Association of Centrioles with the Marginal Band of a Molluscan Erythrocyte

WILLIAM D. COHEN and IRIS NEMHAUSER

Department of Biological Sciences, Hunter College of The City University of New York, New York 10021, and The Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT Continuous circumferential bundles of microtubules, or marginal bands (MBs), are best known as a prominent structural feature of all nonmammalian vertebrate erythrocytes and mammalian blood platelets. Since their discovery in the late 19th century, MBs have been thought to play a cellular morphogenetic role, but no cytological clues to the mechanism of MB biogenesis have been reported.

In previous work we have established the presence of MBs in several invertebrate blood cell types, including amebocytes and coelomocytes of certain Arthropod species and erythrocytes of a Sipunculan. We report here the occurrence of MBs in erythrocytes of the ark *Anadara transversa* (Mollusca) and four closely related species. The MBs of these arks have a striking structural feature; each is physically associated with a pair of centrioles. The centrioles are identified as such on the basis of morphological criteria: size, cylindrical shape, right-angle orientation, pairing, and 9-triplet ultrastructure. This intimate association between centrioles and MBs suggests that centrioles may be MB-organizing centers and invites comparative investigation of their possible role in vertebrate erythrocyte and platelet morphogenesis.

Marginal bands (MBs) are continuous peripheral bundles of microtubules, observed classically in the plane of flattening of all nonmammalian vertebrate erythrocytes and of mammalian platelets (3, 5, 14–16, 30). They are generally believed to function as cytoskeletal elements and to play a role in cellular morphogenesis (2, 7, 17, 19, 22, 31). Since their discovery in 1895 (12), MBs have been studied in numerous animal species by light and electron microscopy, but no cytological clues to their mechanism of formation have been reported.

We have been examining invertebrate blood cells with the objectives of establishing the phylogenetic distribution of the MB system and discovering cell types that are of experimental value for its study. In previous work we have found MBs in Arthropod blood cells, including *Limulus* amebocytes (horseshoe crab clotting cells), coelomocytes of *Homarus americanus* (lobster) and several marine crab species, and in erythrocytes of a Sipunculan (unsegmented marine worm; 9, 10, 25). We report here the presence of MBs in the hemoglobin-containing erythrocytes of the ark *Anadara transversa* and its close relatives ("blood clams"; Mollusca). A striking feature of these molluscan erythrocyte MBs is the direct physical association of each with a pair of centrioles[1] (8).

[1] Use of the term "centriole" in this paper is based upon morphological criteria only. All structures in this category are not necessarily functionally equivalent (for example, mitotic centrioles vs. basal bodies).

MATERIALS AND METHODS

Anadara transversa ("transverse ark") and *Anadara ovalis* ("blood ark") specimens were obtained at the Marine Biological Laboratory, Woods Hole, Mass. *Anadara brasiliana* ("incongruous ark"), *Anadara lienosa floridana* ("cut-ribbed ark") and *Noetia ponderosa* ("ponderous ark") were obtained from the Gulf Specimen Co., Panacea, Florida. Animals were maintained in the MBL running sea water system or in tanks of "Instant Ocean" formula artificial sea water (Aquarium Systems, Inc., Eastlake, Ohio) at Hunter College. Species identification was made by means of published guides (1, 18) in consultation with suppliers.

To prevent premature puncture of tissues and subsequent blood loss or mixture with internal sea water, the animals were opened in the following manner: a small hole was created by chipping or filing the meeting edges of the shells. A probe was inserted and worked in parallel to the shell edge, followed by a clam knife for larger animals. The shells were then pried apart sufficiently by hand or by rotation of the knife to prevent reclosure. Most of the blood was in the mantle cavity lining both shells, from which it was removed by Pasteur pipette (smaller specimens) or by puncture and drainage into a beaker (larger specimens). In all experiments blood samples were used immediately upon removal from the animal. In a few instances the blood of *A. transversa* was murky brown rather than red, and contained relatively few erythrocytes; such blood was not used in experiments. Molluscan Ringer's solution (6, 32) served as blood diluent. No clotting occurred with or without diluent, obviating the need for anticoagulants.

For rapid visualization of the erythrocyte cytoskeletal system, blood was diluted directly (~1:10 vol/vol) into modified microtubule polymerization medium (34) containing Triton X-100 (Triton) as the lytic agent. The lytic medium (LyM) consisted of 100 mM PIPES, 1 mM $MgCl_2$, 10 mM TAME (*p*-tosyl-L-arginine methyl ester HCl), 5 mM EGTA, 0.4% Triton X-100, pH 6.8, as used previously for studies of MBs in a wide range of vertebrates and invertebrates (7, 10, 11, 25) and for mitotic spindle stabilization (23, 28).

When blood samples were examined immediately upon removal from the

THE JOURNAL OF CELL BIOLOGY · VOLUME 86 JULY 1980 286–291

animal, the erythrocytes were consistently flattened, usually elliptical, and smooth in contour. However, they lost this native morphology rapidly during incubation in glassware or in contact with slide/coverslip at room temperature, becoming more spherical and irregular in contour ("potato-shaped"). Therefore, for purposes of photography, freshly drawn cells were fixed in molluscan Ringer's solution containing 1% glutaraldehyde (9 vol Ringer's + 1 vol 10% glutaraldehyde). Living, fixed, and lysed cells were examined and photographed with a Zeiss phase contrast microscope, using × 40 and × 100 oil-immersion objectives (0.75 and 1.3 NA).

Uranyl acetate–stained cytoskeleton whole mounts for TEM were prepared as follows: samples of lysed erythrocytes from *A. transversa* (1 drop of fresh blood/0.5 ml of LyM) were placed on Formvar-coated grids for 20 min. Fluid was drawn off with filter paper (as in subsequent steps) and replaced successively by LyM lacking Triton and TAME (= wash medium), wash medium containing 2.5% glutaraldehyde for 12–15 min, wash medium, water, and 2% aqueous uranyl acetate. The last was drawn off and the grids air-dried.

Cytoskeletal ultrastructure was also examined in thin sections. In initial experiments with sections from whole fixed cells, excessive hemoglobin electron density was found to obscure structural features. Therefore, the following method involving simultaneous lysis and fixation was employed: 0.8 ml of blood (pooled from three specimens of *A. transversa*) was centrifuged gently, yielding a 0.2-ml cell pellet. The supernatant fluid was removed, and the cells were resuspended to 1 ml in LyM containing 1% glutaraldehyde, pH 6.8. After fixation for 1 h at 16°C, the cells were postfixed for 1 h in 0.1 M sodium phosphate buffer containing 0.1% OsO_4, pH 6, at 0°C (20). The material was then rinsed in water at 0°C, stained en bloc in 1% uranyl acetate during ethanol dehydration, infiltrated with propylene oxide–Epon, and embedded in Epon. Sections were cut with a diamond knife on a Sorvall MT-2 ultramicrotome (DuPont Co., Sorvall Biomedical Div., Wilmington, Del.) and stained with uranyl acetate (saturated, in 50% ethanol) and Reynolds' lead citrate. These sections and the whole mounts described above were examined in the Hitachi HS-8 TEM operating at 50 kV.

RESULTS

Living erythrocytes of all ark species examined are highly flattened, nucleated, and usually elliptical as observed in fresh blood samples. As the shape is quite labile with even brief storage, however, it may be preserved in essentially native condition by fixing the cells immediately in molluscan Ringer's solution containing 1% glutaraldehyde (Fig. 1 *a*). Hemoglobin tends to obscure both MB and nucleus in the intact cell, but refractile inclusions are visible. Suspension of the erythrocytes in LyM immediately clears them of hemoglobin, rendering nucleus, granular inclusions, and MB easily visible under phase contrast (Fig. 1 *b* and *c*). The MBs appear as continuous elliptical bands of intermediate phase density, each containing at one location along the ellipse a pair of centrioles observable as two phase-dense "dots" under oil immersion. The centriole pairs were present in association with the MBs of erythrocytes from all of the ark species investigated.

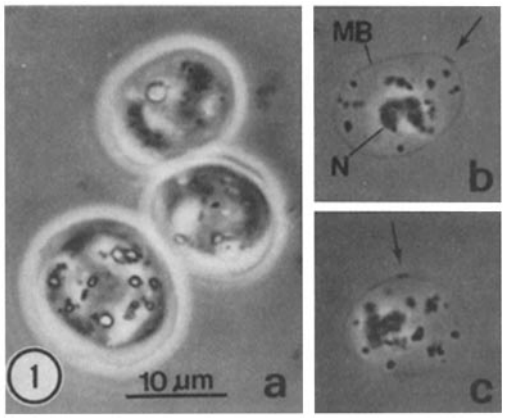


FIGURE 1 Erythrocytes and erythrocyte "cytoskeletons" of the ark *Anadara transversa*, as seen in phase contrast. (*a*) Cells freshly fixed in molluscan Ringer's solution containing 1% glutaraldehyde. The cells are highly flattened, generally elliptical, and contain refractile bodies. Axes are typically in the 10–15 μm range. (*b* and *c*) Examples of cytoskeletons prepared in LyM. Hemoglobin is immediately cleared by Triton, revealing elliptical MB, nucleus (*N*), cytoplasmic particles (organelles?), and a pair of centrioles visible as phase-dense "dots" within or upon each MB (arrows). The centriole pair is usually found near one end of the MB ellipse, as in *b*, but is sometimes observed at one side of the ellipse, as in *c*. Phase contrast, × 1,300.

Further detailed light and electron microscopic observations were made on *A. transversa* erythrocytes. In this species, the centrioles are usually close together on the MB, but occasionally there is a considerable gap between them. There is only one pair per MB, typically occurring near one end of the ellipse, as in Fig. 1 *b*, but sometimes at one side of the ellipse as in Fig. 1 *c*. The nucleus and other organelles/inclusions are trapped within the area circumscribed by the MB, but the structural basis for this entrapment is not evident in phase contrast.

All of these structures are observed in glutaraldehyde-fixed, uranyl acetate–stained lysed cell whole mounts in TEM (Fig. 2). In addition, a trans-MB network of fibrous material can now be seen (Fig. 2 *a*; *Net*) in which all cellular structures appear to be enmeshed, and which presumably accounts for their positional stability after cell lysis. The MB appears as a continuous band, typically twisted at some point into a figure eight. The centrioles are highly visible in the whole mounts because of great electron density, surpassing that of the MB (Fig. 2 *a*). The impression gained from studying numerous whole mounts is that the centrioles are embedded within or attached to the MB. Microtubules are often observed radiating from one or both members of the centriole pair (Fig. 2 *a* and *b*), and these are particularly prominent in the minority of cases in which the centriole pair is attached to the cytoplasmic side of the MB rather than being on or within it (Fig. 3).

In some whole mounts, fortuitous views reveal structural features typical of centrioles in other systems, including approximate right-angle pairing (Fig. 4 *a*), "hollow" cylindrical shape with one closed end (Fig. 4 *a* and *c*), and appropriate size (~0.17 × 0.25 μm). The centrioles are similar in diameter to the sperm flagellum of the same species (Fig. 4 *c* and *inset*) observed as a contaminant in the whole-mount preparations, and exhibiting typical "9 + 2" ultrastructure in our thin sections (not shown).

Varying degrees of separation between members of the centriole pairs can be observed in the TEM whole mounts (Fig. 5), ranging from none (Fig. 5 *a*) to as much as 2 μm (Figure 5 *f*) in terms of intervening length along the MB. The smaller degrees of separation are by far the most common.

Thin sections of cells simultaneously lysed and fixed reveal typical "9-triplet + 0" centriolar construction (Fig. 6 *a*) and right-angle cylinder orientation (Fig. 6 *b*). The centrioles appear to be coated with fuzzy material through which some of the MB microtubules insert or pass (Fig. 6 *a* and *b*). The number of microtubules in the *A. transversa* MB, as counted in cross section, is in the 40–50 range. In longitudinal sections of the MB, wispy bits of material are observed projecting from the microtubule surfaces (Fig. 6 *c*), perhaps accounting for the gap usually evident between adjacent microtubules in both longitudinal and cross section (Fig. 6 *a* and *c*). In cross sections of the MB, the centrioles often appear to be situated among the MB microtubules, with the greatest microtubule density being between the centriole and the trans-MB network (Fig. 6 *a*). The

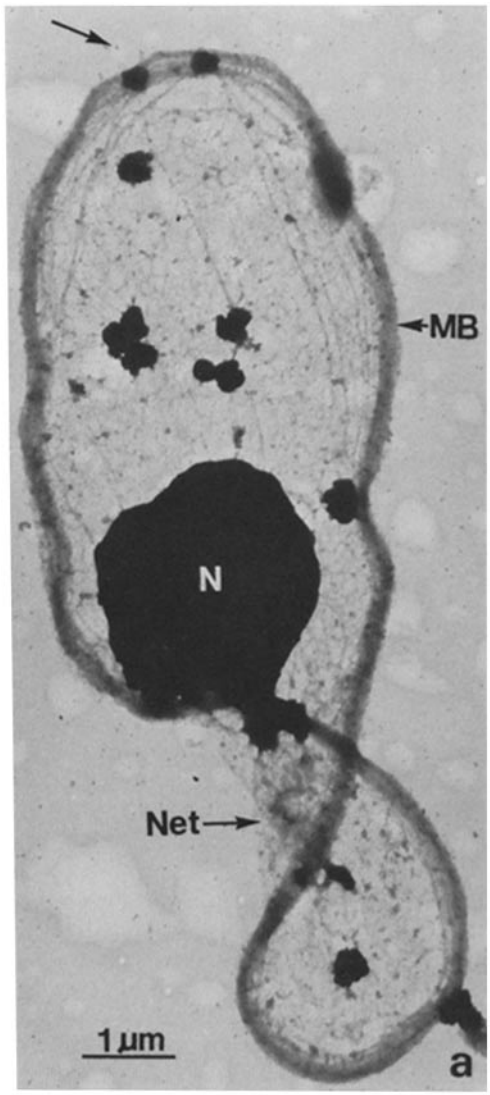
latter is always found in section to be peripheral to the MB.

DISCUSSION

The centrioles observed in association with the MBs of the molluscan erythrocytes examined have structural features typical of centrioles in general: cylindrical morphology with one closed end, 9-triplet + 0 cross-sectional ultrastructure, and (often) right-angle orientation. Their size is similar to that of centrioles and basal bodies in other systems (13). Although their function in this system remains to be demonstrated, the presence of a centriole pair within or upon each MB immediately suggests a role as microtubule-organizing centers (MTOC; 21, 27) for MB formation. The structural observations reported here indicate that each member of the centriole pair is (or can be) functional, as each is associated with radiating microtubules in at least some cells. Their separation in a significant number of cells is suggestive of individual but coordinated function, precedents for which occur in the case of paired basal bodies in certain species (29), and in the early stages of centriole pair separation during animal cell prophase (13). The centrioles are usually found near one end of the MB ellipse, but are occasionally at the side. If they do have a role in MB formation, it would thus appear to be independent of position on the MB or, alternatively, to involve movement along the MB.

Although the most obvious role for these MB-associated centrioles would be in MB formation and perhaps maintenance, other possibilities are worthy of consideration. The centrioles might be inactive remnants of the last mitotic division, banished to the cell periphery. It is also conceivable that these molluscan erythrocytes retain a capacity for mitotic division subsequent to differentiation, and/or that they have more than a respiratory function in the animals (unlikely alternatives; a search of the literature yields no information on them). The developmental history of the MB-associated centrioles is of central importance to the problem, but the hematopoietic organ of the blood arks (if any) has not yet been identified (33).

The ark erythrocyte cytoskeleton is in general respects similar to that of nonmammalian vertebrate erythrocytes in consisting of MB plus trans-MB network (7). Might centrioles be involved in MB formation in these other species? Direct examination of the MB in similarly lysed nonmammalian vertebrate erythrocytes has not revealed such obvious structures. However, centrioles could be present during the period of MB formation in hematopoietic tissue and subsequently disassembled, destroyed, or hidden somewhere in the mature cell. Initial investigation of dogfish erythrocyte cytoskeletons by use of



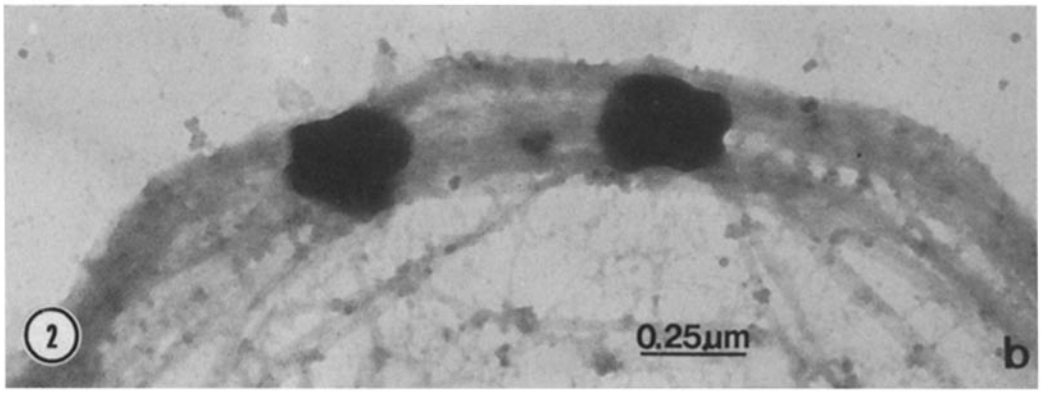


FIGURE 2 (*a*) Typical cytoskeleton whole mount (*A. transversa*) as seen with transmission EM after glutaraldehyde fixation and uranyl acetate staining. The MB is visible as a continuous peripheral fibrous bundle (*MB*) twisted into a figure eight, with the centrioles appearing to be embedded within it at the upper end (arrow). Extra-MB microtubules radiate from at least one of the centrioles, as shown at higher magnification in *b*. Nucleus (*N*) and cytoplasmic particles are densely stained, and surrounded by the second major cytoskeletal component, a trans-MB network (*Net*) most easily seen near the figure eight crossover point. TEM, × 12,000 and × 54,000 respectively.

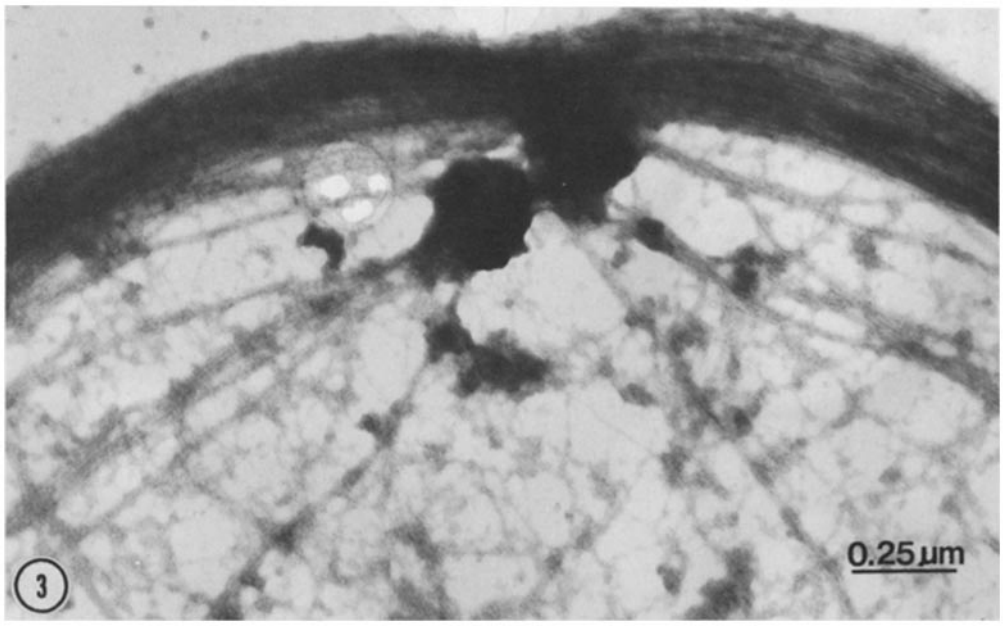


FIGURE 3 A pair of centrioles appearing to be attached to the cytoplasmic surface of the MB rather than embedded within the MB. Each of the centrioles has extra-MB microtubules radiating from it. Whole mount, *A. transversa*; TEM, × 54,000.

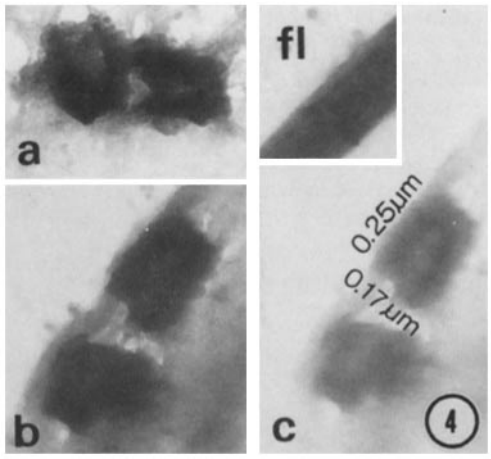


FIGURE 4 Structural features of the centrioles as observed in whole mounts include approximately right-angle orientation in some cases (*a*), and hollow, cylindrical appearance (*a* and *b*). In *c*, an underexposed print of the same pair as in *b* more clearly reveals the electron-translucent interior, and shows one end of each cylinder "closed" by electron-dense material. Typical dimensions are given in *c*; centriole diameter in whole mount is comparable to that of the 9 + 2 sperm flagellum of the same species (*inset, fl*), which is often found contaminating the preparations. *A. transversa*; TEM, × 54,000.

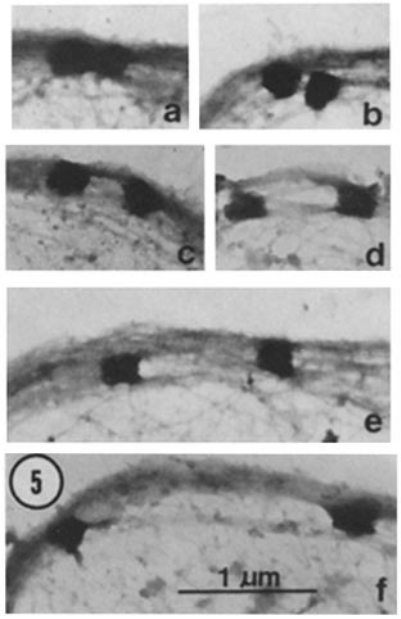


FIGURE 5 Variation in the extent of separation between members of the centriole pairs observed in different cytoskeleton whole mounts. The separation distance is usually small, as in a-c, ranging from none (*a*) to ~0.25 μm (*c*). However, greater separation is sometimes observed, as in *d*, *e*, and *f*, ~0.5, 0.8, and 2.0 μm (length along MB), respectively. *A. transversa*; TEM, × 18,000.

indirect antitubulin immunofluorescence reveals paired fluorescing structures in at least some cells (a minority; 24). Though preliminary, these results indicate that centrioles may be present in vertebrate erythrocytes as well, and a careful search for them in differentiating cells is warranted.

It has long been known that MBs are a prominent structural feature of all mammalian blood platelets (3, 30). Are centrioles involved in platelet morphogenesis? Developing megakaryocytes, the large cells that ultimately produce platelets, are known to undergo unique multipolar mitoses (26) and to contain numerous centrioles (4). Centrioles have also been observed on occasion in platelets in association with the MB (35), but it is not clear what percentage of platelets contain them. Perhaps in the light of the present observations on the blood ark erythrocyte MB, the possible role of centrioles in platelet MB formation should be evaluated.

In the present work, MB-associated centrioles have been found in all five of the closely related ark species examined, including two from colder northern waters and three from warmer southern waters. It is therefore likely that they are a universal feature of erythrocytes of the Arcidae, and will be found in similar "blood clams" available for study worldwide

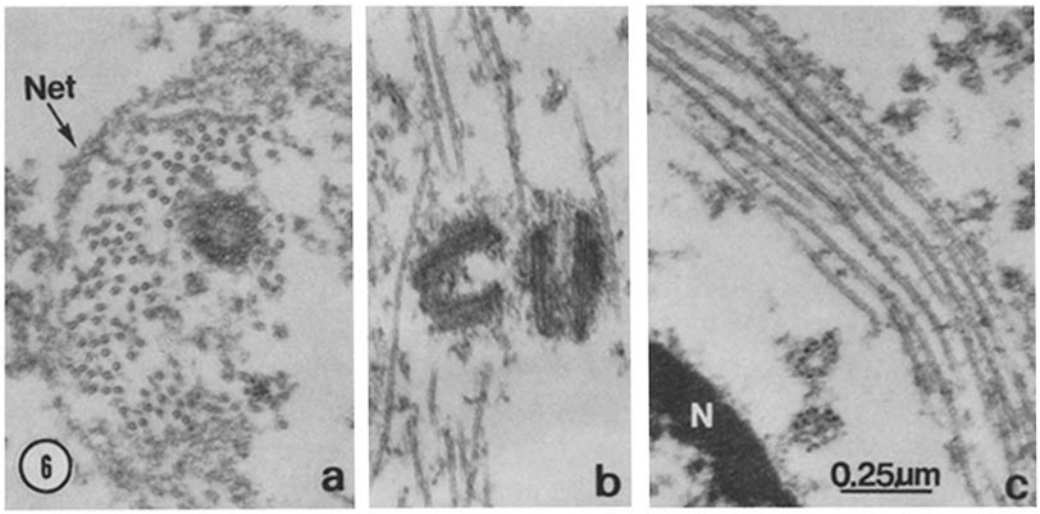


FIGURE 6 Cytoskeletal structure in thin sections of *A. transversa* erythrocytes that were lysed and fixed simultaneously. (*a*) Cross section of MB and one of its associated centrioles. The latter shows typical 9-triplet organization, and has "fuzzy" material coating its surface. About 50 sectioned microtubules are present, a few of which are on the cytoplasmic side of the centriole. The network material (*Net*) appears rather diffuse in section, but characteristically coats the outer surface (plasma membrane side) of the MB microtubule assemblage. (*b*) A centriole pair in near longitudinal section, showing right-angle orientation and cylindrical structure with one "closed" end. Fuzzy material, with which adjacent microtubules make contact, coats the centrioles. (*c*) MB in longitudinal section. Microtubules show gentle curvature, and thin wisps of material are present on microtubule surfaces. *N*, nucleus. TEM, × 47,000.

(for example, *A. senilis*, the "bloody cockle" of West Africa, and *A. trapezia*, the "Sydney cockle" of Australia; 33, 36). Though typical of these species, hemoglobin-containing erythrocytes are a rarity for the Mollusca in general.

We wish to thank Drs. Lynn Amende and Sidney K. Pierce (University of Maryland) for showing us unpublished electron micrographs of *Noetia ponderosa* erythrocytes that independently confirm the presence of MBs in these cells, and for their general advice and assistance during early stages of the work. We are also indebted to Dr. Richard Hoffmann (University of Pittsburgh) and Dr. W. D. Russell-Hunter (Syracuse University) for valuable discussion, to the Marine Biological Laboratory, Marine Resources Department, Woods Hole, Massachusetts, for their efforts in supplying *Anadara ovalis* and *transversa*, and to Dr. Jack Rudloe of the Gulf Specimen Co. for suggesting and supplying the southern species. Finally, W. D. Cohen wishes to express his gratitude to Prof. A. W. Pollister (Columbia University), who imbued his cytology students with his fascination for cellular organization and for centrioles in particular.

This work was supported by City University of New York PSC-BHE grants 12260 and 13051 and by National Institutes of Health grant HL 20902.

Received for publication 21 January 1980, and in revised form 12 March 1980.

REFERENCES

1. Abbott, R. T. 1954. *American Seashells*. D. Van Nostrand Co., Inc., Princeton, N. J.
2. Barrett, L. A., and R. B. Dawson. 1974. Avian erythrocyte development: Microtubules and the formation of the disk shape. *Dev. Biol.* 36:72–81.
3. Behnke, O. 1965. Further studies on microtubules. A marginal bundle in human and rat thrombocytes. *J. Ultrastruct. Res.* 13:469–477.
4. Behnke, O. 1969. An electron microscope study of the rat megakaryocyte. II. Some aspects of platelet release and microtubules. *J. Ultrastruct. Res.* 26:111–129.
5. Behnke, O. 1970. A comparative study of microtubules of disk-shaped blood cells. *J. Ultrastruct. Res.* 31:61–75.
6. Cavanaugh, G. M., editor. 1975. Formulae and Methods VI of the Marine Biological Laboratory Chemical Room. The Marine Biological Laboratory, Woods Hole, Mass. 6th edition.
7. Cohen, W. D. 1978. Observations on the marginal band system of nucleated erythrocytes. *J. Cell Biol.* 78:260–273.
8. Cohen, W. D., and I. Nemhauser. 1979. Association of centriole-like structures with the marginal band of a molluscan red cell. *Biol. Bull. (Woods Hole).* 157:363. (Abstr.).
9. Cohen, W. D., I. Nemhauser, and M. F. Cohen. 1978. Marginal bands of *Homarus americanus* coelomocytes: Disappearance associated with changes in cell morphology. *Biol. Bull. (Woods Hole).* 155:431–432.
10. Cohen, W. D., I. Nemhauser, and R. Jaeger. 1977. Rapid visualization of the marginal band system in blood cells of marine species. *Biol. Bull. (Woods Hole).* 153:420. (Abstr.).
11. Cohen, W. D., and N. B. Terwilliger. 1979. Marginal bands in camel erythrocytes. *J. Cell Sci.* 36:97–107.
12. Dehler, A. 1895. Beitrag zur Kenntnis des feineren Baues der roten Blutkörperchen beim Hühnerembryo. *Arch. Mikrosk. Anat. Entwicklungsmech.* 46:414–430.
13. Dustin, P. 1978. Microtubules. Springer-Verlag, Inc., New York.
14. Fawcett, D. W. 1959. Electron microscopic observations on the marginal band of nucleated erythrocytes. *Anat. Rec.* 133:379.
15. Fawcett, D. W., and F. Witebsky. 1964. Observations on the ultrastructure of nucleated erythrocytes and thrombocytes with particular reference to the structural basis of their discoidal shape. *Z. Zellforsch. Mikrosk. Anat.* 62:785–806.
16. Goniakowska-Witalińska, L., and W. Witaliński. 1976. Evidence for a correlation between the number of marginal band microtubules and the size of vertebrate erythrocytes. *J. Cell Sci.* 22:397–401.
17. Goniakowska-Witalińska, L., and W. Witaliński. 1979. Occurrence of microtubules during erythropoiesis in *Llama glama*. *J. Zool. (Lond.).* 181:309–313.
18. Gosner, K. L. 1971. Guide to Identification of Marine and Estuarine Invertebrates. John Wiley & Sons, Inc., New York.
19. Maser, M., and C. W. Philpott. 1964. Marginal bands in nucleated erythrocytes. *Anat. Rec.* 150:365–382.
20. Maupin-Szamier, P., and T. D. Pollard. 1978. Actin filament destruction by osmium tetroxide. *J. Cell Biol.* 77:837–852.
21. McGill, M., and B. R. Brinkley. 1975. Human chromosomes and centrioles as nucleating sites for the in vitro assembly of microtubules from bovine brain tubulin. *J. Cell Biol.* 67: 189–199.
22. Meves, F. 1911. Gesammelte Studien an den roten Blutkörperchen der Amphibien. *Archiv. Mikroskop. Anat. Entwicklungsmech.* 77:465–540.
23. Milsted, A., W. D. Cohen, and N. Lampen. 1977. Mitotic spindles of *Drosophila melanogaster*: a phase-contrast and scanning electron-microscope study. *J. Cell Sci.* 23:43–55.
24. Nemhauser, I., W. D. Cohen, A. Milsted, and R. D. Goldman. 1979. Marginal band systems in blood cells of marine species: Visualization by indirect immunofluorescence. *Biol. Bull. (Woods Hole).* 157:384–385.
25. Nemhauser, I., R. Ornberg, and W. D. Cohen. 1980. Marginal bands in blood cells of invertebrates. *J. Ultrastruct. Res.* 70:308–318.
26. Penington, D. G. 1979. The cellular biology of megakaryocytes. *Blood Cells.* 5:5–10.
27. Pickett-Heaps, J. D. 1969. The evolution of the mitotic apparatus: An attempt at comparative cytology in dividing plant cells. *Cytobios.* 1:257–280.
28. Rebhun, L. I., J. Rosenbaum, P. Lefebre, and G. Smith. 1974. Reversible restoration of the birefringence of cold-treated, isolated mitotic apparatus of surf clam eggs with chick brain tubulin. *Nature (Lond.).* 249:113–115.
29. Rosenbaum, J. L., J. E. Moulder, and D. L. Ringo. Flagellar elongation and shortening in *Chlamydomonas*. The use of cyclohexamide and colchicine to study the synthesis and assembly of flagellar proteins. *J. Cell Biol.* 41:600–619.
30. Sandborn, E. B., J. LeBuis, and P. Bois. 1966. Cytoplasmic microtubules in blood platelets. *Blood.* 27:247–252.
31. Sekhon, S. S., and H. W. Beams. 1969. Fine structure of the developing trout erythrocytes

and thrombocytes with special reference to the marginal band and the cytoplasmic organelles. *Am. J. Anat.* 125:353–374.
32. Singh, I. 1938. The effect of the interaction of ions, drugs and electrical stimulation as indicated by contraction of the anterior retractor of the byssus of *Mytilus edulis*. *J. Physiol. (Lond.)*. 92:62–90.
33. Sullivan, G. E. 1961. Functional morphology, micro-anatomy, and histology of the "Sydney cockle," *Anadara trapezia* (Deshayes) (Lamellibranchia: Arcidae). *Aust. J. Zool.* 9:219–257.
34. Weisenberg, R. C. 1972. Microtubule formation *in vitro* in solutions containing low calcium concentrations. *Science (Wash. D. C.)*. 177:1104–1105.
35. White, J. G. 1971. Platelet morphology. *In* The Circulating Platelet. S. A. Johnson, editor. Academic Press, Inc., New York.
36. Yoloye, V. 1975. The habits and functional anatomy of the West African bloody cockle, *Anadara senilis* (L.). *Proc. Malacol. Soc. Lond.* 41:277–299.