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Evolution

Centipedal hemocyanin: Its structure and its implications for arthropod phylogeny

(protein structure/subunits)

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ABSTRACT **The oxygen carrier hemocyanin occurs in the blood of *Scutigera coleoptrata*, a uniramous arthropod, as well as the crustaceans and chelicerates. The native polymer appears to be composed of substructures having the same size and electron-dense image as those of other arthropod hemocyanins but assembled into a unique multiple and arranged in a unique configuration. The simplest explanation of these findings is that the arthropod hemocyanins have a common origin, exemplifying a derived (as opposed to primitive) character shared by each of the three living groups.**

In contrast to the hemoglobins (Hbs), the hemocyanins (Hcs) are regarded as coherent in taxonomic distribution. They are found in only two groups: the molluscs, in three of the four classes examined, and the arthropods, in the crustaceans and chelicerates. Also unlike the Hbs, the Hcs are always widespread in a particular taxon.

Therefore, the report by Rajulu (1) of a Hc-like substance in a single order of uniramous arthropods, the scutigeromorph centipedes, is somewhat surprising. All but a few of the Uniramia lack an O_2 carrier in the blood, the exceptions containing Hb rather than Hc. The brief report (1) was based on high levels of blood Cu and N, the blue color of the blood, and its absorption peak at 340 nm, all of which resemble features of the chelicerates. He did not, however, present the decisive evidence—namely, reversible O_2 binding. Later, when the amino acid composition of this protein proved to differ from that of a crustacean Hc, he identified it with less certainty (2). Not unexpectedly, his inference is treated tentatively in a recent volume on centipede biology (3). Moreover, the possibility of Hc in the Uniramia appears to be either unknown to or dismissed by proponents of various points of view in the current debate on arthropod phylogeny (4, 5).

Our findings show that the common house centipede *Scutigera coleoptrata* (Linnaeus) does contain a Hc that resembles the crustacean and chelicerate Hcs in fundamental structural features but differs in superficial respects. The molecule is of both structural and phylogenetic interest.

METHODS AND MATERIALS

Blood, which does not clot, was withdrawn into a glass capillary from the pericardial cavity. With the exception of the samples used for electron microscopy, the blood was of necessity frozen and stored until the volume sufficient for a particular measurement accumulated.

Activities of the inorganic ions in the blood were measured with ion-selective electrodes (6). Absorption of the native blood (in physiological saline) and the Hc subunits (in 10 mM EDTA at pH 8.95) was examined with a Beckman DK-2A recording spectrophotometer. O_2 binding was determined by the cell respiration method (6), using a miniaturized respirometer that accepts samples as small as 300 μl.

The Hc was purified on a 1 × 20 cm column of Sephacryl S-300 equilibrated with Tris buffer (0.1 ionic strength, pH 7.65) containing 50 mM $MgCl_2$ (buffer A). Most of the Hc eluted in the void volume.

Whole blood was electrophoresed on polyacrylamide slab gels (7.5%) in the presence of $NaDodSO_4$. Photographic negatives of the gels were scanned with a Biomed gel scanner. The Hc subunits were also separated on slab gels according to charge. Finally, the $NaDodSO_4$/PAGE was repeated using the purified molecule.

The purified Hc was precipitated with an equal volume of 20% trichloroacetic acid, spun down, and washed twice with 10% trichloroacetic acid. The precipitate was hydrolyzed in 1 ml of 6 M HCl (26 hr, 110°C). The HCl was flashed off and the sample was analyzed on a Beckman 120B analyzer, updated to a 6-mm column system. In sedimentation velocity measurements (Beckman model E ultracentrifuge) scanner optics, in most cases at 345 nm, were used. The coefficients were determined at 20°C and corrected to standard conditions. Sedimentation equilibrium was carried out by the meniscus-depletion method (7) at a rotor speed of 4000 rpm (33 hr, 21.5°C); Rayleigh optics were used. A blank (buffer in both compartments) was run at the same speed and the small fringe displacement was subtracted.

Fresh blood was diluted about 1:1000 with Tris maleate buffer (0.1 ionic strength, pH 7.65) containing 10 mM $CaCl_2$, stained with uranyl acetate (1%), and examined with Zeiss EM-109 and EM-9S2 electron microscopes. The dimensions of the centipede molecule were calculated from the image of *Busycon canaliculatum* Hc on the same grid (8).

RESULTS

Absorption. *Scutigera* blood absorbs at 277.5 and 339 nm (Fig. 1). A crustacean (*Callinectes sapidus*) blood treated similarly absorbs at 277 and 336 nm; a chelicerate (*Limulus polyphemus*) blood absorbs at 278.5 and 340 nm. Following deoxygenation the absorption peak of the active site disappears and after reoxygenation the 336- to 340-nm peaks reappear. In Fig. 1 this peak is somewhat higher and less sharp due to evaporation and interference by residual H_2 bubbles. A negative result was obtained from a species belonging to a different order, indicating that the Hcs are not ubiquitous among the centipedes.

Oxygen Binding. *Scutigera* Hc has an exceptionally low O_2 affinity (Fig. 2). Its O_2 binding is also unusually cooperative; the Bohr shift, which is normal, is unexceptional among the arthropod Hcs ($\Delta \log P_{50}/\Delta pH = -0.87$). By using the extinction coefficient at 340 nm for *Limulus* Hc (9), the absorbance of a solution of dissociated subunits predicts a

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Abbreviation: Hc, hemocyanin.

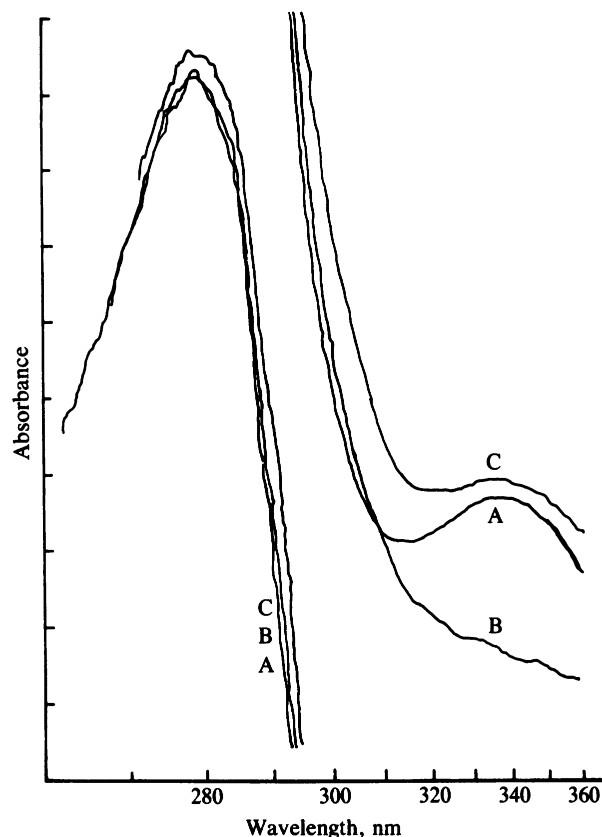

FIG. 1. Absorbance of 7.5 μl of *S. coleoptrata* blood diluted with 3.9 ml of physiological saline. The saline, based on measurements of the activities of Na^+, K^+, Ca^{2+}, Mg^{2+}, and Cl^-, contained 306 mM NaCl, 46 mM $MgSO_4$, 8 mM $CaCl_2$, 7 mM KCl, and 2 mM $NaHCO_2$. Trace A, air equilibrated; trace B, with sodium borohydride; trace C, reequilibrated with air. Scale: 0–1 for 339-nm peak; 1–2 for 277.5-nm peak.

concentration of about 9.3 g of Hc per 100 ml, or a total O_2 carrying capacity at 23°C of >3 ml of O_2 per 100 ml of blood.

Electrophoresis. $NaDodSO_4$/PAGE reveals most of the protein subunits in whole blood at approximately the same position as the subunits of *Callinectes* Hc (72–78 × 10^3 daltons) and slightly retarded relative to those of *Limulus* Hc (67 × 10^3 daltons) (Fig. 3 *A–C*). *Scutigera* subunits migrate to positions corresponding to 72 and 80 × 10^3 daltons. Material from *Scutigera* (30–35% of the total) was also observed at positions ahead of (32, 40, and 48 × 10^3 daltons) and behind (127, 203, and 208 × 10^3 daltons) the Hc subunits. In contrast, only 5% of the material from *Callinectes* and none from *Limulus* migrated to positions other than 67–80 × 10^3 daltons. However, all of the larger and smaller material disappeared when a purified sample of *Scutigera* Hc was electrophoresed (Fig. 3 *D* and *E*). When the electrophoresis was carried out at constant current (10 mM EDTA, pH 8.9) we were still unable to resolve more than two subunits of *Scutigera* Hc.

Amino Acid Composition and Sedimentation Coefficients. The amino acid compositions of the two *Scutigera* Hcs differ from one another at least as much as either differs from those of crustacean or chelicerate Hcs (Table 1).

Two components were observed in sedimentation velocity studies. Most of the material in whole blood sedimented at about 49 S and the remainder at 5 S. A series of dilutions (25, 50, and 100 μl of blood per ml of buffer A) gave $s_{20,w}$ = 48.9, 48.3, and 48.9 S, respectively. The average predicts $s^0_{20,w}$ = 48.7 S. The purified material recovered in the void volume of the Sephacryl column showed only the 49S component. A sedimentation coefficient in this range has never been reported for an arthropod Hc, suggesting an unusual quaternary structure.

At higher pH (9.1) and in the absence of divalent cations (10 mM EDTA) the Hc wholly dissociates into a single component with $s_{20,w}$ = 4.8 S, which is in the range found for other arthropod Hc subunits (10).

Molecular Weight. The sedimentation equilibrium data indicate molecular homogeneity of the purified high molecular weight Hc (Fig. 4). By using the measured amino acid composition, the partial specific volume was $\bar{v}$ = 0.723 (11), which is close to the values for other arthropod Hcs. It indicates a molecular weight of 2.81 × 10^6 (±5%). Given the

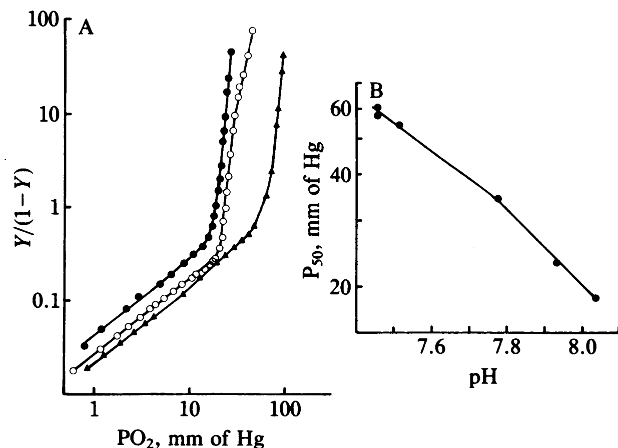

FIG. 2. O_2 binding by *S. coleoptrata* Hc. Blood diluted 9:1 to 1:1 with Tris maleate-buffered saline; 25°C. (*A*) Hill plots: ●, pH 8.04, Hill coefficient h = 10.7; ○, pH 7.93, h = 9.3; ▲, pH 7.52, h = 8.9. (*B*) pH dependence of O_2 affinity.

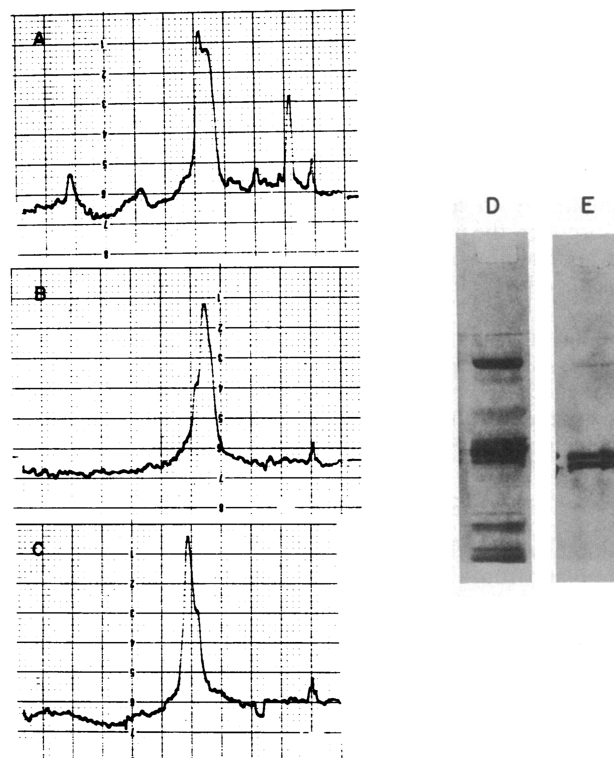

FIG. 3. Densitometer scans of $NaDodSO_4$/PAGE of blood protein subunits. (*A*) *S. coleoptrata*. (*B*) *L. polyphemus*. (*C*) *C. sapidus*. $NaDodSO_4$/PAGE of whole blood (*D*) and purified Hc (*E*) from *S. coleoptrata*.

Table 1. Amino acid composition (mol%) of arthropod Hcs

Residue	*Panulirus interruptus**	*Eurypelma californicum*†	*Scutigera longicornis*‡	*S. coleoptrata*
Lys	4.7	4.9	3.1	5.9
His	6.0	3.6	5.4	4.6
Arg	5.0	5.2	3.6	5.4
Asp	14.2	13.2	10.5	14.6
Thr	5.2	4.6	7.0	5.0
Ser	5.3	8.2	0.0	4.4
Glu	10.2	10.1	12.3	12.2
Pro	4.5	4.3	5.8	4.9
Gly	7.2	9.2	7.1	5.8
Ala	5.3	6.2	7.1	6.2
Cys	0.8	1.0	2.5	0.9
Val	5.7	5.1	5.4	6.1
Met	2.4	1.8	4.2	1.8
Ile	5.4	4.2	3.6	4.1
Leu	8.0	9.3	5.0	8.0
Tyr	4.3	3.4	5.1	4.4
Phe	5.8	4.5	5.0	4.6
Trp	ND	1.2	1.1	ND
Total	100.0	100.0	93.8	100.0

Crustacea: *P. interruptus*. Chelicerata: *E. californicum*. Uniramia: *S. longicornis* and *S. coleoptrata*. ND, not determined.
*Ref. 10.
†Subunit d, ref. 11.
‡Ref. 2.

average subunit size of 76 × 10^3 the estimate corresponds to 37 ± 2 subunits per molecule. Like the sedimentation coefficient, this value is unique among arthropod Hcs.

Electron Microscopy. Both simple and complex images appear in electron micrographs of *Scutigera* Hc. The simple images are (*i*) two pairs of spherical substructures, each member of a pair measuring about 12 nm in diameter and each pair separated by a 2-nm cleft (Fig. 5*A*). One pair is in sharper focus than the other and usually one pair is slightly skewed with respect to the other; (*ii*) two pairs of the 12-nm spheres with a fifth sphere, which encloses a central dot, in the middle of the other four (Fig. 5*B*); (*iii*) a triangle of three substructures surrounding a fourth, which also encloses a central dot (Fig. 5*B*). Often the triangles are not quite equilateral, with two sides measuring about 24 nm and the third as small as 21 nm. Two of the three peripheral units are in sharper focus than the third. On closer inspection additional material even more out of focus can be seen on the flat sides. Complex images, suggesting larger numbers of spheres, are also common.

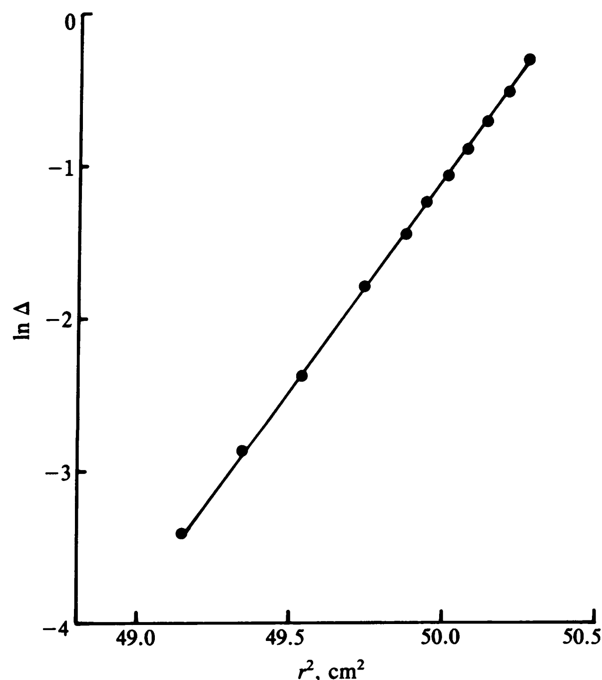

FIG. 4. Sedimentation equilibrium of the purified, high molecular weight Hc of *S. coleoptrata*.

DISCUSSION

Molecular Structure. Other arthropod Hcs exist in the blood as structures with $s^0_{20,w}$ = 16, 24, 37, or 60 S and corresponding molecular weights of about 0.45, 0.9, 1.8, or 3.6 × 10^6 (10). They are multiples of 6, 12, 24, or 48 of the 67–80 × 10^3-dalton chains that are common to all. Although the subunit molecular weight of *Scutigera* Hc is typical, both the sedimentation coefficient and the molecular weight of the major component in the blood are not (although these data suggest 37 ± 2 subunits, the electron micrographs indicate that the actual number is more likely to be 36).

Native crustacean and chelicerate Hcs usually appear in electron micrographs as hexagons measuring 12.5 nm from flat side to parallel flat side, or as multiples of the hexagons, which in different taxa are oriented in different planes (8). Native crustacean polymers are usually one of two sizes. In what is designated a top view of hexamers, the smaller of the two, high-resolution images look like hexagons; a side view shows rectangles with two sharp and two fuzzy edges. These two images represent alternative views of a trigonal antiprism structure that has been defined recently by x-ray diffraction (12). Dodecamers, the larger multiples, usually appear as one hexagon joined to one rectangle. Native chelicerate polymers are one of three sizes: (*i*) dodecamers, in this case, two hexagons joined side by side, (*ii*) icosatetramers consisting of two pairs of hexamers divided by a 2-nm cleft, or (*iii*) multiples of 48 monomers appearing either as 25-nm pentagons, as rectangles with a cleft parallel to one side, or as squares with four subsquares. Regardless of the size or image of the native polymer, an underlying similarity is clear in each case examined in depth. A native multiple of 48 subunits can be dissociated to one of 24 that resembles a native icosatetramer, which, at least in some species, can be dissociated to a multiple that resembles a native dodecamer, etc. (8). The dissociation products do not include intermediate multiples—namely, 18 and 36 monomers. Since dodecamers are formed by the presence in a hexamer of a subunit that tends to dimerize (10) one would not expect to find a decaoctamer (18 monomers), but there seems to be no structural constraint on the formation of a triantahexamer (36 monomers) even though none is known.

The underlying structural features of *Scutigera* Hc resemble those of the other arthropod Hcs. The subunit size is typical, as is the size of electron-dense images believed to be hexamers, the fundamental structural and functional units of arthropod Hcs. On the other hand, a more superficial feature such as the higher order structure of *Scutigera* Hc is unique.

A possible arrangement of the putative hexamers is shown in Fig. 6. The native polymer is envisaged with six hexamers arranged in an octahedral array. Two of the three pairs of hexamers are lined up side by side; the third is located in the center and in an axis perpendicular to the other two. When viewed from the "top" or from one side the molecule appears to be composed of only four substructures (Fig. 6*A*). The two pairs of hexamers are not in exactly the same focus because one pair is on top and the other is underneath. When viewed from the other side five of the six hexamers can be seen (Fig. 6*B*). Slight tilt results in skewness and disappearance of the fifth hexamer (Fig. 6*A*). Additional tilting results in the virtual

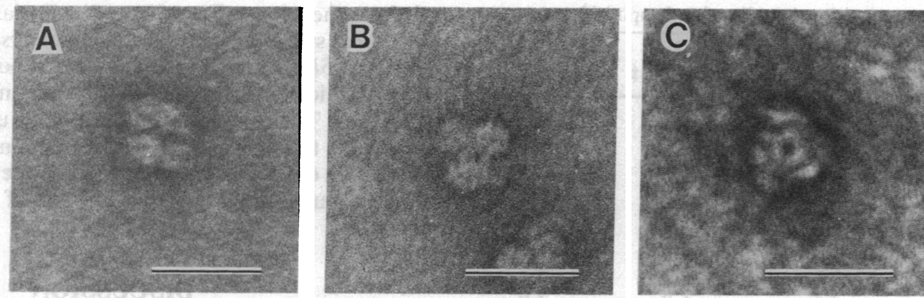

FIG. 5. Electron micrographs of fresh sample of *S. coleoptrata* blood. (*A*) Simple image of four spheres. (*B*) Simple image of five spheres. (*C*) Triangular image. (Scale bar = 30 nm; original magnification = ×490,000.)

disappearance of the fourth substructure, producing the image of an isosceles triangle (Fig. 6*C*). Further details of this image, however, remain somewhat unclear because the central and not the peripheral material has the shape of the substructures observed in other images.

In an alternative arrangement the three pairs of hexamers would be lined up side by side and in the same plane, a somewhat less compact configuration. This arrangement is not preferred because it does not yield the image of five symmetrically placed substructures (Fig. 6*B*) and because it does yield an image of four substructures with all four in the same focus, which was virtually nonexistent in our micrographs.

Phylogeny. For more than two decades Manton (13–15) argued persuasively that the phylum Arthropoda is a polyphyletic assemblage of three or four taxa. She considered the three extant groups to be clearly different in origin and noted that the extinct Trilobita may prove to be so. This conclusion was based on the functional morphology of the limbs, both locomotor and masticator. In her view the Uniramia arose from the Onycophora or a similar stock, which in turn arose from annelid-like lobopods. The ancestry of the Crustacea was regarded as uncertain, but their embryonic development seemed to suggest a distinct origin. The organization and limb mechanisms of the Chelicerata were regarded as fundamentally different from analogous features of both the Crustacea and the Uniramia. The origin and phylogenetic position of the Trilobita were not decided, but she rejected the possibility of their ancestry to the Crustacea and Chelicerata. The di- and probably triphyletic nature of the phylum was strongly supported by Anderson (16), who compared the developmental morphology of the three living groups and concluded that the arthropods lack the common developmental theme that unites other morphologically diverse phyla. These arguments were so convincingly articulated that they have begun to appear in modern textbooks (17).

Recently, however, more conservative views have been revived. Hessler and Newman (18) reconstructed a hypothetical primitive crustacean from living species and concluded that it could have descended from a trilobite, which they also regarded as a viable candidate for ancestor of the chelicerates. They argued that the Arthropoda is at most diphyletic, and their acceptance of the distinctness of the Uniramia and the Trilobitomorpha (trilobites, chelicerates, and crustaceans) was tentative. Most of the contributors to a multiauthor volume on the subject favored the integrity of the phylum as a monophyletic taxon. Strong arguments were advanced by Clarke (4), on the basis of a broad comparison of the internal organ systems, and Boudreaux (19), who attempted to show that some 17 both "derived" (as opposed to primitive) and shared characters are not results of convergent evolution. Boudreaux then used these characters to describe a "ground plan" or the ancestral state from which homologies in modern arthropods arose.

The simplest interpretation of our findings is that arthropod Hc is just such a character. In each of the three living groups the native polymer is built as a multiple of noncovalently linked polypeptide chains of about the same size, each with a single active site. In each group the subunits form hexamers of the same size and general appearance. However, the arrangement of hexamers in larger aggregates is distinctive, the pattern and the number in Uniramia being unique. The status of Hc in the Onycophora is not clearly predicted by this hypothesis because the group is variously regarded as central or peripheral to the mainstream of uniramian evolution. But the hypothesis does suggest that Hcs occurred in the trilobites; the possibility that this important respiratory adaptation was lost from a group that inhabited the aqueous and hence O_2-poor medium is far less probable.

The alternative view, that arthropod Hcs had multiple origins and that their similar substructure is the product of convergent evolution, is a more complex hypothesis though it cannot be rejected on that ground alone. Perhaps more cogent is the difficulty of reconciling it with the respiratory properties of the Hcs. A precedent, a Hc that is more likely to be convergent, actually exists as the molluscan form, which is built along fundamentally different lines. Molluscan

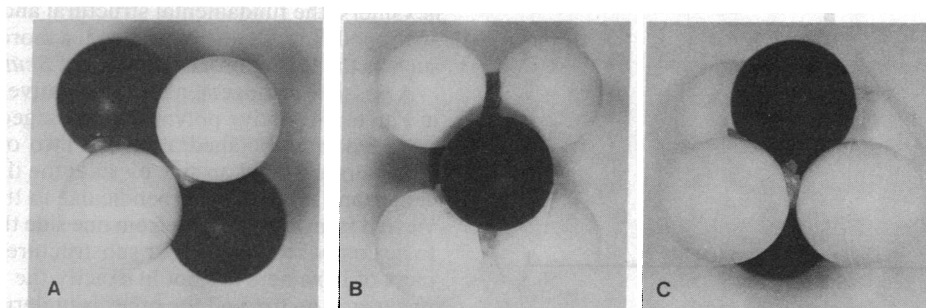

FIG. 6. Possible arrangement of hexamers of *S. coleoptrata* Hc in structures observed in electron micrographs. (*A*) Six spheres arranged and tilted so that only four can be seen. (*B*) Six spheres tilted so that five can be seen. (*C*) Six spheres tilted to produce triangular image with additional material beneath one side.

Hcs are multiples of 450–500 × 10^3-dalton polypeptide chains, each with eight covalently linked O_2 binding domains (10). Their distinctive ratio of binuclear copper sites to protein (1:50 × 10^3 by mass) indicates that the subunits are not merely arthropod monomers linked to one another in a different way. Structurally arthropod and molluscan Hcs have little in common except the active site, and the details of its structure differ (10). If the convergence between the various arthropod Hcs were simply greater than that between the arthropod and the molluscan Hcs, their particular architecture must have resulted from a strong selection pressure, most likely related to respiratory function. And yet none is known. Indeed, the O_2 binding properties of Hcs with very different structures can be more alike than those of similar Hcs. For example, *Limulus* (arthropod) and *Busycon* (mollusc) Hcs both have reversed Bohr shifts, moderate O_2 affinities and cooperativities, and a wide spectrum of inorganic ion sensitivities but no sensitivity to L-lactate (6, 20). In contrast, portunid crab (arthropod) Hcs, which share many aspects of structure with the *Limulus* molecule, have a normal Bohr shift, a lower O_2 affinity and much greater cooperativity, sensitivity to only divalent cations, and sensitivity to L-lactate (21, 22). Thus, the structure of the arthropod Hcs was not evolved repeatedly to preserve a particular set of respiratory properties.

We are tempted to suggest that the two hypotheses can be evaluated further by comparing the primary structures of the arthropod Hc monomers or their transcription–translation system. Their heterogeneity is so great that the comparison of amino acid sequences would be a formidable task indeed. Moreover, large portions of the sequences in a monomeric Hb may resemble those of a species belonging to a distantly related phylum more closely than a member of its own class (23), indicating that a simplistic interpretation of this single structural feature in isolation from other properties can be misleading. The improbability of the relationships thus far inferred from nucleic acid sequences (24) suggests that more extensive refinement of methodology is needed, at least at the level of animal phyla. We suggest that the evolution of the arthropod Hcs is likely to be elucidated further by identifying the structural features that are and are not responsible for a particular set of physiological properties, thus permitting the separation of strongly selected features from less critical ones that have been retained in the course of arthropod radiation.

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