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Subunit Heterogeneity in Arthropod Hemocyanins: II. Crustacea *

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Summary. 1. The hemocyanins of 10 decapod Crustacea were dissociated and their subunits analyzed by high resolution polyacrylamide electrophoresis (PAGE): 5 brachyuran crabs (*Cancer pagurus, Carcinus maenas, Macropipus holsatus, Hyas araneus, Maja squinado*), 3 Astacura (*Astacus leptodactylus, Homarus americanus, Homarus gammarus*) and the spiny lobsters *Palinurus vulgaris* and *Panulirus interruptus*.

2. All of the species save the spiny lobsters possess a major hemocyanin component sedimenting with 24 S. A second hemocyanin component sedimenting with ca. 16 S was found in *H. gammarus, M. squinado, C. pagurus* and *M. holsatus* (about 10 per cent in each case) and in *A. leptodactylus* (about 25 per cent). A second, major blood protein (10–25% of the total blood protein) was observed in *H. gammarus* where its sedimentation coefficient was 24 S, *M. squinado* (16 S), *H. araneus* (24 S) and *C. pagurus* (16 S). This second protein has no respiratory function. Two such non-respiratory proteins sedimenting with 24 S and 16 S were found in *H. americanus*.

3. Between 2 and 7 hemocyanin bands were obtained after incubation with sodium dodecylsulfate (SDS) and β-mercaptoethanol and subsequent electrophoresis in polyacrylamide gradients. The average molecular weight was about 75,000 in the crabs, 80,000 in the crayfishes and 85,000 in the spiny lobsters. The non-respiratory proteins yield between one and four chains with molecular weights ranging from 76,000 to 87,000.

4. The hemocyanins were dissociated at pH 9.6 into "native" subunits, but dissociation was not quantitative in several species. By gel filtration, the products were separated into undissociated material and hemocyanin monomers (5 S). In *Astacus leptodactylus* a dimeric subunit (7 S) was obtained in addition; its components are linked by a disulfide bridge. The subunit mixtures were separated by PAGE into 4 to 6 distinct bands.

5. To establish the total number of different polypeptide chains present in each hemocyanin, the two electrophoretic patterns were related to each other by preparative isolation of "native" subunits and subsequent analysis in SDS-PAGE. The number of different polypeptide chains ranges from four to seven in the species studied by us. In those species which contained both 24 S and 16 S hemocyanin, more different polypeptide chains were found in the 24 S hemocyanin than in the 16 S hemocyanin, the only exception being *Homarus gammarus*.

Introduction

Crustacean hemocyanins consist of polypeptide chains with a sedimentation coefficient of 5 S and a molecular weight of ca. 75,000, which in vivo form aggregates of six (ca. 16 S), twelve (ca. 24 S) and twentyfour (ca. 37 S) subunits (Van Holde and Van Bruggen, 1971). The three types of hemocyanin oligomers are not found together in the hemolymph; on the contrary, the state of aggregation appears to be characteristic for each species and may be characteristic for different taxonomic groups.

Berthet et al. (1964) have studied the hemocyanins of several isopods and found them to sediment with ca. 15 S. The hemocyanin of the isopod *Ligia exotica* was recently studied by Terwilliger et al. (1979) and found to comprise both 16 S and 24 S states. All other available data were obtained from species belonging to the order Decapoda: 16 S hemocyanin appears to be typical for the suborder Natantia, the shrimps,

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which was shown for *Pandalus borealis* (Eriksson-Quensel and Svedberg, 1936), *Palaemon fabricii* (Ghiretti et al., 1973) and *Penaeus setiferus* (Brouwer et al., 1978). Within the suborder Reptantia the situation is less homogeneous: The spiny lobsters (Palinura) possess 16 S hemocyanin (*Palinurus vulgaris,* Eriksson-Quensel and Svedberg, 1936; *Panulirus interruptus,* Johnston et al., 1967; Kuiper et al., 1975; *Jasus* spp., Joubert, 1954; Moore et al., 1968; Ellerton et al., 1977; Robinson and Ellerton, 1977). In the crayfish families Homaridae and Astacidae (both belonging to the Astacura) the lobsters *Nephrops norvegicus* and *Homarus gammarus* possess 24 S hemocyanin and very little 16 S material (Eriksson-Quensel and Svedberg, 1936), whereas *Homarus americanus* (Pickett et al., 1966) and the freshwater crayfishes *Astacus fluviatilis* (Eriksson-Quensel and Svedberg, 1936) and *Cherax destructor* (Murray and Jeffrey, 1974) have 24 S hemocyanin as major component but also an appreciable amount of 16 S hemocyanin. Among the Anomura, 39 S hemocyanin has been reported for the ghost shrimp *Callianassa californiensis* and two other thalassinids (Miller et al., 1977); a small amount of 16 S material was also found. The hermit crabs *Pagurus striatus* and *Eupagurus bernhardus* possess only 16 S hemocyanin (Ghiretti et al., 1973).

In the Brachyura, the crabs, 24 S hemocyanin has been found for *Carcinus maenas* and *Cancer pagurus* (Eriksson-Quensel and Svedberg, 1936), *Eriphia spinifrons* (Di Giamberardino, 1967), *Cancer magister* (Ellerton et al., 1970), *Hyas araneus* and *Maja squinado* (Ghiretti et al., 1973), *Potamon edulis* and *Carcinus mediterraneus* (Chantler et al., 1973), *Ovalipes catharus* (Robinson and Ellerton, 1977) and *Callinectes sapidus* (Hamlin and Fish, 1977). Very minor quantities of 16 S hemocyanin were also observed in many cases.

It is presently not understood why different hemocyanin aggregates should exist in different species, and why some groups should possess only one type of oligomer and others two. It must be assumed that there is also great structural diversity at the level of the polypeptide chains, leading to the different levels of association. Of particular interest in this respect is the heterogeneity of subunits which has been observed in a number of hemocyanins. For the Chelicerata, pertaining literature has been quoted in our recent communication (Markl et al., 1979a). Among the Crustacea, two different hemocyanin fractions were observed in the crabs *Carcinus maenas* (Busselen, 1970), *Cancer magister* (Loehr and Mason, 1971), and *Callinectes sapidus* (Hamlin and Fish, 1977), as well as in the lobster *Homarus americanus* (Waxman, 1975). Three different polypeptide bands were found in the hemocyanins of the spiny lobsters *Panulirus interruptus* (Van den Berg et al., 1977) and *Jasus edwardsii* (Robinson and Ellerton, 1977) and in the crab *Ovalipes catharus* (Robinson and Ellerton, 1977). The hemocyanin subunits of *Callianassa californiensis* and of two other thalassinid shrimps were resolved into maximally six bands by Miller et al. (1977). Incubation of various isopod hemocyanins at pH 9.1 leads to the appearance of 3 to 6 new electrophoretic bands (Sevilla and Lagarrique, 1976; Sevilla, 1977). It must be pointed out, though, that in neither of the preceding examples the true number of different subunits has been established with certainty.

In our laboratory, high resolution polyacrylamide-gradient slabgel techniques have been used to analyze the subunits of tarantula hemocyanin (Schneider et al., 1977). In a subsequent study cheliceratan species of five different orders were compared with respect to subunit heterogeneity of their hemocyanins (Markl et al., 1979a). In no case less than six distinct polypeptide chains were detected, while the maximum number was twelve (in *Limulus polyphemus* hemocyanin).

The present study extends our previous work to the class Crustacea with particular attention to the question whether the state of aggregation and the degree of subunit heterogeneity can be related to each other. Ten decapod species have been investigated, all of the suborder Reptantia but ranging from the Palinura to the Brachyura.

Materials and Methods

Blood Samples

The crabs *Cancer pagurus* (fam. Cancridae), *Carcinus maenas* (fam. Portunidae), *Macropipus* (=*Portunus*) *holsatus* (fam. Portunidae), and *Hyas araneus* (fam. Majidae) were obtained from the Biologische Anstalt Helgoland; *Maja squinado* (fam. Majidae) was collected at the Atlantic coast near St. Malo (France). The freshwater crayfish *Astacus leptodactylus* (fam. Astacidae) and the two lobster species *Homarus americanus* and *Homarus gammarus* (fam. Homaridae), and the European spiny lobster *Palinurus vulgaris* (fam. Palinuridae) were obtained from a local seafood dealer. Blood from the North American spiny lobster *Panulirus interruptus* (fam. Palinuridae) was a gift of Prof. Dr. E. van Bruggen, University of Groningen, Netherlands.

Blood was sampled by puncture of the base of a walking leg. After clotting, the debris was removed by centrifugation. Most experiments were performed on fresh samples, otherwise, the serum was stored at 4 °C.

Dissociation of Proteins

The hemolymph proteins were either dissociated (overnight, room temp.) by glycine/NaOH, pH 9.6, $I=0.05$, or by hydrogen carbonate, pH 10.1, $I=0.1$, at a protein concentration of 10 mg/ml, or denatured by heating for 20 min at 56 °C in 0.1 M Tris/HCl buffer,

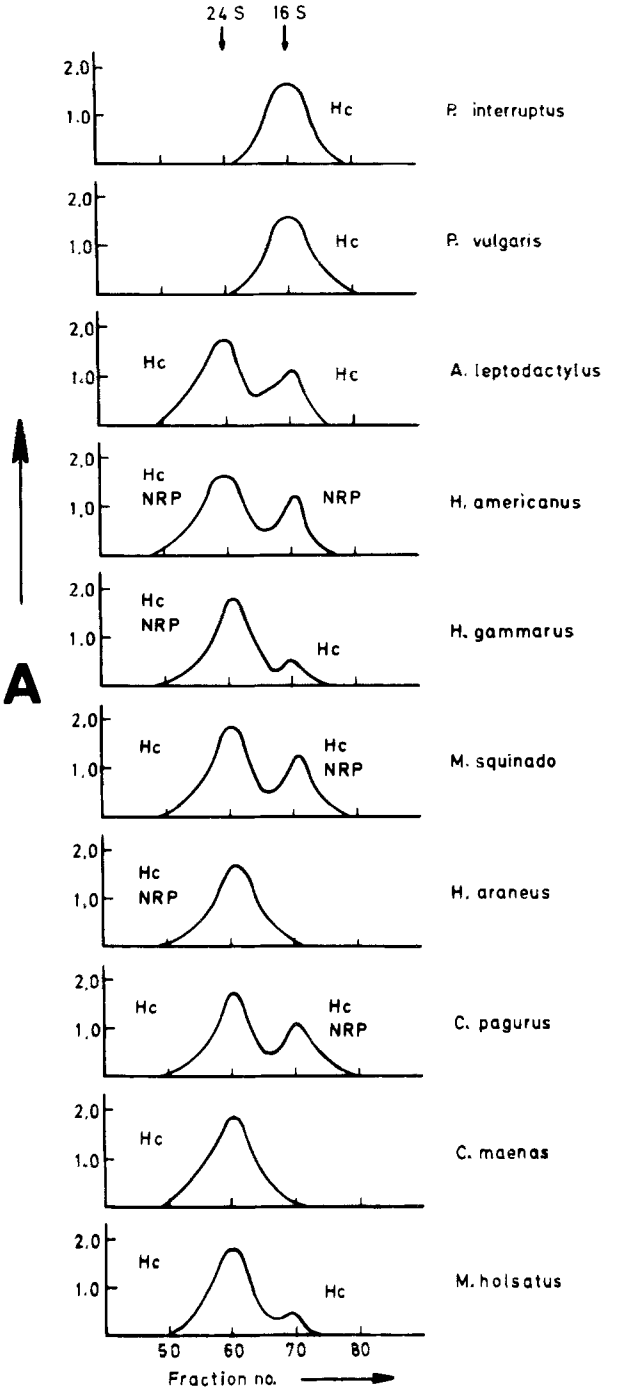

Fig. 1. Gel filtration (over Biogel A-5 m) of the hemolymph of various Crustacea. On the ordinate is the absorbance at 280 nm. *NRP*, non-respiratory protein (function not known). Sedimentation coefficients corresponding to the elution volumes are indicated at the top

pH 8.8, containing 2% sodium dodecylsulfate and 1% β-mercaptoethanol, at a protein concentration of 1 mg/ml.

Separation Procedures

Gel filtration, analytical and preparative polyacrylamide-electrophoresis (PAGE), and molecular weight determinations were exactly as described previously (Markl et al., 1979a).

Other Analytical Procedures

Sedimentation analysis was performed by Dr. W. Schartau and Mr. H. Decker 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 nor extrapolated to zero concentration.

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

Results

Gel Filtration of Native Hemolymph Proteins

By gel filtration of cell-free hemolymph one or two large protein peaks were obtained. The major peak in each case consisted mainly or solely of hemocyanin as shown by light absorption at 340 nm and copper analysis (Fig. 1). Its sedimentation coefficient was near 24 S in all the species, save the spiny lobsters (16 S).

The proportion of the 16 S peak of *Cancer pagurus* and *Maja squinado* hemocyanin rose steadily with storage time of the cell-free blood, indicating a slow dissociation upon ageing. This process was accelerated by freezing and thawing.

The second peak (ranging from ca. 10 to 25 per cent of the total protein) was pure hemocyanin in some cases as judged from its low 280 nm/340 nm absorbance ratio (between 4 and 5); this was the case with *Astacus leptodactylus, Homarus gammarus* and *Macropipus holsatus*. In the case of *Homarus americanus* it did not absorb light at 340 nm, whereas for *Maja squinado* and *Cancer pagurus* the 280/340 ratio was high (approaching 15) indicating a mixture of hemocyanin with some other protein(s). This second protein will be called "non-respiratory protein" for the time being. We do not have evidence that it should represent apo-hemocyanin.

The non-respiratory protein was eluted along with the 24 S fraction of *Homarus americanus, Homarus gammarus*, and *Hyas araneus* hemocyanin.

Analysis of Polypeptide Chains by SDS-PAGE

After denaturation of the hemolymph proteins by incubating with SDS and β-mercaptoethanol, in each of the species a series of bands was obtained upon electrophoresis in polyacrylamide gradient gels. An example is shown in Fig. 2, while a synopsis which also shows the molecular weights, is presented in Fig. 3.

As in our study of cheliceratan hemocyanins, there is considerable heterogeneity in each of the species

investigated. The number of distinct protein bands ranged from 3 in *Astacus leptodactylus* to 8 in *Cancer pagurus*. In several species one to four of the slower moving bands were shown to be the polypeptide chains of the non-respiratory proteins. These have been labelled with greek letters. The number of hemocyanin bands ranged from two in *Hyas araneus* to seven in *Macropipus holsatus*. Among the hemocyanin chains, the lowest molecular weight was 70,000 (in *Hyas araneus*), the highest 90,000 (in *Panulirus vulgaris*). The average molecular weights appear to be above the figures reported for the cheliceratan hemocyanins. This is readily borne out by Fig. 3.

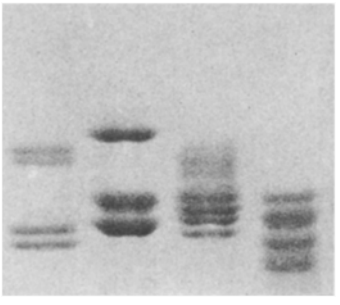

Fig. 2. Examples of crustacean blood proteins denatured in SDS/β-mercaptoethanol and separated on linear polyacrylamide gradients (5-10%), pH 8.8, according to Laemmli (1970). From left to right: *Hyas araneus, Carcinus maenas, Cancer pagurus, Eurypelma californicum* (for comparison). Cf. Fig. 3 for identification of non-hemocyanin proteins. Slightly enlarged photograph

The second blood protein yielded from one to four bands in the SDS system, thus differing from the Chelicerata. In addition, the molecular weights of these bands were only little different from the hemocyanin bands (by ca. 1,000 to 10,000 higher) so that they were sometimes mistaken for the latter.

The Pattern of "Native" Subunits

The dissociation of hemocyanins in alkaline media leads to subunits, either mono- or dimeric, which are still able to bind oxygen and will be called "native" subunits. Alkaline dissociation met with difficulties in some of the crustacean hemocyanins. E.g. *Astacus leptodactylus* hemocyanin was completely dissociated only in hydrogen carbonate buffer of pH 10.1. Even under these conditions, dissociation of *Homarus gammarus, Maja squinado,* and *Macropipus holsatus* hemocyanins was only partial. The addition of EDTA did not improve the result. However, if such undissociated material was isolated and once more subjected to the same procedure, the same dissociation products were again formed.

Dissociation products were filtered through a column of Sephadex G-100 sf (Fig. 4). In each species a slow moving component was obtained which represented the mixture of monomeric hemocyanin subunits (5 S). For the two spiny lobsters, this was the only peak. In the other species a second, fast moving

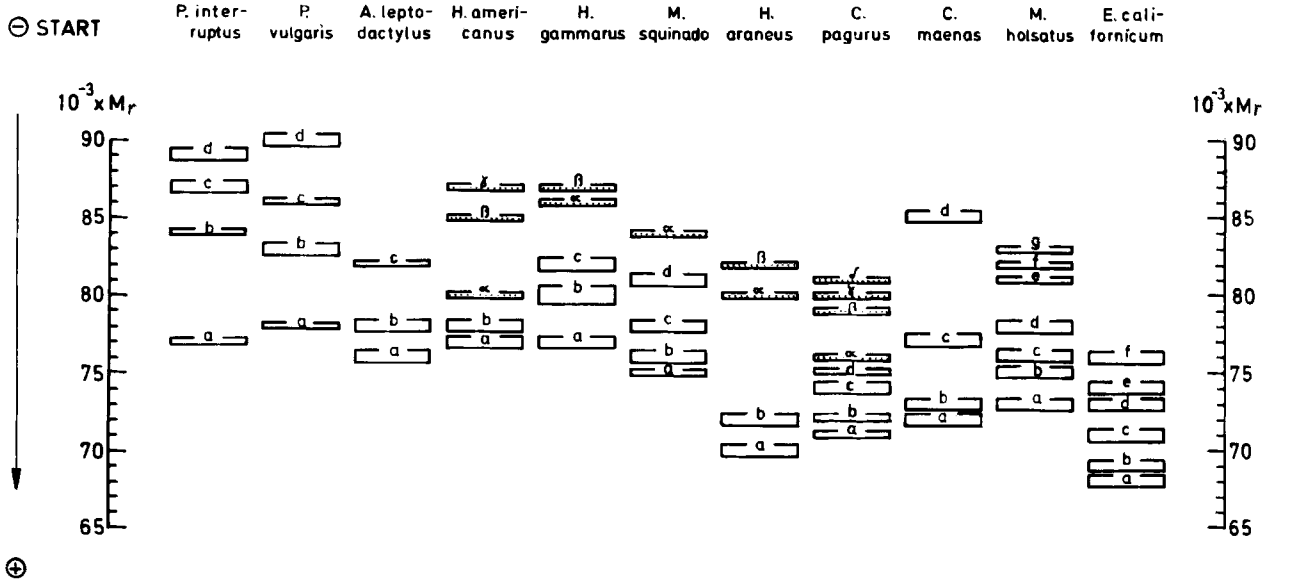

Fig. 3. Schematic presentation of protein patterns obtained by electrophoresis of SDS/β-mercaptoethanol-denatured crustacean blood proteins. A linear polyacrylamide gradient from 5–10% was used, pH was 8.8 (Laemmli, 1970). For easy comparison, a linear molecular weight scale is included; the actual patterns were redrawn and slightly distorted to conform to the scale. Their appearance was negligibly altered, however, since the scale covers only a distance of about 15 mm in the gel. Hemocyanin chains are labelled with roman letters, chains of the non-respiratory protein are hatched and labelled with greek letters

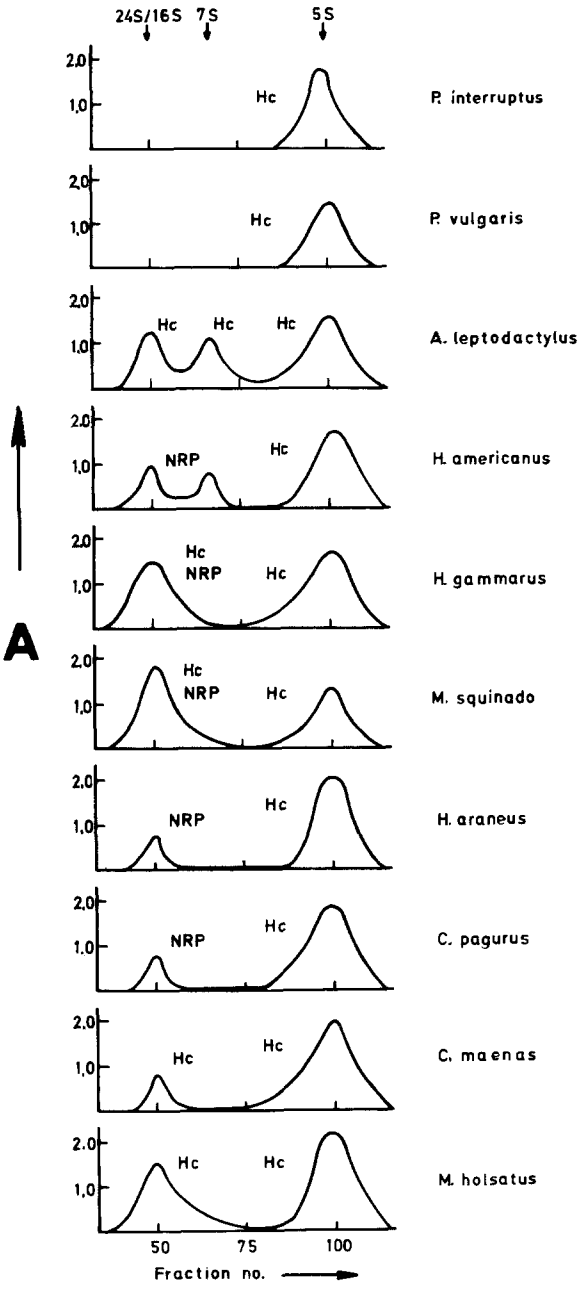

Fig. 4. Elution patterns of crustacean blood proteins after alkaline dissociation (pH 9.6) and gel filtration through Sephadex G-100 sf. Undissociated material and the non-respiratory protein emerge at the exclusion volume. The 7 S-peak represents a hemocyanin dimer in the case of *Astacus leptodactylus* and a dissociation product originating from the non-respiratory protein in the case of *Homarus americanus*. Absorbance was monitored at 280 nm

peak was obtained. It contained either undissociated hemocyanin, or non-respiratory protein, or both. In the cases of *Astacus leptodactylus* and *Homarus americanus* a third peak was found with intermediate mobility (7 S), corresponding to a dimer. The subunit mixtures were resolved by electrophoresis in polyacrylamide gradients at alkaline pH (Figs. 5 and 6). In each case several bands were obtained. Their number ranged from 4 to 6 (*Macropipus holsatus*). The 16 S hemocyanins present in the hemolymph of *Maja squinado, Macropipus holsatus, Cancer pagurus* and *Astacus leptodactylus* contained one or two subunits less than the 24 S-hemocyanin (Table 1, Fig. 7). In *Homarus gammarus* hemocyanin the composition was the same in both states of aggregation.

Band 5 of *Astacus leptodactylus* hemocyanin appears to be a heterodimer. By dialysis against glycine buffer, pH 9.6, containing 5 mM cysteine and 10 mM EDTA it was split up into two monomers migrating at the position of subunits 3 and 4 (Fig. 6). Dimers were not observed in any of the other crustacean hemocyanins.

The second, non-respiratory blood protein did not dissociate at pH 9.6, with one exception, viz. the 24 S protein of *Homarus americanus* hemolymph which was broken down to a protein with a sedimentation coefficient of 7 S. The corresponding polypeptide chain in the SDS system (chain α) having a molecular weight of ca. 80,000, it may be presumed that the 7 S material represents a dimer of this chain. The 16 S non-respiratory protein of *H. americanus* which consists of two different chains, β and γ, does not dissociate at pH 9.6.

Cross-Reference of PAGE Protein-Patterns

By the two different electrophoretic procedures we obtained two patterns of hemocyanin bands, of which one mainly arose by charge differences ("alkaline" system) and the other by differences in molecular weight (SDS-system). Considering the high resolution conveyed by the polyacrylamide gradients it appears unlikely that any one component is obscured in both systems. To determine the true number of different polypeptide chains in each hemocyanin with some reliability, we isolated the distinct "alkaline" components by preparative PAGE and analyzed them in the SDS-system so that the two patterns could be referred to each other crosswise. Individual subunits have been obtained in a pure state in many cases. This was, however, not a prerequisite, since the different components of a mixture (usually not more than two) could be easily distinguished by means of their quantitative proportion. A summary of the results is given in Fig. 7. In most of the hemocyanins the total number of polypeptide chains agreed with the number of subunits in the alkaline PAGE system. Only in the case of *Macropipus holsatus, Homarus gammarus,* and *Palinurus vulgaris* hemocyanin, some subunits which appeared homogeneous in the alkaline system were shown to be heterogeneous by means

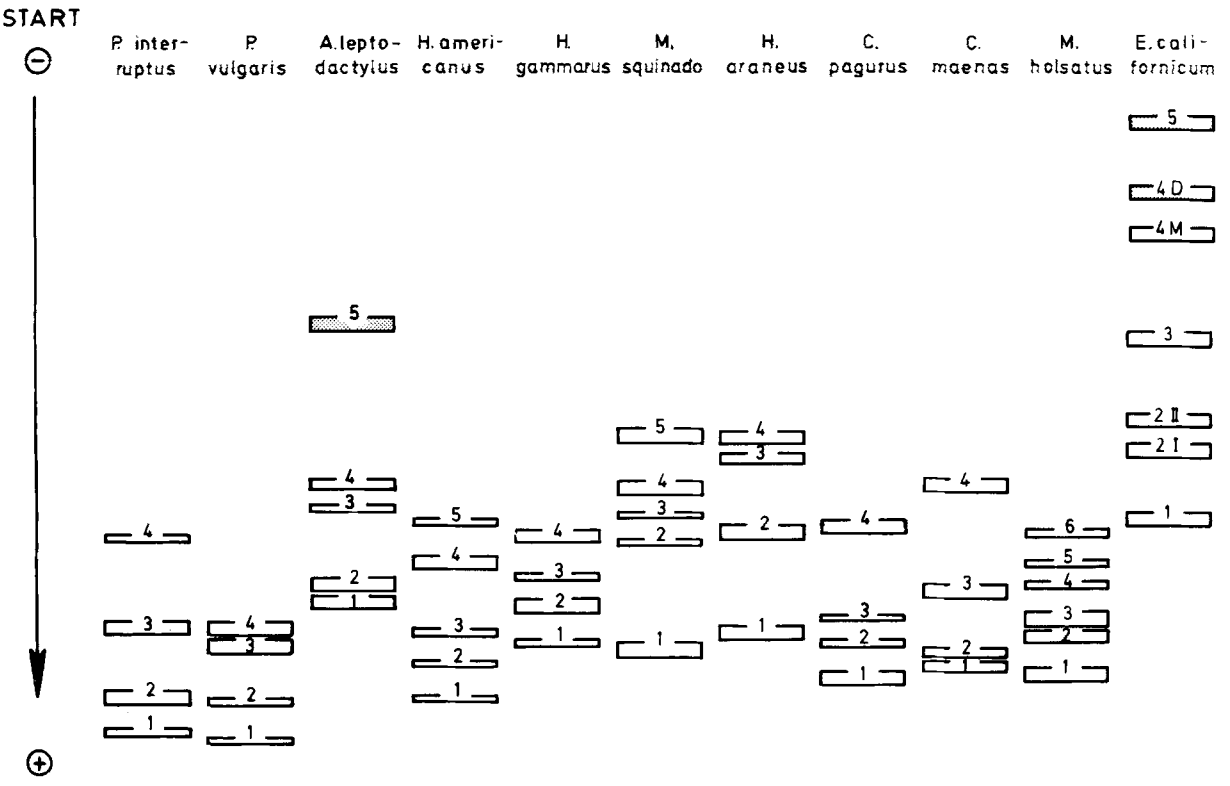

Fig. 5. Electrophoretic patterns (PAGE at pH 9.6, cf. Methods section) of crustacean hemocyanins after alkaline dissociation and gel filtration (cf. Fig. 4). *Eurypelma californicum* is included for comparison. Band 5 (stippled) of *Astacus leptodactylus* is a dimer

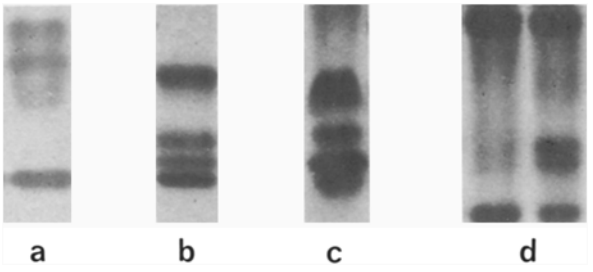

Fig. 6a-d. Examples of electrophoretic patterns (PAGE at pH 9.6) of crustacean hemocyanin subunits dissociated at alkaline pH (cf. Fig. 5). **a** *Maja squinado*, **b** *Cancer pagurus*, **c** *Homarus gammarus*, **d** *Astacus leptodactylus* dissociated in the absence (left) and in the presence (right) of 5 mM cysteine

of the SDS system. The total number of different hemocyanin polypeptide chains obtained by this kind of cross-reference ranges from 4 to 7 (Table 1).

Discussion

As the cheliceratan hemocyanins, crustacean hemocyanins show a surprising complexity in the pattern of their building stones. Non of the species studied by us, had less than four different polypeptide chains, and the maximum was seven. While most of the figures reported in our preliminary communication (Markl et al., 1978) have been confirmed, there are a few which must now be corrected by the data of Table 1 (*Astacus leptodactylus, Hyas araneus, Cancer pagurus, Macropipus holsatus,* and *Carcinus maenas*). Since in the majority of these cases the number of different polypeptide chains had to be increased (e.g. in *Astacus* by the finding that the dimeric subunit is composed of two different entities), we should not exclude the possibility that still more distinct chains will be found by future analysis.

On the other hand it must be considered that some of the electrophoretic protein bands could have arisen by degradation of a native protein, in particular at the alkaline pH used for dissociation, thus giving rise to a delusive presentation of heterogeneity. We are rather confident though, that our figures are not seriously biased by protein degradation, as essentially identical results have been obtained in repeated experiments and with fresh or aged samples. The true number of distinct polypeptide chains can be reached only after isolation and chemical characterization, preferably backed by immunological analysis. These methods have recently been applied to the hemocyanins of the spider, *Eurypelma californicum* (Markl et al., 1979b; Lamy et al., 1979a) and of the scorpion, *Androctonus australis* (Lamy et al., 1979b) and have fully confirmed the figures derived from electrophoretic analysis.

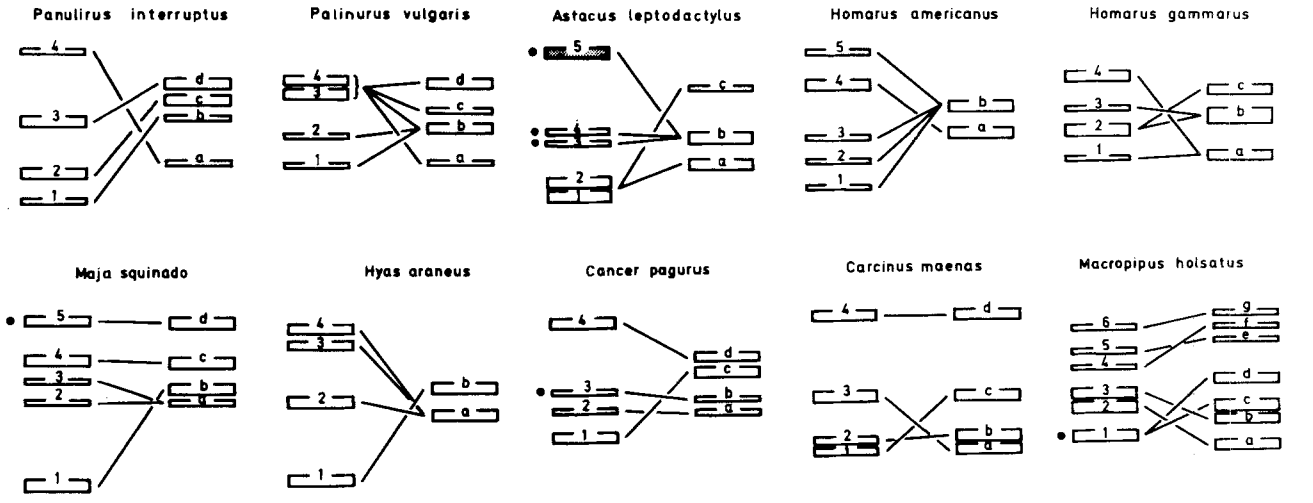

Fig. 7. Cross-reference of electrophoretic patterns of crustacean hemocyanin subunits. Left hand patterns: subunits obtained by alkaline dissociation; right hand patterns: polypeptide chains obtained with SDS/β-mercaptoethanol. The number of connecting lines equals the number of distinct monomers except for *Astacus leptodactylus* hemocyanin where the dimer 5 is made up of subunits 3 and 4. Subunits labelled with a black dot were observed only in the 24 S-hemocyanin

Table 1. The subunit composition of various crustacean hemocyanins. The number of subunits per oligomer and the total number of different polypeptide chains as derived from Fig. 7, are given

Species	Subunits in oligomer	Number of different chains
Panulirus interruptus	6	4
Palinurus vulgaris	6	6
Homarus americanus	12	5
Homarus gammarus	12	5
	6	5
Astacus leptodactylus	12	4
	6	2
Maja squinado	12	5
	6	4
Hyas araneus	12	4
Cancer pagurus	12	4
	6	3
Carcinus maenas	12	4
Macropipus holsatus	12	7
	6	5

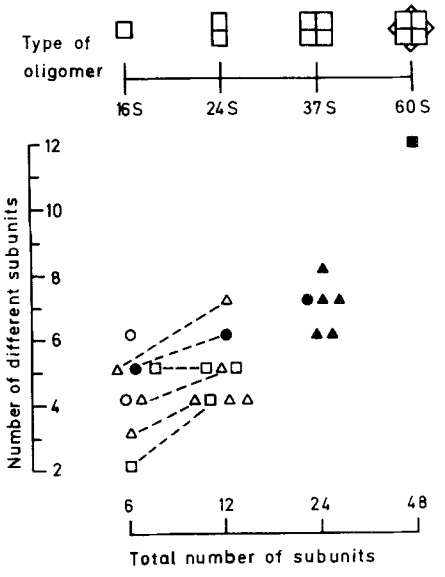

Fig. 8. Relationship between aggregation state and number of distinct polypeptide chains in arthropodan hemocyanins (data taken from this study and from Markl et al., 1979a). Filled symbols: Chelicerata; open symbols: Crustacea. ■ *Limulus*, ● spiders, ▲ other arachnids (scorpions, whipspiders, a whipscorpion), ○ spiny lobsters, □ lobsters and crayfish; △ crabs. Broken lines connect hexameric and dodecameric hemocyanins when they occur in a single species. The type of aggregate and approximate sedimentation coefficients are indicated at the top

It must be asked then, what are the causes and the significance of the heterogeneity of hemocyanin chains. As the blood samples used in this study were withdrawn from individual specimens, it appears that genetic polymorphism is not the cause of the observed heterogeneity. It is more likely that structural and/or functional advantages brought about by heterogeneity have favored the evolution of multiple structural genes. Figure 8 indicates that there is a correlation between state of aggregation in the hemocyanin particle and the number of distinct polypeptide chains.

The formation of the oligomeric hemocyanin molecule is presently not well understood, although some important information has already been obtained by reassembly experiments. For reassembly of cheliceratan hemocyanins various subunits must be present simultaneously (Lamy et al., 1977; Bijlholt et al., 1979). These hemocyanins are, however, very large structures comprising 24 or 48 subunits. The simple

hexameric structure which appears to be the basic physiological unit of the arthropodan hemocyanins, has been obtained with certain individual subunits (e.g. fraction IV of *Limulus* hemocyanin; Bijlholt et al., 1979; fraction 4 of *Androctonus* hemocyanin; Lamy et al., 1977). While these reassociated cheliceratan hemocyanin hexamers do certainly not correspond to the native molecules, there is, on the other hand, a crustacean hemocyanin which can doubtlessly be formed from a single type of subunit: the 16 S hemocyanin of the Australian freshwater crayfish, *Cherax destructor* (Jeffrey et al., 1976). It appears therefore, that a single type of protein would suffice to build the typical hemocyanin hexamer.

Looking now again at our data it is intriguing to find that the 16 S hemocyanin of *Astacus leptodactylus* can be resolved into two distinct proteins, but the 16 S hemocyanin of *Palinurus vulgaris* into six, and others into four or five. It would be most important to find out whether the heterogeneity at the subunit level extends to the level of the native hexamers. It might turn out that some of the hexameric hemocyanins have indeed a rather complicated structure.

Turning to the 24 S hemocyanins, there is strong evidence that their formation requires at least one additional type of subunit, as more distinct polypeptide chains were found in this higher aggregation state, if both 16 S and 24 S were present in a species. These additional subunits are presumably located at the site of contact between the two hexamers, and it may be reasonably assumed that they tend to dimerize. However, dimers were usually not detected in crustacean hemocyanins after alkaline dissociation, save in *Astacus* hemocyanin. The distinguishing feature in this case is that the dimer is formed by a disulfide bridge between the two polypeptide chains. Such a type was also found in *Cherax destructor* hemocyanin (Murray and Jeffrey, 1974) and in *Cupiennius salei* (a spider) hemocyanin (Markl et al., 1976). While the dimer of *Cherax* appears to consist of identical or very similar polypeptides, the *Astacus* dimer is clearly asymmetrical. The best studied cheliceratan hemocyanins yield dimers, which are held together by strong hydrophobic interactions. It is tentatively concluded that these are the elements typically required to form the large 37 S structures of this group. It is remarkable that many crustacean hemocyanins are difficult to dissociate quantitatively, even at pH 10.5.

With respect to evolutionary relationships there is only little basis for speculation. Within the higher Crustacea which have solely been studied hitherto, both hexameric and dodecameric hemocyanins are widespread, and the number of distinct chains is typically near five. If the cheliceratan hemocyanins are compared to the former, it appears that the more ancient groups are characterized by the higher levels of hemocyanin aggregation: The largest structure (48 subunits) is found in the living fossil, *Limulus polyphemus*. 37 S hemocyanin (24 subunits) occurs in the rather primitive scorpions, whipscorpion, whipspiders and tarantulas ("bird-eating spiders") (Markl et al. 1979a), whereas more recent spider representatives, as *Tegenaria* species (Wibo, 1966) and *Cupiennius salei* (Markl et al., 1976) possess 24 S and 16 S hemocyanins.

While the other hemolymph proteins were not in the focus of this study, there are nonetheless some features which should be commented on. First, the native "non-respiratory" proteins have sedimentation properties very similar to those of the hemocyanins in the same species. This is striking in *Homarus americanus* where two such proteins were encountered. They were eluted from the gel filtration column at the positions of 24 S and 16 S hemocyanin. The significance of this similarity in oligomeric molecular weight is not understood. These proteins did not occur in all species, and it is likely that their concentration in the hemolymph is subject to greater fluctuations, in comparison to the hemocyanins. Considerable variations in concentration have been observed for various proteins, unrelated to hemocyanin, in the hemolymph of crayfish (Vranckx and Durliat, 1976, and references cited by these authors).

The polypeptide composition of these other proteins showed greater variation as in most of the Chelicerata (cf. Markl et al., 1979a). In one species, *Maja squinado,* the non-respiratory protein was apparently made up of only one type of polypeptide chain, while four chains were encountered in *Cancer pagurus* (Fig. 3). Finally, there is very little difference in molecular weight (as judged from SDS gels) between these chains and the hemocyanin chains. In the earlier phase of our study these polypeptides were sometimes mistaken for hemocyanins. In contrast, the major non-respiratory blood proteins of all terrestrial Chelicerata studied (Markl et al., 1979a) are composed of only two polypeptide species which differ by M_r 20,000–40,000 from the polypeptide chains of the hemocyanin.

While further interesting details of hemocyanin quaternary structure and its variability might be discovered by comparing still a greater number of species, and particularly by turning to the entomostracan Crustacea, we feel that the imminent question to be answered, is the participation of the individual subunits in the compound hemocyanin particle. To obtain this answer, research will again have to be centered upon a single or very few selected species.

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