Histol Histopath (1990) 5: 513-525

Histology and Histopathology

Invited Review

The phylogenetic odyssey of the erythrocyte. II. The early or invertebrate prototypes

Chester A. Glomski and Judith Tamburlin

Department of Anatomical Sciences, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York, USA

Summary. Freely existing hemoglobin-bearing cells suspended in a plasmic milieu (erythrocytes) are found in a relatively small number of taxanomically scattered invertebrates. These species include some annelids, echiurids, molluscs, phoronids, nemerteans and echinoderms, e.g. *Pista pacifica, Urechis caupo, Noetia ponderosa, Phoronis australis, Lineus fuscoviridis* and *Cucumaria miniata* respectively. The typical invertebrate erythrocyte (hemocyte, coelomocyte) can be described as permanently nucleated, considerably larger than the human red cell, oval or circular in configuration and spherical, biconvex or flattened in profile. The marginal band of the erythrocyte, a bundle of subplasmalemmal microtubules that circumscribes the periphery of the cell and lies in the plane parallel to its flat surface makes its first appearance in certain invertebrates. This structure in association with the cell surface-associated cytoskeleton is responsible for the flattened elliptical shape seen in some invertebrate erythrocytes and endows them with flexibility and resilience to mechanical forces. This in an evolutionarily persistent characteristic that is retained throughout the submammalian vertebrates. The erythrocytes of invertebrates are more morphologically and functionally diversified than the mammalian model. In addition to respiratory activities (oxygen storage and transport) they can sometimes function as vendors of nutrients and participate in other less obvious processes. These cells therefore frequently not only retain organelles that are usually discarded by vertebrate erythrocytes (ribosomes, golgi apparatus, etc.) but may also depending upon the species, manifest in their cytoplasm organelles and inclusions that are not a normal component of developing or mature submammalian vertebrate and mammalian erythroid cells. Examples of the latter are pigment granules, lipid droplets, extensive glycogen stores and prominent Prussian blue positive inclusions. Erythrocytes in the invertebrates, though presenting certain cytologic and functional features in common, are a heterogenous collection of cells, each tailored for a specific species or group of organisms.

Key words: Invertebrate erythrocytes, Erythrocyte, Hemocyte, Hemoglobin, Coelomocyte

Introduction

The erythrocytes found in invertebrates, submammalian vertebrates and mammals (including man) have in common certain unifying characteristics. Among these are free, independent existence in a suspending plasmic mileu, high intracellular content of hemoglobin capable of long term repetitive oxygen uptake and release, and resilience to potentially deforming external mechanical forces. Superimposed upon this phylogenetic spectrum of erythrocytes are various cytologic modifications that further enhance the red cell's respiratory functions. Examples of the latter, particularly among the vertebrates, include the elimination of the hemoglobin-synthesizing machinery (ribosomal RNA) from the mature cell, the disposal of other cytoplasmic organelles, alteration of the erythrocytic cytoskeleton and revision of the geometric configuration of the red cell.

The purpose of the present discussion is to identify the phylogenetically primitive extant animal species that manifest erythrocytes, consider the structural and ultrastructural features of their red cells and examine the roles of those organelles that are particularly pertinent to erythrocellular function. Having established this invertebrate profile, the intent is to eventually consider the parallel evolved erythrocytic counterparts in the lower and higher vertebrates that lead to the red cell present in man. Hopefully this will result in a synoptic, lucid overview of invertebrate and vertebrate erythroid cytoarchitecture and bring into focus the evolutionary adaptations expressed by this cell.

Offprint requests to: Chester A. Glomski, Department of Anatomical Sciences, School of Medicine and Biomedical Sciences, State University of New York at Buffalo, Buffalo, New York 14214, USA

The phylogenetic aspects of the hemoglobin molecule are discussed in the initial segment of this series (Glomski and Tamburlin, 1989).

Discussion

Permanently nucleated, hemogoblin-containing cells suspended in the hemolymph, coelomic fluid or blood of invertebrates are the earliest identifiable erythrocytes. Such cells are found in a limited number of taxonomically scattered invertebrates. In terms of respiratory pigment hierarchy they can be viewed as a logical cytologic progression that supersedes the more primitive occurrence of free hemoglobin dissolved in an extracellular fluid (e.g. that present in the plasma of the common earthworm *Lumbricus terrestris*). In tracing the lineage of the red cell, the freely existing erythrocyte can also be considered an advance beyond fixed, static hemoglobin-containing cells (seen in some insects as the bot fly larva, *Gasterophilus equi*) and a higher level of erythrocellular development than blood cells which manifest an evolutionarily impersistent respiratory pigment as hemerythrin. The latter, for example, is found in the hemocytes of the sipunculans, a small phylum of unsegmented marine worm-like animals, e.g. *Phascolopsis gouldii*, the peanut worm.

The typical invertebrate erythrocyte can be described as permanently nucleated, considerably larger than the human red cell, having a flattened, biconvex or spherical profile, oval or circular in configuration and manifesting less hemoglobin per unit cellular volume than the mammalian counterpart. The retention of the nucleus is a stable feature that persists throughout the phyla until the appearance of mammals. The oval shape, once evolutionarily established, is similarly a strongly entrenched characteristic and one that has become a hallmark of submammalian vertebrate erythrocytes. This configuration is directly related to an ultrastructural organelle, the marginal band, which is discussed in detail below. A red cell's spheroidal or biconvex lateral profile can be viewed as frequently dependent in part upon the retention of the nucleus, an often recognizably prominent space-occupying structure. It may also be promoted by the physical tendency for free, independently existing structures to assume a shape that approaches one that offers the lowest surface area to volume ratio as well as affording a stable internal organization, i.e. a sphere. The low content of hemoglobin reflects the concomitant presence of cytoplasmic inclusions (granules etc.) in some invertebrate erythrocytes as well as the absence of an organismal physiologic need for intracellular adaptations permitting higher oxygen carrying capabilities.

A survey of the dimensions of erythrocytes in the relatively few invertebrates that manifest these free, fluid-suspended hemoglobin-containing cells reveals with rare exception that they are larger than those observed in mammals and have a more variable configuration. Their sizes can demonstrate a great deal of variation within a species and even in a given individual (Ohuye, 1937-1938a). This is unlike the erythrocytes of the vertebrates where cellular uniformity is the norm. *Pista pacifica* (a member of the Annelida, a phylum comprised mostly of marine segmented, typically multibristled worms) has hemoglobiniferous spherical coelomocytes (hence erythrocytes) 27-35 µm in diameter (Figs. 1-3) (Terwilliger et al., 1985). Another marine terebellid (tube dwelling) annelid *Terebella ehrenbergi* has even larger erythrocytes. These are spherical or oval and sometimes have a surface indentation giving them "a heart-shaped" appearance under light and electron microscopy (Ochi, 1969). They are 30-70 µm in length and contain a small amount of hemoglobin. The ophelid polychaete *Travisia japonica* also has typically large oval discoid erythrocytes, larger in the male (50 x 40 µm) than in the female (40 x 30 µm) according to the observations of Ochi (1969). In Ohuye's experience, however, the dimensions of these slightly biconcave cells were 20 x 15 x 4 µm. This most likely reflects the size and hence the age of the worms studied. An interesting feature of this species is the occurrence of occasional very small erythrocytes (about one half the usual size) that do not have a nucleus (Ohuye, 1937-1938b). Although these could be damaged, sheared erythrocytic fragments, they could alternatively be an early presentation of erythroplastids. The bloodworm *Glycera dibranchiata* (an extensively studied marine annelid, Figs. 4, 5) has erythrocytes that are spherical with a diameter of 17-27 µm; conversely, two other members of this genus *G. americana* and *G. decipiens* exhibit round or oval discoid erythrocytes in their blood vessels which measure in the range of 20 µm (Cowden, 1966; Ochi, 1969). The coelomocytes of *Amphitrite johnstoni*, a tentacled, terebellid annelid, are somewhat flattened oval discs approximately 40 µm in diameter (Fig. 6). The numbers of these cells attain their highest and lowest levels during periods of gamete production and maturation respectively. *Amphitrite ornata*, a similar organism, has erythrocytes that are described as irregular in shape (17-35 µm diameter); those of

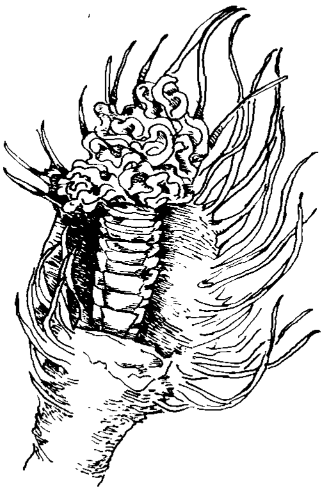

Fig. 1. *Pista pacifica.* This annelid is depicted within its vertical sandy tube which terminates in an overhanging hood with many marginal tendrils. The segmented structure of the worm and its tentacles are identifiable. The entire length of the tube and its enclosed worm are not illustrated.

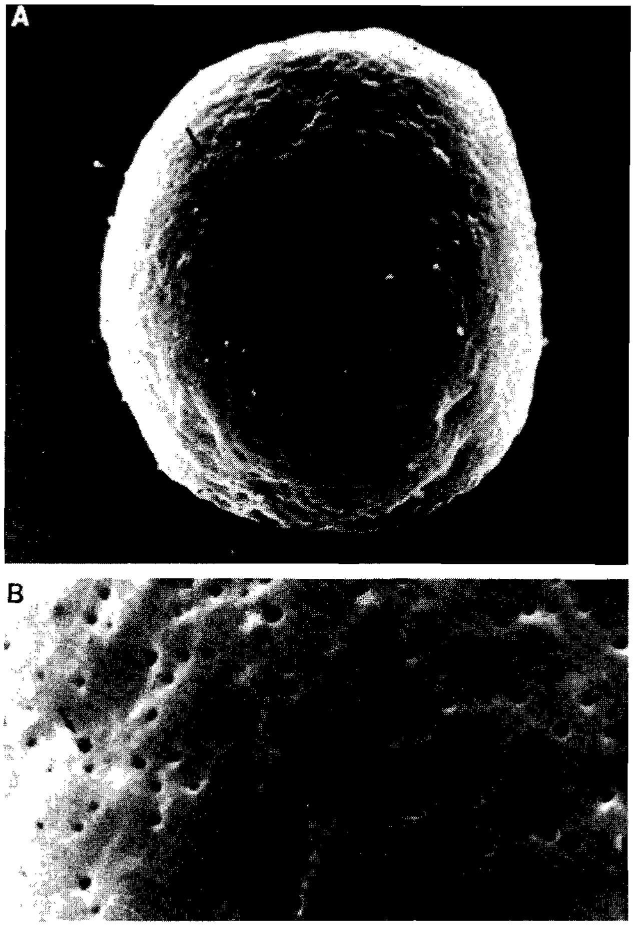

Fig. 2. *Pista pacifica* coelomic hemoglobin-containing cell. **A.** SEM demonstrating the spherical configuration of the cell and the numerous pinocytotic invaginations of the plasmalemma (arrow). **B.** A higher magnification further illustrating the particularly large number of pinocytotic invaginations and their uniform distribution over the surface of the cell (arrow). Courtersy of Terwilliger et al., and Alan R. Liss Inc.

Enoplobranchus sanguineus are smaller, 11-15 µm, but still considerably larger than human red cells.

Erythrocytes are the most numerous cells in the coelomic fluid of the members of the phylum Echiurida (a limited group of small marine worms that live in burrows in mud or sand- -they have an extendable proboscis that is used for exploration and feeding, leading to their title of spoonworms). They are annelid-like creatures and have a closed circulatory system with colorless plasma since their respiratory pigment is limited to the free hemoglobin and red cells in the coelomic fluid. These serve as a means of oxygen storage for periods of low oxygen availability (e.g. when the tide is out). The most thoroughly studied echiurid, *Urechis caupo* the «innkeeper worm» (so called because commensal organisms such as some crabs or an annelid often co-reside in its burrow) has spherical coelomic erythrocytes with a diameter ranging 30-45 µm as demonstrated by bright field, scanning and transmission electron microscopy, (Fig. 7-10) (Terwilliger et al., 1985).

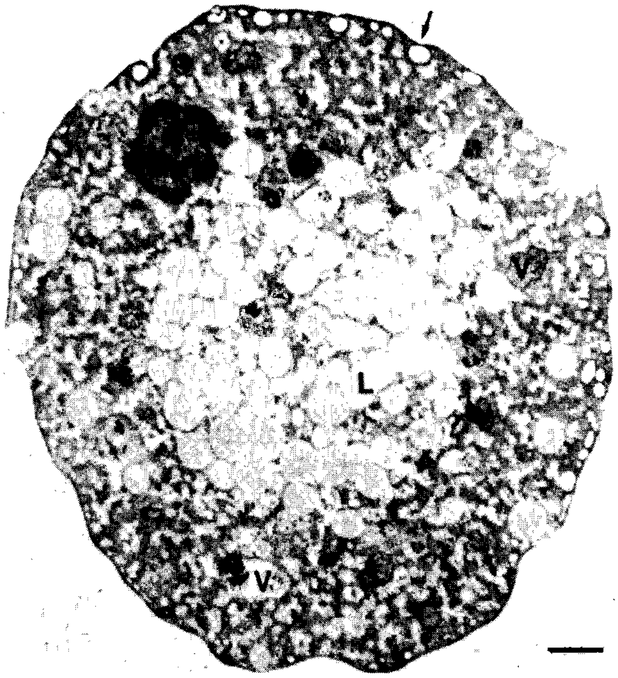

Fig. 3. *Pista pacifica* coelomic hemoglobin-containing cell. TEM. A large number of lipid granules (L) and a small eccentric nucleus (N) are present. Numerous forming and formed pinocytotic vesicles are identifiable along the periphery of the cell (arrows). The homogenous grey electron-dense material distributed throughout the cytoplasm, in the main, is believed to be hemoglobin. Glycogen, though abundantly present, is not resolveable at the present magnification. Bar equals 2 µm. Courtesy of Terwilliger et al., and Alan R. Liss Inc.

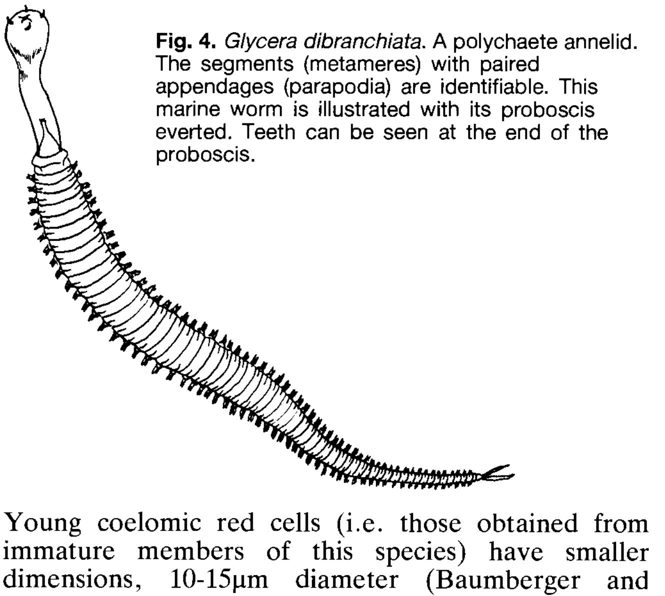

Fig. 4. *Glycera dibranchiata*. A polychaete annelid. The segments (metameres) with paired appendages (parapodia) are identifiable. This marine worm is illustrated with its proboscis everted. Teeth can be seen at the end of the proboscis.

Young coelomic red cells (i.e. those obtained from immature members of this species) have smaller dimensions, 10-15µm diameter (Baumberger and Michaelis, 1931). Another member of this group *Urechis unicinctus* has circular, slightly biconvex, disc-shaped cells measuring 10-36 by 3-6µm (Ohuye, 1937-1938a). *Thalassema gogoshimense*, also an echiurid, has corpuscles with the same dimensions while those of *Thalassema mellita* (an Atlantic coast echiurid that often

Fig. 5. *Glycera dibranchiata* hemoglobin-bearing coelomocyte. TEM. The cell contains inclusions composed of amorphous material (arrows), mitochondria, and vacuoles. Courtesy of Pierce and Maugel, and Alan R. Liss Inc.

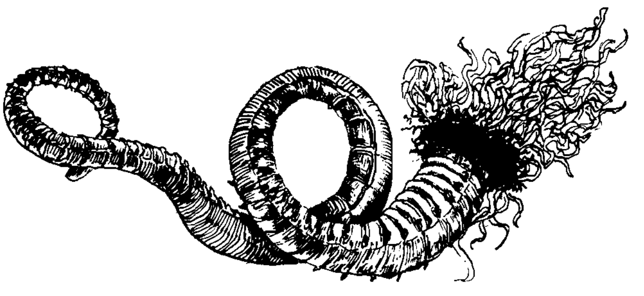

Fig. 6. *Amphitrite johnstoni.* A terebellid (tube dwelling) annelid. The light, wavy, hair-like structures at one end are its tentacles and the dark subadjacent region are its gills.

inhabits the shells of dead sand dollars) are spherical and approach the size range of mammalian rbc, 8-9µm. Some sand dollars belong to the genus *Mellita* hence the name for the worm that is found sheltering in its shell. Two other members of this phylum *Ochetostoma capensis and Thalassema neptuni* similarly have spherical erythrocytes (Dybas, 1981).

Phoronis australis is a representative species of Phoronida a minor phylum consisting of approximately fifteen species of sessile marine worms. Members of this

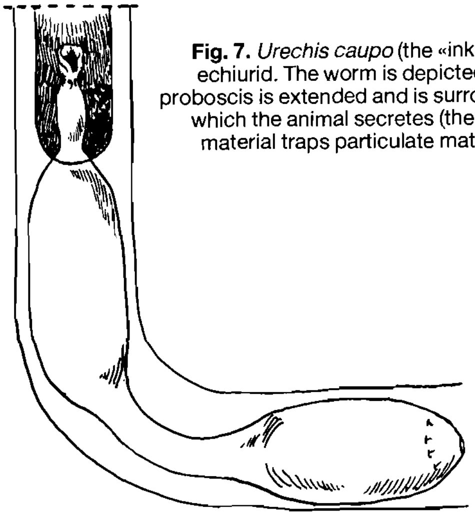

Fig. 7. *Urechis caupo* (the «inkeeper worm»). An echiurid. The worm is depicted in its burrow. Its proboscis is extended and is surrounded by mucus which the animal secretes (the shade area). This material traps particulate matter to be ingested by the organism.

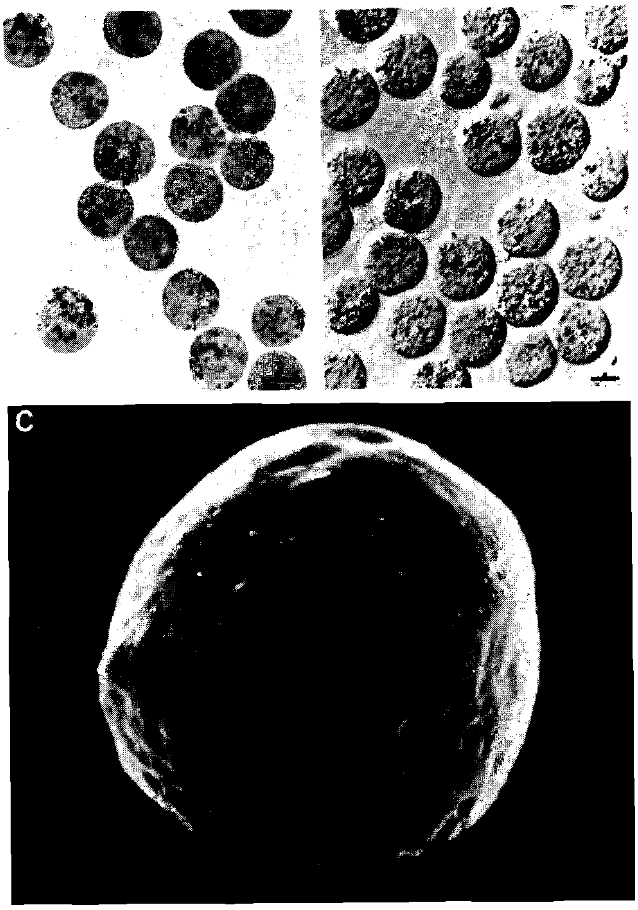

Fig. 8. *Urechis caupo* coelomic erythrocytes. **A.** Bright field and **B.** differential interference contrast microscopy of living cells in coelomoic fluid. The granular content of the cells is readily apparent. Bars equal 20 µm. **C.** SEM demontrating the spherical configuration of the cell and the pits in the plasmalemma (arrow). Courtesy of Terwilliger et al., and Alan R. Liss Inc.

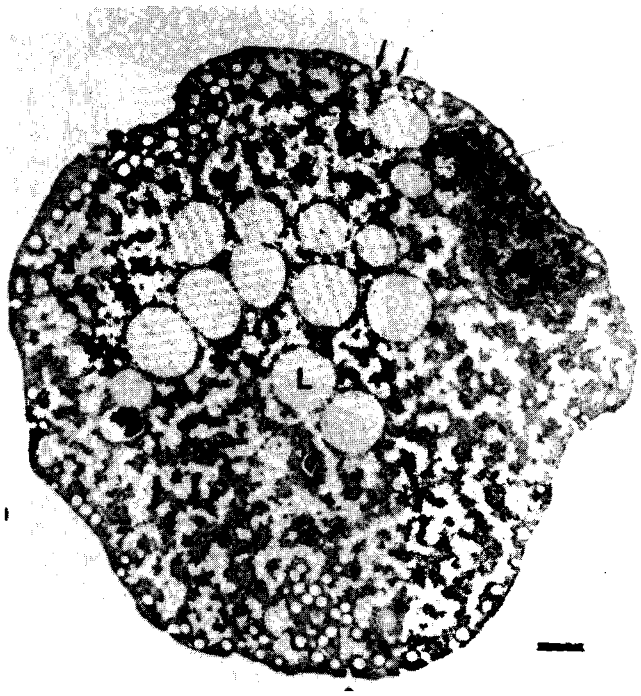

Fig. 9. *Urechis caupo* coelomic erythrocyte. Low magnification TEM demonstrating a large number of prominent lipid granules (L) and lysosome-like vacuoles containing electron dense material (V). Arrow indicates one of many subplasmalemmal vesicles. The nucleus is small and eccentric (N). Bar equals 2 um. Courtesy of Terwilliger et al., and Alan R. Liss Inc.

phylum have a ring of tentacles termed a lophophore surrounding the mouth, possess a closed vascular system, and inhabit secreted chitinous tubes in shallow water (Fig. 11). Their erythrocytes have been reasonably well investigated. Those of *Phoronis australis* typically are circular discs, slightly biconvex, 8-12 µm in diameter and 2-4 µm in thickness (Ohuye, 1942). Its European counterpart, *Phoronis hippocrepia*, has red cells of similar diameter and structure. Unlike most red blood cell-bearing invertebrates the origin and development of the blood cells of *P. australis* and to a lesser extent *P. hippocrepia* have been established (Ohuye, 1942; Hayward, 1981). Erythrocytes were seen to arise from endothelium of tentacular blood vessels and from mesothelium (peritoneum) within the tentacles. They became enlarged, rounded up, developed a cytoplasmic attachment stalk and subsequently disassociated themselves from their origination site. The isolated cells demonstrated a sequence of nuclear and cytoplasmic changes; the terms proerythroblast, polychromatophilic erythroblast and normoblast were assigned to the maturational stages. The last cell was considerably smaller than the proerythroblast as a result of mitotic activity. It developed into a mature (nucleated) erythrocyte by subsequently increasing its cytoplasmic volume and condensation of the nucleus. Erythrocytes have also been described in *Phoronis architecta*, an inhabitant of the intertidal coast of southeastern U.S.A. and adjoining Gulf of Mexico (Vandergon and Colacino,

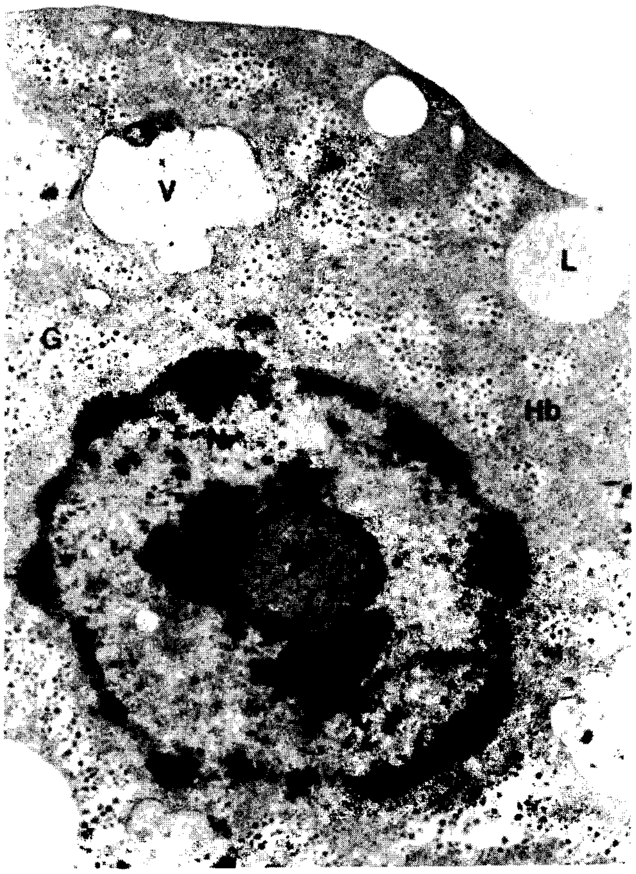

Fig. 10. *Urechis caupo* coelomic erythrocyte. High magnification TEM demonstrating Nucleus (N) with nucleolus (Nu). A lysosome-like vacuole (V), lipid granule (L) and prominent patches of glycogen rosettes (G) are indicated. The homogenous, amorphous, electron dense areas of the cytoplasm are believed to represent hemoglobin (Hb). Bar equals 1 µm. Courtesy of Terwilliger et al., and Alan R. Liss Inc.

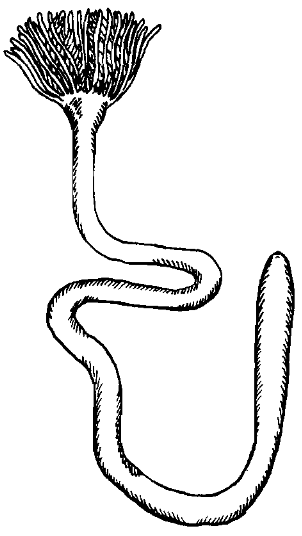

Fig. 11. *Phoronis australis*. A member of the phylum Phoronida. The worm-like, cylindrical, unsegmented body and ring of hollow tentacles (termed the lophophore) surrounding the mouth of this marine worm are identifiable.

1989). *Phoronis mülleri* and *Phoronopsis viridis* are two additional members of this group with rbc containing hemoglobin.

Nemertina (Rhynchocoela) is a phylum of elongate cylindrical or flattened marine scavenger worms (ribbon worms, Fig. 12). They are notable because they are the only animals without a coelomic cavity that nevertheless have blood contained within a closed circulatory system. Thus, the first (earliest) definitive circulatory system is found in nemertines. A few members of this group have hemoglobin-containing cells in their vasculature (Meglitsch, 1972). *Lineus fuscoviridis*, a member of this phylum, has rounded or oval, flattened discoid erythrocytes measuring about 12-20 μm in diameter (Ohuye, 1943). They are described as similar to the red cells of holothurians *Caudina* and *Molpadia*. *Drepanophorus* is another example of this group, it manifests small, oval, biconvex red cells (Gontcharoff, 1961). Giemsa-stained dry film smears of blood from *L. fuscoviridis* reveal the maturational steps of its erythrocytes (Fig. 13). Young cells (erythroblasts) are small, have a relatively large nucleus with an open chromatin pattern, and exhibit a basophilic cytoplasm. With maturation the nucleus undergoes pyknosis and reduction in size, the cytoplasm becomes polychromatophilic and then eosinophilic. The total cytoplasmic volume concurrently increases. A small number of basophilic cytoplasmic granules are present from the basophilic erythroblast stage onward.

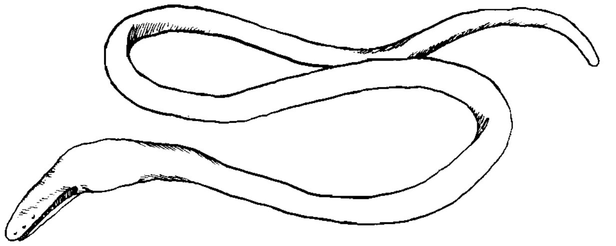

Fig. 12. *Lineus ruber*, a representative of the phylum Nemertina, (ribbon worms), a group of fragile, elongated, unsegmented worms. Its characteristic long eversible proboscis (used for defense and capture of prey) is not exposed having been withdrawn into the body. The cephalic end has multiple eyes (seen as small dots on the dorsal surface) as well as a lateral furrow.

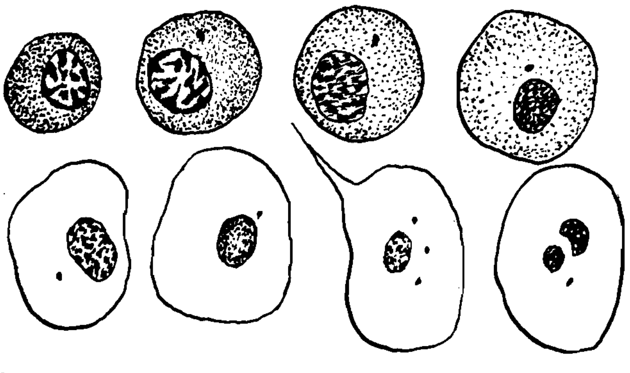

Fig. 13. *Lineus fuscoviridis*. Giemsa-stained erythrocytes as seen in dry film blood smears. The cells were selected to demonstrate the maturational sequence. The youngest cell is in the upper left with developmental progression to the right. The third cell in the bottom row has a spine shaped cytoplasmic projection. Such structures (often multiple) as well as a change in the entire shape of the cell are commonly observed when the erythrocytes are removed from the worm. The last cell illustrated is binucleate. After Ohuye, 1943.

The erythrocytes present in the hemolymph of the arcid or «blood» clams (phylum Mollusca) *Noetia ponderosa*, *Anadara ovalis* and *Anadara transversa* are flattened and usually elliptical, a configuration that can be attributed to the presence of a documented marginal band (Cohen and Nemhauser, 1985). The length of these hemoglobiniferous cells in *Noetia ponderosa* when examined in a viable state under ideal conditions with phase contrast microscopy is 15-20 μm, the breadth is 25% less (Figs. 14-16). The dimensions of the red cells in another clam *Venus verrucosa* are in the order of 20 x 30 μm (Fig. 17). The thermal vent clam *Calyptogena magnifica*, a species that lives at oceanic depths of 2.5 km, also has erythrocytes that contain hemoglobin (Terwilliger and Terwilliger, 1983). It is the only identified heterodont clam that has a circulating intracellular hemoglobin. Not surprisingly its body tissues have a red color.

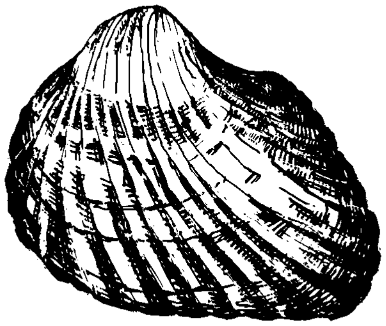

Fig. 14. *Noetia ponderosa*, a mollusc. A «blood» clam.

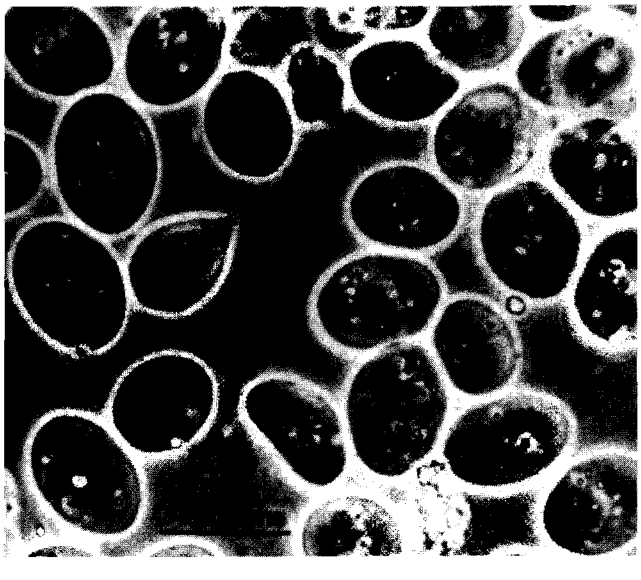

Fig. 15. *Noetia ponderosa*. Living erythrocytes observed under phase contrast microscopy. The cells are ideally presented. Their characteristic flattened, elliptical shape is readily apparent. The arrowhead indicates one cell with a pointed end, the result of a modification of the usual pattern of the marginal band. Courtesy of Nemhauser et al. Reproduced from the Journal of Cell Biology, 1983, vol. 96, pp. 980 by copyright permission of the Rockefeller University Press.

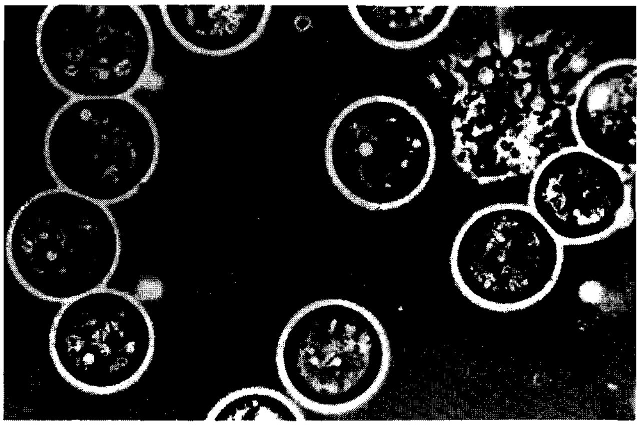

Fig. 16. *Noetia ponderosa.* Unfixed erythrocytes observed under phase contrast microscopy. The erythrocytes have assumed a circular configuration as compared to their initial oval form. A small eccentric nucleus is identifiable in some cells. An amoeboocyte (leukocyte) is present in the upper right field. Courtesy of Pierce and Maugel, and Alan R. Liss Inc.

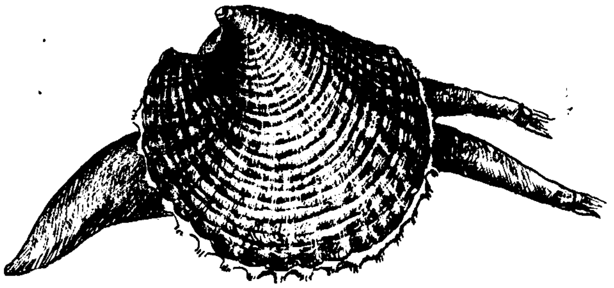

Fig. 17. *Venus Verrucosa.* A mollusc. This clam is illustrated with its siphons and foot extended out of the shell.

Some holothurians (sea cucumbers, members of the phylum Echinodermata) have hemoglobin-bearing coelomocytes. They are variable in size and shape but are generally about 10-20 µm in diameter and usually spherical or biconvex. The red cells of the U.S.-Canadian Pacific coast sea cucumber *Cucumaria miniata* are biconvex, flattened, elliptical discs 18 x 14 x 5 µm (Fontaine and Lambert, 1973). The mature erythrocytes of another sea cucumber *Eupentacta quinquesemita* are subspheroidal and measure 19 x 18 µm (Figs. 18-21). Their very immature counterparts are similarly spherical but smaller. Conversely, intermediate-age red cells are flattened ellipsoids (up to 40 µm in length) and consequently longer and flatter than both the younger and older forms (Fontaine and Hall, 1981).

The dimensions of the erythrocytes in some invertebrates are dependent upon the observed phenomenon that smaller and presumably younger animals may have smaller red cells than their larger peers. The younger (smaller) cells can also lack some morphologic features present in the fully developed cells. Sex-related and seasonal variations have also been described.

The configuration of erythrocytes from invertebrates can be subject to significant, often asymmetrical alteration when they are removed from their normal

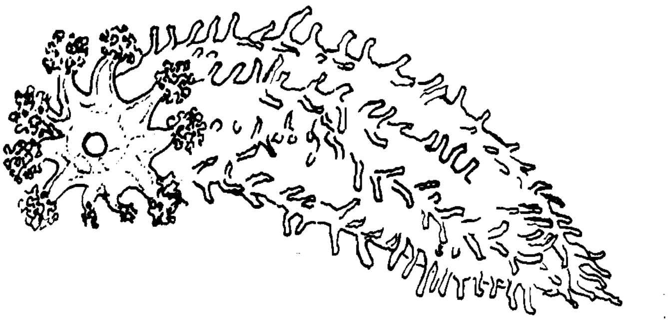

Fig. 18. *Eupentacta quinquesemita.* A member of the phylum Echinodermata (a holothurian, a sea cucumber). The circle of ten tentacles surrounding the oral opening and the numerous podia covering the tubular body of this marine animal are identifiable.

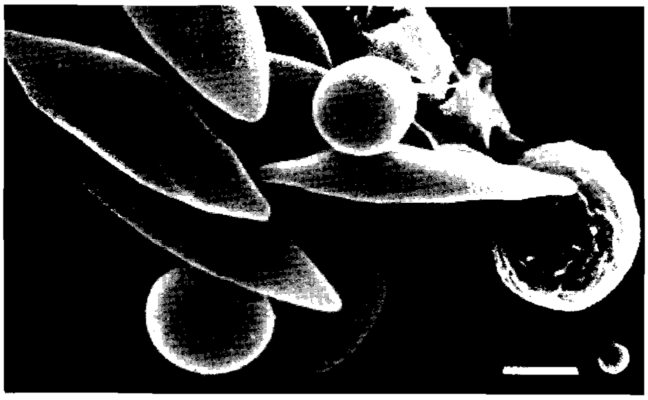

Fig. 19. *Eupentacta quinquesemita.* SEM. An aggregate of erythrocytes of various ages. The most immature cells are spherical, smooth surfaced and smaller (center field) than the most mature cell (far right) which is also spherical but is larger and has a wrinkled plasmalemma. The prominent, ellipsoidal cells in the central and left fields are of intermediate age. The tiny sphere in the lower right is a sperm head. Bar equals 5µm. Courtesy of Fontaine and Hall, and the Canadian Journal of Zoology.

environment. The cause for this sensitivity is usually unknown but it is a recognized entity in the clams *Anadara ovalis* and *A. transversa* (arcids that inhabit the sea coast of Eastern U.S.A.), in the echiurid worm *Thalassema gogoshimense* (so called because it is collected in the region of Gogoshima Island, Japan), and the ribbon worm *Lineus fuscoviridis* (Fig. 13). An example of this activity can be seen in the comparison of two samples of unfixed erythrocytes from the blood clam *Noetia ponderosa* (Figs. 15, 16). In one instance the cells have maintained their oval shape, in another they have not and have become circular. The well recognized crenation of mammalian erythrocytes in the preparation of dry film smears attests to the potentially disruptive forces that can be imposed upon red cells during routine laboratory manibulations. This propensity for deformation, nevertheless, does appear to be intensified among the invertebrates.

It does seem appropriate to suggest, as shown by Ohuye (1942) in the development of the erythorycte of *Phoronis australis*, that the most undifferentiated, primitive, freely existing cells of an organism destined to synthesize and retain hemoglobin are likely to be circular (spherical?). As they undergo their maturational processes these cells may subsequently retain their

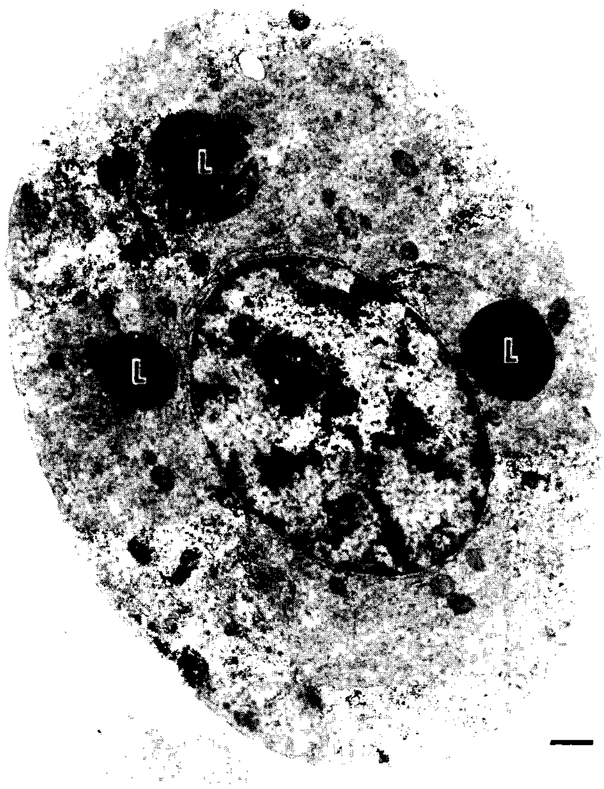

Fig. 20. *Eupentacta quinquesemita.* TEM of immature erythrocyte. A prominent nucleus, minimal nuclear membrane-associated heterochromatin, very large lysosomes (L) and mitochondria are identifiable. Bar equals 0.5 µm. Courtesy of Fontaine and Hall, and the Canadian Journal of Zoology.

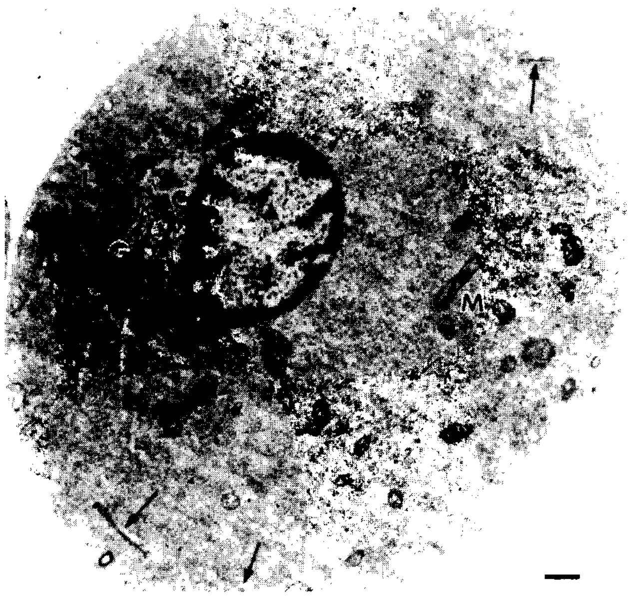

Fig. 21. *Eupentacta quinquesemita.* TEM. A mature erythrocyte demonstrating abundant retained cytoplasmic organelles i.e. mitochondria (M), centrioles (C), golgi apparatus (G), rough endoplasmic reticulum (arrows), and small lysosome-like bodies (L). Bar equals 0.5 µm. Courtesy of Fontaine and Hall, and the Canadian Journal of Zoology.

original spherical or round discoid shape or alternatively evolve into an ellipsoid. This concept is supported by the observation that higher developed species that consistently manifest biconvex ellipsoidal erythrocytes (e.g. avians), have red cells that arise from round (spherical?) precursors (proerythroblasts) (Lucas and Jamroz, 1961). The appearance of ellipsoidal rbc, first in some invertebrates and the subsequent retention of this form throughout the lower vertebrates (fish, reptiles, etc.), can be considered from a conceptual viewpoint as an evolutionary selection of a cellular configuration that offers advantages to fluid-suspended, freely existing, potentially circulating cells.

Investigations within the past decade have fostered an improved understanding of the cytoskeletal and other ultrastructural features of the erythrocytes of invertebrates (as well as vertebrates). It has been established by Cohen and Nemhauser (1985) and other workers that some erythrocytes in invertebrates (as well as the erythrocytes of essentially all submammalian vertebrates) possess a rather elaborate ultrastructural cytoskeleton composed of a marginal band of microtubules (MB) and a cell surface-associated cytoskeleton (SAC). These structures are believed to be responsible for the flattened, elliptical configuration of the red cells in these species. Hemoglobin-containing erythrocytes with this morphology have been identified in at least three invertebrate phyla, Annelida (e.g. *Travisia japonica*, a marine polychaete worm), Mollusca (e.g. *Anadara transversa* and *Noetia ponderosa*, blood clams) and Echinodermata (e.g. *Cucumaria miniata*, and *Molpadia arenicola*, sea cucumbers, (Fig. 22).

Fig. 22. *Molpadia arenicola*, a sea cucumber, the "sweet potato". This species, unlike many other holothurians, has essentially no podia over its body. This specimen has presumably withdrawn its tentacles and closed its body wall over them thereby hiding them from view.

The marginal band was initially observed under light microscopy in amphibian red cells in approximately 1900 but its significance was not keenly appreciated until the advent of analytical electron microscopy. It is composed of a bundle of perhaps 10-40 subplasmalemmal microtubules that circumscribes the periphery of an erythrocyte and lies in the plane parallel to the flat surface of the cell. The assembly of this circumferential band, at least in the blood clam erythrocyte, originates at or from two centrioles which have consistently been shown to act as organizing centers for the microtubules that comprise it. Its molecular structure is primarily the protein tubulin. The marginal band, in turn, is completely enclosed in an irregular network, the cell surface-associated cytoskeleton (SAC), which envelopes it as well as the nucleus and all the cytoplasmic organelles (Fig. 23). The SAC is a latticework of microfilaments

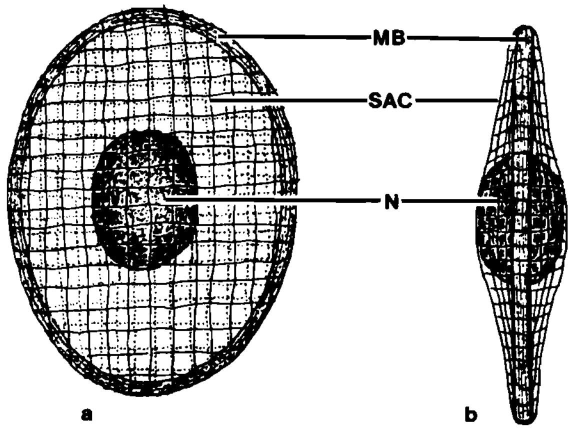

Fig. 23. Model of the nucleated erythrocyte cytoskeleton illustrating the relationship between the marginal band (MB) and the cell surface-associated cytoskeleton (SAC). Face view (a) and Edge view (b). The SAC completely encloses the MB and holds it under asymmetrical tension generating an oval configuration. The presence of a prominent nucleus (N) results in a central bulge and the biconvex profile typical of elliptical cells. Courtesy of Joseph-Silverstein and Cohen. Reproduced from the Journal of Cell Biology, 1984, Vol. 98, p 2124 by copyright permission of the Rockefeller University Press.

comprised predominantly of actin and «spectrin». It underlies and is associated (structurally linked) with the bilipid layer that makes up the plasmalemma. The cell surface-associated cytoskeleton is normally under tension and applies forces asymmetrically across the circumferential marginal band. The net effect of these forces is believed responsible for converting a potentially spheroidal erythrocyte into an ellipse as well as giving it a flatter profile. The flattened shape of some nucleated invertebrate (and lower vertebrate) erythrocytes is thus fostered by the development and orientation of the marginal band. The elliptical shape, conversely, is the result of two forces: the outward force of the coiled microtubules and the asymmetric force generated by the lattice of the SAC. The pressure that the SAC exerts against the nucleus accounts for its positional stability and the appearance of the nuclear bulge of «on edge» views of red cells. The marginal band of microtubules (and its surface-associated cytoskeleton) can be considered a structural component of cells that exist in a fluid compartment on a long term basis. When it appears in erythrocellular morphogenesis it is accompanied by the adoption of a flat ellipsoidal shape that apparently adapts the cell for existence in a flowing environment.

The marginal band is flexible and resilient to mechanical forces. It thus permits an erythrocyte to resist permanent deformation by springing back into its original shape following traumatic indentation, folding, etc. Experimental depolymerization of the marginal band (in most species it disassembles at 0° C within 1 hr) will not cause an erythrocyte to lose its oval shape; however if a such-treated cell is subsequently subjected to mechanical trauma it will buckle and fold and will be unable to return to the original shape (Joseph-Silverstein and Cohen, 1984). Conversely, digestion of the cell surface-associated cytoskeleton apparently releases the tension on the marginal band and results in the loss of the elliptical configuration and the assumption of a round, disc shape. It can be therefore stated that the SAC maintains the oval shape of a red cell when the cell is static while the MB imparts to the cell the capability of returning to this original form following physical trauma.

Although the marginal band has been demonstrated in some invertebrate erythrocytes, its absence has also been confirmed in the hemoglobin-laden cells of others, e.g. the polychaete annelid *Glycera dibranchiata*. As noted previously its red cells are spherical. Invertebrates that utilize hemerythrin as the respiratory pigment in their erythrocytes similarly may or may not incorporate a marginal band in their cells' cytoarchitecture (e.g. the marine worm *Phascolopsis gouldii*, the peanut worm, which exhibits a marginal band and *Themiste dyscrita* which does not). A particularly enlightening and intriguing erythrocyte is that found in the sea cucumber *Eupentacta quinquesemita* (Fig. 19). It not only expresses a maturational conversion from a sphere to an ellipsoid and back to a sphere again but also reveals an alternating arrangement of its cytoplasmic microtubules that is consistent with the shape of the cell (Fontaine and Hall, 1981). The youngest erythroid cell of this holothurian is spheroidal and its microtubules radiate from centriolar satellites and girdle the cell like the meridians of longitude on a geographer's globe. This arrangement would theoretically disperse any microtubular forces equally over the surface of the cell and permit a spherical shape. With further, but not total, maturation the cell assumes an ellipsoidal shape and at that time it has a distinct marginal band with characteristic bundling of the microtubules. Complete maturation is associated with a reversion to the spherical conformation and the symmetrical, dispersed distribution of the microtubules. Another characteristic feature of the young cells in this species, as seen under scanning electron microscopy, is the smooth surfaced plasmalemma as opposed to the mature forms whose plasma membrane has many surface foldings and a wrinkled appearance.

Although the marginal band is responsible for the ellipsoidal form of many erythrocytes the presence of a uniformly distributed system of microtubules has not been shown to be required for a spheroidal shape. Indeed, the assumption of a spheroidal vs. an ellipsoidal shape is not necessarily determined unifactorially, that is, on the basis of the presence or absence of a marginal band with or without nuclear retention. Although it is customary for lower vertebrates to have nucleated oval erythrocytes with a marginal band there are rare instances of amphibians (e.g. *Batrachoseps*) that manifest denucleated oval red cells that retain their marginal band, as well as mammals (e.g. camels) which extrude their erythrocytic nuclei and demonstrate a marginal band only in their normoblasts and reticulocytes but nevertheless elaborate permanently ellipsoidal red cells (Cohen, 1982; Cohen and Terwilliger, 1979). Finally, some humans have oval red cells (e.g. in hereditary elliptocytosis, megaloblastic anemia and severe iron deficiency anemia) even though, like other humans, they fail to develop a marginal band at any point of erythrocytic morphogenesis.

A sometimes dramatic surface feature that distinguishes certain invertebrate erythrocytes is their morphologic evidence of intense pinocytotic activity. This is evidenced in scanning electron microscopic studies (SEM) of the plasmalemmal surface as well as in transmission electron microscopy (TEM) of sectioned specimens. Erythrocytes from *Pista pacifica* studied under SEM reveal exceedingly numerous tiny surface «pits» which cover the entire surface of the cell (presumptive forming pinocytotic vesicles); TEM studies support this interpretation by revealing both open (forming) as well as formed, intact intracellular vesicles (Figs. 2, 3). Similar structures have been noted in red cells from the California Pacific coast sea worm *Urechis caupo* (Figs. 8-10). Pinocytotic activity is also moderately intense along the cell margin of *Cucumaria miniata* and *Glycera dibranchiata* erythrocytes. Of possible morphofunctional relationship is the pinocytotic activity in erythrocytes of higher evolved species. This can be seen in the spectrum extending from the primitive vertebrates (e.g. the hagfish *Eptrateus stoutii*) to the primates (e.g. *Homo sapiens*). Indeed, it is now established that even denucleated human erythrocytes devoid of cytoplasmic organelles manifest pinocytosis *in vivo* and *in vitro* (Sills et al., 1988).

Transmission electron microscopy in particular as well as other microscopic techniques reveal that the cytoplasmic structure of invertebrate erythrocytes is likely to be more complex and functionally more diversified than the mammalian model. In addition to the well-recognized persistence of the nucleus in invertebrate erythrocytes, some of these cells also tend to retain the cytoplasmic organelles that are otherwise discarded by maturing mammalian (human and non-human) normoblasts, e.g. golgi apparatus, mitochondria, and centrioles. Furthermore, it is not at all unusual to identify organelles and inclusions that are not a normal component of developing or mature mammalian red cells. Common examples include pigment granules, lysosomes, lipid droplets, a prominent glycogen component, large Prussian-blue positive masses, vacuoles and multivesicular structures. In addition to respiratory activities (storage or transportation of oxygen) it has been suggested that some of these cells may function as vendors of nutrients and even as scavengers. A specific example is the nutritive support provided for maturing germ cells which in some annelids reside in the coelomic fluid accompanied by hemoglobinized coelomocytes.

The erythrocytes (coelomocytes) observed in the polychaete families Terebellidae, Opheliidae and Cirratulidae contain large lipid stores and other materials in addition to hemoglobin and are therefore believed to have multiple functions (Dales and Dixon, 1981; Terwilliger et al., 1985). Representative animals having red cells with such characteristics include the terebellids (tube dwelling sea worms) *Pista pacifica* and *Terebella ehrenbergi* (Fig. 3). The latter worm has rbc with two populations of granules, colorless (lipid) and some with an orange color. The lipid granules, it is proposed, are eliminated by the action of lysosomes (Ochi, 1969). It is also observed that the red cells are largest when filled with lipid granules and smallest when the lysosomes are most abundant. Conversely, the (families) Capitellidae and Glyceridae have red cells that are apparently more specialized for respiratory activity. These erythrocytes have neither extensive lipid nor other marked granule stores (e.g. *Glycera americana*, *G. Decipiens* and *G. dibranchiata*). *Glycera siphonostoma* erythrocytes are remarkable in that they are ameboid and capable of phagocytic activity (e.g. uptake of carmine and india ink). The segmented, bristled worm *Amphitrite johnstoni* has also been cited as having red cells that appear phagocytic. Granular structures have been described in the red cells of the spoonworm *Urechis caupo* (Figs. 8-10). These cells have a large number of colorless lipid granules which are subsequently replaced by (or modified into) a population of brown, hematin-positive granules, another population of darkly pigmented granules (in male worms), and scattered glycogen deposits. A noteworthy activity of *Urechis caupo* rbc is the proposed transfer of granular hematin from mature erythrocytes in the female to its developing eggs (Baumberger and Michaelis, 1931). Comparable activity is not seen in the male. This translocation could serve as a source of recyclable material for the synthesis of hemoglobin for the benefit of the next generation. Two other echiuroids whose erythrocellular cytoplasm have a morphologic complexity comparable to *Urechis caupo* are *Urechis unicinctus* and *Thalassema gogoshimense* (Ohuye, 1937-1938a). Refractile granules in the latter two species' cells are described as soluble in fat solvents and stainable by the fat soluble stain Sudan III and osmium. Their lipid composition is consequently firmly documented. The rbc of the male in *Travisia japonica* have large colorless granules and lipid granules (some females' cells may have a few of each) in addition to the brownish non-lipid ones seen in both sexes. The number of the colorless granules has a seasonal variation with the maximum seen in June or July just prior to the breeding season. The previously described sex-linked difference in the size of this annelid's erythrocytes is attributable to their presence in the male (Ochi, 1969). The presence of oxidase-positive granules in red cells has been reported for at least some species of echiurids, polychaetes, molluscs, and holothurians. This is in contrast to vertebrates where the appearance of oxidase-positive granulation is restricted to leukocytes of myeloid origin. This is another feature that illustrates the differences between erythrocytes in lower and higly developed animals.

Another cytoplasmic organelle development and distribution pattern observed in certain invertebrate erythrocytes is the attainment of a maximal complement of cytoplasmic structures at the peak of synthetic activity followed by its reduction to a lower level which persists throughout the life span of the cell. A case in point is the echinoderm (holothurian) *Eupentacta quinquesemita* which retains a considerable quantity of cytoplasmic organelles throughout its existence (Fontaine and Hall, 1981). These include numerous mitochondria, lysosomes, some rough endoplasmic reticulum, polyribosomes, golgi apparatus, and centrioles (Figs. 20, 21). On the other hand the temporal continuity of erythrocytic organelles, though demonstrable, is reduced in its fellow sea cucumber *Cucumaria miniata* (Fontaine and Lambert, 1973). The total elimination of formed cytoplasmic structures from mature erythroid cells as seen in the higher vertebrates can be viewed as the ultimate extension of this reduction process. Another morphologic feature of occassional invertebrate erythrocytes is the presence of prominent cytoplasmic, iron-containing, Prussian blue-positive inclusions (siderosomes), e.g. *Cucumaria miniata* and particularly the blood clam *Anadara ovalis* (Nagel, 1985). It has been proposed that since some of the masses have been identified in lysosomes they may represent a mechanism for the digestion and exocytosis of effete hemoglobin. On the other hand, in some instances this material could be coupled or associated with an intracellular synthesis of new hemoglobin, particularly in those cells with a long life span.

Retention of the nucleus by the erythrocyte throughout the duration of its existence is a feature characteristic of invertebrate (and submammalian vertebrate) red cells. Nuclear morphology is variable and is dependent upon the level of cellular maturation. Nucleoli may or may not be present; their existence is considered evidence of active nuclear processing. An increased amount of heterochromatin and nuclear condensation are typically correlated with decreased nuclear synthetic activity and increased maturity (Figs. 13, 20, 21). The nucleus frequently condenses into a small pyknotic mass. At this point it is probably equivalent to the end stage mammalian normoblast nucleus which is synthetically inactive and awaits extrusion from the cell. In many species the nucleus is reduced to relatively disminutive dimensions. In *Phoronis*, *Lineus*, *Urechis* and *Thalassema* it can represent only 20% of the mature cell volume.

The nuclei may be round or oval. In ellipsoidal cells the nucleus is likely to conform to the cell's general configuration and assume an oval shape, e.g. hemocytes of *Cucumaria miniata*. The asymmetrical forces applied to a cell and its contents via the marginal band and the cell surface-associated cytoskeleton are no doubt important factors generating an oval shaped nucleus in a correspondingly ovoid cell. Of interest is the observation that a mature condensed spherical nucleus in a round red cell is not predictably centrally located. Although it is central in *Glycera decipiens* it is often eccentrically positioned in the cytoplasm of *Urechis caupo*, *Pista pacifica*, *Glycera dibranchiata*, *Phoronis australis* and *Thyone* (Figs. 3, 9). Binucleate cells are similarly not uncommon, e.g. *Eupentacta quinquesemita*, *Arca inflata*, and *Lineus fuscoviridis* (Fig. 13).

Intracellular crystallization of hemoglobin following a laboratory procedure such as supravital staining has been observed in some species. The crystals have been described as rhombic and in radial or parallel arrangement. They are seen in *Urechis unicinctus* and *Arca inflata* (Ohuye, 1937-1938a). Such affected cells have altered cell configurations and the crystals may even perforate the plasmalemma. Although this activity is typically not manifested by vertebrate red cells it has been observed in the amphibian *Necturus* (mud puppy). This also appears to be comparable to the intracellular crystallization of human hemoglobins S and C.

The simultaneous occurrence of both intra and extracellular (free) hemoglobin in the same organism has been observed (e.g., some terebellids, ophelids and arcids). A specific instance is the sand dwelling polychaete *Pista pacifica*. It has a vascular extracellular high molecular weight hemoglobin as well as its previously described hemoglobin-bearing coelomocyutes (Terwilliger and Koppenheffer, 1973). Another worm with free circulating hemoglobin and hemoglobinferous erythrocytes is *Travisia japonica*. In this instance hemoglobin is not only incorporated in the coelomocytes but it also occurs in the free state in the closed blood vascular system as well as the coelomic fluid (Ochi, 1969). This tantalizing phenomenon when viewed from a broad evolutionary perspective can be accepted as one of the many variable manifestations of respiratory pigment utilization. Though useful to a limited group of primitive animals this combination was not found necessary or advantageous for higher species.

Red cell counts, hematocrit and hemoglobin levels as well as other hematologic indices have been determined in some invertebrates. The results are incomplete partly because of the limitations in obtaining adequate samples of hemolymph or coelomic fluid from these frequently tiny subjects. It is also understood that the derived data are not necessarily comparable with those of mammalian blood since these fluids are not its physiologic or biochemical equivalent in all aspects. Nevertheless some interesting information has been generated. The number of erythrocytes in the coelomic fluid or hemolymph of invertebrates is characteristically small compared with that observed in the blood of mammalian vertebrates (approximately 5-8 x $10^6/\mu l$). Thus although the hemoglobin-bearing coelomocytes in the echinoderms *Eupentacta quinquesemita* and *Cucumaria miniata* are considered quite numerous their quantitative values are only in the order of 55 x 10^3 hemocytes/μl of coelomic fluid. *Paracaudina*, another echinoderm, has 15 x 10^4 erythrocytes/μl in its coelomic fluid. The blood or coelomic fluid of representatives from the echiurid (*Urechis caupo*), annelid *(Pista pacifica*, *Amphitrite ornata*, *Glycera dibranchiata*), and molluscan (*Noetia ponderosa*, *Anadara ovalis*) phyla have been described as having mean hematocrits in the order of 6-40 vol%. These values can be understandably relatively high at times even in the face of low red cell counts because of the large dimensions of the individual invertebrate erythrocytes. Hemoglobin concentrations in spite of some rather high hematocrits, however, are disproportionately low, characteristically in the 2-6gm/dl range as opposed to 12-15gm/dl for mammals. This can be ascribed primarily to a low concentration of respiratory pigment in the cytoplasm. In addition, the nucleus and perhaps other organelles acting as space occupying masses effect a further reduction in the amount of hemoglobin per unit cell volume. A case in point, *Glycera americana* erythrocytes, though recognized for their high level of hemoglobin by invertebrate standards, nevertheless manifest lower concentrations of hemoglobin than vertebrate rbc (Cowden, 1966). Some mean corpuscular hemoglobin concentrations (MCHC) were determined (apparently without an adjustment for nuclear volume) and typical values such as 6gm/100ml for *Pista pacifica*, 0.1-1.4gm/100ml for *Amphitrite ornata*, and 9-27gm/100ml for *Enoplobranchus sanguineus* were cited (Terwilliger et al., 1985). In one estimation of the MCHC in red cells of *Noetia ponderosa* that attempted to take into account the volume occupied by the nucleus the derived cellular hemoglobin concentration was approximately 40gm/100ml (Nagel, 1985). This is a rather remarkable concentration in view of the fact that it would be a high value even for well evolved species whose erythrocytes maximize the concentration of hemoglobin (e.g. the MCHC for human erythrocytes is 33gm/100ml RBC). It is apparent that erythrocytes in invertebrates do not achieve a capacity/efficiency for hemoglobin transport comparable to that of higher vertebrates.

The erythrocytes of the invertebrates and vertebrates have fundamental features in common. According to accepted evolutionary hypotheses the development of vertebrates and invertebrates bifurcated at an early point in phylogeny and thereafter manifested parallel evolution. As a result the invertebrate erythrocyte is not considered the progenitor of the red cell in the vertebrates. Even the erythrocyte of the echinoderm, a phylum which some authorities hold as in direct lineage with the chordates, is not necessarily ancestral to the subsequently evolved amphibian, reptilian, and mammalian red cells. Nevertheless, the spectrum of morphologic and functional erythrocellular characteristics observed among the invertebrates offers an exposition of the prototypes that are biologically attainable and metabolically supportive under different physiologic conditions. An appreciation of the invertebrate red cell consequently serves as a basis for understanding the erythrocyte of the higher evolved members of the animal kingdom.

Acknowledgements. The illustrations were rendered by Ms. Kathleen Long, medical illustrator and the typescript was prepared by Ms. Theresa D'Angelo. Colleagues Thomas Welch, Stephanie Iovino and Meena Chainani assisted in critical analysis of the text. The authors are greatly indebted to all these individuals for their professional interest.

References

Baumberger J.P. and Michaelis L. (1931). The blood pigments of urechis caupo. Biol. Bull. 61, 417-421.

Cohen W.D. (1982). The cytomorphic system of anucleate non-mammalian erythrocytes. Protoplasma 113, 23-32.

Cohen W.D. and Nemhauser I. (1985). Marginal bands and the cytoskeleton in blood cells of marine invertebrates. In: Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology. Cohen W.D. (ed). Alan R. Liss, New York. pp 3-49.

Cohen W.D. and Terwilliger N.B. (1979). Marginal bands in camel erythrocytes. J. Cell Sci. 36, 97-107.

Cowden R.R. (1966). A cytochemical study of the nucleated hemoglobin-containing erythrocytes of *Glycera americana*. Trans. Am. Microsc. Soc. 85, 45-53.

Dales R.P. and Dixon L.R.J. (1981). Polychaetes. In: Invertebrate Blood Cells. Vol. 1. Ratcliffe N.A. and Rowley A.F. (eds). Academic Press. New York. pp 35-74.

Dybas L. (1981). Sipunculans and echiuroids. In: Invertebrate Blood Cells. Vol. 1. Ratcliffe N.A. and Rowley A.F. (eds). Academic Press. New York. pp 161-168.

Fontaine A.R. and Hall B.D. (1981). The haemocyte of the holothurian *Eupentacta quinquesemita*: ultrastructure and maturation. Can. J. Zool. 59, 1884-1891.

Fontaine A.R. and Lambert P. (1973). The fine structure of the haemocyte of the holothurian, *Cucumaria miniata* (Brandt). Can J. Zool. 51, 323-332.

Glomski C.A. and Tamburlin J. (1989). The phylogenetic odyssey of the erythrocyte. I. Hemoglobin: the universal respiratory pigment. Histol. Histopath. 4, 509-514.

Gontcharoff M. (1961). Embrachement des némertiens. In: Traité de Zoologie. Tome IV. Grassé P. (ed). Masson et Cie., Editeurs, Paris. pp 783-886.

Hayward P.J. (1981). Lophophorates. In: Invertebrate Blood Cells. Vol. 2. Ratcliffe N.A. and Rowley A.F. (eds). Academic Press. New York. pp 491-509.

Joseph-Silverstein J. and Cohen W.D. (1984). The cytoskeletal system of nucleated erythrocytes. III. Marginal band function in mature cells. J. Cell Biol. 98, 2118-2125.

Lucas A.M. and Jamroz C. (1961). Aatls of Avian Hematology. United States Department of Agriculture Monograph 25, Superintendent of Documents. U.S. Government Printing Office. Washington, D.C. pp 1-271.

Meglitsch P.A. (1972). The acoelomate bilateria. In: Invertebrate Zoology. 2nd. ed. Oxford University Press. New York. pp 201-209.

Nagel R. (1985). Molluscan hemoglobins. In: Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology. Cohen W.D. (ed). Alan R. Liss. New York. pp 227-247.

Ochi O. (1969). Blood pigments and erythrocytes found in some marine annelida. Mem. Ehime Univ., Sci. Ser. B (Biol.) VI: pp 23-131.

Ohuye T. (1937-1938a). On the coelomic corpuscles in the body of some invertebrates. VII. On the formed elements in the body fluid of some marine invertebrates which possess the red blood corpuscles. Sci. Rep. Tohoku Imp. Univ. 12, 203-239.

Ohuye T. (1937-1938b). On the coelomic corpuscles in the body fluid of some invertebrates. XI. Supplementary observations on the cytoplasmic inclusions of red coloured corpuscles in the blood of some marine invertebrates. Sci. Rep. Tohoku Imp. Univ. 12, 623-628.

Ohuye T. (1942). On the blood corpuscles and the blood formation in Phoronis. Sci. Rep. Tohoku Imp. Univ. 17, 167-185.

Ohuye T. (1943). On the blood corpuscles and the hemopoiesis of a nemertean, *Lineus fuscoviridis*, and of a sipunculus, *Dendrostoma minor*. Sci. Rep. Tohoku Imp. Univ. 17, 187-196.

Pierce S.K. and Maugel T.K. (1985). A comparison of the water-regulatingresponses of bivalve and polychaete red cells to osmotic stresses. In: Blood Cells of Marine Invertebrates: Experimental Systems in Cell Biology and Comparative Physiology. Cohen. W.D. (ed). Alan R. Liss. New York. pp 167-189.

Sills R.H., Tamburlin J.H., Barrios N.J., Yeagle P.L. and Glomski C.A. (1988). Formation of intracellular vesicles in neonatal and adult erythrocytes: Evidence against the concept of neonatal hyposplenism. Ped. Res. 24, 703-708.

Terwilliger R.C. and Koppenheffer T.L. (1973). Coelomic cell hemoglobins of the polychaete annelid, *Pista pacífica* Berkeley. Comp. Biochem. Physiol. 45B, 554-556.

Terwilliger R.C. and Terwilliger N.B. (1983). Thermal vent clam *(Calyptogena magnífica)* hemoglobin. Science 219, 981-983.

Terwilliger N.B., Terwilliger R.C. and Schabtach E. (1985). Intracellular respiratory proteins of Sipuncula, Echiura and Annelida. In: Blood cells of marine invertebrates: Experimental systems in cell biology and comparative physiology. Cohen W.D. (ed). Alan R. Liss. New York. pp 193-225.

Vandergon T.L. and Colacino J.M. (1989). Characterization of hemoglobin from *Phoronis architecta* (phoronida). Comp. Biochem. Physiol. 94B, 31-39.