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</table>
| **G-2 S-2 el-A-7** | Glu 2.72 Ala 2.43 Met 0.75 Leu 1.03 Phe 1.08 (68% Gly; 42% Ser)  
(Glu, Glu, Glu, Leu, Phe) (Ala-Ala-Met) |
| **G-2 S-2 el-N-2** | Asp 1.16 Ser 0.80 Gly 0.95 Ala 2.10 (28% Glu) (Asn, Ser, Gly, Ala, Ala) |
| **G-2 S-2 el-N-3** | Asp 2.00 Ala 5.06 Tyr 0.93 (66% Glu; 53% Ser) (Asn, Asn, Ala, Ala, Ala)- (Ala-Ala-Tyr) |
| **G-2 S-2 el-N-5** | Glu 2.06 Pro 2.29 Gly 0.85 Val 1.96 Leu 1.05 Tyr 0.76 Phe 1.06 (19% Gly) Gly-x-Val-x-(Pro)-(Gln, Pro, Phe)-(Gln-Leu)-Val-Tyr |
| **G-2 S-4 el-A** | Lys 1.01 His 1.00 Asp 3.91 Glu 2.33 Leu 1.92 Tyr 0.95 Phe 0.87 (11% Gly) Tyr-Asp-Asp-Leu-Asp-Asp-Gln-Asn-(Lys, Glx)-Leu-Phe |
| **G-2 S-5 el-B-1** | Lys 0.83 Glu 1.16 Gly 1.00 Leu 1.01 (8% Ser) Lys-Gly-Gln-Leu |
| **G-2 S-5 el-N-2** | Lys 1.00 Asp 2.16 Ile 1.99 Phe 0.89 (16% Leu) (Lys, Ile, Asn)-Ile-Asp-Phe |
| **G-2 S-5 el-N-3** | Lys 1.01 Asp 1.06 Thr 0.98 Ile 1.89 Phe 1.06 (22% Glu) Ile-x-Thr-(Ile, Asp, Lys)-Phe |
| **G-2 S-7 Ch-1** | Lys 1.12 His 1.59 Asp 0.94 Glu 0.99 Gly 1.04 Ala 3.09 Phe 0.85 (4% Ser) Asp-Ala-His-Lys-Glu-Ala-Ala-His-Gly-Phe |
| **G-2 S-9 el-N-1** | Lys 1.00 His 0.80 Asp 1.46 Thr 0.64 Ile 1.93 Phe 1.18 (14% Gly) His-Ile-Lys-Thr-Ile-Asp-Phe Asn |
| **G-2 S-9 el-B-2** | Lys 1.98 Gly 1.03 Leu 0.99 (11% Tyr) Lys-Gly-Lys-Leu |
| **G-2 S-12** | Lys 0.96 His 1.02 Glu 0.99 Gly 1.00 Ala 1.06 Phe 0.97 (11% Asp) (Lys, His, Glx, Gly, Ala)-Phe |
| **G-3 S-1 el-B** | Lys 1.05 Thr 0.90 Phe 1.20 (27% Leu) Lys-Thr-Phe |
G-3 S-1 el-B\textsuperscript{e} & Lys 1.10 Ala 0.96 Val 0.85 Tyr 0.95 & Val$\rightarrow$Ala$\rightarrow$(Lys)$\rightarrow$Tyr \\
& (8% Leu) & \\
G-3 S-2 el-N & Lys 1.09 Ala 0.88 Asp 1.03 Trp (1)\textsuperscript{b} & Ala$\rightarrow$Lys$\rightarrow$Asp$\rightarrow$Trp  \\
& (3% Gly) & \\
G-3 S-2 el-B & Lys 1.06 Tyr 0.94 (7% Gly) & Lys$\rightarrow$Tyr\textsuperscript{d}  \\
G-4 Ch-1 & Arg 0.88 Asp 1.05 Ala 1.06 Trp (1)\textsuperscript{b} & (Ala, Arg, Asp, Trp)  \\
& (21% Ser) & \\
G-4 Ch-2 & Ser 1.01 Gly 1.14 Tyr 0.85 (36% Asp) & Ser$\rightarrow$(Gly)$\rightarrow$Tyr  \\
G-6 Ch-1 & Thr (1) Trp (1)\textsuperscript{b,c} (3% Gly) & Thr$\rightarrow$Trp\textsuperscript{c}  \\
G-7 Ch-3 & Val (1) Trp (1)\textsuperscript{b,c} (4% Gly) & Val$\rightarrow$Trp\textsuperscript{c}  \\
\textsuperscript{a}Pauly positive for Tyr.  \\
\textsuperscript{b}Ehrlich positive for Trp.  \\
\textsuperscript{c}Quantitated by 280 nm absorption.  \\
\textsuperscript{d}Sequenced and quantitated by amino acid analysis of N-1 peptide.  \\
\textsuperscript{e}These peptides were isolated and sequenced together. This was possible because the ratio of the tripeptide to the tetrapeptide was 1:3 and Lys was the only residue in common to both peptides.  \\
\textsuperscript{f}A high level of contamination may actually be indicative of variance in the peptide.
TABLE VI. Properties and yields of the pooled coelomic *P. agassizii* hemerythrin chymotryptic peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>% Yield</th>
<th>Electrophoretic mobility</th>
<th>Chromatographic mobility</th>
<th>Color reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1 S-2 e1-A-1</td>
<td>1.97</td>
<td>+4.0</td>
<td>2.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-1 S-2 e1-A-2</td>
<td>1.83</td>
<td>+4.5</td>
<td>7.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-1 S-7 e1-A-1</td>
<td>2.09</td>
<td>+8.0</td>
<td>5.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-1 S-7 e1-N-2</td>
<td>0.56</td>
<td>-1.0</td>
<td>12.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-1 S-8 e1-N-1</td>
<td>0.96</td>
<td>-1.0</td>
<td>2.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-1 S-11</td>
<td>4.29</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-2 S-2 e1-A-2</td>
<td>0.86</td>
<td>+9.5</td>
<td>9.0</td>
<td>Ehrlich</td>
</tr>
<tr>
<td>G-2 S-2 e1-A-3</td>
<td>1.04</td>
<td>+6.5</td>
<td>16.0</td>
<td>None</td>
</tr>
<tr>
<td>G-2 S-2 e1-A-4</td>
<td>3.62</td>
<td>+6.5</td>
<td>18.5</td>
<td>None</td>
</tr>
<tr>
<td>G-2 S-2 e1-A-6</td>
<td>3.72</td>
<td>+10.0</td>
<td>23.5</td>
<td>Toennis-Kolb</td>
</tr>
<tr>
<td>G-2 S-2 e1-A-7</td>
<td>1.03</td>
<td>+11.0</td>
<td>32.0</td>
<td>Toennis-Kolb</td>
</tr>
<tr>
<td>G-2 S-2 e1-N-2</td>
<td>1.28</td>
<td>-2.0</td>
<td>8.0</td>
<td>None</td>
</tr>
<tr>
<td>G-2 S-2 e1-N-3</td>
<td>0.48</td>
<td>-2.0</td>
<td>13.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-2 S-2 e1-N-5</td>
<td>2.26</td>
<td>-2.0</td>
<td>25.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-2 S-4 e1-A</td>
<td>6.13</td>
<td>+3.0</td>
<td>8.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-2 S-5 e1-B-1</td>
<td>3.28</td>
<td>-11.5</td>
<td>8.0</td>
<td>None</td>
</tr>
<tr>
<td>Sample</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>G-2 S-5 el-N-2</td>
<td>0.67</td>
<td>-2.0</td>
<td>13.0</td>
<td>None</td>
</tr>
<tr>
<td>G-2 S-5 el-N-3</td>
<td>0.97</td>
<td>-2.0</td>
<td>17.0</td>
<td>None</td>
</tr>
<tr>
<td>G-2 S-7 Ch-1</td>
<td>2.46</td>
<td>N.D.</td>
<td>2.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-2 S-9 el-N-1</td>
<td>2.98</td>
<td>+0.5</td>
<td>6.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-2 S-9 el-B-2</td>
<td>9.52</td>
<td>-18.0</td>
<td>7.0</td>
<td>None</td>
</tr>
<tr>
<td>G-2 S-12</td>
<td>10.91</td>
<td>-11.0</td>
<td>0.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-3 S-1 el-B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.9</td>
<td>-19.0</td>
<td>7.0</td>
<td>None</td>
</tr>
<tr>
<td>G-3 S-1 el-B&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.3</td>
<td>-19.0</td>
<td>7.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-3 S-2 el-N</td>
<td>3.4</td>
<td>-3.0</td>
<td>7.5</td>
<td>Ehrlich</td>
</tr>
<tr>
<td>G-3 S-2 el-B</td>
<td>8.5</td>
<td>-15.0</td>
<td>7.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-4 Ch-1</td>
<td>3.5</td>
<td>-3.5</td>
<td>6.0</td>
<td>Ehrlich, Sakaguchi</td>
</tr>
<tr>
<td>G-4 Ch-2</td>
<td>2.7</td>
<td>-3.5</td>
<td>14.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>G-6 Ch-1</td>
<td>11.0</td>
<td>-2.0</td>
<td>24.0</td>
<td>Ehrlich</td>
</tr>
<tr>
<td>G-7 Ch-3</td>
<td>16.0</td>
<td>-3.5</td>
<td>33.0</td>
<td>Ehrlich</td>
</tr>
</tbody>
</table>

<sup>a</sup>Yields were calculated as the percentage of peptide recovered of the original 15 μmoles of protein used for the chymotryptic digestion.

<sup>b</sup>These two peptides cochromatographed and coelectrophoresed in the systems used for peptide isolation and purification.
N.D. = data not obtained.
Figure 31: Elution of pooled coelomic hemerythrin from DEAE-cellulose, 8 M in urea. The flow rate of the 0.01 M Tris-HCl buffer, pH 8.0, 8 M in urea, was initially 252 ml/hr which was lowered to 126 ml/hr. Arrows indicate where the gradient steps of NaCl began; 1, 0.0044 M; 2, 0.0366 M; and 3, 0.0662 M.
Figure 32: Disc gels of peaks A, B, and C isolated via the DEAE-cellulose column, 8 M in urea (Fig. 31), and pooled unfractionated hemerythrin. The gels were 7% acrylamide and 8 M in urea.
TABLE VII

Gel Electrophoretic Mobilities of Hemerythrin Subunit Types Isolated by DEAE-Cellulose Chromatography in 8 M Urea

<table>
<thead>
<tr>
<th>DEAE-Cellulose</th>
<th>Mobility on Gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak Designation</td>
<td>8 M in Urea</td>
</tr>
<tr>
<td>A</td>
<td>.25</td>
</tr>
<tr>
<td>B</td>
<td>---</td>
</tr>
<tr>
<td>C*</td>
<td>---</td>
</tr>
<tr>
<td>Pooled Hemerythrin</td>
<td>.25</td>
</tr>
</tbody>
</table>

Subunit Types: A, B, C

*Hemerythrin subunit type C not cleanly separated from B with DEAE-cellulose chromatography 8 M in urea.
Peak C consisted of a mixture of subunit types B and C, while peaks A and B represented their respective purified subunit types. The recovery of hemerythrin from the DEAE-cellulose column was about 9.5% as subunit type A (based on amino acid analysis of the peptide fractions from the tryptic digestions), 6% as subunit type B, and 9% as the mixture of subunit types B and C (all recoveries based on total protein initially applied to the column). The latter recoveries were based on direct hydrolysis of aliquots of the peaks and quantitation by amino acid analysis.

**Tryptic Digestion of Subunit Type A, Peptide Separation and Purification**

A peptide map of the tryptic digest of subunit type A is shown in Fig. 33. The digest was fractionated by gel filtration on a Sephadex G-15 column into seven fractions (Fig. 34). Peptide maps were prepared for each of these fractions and were used as guides to further purification steps, if necessary. The μmoles of amino acids in the Sephadex fractions were as follows: A-TT-1, 87.1; A-TT-2, 57.0; A-TT-3, 25.3; A-TT-4, 20.7; A-TT-5, traces; A-TT-6, 0.78; and A-TT-7, traces.

Fraction A-TT-1 was a complex peptide mixture and was further fractionated by Dowex-50 cation exchange chromatography which resulted in fractions S-1 through S-11. Of these, only S-8 contained a pure peptide. Fractions S-5 and S-10 were purified by preparative chromatography, while fractions S-6 and S-7 required purification by preparative electrophoresis. The remaining fractions were of too low a yield for further characterization.

The peptides of fraction A-TT-2 were further fractionated by
Figure 33: Peptide map of the tryptic peptides of *P. agassizii* coelomic hemerythrin subunit type A isolated by DEAE-cellulose chromatography. Conditions of electrophoresis and chromatography identical to those of Fig. 29. Ninhydrin positive spots shown.
Figure 34: Elution pattern of the tryptic peptides of *P. agassizii* coelomic hemerythrin subunit type A. Conditions for gel filtration the same as in Fig. 30. The fractions were pooled as follows: A-TT-1, 39-53; A-TT-2, 54-66; A-TT-3, 67-75; A-TT-4, 76-99; A-TT-5, 100-109; A-TT-6, 110-128; and A-TT-7, 133-148.
Dowex-50 chromatography which resulted in fractions S-1 through S-17. Of these, S-4 was a pure peptide, S-6 free lysine, and S-8 a mixture of three peptides which were separated by preparative electrophoresis. The remaining fractions did not contain enough material for further characterization.

Sephadex fraction A-TT-3 was also further fractionated by Dowex-50 chromatography yielding fractions S-1 through S-8. Fractions S-6 and S-8 were pure peptides. The remaining fractions were impure and of too low a yield for further characterization.

Fraction A-TT-4 was purified via preparative electrophoresis-chromatography yielding fractions el-1 through el-8. Of these, el-1, el-2, and el-4 were pure peptides of sufficient quantity for sequence analysis. No further characterization was done on the remaining fractions.

Sephadex fraction A-TT-6 was a tetrapeptide which was sequenced without further purification.

The insoluble portion of the tryptic digestion had, after hydrolysis and amino acid analysis of an aliquot, a very low yield of amino acids. Four Sephadex fractions were obtained, but mapping and amino acid analysis of aliquots of these fractions showed too little material in them for further characterization.

The composition, sequence, and major contamination of the characterized tryptic peptides of subunit type A are shown in Table VIII. The yields, chromatographic properties and color reactions of these peptides are shown in Table IX.
### TABLE VIII. The main tryptic peptides of *P. agassizii* coelomic hemerythrin subunit type A

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Composition</th>
<th>Contamination Maximum (%)</th>
<th>Proposed Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-TT-1 S-7</td>
<td>Lys 1.11 His 0.99 Asp 3.02 Thr 0.98 Glu 2.12 Met 0.91 Leu 1.03 Tyr 0.83 (40% Ala)</td>
<td>Thr-Met-Tyr-Glu-Asp-(Leu, Asp, Asp) → Gln-His-Lys</td>
<td></td>
</tr>
<tr>
<td>A-TT-1 S-8</td>
<td>Lys 0.89 His 0.98 Glu 1.88 Gly 2.17 Ala 2.19 Val 1.65 Leu 1.12 Phe 1.14 (20% Thr)</td>
<td>Ala-His-x-Glu-Gly-(Gly)-Gln-(Lys, Ala, Val, Val, Leu, Phe)</td>
<td></td>
</tr>
<tr>
<td>A-TT-2 S-4</td>
<td>Glu 1.04 Gly 1.04 Leu 0.92 (8% Ser)</td>
<td>Gly-(Gln)-Leu</td>
<td></td>
</tr>
<tr>
<td>A-TT-2 S-6</td>
<td>Lys (1)</td>
<td>Lys (Free)</td>
<td></td>
</tr>
<tr>
<td>A-TT-2 S-8 el-1</td>
<td>Lys 0.94 Ser 1.00 Gly 1.05 Ala 2.12 Tyr 0.90 (2% Glu)</td>
<td>Gly-(Ser)-Ala-Tyr-Ala-Lys</td>
<td></td>
</tr>
<tr>
<td>A-TT-2 S-8 el-2</td>
<td>Lys 1.01 Asp 0.98 Thr 0.98 Ile 1.02 Phe 1.01 (11% Gly)</td>
<td>Thr-Ile-(Asp)-Phe-Lys</td>
<td></td>
</tr>
<tr>
<td>A-TT-2 S-8 el-3</td>
<td>Lys 0.72 Asp 1.10 Ser 1.00 Glu 0.94 Pro 3.18 Gly 0.99 Ala 1.02 Val 2.16 Tyr 0.71 Phe 1.86 Trp (1) (8% Ile)</td>
<td>Gly-Phe-Pro-Val-Pro-Glu-Pro-Tyr-Val-Trp-(Trp)-Asp-(Ala, Ser, Phe, Lys)</td>
<td></td>
</tr>
<tr>
<td>A-TT-3 S-6</td>
<td>Lys 1.00 Glu 1.15 Gly 1.12 Leu 1.11 Tyr 0.80 (13% Asp)</td>
<td>Tyr-(Lys)-Gly-Gln-Leu</td>
<td></td>
</tr>
<tr>
<td>A-TT-3 S-8</td>
<td>Lys 1.03 His 0.97 Asp 0.91 Glu 1.24 Val 0.88 Ile 0.95 Leu 1.04 Trp (1)b</td>
<td>Asp-Trp-Leu-Val-(Lys, His, Glu, Ile)</td>
<td></td>
</tr>
<tr>
<td>A-TT-4 el-1</td>
<td>Lys 1.02 His 0.73 Gly 1.92 Ala 2.04 Tyr 1.31c (6% Ser)</td>
<td>Tyr-Gly-Gly-Tyr-Ala-Ala-His-(Lys)</td>
<td></td>
</tr>
</tbody>
</table>
A-TT-4 el-2
Lys 1.12 Tyr 0.87 (21% Gly)  
Tyr-(Lys)

A-TT-4 el-4
Lys 1.02 His 0.85 Asp 1.91 Ser 1.23  
Glu 1.15 Ala 1.37 Val 1.79 Leu 0.88  
Phe 0.82 Trp (1)\textsuperscript{a} (42% Gly)  
(Lys, His, Asp, Asp, Ser, Glu,  
Ala, Val, Val, Leu, Phe, Trp)

A-TT-6
Gly (2) Tyr (2)\textsuperscript{c}  
Try-Gly-Gly-Tyr

\textsuperscript{a} Present on amino acid analysis and Ehrlich positive.
\textsuperscript{b} Presence indicated by 280 nm absorption.
\textsuperscript{c} Low tyrosine values due to degradation during hydrolysis. Sequence data confirmed 2 residues in each case.
TABLE IX. Properties and yields of the tryptic peptides of *P. agassizii* coelomic hemerythrin subunit type A

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Yield$^a$</th>
<th>Electrophoretic mobility</th>
<th>Chromatographic mobility</th>
<th>Color reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-TT-1 S-7</td>
<td>46.7</td>
<td>+3.0</td>
<td>4.0</td>
<td>Pauly, Toennis-Kolb</td>
</tr>
<tr>
<td>A-TT-1 S-8</td>
<td>39.1</td>
<td>0.0</td>
<td>7.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>A-TT-2 S-4</td>
<td>35.4</td>
<td>-3.0</td>
<td>22.5</td>
<td>None</td>
</tr>
<tr>
<td>A-TT-2 S-6</td>
<td>20.9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>None</td>
</tr>
<tr>
<td>A-TT-2 S-8 el-1</td>
<td>22.7</td>
<td>-13.0</td>
<td>3.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>A-TT-2 S-8 el-2</td>
<td>27.4</td>
<td>-3.0</td>
<td>14.4</td>
<td>None</td>
</tr>
<tr>
<td>A-TT-2 S-8 el-3</td>
<td>9.2</td>
<td>+1.0</td>
<td>18.0</td>
<td>Pauly, Ehrlich</td>
</tr>
<tr>
<td>A-TT-3 S-6</td>
<td>14.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Pauly</td>
</tr>
<tr>
<td>A-TT-3 S-8</td>
<td>19.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>Ehrlich, Pauly</td>
</tr>
<tr>
<td>A-TT-4 el-1</td>
<td>25.4</td>
<td>-11.5</td>
<td>1.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>A-TT-4 el-2</td>
<td>28.9</td>
<td>-13.0</td>
<td>6.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>A-TT-4 el-4</td>
<td>7.4</td>
<td>-2.5</td>
<td>12.0</td>
<td>Pauly, Ehrlich</td>
</tr>
<tr>
<td>A-TT-6</td>
<td>10.9</td>
<td>-2.0</td>
<td>21.0</td>
<td>Pauly</td>
</tr>
</tbody>
</table>
Yields were calculated as the percentage of peptide recovered of the estimated 1.8 μmoles of hemerythrin A used for the tryptic digestion.

N.D. = data not obtained
Tryptic Digestion of Pooled P. agassizii Coelomic Hemerythrin

A tryptic digestion of pooled coelomic P. agassizii hemerythrin was performed in this laboratory in a manner identical to the previously described digests (S. Ludlam, personal communication). The methodology for the peptide separations, purifications, and characterizations was identical with that already described.

A tryptic peptide map is shown in Fig. 35. A summary of the pooled tryptic peptide data can be found in Tables X and XI. These tables do not include peptides which were isolated in very low yields or which were highly contaminated.

Peptide Compositions, Sequences, and Assignments

Aliquots of all isolated peptides were hydrolyzed and analyzed for amino acid composition. Those which were isolated in sufficient quantity and reasonable purity were subjected to sequence analysis from the amino terminus by the dansyl Edman technique or the sequential Edman reaction with direct identification of the PTH amino acid derivatives. The carboxyl termini were sequenced by use of the carboxypeptidase enzymes. Determination of the amide amino acids in peptides was based on net electrophoretic mobility, direct PTH identification, and elution time on amino acid analysis when removed by carboxypeptidase.

The tryptic peptides of hemerythrin subunit type A, and the tryptic and chymotryptic peptides of pooled hemerythrin were assigned as shown in Fig. 36 according to their homology with the known primary structures of G. gouldii coelomic hemerythrin (Klippenstein et al., 1968) and D. pyroides myohemerythrin (Cote, 1973). Overlapping peptides helped to confirm assignments based on homology, especially in the
Figure 35: Peptide map of the tryptic peptides of pooled coelomic *P. agassizii* hemerythrin. Conditions of electrophoresis and chromatography identical to those of Fig. 29. Ninhydrin positive spots shown.
TABLE X. The main tryptic peptides of pooled coelomic *P. agassizii* hemerythrin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Composition</th>
<th>Contamination Maximum (%)</th>
<th>Proposed Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT-1 S-3 el-4</td>
<td>Lys 0.98 Glu 2.88 Ala 2.75 Val 1.02</td>
<td>Met 0.72 Leu 0.82 Phe 0.84 (18% Gly)</td>
<td>Leu-(Phe)-Gln-Glu-(Glx)-Ala-(Met, Ala, Val, Ala)-Lys</td>
</tr>
<tr>
<td>TT-1 S-4 el-2</td>
<td>Lys 1.03 His 1.05 Asp 3.25 Thr 0.96</td>
<td>Glu 1.86 Met 0.85 Leu 1.02 Tyr 0.98 (23% Phe)</td>
<td>Thr-Met-Tyr-Glu-Asp-Leu-Asp-Asp-Gln-His-Lys</td>
</tr>
<tr>
<td>TT-1 S-5 el</td>
<td>Lys 1.07 His 1.00 Asp 3.87 Thr 1.00</td>
<td>Glu 1.00 Leu 1.04 Tyr 0.92 Phe 1.09 (15% Ser)</td>
<td>Val-Thr-Phe-Tyr-Asp-Asp-Leu-Asp-Asp-Gln-His-(Lys)</td>
</tr>
<tr>
<td>TT-1 S-6 el-0</td>
<td>Lys 0.95 His 0.89 Asp 0.99 Ser 0.91</td>
<td>Glu 1.19 Gly 1.06 Ala 2.70 Val 1.32 Leu 0.98 Phe 1.02 (5% Ile)</td>
<td>Asp-Ala-His-Glu-Gly-Phe-Ser-(Ala, Leu, Val, Ala, Lys)</td>
</tr>
<tr>
<td>TT-1 S-6 el-1</td>
<td>His 0.96 Arg 0.91 Asp 1.08 Glu 2.03</td>
<td>Gly 1.09 Ala 1.05 Leu 0.94 Phe 0.95 (12% Ser)</td>
<td>Glu-Ala-His-Glu-Asn-(Phe, Leu) Gly-(Arg)</td>
</tr>
<tr>
<td>TT-1 S-7 el-1</td>
<td>Lys 1.17 His 1.08 Glu 1.13 Gly 2.02</td>
<td>Ala 1.92 Val 1.68 Leu 1.11 Phe 0.94 (14% Asp)</td>
<td>Ala-His-Glu-Gly-x-Phe-Gly-x-(Leu, Val, Val, Ala, Lys)</td>
</tr>
<tr>
<td>TT-2 S-2 el-1</td>
<td>Glu 1.03 Gly 0.97 Leu 0.69 (36% Ser)</td>
<td>(Gly) (Gln) (Leu)</td>
<td></td>
</tr>
<tr>
<td>TT-2 S-3 el-3</td>
<td>Ser 0.99 Gly 1.00 Ala 1.58 Tyr 0.95 (28% Asp)</td>
<td>Gly-Tyr-Ala-Ser</td>
<td></td>
</tr>
<tr>
<td>TT-2 S-3 el-4</td>
<td>Glu 0.98 Gly 0.89 Leu 1.18 (6% Lys)</td>
<td>Gly-Gln-(Leu)</td>
<td></td>
</tr>
<tr>
<td>TT-2 S-4</td>
<td>Lys (1) (17% Gly)</td>
<td>Lys (Free)</td>
<td></td>
</tr>
</tbody>
</table>

133
<table>
<thead>
<tr>
<th>protein</th>
<th>sequence</th>
<th>composition</th>
<th>reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT-2 S-6 e1-1</td>
<td>Lys 0.98 Asp 2.04 Ile 1.01 Phe 0.97 (8% Glu)</td>
<td></td>
<td>Asx-Ile-Asx-Phe-Lys</td>
</tr>
<tr>
<td>TT-2 S-6 e1-7</td>
<td>Lys 2.05 Gly 0.99 Leu 1.95 (7% Ser)</td>
<td></td>
<td>Gly-Lys-Leu-Leu-Lys</td>
</tr>
<tr>
<td>TT-2 S-8 e1-7</td>
<td>Arg 1.02 Ala 0.98 (42% Lys)</td>
<td></td>
<td>Ala-Arg</td>
</tr>
<tr>
<td>TT-2 S-9 e1-1</td>
<td>Arg 0.98 His 0.98 Asp 1.03 Glu 1.06 Gly 1.06 Ala 0.93 Leu 0.98 Phe 0.98 (6% Lys)</td>
<td></td>
<td>Ala-His-Gly-Asp-(Phe, Leu, Gly, Arg)</td>
</tr>
<tr>
<td>TT-2 S-9 e1-8</td>
<td>Lys 1.03 His 0.98 Ala 1.39 (13% Gly)</td>
<td></td>
<td>Ala-(His, Lys)</td>
</tr>
<tr>
<td>TT-3 S-9 e1-1</td>
<td>Lys 0.98 His 0.90 Asp 1.01 Glu 1.15 Val 1.11 Ile 0.92 Leu 0.95 Trp (1) a (17% Gly)</td>
<td></td>
<td>Asp-Trp-Leu-Val-Gln-His-(Ile, Lys)</td>
</tr>
<tr>
<td>TT-3 S-9 e1-3</td>
<td>Lys 1.09 His 0.97 Asp 1.16 Ser 0.82 Gly 1.12 Ala 1.01 Tyr 1.90 (13% Ile)</td>
<td></td>
<td>Tyr-Ser-Gly-Tyr-Asn-Ala-His-Lys</td>
</tr>
<tr>
<td>TT-3 S-10</td>
<td>Lys 1.28 His 0.95 Asp 1.16 Gly 2.08 Ala 0.87 Tyr 1.65 (32% Glu)</td>
<td></td>
<td>Tyr-(Asp)-Gly-Tyr-Gly-Ala-His-(Lys)</td>
</tr>
<tr>
<td>TT-3 S-11</td>
<td>Lys 1.03 His 1.01 Gly 2.19 Ala 1.78 Tyr 1.99 (15% Ser)</td>
<td></td>
<td>Tyr-Gly-Gly-Tyr-Ala-Ala-His-Lys</td>
</tr>
<tr>
<td>TT-4</td>
<td>Gly 2.32 Tyr 1.68 (10% Gly)</td>
<td></td>
<td>Tyr-Gly-Gly-Tyr</td>
</tr>
<tr>
<td>TT-2 S-8 e1-6</td>
<td>Arg 0.85 Asp 0.96 Gly 1.28 Ala 1.91 Tyr 1.04 (42% Lys)</td>
<td></td>
<td>Gly-Tyr-Ala-Ser-Ala-Arg</td>
</tr>
</tbody>
</table>

*a Assumed present by a positive Ehrlich reaction
TABLE XI. Properties and yields of the tryptic peptides of pooled *P. agassizii* coelomic hemerythrin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Yield</th>
<th>Electrophoretic mobility</th>
<th>Chromatographic mobility</th>
<th>Color Reactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TT-1 S-3 e1-4</td>
<td>1.34</td>
<td>-2.0</td>
<td>19.5</td>
<td>Toennis-Kolb</td>
</tr>
<tr>
<td>TT-1 S-4 e1-2</td>
<td>3.44</td>
<td>+3.0</td>
<td>4.5</td>
<td>Pauly, Toennis-Kolb</td>
</tr>
<tr>
<td>TT-1 S-5 e1</td>
<td>2.85</td>
<td>+3.5</td>
<td>5.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>TT-1 S-6 e1-0</td>
<td>2.35</td>
<td>0.0</td>
<td>7.0</td>
<td>Pauly</td>
</tr>
<tr>
<td>TT-1 S-6 e1-1</td>
<td>1.52</td>
<td>+5.0</td>
<td>6.5</td>
<td>Pauly, Sakaguchi</td>
</tr>
<tr>
<td>TT-1 S-7 e1-1</td>
<td>1.86</td>
<td>-0.5</td>
<td>9.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>TT-2 S-2 e1-1</td>
<td>0.96</td>
<td>-2.5</td>
<td>19.0</td>
<td>None</td>
</tr>
<tr>
<td>TT-2 S-3 e1-3</td>
<td>0.67</td>
<td>-2.0</td>
<td>13.5</td>
<td>Pauly</td>
</tr>
<tr>
<td>TT-2 S-3 e1-4</td>
<td>1.33</td>
<td>-2.0</td>
<td>19.0</td>
<td>None</td>
</tr>
<tr>
<td>TT-2 S-4</td>
<td>12.44</td>
<td>-20.5</td>
<td>4.5</td>
<td>None</td>
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<tr>
<td>TT-2 S-6 e1-1</td>
<td>2.16</td>
<td>-5.0</td>
<td>11.0</td>
<td>None</td>
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<tr>
<td>TT-2 S-6 e1-7</td>
<td>1.68</td>
<td>-2.0</td>
<td>10.0</td>
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<tr>
<td>TT-2 S-8 e1-6</td>
<td>0.75</td>
<td>-11.5</td>
<td>4.0</td>
<td>Pauly, Sakaguchi</td>
</tr>
<tr>
<td>TT-2 S-8 e1-7</td>
<td>1.38</td>
<td>-19.5</td>
<td>3.5</td>
<td>Sakaguchi</td>
</tr>
<tr>
<td>TT-2 S-9 e1-1</td>
<td>1.07</td>
<td>0.0</td>
<td>6.0</td>
<td>Pauly, Sakaguchi</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>--------</td>
<td>-------</td>
<td>-----</td>
<td>-------</td>
</tr>
<tr>
<td>TT-2</td>
<td>S-9 e1-8</td>
<td>0.66</td>
<td>-18.0</td>
<td>0.0</td>
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<tr>
<td>TT-3</td>
<td>S-9 e1-1</td>
<td>0.72</td>
<td>0.0</td>
<td>9.0</td>
</tr>
<tr>
<td>TT-3</td>
<td>S-9 e1-3</td>
<td>2.26</td>
<td>-7.5</td>
<td>1.5</td>
</tr>
<tr>
<td>TT-3</td>
<td>S-10</td>
<td>3.52</td>
<td>-6.0</td>
<td>1.5</td>
</tr>
<tr>
<td>TT-3</td>
<td>S-11</td>
<td>3.84</td>
<td>-13.0</td>
<td>1.0</td>
</tr>
<tr>
<td>TT-4</td>
<td></td>
<td>6.50</td>
<td>-0.5</td>
<td>16.5</td>
</tr>
</tbody>
</table>

*Yields were calculated as the percentage of peptide recovered of the original 15 μmoles of protein used for the tryptic digestion.*
Figure 36: Assignments of *P. agassizii* coelomic hemerythrin peptides based on homology with known hemerythrin primary structures and peptide overlaps where available. Data from both tryptic digestions and the chymotryptic digestion are presented. The numbering is based on that used for *G. gouldii* coelomic hemerythrin (Klippenstein *et al.*, 1968).
N-Terminus

Pooled Hemerythrin

Subunit Type A

Tryptic A

Chymotryptic Pooled

Chymotryptic Pooled

Tryptic Pooled

Sequence incomplete for residues 30-63
Sequence incomplete for residues 82-89
Tryptic A

Tryptic Pooled

Chymotryptic pooled

COOH-Terminus

Varial probably present in A along with Phe

a Variant probably present in A along with Phe

b Variants found in hemerythrin A in place of a pooled residue

c Variants found in chymotryptic peptides
Chymotryptic peptide Lys-Thr-Met-Tyr not isolated

Chymotryptic peptide G-2 S-9 e1-N-1 contained 0.5 Asn and 0.5 Thr which were sequenced at position 105.

Chymotryptic peptide Gly-Gly-Tyr not isolated

Amino terminus of chymotryptic peptide G-2 S-7 Ch-1 Asp and G-1 S-11 Gly

Ala at position 71

Asn at position 71

Amino terminal Ser

Personal communication S. Ludlam and G. L. Klippenstein

Peptide G-2 S-5 e1-N-3 contained Thr at position 104 and G-2 S-5 e1-N-2 contained Ser at the same position

Peptide G-2 S-5 e1-B-1 contained Gln at position 112 and G-2 S-9 e1-B-2 contained Lys at the same position

Variant found in pooled tryptic peptide TT-2 S-8 e1-7 which cannot be in hemerythrin A which lacks arginine.

Peptide TT-4 had Gly at position 68.
carboxyl terminal region of the protein.

Direct sequence work on pooled hemerythrin and subunit type A gave reliable information on the first 12 residues of the amino terminus and tentative identification on the next two residues.

**Peptide A-TT-3 S-8 el-3** The composition and partial sequence of this tryptic peptide from the hemerythrin A digest suggested that it was the amino terminal peptide. The sequence of the first nine residues had a close homology with that of the known hemerythins. The placement of tryptophan at position 10 was based on composition, homology, and the chymotryptic peptide G-7 Ch-3. Residue 11 was sequenced and 12-14 tentatively placed on the basis of composition and the sequence of the chymotryptic peptide G-2 S-2 el-A-4. Lysine was assumed to be the carboxyl terminus based on composition and tryptic specificity.

**Peptides A-TT-1 S-7 and TT-1 S-5** These peptides were assigned to positions 16-26 on the basis of close homology with the previously sequenced hemerythins. The hemerythrin A peptide, A-TT-1 S-7, contained methionine in place of phenylalanine at position 17 and glutamic acid in place of aspartic acid at position 19. The overlapping chymotryptic peptide, G-3 S-1 el-B, had a carboxyl terminus of phenylalanine. The potential overlapping chymotryptic peptide containing methionine, and a carboxyl terminal tyrosine was not isolated from the pooled chymotryptic digest.

**Peptide G-2 S-4 el-A** This overlapping chymotryptic peptide had been cleaved between positions 17 and 18. The potential peptide from a chymotryptic cleavage after position 18 was not isolated. The first nine residues of this peptide were sequenced by direct identification of the PTH amino acid derivatives. On the basis of homology with the tryptic
peptides, and the known hemerythrin sequences, these residues were assigned to positions 18-26. The remaining residues of this peptide were sequenced by carboxypeptidase digestion which allowed extension of the total amino terminal hemerythrin sequence to position 29.

No peptides could be assigned to positions 30-63 of the total hemerythrin sequence with the information available.

**Peptide G-3 S-1 el-B**  This designation actually covers two peptides which co-electrophoresed and co-chromatographed with the systems and conditions used for peptide isolation and purification. Amino acid analysis revealed the presence of two peptides, in about a 3:1 ratio, with only one residue in common, lysine. The dansyl Edman sequence procedure gave two different spots which differed noticeably in intensity. The amino terminus and an additional residue of each peptide was sequenced in this manner. Carboxypeptidase digestion released the carboxyl terminal residue of each peptide in nearly the expected ratio. These data allowed the determination of the total sequence of the tripeptide which has already been discussed as a critical chymotryptic overlap peptide in the first 29 positions of this hemerythrin's primary structure. The tetrapeptide had only lysine unaccounted for, and by difference, it had to be the third residue in this peptide.

The placement of the tetrapeptide in G-3 S-1 el-B at positions 64-67 was based on homology with the known hemerythrins and a one residue overlap with the tryptic peptides discussed in the following section. This assignment should be considered tenuous at best.

**Peptides A-TT-4 el-1, A-TT-6, and TT-4**  The hemerythrin A tryptic peptides were assigned to positions 67-74 based on a reasonable homology with the known hemerythrin primary structures. Peptide A-TT-6 and TT-4
represented unexpected cleavages for trypsin since the carboxyl terminus of tyrosine would normally be associated with a chymotryptic peptide. Also, these tetrapeptides form the amino terminal half of A-TT-4 el-1.

**Peptide TT-3 S-11** This peptide was identical in composition and sequence to A-TT-4 el-1 of the hemerythrin A trypptic digest and differed only in its source, pooled hemerythrin.

**Peptide TT-3 S-9 el-3** Variance at positions 68 and 71 was noted in this pooled hemerythrin peptide. Serine replaced glycine and asparagine replaced alanine in this peptide which was assigned to positions 67-74 in the hemerythrin sequence. Otherwise, this peptide was identical in composition and sequence to the already described trypptic peptides of this region.

**Peptide G-4 Ch-2** This was an expected chymotryptic cleavage within the trypptic peptide TT-3 S-9 el-3; however, the expected chymotryptic peptide for A-TT-4 el-1 of hemerythrin A was not isolated.

**Peptides G-2 S-7 Ch-1 and G-1 S-11** These peptides were assigned to positions 75-80 based on a four residue overlap and homology with the known hemerythrin primary structures. The former had an amino terminus of aspartic acid and the latter glycine which indicated still more variation at position 71. Between positions 75-77, and at position 79, these peptides differed in composition and sequence from one another.

No peptides could be assigned to positions 82-89 of the total hemerythrin sequence with the information available.

**Peptide A-TT-2 S-8 el-1** This hemerythrin A peptide was assigned to positions 90-95 on the basis of homology with the known hemerythrin primary structures and a two residue carboxyl terminal overlap with the amino terminus of the chymotryptic peptide G-3 S-2 el-N. An unusual
pooled tryptic peptide, TT-2 S-8 el-1, represented a chymotryptic cleavage at the amino terminus and a typical tryptic cleavage at the carboxyl terminus. This peptide allowed the tentative assignment of arginine to position 95. The chymotryptic peptide G-3 S-2 el-N had the lysine variant at this position and since hemerythrin A lacks arginine, this peptide must have been cleaved from this subunit type.

Peptides A-TT-3 S-8 and TT-3 S-9 el-1 These tryptic peptides were identical in composition and sequence, the former having been isolated from the hemerythrin A digest, and the latter from the pooled hemerythrin digest. Based on a nearly perfect homology with the known hemerythrin primary structures, and two to three residue overlaps at the amino and carboxyl termini with chymotryptic peptides, these peptides were assigned to positions 96-103.

Peptides A-TT-2 S-8 el-2 and TT-2 S-6 Except for the amino termini, these peptides were of the same composition and sequence. In A-TT-2 S-8 el-2, a hemerythrin A peptide, the amino terminus was threonine while that of TT-2 S-6 el-1, a pooled hemerythrin peptide, had asparagine as the amino terminus. The nearly perfect homology of these peptides with those of the known hemerythrin structures, and overlaps with chymotryptic peptides for both the amino and carboxyl termini, allowed the placement of these peptides at positions 104-108.

Peptides G-2 S-5 el-N-3 and G-2 S-5 el-N-2 Both of these peptides represented an unusual chymotryptic cleavage that occurred just after histidine 101. With the exception of the expected variance in composition, threonine for asparaginc, the peptides had identical compositions and sequences.

Peptide G-2 S-9 el-N-1 There were two unusual features noted for this
peptide. The chymotryptic cleavage at the amino terminus occurred just after position 100, a glutamine. Also, the peptide contained 0.5 residues of threonine and 0.5 residues of asparagine. These were clearly located at position 104 by direct identification of the PTH derivatives. This peptide contains the sequences of the just described chymotryptic peptides and provides the overlap for the tryptic peptides of the region. It was assigned to positions 101-107.

**Peptides A-TT-3 S-6, A-TT-4 el-2, and A-TT-2 S-4** These hemerythrin A peptides represented the expected tryptic peptides for the carboxyl terminus of hemerythrin A. Peptide A-TT-3 S-6 was assigned to positions 109-113 based on excellent homology with the known hemerythrins. The other two tryptic peptides represent an expected internal cleavage of A-TT-3 S-6 by trypsin.

**Peptides G-2 S-5 el-B-1, G-2 S-9 el-B-2, and G-3 S-2 el-B** These peptides covered the expected chymotryptic cleavages between positions 108-113. Peptides G-2 S-5 el-B-1 and G-2 S-9 el-B-2 were identical in sequence and composition except for a glutamine-lysine interchange at position 112. Since glutamine occurred in hemerythrin A, at this position, and lysine in one of the pooled chymotryptic peptides, the lysine variant must occur in the other subunit types. The dipeptide G-3 S-2 el-B was an expected chymotryptic overlap peptide of the carboxyl terminal region for positions 108 and 109.

**Unassigned Peptides**

A number of peptides were isolated in relatively high yields but could not be placed in the proposed hemerythrin sequence with any degree of certainty. Some resisted all attempts to sequence them while
others gave ambiguous or contradictory results.

Free lysine was found after both tryptic digests and may belong at position 49. Peptides TT-1 S-3 e1-4, G-2 S-2 e1-A-6, and G-2 S-2 e1-A-7 all contained methionine and were suspected of being from the same region of the total sequence. Unfortunately, the sequence data for the amino and carboxyl termini of these peptides were contradictory.

The chymotryptic peptide G-2 S-12, when sequenced by both the dansyl Edman technique and the Edman degradation with direct identification of the PTH derivatives, gave totally confusing results. Carboxypeptidase digestion clearly indicated a carboxyl terminal phenylalanine but nothing conclusive beyond this residue.

The chymotryptic peptide G-6 Ch-1 may be a variant of G-7 Ch-3 and on this basis could be assigned to positions 9 and 10 in the total sequence.

The remaining peptides, for which complete or nearly complete sequence data were available, were not placed in the total hemerythrin sequence due to an absence of overlapping peptides and the lack of recognizable homology with the known hemerythrin primary structures.

Partially sequenced peptides, and those known by their compositions only, were not placed in the overall sequence unless a composition homology existed with the known hemerythrins or already sequenced P. agassizii peptides.

P. agassizii Myohemerythrin

P. agassizii metazidemｙohemerythrin had the absorption spectrum shown in Fig. 37 which closely resembles that of D. pyroides metazidemｙohemerythrin (Van Riper, 1972). Analytical disc gel electrophoresis,
Figure 37: The absorption spectrum of *P. agassizii* metazidemyohemerythrin (---) compared with that of *D. pyroides* metazidemyohemerythrin (---) (Van Riper, 1972) between 300 and 600 nm.
on 7% acrylamide gels, revealed a darkly stained band of high mobility which was probably myohemerythrin, and a lightly stained band of lower mobility which was probably a higher molecular weight impurity (see Fig. 38).

Ultracentrifugation gave a major slowly migrating peak, and a small amount of a rapidly migrating peak. The major peak had an $s_{20,w}$ of 1.87 S which was in the same range as that of *D. pyroides* myohemerythrin, $s_{20,w} = 1.5$ S (Van Riper, 1972).

SDS-polyacrylamide gel electrophoresis (Fig. 38) revealed two bands with molecular weights of 19,700 and 13,800 (Fig. 39). The lightly stained band of lower mobility probably contained subunits of the high molecular weight impurity suspected of being present. The darkly stained band of higher mobility had a molecular weight very close to that of *D. pyroides* myohemerythrin which has been calculated as 13,900 based on the known sequence (G. L. Klippenstein, personal communication).

**P. agassizii Vascular System**

Although Manwell (1963) reported that *P. agassizii* does not have a vascular system, I have observed one in this organism. Along the retractor muscles a colorless tube becomes a purple-pink on exposure to air as do the muscles to a lesser degree. The same phenomenon has been observed in *D. pyroides* when the more extensive vascular system of this worm was exposed to air.

Erythrocytes in the small vascular system of *P. agassizii* were about 15 microns in diameter while those of the coelomic fluid were about 25 microns in diameter. Similar size differences have been observed for the coelomic and vascular erythrocytes of the sipunculid
Figure 38: The *P. agassizii* myochemerythrin band pattern on an SDS-polyacrylamide molecular weight gel (left) and on a standard 7% acrylamide disc gel (right).
Figure 39: Calibration curve of SDS-polyacrylamide electrophoresis gels used for myohemerythrin molecular weight determination. The standards were identical to those used in Fig. 11 and protein mobilities were determined in the same manner. Arrows mark the mobilities of the bands observed.
Dendrostomum pyroides (Manwell, 1960b).
DISCUSSION

All native coelomic hemerythrins previously studied have been found to be octameric proteins with molecular weights around 100,000 and monomer molecular weights near 13,500 (Klotz and Keresztes-Nagy, 1963; Ferrell and Kitto, 1970; Bates et al., 1968; Joshi and Sullivan, 1973). The coelomic hemerythrin of *P. agassizii* has a monomer molecular weight of about 12,700 which is in the expected weight range. However, the molecular weight of the native *P. agassizii* coelomic hemerythrin from gel chromatography is 32,200, a value completely inconsistent with an octameric structure. The sedimentation coefficient of 3.65 S is also significantly lower than the 6.6 S reported for *G. gouldii* coelomic hemerythrin (Klotz and Keresztes-Nagy, 1963). Since neither gel chromatography (Fig. 7) nor the sedimentation studies (Figs. 8 and 9) show any anomalous concentration dependence, the possibility of obtaining low values because of dissociation of a larger oligomer seems to be ruled out.

Nevertheless, it is true that a molecular weight of 32,200 is somewhat low for a protein of 3.65 S. Use of the Stoke's radius and the sedimentation coefficient gave a calculated molecular weight of 40,600. This difference can be explained if one assumes that the molecule is extremely compact, due, for instance, to close packing of the subunits. In this case, the frictional ratio would be nearly 1 and as a result the molecular weight from gel chromatography would be lower than expected. Also, the sedimentation coefficient would be slightly higher than for an "average" protein in this molecular weight range.
Sedimentation equilibrium ultracentrifugation has been performed on native pooled P. agassizii coelomic metazidehemerythrin at another laboratory on samples sent from this laboratory. A molecular weight of 36,400 was obtained in these experiments (N. V. Beaudette and N. R. Langerman, personal communication, 1974).

It was therefore concluded that P. agassizii coelomic hemerythrin exists as a trimer of 12,700 molecular weight subunits. Additional support for this conclusion was obtained by cross-linking with dimethyl suberimidate. Cross-links are formed between polypeptide chains within the oligomer in this treatment and give rise, upon denaturation, to covalently linked species, the molecular weights of which are multiples of the monomer molecular weight. The correct number of monomers in the oligomer is indicated by the number of principal species detected (Davies and Stark, 1970). The appearance of three bands on the SDS-polyacrylamide gels of cross-linked hemerythrin indicated that the oligomer consists of three subunits (Fig. 13). The bands are multiples of a monomer molecular weight near 13,000 (Fig. 14). The smear of higher molecular weight species in overloaded SDS-polyacrylamide gels has been observed with other proteins (Davies and Stark, 1970; Carpenter and Harrington, 1972).

A model based on the different charge types has been formulated (Table II). The model was confirmed by the observation that the relative mobility of a native trimer during gel electrophoresis corresponded to the proportion of each subunit charge type it contained. Two trimer types (AC₂ and B₃) were predicted to have nearly identical mobilities. The stoichiometry of the corresponding band (Table II, band 4) was consistent with this conclusion. One predicted trimer type was missing (C₃).
and an additional rapidly migrating hemerythrin was observed (D). The subunit of this latter protein, detected in only small quantities, had a much greater mobility on both analytical and preparative electrophoresis gels, 8 M in urea, than the most rapidly migrating of the three major subunit types. The absence of trimer type C₃ is unexplained.

Using the formula: \[ N = \frac{(M+S-1)!}{S!(M-1)!} \] (Shaw, 1964) where N is the number of possible oligomers, M the number of subunit types, and S the number of subunits per oligomer, the number of oligomeric types for a dimer, trimer, and tetramer of three subunit types was computed. A dimer of three subunit types has a maximum of 6 possible combinations, a trimer 10 possible combinations, and a tetramer 15 possible combinations. Only a trimer of three subunit types would explain the number of bands observed during disc gel electrophoresis. Eight bands have been observed during disc gel electrophoresis with one of the bands having been shown to represent two trimer combination types. Of the ten possible trimers, nine have been accounted for. The tenth possible combination (C₃) has not been observed.

Most oligomeric proteins discovered to date have contained an even number of subunits. Oligomeric proteins with an odd number of subunits are represented by a few trimers and pentamers (Darnall and Klotz, 1972). By making the assumption that \textit{P. agassizii} coelomic hemerythrin subunits exist in identical environments, only one form of quaternary geometry is possible for them, triangular (Klotz et al., 1970). Recently, a 2-oxo-3-deoxy-6-phosphogluconate aldolase from \textit{Pseudomonas putida} was shown by x-ray diffraction studies to have a space group with a 3-fold rotation axis (Vandlen et al., 1973). This finding confirmed the physical and chemical studies which had already shown this enzyme to
exist as a trimer of three identical or nearly identical subunits.

Isoelectric focusing of native pooled *P. agassizii* coelomic metazidehemythrin revealed more than twice as many bands as had been observed during disc gel electrophoresis. Additional isoelectric focusing, on gels 8 M in urea, with ampholytes of the same pH range as those used for the native pooled electrofocusing gels, revealed not three but five major subunit types (Fig. 23). The five major isoelectric focusing subunit types have been correlated with the electrophoretic subunit types (Table IV).

With the information available from gel isoelectric focusing in 8 M urea, it became possible to explain the appearance of the 20 plus bands when native coelomic hemerythrin was subjected to isoelectric focusing. Using the previously cited formula, a dimer of five subunit types would have a maximum of 15 possible combinations, a trimer 35 possible combinations, and a tetramer 70 possible combinations. A trimer of five subunit types comes closest to explaining the 20 plus bands observed on the isoelectric focusing gel of the native hemerythrin. The nearly identical isoelectric points of subunit types 2 and 3, and 4 and 5 would lead one to expect some trimers of identical, or nearly identical isoelectric points. This probably explains the failure to observe the maximum number of trimers predicted. Also, some trimer types may simply not be present (*C*₃ is inexplicably missing during electrophoresis).

On the 8 M urea gels which contained ampholytes of the narrow range, pH 5-8, the five major isoelectric focusing subunit types were visible, in addition to a number of minor bands. These minor bands were indicative of small quantities of additional variant subunit types in this hemerythrin. This type of microheterogeneity has been observed in
other proteins, for example, the iron storage protein apoferritin (Righetti and Drysdale, 1973).

While the band patterns of the isoelectric focusing gels could be easily reproduced, the pH curves from the eluted gel slices were not as easily replicated. All of the pI values in the results should be regarded as approximate values due to these replication difficulties (Wrigley, 1968).

Individual *P. agassizii* worms had coelomic hemerythrin gel electrophoresis band patterns which were variants of the pooled pattern in about half the animals examined. These variant electrophoretic patterns were grouped into five classes and when pooled maintained their respective variant patterns. The most probable explanation for these variant electrophoretic patterns is the absence of one of the subunit types seen on the 8 M urea isoelectric focusing gels. A lack of subunit type 1 would cut down the mobility range of the bands observed during electrophoresis and this was not the case at all. Thus, one of the other subunit types, 2-5, was probably missing in the variant animals.

An equilibrium has been detected between the octamer and monomer of *G. gouldii* hemerythrin by succinylation of the octameric protein and then mixing with unmodified octamer and allowing hybridization to take place. The detection of octamer of an intermediate mobility, on electrophoresis, was regarded as proof that an octamer-monomer equilibrium existed (Keresztes-Nagy et al., 1965). A similar equilibrium probably exists with *P. agassizii* coelomic hemerythrin, except that the oligomer is a trimer and the differently charged subunits allow electrophoretic detection of the equilibrium caused hybridization, without having to modify some of the protein chemically. The evidence for this equilibrium
was found in the gel electrophoresis patterns of individual hemerythrin trimer charge types, which had been separated by preparative electrophoresis, and initially had one band when electrophoresed again. Storage for a week or more apparently allowed sufficient time for the equilibrium and rehybridization to produce other trimer combinations which could then be detected by gel electrophoresis. The vast difference in mobility of some of the new trimers on these gels, when compared with the originally isolated trimer type, excluded the possibility that the new bands represented undetected cross-contamination from the initial preparative electrophoresis. When only one subunit charge type was present (A₃), additional bands did not appear (see Fig. 28, gel 9).

The circular dichroic spectrum of P. agassizii coelomic hemerythrin was typical of a protein having a high content of α-helix. Its α-helical content, estimated at 82%, was slightly higher than that reported for any other hemerythrin examined to date: G. gouldii coelomic, 75% (Darnall et al., 1969; Garbett et al., 1969); S. nudus coelomic, 71% (Bossa et al., 1970); and D. pyroides; coelomic, 69%; vascular, 66%; and myohemerythrin, 69% (Klippenstein et al., 1972). Uncertainties as to the exact monomer molecular weight (about 12,700) and the number of amino acid residues per monomer (about 113) may explain the slightly higher percentage of α-helix found for P. agassizii coelomic hemerythrin.

A comparison of the amino acid composition of pooled P. agassizii coelomic hemerythrin with that of G. gouldii revealed a number of important differences. A lack of cysteine explained the failure of sulfhydryl reagents to produce a monomer from pooled P. agassizii trimeric hemerythrin. Overall, P. agassizii coelomic hemerythrin, when compared to that of G. gouldii, contains much less arginine (1 residue
only), isoleucine, tryptophan, and aspartic acid; while it contains much more alanine, serine, glycine, and glutamic acid (see Table III).

When the amino acid composition of the "purified" subunit types of P. agassizii coelomic hemerythrin are compared with one another, it becomes obvious that they represent proteins of different compositions. Subunit types B and C closely resemble one another, except that C has at least one more acidic and one fewer basic residue than B. This is a reasonable composition in view of the fact that subunit type C migrated in electrophoresis slightly more rapidly than B. Regardless of the source, subunit type A contains fewer potential negative charges when the positive charges are subtracted. Thus, at the normal electrophoresis pH, subunit type A would have a much lower mobility than the other hemerythrin subunit types. This is exactly the electrophoretic behavior observed.

Hemerythrin D, a very minor band (around 3%) detected during electrophoresis of both native and 8 M urea denatured protein, has an amino acid composition which differs greatly from that of the three major subunit types. This hemerythrin contains several fewer basic residues (however, one more arginine) and several more potential acidic residues. It has a much greater electrophoretic mobility than any of the confirmed trimer bands, and on electrophoresis in 8 M urea, migrated far ahead of the three major subunit types. In addition, seven fewer alanines are present in this hemerythrin than in any of the major subunit types. Finally, although pooled P. agassizii coelomic hemerythrin and subunit types A, B, and C lack cysteine, hemerythrin D definitely contains a residue of cysteine. Considerable differences in amino acid composition have been noted between the coelomic and vascular hemery-
thins of *D. pyroides* (Klippenstein *et al.*, 1972).

Based on the differences in amino acid composition and mobility from the major subunit types of *P. agassizii* coelomic hemerythrin, it is probable that hemerythrin D represents a trace of vascular hemerythrin from the small vascular system found along the retractor muscles of *P. agassizii*.

The dissociation of *P. agassizii* coelomic hemerythrin into native subunits has not been accomplished. All attempts, including succinylation, changes in pH, and use of sulfhydryl reagents have failed. The preparation of 8 M urea denatured subunits, which were separated on the basis of charge, has been accomplished. Initially, preparative electrophoresis in 8 M urea was used for subunit separation. However, attempts to separate larger quantities of protein by this method failed, probably due to the insolubility of the denatured hemerythrin during electrophoresis.

Chromatography on DEAE-cellulose columns, 8 M in urea, was more convenient and allowed the use of larger quantities of hemerythrin for subunit preparation. The total protein recovered did not amount to more than 30% of that initially placed onto the column. Again, precipitation of the denatured protein, even in 8 M urea, was the suspected reason for the low overall recovery.

As expected, the tryptic and chymotryptic digestions of pooled *P. agassizii* coelomic hemerythrin resulted in many low yield peptides. Those peptides which were recovered in larger quantities from the pooled digestions are suspected of being from areas of the molecule essential to the tertiary structure and maintenance of the iron binding site. This hypothesis is based on the fact that the two fully characterized
hemerythrin primary structures, *G. gouldii* coelomic (Klippenstein et al., 1968) and *D. pyroides* myohemerythrin (Cote, 1973) which differ substantially in composition and quaternary structure, are quite similar in primary structure near the amino and carboxyl terminal regions (Cote, 1973). The coelomic hemerythrins of *P. agassizii* differ from these hemerythrins both in composition and quaternary structure; and yet, the homologous regions at the termini remain. The many low yield peptides represent the nonhomologous regions among the *P. agassizii* subunits which are presumed not to be essential for the tertiary structure.

Histidines 25, 54, 73, and 101 (Morrissey, 1971) and tyrosines 8 and 109 (Rill and Klotz, 1971), the suspected iron ligands of *G. gouldii* hemerythrin, are all preserved in the primary structure of *D. pyroides* myohemerythrin (Cote, 1973). The peptides assigned to the amino and carboxyl regions of *P. agassizii* coelomic hemerythrin show a preservation of tyrosine at positions 8 and 109, and histidine at positions 25 and 101 (Fig. 40). In addition, if the assignment by homology of peptides A-TT-4 el-1, TT-3 S-11, and TT-3 S-9 el-3 is correct, the histidine at position 73 has been preserved in the primary structure of *P. agassizii* coelomic hemerythrin. This region does not show a homology as close as that observed for the termini; however, the tyrosines and their spacing in these peptides, as well as the histidine at position 73 makes their assignments to this region fairly certain. Histidine 77 is probably not an iron ligand (Morrissey, 1971), but some doubt about this remains since this residue was preserved in the primary structure of myohemerythrin (Cote, 1973). Alanine and glutamine have been found at this position in the primary structure of *P. agassizii*; however, histidine is the very next residue at position 78. The exact ligand
Figure 40: A comparison of the *P. agassizii* sequence data with the primary structures of *G. gouldii* coelomic hemerythrin (Klippenstein *et al.*, 1968; Klippenstein, 1972a) and *D. pyroides* myohemerythrin (Cote, 1973).
Figure 40

<table>
<thead>
<tr>
<th>Protein Type</th>
<th>Sequence (N terminus)</th>
<th>Sequence (C terminus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. gouldii hemerythrin</td>
<td>Gly-Phe-Pro-Ile-Pro-Asp-Pro-Tyr-Val-Trp-Asp-Pro-Ser-Phe-Arg-</td>
<td>Trp Leu</td>
</tr>
<tr>
<td>D. pyroides myohemerythrin</td>
<td>Gly-Trp-Glu-Ile-Pro-Glu-Pro-Tyr-Val-Trp-Asp-Glu-Ser-Phe-Arg-</td>
<td></td>
</tr>
<tr>
<td>P. agassizii hemerythrin A</td>
<td>Gly-Phe-Pro-Val-Pro-Glu-Pro-Tyr-Val-Trp-Asp-Ala-Ser-Phe-Lys-</td>
<td></td>
</tr>
<tr>
<td>P. agassizii pooled</td>
<td>Gly-Phe-Incomplete-Val-Trp-Asp-Ala-Ser-Phe-Lys Thr-Trp-Thr-Thr-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trp-Thr-Phe-Tyr-Ser-Ile-Ile-Asp-Asp-Glu-His-Lys-Thr-Leu-Phe-Asn-Gly-Ile-Phe-His-Leu-Ala-Ile-</td>
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</tr>
<tr>
<td></td>
<td>Val-Phe-Tyr-Glu-Gln-Leu-Asp-Glu-Glu-His-Lys-Lys-Ile-Phe-Lys-Gly-Ile-Phe-Cys-Asp-Ile-Arg-</td>
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<td></td>
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<tr>
<td></td>
<td>Thr-Phe-Tyr-Asp-Asp-Leu-Asp-Gln-His-Lys-Incomplete</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Incomplete</td>
<td>Incomplete</td>
</tr>
</tbody>
</table>
---Incomplete--- Tyr Gly Gly Tyr Ala Ala His Lys ---Incomplete---
---Incomplete--- Val Ala Lys Tyr Ser Gly Tyr Asn Ala His Lys Glu Ala Ala His Gly Phe---
---Incomplete---
Asn His-Ala-Leu-Asp-Asn-Trp-Lys-Gly-Asp--- (footnote a) Val-Lys-Trp-Ala-Lys-Ser-Trp-Leu-
Glu-Lys-Ile-Gly-Gly-Leu-Ser-Ala-Pro-Ala-Lys-Asn-Val-Asp-Tyr-Cys-Lys-Glu-Trp-Leu-
---Incomplete--- Gly- a Ser-Ala-Tyr-Ala-Lys-Asp-Trp-Leu-
---Incomplete--- a ---Incomplete--- Ala-Arg-Asp-Trp-Leu-

---

Val-Asn-His-Ile-Lys-Thr-Ile-Asp-Phe-Lys-Tyr-Lys-Gly-Lys-Ile C-terminus
Val-Asn-His-Ile-Lys-Gly-Thr-Asp-Phe-Lys-Try-Lys-Gly-Lys-Leu
Val-Gln-His-Ile-Lys-Thr-Ile-Asp-Phe-Lys-Tyr-Lys-Gly-Gln-Leu

---

*Numbering of G. gouldii coelomic hemerythrin followed in this figure.

*Peptide G-6 Ch-1 (Thr-Trp) may belong here, allowing a Thr variant at this position.*
status of histidine 73 versus 77 remains unclear with the data presently available.

The replacement of lysine by glutamine at position 112 in hemerythrin subunit type A (Fig. 40) is the only apparently significant substitution from residues 95-113 of the carboxyl terminus. All of the lysines in _G. gouldii_ hemerythrin, and the amino terminus, have been chemically modified with neither iron loss nor spectral absorption loss. They were concluded to be nonessential for the maintenance of tertiary structure and the iron binding site (Fan and York, 1969; Morrissey, 1971). Thus, the replacement of a positively charged side chain with an uncharged but polar one remains conservative in terms of the tertiary structure of this protein. The same interchange occurs so often among the globins that it has been termed very conservative (Zuckerkandl and Pauling, 1965).

Either or both of the tyrosines at positions 18 and 70 of _G. gouldii_ coelomic hemerythrin are suspected of being involved in subunit binding (Rill and Klotz, 1970). Tyrosine 70 is replaced by valine in the primary structure of myohemerythrin which lacks a quaternary structure. In _P. agassizii_ hemerythrin, tyrosine is clearly present at position 70. Thus it is possible that the tyrosine at position 70 is involved in the subunit interactions of _G. gouldii_ and _P. agassizii_ coelomic hemerythrins. Tyrosine 18, which is present in both _G. gouldii_ and _P. agassizii_ coelomic hemerythrin, and the myohemerythrin of _D. pyroides_, may play a part in the subunit interactions of these coelomic hemerythrins, but it has no such role in myohemerythrin.

Positions 71 and 72 of both coelomic hemerythrins, compared to myohemerythrin, show a wide range of variance (Fig. 40). The methionine
at position 62 in *G. gouldii* coelomic hemerythin is believed to play an important part in the maintenance of tertiary structure, but not in binding the iron (Morrissey, 1971). Methionine is also found at position 62 in the myohemerythin of *D. pyroides* (Cote, 1973). From the pooled tryptic and chymotryptic digestions of *P. agassizii* coelomic hemerythin, methionine containing peptides, whose compositions had some homology with the known hemerythrins in the region around position 62, have been isolated. However, ambiguous and contradictory sequence information precluded assignment of these peptides (G-2 S-2 el-A-6 and TT-1 S-3 el-4) to this region of the primary structure.

Even in subunit type A which was believed to be a relatively pure subunit, variance was noted. At the amino terminus, there is a leucine at position 3 when undigested hemerythin is sequenced, while proline is present at the same position in the amino terminal peptide isolated from the digestion of hemerythin A. This amino acid interchange is not detectable or separable by any technique which depends on charge differences. Additional neutral, or near neutral interchanges were noted at several positions in the partial sequence of *P. agassizii* coelomic hemerythin (see Fig. 40; positions 12, 17, 19, 68, 71, 99, and 104).

Isoelectric focusing, especially with ampholytes of the narrow pH range, can separate some of the electrophoretically near-neutral interchanges, for example, glutamic for aspartic acid, or lysine for arginine. The many minor bands observed during isoelectric focusing with the narrow range ampholytes, and 8 M urea, are probably due to the separation of subunits exhibiting this type of variance.

Amino acid invariance in the same protein from different species
has been interpreted as indicating the necessity of these residues for maintaining the tertiary structure or, their involvement in other essential protein interactions (Dickerson, 1972). When homology is maintained for certain amino acid residues among hemerythrins which differ widely from one another (composition, source, and quaternary structure), especially when chemical modification studies have suggested they are potential ligands, the case for their essential involvement in iron binding, and thus, maintenance of the tertiary structure is greatly strengthened. However, the necessity of maintaining the conformation of the mRNA may also cause an invariant residue in a protein. Or, the second strand of DNA may at that coding point be essential for the structure of another mRNA or protein (North, 1972). Ideally, the conclusion that an invariant residue is essential for the functioning of a protein should be based on chemical modification studies with the final conclusion being reserved for x-ray diffraction data.

The assembled peptide data, especially in the central region of the molecule, suggests a minimum of four subunit types being present. Since half the animals examined individually had a gel electrophoretic pattern virtually identical to the pattern of pooled hemerythrin, these individuals probably have in their genomes a sufficient number of different alleles of the hemerythrin structural gene(s) to account for all of the subunit types observed (five major types on isoelectric focusing of pooled hemerythrin). Posttranslational modifications may also be responsible for some of the variants observed in this protein.

In terms of tertiary structure, the subunits are probably quite similar based on their sequence homologies with one another and the known hemerythrins. The subunits may be functionally equivalent, but
this is speculation until a method to make native monomer is found and oxygen binding studies are performed on them.

Heterogeneity among functionally identical enzyme subunits and the formation of differently charged oligomers from these subunits, via hybridization, has been an established fact for some time (Vessell, 1968). Lactate dehydrogenase is the classic example of this. It is a tetramer which can be formed from any combination of the two electrophoretically different subunit types (Markert, 1968). Although the tetramer types are functionally similar, they require different conditions for optimum activity. The same could be true for oxygen affinity in the different trimers of P. agassizii coelomic hemerythrin.

In a number of properties, P. agassizii coelomic hemerythrin resembles the hemerythrin found in the brachiopod L. unguis more than those examined in other sipunculids to date. The brachiopod hemerythrin is octameric and has two electrophoretic species on disc gels, even from an individual animal. Each of these bands shows additional heterogeneity when electrophoresed on gels 8 M in urea (Joshi and Sullivan, 1973). Heterogeneity of the L. unguis hemerythrins was also indicated by the low yields of tryptic peptides obtained. Finally, although the Lingula hemerythrins each contained a cysteine, no mention was made of the obvious experiment of producing native monomers by chemically blocking the cysteine. The separation of different native monomer types, the monomer having been made by chemically blocking the cysteine, has already been reported for the coelomic hemerythrin of G. gouldii (Klippenstein, 1972a). In addition, the authors (Joshi and Sullivan, 1973) produced monomer for a sedimentation equilibrium molecular weight determination by using harsh denaturing conditions (6 M guanidine hydro-
chloride and 0.1 M mercaptoethanol). Although not stated concerning
the Lingula hemerythrins, it can be deduced that as with *P. agassizii*
coelomic hemerythrin, the *Lingula* hemerythrins resisted all attempts
to produce a native monomer. In addition, 21 residues in from the
carboxyl termini were reported as having been sequenced for each of the
*Lingula* hemerythrins (Joshi and Sullivan, 1973). It is quite likely
that the *Lingula* hemerythrins resemble the coelomic hemerythrins of *P.
agassizii* in having termini which show a strong homology with other
hemerythrins and highly variable central regions.

The primary structures of the coelomic hemerythrins from worms
in the two genera which have been examined are remarkably similar to
each other. The primary structure of *G. gouldii* hemerythrin (Klippenstein
*et al.*, 1968), and that of *D. pyroides* (Ferrell and Kitto, 1971) differ
at only four points in their total primary structures. Both are octa-
The close homology of these respiratory proteins led to the conclusion
that these genera had diverged from a common ancestor fairly recently
in sipunculid evolutionary history (Ferrell and Kitto, 1971). The
coelomic hemerythrin of *P. agassizii* differs radically from either of
these coelomic hemerythrins in terms of primary and quaternary structure.
Having a trimeric quaternary structure, *P. agassizii* coelomic hemerythrin
differs from all other sipunculid coelomic hemerythrins studied to date
(three species from three genera, all octameric), even from those of
another phyla (*Brachiopoda*, *Lingula unguis* and *Lingula reevi*, both octa-
meric) (Joshi and Sullivan, 1973; Klotz, 1971). This marked difference
in quaternary structure, plus the observed and suspected lack of homology
in the primary structure of the *P. agassizii* coelomic hemerythrins with
those of *G. gouldii* and *D. pyrroides*, strongly suggests that the genus *Phascolosoma* belongs in at least a different grouping in the next step of the Linnean classification scheme. *Dendrostomum* and *Golfingia* as genera probably belong in the same family, while *Phascolosoma* should be in another family. This presupposes that the hemerythrins examined are typical of the genus involved. Of course, the inherent danger of classifying an organism on the basis of a single trait renders the above conclusion tentative (Cronquist, 1965).

A myohemerythrin does occur in the muscles of *P. agassizii* and appears to be similar to that characterized in *D. pyrroides* (Klippenstein *et al.*, 1972) in terms of its uv-visible spectrum, estimated molecular weight on SDS-polyacrylamide gels, and sedimentation behavior.

Although reported to not contain a vascular system (Manwell, 1963), *P. agassizii* worms do contain a small vascular system. Hemerythrin D, the minor and unusual hemerythrin found with the coelomic hemerythrin of *P. agassizii*, is suspected of being an accidental contamination from this vascular system. Since it went through the coelomic hemerythrin purification procedure, it probably has a molecular weight in the same range as that of the trimeric coelomic hemerythrin.

Burrowing sipunculids have smaller vascular systems than those which live near the surface in loose sand and obtain their oxygen via the vascular system associated with the tentacles, rather than by diffusion through the body wall, the method of the burrowing worms (Manwell, 1960b). It has been reported that the largest *P. agassizii* worms collected for classification purposes (Fisher, 1952) were found in the mud of Humboldt Bay, California, which was suspected of being a very favorable environment for this species of sipunculid. Thus, a small
vascular system in *P. agassizii* seems reasonable in view of the ecology of this worm.

Much remains to be done with the coelomic hemerythrin of *P. agassizii*. Work is progressing in this laboratory on completing the sequence of hemerythrin subunit type A. Also, the sequences of subunit types B and C, and as many of their variants as possible, should be completed. This data would be very valuable when the tertiary structure of any hemerythrin becomes available. (*P. pyroides* myohemerythrin is presently being studied by x-ray diffraction techniques, Hendrickson and Klippenstein, 1974).

The octameric hemerythrins probably have a cuboidal quaternary structure (Klotz and Keresztes-Nagy, 1963) which requires three binding surfaces per subunit (Klotz et al., 1970). Recent x-ray diffraction data of octameric hemerythrin was consistent with the placement of the subunits in an approximately cubic structure (North and Stubbs, 1974). The trimeric structure of *P. agassizii* coelomic hemerythrin would allow only two binding sites per subunit (Klotz et al., 1970). Combining primary, secondary, tertiary, and quaternary structural data of the hemerythrins should allow a determination of which residues are involved in the subunit binding sites and the nature of the interactions involved. Considering the difficulties involved in dissociating *P. agassizii* coelomic hemerythrin, hydrophobic and hydrogen bond interactions are predicted to be the major forces involved in its subunit binding.

Hemerythrin-type active centers are not confined to marine invertebrates only. Recently, a subunit of the ribonucleotide reductase system of *Escherichia coli* was isolated which contained two irons per subunit and had Mössbauer and uv-visible spectra that strongly resembled
those of the known hemerythrins (Aiken et al., 1973). Since eucaryotic cells also reduce ribonucleotides, they may contain oxidation-reduction type proteins with hemerythrin-like active centers.
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