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# THE QUATERNARY STRUCTURE OF *SCORPIO MAURUS* HEMOCYANIN: COMPARISON WITH *ANDROCTONUS* *AUSTRALIS* HEMOCYANIN

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**Abstract**—The quaternary structure of the 4 × 6-meric hemocyanin from the scorpion *Scorpio maurus* (Scorpionidae) was determined and compared to that of *Androctonus australis* (Buthidae).

1. Eight immunologically pure 70–75 kDa subunits were isolated, characterized, and used to prepare subunit-specific antisera.
2. The intramolecular locations of the subunits were determined by immunoelectron microscopy.
3. Each subunit of *Scorpio* hemocyanin was immunologically related to a single subunit of *Androctonus* hemocyanin, having the same intramolecular location.
4. The presence of two subunits near the middle of the lateral edge of the 4 × 6-meric appears to be a characteristic of the scorpion order.

## INTRODUCTION

Since 1981, the architecture of three of the most complex arthropod hemocyanins, the 4 × 6-mers from the scorpion *Androctonus australis* (Lamy *et al.*, 1981) and the spider *Euryopelma californicum* (Markl *et al.*, 1981), and the 8 × 6-mer from the horseshoe crab *Limulus polyphemus* (Lamy *et al.*, 1983a; Lamy, 1987) have been resolved by immunoelectron microscopy. In these species, hemocyanin comprises seven or eight different polypeptide chains. Isolated subunits from these hemocyanins were used for the reconstruction of oligomers (Lamy *et al.*, 1977, 1981; Bijlholt *et al.*, 1979; Markl *et al.*, 1982) which were labeled by subunit-specific Fab fragments and observed in the electron microscope (Lamy *et al.*, 1983a). It was also demonstrated that a given type of polypeptide chain can be interchanged from species to species (Van Bruggen *et al.*, 1980) and that the various polypeptides have specific locations and structural roles.

In the current research program, we plan to study the mechanism of the reassembly process using precise methods of intramolecular labeling of the subunits with monoclonal antibodies. The method is based on a determination of the quaternary structure of molecules reconstructed from subunits isolated from two hemocyanin species, evolutionarily related in order to reassemble readily, but distant enough to be characterized easily. In the scorpion order, the Buthidae family and Scorpionidae family perfectly fulfilled these conditions. Indeed, hemocyanins from the scorpions *Androctonus australis* and *Scorpio maurus* look remarkably similar in the electron microscope and they belong to taxonomic groups which are known to be distant within the scorpion order.

However, very little information about *Scorpio* hemocyanin being available, the first step of the program was the determination of its quaternary structure at the same level of precision as that of *Androctonus* hemocyanin. This report shows how this was accomplished.

## MATERIALS AND METHODS

### Hemocyanin

Hemolymph of the scorpion *Scorpio maurus*, drawn by cardiac puncture, was first cleaned of the cellular fragments by a 10 min centrifugation at 800 g. Then, the concentration was adjusted to about 40 mg/ml and the hemolymph was centrifuged at 38,000 g for 200 min in the Sorvall SV-80 vertical superspeed rotor of a RC5B centrifuge (DuPont Instruments, Newtown, CT), and the blue pellet was resuspended in 0.05 M Tris-HCl buffer, pH 7.5, 10 mM CaCl<sub>2</sub>. After a second centrifugation and redissolution, the “whole hemocyanin” preparation, still contaminated by high molecular weight material, was stored in the cold.

“Dissociated hemocyanin” was obtained by an overnight dialysis of whole hemocyanin against a 0.05 M Tris-HCl buffer, pH 8.9, 10 mM EDTA.

For the study of the monomer-dimer equilibrium, dissociated hemocyanin was filtered on a HiLoad® 16/60 Superdex® 200 prep-grade column (Pharmacia, Uppsala, Sweden) using FPLC® equipment (Pharmacia). The volume of the chromatographic bed was 120 ml and the flow rate was 1.0 ml/min.

For purification of the subunits, dissociated hemocyanin was submitted to ion-exchange chromatography on a DEAE-Sepharose CL-6B® (Pharmacia)

equilibrated in 0.05 M Tris-HCl buffer, pH 8.9, 10 mM EDTA. The elution was carried out using a linear 0–0.5 M NaCl gradient in the same buffer. The impure fractions were readSORBED on the same gel equilibrated in 0.1 M sodium phosphate buffer, pH 6.8, and eluted under various conditions of ionic strength and pH. The subunit compositions of the elution profiles were estimated by fused-rocket immunoelectrophoresis.

#### *Antibodies*

Nine rabbit antisera were prepared according to a previously published protocol (Lamy *et al.*, 1979a). The anti-dissociated hemocyanin antiserum was mainly used at the immunoprecipitation step of the immunoelectrophoreses. The other eight antisera were specific for the pure hemocyanin subunits. For the preparation of anti-*Sma*, *b*, *c*, *f*, *g* and *h* antisera, the antigen solutions (100 µg per plate) were first submitted to crossed-line immunoelectrophoreses vs the anti-dissociated hemocyanin antiserum. The minor impurities producing precipitin lines with low migration distances and the fast lines corresponding to the major components, were dug from the agarose plate, homogenized with Freund complete adjuvant and injected into the rabbits. For subunits *Smd* and *e*, the same method was used, but antisera specific for subunits *Aa3C* and *Aa5B* of *Androctonus australis* hemocyanin were used for precipitation of the impurities instead of the anti-dissociated hemocyanin antiserum.

The crude antisera were purified by precipitation of the immunoglobulins with ammonium sulfate; they were then dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, 0.15 M NaCl, sodium azide 0.02% and stored at -20°C.

Prior to the preparation of Fab fragments, IgGs were purified by affinity chromatography on Protein A-Sepharose CL-4B® (Pharmacia) according to the method recommended by the manufacturer, except that the 0.05 M Tris-HCl buffer, pH 7.5, 0.15 M NaCl, replaced the phosphate buffer, pH 7.0. Fab fragments were prepared by papainolysis according to Porter (1959), and their activity was studied in immunoelectrophoresis by a modification of a previously published method (Loft, 1975).

#### *Immunocomplex preparation*

One hundred micrograms of native hemocyanin in 0.05 M Tris-HCl buffer, pH 7.5, 10 mM CaCl<sub>2</sub>, were incubated for 1 hr at 37°C and for one night at 4°C with 200 µg of subunit-specific Fab fragments. Then, immunocomplexes were freed from unbound material by gel filtration on an AcA 34 Ultrogel® (Sepracor, Villeneuve la Garenne, France) in the same buffer.

#### *Electron microscopy*

Specimens for electron microscopy were negatively stained with 2% uranyl acetate and examined in a CM12 Philips electron microscope at an accelerating

voltage of 80 kV. In the microscope, the grid was orientated with the carbon film facing the emulsion of the photographic film. This procedure restores the correct handedness of the molecules on the print.

Image processing was carried out as recently reported (Boisset *et al.*, 1991).

#### *Polyacrylamide gel electrophoreses*

Polyacrylamide gel electrophoreses were carried out under non-denaturing (Davis, 1964) and denaturing conditions (Laemmli, 1970) using a slab gel electrophoresis unit SE 250® (Hoefer Scientific Instruments, San Francisco, CA). The thickness of the gel was 0.75 mm. The acrylamide and bis-acrylamide concentrations were 7 and 0.185%, respectively. The calibration curve was determined using the Electrophoresis Calibration Kit for low molecular weight proteins® (Pharmacia).

#### *Immunoelectrophoreses*

Crossed immunoelectrophoreses, line immunoelectrophoresis and fused-rocket immunoelectrophoreses were carried out as described by Axelsen *et al.* (1973).

## RESULTS

#### *Strategy*

Determination of the quaternary structure of an oligomeric protein requires: (i) the identification of the various subunits composing the oligomer, (ii) the determination of the number of copies of the various polypeptide chains, (iii) the determination of the molecular architecture, and (iv) the intramolecular localization of copies of the various subunits within the native oligomer.

In this work, we were considerably helped by the previous resolution of the quaternary structures of the 4 × 6-meric hemocyanins of *Androctonus australis*, *Eurypelma californicum* and *Limulus polyphemus* by the method of Lamy *et al.* (1981). In this method, subunit-specific Fab fragments are incubated with the native oligomer, then the immunocomplexes are purified by gel filtration and examined in the electron microscope. The positions of the Fab fragments, pointing out the contour line of the hemocyanin molecule, reveal the approximate intramolecular locations of the subunit.

#### *Architecture of the native molecule*

In the electron microscope *Scorpio maurus* hemocyanin produces exactly the same stain exclusion pattern as *Androctonus australis* with top, side and 45° views. In the top view, the molecules lie on their flip or flop faces producing the so-called flip and flop views. According to Van Heel and Frank (1981), when the interdodecamer cleft is top-bottom oriented, the long diagonal of the molecule passes by the lower right and upper left hexamers in the flop view and by the lower left and upper right hexamer in the flip view. A rocking effect around an axis passing by

the lower right and upper left hexamers is present, as in *Androctonus hemocyanin*. The dimensions of the *Scorpio* and *Androctonus* molecules are identical, as demonstrated by the observation in the electron microscope of a mixture of the two hemocyanins on the same grid. The images of the molecules were indistinguishable even after image-processing by correspondence analysis and hierarchical ascendant classification. On the basis of these arguments, the two molecules were considered to have identical architecture.

#### Dissociation

As shown in Fig. 1(A), dissociated *Scorpio maurus* hemocyanin produced a complex immunoprecipitation pattern in crossed immunoelectrophoresis vs the anti-dissociated *Scorpio* hemocyanin antiserum. The peaks were arbitrarily termed *a*, *b*, *c*, *d*, *e*, *f*, *g* and *h*. In the following, the subunits corresponding to these peaks are prefixed *Sm* for *Scorpio maurus*. For comparison, Fig. 1(B) shows the immunoprecipitation pattern obtained under the same conditions with *Androctonus* hemocyanin vs an anti-dissociated *Androctonus* hemocyanin antiserum. The overlaps between the immunoprecipitation peaks, due to a greater similarity in the net charge of the *Scorpio* subunits at pH 8.6, are more important in *Scorpio* than in *Androctonus* hemocyanin.

When submitted to a gel permeation on a HiLoad 16/60 Superdex 200 prep-grade<sup>®</sup> column, dissociated *Scorpio* hemocyanin was fractionated into three well-separated chromatographic zones [Fig. 2(A)]. Zones II and III had absorption peaks in ultraviolet at 340 nm, with  $A_{280}/A_{340}$  ratio of 4.75 and 4.14, respectively, suggesting that they contain hemocyanin subunits. Zone I had no absorption peak at 340 nm. In polyacrylamide gel electrophoresis under denaturing conditions (in the presence or absence of thiol groups) the polypeptide chains of zones II and III had

molecular weights in the range 70–75,000 kDa. Zone I produced 238 and 105 kDa bands in the absence of thiol groups [Fig. 2(B)], and two bands of 126 and 106 kDa in the presence of  $\beta$ -mercaptoethanol [Fig. 2(C)]. In crossed immunoelectrophoresis vs the anti-dissociated hemocyanin antiserum, zone I gave two immunologically identical immunoprecipitation peaks, the anodic peak being sharper than the cathodic one [Fig. 2(D)]. Zone II led to a major anodic peak and a minor cathodic shoulder, antigenically deficient compared to the anodic peak [Fig. 2(E)]. Zone III contained the rest of the antigens separated by crossed immunoelectrophoresis in dissociated hemocyanin [Fig. 2(F)].

These experiments demonstrate that the protein material contained in zone I is not a respiratory pigment because of (i) the high  $M_r$  of its subunits and (ii) its lack of characteristic absorption spectrum at 340 nm. Zone II is probably a dimeric hemocyanin subunit because of (i) its intermediate  $R_f$  value on Superdex 200, (ii) the molecular weight of its subunits (70–75 kDa), similar to those of other arthropod hemocyanins, and (iii) its absorption band at 340 nm. Zone III, which has the same characteristics as zone II, but which is more retarded on Superdex, probably corresponds the monomeric hemocyanin subunits.

#### Purification of the subunits

In the cases of *Androctonus*, *Euryptelma* and *Limulus* hemocyanins the subunits had been identified by polyacrylamide gel electrophoresis and by crossed immunoelectrophoresis and had then been purified by ion-exchange chromatography. The same protocol was used for *Scorpio* hemocyanin.

Dissociated hemocyanin was first adsorbed on DEAE-Sepharose CL-6B in 0.05 M Tris-HCl, pH 8.9, 10 mM EDTA. Then, the subunits were eluted by linear 0–0.5 M NaCl gradient, and the

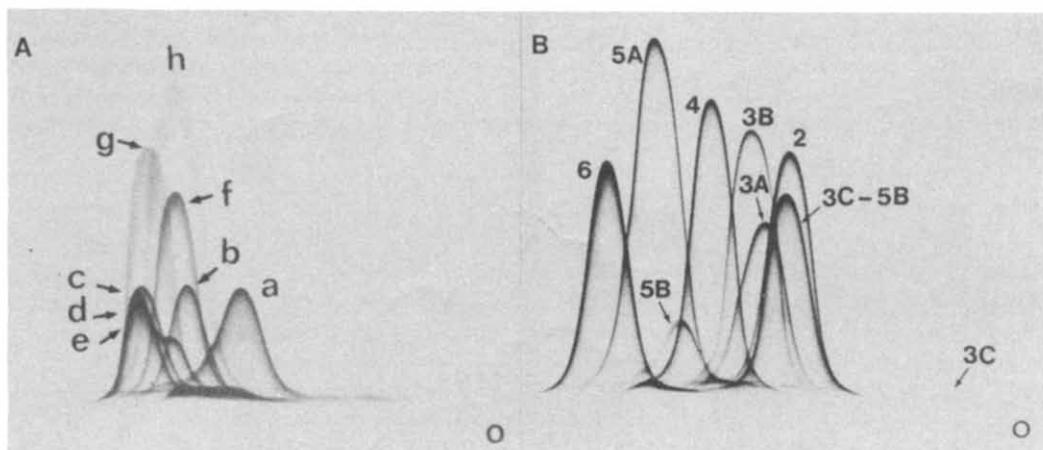


Fig. 1. Crossed immunoelectrophoresis pattern of (A) *Scorpio maurus* and (B) *Androctonus australis* dissociated hemocyanins vs their homologous anti-dissociated hemocyanin antiserum. The nomenclatures of the *Androctonus* and *Scorpio* subunits are from Lamy *et al.* (1973) and this paper respectively. In the first (horizontal) electrophoretic migration the anode and cathode are respectively located on the left and on the right.

elution profile was monitored by fused-rocket immunoelectrophoresis [Fig. 3(A)]. This procedure yields roughly purified fractions which were re-chromatographed under different conditions of pH and ionic strength. Finally, as schematically

shown in Fig. 3(B), eight immunologically pure sub-units were obtained and their position in the immunoprecipitation pattern of dissociated hemocyanin was identified by crossed line immunoelectrophoresis (Fig. 4).

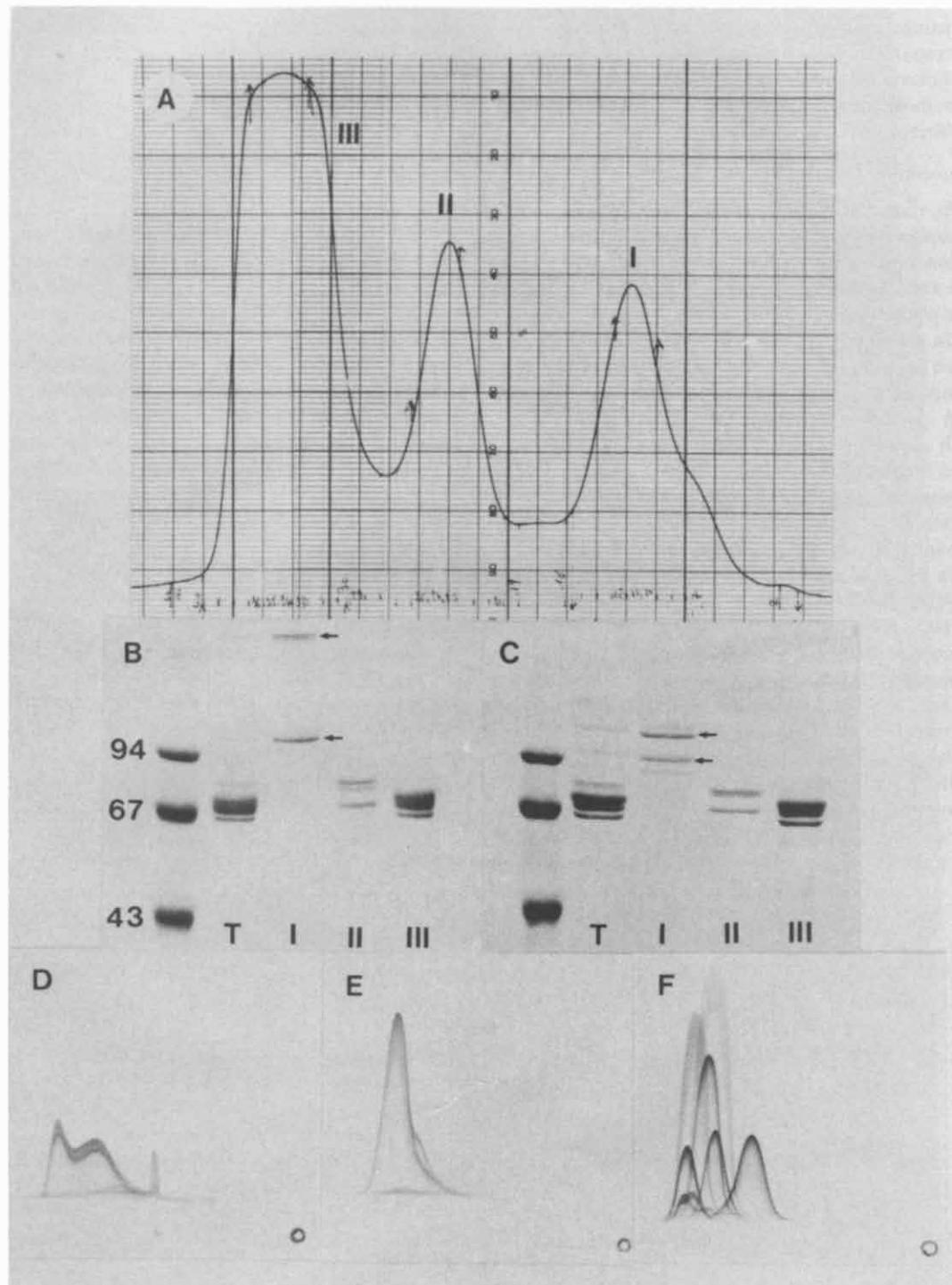


Fig. 2. Separation of dissociation *Scorpio maurus* hemocyanin on Superdex 200. (A): elution profile in 0.05 M Tris-HCl, pH 8.9, 10 mM EDTA. (B, C): polyacrylamide gel electrophoresis in presence and absence of *b*-mercaptoethanol; 43, 67 and 94 correspond to the molecular weight of the calibration proteins; T: dissociated hemocyanin, I-III: zones I-III collected from the separation of Superdex 200. (D-F): immunoelectrophoreses of zones I-III vs the anti-dissociated hemocyanin antiserum.

The purity of the eight subunits was determined by crossed immunoelectrophoresis vs the anti-dissociated hemocyanin antiserum. As shown in Fig. 5, in all cases a major single immunoprecipitation peak was observed and only small amounts of contaminants were present.

#### Characterization of the subunits

The subunits were characterized by their molecular weight determined in polyacrylamide gel electrophoresis, by their stain exclusion pattern in the electron microscope, and by their immunological properties.

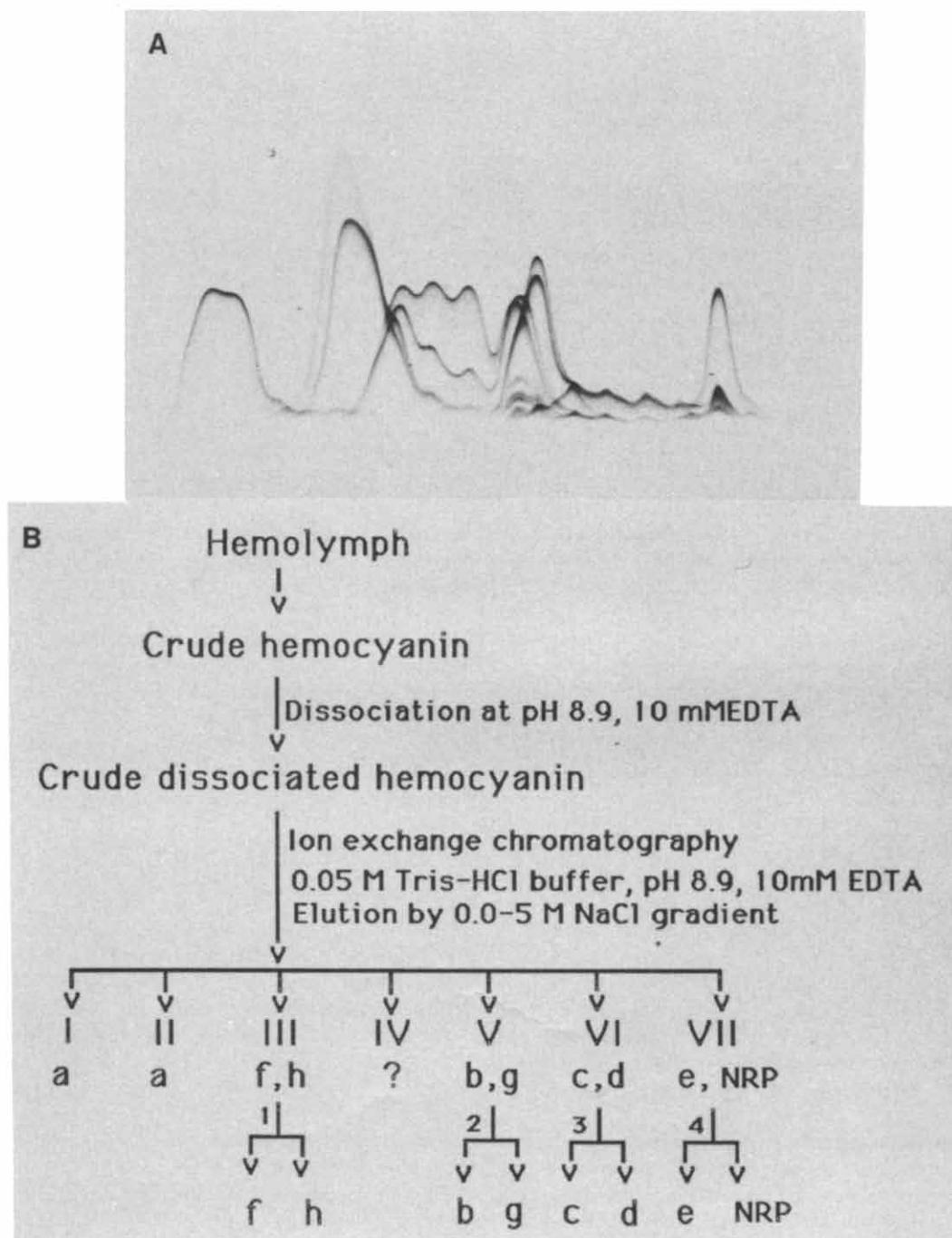


Fig. 3. Isolation of eight immunologically pure subunits in dissociated hemocyanin of *Scorpio maurus*. (A): fused-rocket immunoelectrophoresis of the elution pattern on DEAE-Sepharose CL-6B. (B): schematic description of the purification of the subunits. I-VII correspond to the main fractions of the chromatography on DEAE-Sepharose CL-6B. 1-4 correspond to four chromatographies under different elution conditions. NRP: non-respiratory protein.

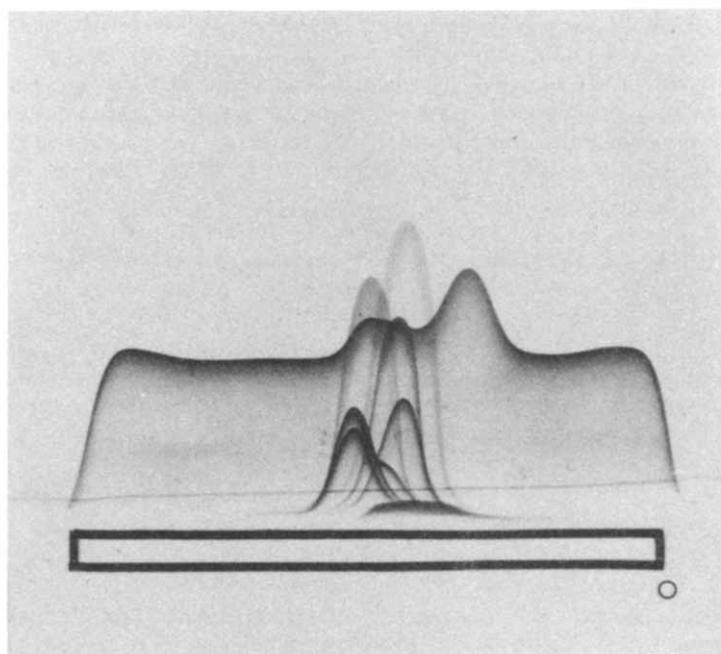


Fig. 4. Example of identification of a purified fraction containing subunit *Sma* by crossed line immunoelectrophoresis vs the anti-dissociated hemocyanin antiserum. The deposit hole contains crude dissociated hemocyanin and the trough an agarose gel containing the subunit to be identified. This immunoelectrophoresis is to be compared to Fig. 1(A).

The molecular weight of the monomeric subunits was determined by polyacrylamide gel electrophoresis under denaturing conditions in the presence and in the absence of  $\beta$ -mercaptoethanol. As shown in Figs 2(B) and (C), the presence of the thiol groups did not change the electrophoretic mobility. The  $M_r$  values obtained by this method were comparable to those obtained with other chelicerate hemocyanins and were in the range 70–75,000 for the eight subunits.

The electrophoretic mobilities of the monomeric subunits in polyacrylamide gel electrophoresis under non-denaturing conditions were significantly greater with *Scorpio* (seven of the eight subunits had an electrophoretic mobility value  $>0.75$ ) than with *Androctonus* hemocyanin (three subunits only had a mobility  $>0.75$ ). The origin of this difference is not clear.

After negative staining, the monomeric subunits appeared in the electron microscope as small globular irregular structures, while the dimeric subunits had characteristic elongated shapes. For example, Fig. 6 shows an electron micrograph of zone II of the chromatography on Superdex 200.

Each purified subunit was used as an antigen to prepare rabbit subunit-specific antiserum whose specificity was studied in crossed immunoelectrophoresis vs dissociated *Scorpio maurus* hemocyanin (Fig. 7). In all cases, a single major immunoprecipitation peak was obtained. In some cases, very faint and blurred ghost peaks corresponding to authentic weak crossreactivities were hardly visible [Fig. 7(E)]. With the anti-*Sma* and Anti-*Sme* antisera, the major

peaks were deformed by shoulders corresponding to dimers of these subunits in equilibrium with the monomers.

The eight subunit-specific antisera were also used to study, in rocket immunoelectrophoresis, cross-reactivities with the *Androctonus australis* subunits. The results were confirmed by investigating the precipitation of the *Scorpio* subunits by antisera raised against the subunits of *Androctonus* hemocyanin (Table 1). Although the crossreactivities were more or less important, the results were not ambiguous. Each subunit of the *Scorpio* system markedly crossreacted with a single subunit of the *Androctonus* system.

#### *The heterodimeric subunit*

In all the chelicerate 4  $\times$  6-meric hemocyanins so far studied, a stable heterodimeric subunit was responsible for the cohesion of the half-molecules and of the interdodecameric bridges. In *Scorpio* hemocyanin such a heterodimer was searched for and found. As described above, the crossed immunoelectrophoresis of zone II of the separation on Superdex 200 possessed a single asymmetric peak with a double shoulder on the cathodic side, suggesting that the heterodimer may be unstable [Fig. 2(E)]. After dialysis against 3 M urea, zone II produced two immunoprecipitation peaks [Fig. 8(A)] which were further identified as subunits *Smd* and *Sme* in immunoelectrophoresis. The *Sme* peak had a small shoulder on the anodic side suggesting that, despite the presence of 3 M urea, two forms of *Sme* were in equilibrium.

Another approach to the structure of zone II was done by bidimensional polyacrylamide gel electrophoresis [Fig. 8(B)]. In the first dimension, the dimer(s) were separated by a non-denaturing polyacrylamide gel electrophoresis, then the plate was

turned 90° and a SDS-polyacrylamide gel electrophoresis was run. A major cathodic and a minor anodic spot are visible on the control migration, corresponding to the first dimension [top of Fig. 8(B)]. The two fractions are linked by a blurred

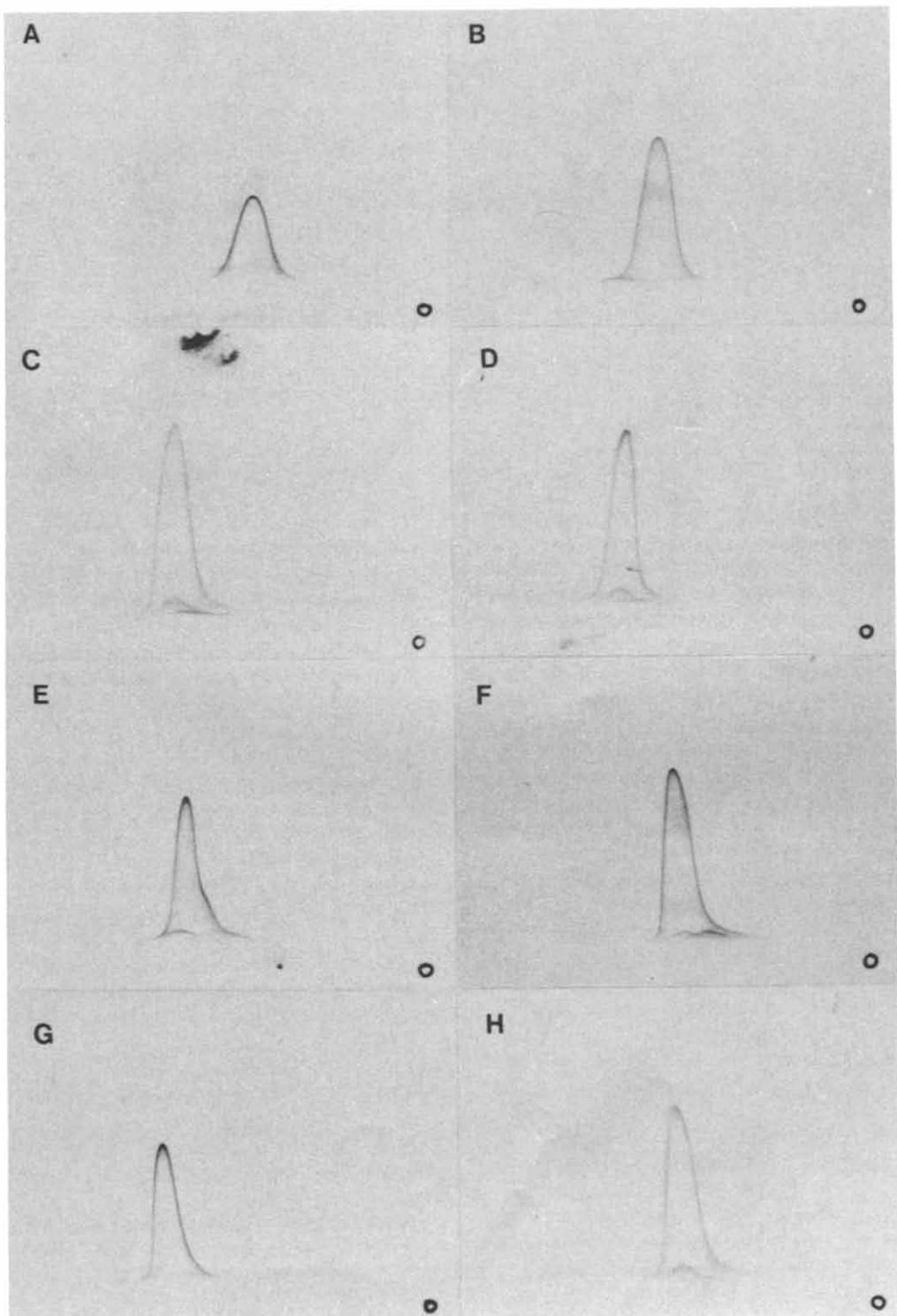


Fig. 5. Purity of the eight subunits of *Scorpio maurus* hemocyanin determined by crossed immunoelectrophoresis vs the anti-dissociated hemocyanin antiserum. (A-H): purified subunits *Sma-h* were deposited in the wells located in the lower right corner of the gels.

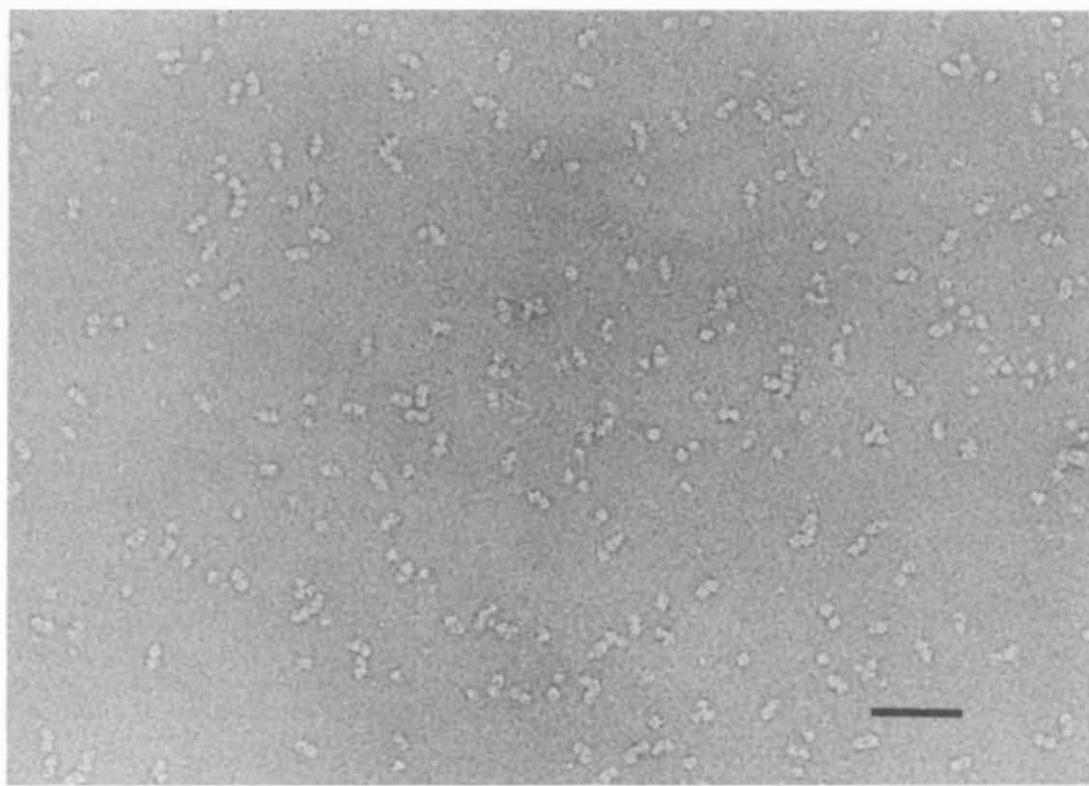
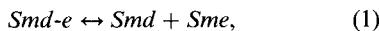


Fig. 6. Electron micrograph of negatively stained zone II obtained by gel permeation on Superdex 200.  
The scale bar is 50 nM.

trail which suggests that the dimer is in equilibrium with its monomeric components. The second dimension shows that both the monomeric and the dimeric fractions comprise the *Smd* and *Sme* subunits in different proportions. Clearly, the dimeric fraction contains a major *Sme-e* homodimer and a minor component which could be either a *Smd-d* homodimer or a *Smd-e* heterodimer. Conversely, the monomeric components of zone II are *Smd* (major component) and *Sme* (minor component). This experiment suggests that *Smd-e* is unstable and in equilibrium with its components.

This hypothesis was verified by a rechromatography of zone II on Superdex 200 which also produced a monomeric (M) and a dimeric (D) fraction, each containing the *Smd* and *Sme* subunits [Fig. 8(C)]. In turn, these fractions were isolated and rechromatographed. Again, each of them yielded a monomeric [Fig. 8(D)] and a dimeric [Fig. 8(E)] fractions containing the *Smd* and *Sme* subunits.

This experiment clarifies the fact that *Smd-e* is in equilibrium with its components and that the *Smd* and *Sme* monomers are in equilibrium with their homodimers. However, while the equilibrium is shifted toward the monomer for subunit *Smd*, it is shifted toward the dimer in the case of *Sme*.



The equilibria of equations (1)–(3) are in agreement with all the results described in this paper, and with all the previous data collected for chelicerate hemocyanins.

#### Immunolabeling

The intramolecular localization of the subunits was carried out by immunoelectron microscopy using Fab fragments prepared by papainolysis of the subunit-specific IgGs. The results, shown in Fig. 9, were exactly those which were expected from the immunological relatedness with the subunits of *Androctonus australis* (Table 1). For example, in the side view, anti-*Sma* Fab fragments bound to *Scorpio* hemocyanin were visible near the top/bottom edges of the dodecamer in the same position as anti-*Aa5A* Fab fragments bound to *Androctonus* hemocyanin. *Smb* and *Smc* subunits were labeled with prominent Fab fragments located slightly apart from the middle of the lateral edges and easily recognizable in the top view. This structure was also obtained with anti-*Aa3A* and anti-*Aa3B* Fab fragments in the *Androctonus* system. Binding Fab fragments to subunits *Smd* and *Sme* often induced a dissociation of the 4 × 6-mers into dodecamers. In this structure, the Fabs were distinctly observable in the bridge area, exactly where the anti-*Aa3C* and anti-*Aa5B* Fabs bind *Androctonus* hemocyanin. Occasionally, anti-*Smd* and anti-*Sme* Fabs were observed in the interdodecamer cleft of the 4 × 6-mer

side view (first two views of the *Sme* row in Fig. 9). Anti-subunit *Smf*, *g* and *h* antibodies produced the same types of immunocomplexes with *Scorpio*

hemocyanin as anti-*Aa2*, *4* and *6* antibodies with *Androctonus* hemocyanin. In these complexes the Fabs were located in the top view on the top of

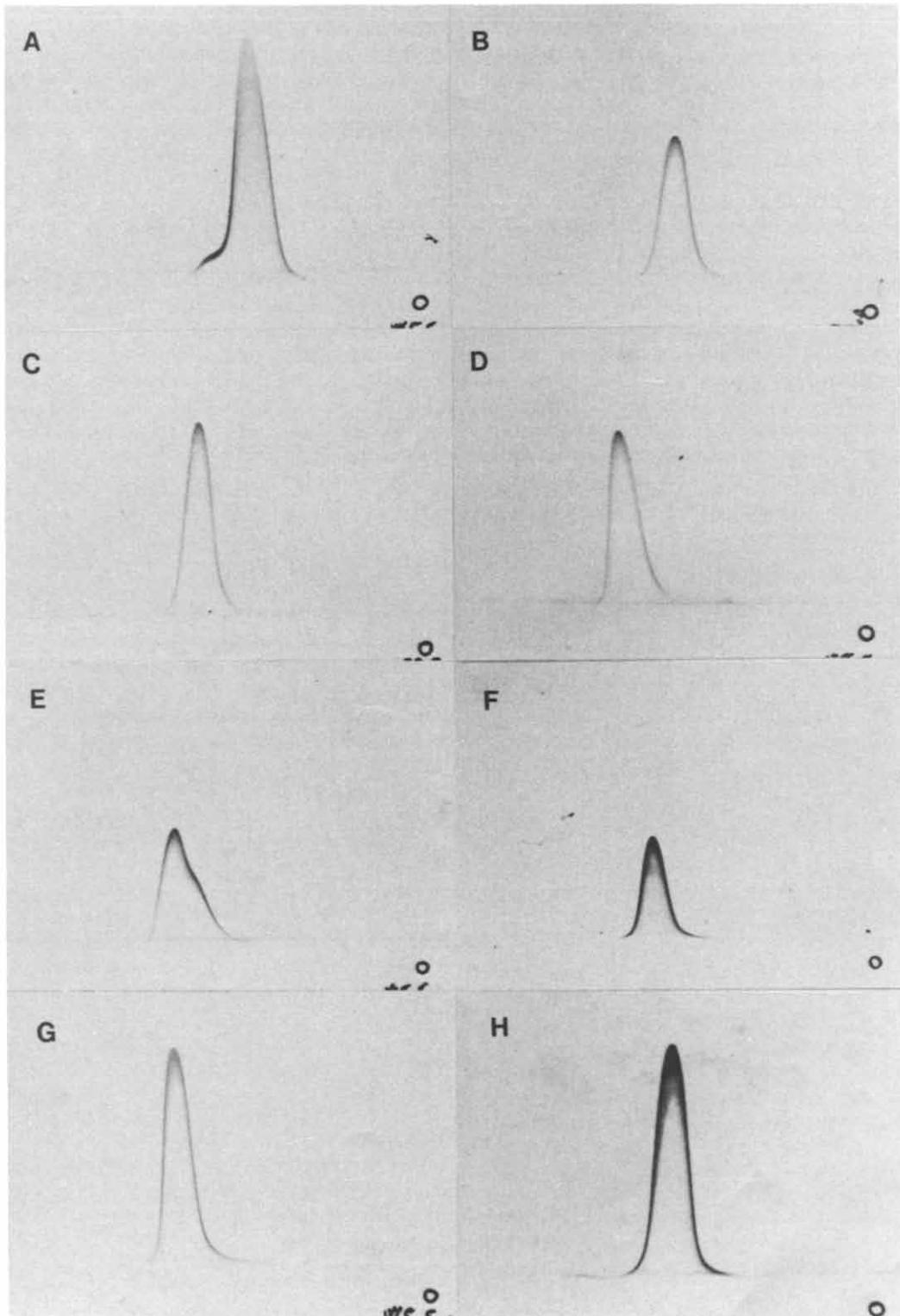


Fig. 7. Crossed immunoelectrophoresis of dissociated *Scorpio maurus* hemocyanin vs subunit-specific antisera. A-H: antisera specific for subunits *Sma-h*. The deposit well is located in the lower right corner of each plate.

Table 1. Immunological correspondence between the subunits of *Scorpio maurus* and *Androctonus australis* hemocyanin

<i>Scorpio maurus</i> subunits	<i>Androctonus australis</i> subunits
<i>Sma</i>	<i>Aa5A</i>
<i>Smb</i>	<i>Aa3B</i>
<i>Smc</i>	<i>Aa3A</i>
<i>Smd</i>	<i>Aa3C</i>
<i>Sme</i>	<i>Aa5B</i>
<i>Smf</i>	<i>Aa4</i>
<i>Smg</i>	<i>Aa2</i>
<i>Smh</i>	<i>Aa6</i>

the dodecamer (*Smf*), near the interdodecamer cleft (*Smg*), and near the corners of the dodecamers (*Smh*).

#### Quaternary structure

A model of the quaternary structure of *Scorpio maurus* hemocyanin was built on the same architectural basis as the model of *Androctonus* hemocyanin (Lamy *et al.*, 1985; Boisset *et al.*, 1990). The molecule is a  $4 \times 6$ -meric structure composed of two dodecamers of the right isomeric type

(Lamy *et al.*, 1985). The model also possesses a rocking effect and flip/flop faces, as first described by Van Heel and Frank (1981) in *Androctonus* hemocyanin. Figure 10 shows the top view of this model lying on its flop face and the corresponding view of the model of *Androctonus australis* hemocyanin.

The model assumes that the number of copies of the various subunits is the same in *Androctonus* and *Scorpio* hemocyanins (four copies of subunits *Sma*, *f*, *g* and *h* and two copies of subunits *Smb*, *c*, *d* and *e*). These data, determined in *Androctonus* hemocyanin, were extrapolated to *Scorpio* hemocyanin on the basis (i) of the subunit diversity (eight different polypeptide chains), (ii) of the immunological relatedness between the subunits of the two species, (iii) of the common intramolecular location of immunologically related subunits, and (iv) of the observation that the copy numbers assumed to be present are sometimes reached on a single molecule, but never overcome. For example, two copies of *Smc* and four copies of *Smh* are labeled by Fab fragments.

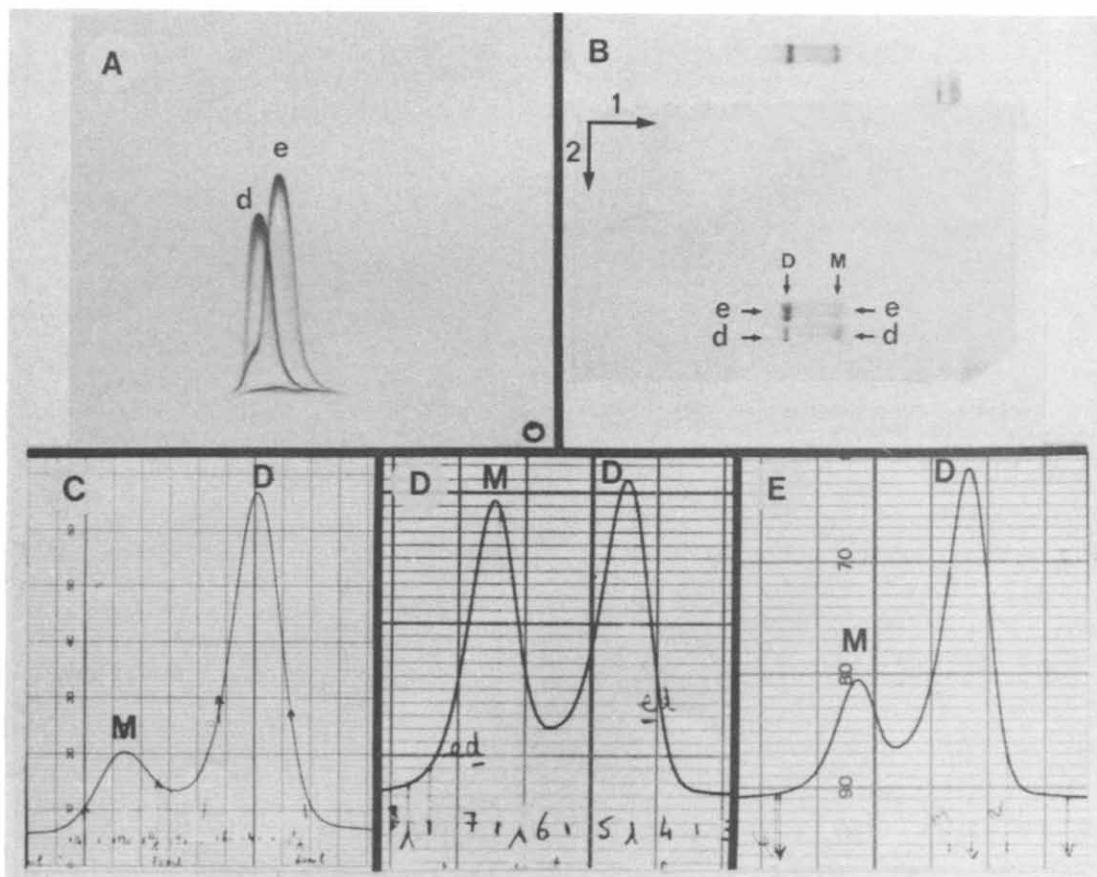


Fig. 8. Hemocyanin in zone II of the separation on Superdex 200. (A): crossed immunoelectrophoresis after dialysis against 3M urea. (B): bidimensional electrophoresis in polyacrylamide gel. 1: non-denaturing electrophoresis; 2: SDS-gel electrophoresis; (C): rechromatography of zone II on Superdex 200; (D): rechromatography on Superdex 200 of fraction M of Fig. 8(C); (E): rechromatography on Superdex 200 of fraction D of Fig. 8(C); M: monomers; D: dimers; d: *Smd*; e: *Smc*.

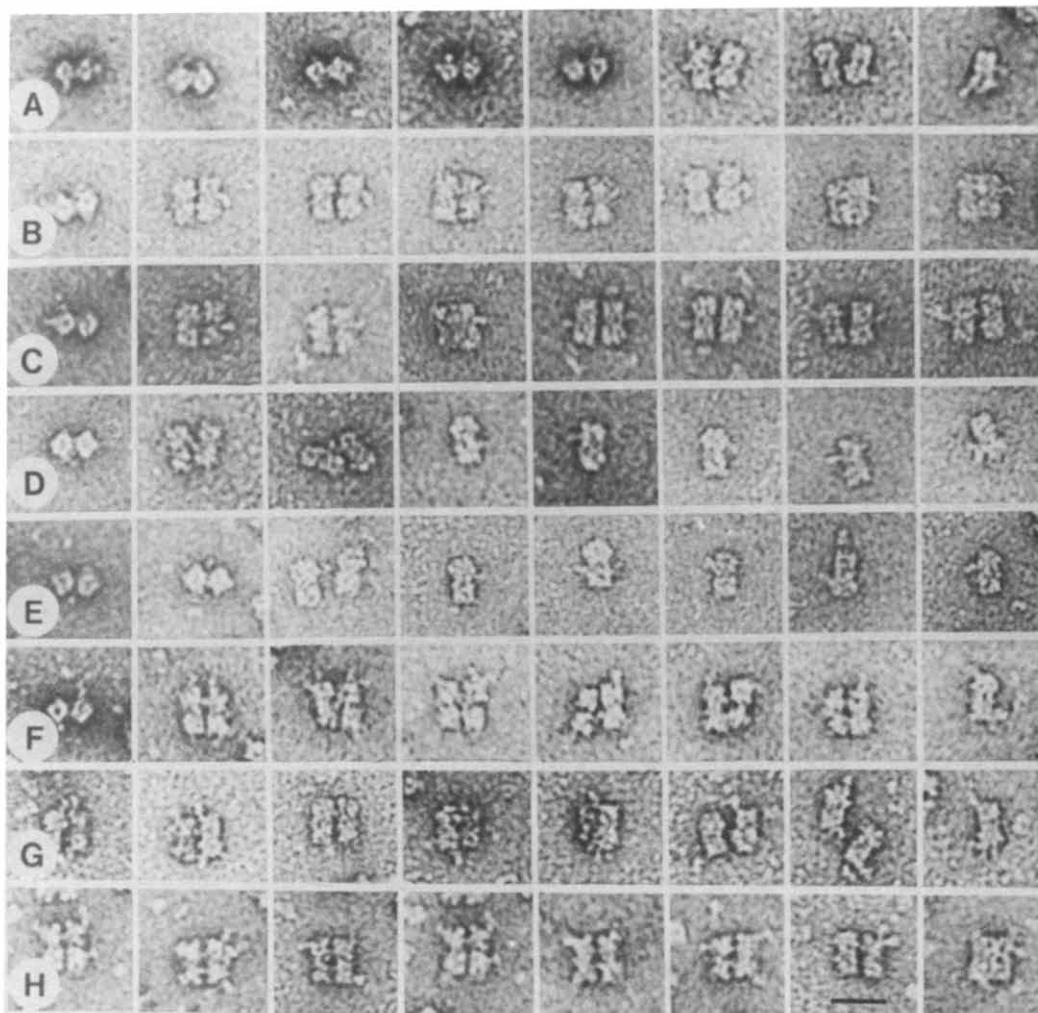


Fig. 9. Gallery of selected electron microscopic views of immunocomplexes resulting from the incubation of native *Scorpio maurus* hemocyanin with subunit-specific Fab fragments. (A–H): antisera specific for subunits *Sma-h*. The scale bar is 25 nm.

## DISCUSSION

### Structural resemblance in scorpion hemocyanins

As described above, the hemocyanins of *Scorpio maurus* and *Androctonus australis* are indistinguishable in the electron microscope. This structural resemblance is in agreement with the immunological relatedness and the intramolecular location of the subunits of *Androctonus* and *Scorpio* hemocyanins. One can conclude from these convergent arguments that the hemocyanins of the two species have similar quaternary structure.

Eight subunits isolated from *Androctonus* and *Scorpio* hemocyanins were characterized on the basis of their immunoprecipitation pattern in crossed immunoelectrophoresis and by their electrophoretic mobilities in polyacrylamide gel electrophoresis under non-denaturing conditions. Their molecular weights of 70–75 kDa were comparable with those of all the other chelicerate subunits so far isolated (32 subunits

from four species). The subunits were not characterized by their *N*-terminal sequence of amino acids because previous studies on *Androctonus australis* (Lamy *et al.*, 1979b; Jollès *et al.*, 1979), *Eurypelma californicum* (Lamy *et al.*, 1979c; Linzen *et al.*, 1985), *Limulus polyphemus* and *Tachypleus tridentatus* (Lamy *et al.*, 1979a, 1983; Takagi and Nemoto, 1980) had shown that all the immunologically different subunits had different *N*-terminal sequences.

An interesting feature is that subunits *Smb* and *c*, which are antigenically related to subunits *Aa3B* and *3A* of *Androctonus* hemocyanin, have exactly the same intramolecular locations as these subunits. In other chelicerate 4 × 6-mers (*Eurypelma californicum*, *Limulus polyphemus*, *Tachypleus tridentatus*), the two positions occupied by *Aa3A* and *3B* are always occupied by two copies of a single polypeptide chain. Therefore, it appears that the occurrence of two different polypeptide chains in the two positions apart from the middle of the lateral edge, is a

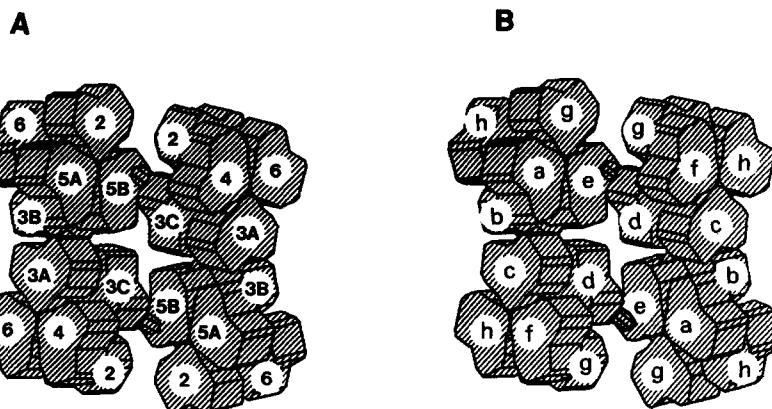


Fig. 10. Model of quaternary structure of *Scorpio maurus* and *Androctonus australis* hemocyanins. The models are represented in their top view and lies on their flop faces (the long diagonal is orientated lower right-upper left).

character of scorpion hemocyanins. *Androctonus* and *Scorpio* being phylogenetically distant, it would be surprising if the phenomenon were limited to these species.

At this point it appears that *Scorpio* and *Androctonus* hemocyanins are quite similar and that they only differ in minor characteristics. The most obvious difference is the electrophoretic mobility of the subunits in polyacrylamide gel electrophoresis. Indeed, the electrophoretic mobilities of the *Scorpio* subunits are higher and more homogeneous than those of *Androctonus*. The same phenomenon has been observed in *Limulus* hemocyanin, but not in *Tachypleus tridentatus*. Whether or not this electrical homogeneity is due to a better preservation of the charged residues in some taxonomic groups is not clear.

#### The heterodimer problem

Another important difference between *Androctonus* and *Scorpio* hemocyanins is the behavior of the heterodimeric subunit. In *Androctonus* and *Eurypelma* hemocyanins, a stable heterodimer, involved in the interhexamer and interdodecamer contacts is required for the reassembly of oligomers higher than hexamers (Van Bruggen *et al.*, 1980). In *Xiphosura* the situation is more complex (Lamy *et al.*, 1979a; Van Bruggen *et al.*, 1980). A heterodimer is also required for the autoassembly of 4 × 6-mers and 8 × 6-mers, but it seems less stable than in *Arachnida*. It is composed of two highly antigenically related subunits [*LpV* and *VI* in *Limulus polyphemus*, and *TtVI<sub>A</sub>* and *VI<sub>B</sub>* in *Tachypleus tridentatus*, after the nomenclature of Lamy *et al.* (1979a) or  $\zeta$  and  $\zeta'$  in the nomenclature of Takagi and Nemoto (1980)]. Furthermore, in dissociated *Limulus* hemocyanin, subunit *LpV* occurs as a monomer in equilibrium with its homodimer, and with the heterodimer *LpV–VI*. In the *Scorpio* system the heterodimer is also in equilibrium with its free components *Smd* and *Sme*, both of them being in equilibrium with their homodimer. Furthermore, the fact that *Sme* equilibrium is shifted toward the homodimer is strongly

reminiscent of the case of the subunit *LpV*. These data, together with the fact that the antigenic structure of both components of the heterodimer are better preserved by evolution than those of other subunits (Lamy *et al.*, 1983b), suggest that, in chelicerates, a heterodimer, required for the 4 × 6-mer aggregation, is in equilibrium with two monomers and that each of them is in equilibrium with its homodimer. Therefore, the heterogeneity and size of the subunits found in dissociated hemocyanins depends on the direction towards which the equilibria (i) between the heterodimer and the monomers and (ii) between the monomers and their homodimers are shifted.

Because of the instability of the *Scorpio* heterodimer, the purification of its monomers (*Smd* and *e*) was readily achieved, while in the *Androctonus* system the isolation of the *Aa3C* and *Aa5B* subunits could not be carried out quantitatively by non-denaturing methods. Therefore, isolated subunits *Smd* and *Sme* seem to be favorable material for studying the precise structural and possibly functional roles of the heterodimer. This has not been feasible with the other chelicerate heterodimers so far studied because of too high a stability.

#### CONCLUSION

Though *Scorpio maurus* and *Androctonus australis* are two evolutionarily distant scorpion species, the architecture of their hemocyanins is remarkably similar. (i) Both of them are composed of eight different polypeptide chains which occupy the same intramolecular positions as demonstrated by immunoelectron microscopy using polyclonal subunit-specific Fab fragments. (ii) The eight polypeptide chains are evolutionarily preserved as demonstrated by immunological crossreactivities occurring between the subunits occupying the same intramolecular positions in both species. (iii) The observation that two different polypeptide chains occupy the two positions located apart from the middle of the lateral edge

(*Aa3A*, *Aa3B* and *Smb*, *Smc*) seems to be characteristic of the scorpion order. (iv) The difference of behavior between the various chelicerate heterodimeric subunits involved in the interhexamer and interdodecamer contacts is spectacular, but it can be explained simply as minor differences in the equilibria between the heterodimers and their monomers and between the monomers and their homodimers.

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