Reference: *Biol. Bull.* **173:** 205–221. (August, 1987)

BIVALVE HEMOCYANIN: STRUCTURAL, FUNCTIONAL, AND PHYLOGENETIC RELATIONSHIPS

C. P. MANGUM[1], K. I. MILLER[2], J. L. SCOTT[1], K. E. VAN HOLDE[2], AND M. P. MORSE[3]

[1]*Department of Biology, College of William and Mary, Williamsburg, Virginia 23185;* [2]*Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331; and* [3]*Marine Science Center and Biology Department, Northeastern University, Nahant, Massachusetts 01980*

Abstract

The hemocyanin-like molecule found in the blood of the most primitive bivalves (protobranchs) reversibly binds O_2. Its respiratory properties and its sedimentation behavior are both distinctive. Although its electron-dense image looks like that of the gastropod hemocyanins, its molecular weight differs from those of all other molluscan Hcs and is more consistent with the concept of bivalve hemocyanin as a pair of octopod hemocyanins. Bivalve hemocyanin occurs in the solemyoids as well as the nuculoids, which argues for the integrity of the Protobranchia as a natural taxon. The ancestral bivalve O_2 carrier was previously believed to be a simple intracellular hemoglobin, which is found in the less primitive Pteriomorpha. The most obvious interpretation of the present results, however, is that hemocyanin is the primitive bivalve O_2 carrier and that it was replaced by the red blood cell, which originated at least twice: once in the pteriomorph bivalves and at least once in other taxa.

Introduction

Recently Morse *et al.* (1986) reported the presence of electron dense images that resemble molluscan hemocyanins (Hcs) in the blood of two nuculoid bivalves. In addition, the denatured subunits exhibited the same anomalous behavior during sodium dodecyl sulfate polyacrylamide electrophoresis as those of some other molluscan Hcs, *viz.*, they migrated to a position corresponding to a lower molecular weight than expected from other aspects of quaternary structure (Van Holde, 1983; Ryan *et al.*, 1985). Finally, copper electrons were identified in the blood of one species by X-ray spectroscopy of sections of the auricle. Thus the evidence indicates the existence of a molecule that closely resembles molluscan Hc in the most primitive members of the class Bivalvia, which was formerly believed to use either heme proteins or no O_2 carrier at all (Mangum, 1980a; Terwilliger and Terwilliger, 1985).

This finding has considerable phylogenetic importance. First, the Hcs found in the various molluscan classes are believed to exhibit differences, albeit quite subtle ones, in quarternary structure (Ryan *et al.*, 1985). Therefore a comparison may elucidate evolutionary relationships between them. Second; it has been suggested that red blood cells (RBCs), which occur in the somewhat less primitive pteriomorphs, may represent the ancestral condition among the bivalves (*e.g.*, Mangum, 1980a). Third, if instead Hc is the primitive O_2 carrier in the class, then the RBC must have originated independently on at least two occasions, within the bivalves and in other taxa.

In the present contribution we report evidence of reversible O_2 binding, which demonstrates that the molecule in the blood of both groups (solemyoid as well as

Received 2 March 1987; accepted 1 May 1987.

nuculoid) of protobranch bivalves is a typical O_2 carrier, not a Hc-like precursor. We also describe additional aspects of molecular structure and respiratory function that enable comparison of the protobranch blood O_2 carrier with the Hcs found in other molluscs. Finally, we explore the implications of our findings for RBC phylogeny.

Materials and Methods

Acila castrensis (Hinds) and *Cyclocardia* (=*Venericardia*) *ventricosa* (Gould) were collected near San Juan Island, Washington. *Yoldia limatula* (Say), *Nucula proxima* Say, and *Solemya velum* Say were purchased from commercial sources in Massachusetts.

O_2 uptake ($\dot{V}O_2$) of intact animals was determined as the depletion of O_2 in the PO_2 range 120–159 mm Hg, measured with a self-stirring polarographic electrode (Yellow Springs Instrument Co. Model 54). To prevent extraneous O_2 uptake by shell epibiota, the animals were disinfected by immersion for a few seconds in benzalkonium chloride (0.13%). Vacant shells given this treatment do not take up appreciable volumes of O_2.

Blood was obtained by first inducing the animals to empty their mantle cavities and then prying open the valves about 1 mm or less with a jeweler's screwdriver. The valves were reflected backwards about 270° and the animals placed in a small funnel draining into an Eppendorf tube. When the blood ceased to drain, additional volumes were obtained by centrifuging the animals at a very low speed. After repeated problems with apparent proteolysis during sedimentation analysis, we collected the blood of *Y. limatula* by draining it directly into a mixture of protease inhibitors, which appeared to enhance the stability of the preparation. About 1 ml blood was drained into a 60 μl solution containing 30 pg leupeptin, 30 pg pepstatin A, and 3 μmoles phenylmethylsulfonyl fluoride.

The gills of *Y. limatula* were dissected and extracted with 0.5% Na_2CO_3. The extract was diluted by 10% with pyridine, reduced with a few grains of sodium dithionite, and, due to its very small volume, examined with Zeiss micro- and Hartridge reversion spectroscopes (Mangum and Dales, 1965).

The bloods were centrifuged and immediately prepared for electron microscopy (Zeiss EM-109) by negative staining with uranyl acetate (Mangum *et al.*, 1985; Morse *et al.*, 1986). In the present investigation the blood was diluted with 0.05 *M* Tris maleate buffer + 10 m*M* $CaCl_2$ (pH 7.63) by factors ranging from 1:9 to only 1:50, because its appearance suggested a low Hc concentration. The sample and the stain were applied to the grid with an atomizer.

O_2 binding was determined on fresh (never frozen) blood samples from *A. castrensis, S. velum,* and *Y. limatula* by the cell respiration method (Mangum and Lykkeboe, 1979). Due to the size of the *N. proxima* individuals (2–5 mm), it was necessary to stockpile frozen material until the requisite volume (300 μl) accumulated. About half of the material on which the measurements were performed had been frozen. The samples were diluted by 10% with Tris maleate (final concentration 0.05 *M*) buffered seawater (32‰) containing commercial yeast cells. An attempt to first concentrate *A. castrensis* blood by membrane filtration was unsuccessful due to precipitation of some of the material, which was also noted during the O_2 binding measurements. No precipitation of the *Y. limatula, S. velum,* or *N. proxima* material was observed.

The O_2 affinities of heme proteins are often concentration dependent. Because most experimental procedures require dilute solutions, the results do not accurately reflect *in vivo* values. To obtain physiologically meaningful data for the branchial

heme protein of *Y. limatula,* O_2 binding in the present investigation was also determined using whole gills, dissected intact. An absorption spectrum of the bathing medium indicated that there was no loss from the gills during the measurement. It was necessary to modify the cell respiration procedure because the method requires that the rate of free O_2 depletion be linear. This is achieved by lowering the PO_2 with particles such as isolated mitochondria or yeast cells which are so small that the diffusion distance is not limiting. If the O_2 uptake of whole unperfused gills had been allowed to make an appreciable contribution to total O_2 uptake, this condition would clearly have been violated and the apparent O_2 affinity would have been erroneously low due to an extraneous departure from linearity. The problem was circumvented by first determining the $\dot{V}O_2$ of the gills and then adding large numbers of yeast cells so that yeast $\dot{V}O_2$ was more than 10 times gill $\dot{V}O_2$. The cell respiration method also requires that the rate of O_2 depletion be slow enough to permit equilibration of the electrode at any PO_2. If this condition had been violated, the result would have been an erroneously high O_2 affinity, because an apparent oxygenation state would have coincided with a PO_2 that actually had obtained earlier, at a higher oxygenation state. The possibility was eliminated by ascertaining equilibration under the following experimental conditions: using the Hc of the crab *Cancer magister,* whose respiratory properties are well known (*e.g.,* Graham *et al.,* 1983), the rate of O_2 depletion was increased until an erroneously high O_2 affinity was obtained. The period for depletion of free O_2 from 100 to 0% air saturation was considerably less than 25 s. In the measurements on gills, much longer periods (87–233 s) were employed.

Absorption spectra of the medium and of fresh Hcs were determined with Beckman DK-2A and Varian 2200 spectrophotometers. To eliminate light scatter, the Hc samples were first diluted with dissociating buffer (0.05 *M* Tris HCl, pH 8.95 + 0.05 *M* EDTA) by 50 to 97% depending on color intensity.

All sedimentation experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with scanner optics. Wavelengths in the vicinity of the Hc bands at 280 and 345 nm were used. Temperature was controlled to <0.1°C. Sedimentation coefficients were measured from the midpoints of the well-defined boundaries and corrected to $S_{20,w}$ in the usual way. The sedimentation equilibrium experiment was conducted at 1500 rpm, using the heavy J rotor. Equilibrium was attained when no difference could be noted between scans approximately 6 h apart. After equilibrium the rotor was accelerated to 6000 rpm and a baseline recorded approximately 4 h later.

Results

O_2 uptake

Intact individuals of *Acila castrensis* take up O_2 measurably, but $\dot{V}O_2$ is more than two orders of magnitude lower than in the heterodont *Cyclocardia ventricosa* (Table I), which was collected from the same bottom on the same occasion and held in the laboratory in the same container for the same period. The difference in body size can account for only a small fraction of the difference in O_2 uptake. Moreover, $\dot{V}O_2$ is also orders of magnitude lower in *A. castrensis* than in the pterimorph *Noetia ponderosa,* a much larger animal measured at a slightly lower temperature (Table I).

Absorption

The dissociated subunits of the four bivalve Hcs absorb at 280 and 345 nm (Fig. 2). Other molluscan Hcs absorb in the same regions (Nickerson and Van Holde,

TABLE I

Oxygen uptake in bivalves with specialized oxygen carriers

Species	O_2 carrier	$\dot{V}O_2$ (μl/g dry wt-h)	Dry wt. (mg)	Temp. (°C)	Source
Acila castrensis	Hc	2.0 ± 0.9 (6)	107.9–122.8	11.5	present data
Venericardia ventricosa	extracellular Hb	269.5 ± 15.5 (6)	33.5–34.9	11.5	present data
Noetia ponderosa	intracellular Hb	148	5×10^3	10	Deaton and Mangum, 1976
Glycimeris nummaria	intracellular Hb	*ca.* 49.5[a]	2.7×10^3	+16–20	Kruger, 1957

[a] Converted from original data assuming that dry wt. = 20% wet wt. Mean ± SE (n).

1971). R. C. Terwilliger kindly communicated data for polyplacophoran Hc, which have not been reported in the literature. Observations on the Hc of the chiton *Chaetopleura apliculata* were also made together with those reported here. The peak at 345 nm disappears in the presence of sodium borohydride (*e.g.*, Fig. 1).

Extracts of the gill of *Y. limatula* clearly form a pyridine hemochromagen with absorption bands at 542 and 556 nm. High concentrations of red granules also were observed in the nerve ganglia and connectives of this species but not in *A. castrensis* or *N. proxima*, which appeared to lack branchioglobin (Bb) as well. However, a seawater extract of the whole bodies of *N. proxima* appeared to form a pyridine hemochroma-

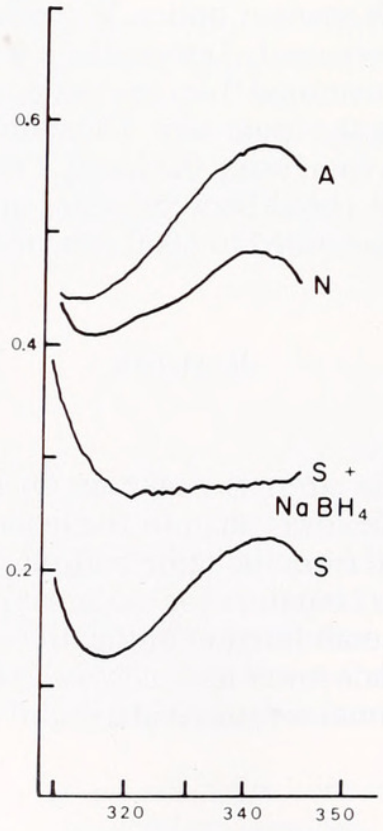

FIGURE 1. Absorption spectra of protobranch Hcs. (A) *Acila castrensis*, (N) *Nucula proxima*, and (S) *Solemya velum*. Abscissa is wavelength in nm and ordinate is absorbance.

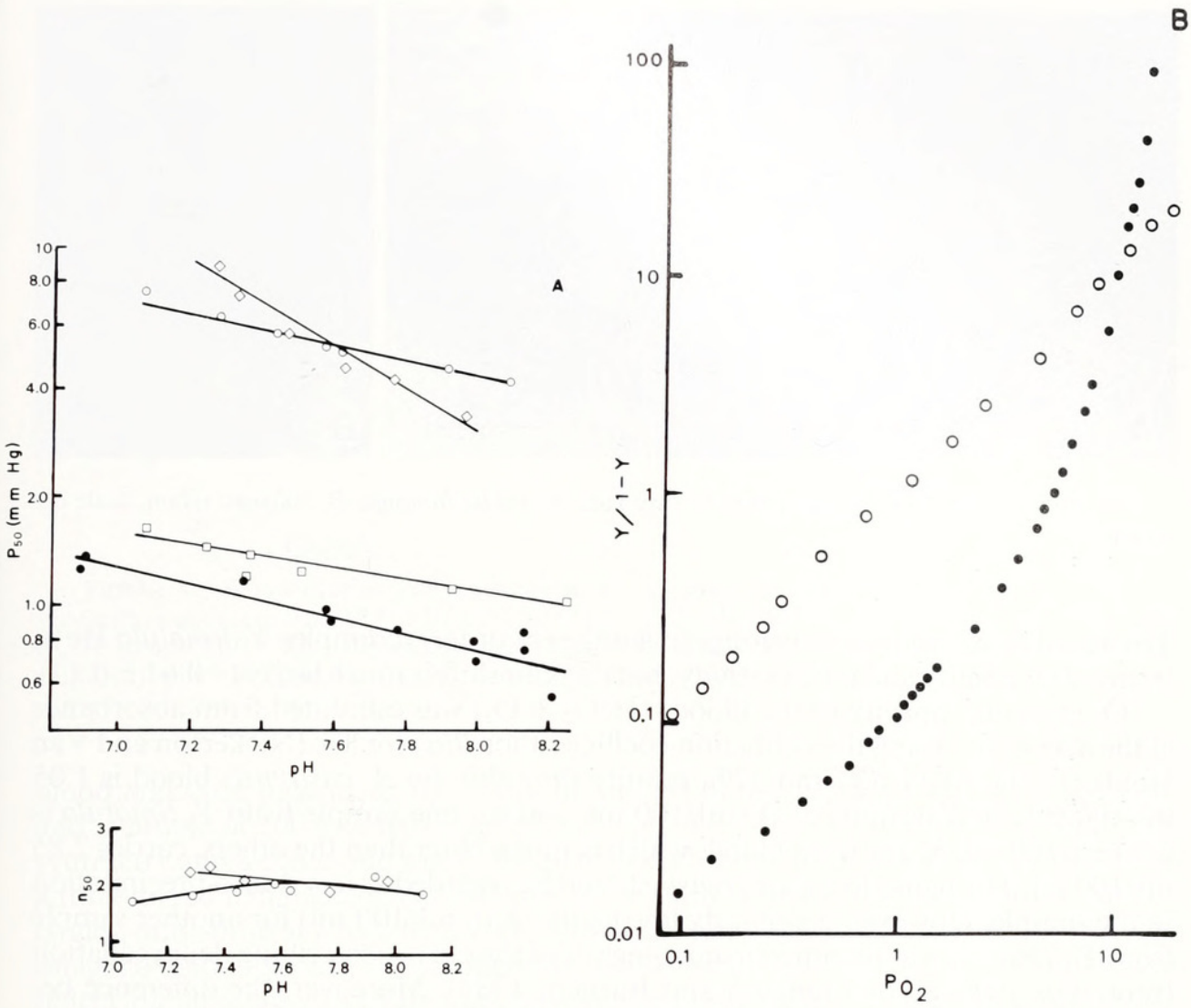

FIGURE 2. A. PH dependence of O_2 binding by *Acila castrensis* (●), *Nucula proxima* (□), *Solemya velum* (◇), and *Yoldia limatula* (○) Hcs. 20°C, 0.05 *M* Tris maleate buffered blood. B. Hill plot of O_2 equilibrium of *Yoldia limulata* (●, pH 8.10.) and *Acila castrensis* (○, pH 7.78) Hcs.

gen, although the visual observation could not be confirmed even by microspectroscopic observation due to the very small volume (*ca.*, 10 μl) obtained.

O_2 binding of the bloods

Unlike other Hcs, *A. castrensis* Hc binds O_2 non-cooperatively (*e.g.*, Fig. 2). The Hill coefficient (n_{50}), which is independent of pH, is 1.03 (±0.03 SE, n = 11). Among the Hcs, *A. castrensis* also has an unprecedentedly high O_2 affinity (Fig. 2), thus resembling tissue O_2 carriers more than most blood O_2 carriers. Unlike tissue O_2 carriers, however, *A. castrensis* Hc has a small but significant normal Bohr shift. The slope of the regression line describing the data in Figure 2, or Δ log P_{50}/Δ pH, is −0.23 (±0.08 95% C.I.). HcO_2 binding in *N. proxima* (which belongs to the same family as *A. castrensis*) is also non-cooperative (0.94 ± 0.09 SE; n = 7) and it has a similar Bohr shift (−0.16 ± 0.07 95% C.I.) although its O_2 affinity is somewhat lower. The Hc of *Y. limatula* (which belongs to a different family) has a much lower O_2 affinity, though still fairly high for a molluscan Hc, and it is moderately cooperative (Fig. 2). Its Bohr shift is indistinguishable from that of the other nuculoid Hcs (−0.24 ± 0.05).

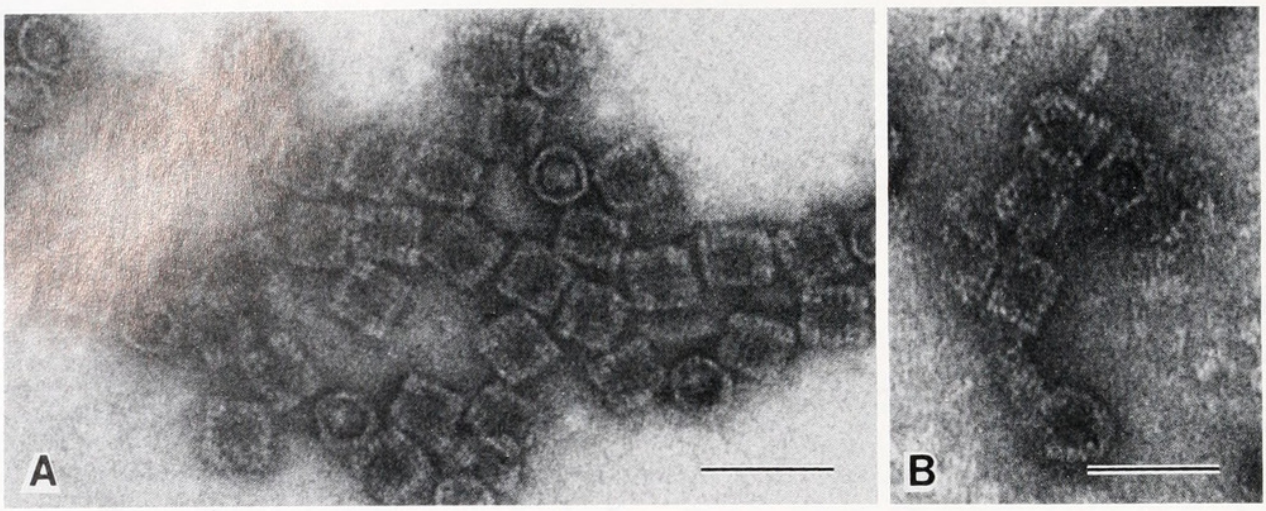

FIGURE 3. Electron micrographs of bivalve Hcs. A. *Yoldia limatula.* B. *Solemya velum.* Scale bar 50 nm.

The Hc of *S. velum* (which belongs to a different order) resembles *Y. limatula* Hc in terms of O_2 affinity and cooperativity, but its Bohr shift is much larger (-0.61 ± 0.17).

O_2 carrying capacity of the bloods ($HcO_2 + O_2$) was estimated from absorbance at the active site, using the extinction coefficient for *Busycon* Hc (Nickerson and Van Holde, 1971). At 11.5°C and 32‰ salinity the value for *A. castrensis* blood is 1.05 ml/100 ml, for *S. velum* is 1.00 ml/100 ml, and for one sample from *Y. limatula* is 0.96 ml/100 ml. *N. proxima* blood, which is much bluer than the others, carries 2.85 ml/100 ml. The figure for *A. castrensis* should be regarded as low due to precipitation in the sample. However, a similarly low figure (0.76 ml/100 ml) for another sample from *Y. limatula* was obtained from integrals of the curves describing deoxygenation (procedure detailed by Mangum and Burnett, 1986). Moreover, the difference between *N. proxima, S. velum,* and *Y. limatula* cannot be due to starvation of the latter two in the laboratory (which, in fact, has not been reported for molluscan Hcs) since they were held for the same period (<2 days).

O_2 binding by gills

Two determinations of O_2 binding by intact *Y. limatula* gills, which should provide physiologically meaningful information, gave P_{50} values of 0.43 and 0.46 mm Hg and n values of 0.98 and 1.02 (20.3°C, ambient pH 8.01).

Electron microscopy

Since *N. proxima* is so closely related to *A. castrensis,* the small amount of material available was used for other purposes. The shapes of *S. velum* and *Y. limatula* Hcs (Fig. 3) are indistinguishable from that of *A. castrensis* Hc, which was described earlier (Morse *et al.,* 1986). All three molecules are six-tiered cylinders and, like many gastropod Hcs, appear as circles in top view and as squares in side view (see Ghiretti-Magaldi *et al.,* 1979, van Bruggen *et al.,* 1981). They lack the "belt," or unequal spacing of the six tiers, found in one species (van der Laan *et al.,* 1981). The width (31 nm) of the *Y. limatula* squares appears to be slightly but significantly ($P < .001$ according to Student's t test) smaller than that of 12 tiered cylinders found in the blood of the gastropod *Busycon canlicutatum.* These dimensions were determined by mixing a small volume of *B. canaliculatum* blood with a large volume of *Y. limatula*

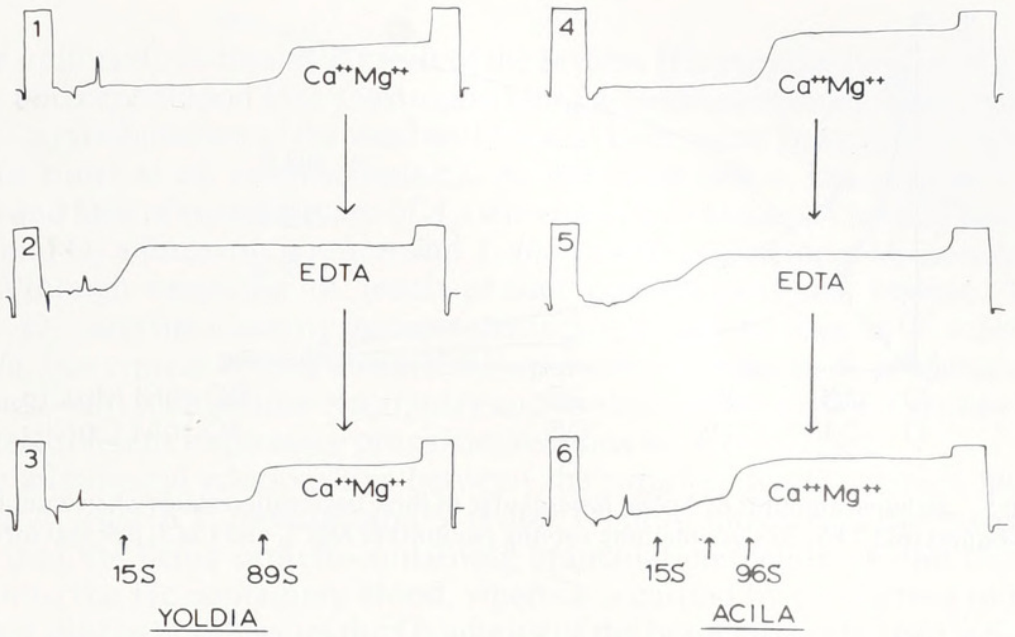

FIGURE 4. Scanner traces showing dissociation and reassociation of bivalve Hcs at pH 7.65, 20°C. *Yoldia limatula:* (1) in 0.05 *M* Tris-HCl, 50 m*M* $MgCl_2$, 10 m*M* $CaCl_2$; (2) dialyzed against 0.05 *M* Tris-HCl + 10 m*M* EDTA; (3) dialyzed back again against the original buffer. *Acila castrensis:* (4) as in 1; (5) as in 2; (6) as in 3. In 5 and 6 the middle boundary sediments at about 55S.

blood and then measuring the width of all (24) of the 12-tiered cylinders observed and a sample of 100 6-tiered cylinders. The bivalve circles have a five-fold rotational symmetry and a collar and a cap. When dissociated to halves, the molecule looks like a three-tiered rectangle, which absorbs more stain at one end than the other, and as circles, only some of which have collars and caps (see Fig. 3 in Morse *et al.*, 1986). The images of half molecules, which have also been described for *Helix* Hc, are believed to reflect the absence of collars and caps at the broken surfaces (van Bruggen *et al.*, 1981). Like gastropod Hcs, the bivalve squares are about 35 nm long.

Physical characterization

Sedimentation velocity experiments with *A. castrensis* and *Y. limatula* Hcs were performed at room temperature and under a variety of solvent conditions. The results are summarized as follows:

(1) In 0.05 *M* Tris-HCl buffer (pH 7.65) containing 50 m*M* $CaCl_2$ and 10 m*M* $MgCl_2$, both Hcs exhibited single, sharp boundaries. The sedimentation coefficients ($S^\circ_{20,w}$), when corrected to standard conditions and extrapolated to zero Hc concentration, were 95.8 for *A. castrensis* Hc and 88.8 for *Y. limatula* Hc.

(2) When the two Hc solutions were dialyzed exhaustively against 10 m*M* EDTA in 0.05 *M* Tris-HCl (pH 7.65), they behaved differently (Fig. 4). *Y. limatula* Hc dissociated completely to yield a single boundary with $S_{20,w}$ = 15.9S. Under the same conditions *A. castrensis* Hc showed incomplete dissociation, yielding two components with $S_{20,w}$ = 54 and 18S. Attempts at reassociation also gave quite different results. Upon dialysis of the Tris EDTA treated material back to Tris Ca^{+2} + Mg^{+2}, *Y. limatula* Hc quantitatively reassociated to the 89S component. Under the same conditions only partial reassociation could be attained with *A. castrensis* Hc.

(3) When *Y. limatula* Hc was dialyzed against a series of dilutions of the Tris buffer in which the divalent cations were reduced to $^1/_{10}$, $^1/_{20}$, $^1/_{40}$, and finally $^1/_{100}$ of

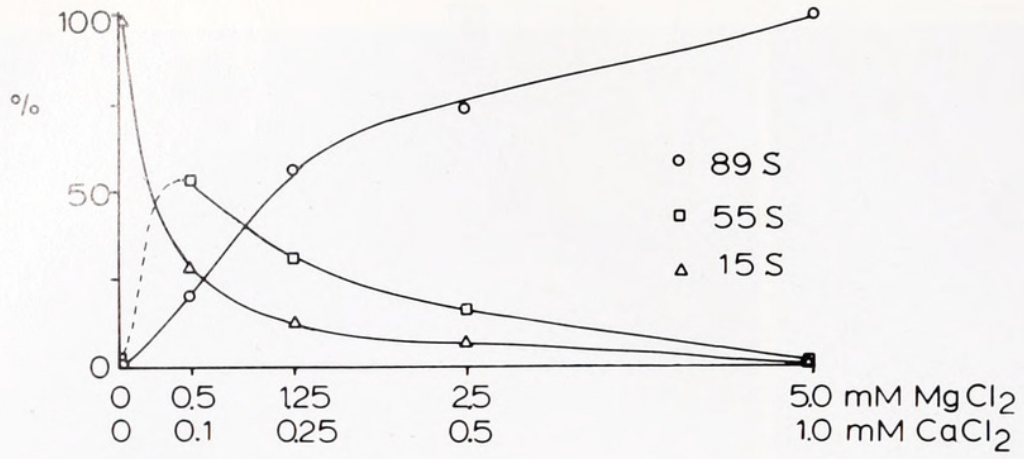

FIGURE 5. Relative amounts of *Yoldia limatula* Hc of three aggregation states when equilibrated to 0.05 *M* Tris buffers (pH 7.65; 20°C) containing varying amounts of $MgCl_2$ and $CaCl_2$ (see text for details).

their original concentrations, the resultant dissociation yielded a mixture of three components: the 89S Hc (the whole molecule), another with a sedimentation coefficient of 55S (probably a half molecule), and a third with a sedimentation coefficient of about 15S (Fig. 5). The present data do not indicate just what multiple of the polypeptide chain this smallest product represents, but the sedimentation coefficient corresponds to that of the dissociation product in the presence of EDTA.

The reversible dissociation behavior of *Y. limatula* Hc strongly suggests that, like *Octopus* Hc (Van Holde and Miller, 1985), it is composed of a single type of subunit. In contrast, the incomplete reassociation of *A. castrensis* Hc is more like that of other molluscan Hcs (Van Holde and Miller, 1982). Furthermore *Y. limatula* Hc, like *Octopus* Hc, dissociates in the presence of EDTA at a much lower pH than normally required for other molluscan Hcs. Although divalent cation levels must be reduced to extremely low levels before dissociation begins, *Y. limatula* Hc dissociates at pH 7.65, which is probably close to the physiological value. In all likelihood, at higher pH it would dissociate at higher divalent cation levels.

DISCUSSION

Respiratory properties and their relationships to protobranch biology

Allen (1978) suggested that protobranch bivalves are able to exist with their small and, in his view, relatively inefficient feeding organs because they have low metabolic rates. The present findings support his suggestion, at least with respect to aerobic metabolism. However, we should point out that, relative to bivalves that both use the gill as a filter-feeding organ and lack an O_2 carrier, the branchial surface area is also small in the pteriomorph *Noetia ponderosa,* whose feeding has not been investigated and whose $\dot{V}O_2$ is not especially low (Mangum, 1980a). Almost certainly $\dot{V}O_2$ is influenced by other factors in addition to feeding efficiency. While it is believed that conventional feeding in *Solemya* is supplemented or perhaps even supplanted by a symbiotic relationship with chemoautotrophic bacteria (Cavanaugh, 1980, 1983; Felbeck, 1983; Doeller, 1984; Fisher and Childress, 1984; Reid and Brand, 1986), no sign of bacteria can be found in electron micrographs of the gills of protobranchs such as *A. castrensis* (mentioned by Reid and Brand, 1986) and *Y. limatula* (M. P. Morse, unpub. obs.).

The uniformly normal Bohr shift of the bivalve Hcs resembles those of polyplacophoran and cephalopod Hcs. Gastropod Hcs have either reversed Bohr shifts (prosobranch), a combination of reversed and normal Bohr shifts (prosobranch and pulmonate), or none at all (opisthobranch). As indicated above, the extremely high O_2 affinity and lack of cooperativity of *A. castrensis* Hc is unique. The moderate cooperativity and O_2 affinity of *S. velum* and *Y. limatula* Hcs are common among the molluscs, although examples of much greater cooperativity are known (Mangum, 1980b). O_2 carrying capacity appears distinctively low, at least in *S. velum* and *Y. limatula,* but typical of molluscan HcO_2 transport systems in *N. proxima.* Why the nuculoids, with such similar respiratory and cardiovascular systems, should have Hcs with such different respiratory properties remains to be elucidated.

The anatomical relationships between the protobranch O_2 carriers also are intriguing. At least in *Y. limatula* and *S. velum,* O_2 must move from the environmental source into the heme protein-containing branchial epithelium. From there the O_2 moves into the Hc-containing blood, where it is carried by convection to the metabolic sink. But in both species the O_2 affinity of the branchioglobin (Bb) is higher than that of the Hc (see Doeller *et al.,* 1983, for values for *S. velum* Bb). The arrangement violates the fundamental design principle of an O_2 transfer system, which mandates the highest O_2 affinity in the compartment most remote from the environmental source. Bb must actually be a barrier to O_2 influx as long as it is not fully oxygenated.

The physiological question is complicated by uncertainty surrounding the function of bivalve Bb and other tissue heme proteins. Doeller (1984) suggested that *S. velum* Bb transports sulfide to the chemoautotrophic bacteria in the gills; the sulfide-oxidizing bacteria are believed to serve as key components of a newly discovered mode of animal nutrition. As pointed out by Dando *et al.* (1985), this function does not preclude the possibility of others, such as facilitated diffusion or O_2 storage.

We noted that the period from onset to completion of nonlinearity of O_2 uptake by *Y. limatula* gills (in the absence of yeast cells) was only 162 s. This period includes both the diffusion-limited and BbO_2-supplied components. The molecule cannot be an O_2 store of significant longevity. We suggest that BbO_2 carrying capacity also be considered in the continuing debate on functions of tissue heme proteins.

Structural properties

The sedimentation coefficients observed for the bivalve Hcs are surprisingly low. The Hcs that would seem to resemble bivalve Hc in shape are the six-tiered cylinders found in the prosobranch whelk *Kelletia* and the pulmonate snail *Helix* (van Bruggen *et al.,* 1981). Opisthobranch and the other prosobranch Hcs studied tend to form larger aggregates or have special features such as unequal spacing of the six tiers. Cephalopod and polyplacophoran Hcs are three-tiered cylinders.

As Table II shows, however, almost all reliable measurements of the sedimentation coefficients of the gastropod six-tiered multiple yield values of $S^\circ_{20,w}$ between 100 and 105S. We were struck by the value for *Y. limatula* Hc, which is 10–15% lower. A lower value might be explained by either a looser quaternary structure, greater hydration, or a lower molecular weight. The molecular weight obtained from Figure 6 depends on the value assumed for the partial specific volume (ν) (see Van Holde, 1985). Unfortunately we have neither an experimentally determined value for ν for *Y. limatula* Hc nor an amino acid composition, from which it might be estimated. Values reported for molluscan Hcs range from about 0.73 (gastropod) to 0.74 (cephalopod). The former yields a molecular weight of 6.5×10^6 and the latter yields 6.8×10^6. Either is much lower than the values reported for the gastropod 100–105 S Hcs (Table II).

TABLE II

Comparative properties of native hemocyanin molecules of bivalves and gastropods

Species	$S^{\circ}_{20,w}$ (svedbergs)	M (g/mol × 10^6)	$\bar{v}$ (cm^3/g)
Class: Gastropoda[1]			
Archachatina marginata	102.3	9.1	—
Buccinum undatum	101.1	9.0	—
Busycon canaliculatum	103.2	8.8	.727
Helix pomatia (α)	104.3	8.7	.727
Helix pomatia (β)	105.8	9.0	—
Murex trunculus	102.7	8.9	—
Paludina vivipara	102.5	8.7	—
Pila leopoldvillensis	101.2	8.7	—
Class: Bivalvia[2]			
Acila castrensis	95.8	—	—
Yoldia limatula	88.8	6.5–6.8	—

[1] Data from Van Holde and Miller (1982). Original references are given therein.

[2] Present data. The value of M for *Yoldia* hemocyanin depends upon whether a value of 0.73 (as for gastropods) or 0.74 (as for cephalopods) is assumed.

The data are more consistent with the concept of bivalve Hc as a pair of cephalopod Hcs. A pair of polyplacophoran Hcs would have a much higher molecular weight (Ryan *et al.*, 1985; Herskovits *et al.*, 1986). The cephalopod 51–60S particles, however, do not pair to form six-tiered cylinders. If they did, they might give rise to a particle with a sedimentation coefficient of about 90S and an electron-dense image much like that of *Y. limatula* Hc. If we assume that native *Y. limatula* Hc is a 20-mer of polypeptide chains, like other 6-tiered molluscan Hcs, then the chain weight must be approximately 3.4×10^5. This is very close to the weight of octopod chains

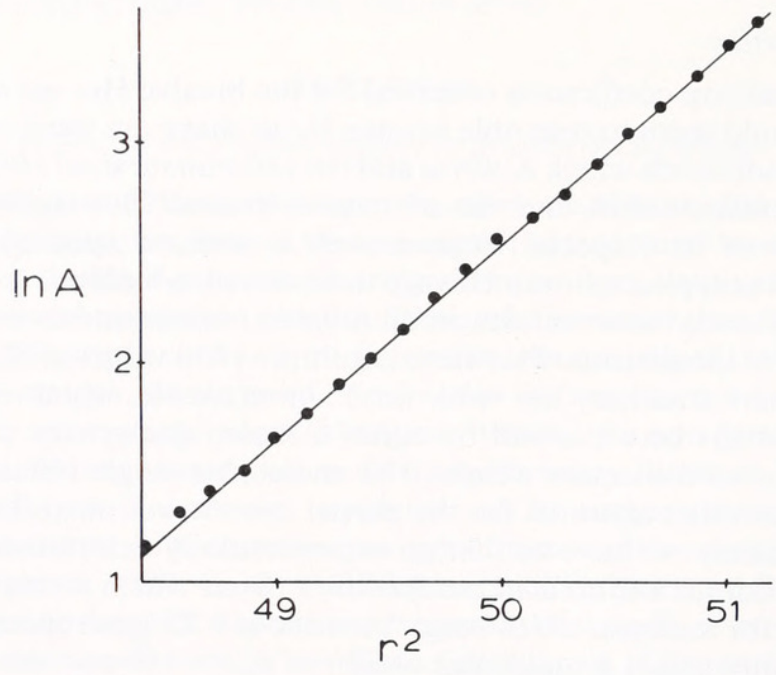

FIGURE 6. Determination of molecular weight of *Yoldia limatula* Hc by sedimentation equilibrium. A represents concentration in arbitrary units of A_{345} nm; r is the distance from center of rotation.

(Gielens *et al.*, 1986; Lamy *et al.*, 1986), and substantially smaller than that of gastropod chains ($4.0–4.5 \times 10^5$). Such a conclusion seemingly contradicts the observation that bivalve chains (Morse *et al.*, 1986) run more slowly than cephalopod chains (Van Holde and Miller, 1982) on SDS gels. However, SDS gel electrophoresis is notoriously unreliable for glycoproteins such as Hcs. It has been frequently reported that estimates of subunit molecular weight are in substantial error for these proteins.

On the basis of the available information we suggest that the bivalve Hcs may resemble octopod Hcs in having a small (relative to other molluscan Hcs) subunit, but share with gastropod Hcs the capacity to associate to 20-mers. This conclusion is supported by the apparently smaller width of the bivalve cylinders. In possible contradiction, however, we should mention that Ellerton and Lankovsky (1983) reported a 26–30 nm wide and 28–34 nm long Hc in the primitive archaeogastropod *Haliotis iris*.

Phylogeny of the molluscan Hcs

The most recent discussions of molluscan phylogeny suggest two major phyletic lines leading from the postulated ancestor, which is in turn descended from an acoelomate animal at the turbellarian-nemertine level of organization (Runnegar and Pojeta, 1986; Salvini-Plawen, 1986). One of these phyletic lines is an aplacophoran-polyplacophoran lineage and the other leads through the monoplacophorans to the gastropods, the rostroconch-bivalve-scaphopods, and the cephalopods (Fig. 7). In view of the equally recent conclusion that the nemertines are, in fact, descendants of an annelid-like coelomate (and, we suggest, RBC-containing) animal (Turbeville and Ruppert, 1986), the condition of the coelom in the ancestral mollusc probably should be reconsidered. Regardless, the present findings emphasize the importance of ascertaining the properties of O_2 carriers (if any) in the poorly known molluscan classes such as the monoplacophorans and the scaphopods. We also look forward to the results of the sedimentation equilibrium studies of archaeogastropod Hc which were underway at the time of Ellerton and Lankovsky's 1983 report. The elucidation of the structures of the Hcs (if any) in these three groups and also additional members of other molluscan groups may have important implications for molluscan phylogeny. In our view, the detail available at present does not permit a very confident conclusion concerning the evolutionary relationships of the molluscan Hcs.

Origins of the red blood cell and its simple hemoglobins

If the status of postulated transitional group is disregarded, there is some consensus among molluscan systematists concerning the ancestry of the bivalves. Along with the Gastropoda, Scaphopoda, and Cephalopoda, the class Bivalvia is believed to be descended from the Monoplacophora (Cox *et al.*, 1969; Newell, 1969; Stasek, 1972; Pojeta, 1978; Runnegar, 1978; Runnegar and Pojeta, 1986; Salvini-Plawen, 1986). There is considerable disagreement, however, on the relationships of the different groups of bivalves. Newell (1969) described six subclasses and assigned the solemyoids and the nuculoids to separate ones. However, Allen (1986) argued for the integrity of the Protobranchia as a subclass that includes all bivalves with simple, pectinate gills and described only one other subclass, the Lamellibranchia. Our findings strongly support Allen's (1986) view. While we cannot provide evidence for the absence of Hc in all lamellibranchs, we can provide evidence of the absence of any O_2 carrier in the blood of one pteriomorph (*Modiolus demissus*) and two heterodonts (*Crassostrea virginica* and *Rangia cuneata*): When measured with a Lexington In-

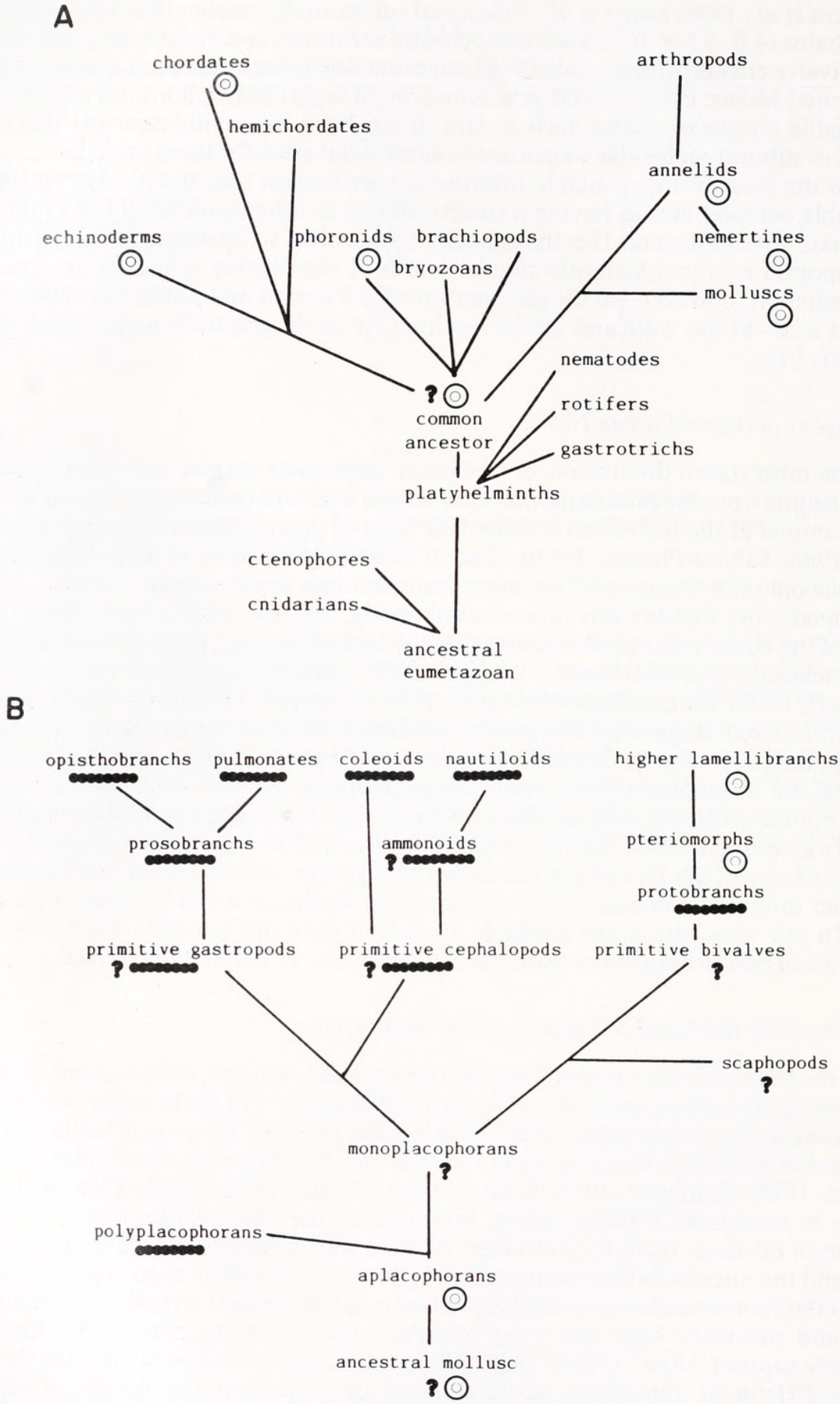

FIGURE 7. Phylogeny of: A. The red blood cell; B. The molluscan O_2 carriers. (◎) symbolizes RBCs and (●●●●) symbolizes molluscan Hc. Question mark indicates uncertainty.

struments Co. analyzer, the total O_2 contents of these bloods did not differ from that of the seawater to which the animals were acclimated. Moreover, these bloods also lacked absorption maxima in the region of 345 nm, as did plasma of the RBC-containing pteriomorph *Noetia ponderosa* (C. P. Mangum, unpub. obs).

The higher bivalve taxa, including the Heterodonta (which contains most of the familiar species), are regarded as suspect (Newell, 1969). But the Pteriomorpha, a relatively primitive group consisting of the anisomyarians, the extinct cyrtodonts, and the RBC-containing arcoids, appears to be a natural taxon. In addition, there is general agreement that the protobranch bivalves are even more primitive than the pteriomorphs (Newell, 1969; Allen, 1978, 1986). This relationship has several implications for the question of the origin and further evolution of O_2 transport systems, because it is among the pteriomorphs that one finds RBCs resembling counterparts and containing Hbs similar to those in other phyla at comparable levels of organization. The subject is of such importance that it is discussed in detail below.

Nucleated RBCs containing either monomeric or oligomeric Hbs are found in seven animal phyla (summarized by Mangum and Mauro, 1985), including five (Phoronida, Annelida, Echiura, Nemertina, and Mollusca) that are often regarded as not too distantly related to one another and at an intermediate stage of phylogenetic development (Fig. 7A). While the limited anatomical information indicates the possibility of some distinctly different features of the RBCs in each group, it also indicates many similarities. The physiological information, also limited, indicates a similar metabolic organization of at least annelid and molluscan RBCs, which differs from that of avian and mammalian RBCs and, possibly, the sipunculid pink blood cell (Mauro and Isaacks, 1984; Mangum and Mauro, 1985).

Within the molluscs, RBCs occur widely in one order of pteriomorph bivalves (as well as in a single species of heterodonts; Terwilliger *et al.*, 1983), and they almost certainly occur in the Aplacophora, which was regarded by Hyman (1967; p. 69) as the "genuinely primitive" molluscan class (Fig. 7B). Hyman (1967; p. 65) noted that "the coelomic fluid has a reddish hue invested in the corpuscles, except in the Chaetodermidae, where the red substance, not proved to be hemoglobin, is dissolved in the fluid itself." Despite the caveat, it is highly likely that this pattern reflects yet another instance of O_2 transport by RBCs (see also Baba, 1940) as the primitive condition and of multidomain, extracellular Hb as the derived condition. Well known examples include the annelids as well as the lamellibranch bivalves. RBCs containing simple heme proteins are found in more primitive species and extracellular Hbs that differ fundamentally from one another as well as from the simple heme proteins occur in more advanced taxa. One pteriomorph bivalve, believed to represent the transitional stage, has both kinds of Hbs in its RBCs (Grinich and Terwilliger, 1980).

There is a strong possibility that the nemertines exhibit the same trend. Hyman (1951; p. 490) believed that the red color of the blood "resides in the corpuscles," which is true of a few marine species (Vernet, 1979). In support of this contention Hyman (1951) cited the 1872 report by Lankester, whose words indicate otherwise: "the colour is due to Haemoglobin *diffused* in the liquid" (p. 73, italics ours). Poluhowich (1970; also pers. comm.), who reported Hb in freshwater (and therefore not primitive) nemertines, did not detect RBCs. Outside of the vertebrates, RBCs are unknown in freshwater animals, which is believed to be due to their osmotic fragility (Mangum, 1980a).

On the basis of the distribution of the RBC summarized above and illustrated in Figure 7, there is no compelling reason to postulate more than one origin of the RBC and its simple hemoglobins. One need only to suppose that the RBC originated

shortly after circulating body fluids arose (Fig. 7). In more advanced groups it was repeatedly replaced by extracellular O_2 carriers due to the greater viscosity of the large bore tubes that dominate primitive cardiovascular systems (Mangum, 1976), and it was inherited by two more advanced deuterostome groups: the echinoderms and the chordates.

The most obvious interpretation of the existence of Hc in the blood of protobranch bivalves is that the hypothesis of a common origin of the RBC is incorrect. This interpretation has the following implications: Protobranchs represent the ancestral bivalve condition and their HcO_2 transport system was either lost (anisomyarians) or replaced (arcoids) in the pteriomorphs by an intracellular HbO_2 transport system of *de novo* origin. This interpretation is consistent with the presence of neuro- and branchioglobin in the same individuals that contain Hc. It is also consistent with the recent finding that the tertiary structures of the simple Hbs found in the annelids, bivalves, and primitive vertebrates are similar to one another and to mammalian myoglobin, and different from that of higher vertebrates Hbs (Perutz, 1985; Royer *et al.*, 1985; W. E. Love, pers. comm.). According to this multiple origin hypothesis, structural similarity of the simple Hbs of the lower animals is due to two separate origins from their tissue heme proteins, which also have the same tertiary structure (presently unknown, but probable), not a common origin.

The weaknesses of this interpretation include uncertainty about the integrity of the class Bivalvia (McAlester, 1966; Cox *et. al.*, 1969; Newell, 1969; Runnegar, 1978) and the absence of a clear selection pressure for the replacement of Hc with RBCs. The simple heme proteins found in pteriomorph RBCs would seem to offer no clear advantages over protobranch Hcs. Indeed, their respiratory properties are not nearly as plastic, at least under physiological conditions (Mangum, 1980a), and therefore the selection pressure would seem to be negative.

Two alternative interpretations seem so unlikely that they can be dismissed with some confidence. (1) The hypothesis that the RBC and its simple Hbs had a common origin and that protobranch RBCs were lost in favor of a HcO_2 transport system after the divergence of the protobranchs and the pteriomorphs entails at least two independent origins of molluscan Hc culminating in similar quaternary structures with very different functional properties. As pointed out in detail earlier (Mangum *et al.*, 1985), this absence of correlation between known aspects of quaternary structure and respiratory function fails to provide a selection pressure for convergent evolution of those aspects of structure and it implies that they are conservative, ancestral characters. The point is strengthened by the present finding of a similar quaternary structure of bivalve and other molluscan Hcs, with a strikingly different combination of respiratory properties. (2) The notion that the RBC had a common origin and that the pteriomorphs, not the protobranchs, are the more primitive bivalves can also be dismissed. Abundant morphological evidence indicates otherwise.

A third alternative, the hypothesis that the RBC had a common origin and that the Protobranchia and the Pteriomorpha did not have a common ancestor, is somewhat more difficult to reject. While the possibility of a di- and even polyphyletic origin of the bivalves is frequently mentioned (*e.g.*, Cox *et al.*, 1969; Newell, 1969; Runnegar, 1978), the position has been advocated positively and forcefully only with respect to a separate origin of the Lucinacea (McAlester, 1966), not separate origins of the Protobranchia and at least one other lineage containing the Pteriomorpha and the (infrequently) Hb-containing Heterodonta. The strongest supporting evidence may be the results reported by Purchon (1978), who employed a matrix analysis based on set theory to cluster and thus to gauge the degree of relatedness between the 40 recent bivalve superfamilies whose taxonomic integrity is uncontroversial. Using the

nine (of 17) characters that Newell (1969) had designated as diagnostic of the superfamilies and that were either practical or suitable for the analysis, Purchon (1978) identified only two major clusters of bivalves, the nuculoids and the rest. His conclusion is reflected in Allen's (1986) diagnosis of the two quite different bivalve subclasses. With the stipulation that the nuculoid cluster should include the solemyoids, as in Allen's (1986) scheme, the present findings identify a tenth character that supports the notion of one and only one major "taxochasm" among the bivalves (Purchon, 1978). We mention the possibility of diphyly less in advocacy of it than as an alternative that circumvents the weaknesses of the hypothesis of multiple origins of the RBC and which, therefore, should not be ignored.

Further progress in understanding the evolution of O_2 transport systems awaits further elucidation of the structure and function of tissue heme proteins and also further understanding of how bivalve O_2 transport systems work. When details such as blood gas levels, pH, responses to hypoxia, respiratory and cardiovascular design constraints, etc. are known in protobranchs as well as additional Hb-containing bivalves, then the selection pressures favoring the evolution of systems with particular properties will become clearer.

ACKNOWLEDGMENTS

Supported by NSF DCB 84-14856 (Regulatory Biology), BSR 83-07714 (Systematic Biology), and DMB 17310 (Biochemistry). CPM is grateful to M. J. Greenberg for leads to the literature on bivalve phylogeny and to R. D. Barnes for helpful discussion. For MPM this is Contribution No. 153 from the Marine Science Center of Northeastern University.

LITERATURE CITED

ALLEN, J. A. 1978. Evolution of deep sea protobranchs. *Phil. Trans. R. Soc. Lond. B* **284:** 387–401.

ALLEN, J. A. 1986. The recent Bivalvia: their form and evolution. Pp. 337–407 in *The Mollusca,* Vol. 10, K. M. Wilbur, ed. Academic Press, Orlando, Florida.

BABA, K. 1940. The mechanisms of absorption and excretion in a solenogastre, *Epimenia verrucosa* (Nierstrasz) studied by means of injection methods. *J. Dep. Agric. Kyusyu Imperial Univ.* **6:** 119–166.

VAN BRUGGEN, E. J. F., W. G. SCHUTTER, J. F. L. VAN BREEMEN, M. M. C. BIJHOLT, AND T. WICHERTJES. 1981. Arthropodan and Molluscan haemocyanins. Pp. 1–38 in *Electron Microscopy of Proteins,* Vol. 1, J. R. Harris, ed. Academic Press, London.

CAVANAUGH, C. 1980. Symbiosis of chemoautotrophic bacteria and marine invertebrates. *Biol. Bull.* **159:** 457.

CAVANAUGH, C. 1983. Symbiotic chemoautotrophic bacteria in marine invertebrates from sulphide-rich habitats. *Nature* **302:** 58–61.

COX, L. R., C. P. NUTTALL, AND E. R. TRUEMAN. 1969. General features of Bivalvia. Pp. N2–N129 in *Treatise on Invertebrate Palaeontology,* Part N, Vol. 1, R. C. Moore, ed. Geol. Soc. Am., Lawrence, Kansas.

DANDO, P., A. J. SOUTHWARD, E. C. SOUTHWARD, N. B. TERWILLIGER, AND R. C. TERWILLIGER. 1985. Sulphur-oxidising bacteria and haemoglobin in gills of the bivalve mollusc *Myrtea spinifera. Mar. Ecol. Prog. Ser.* **23:** 85–98.

DEATON, L. E., AND C. P. MANGUM. 1976. The function of hemoglobin in the arcid clam *Noexia ponderosa.* II. Oxygen uptake and storage. *Comp. Biochem. Physiol.* **53A:** 181–186.

DOELLER, J. E. 1984. A hypothesis for the metabolic behavior of *Solemya velum,* a gutless bivalve. *Am. Zool.* **24:** 57A.

DOELLER, J. E., D. W. KRAUS, AND J. M. COLACINO. 1983. The presence of hemoglobin in *Solemya velum.* (Bivalvia, Protobranchia). *Am. Zool.* **23:** 976.

ELLERTON, H. D., AND T. LANKOVSKY. 1983. Structure of the hemocyanin from the paua (abalone), *Haliotis iris. Life Chem. Rep.,* Suppl. **1:** 129–132.

FELBECK, H. 1983. Sulfide oxidation and carbon fixation by the gutless clam *Solemya reidi:* an animal-bacteria symbiosis. *J. Comp. Physiol.* **152:** 3–11.

FISHER, C. R., AND J. J. CHILDRESS. 1984. Carbon fixation and translocation by symbiotic bacteria in *Solemya reidi* (Bivalvia: Protobranchia). *Am. Zool.* **24:** 57A.

GIELENS, C., C. BENOY, G. PREAUX, AND R. LONTIE. 1986. Presence of only seven functional units in the polypeptide chain of the haemocyanin of the cephalopod *Octopus vulgaris.* Pp. 223–226 in *Invertebrate Oxygen Carriers,* B. Linzen, ed. Springer-Verlag, Berlin.

GHIRETT-MAGALDI, A., B. SALVATO, L. TALLANDINI, AND M. BELTRAMINI. 1979. The hemocyanin of *Aplysia limacina:* chemical and functional characterization. *Comp. Biochem. Physiol.* **62A:** 579–584.

GRAHAM, R. A., C. P. MANGUM, R. C. TERWILLIGER, AND N. B. TERWILLIGER. 1983. The effect of organic acids on oxygen binding of hemocyanin from the crab *Cancer magister. Comp. Biochem. Physiol.* **74A:** 45–50.

GRINICH, N. P., AND R. C. TERWILLIGER. 1980. The quaternary structure of an unusual high-molecular-weight intracellular haemoglobin from the bivalve mollusc *Barbatia reeveana. Biochem. J.* **189:** 1–8.

HERSKOVITS, T. T., M. G. HAMILTON, AND L. J. MAZELLA. 1986. Hemocyanin of the chiton *Acanthopleura granulata. Biochemistry* **25:** 3612–3619.

HYMAN, L. H. 1951. *The Invertebrates,* Vol. II. McGraw-Hill, New York. 550 pp.

HYMAN, L. H. 1967. *The Invertebrates,* Vol. VI. McGraw-Hill, New York. 763 pp.

KRUGER, F. 1957. Beitrage zur Physiologie des Hamoglobins wirbelloser Tiere. IV. Zur Atmungsphysiologie von *Glycimeris nummaria* (Linne) (Mollusca: Lammellibranchiata). *Zool. Jahrb.* **67:** 311–322.

VAN DER LAAN, J. M., W. G. SCHUTTER, R. TORENSMA, AND E. J. F. VAN BRUGGEN. 1981. *Neptunea antiqua* haemocyanin: a different structure. Pp. 415–424 in *Invertebrate Oxygen-Binding Proteins,* J. Lamy and J. Lamy, eds. Marcell Dekker, Inc., New York.

LAMY, J., J. N. LAMY, M. LECLERC, S. COMPIN, K. I. MILLER, AND K. E. VAN HOLDE. 1986. Preliminary results on the structure of *Octopus dofleini* hemocyanin. Pp. 231–234 in *Invertebrate Oxygen Carriers,* B. Linzen, ed. Springer-Verlag, Berlin.

LANKESTER, E. R. 1872. A contribution to the knowledge of hemoglobin. *Proc. R. Acad. Sci. Lond.* **21:** 70–80.

MANGUM, C. P. 1976. Primitive respiratory adaptations. Pp. 191–278 in *Adaptation to Environment: Physiology of Marine Animals,* R. C. Newell, ed. Butterworth's & Co., Ltd, London.

MANGUM, C. P. 1980a. Distribution of respiratory pigments and the role of anaerobic metabolism in the lamellibranch molluscs. Pp. 171–184 in *Animals and Environmental Fitness,* R. Gilles, ed. Pergamon Press, Oxford.

MANGUM, C. P. 1980b. Respiratory function of the hemocyanins. *Am. Zool.* **20:** 19–38.

MANGUM, C. P., AND G. LYKKEBOE. 1979. The influence of inorganic ions and pH on the oxygenation properties of the blood in the gastropod mollusc *Busycon canaliculatum. J. Exp. Zool.* **207:** 417–430.

MANGUM, C. P., AND L. E. BURNETT. 1986. The CO_2 sensitivity of the hemocyanins and its relationship to Cl^- sensitivity. *Biol. Bull.* **171:** 248–263.

MANGUM, C. P., AND R. P. DALES. 1965. Products of haem synthesis in polychaetes. *Comp. Biochem. Physiol.* **15:** 237–257.

MANGUM, C. P., AND N. A. MAURO. 1985. Metabolism of invertebrate red cells: a vacuum in our knowledge. Pp. 280–289 in *Circulation, Respiration and Metabolism,* R. Gilles, ed. Springer-Verlag, Berlin.

MANGUM, C. P., J. L. SCOTT, R. E. L. BLACK, K. I. MILLER, AND K. E. VAN HOLDE. 1985. Centipedal hemocyanin: its structure and its implications for arthropod phylogeny. *Proc. Nat. Acad. Sci. U.S.A.* **82:** 3721–3725.

MAURO, N. A., AND R. E. ISAACKS. 1984. Metabolic characteristics of *Glycera* and *Noetia* erythrocytes. *Am. Zool.* **24:** 120A.

MCALESTER, A. L. 1966. Evolutionary and systematic implications of a transitional ordovician lucinoid bivlve. *Malacologia* **3:** 433–439.

MORSE, M. P., E. MEYHOFER, J. J. OTTO, AND A. M. KUZIRIAN. 1986. Hemocyanin respiratory pigment in bivalve mollusks. *Science* **231:** 1302–1304.

NEWELL, N. D. 1969. Classification of Bivalvia. Pp. N205–N222 in *Treatise on Invertebrate Palaeontology,* Part N, Vol. 1, R. C. Moore, ed. Geol. Soc. Am., Lawrence, Kansas.

NICKERSON, K. W., AND K. E. VAN HOLDE. 1971. A comparison of molluscan and arthropod hemocyanin. I. Circular dichroism and absorption spectra. *Comp. Biochem. Physiol.* **39B:** 855–872.

PERUTZ, M. 1985. Haemoglobin structure: a clam with a difference. *Nature* **316:** 210.

POJETA, J. 1978. The origin and early taxonomic diversification of the bivalves. *Phil. Trans. R. Soc. Lond. B* **284:** 225–246.

POLUHOWICH, J. J. 1970. Oxygen consumption and the respiratory pigment in the fresh-water nemertean, *Prostoma rubrum. Comp. Biochem. Physiol.* **36:** 817–821.

PURCHON, R. D. 1978. An analytical approach to a classification of the Bivalvia. *Phil. Trans. R. Soc. Lond. B* **284:** 425–436.

REID, R. G. B., AND D. G. BRAND. 1986. Sulfur-oxidizing symbiosis in lucinaceans: implications for bivalve evolution. *Veliger* **29:** 3–24.

ROYER, W. E., W. E. LOVE, AND F. F. FENDERSON. 1985. The cooperative dimeric and tetrameric clam hemoglobins are novel assemblages of myoglobin folds. *Nature* **316:** 227–230.

RUNNEGAR, B. 1978. Origin and evolution of the class Rostroconchia. *Phil. Trans. R. Soc. Lond. B* **284:** 319–333.

RUNNEGAR, B., AND J. POJETA. 1986. Origin and diversification of the Mollusca. Pp. 1–57 in *The Mollusca,* Vol. 10, E. R. Trueman and M. R. Clarke, eds. Academic Press, Orlando, Florida.

RYAN, M., N. B. TERWILLIGER, R. C. TERWILLIGER, AND E. SCHABTACH. 1985. Chiton hemocyanin structure. *Comp. Biochem. Physiol.* **80B:** 647–656.

SALVINI-PLAWEN, L. V. 1986. Early evolution and the primitive groups. Pp. 59–150 in *The Mollusca,* Vol. 10, E. R. Trueman and M. R. Clarke, eds. Academic Press, Orlando, Florida.

STASEK, C. R. 1972. The molluscan framework. Pp. 1–44 in *Chemical Zoology,* Vol. VII, M. Florkin and B. T. Scheer, eds. Academic Press, New York.

TERWILLIGER, R. C., AND N. B. TERWILLIGER. 1985. Molluscan hemoglobins. *Comp. Biochem. Physiol.* **81B:** 255–262.

TERWILLIGER, R. C., N. B. TERWILLIGER, AND A. ARP. 1983. Thermal vent clam (*Calyptogena magnifica*) hemoglobin. *Science* **19:** 981–982.

TURBEVILLE, J. M., AND E. E. RUPPERT. 1986. Comparative ultrastructure and the evolution of nemertines. *Am. Zool.* **25:** 53–71.

VAN HOLDE, K. E. 1983. Some unresolved problems concerning hemocyanins. Pp. 403–412 in *Structure and Function of Invertebrate Respiratory Proteins,* E. J. Wood, ed. *Life Chem. Rept.,* Suppl. **1:** 403–412.

VAN HOLDE, K. E. 1985. *Physical Biochemistry.* Prentice-Hall, New York. 287 pp.

VAN HOLDE, K. E., AND K. I. MILLER. 1982. Haemocyanins. *Q. Rev. Biophys.* **15:** 1–129.

VAN HOLDE, K. E., AND K. I. MILLER. 1985. Association-dissociation equilibria of *Octopus* hemocyanin. *Biochemistry* **24:** 4577–4582.

VERNET, G. 1979. Fine structure of nemertean worm *Lineus lacteus* red blood corpuscles. *Cytobios* **24:** 43–46.