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Subunit Heterogeneity in Arthropod Hemocyanins: I. Chelicerata*

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Summary. 1. The hemocyanins of 2 spiders (*Eurypelma californicum, Cupiennius salei*), 2 scorpions (*Pandinus pallidus, Androctonus australis*), a whip-scorpion (*Mastigoproctus brasilianus*) and 2 whip-spiders (*Tarantula palmata, Trichodamon froesi*) were analyzed for subunit heterogeneity by high resolution polyacrylamide electrophoresis (PAGE). For comparison, *Limulus polyphemus* hemocyanin was subjected to the same analytical scheme.

2. All of the species, except for *Limulus* (predominantly 60 S-hemocyanin) and *Cupiennius* (16 S- and 24 S-hemocanin) possess only hemocyanin sedimenting in the 33 S to 37 S range. A second, major blood protein was observed in each species, safe *Mastigoproctus*. This second, non-respiratory protein sediments with ca. 16 S, but with about 24 S in the case of the scorpion species.

3. Upon incubation with sodium dodecylsulfate (SDS) and β-mercaptoethanol and electrophoresis in polyacrylamide gradients, between 2 and 5 hemocyanin bands are obtained, with average molecular weights between 70,000 and 75,000. The non-respiratory proteins yield two chains in each case, with molecular weights between 95,000 and 130,000.

4. Each hemocyanin could be dissociated at pH 9.6 to yield "native" subunits. By gel filtration, these were separated into monomers (4.5 S) and dimers (6 S). No dimers were observed after dissociation of *Pandinus* and of *Limulus* hemocyanin. Only in the case of *Cupiennius* the dimer is formed by a disulfide bridge. – PAGE of the dissociation mixture shows complex patterns which display 6–7 bands.

5. By preparative isolation of "native" subunits and subsequent analysis in SDS-PAGE, the two patterns of hemocyanin bands could be related to each other, and the total number of different polypeptide chains established with some certainty. It ranges from 5 in *Cupiennius* 16 S-hemocyanin to 8 in *Androctonus* hemocyanin and possibly 12 in *Limulus* hemocyanin. A loose correlation between oligomer size and number of different chains is suggested.

Introduction

Arthropod hemocyanins occur *in vivo* as oligomeric molecules with sedimentation coefficients near 16 S, 24 S, 37 S and 60 S, which are made up of 6, 12, 24 and 48 subunits, respectively. The subunits sediment with about 5 S, and their molecular weight is near 70,000 (van Holde and van Bruggen, 1971); there is now nearly general consensus that they represent a single polypeptide chain each. During recent years evidence has been obtained for a number of hemocyanins, that they are composed of more than one type of polypeptide chain. Among the Chelicerata, Lamy et al. (1977) isolated 6 fractions from the hemocyanin of the scorpion *Androctonus australis*. Scorpion species could be readily differentiated by the gel electrophoresis patterns obtained after freezing and thawing of hemolymph proteins (e.g. Lamy et al., 1971; Goyffon et al., 1973). *Limulus polyphemus* hemocyanin was fractionated by ion exchange chromatography to yield 5 bands (Sullivan et al., 1974) three of which were subsequently (Sullivan et al., 1976) shown to be heterogeneous. Sugita and Sekiguchi (1975) compared blood serum isolated from another xiphosuran (*Tachypleus*) with sera from a spider (*Argiope*) and a scorpion (*Heterometrus*) and found them all to yield several bands. Finally, a detailed study of the hemocyanin from the tarantula, *Eurypelma californicum*, has revealed the presence of 6, possibly 7 different polypeptide chains (Schneider et al., 1977).

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Similar results have been obtained for crustacean species; pertaining literature will be cited in a subsequent paper (Markl et al., in preparation).

The methods employed for dissociation and analysis of the ensuing subunit mixtures have varied to some degree. From the results obtained by the combination of alkaline dissociation and electrophoresis at pH 9.6, with denaturation by sodium dodecyl sulfate (SDS) and electrophoresis in gradient polyacrylamide gels (Schneider et al., 1977) it appeared to us that this system was particularly sensitive to detect subunit heterogeneity. It is not too likely that two bands migrating at the same position in the alkaline system would also have the same molecular weight and thus fall together in the SDS system.

The aim of the present study is to compare – within the class of the Chelicerata – hemocyanin subunit heterogeneity by this more refined method, to seek further support for the notion that hemocyanins are principally composed of different subunits, and to include groups (the orders Uropygi and Amblypygi) not studied previously in this respect.

Materials and Methods

Blood Samples

The spiders, *Cupiennius salei* (fam. Lycosidae) and *Eurypelma californicum* (fam. Avicullariidae) were raised and maintained as described previously (Melchers, 1963; Loewe and Linzen, 1973). *Pandinus pallidus* (fam. Scorpionidae; from Kenya) was obtained commercially; the whip-scorpion, *Mastigoproctus brasilianus* (order Uropygi; from Brasil), the whip-spiders *Trichodamon froesi* (order Amblypygi; from Brasil) and *Tarantula palmata* (order Amblypygi; from Colombia) were collected by Prof. P. Weygoldt. Blood was sampled by heart puncture after the animals had been cooled to 4 °C. The blood was immediately centrifuged to remove cells; in most cases, the experiments were performed on fresh samples. Otherwise, the samples were stored at 4 °C. *Androctonus australis* (Scorpiones, fam. Buthidae) cell free blood was sent to our laboratory from Tours, France. *Limulus polyphemus* blood was airshipped to Munich from the Duke University Marine Laboratory, Beaufort, N.C.; chromatographic fractions of *Limulus* hemocyanin were a gift of Drs. J. and C. Bonaventura. No special precautions – except storage in the cold – were taken to prevent proteolysis.

Dissociation of Proteins

The hemolymph proteins were either dissociated (overnight, room temp.) by glycine/NaOH, pH 9.6, I = 0.05, at a protein concentration of 25 mg/ml, or by heating for 20 min at 56 °C in 0.1 M Tris/HCl buffer of pH 8.8, containing 2% sodium dodecylsulphate and 1% β-mercaptoethanol. For denaturation in SDS the protein concentration was 1 mg/ml.

Separation Procedures

Cell-free blood samples were passed through a column (160 × 1,5 cm) of Biogel A 5 m (Bio Rad Laboratories), equilibrated with 0.1 m Tris/HCl buffer, pH 7.5, containing 10 mM Mg^{2+} and 5 mM Ca^{2+}.

The separation of monomer and dimer fractions after alkaline dissociation of the proteins was achieved by passing the mixture through a bed of Sephadex G 100 sf, equilibrated with dissociation buffer (pH 9.6); fractions of 2 ml were collected.

Preparative polyacrylamide electrophoresis was performed in an "Ultra Phor" apparatus (manufactured by COLORA, Lorch) using a 6% gel and the buffer system No. 1 in the list of Maurer (1968). The electrode buffer was concentrated 10-fold, however. Protein samples were applied in Tris/HCl buffer, pH 8.9, I = 0.01. About 3 mg of protein were applied per cm^2 of gel surface.

Analytical electrophoresis was done in vertical gel slabs in linear (5–10%) gel gradients by the method of Laemmli (1970) at pH 8.8 (SDS runs), or, with the same gel gradient, at alkaline pH with a 0.325 M Tris/borate buffer, pH 9.6, as gel buffer, and 0.065 M Tris/borate, pH 9.6 as electrode buffer. Electrophoresis was at 300 V, 10 mA, 6 °C for 2 or 3 h. The calibration proteins, phosphorylase *a* (97,000), bovine serum albumin (68,000), catalase (58,000), and aldolase (40,000) were purchased from Boehringer, Mannheim. In addition, the polypeptide chains of *Eurypelma californicum* hemocyanin, ranging from 67,000 to 76,000 (Schneider et al., 1977), were employed.

Gels were stained in 25% TCA, containing 1% (for SDS runs) or 0.1% (all others) Coomassie Brilliant Blue. Destaining was by a 3:2:35 mixture of glacial acetic acid, methyl alcohol, and water.

Other Analytical Procedures

Sedimentation analysis was performed in a Beckman Model E analytical ultracentrifuge equipped with scanner optics (wavelength set at 280 nm), at protein concentrations between 0.3 and 0.8 mg/ml. Sedimentation coefficients were not corrected or extrapolated to zero concentration.

Copper was determined with a Perkin-Elmer model "400" atomic absorption spectrometer.

Results

The Pattern of Native Hemolymph Proteins

When the cell-free hemolymph was filtered through an agarose column, a very simple elution pattern resulted in each case. Only one or two major peaks were found, although the occurrence of other proteins in minute quantities has not been excluded (Fig. 1). Light absorption at 340 nm indicated that the first peak contained hemocyanin in each sample while the second peak showed either a much reduced 340 nm/280 nm ratio or did practically not absorb light at 340 nm, both indicating the presence of a second major blood protein. This was confirmed by copper analysis.

The presence of proteins not related to oxygen transport in blood of *Limulus* has been known for some time; two of them have been assigned the function of a heteroagglutinin (Roche and Monsigny, 1974). Non-respiratory proteins of *Eurypelma* and of *Cupiennius* have been described recently (Markl et al., 1976); their function is not known. The additional

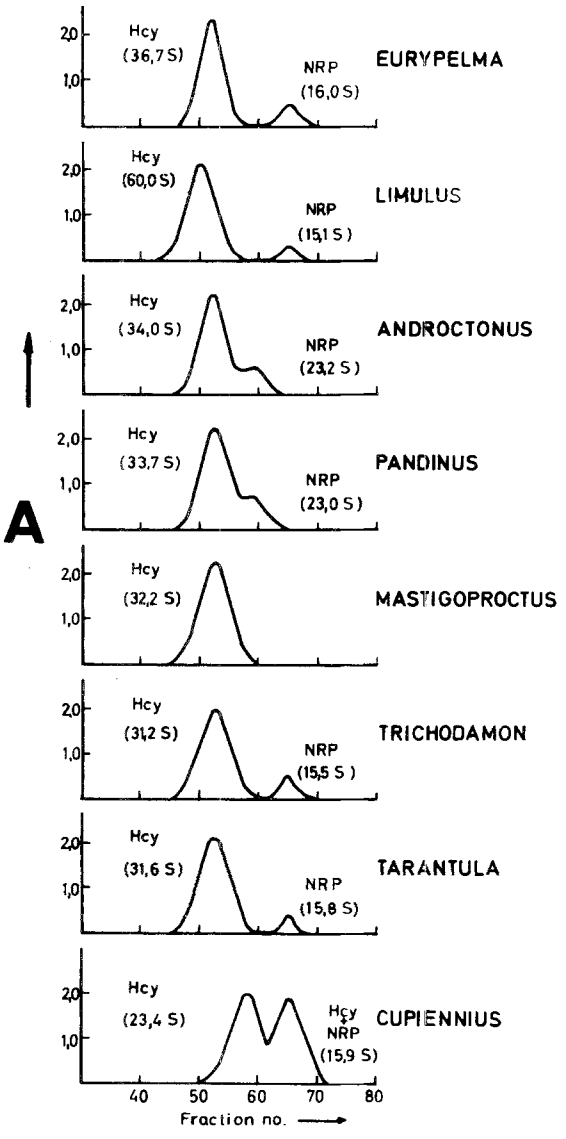

Fig. 1. Elution pattern upon gel filtration of the major hemolymph proteins of various Chelicerata (for species, see Materials and Methods). Absorbance was measured at 280 nm. *Hcy*, hemocyanin; *NRP*, a non-respiratory protein. Sedimentation coefficients are given in brackets

hemolymph proteins comprise from 10 to 20 per cent in each species examined, with the exception of the whip-scorpion *Mastigoproctus* where no such protein was found.

In Fig. 1 the sedimentation coefficients are also incorporated. They fall into the well known classes of 16 S, 24 S, 34 S and 60 S, with a preponderance of the 34 S class. Only one species, *Cupiennius salei*, contains two hemocyanin species which sediment at 16 S and 24 S. The additional, non-respiratory blood proteins had sedimentation coefficients near 16 S with the exception of the two scorpion species where these proteins were found to sediment at 24 S. It is of particular interest that these molecules should fall into the same size classes as the hemocyanins themselves.

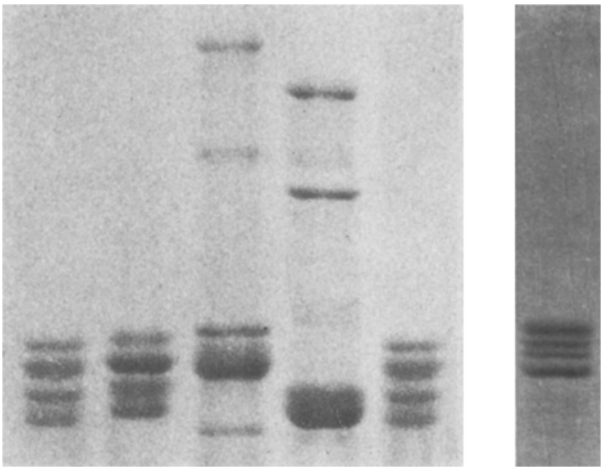

Fig. 2. Electrophoresis of SDS denatured hemocyanins in a polyacrylamide gradient. Left: Five samples of *Eurypelma californicum*, *Pandinus pallidus*, *Androctonus australis*, *Cupiennius salei* and, again, *Eurypelma* (from left to right). The α and β chains of the second, major hemolymph protein are also present in the case of *Androctonus* and *Cupiennius*. Right: A particularly good separation of the polypeptide chains from *Mastigoproctus brasilianus* hemocyanin

Analysis of Polypeptide Chains by SDS-PAGE

Figure 2 depicts several examples of the protein patterns obtained after denaturation by SDS and β-mercaptoethanol and Fig. 3 shows all the patterns schematically and including the molecular weight data. It is clear that each single hemocyanin yields a series of polypeptide chains of similar molecular weight, the number of bands ranging from 2 in the 16 S-hemocyanin of *Cupiennius* to 5 in the case of the *Limulus*, *Androctonus*, *Tarantula*, and *Eurypelma* hemocyanins. It should be noted that the two hemocyanins of *Cupiennius salei* differ by one additional band in the 24 S component, and that dissociation of the latter in the absence of the reducing agent, β-mercaptoethanol, resulted in the disappearance of band *c*, and the appearance of a new band migrating at the position of a dimer.

For the molecular weight determinations, the polypeptide chains of *Eurypelma* hemocyanin served as a handy reference since these had been determined previously both by sedimentation equilibrium centrifugation and in SDS gels. In the hemocyanins of the other species the molecular weights of the individual polypeptide chains differed maximally by 12,000 (*Androctonus*) and minimally, 3,000 (*Cupiennius*). The average molecular weight is between 70,000 and

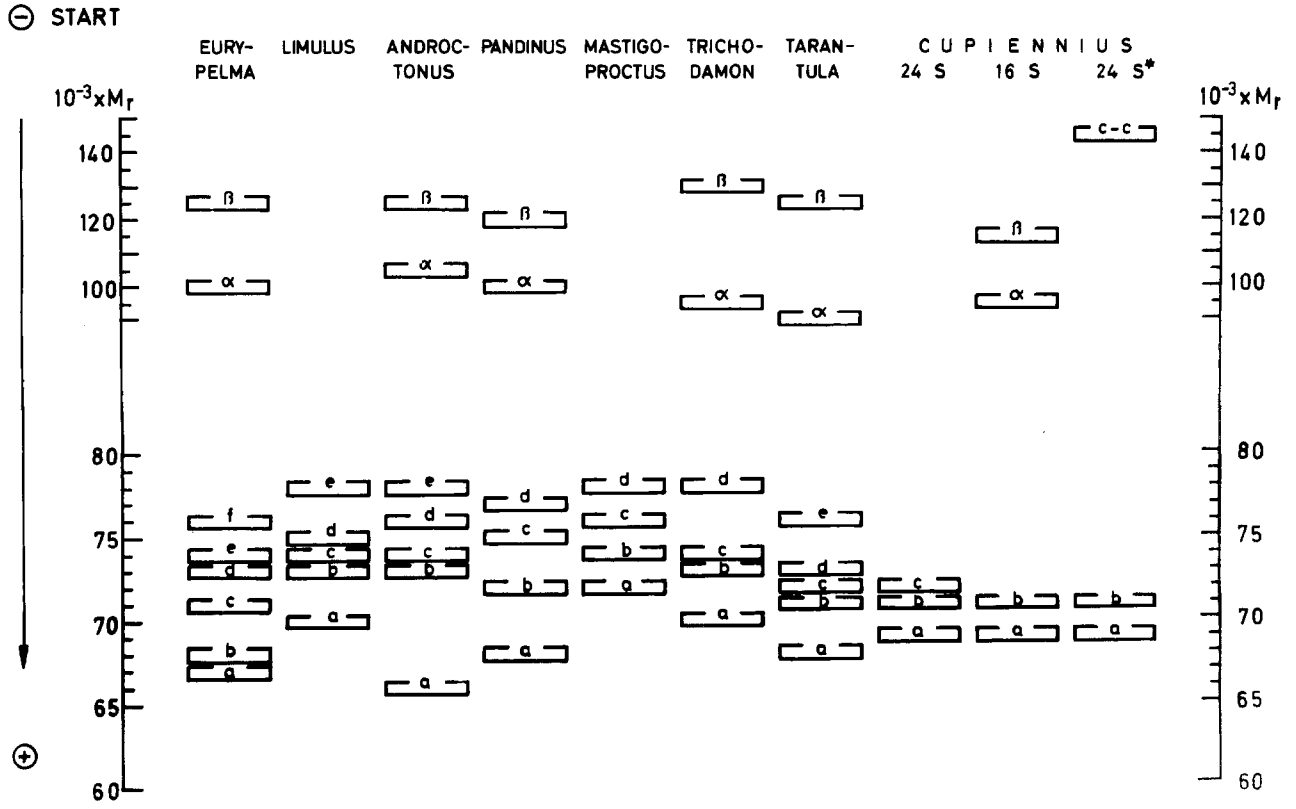

Fig. 3. Patterns of protein bands obtained after sodium dodecylsulphate/β-mercaptoethanol denaturation of hemolymph proteins and electrophoresis in a polyacrylamide gradient. A molecular weight scale is included. Hemocyanin chains are labelled with letters, chains of the second, non-respiratory protein with greek letters. In the case of *Limulus* this protein is of low molecular weight, in *Mastigoproctus* none was detected. In the case of *Cupiennius*, the 24S and 16S peaks of Fig. 1 were analyzed individually, in addition the 24S protein was denatured without adding β-mercaptoethanol (asterisk); note disappearance of band c and appearance of a dimer c-c

75,000 in each species. For *Limulus* hemocyanin this is slightly higher than the value of ca. 66,000 given by Sullivan et al. (1974).

The second, major blood proteins yielded two polypeptide chains with molecular weights between 95,000 and 130,000. Only in the case of *Limulus* this second protein, which is known to be a heteroagglutinin (Roche and Monsigny, 1974) yields low molecular weight bands (18,000 and 20,000).

The Products of Alkaline Dissociation

By incubation in glycine/NaOH, pH 9.6, the hemocyanins were dissociated into "native" subunits, i.e. subunits still capable of binding oxygen and of reassociation in appropriate media. The dissociation mixtures were chromatographed on Sephadex G-100 sf (Fig. 4). A single peak was obtained after dissociation of *Limulus* and *Pandinus* hemocyanin whereas all other hemocyanins yielded two peaks, one (minor) corresponding to subunit dimers, the other (major) to the monomers of molecular weight 70,000. The sedimentation coefficients of the monomer fractions were about 4.5 S, and the coefficients of the dimers ranged near 6 S.

Since the 16 S-hemocyanin of *Cupiennius* still contained the non-respiratory blood protein which also sediments with 16 S a third peak was found after chromatography. This contained the intact non-respiratory protein which does not dissociate at pH 9.6.

The dimer fractions isolated from *Mastigoproctus, Trichodamon, Tarantula,* and *Androctonus* hemocyanin appear to be perfectly stable under these conditions. Some dissociation occurred upon addition of 10 mM EDTA or 3 M urea. The dimer obtained from *Cupiennius* hemocyanin was unaffected by these agents, but could be easily split by 5 mM cysteine.

The dissociation mixtures were resolved by electrophoresis in polyacrylamide gradients (Figs. 5 and 6). *Cupiennius* 16 S-hemocyanin yielded five bands, while a sixth band was observed after dissociation of the 24 S-hemocyanin. The hemocyanins of *Pandinus, Mastigoproctus, Trichodamon* and *Tarantula* could each be resolved into six bands, those of *Eurypelma, Androctonus* and *Limulus* into seven. In each

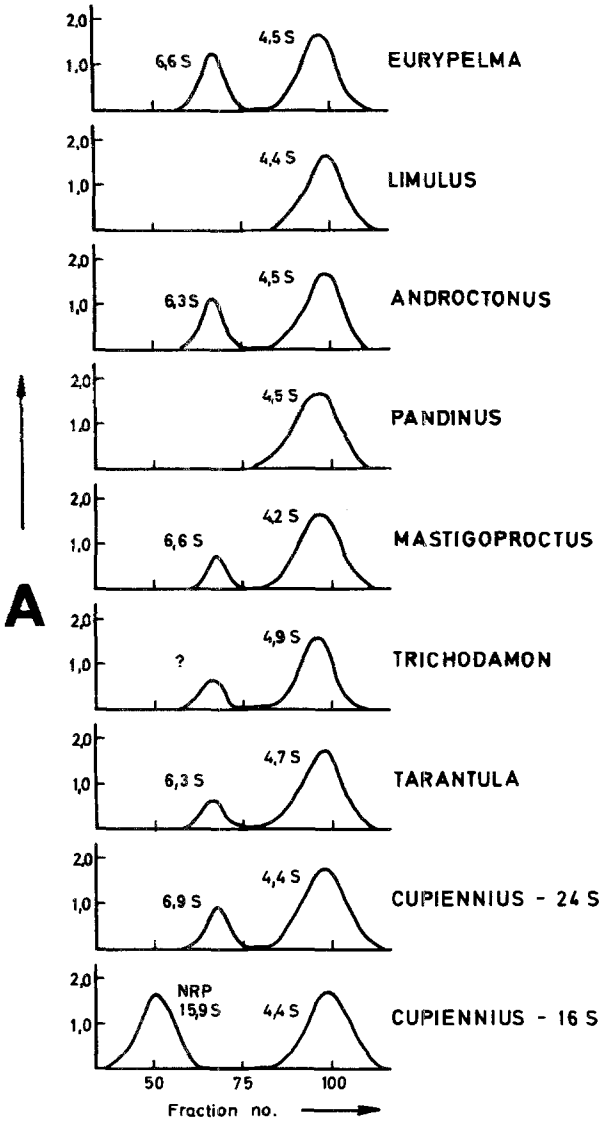

Fig. 4. Gel filtration patterns after alkaline dissociation of Cheliceratan hemocyanins. Absorbance was recorded at 280 nm. Sedimentation coefficients are given for each peak, except for the *Trichodamon* dimer fraction which was lost. The 16 S fraction of *Cupiennius* blood still contained the non-respiratory blood protein; note the absence of hemocyanin dimer from this fraction

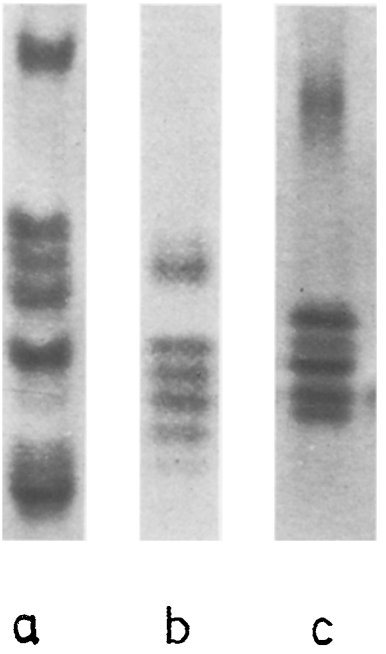

Fig. 5a–c. Examples of electrophoretic patterns obtained after alkaline dissociation of Cheliceratan hemocyanins. **a** *Androctonus australis;* **b** *Trichodamon froesi;* **c** *Cupiennius salei.* In each case the slowest migrating band represents a dimer

case, the slowest migrating band represented the dimeric subunits, i.e. the 6 S-peak of the gel chromatography.

Cross-Reference of PAGE-Protein Patterns

The hemocyanin bands obtained by the two different electrophoretic procedures cannot be compared *a priori*, as they are separated by charge in one system, and by chain length in the other. Furthermore, it must be expected that in either system two or more fractions are not separated but obscure each other. On the other hand, it is not too likely, that two subunits will behave identically in both systems. Thus, by cross-reference of the two types of subunit protein patterns one should be able to establish with some reliability the true number of different polypeptide chains in each hemocyanin.

In order to assign each protein band of one pattern to a band, or a set of bands, in the other we dissociated somewhat larger hemocyanin samples and separated the subunits by preparative PAGE. The individual fractions were then analyzed in the SDS system. Figure 7 illustrates the preparative fractionation of *Androctonus* hemocyanin dissociated at pH 9.6. The dimer fraction had been isolated before as described above.

Single subunits have been obtained in a pure state in many cases. Even if one peak was not homogeneous, it was not difficult to distinguish the major component from the minor and to identify each in the SDS system.

A synopsis of the results is given in Fig. 8. The results for *Eurypelma* confirm a previous study (cf. Fig. 6 in Schneider et al., 1977). Note that chain *f* is derived both from the monomeric subunit 3 and the dimer 5, which is a symmetrical or homodimer of 3. A homodimer is also found in *Cupiennius* hemocyanin and in *Mastigoproctus* hemocyanin (though further criteria would be required to ascertain the

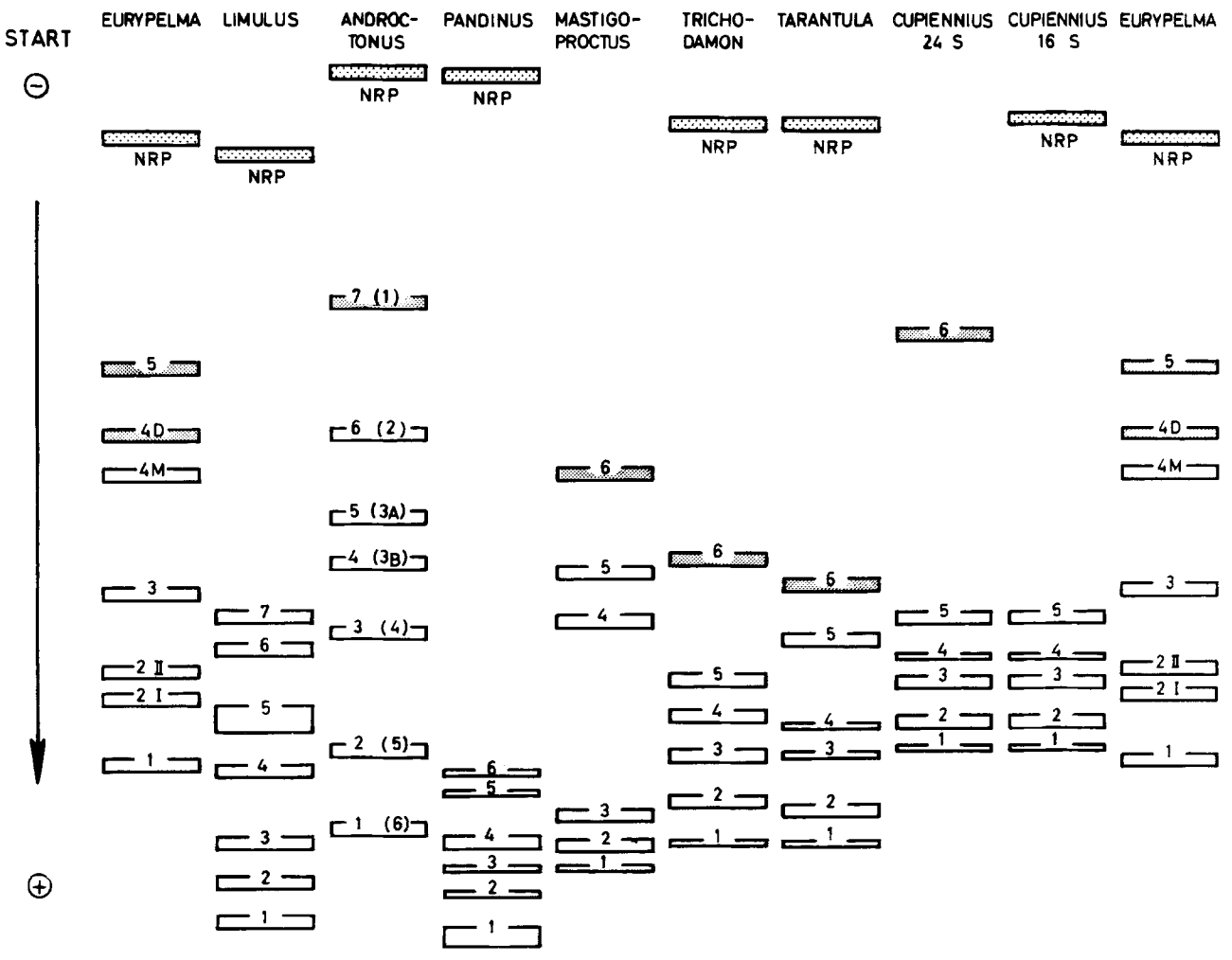

Fig. 6. Scheme of electrophoretic patterns obtained after alkaline dissociation of Cheliceratan blood proteins. *NRP*, non-respiratory protein (stippled). Hemocyanin bands are labelled with arabian numerals; for *Eurypelma* (presented twice for easy comparison) the nomenclature of Schneider et al. (1977) is adopted, for *Androctonus* the nomenclature of Lamy et al. (1977) included for comparison (Lamy's band 3 was resolved into two bands, see Note added in proof). Dimer bands are shaded

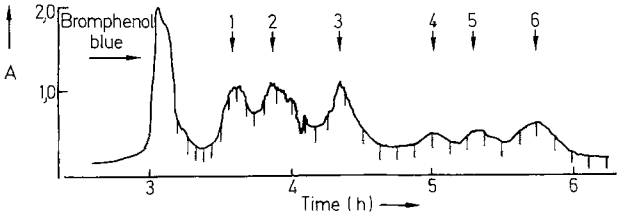

Fig. 7. Preparative polyacrylamide gel electrophoresis of *Androctonus australis* hemocyanin subunits. Only the fraction of monomers was applied to the gel. A 6 per cent gel and buffer system No. 1 in the list of Maurer (1968) were employed

nature of this dimer more firmly). Asymmetrical or heterodimers were obtained from *Eurypelma, Androctonus,* and *Tarantula* hemocyanin. The *Trichodamon* dimer was unfortunately lost by a technical failure.

Resolution in the alkaline PAGE system was generally better than in the SDS system. This may reflect the necessity to keep the dimensions of the subunits within rather narrow limits in order to make them fit well into the hexameric building block of arthropodan hemocyanins. Nevertheless it is clear that in most cases the total number of different polypeptide chains exceeds the number of bands observed in the alkaline PAGE system.

Difficulties were encountered in the analysis of *Limulus* hemocyanin. Bands 1, 6 and 7 of the alkaline pattern each yielded a single band in the SDS-pattern (*d*, *a* and *a*, respectively), "alkaline" bands 2, 3 and 4 yielded two chains each ($d+e$, $c+e$, and $a+d$, respectively), while SDS analysis of band 5 resulted in three different chains (*a*, *b* and *d*). Thus there are twice four chains of practically the same chain length (*a* and *d*; estimated molecular weights 70,000 and

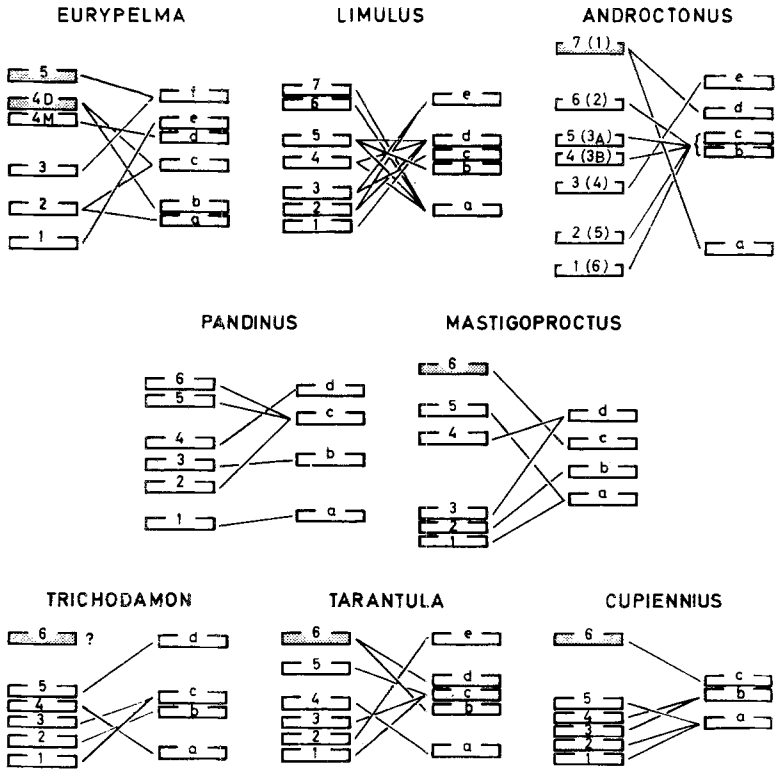

Fig. 8. Cross-reference of electrophoretic patterns obtained after alkaline dissociation (left patterns) and SDS/β-mercaptoethanol denaturation (right patterns), respectively, of cheliceratan hemocyanins. The corresponding protein bands are connected by lines; the number of these lines is equal to the total number of different polypeptide chains. Shaded bands in the "alkaline" patterns represent dimers. Analysis of *Trichodamon* hemocyanin could not be completed due to a technical failure and loss of the dimer fraction. For *Androctonus* hemocyanin, the nomenclature of Lamy et al. (1977) is included; subunits 1, 2, 4, 5 and 6 could not unequivocally be assigned to SDS bands b and c, see also Note added in proof

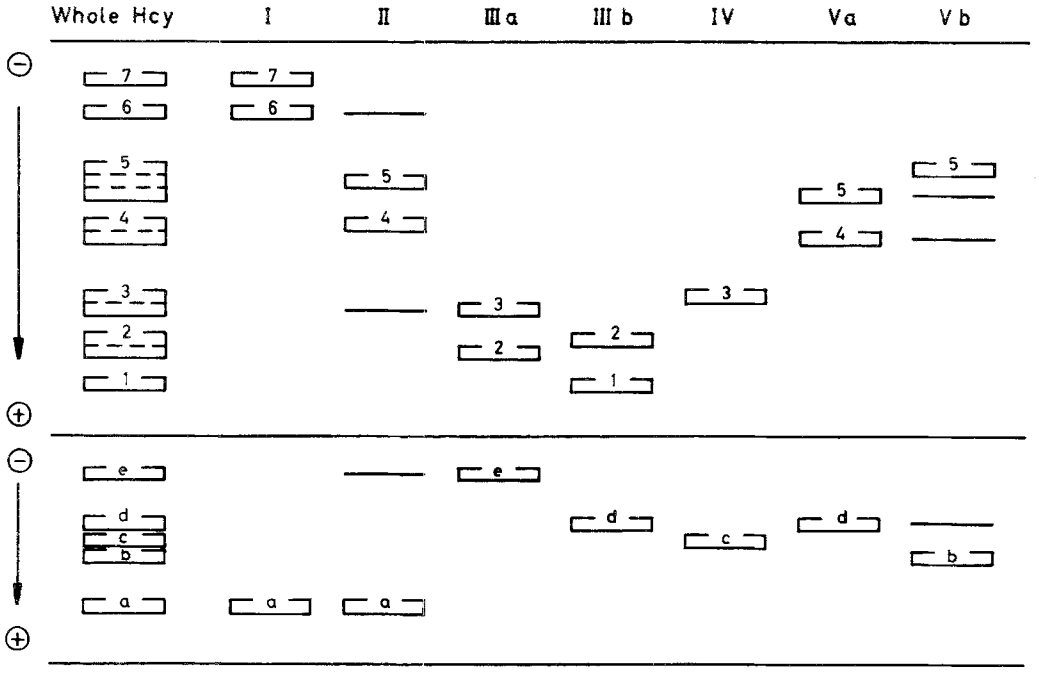

Fig. 9. Polyacrylamide gel electrophoresis of fractions obtained by ion exchange chromatography of *Limulus polyphemus* hemocyanin. The fractions were kindly provided by Drs. C. and J. Bonaventura. Upper panel: Alkaline dissociation and electrophoresis at pH 9.6; lower panel: treatment with SDS/β-mercaptoethanol and electrophoresis in the system of Laemmli (1970)

Table 1. State of association and number of different polypeptide chains in various cheliceratan hemocyanins

Order and species	Subunits in Hcy particle	Number of different chains
Xiphosura		
Limulus polyphemus	48[a]	12
Scorpiones		
Androctonus australis	24	8
Pandinus pallidus	24	6
Uropygi		
Mastigoproctus brasilianus	24	6
Amblypygi		
Trichodamon froesi	24	6 (7?)
Tarantula palmata	24	7
Araneae		
Eurypelma californicum	24	7
Cupiennius salei	12	6
	6	5

[a] predominant hemocyanin fraction

75,000, respectively), while chains *b*, *c* and *e* are represented once, once and twice in the alkaline pattern. This would lead to a total of 12 different polypeptide chains.

This pattern of *Limulus* hemocyanin could be confirmed by analysis of the chromatographic fractions I–V (with III and V divided each into subfractions a and b) kindly put at our disposal by Drs. C. and J. Bonaventura. Fractions I through IIIb and fraction Va yielded two bands each in the alkaline system, while fractions IV and Vb yielded a single band (Vb was contaminated though by Va). This is schematically shown in Fig. 9.

Table 1 shows the number of hemocyanin subunits in each species. It is of interest to note that in *Cupiennius* the 24 S-hemocyanin (dodekamer) and 16 S-hemocyanin (hexamer) differ by one polypeptide chain.

Discussion

1. Absolute Number of Different Subunits

The concept of subunit heterogeneity in arthropod hemocyanins has arisen as a consequence of the higher resolution conveyed by modern analytical methods. There are various examples showing that after the first notion of heterogeneous subunits in a hemocyanin, the number of different entities did further increase in later analyses: In *Limulus* hemocyanin from 5 (Sullivan et al., 1974) to 8 (Sullivan et al., 1976) to 12 (present study); in *Carcinus maenas* hemocyanin, from 2 (Busselen, 1970) to 4 (unpublished results); in *Androctonus* hemocyanin, from 6 (Goyffon et al., 1970) to 8 (this study; Lamy et al., 1979). It is not unreasonable then, to assume that further minor differences between chains will be detected by applying still more refined techniques in the future.

In *Eurypelma*, the question whether there are 6 or 7 different chains hinges on the identity or non-identity of the two chains "*c*" obtained from the "alkaline" fractions 2 and 4_D (Schneider et al., 1977). By chromatographic fractionation of 2 and 4_D, the constituent polypeptide chains have recently been isolated, and by chemical analysis shown to be not identical (Markl et al., in preparation). The number of 7 different subunits is further corroborated by immuno-electrophoresis (Lamy et al., to be published). As the same number of subunits is derived from such different methods and with a number of different samples, we are confident that no further revision will be necessary.

In *Androctonus australis* hemocyanin too, the immunochemical analysis points to the same number of polypeptide chains as found in the present study (J. Lamy et al., 1979).

We are on less firm grounds in the case of *Limulus* hemocyanin. The results of Sullivan et al. (1976) indicate the presence of 8 different subunits. Although the number of 12 found in this study has been arrived at in two sets of experiments with different starting material, one might argue that some of the bands are artifactual. The hemolymph samples and hemocyanin fractions used had been prepared at least many weeks prior to our analysis, and deterioration cannot be rigidly excluded. We have recent evidence that some isolated hemocyanin subunits can be very stable, while others may slowly deteriorate. If such gradual proteolysis had been occurring in the *Limulus* samples, one should have expected a clearer quantitative distinction between the original, native band and the product derived from it. However, all subunits were found in comparable quantities. For the moment, then, we believe that the likely number of subunits in *Limulus* hemocyanin is twelve.

2. The Nature of Heterogeneity in Hemocyanin Subunits

As mentioned, a possible cause of polypeptide heterogeneity may be sought in the stepwise degradation of an originally homogeneous "mother chain". This could lead to the observed differences in molecular weight. A first argument against this is found in the reproducibility of the patterns irrespective of the method of hemocyanin isolation, of the specimen and (within limits) the age of the sample. A definitive proof against the hypothesis, however, stems from

results obtained with *Limulus* hemocyanin (Sullivan et al., 1976) and *Eurypelma* hemocyanin (Markl et al., in preparation): If isolated subunits are subjected to cleavage by cyanogen bromide, formic acid, or trypsin, very different peptide patterns ensue, which demonstrates that the heterogeneity is due to major differences in the primary structure of the chains. Further support stems from differences in the amino acid analysis. It is, of course, not ruled out that secondary modifications, such as substitution by carbohydrates, phosphate or other groups contribute to heterogeneity but this has not been studied.

3. *The Significance of Subunit Heterogeneity*

It will not be attempted to discuss the possible physiological rôle played by different hemocyanin subunits, the experimental evidence being too scarce. There is, however, increasing evidence with various hemocyanins that subunit heterogeneity is a prerequisite for formation of the higher association products. The basic physiological unit appears to be the hexamer (16 S-particle). This can be formed, upon incubation in suitable media, by a number of the isolated subunits: by M_1 and M_2 of *Cherax destructor* hemocyanin (Jeffrey et al., 1976); by fractions II, III (both not homogeneous, though) and IV of *Limulus* hemocyanin (Schutter et al., 1977; and unpublished experiments); by subunit 4 of *Androctonus* hemocyanin (Lamy et al., 1977), and by subunit 3 of *Eurypelma* hemocyanin (unpublished experiments). In case of the latter three hemocyanins, the majority of subunits is not capable of hexamer formation. However, certain mixtures will form hexamers most readily.

To build 24 S and 37 S particles, other subunits are required. In *Cherax destructor* hemocyanin, a dimeric fraction, M'_3 is responsible for dodekamer formation. M'_3 is stabilized by disulfide bonds (Murray and Jeffrey, 1974). A similar condition may prevail in the hemocyanin of *Cupiennius salei*, where a dimer which is formed by a disulfide bridge, is obtained only from the 24 S species. For the formation of the larger aggregates sedimenting with ca. 34 to 37 S, again certain subunits must be present: Subunit 1 in *Androctonus* hemocyanin (Lamy et al., 1977), subunit 4_D in *Eurypelma* hemocyanin (to be published), both of which are dimers composed of different polypeptide chains. These heterodimers are not stabilized by disulfide bonds but are held together by non-covalent bonds, probably hydrophobic interaction. In these species, 24 S particles are normally not observed. Dimers with similar properties as those of *Eurypelma* and *Androctonus* hemocyanin were found upon dissociation of *Mastigoproctus*, *Trichodamon*, and *Tarantula* hemocyanin. It would be intriguing to study their rôle in the formation of the 37 S particles.

It is quite clear, then, that subunit heterogeneity can be explained, in part, by the complexity of the oligomeric hemocyanin molecules which hinges on certain linking components. Even without regard to the reassociation experiments just mentioned this might be inferred from the correlation between the degree of association of the native hemocyanin molecules (16 S, 24 S, 37 S, 60 S) and the number of different constituent polypeptide chains. This is most evident from a comparison on a broader scale, including also the crustacean species (Markl et al., in press) but is also indicated by the more limited scope of the present work: *Cupiennius* 16 S hemocyanin yields 5, the 24 S hemocyanin 6 chains; all the 37 S hemocyanins yield 6–8 chains, while *Limulus* hemocyanin (60 S) yields 12 chains.

It must be pointed out that there is no reason to visualize all the different chains incorporated into a single hemocyanin molecule. The native hemocyanin particles might well be heterogeneous, as has indeed been demonstrated for the crayfish *Cherax destructor* (Jeffrey et al., 1976). Heterogeneity at this level might be physiologically significant, but it is difficult to envisage – other than by very general and vague terms – how such multiple respiratory proteins should serve the animal better than one single oligomer, in particular, if the wide range of allosteric modulation is considered.

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References

Busselen, P.: The electrophoretic heterogeneity of *Carcinus maenas* hemocyanin. Arch. Biochem. Biophys. **137**, 415–420 (1970)

Goyffon, M., le Fichoux, Y., Lamy, J.: Hétérogénéité du protéinogramme de l'hémolymphe des Scorpions *Androctonus australis* L., *Androctonus mauretanicus* Pocock et *Androctonus amoreuxi* Aud. et Sav. en gel de polyacrylamide. C.R. Soc. Biol. **164**, 374–378 (1970)

Goyffon, M., Stockmann, R., Lamy, J.: Valeur taxonomique de l'électrophorèse en disques des protéines de l'hémolymphe chez le Scorpion: étude du genre *Buthotus* (Buthidae). C.R. Acad. Sci. Paris **277** D, 61–63 (1973)

Jeffrey, P.D., Shaw, D.C., Treacy, G.B.: Hemocyanin from the Australian freshwater crayfish *Cherax destructor*. Studies of two different monomers and their participation in the formation of multiple hexamers. Biochemistry **15**, 5527–5533 (1976)

Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T 4. Nature (Lond.) **227**, 680–685 (1970)

Lamy, J., Goyffon, M., Sassé, M., Vachon, M.: L'électrophorèse des protéines de l'hémolymphe en gel de polyacrylamide, premier critère biochimique pour l'identification des scorpions. Biochimie **53**, 249–251 (1971)

Lamy, J., Lamy, J., Baglin, M.-C., Weill, J.: Scorpion hemocyanin subunits: properties, dissociation, association. In: Structure and function of haemocyanin. Bannister, J.V. (ed.), pp. 37–49. Berlin, Heidelberg, New York: Springer 1977

Loewe, R., Linzen, B.: Haemocyanins in spiders. I. Subunits and stability region of *Dugesiella californica* haemocyanin. Hoppe-Seyler's Z. Physiol. Chem. **354**, 182–188 (1973)

Markl, J., Schmid, R., Czichos-Tiedt, S., Linzen, B.: Haemocyanins in spiders. III. Chemical and physical properties of the proteins in *Dugesiella* and *Cupiennius* blood. Hoppe-Seyler's Z. Physiol. Chem. **357**, 1713–1725 (1976)

Markl, J., Markl, A., Hofer, A., Schartau, W., Linzen, B.: Heterogene Untereinheiten bei Arthropodenhämocyaninen. Verh. Dtsch. Zool. Ges. **1978**, 265 (1978)

Maurer, R.: Disc-Elektrophorese. Berlin: de Gruyter 1968

Melchers, M.: Zur Biologie und zum Verhalten von *Cupiennius salei* (Keyserling), einer amerikanischen Ctenide. Zool. Jahrb. Abt. Syst. Ökol. Geogr. Tiere **91**, 1–90 (1963)

Murray, A.C., Jeffrey, P.D.: Hemocyanin from the Australian freshwater crayfish, *Cherax destructor*. Subunit heterogeneity. Biochemistry **13**, 3667–3671 (1974)

Roche, A.C., Monsigny, M.: Purification and properties of limulin: a lectin (agglutinin) from hemolymph of *Limulus polyphemus*. Biochim. Biophys. Acta **371**, 242–254 (1974)

Schneider, H.-J., Markl, J., Schartau, W., Linzen, B.: Hemocyanins in spiders. IV. Subunit heterogeneity of *Eurypelma* (*Dugesiella*) hemocyanin, and separation of polypeptide chains. Hoppe-Seyler's Z. Physiol. Chem. **358**, 1133–1141 (1977)

Schutter, W.G., van Bruggen, E.F.J., Bonaventura, J., Bonaventura, C., Sullivan, B.: Structure, dissociation and reassembly of *Limulus polyphemus* hemocyanin. In: Structure and function of haemocyanin. Bannister, J.V. (ed.), pp. 13–21. Berlin, Heidelberg, New York: Springer 1977

Sugita, H., Sekiguchi, K.: Heterogeneity of the minimum functional unit of the hemocyanins from the spider (*Argiope bruennichii*), the scorpion (*Heterometrus* sp.), and the Horseshoe Crab (*Tachypleus tridentatus*). J. Biochem. (Tokyo) **78**, 713–718 (1975)

Sullivan, B., Bonaventura, J., Bonaventura, C.: Functional differences in the multiple hemocyanins of the Horseshoe Crab, *Limulus polyphemus* L.. Proc. Nat. Acad. Sci. USA **71**, 2558–2562 (1974)

Sullivan, B., Bonaventura, J., Bonaventura, C., Godette, G.: Hemocyanin of the Horseshoe Crab, *Limulus polyphemus*. Structural differentiation of the isolated components. J. Biol. Chem. **251**, 7644–7648 (1976)

Van Holde, K.E., van Bruggen, E.F.J.: The hemocyanins. In: Subunits in biological systems. Timasheff, S.N., Fasman, G.D., (eds.), pp. 1–53. New York: Marcel Dekker 1971

Note Added in Proof

Since this paper was submitted we have learned about a paper describing the isolation of the individual subunits of *Androctonus australis* hemocyanin (Lamy et al., 1979). There are eight subunits which were obtained in immunologically pure state; two of these form a hetero-oligomer (=fraction 1, corresponding to fraction 7 of our pattern). The authors found that there is no immunological cross-reactivity between subunits, and were able to raise specific antibodies. – Thus our own results obtained by different methods, are in complete accord with respect to the total number of subunits.

Reference

Lamy, J., Lamy, J., Weill, J.: Arthropod hemocyanin structure: isolation of eight subunits in the scorpion. Arch. Biochem. Biophys. **192** (in press) (1979)