Centrioles as Microtubule-organizing Centers for Marginal Bands of Molluscan Erythrocytes

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ABSTRACT   The erythrocytes of blood clams (Arcidae) are flattened, elliptical, and nucleated.
They contain elliptical marginal bands (MBs) of microtubules, each physically associated with
a pair of centrioles (Cohen, W., and I. Nemhauser, 1980, J. Cell Biol., 86:286–291). The MBs
were found to be cold labile in living cells, disappearing within 1–2 h at 0°C. After the cells had
been rewarmed for 1–2 h, continuous MBs with associated centrioles were once again present.
Time-course studies utilizing phase contrast, antitubulin immunofluorescence, and electron
microscopy of cytoskeletons prepared during rewarming revealed structural evidence of cen-
triole participation in MB reassembly. At the earliest stage of reassembly, a continuous MB was
not present. Instead, relatively short and straight microtubules focused on a pointed centriolar
"pole," and none were present elsewhere in the cytoskeleton. Thin continuous MBs then
formed, still pointed in the centriolar region. Subsequently, the MBs regained ellipticity, with
their thickness gradually increasing but not reaching that of controls even after several hours
of rewarming. At these later time points, microtubules still radiated from the centrioles and
joined the MBs some distance away. In the presence of 0.1 mM colchicine, MB reassembly was
arrested at the pointed stage. Electron microscopic observations indicate that pericentriolar
material is involved in microtubule nucleation in this system, rather than the centriolar triplets
directly. The results suggest a model in which the centrioles and associated material nucleate
assembly and growth of microtubules in diverging directions around the cell periphery.
Microtubules of opposite polarity would then pass each other at the end of the cell distal to
the centrioles, with continued elongation eventually closing the MB ellipse behind the centriole
pair.

Spatial and temporal control of microtubule (MT) assembly in living cells remains an area of critical importance for understanding cellular morphogenesis. In the case of cilia, flagella, mitotic spindles of animal cells, and cytoplasmic MT networks, centrioles and basal bodies are believed to function as MT-organizing centers (32), but the mechanisms involved are unknown. In many instances including both living cells and in vitro systems, centriole function appears to be indirect in that MT-organizing activity has been localized to pericentriolar material (10, 22, 35, 38). Theoretical and experimental analysis of MT polarity supports the view that the "+", or assembly end, of the MT is distal to such centriole-containing organizing centers (9, 15, 24, 26).

The marginal band (MB) of nonmammalian vertebrate erythrocytes (17), mammalian blood platelets (4), and various other blood cell types (1, 18, 21, 30, 39) is a distinctive MT system with respect to spatial organization. It is restricted to a circumferential location in the plane of cell flattening, just beneath (but not touching) the plasma membrane bilayer. Cells containing MBs have few, if any, additional cytoplasmic MTs. Thus the MB appears to have potential value for studying the genesis of MT systems. Although little is known about MB formation in any cell type, we reported previously that centrioles were associated with the MBs of "blood clam" erythrocytes, and suggested that they may serve as MB-organizing centers in these species (14). In the work described here we have utilized low temperature MB lability in these cells to test that proposal.
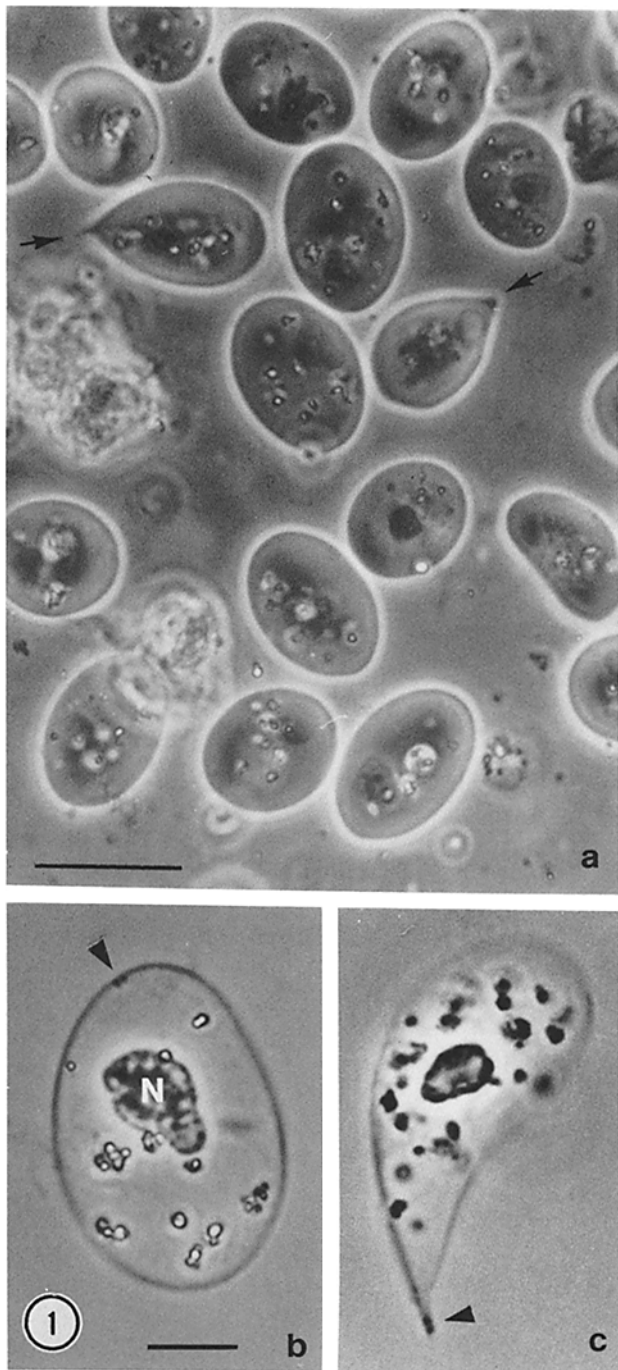
FIGURE 1 "Blood clam" erythrocytes and their cytoskeletons. (a) Living erythrocytes of *Noetia ponderosa*. Nearly all of the cells are flattened and elliptical, but in rare instances they have a point at one end. This field is exceptional in having two such cells (arrows). (b) *N. ponderosa* erythrocyte lysed in LyM. Arrowhead points to the MB-associated centrioles, which are usually located at or near one end of the MB ellipse. Nuclei (*N*) and other organelles remain suspended in position by phase-transparent material. (c) Pointed erythrocyte cytoskeleton of *Anadara spp.* In this species most of the erythrocytes are elliptical, but a significant number have points at one end as shown for two of the *N. ponderosa* cells in (a). A comparable number of single-pointed MBs are seen when the cells are lysed, and the centrioles are invariably located at the points (arrowhead). Centrioles are similarly located in pointed *Noetia* cytoskeletons (not shown). Phase contrast: (a) Bar, 20 μm; × 960. (b and c) Bar, 5 μm; × 2,300.
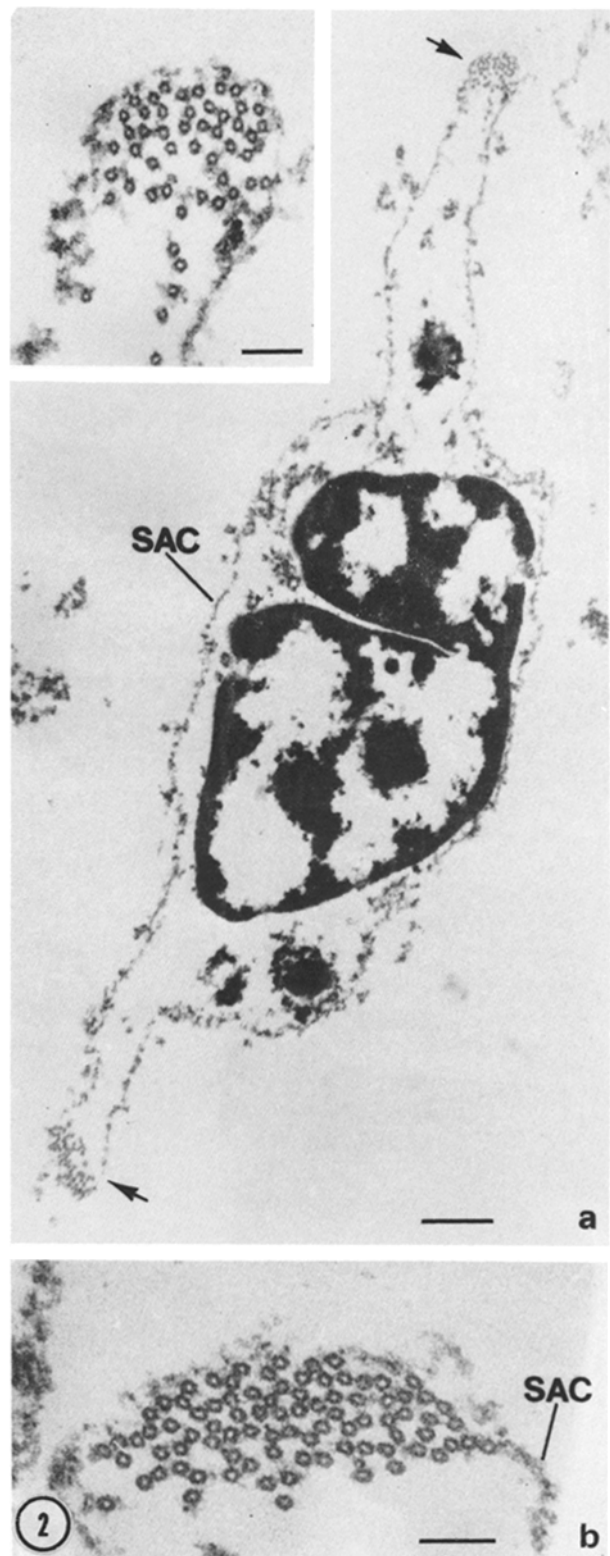

FIGURE 2 *N. ponderosa.* A typical lyse-fixed erythrocyte in thin section. (a) The cross-sectioned MB (arrows) is positioned such that it appears to press against the overlying continuous cell surface-associated cytoskeleton (*SAC*), maintaining it in an extended configuration and resulting in the flattened shape of the cell. *Inset:* Higher magnification of the upper MB cross section, showing ~50 cross-sectioned MTs. Cross-bridges are present between some MTs, and there appears to be an association between some peripheral MTs and the SAC. (b) MB cross section containing 85 MTs in a protrusion of the SAC. Inter-MT bridges are evident. TEM. (a) Bar, 0.5 μm; × 18,800. *Inset:* Bar, 0.1 μm; × 76,500. (b) Bar, 0.1 μm; × 95,000.

MATERIALS AND METHODS

Biological Material: *Anadara ovalis* (the "blood ark") was obtained from The Marine Biological Laboratory (MBL), Woods Hole, MA, and *Noetia ponderosa* (the "ponderous ark") and "*Anadara spp*" from the Gulf Specimen Co. Inc., Panacea, FL. The "*Anadara spp*" were incompletely identified, but were probably small specimens of *A. lienosa* (the "cut-ribbed ark"). The animals were maintained at MBL in running sea water or at Hunter College in cooled (15°C or 18°C), aerated tanks of "Instant Ocean" artificial sea water (Aquarium Systems, Eastlake, OH) supplemented with "marine invertebrate diet" and "trace elements" (Hawaiian Marine Imports, Inc., Houston, TX). The blood, containing primarily hemoglobin-bearing erythrocytes (33, 34) in addition to amebocytes and sometimes gametes, was removed via Pasteur pipette from between mantle and shell. Fresh samples were used for each experiment. Blood clam erythrocytes tend to lose their native flattened, elliptical shape shortly after removal from the animal, becoming less flattened and lumpy. Those of *N. ponderosa* are more resistant to this shape change than erythrocytes of the *Anadara* species. The shape change could be prevented by immediate dilution of blood about 1:10 into artificial sea water at 0°C or 1:200 into artificial sea water at room temperature. "MBL formula" artificial sea water (11), "Instant Ocean", and marine molluscan Ringer's solution (11) all served well as blood diluents, without noticeable effect on cell morphology.

Light Microscopy: Cell and cytoskeletal morphology were routinely examined in phase contrast, using a Zeiss microscope equipped for photomicrography. Observations were made on both living cells and cells fixed by dilution approximately 1:10 or 1:20 into molluscan Ringer's solution or artificial sea water containing 1% glutaraldehyde. "Cytoskeletons" were obtained by dilution of blood or cell suspensions approximately 1:10 into lysing medium (LyM) consisting of 100 mM piperazine-N-N'-bis(2 ethane sulfonic acid) (PIPES), 1 mM $MgCl_2$, 5 mM ethyleneglycol-bis-(b-aminoethyl ether) N-N'-tetraacetic acid (EGTA), 10 mM p-tosyl arginine methyl ester HCl (TAME), and 0.4% Triton X-100, pH 6.8 (12). In some cases, phase-contrast examination of cytoskeletons was done through small air bubbles under the coverslip, thus enhancing contrast (13).

Antitubulin immunofluorescence observations during MB reassembly (*Noetia*) were made as follows: Erythrocytes were incubated at 0°C for 4 h and rewarmed at 18°C. Cytoskeletons were prepared from time-course samples as described above, except that LyM contained 0.1 mM phenylmethyl sulfonyl fluoride (PMSF). Samples included noncooled control cells lysed before cooling of experimentals and at the end of the rewarming period. Cytoskeleton suspensions were allowed to settle onto ethanol-cleaned coverslips for 5 min. The coverslips with adhering material were washed in the lysis medium lacking Triton and TAME, fixed 5 min in the same medium containing 1% glutaraldehyde, rinsed in 0.05 M sodium phosphate buffer, pH 7.4, and incubated 30 min at 37°C with monoclonal antitubulin (J. Kilmartin, Medical Research Council, Laboratory of Molecular Biology, Cambridge, England; antibody YOL1/34; reference 25) in phosphate-buffered saline containing 1% BSA (BSA-PBS). After rinsing in 0.05 M phosphate buffer they were incubated as before with FITC-rabbit anti-rat IgG (Miles Laboratories Inc., Elkhart, IN) in BSA-PBS, rinsed in phosphate buffer, and mounted in the same medium containing 50% glycerol. In both experimental and control preparations (second antibody only) some nonspecific fluorescence of nuclei occurred. This did not interfere with the observations, but rather proved useful for making cell counts in certain experiments. Specimens were viewed and photographed with a Zeiss phase-contrast microscope equipped with an epifluorescence illuminator.

The effect of colchicine on MB reassembly was tested on cells (*N. ponderosa*) incubated at 0°C for 2 h to disassemble MBs. After adding colchicine to 0.1 mM (experimentals) or colchicine solvent only ("Instant Ocean"; controls), cells were rewarmed to ambient temperature (20–23°C). Cytoskeletons were prepared and examined in phase contrast at intervals between 1 and 3 h after rewarming.

Both phase and fluorescence observations on cytoskeletons from control (uncooled) cell populations revealed some that did not appear to contain MBs or any microtubules. Such cytoskeletons represented between 10% and 40% of the population in different preparations; their origin and significance are unknown.

Electron Microscopy: Time-course samples during temperature-induced MB reassembly (*N. ponderosa*) were prepared for thin sectioning using the technique of simultaneous lysis and fixation (14). A noncooled control blood sample was immediately fixed at 15°C by 1:10 dilution into LyM containing 1% glutaraldehyde, and the remaining blood immediately diluted into artificial sea water at 0°C. Aliquots of the latter were prepared and incubated overnight at 2°C. After removal of the sea water from above the samples of settled cells, lysis and glutaraldehyde fixation were carried out as above at zero time (no rewarming), and after 2, 5, 15, and 120 min rewarming at 25°C. Subsequently all samples were washed in 0.1 M sodium phosphate buffer, pH 6, and postfixed in 0.1% OsO_4 in the same buffer at 0°C for 45 min (27). Prestaining in 1% aqueous uranyl acetate for 30 min and dehydration in ethanol were carried out at 0°C, with all centrifugation steps in the cold. After propylene oxide infiltration, the material was embedded in Epon. Cell morphology and cytoskeletal structure were moni-
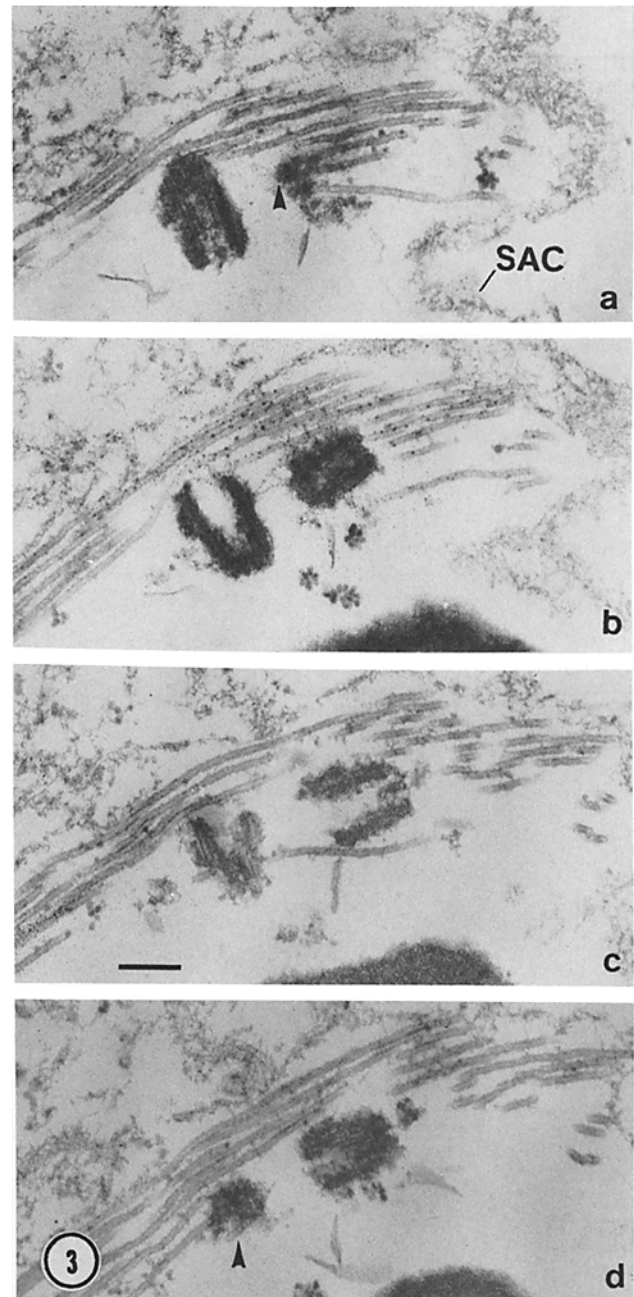

FIGURE 3   *N. ponderosa.* Serial sections through the MB-associated centrioles. (a) Microtubules (MTs) appear to insert into or end at dense, amorphous material (arrowhead) associated with the surface of the centriole observed in (b–d). Note that the centrioles are orthogonally arranged (b and c), with one lying perpendicular to the MB. Microtubules oriented at right angles to this centriole also appear to insert into amorphous material associated with its surface (d). The region between the centrioles is generally devoid of MTs. TEM, Bar, 0.2 μm; × 44,600.

tored in phase contrast during MB disassembly/reassembly experiments, essentially as described under "light microscopy" above.

Thin sections were cut with diamond knives on the Sorvall MT-2 ultramicrotome (DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Newtown, CT), and stained with saturated uranyl acetate in 50% ethanol followed by Reynold's lead citrate. Control material for serial sectioning was processed essentially as above, except that 0.2% tannic acid was added during glutaraldehyde fixation (3, 20). Ribbons were picked up on Formvar-coated wire loops and mounted on slot grids.

Whole mounts of cytoskeletons for transmission electron microscopy were prepared on Formvar-coated grids pretreated with polylysine to enhance retention of material (1% solution of mol wt >400,000, followed by water washes and air drying). Cytoskeleton suspensions in LyM were placed on grids for 10–15 min, followed by a wash in LyM lacking Triton and TAME, and 10-min fixation in the latter medium containing 1% glutaraldehyde. After subsequent washes in LyM without Triton and TAME, and in water, the material was stained with 1% aqueous uranyl acetate and the grids were air dried. Whole mounts and thin sections were examined in the Hitachi HS-8 transmission electron microscope at 50 kV.

EXPERIMENTS AND RESULTS

Cell Morphology and Cytoskeletal Structure

The erythrocytes of all of the species used in the present experiments were nucleated, flattened, and generally elliptical (Fig. 1 a). The cytoskeletons contained MBs conforming to the original cells in size and shape, while circumscribing the nucleus and remaining organelles. When the cytoskeletons were flattened under the coverslip, every MB that could be observed in its entirety was found to have associated centrioles visible as phase-dense "dots" (Fig. 1 b), as reported previously (14). In *N. ponderosa* (Fig. 1 a) and *A. ovalis*, a small percentage of the cells (1–2%) were pointed at one end. In the "*Anadara spp*", however, pointed erythrocytes were often present in great numbers (8–51% in different animals). Cytoskeleton preparations from these blood samples contained a similar percentage of pointed MBs, each with the centrioles located at the apex (Fig. 1 c). Neither the pointed cells nor the pointed cytoskeletons appeared to be artifacts of handling, since the former were observed in blood preserved by in vivo injection of fixative into the mantle cavity, and the latter were present in blood that was poured (rather than pipetted) directly into LyM.

The MB was located just beneath a continuous surface-associated cytoskeleton (SAC) comparable to that of vertebrate erythrocytes (12, 13). In favorable views, the MB was present in cross section at opposite ends of typical cytoskeletons (*N. ponderosa*; Fig. 2 a). MBs of this species observed in cross-section contained from 51 to 85 MTs, many of which were cross-bridged (Fig. 2 a inset and 2 b). Peripherally located MTs were in close association with the SAC.

The relationship between centrioles and MB MTs was further investigated by serial thin sectioning (*N. ponderosa*; Fig. 3). MTs appeared to insert into, or at least end at, the amorphous electron-dense material around each of the centrioles in the right-angle pair (Fig. 3 a and d). Other MT profiles were continuous in this region, generally passing peripherally between centrioles and SAC. MTs were not continuous with those of the centriolar cylinders, and they were not present
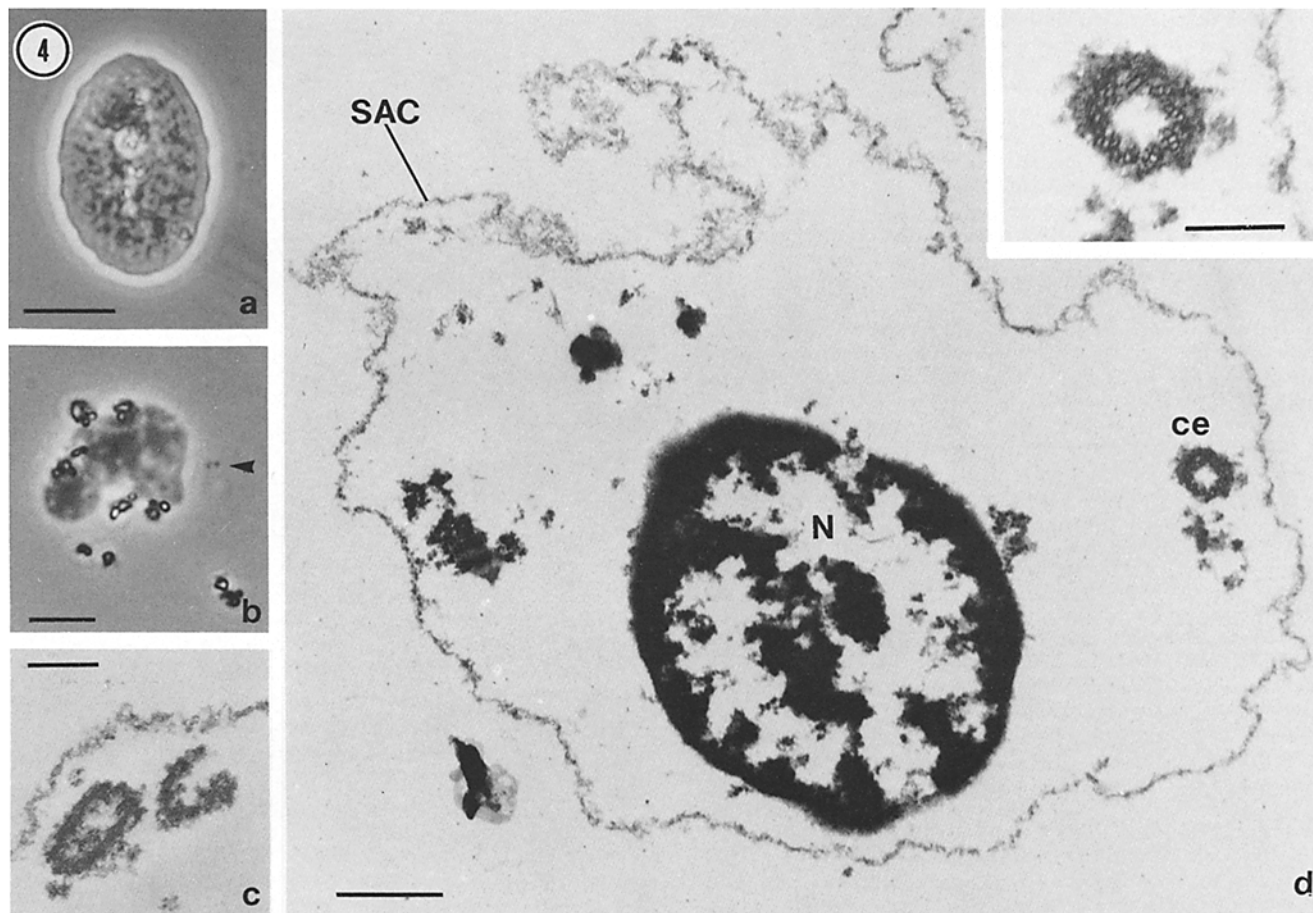

FIGURE 4 *N. ponderosa.* Disappearance of the MB on cooling erythrocytes to 0°C. (a) Intact fixed cell, still flat and elliptical but with wrinkled edges. (b) Cytoskeleton as seen under phase contrast. Centrioles are visible as two phase-dense dots (arrowhead). (c) Centriole pair devoid of MTs, still located near the *SAC.* (d) A typical cytoskeleton in thin section showing cross-sectioned centriole (*ce*) surrounded by electron-dense material. The SAC is present, but assumes a random configuration in the absence of the MB. *Inset:* higher magnification view of the centriole, showing triplets. (a) Phase contrast, bar, 10 μm; × 1180. (b) Bar, 5 μm; × 1700. (c and d) Thin sections, bar, 0.2 μm; TEM × 46,200; and bar, 0.5 μm; × 27,300; respectively. *Inset:* bar, 0.2 μm; × 65,000.

between the centrioles, presumably accounting for the reduced phase density often seen between separated centrioles in phase contrast (14).

MB Disassembly/Reassembly

Assessment of the possible role of the centrioles as MB-organizing centers required either examination of erythropoiesis or experimental induction of MB disassembly/reassembly in living cells. Since the site of erythropoiesis appeared to be unknown in these species (36), the cells were tested for low-temperature MB lability. In *N. ponderosa* erythrocytes, MBs were no longer visible in phase contrast when cytoskeletons were prepared from cells that had been incubated 1.5 h at 0°C. Furthermore, MBs with their associated centrioles reappeared after 1–2 h of rewarming at ambient temperature, even after storage of cells at 0°C–2°C for as long as 23 h. Thus, temper-

ature cycling permitted a detailed examination of the reassembly sequence for structural evidence of centriole participation.

MB Reassembly: Phase Contrast and Thin Section Observations

N. ponderosa erythrocytes were incubated at 0°C until they had lost their MBs (1.5 h). The cells retained approximately normal morphology, except that their edges appeared somewhat wrinkled in fixed samples (Fig. 4*a*). In cytoskeletons observed in phase contrast, MBs were absent and the SAC was not visible, but the centrioles were sometimes seen as a free pair of phase-dense dots (Fig. 4*b*). In thin sections, MTs were not found anywhere in the lysed cells, but centrioles with closely associated, electron-dense amorphous material were present (Fig. 4*c* and *d*). The cytoskeleton of the 0°C-incubated cells consisted essentially of the SAC which, apparently no
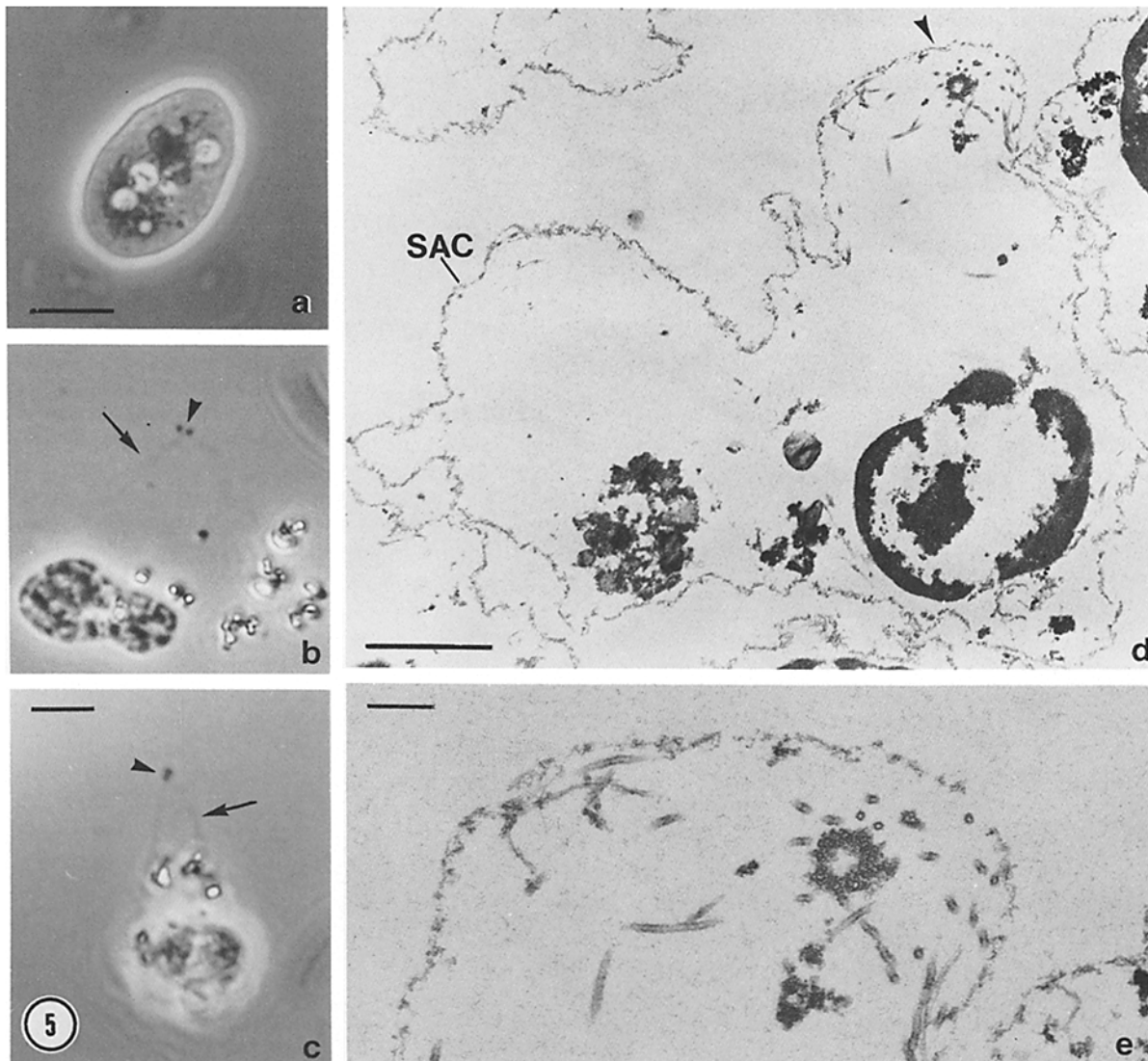

FIGURE 5   *N. ponderosa.* Early stage of MB reassembly upon rewarming cells 2 min at 25°C. (*a–c*) Phase contrast. (*a*) Typical intact fixed cell, still flattened and elliptical, with one end smooth in contour. (*b*) Cytoskeleton exhibiting very thin fibers (arrow) focusing on the centriole pair (arrowhead), and absence of a complete MB. (*c*) Cytoskeleton illustrating the "point" produced by thin fibers (arrow) emanating from the centriolar region (arrowhead). Only the thicker MT bundles are visible at this stage in phase contrast. (*d*) Thin section, showing numerous MTs only in the vicinity of the centriole (arrowhead). The cytoskeleton boundary is delineated by the SAC; MTs are not present in regions of the cytoskeleton distant from the centriole. (*e*) Higher magnification view of the centriolar region in (*d*). (*a*) Phase contrast: bar, 10 μm, × 960; (*b* and *c*) bar, 5 μm, × 1,700. (*d*) TEM: bar, 1 μm × 17,200; (*e*) bar, 0.2 μm × 46,000.

longer stretched by the MB, assumed a random configuration in the lysed cell preparation (Fig. 4 d).

Centriole involvement in MB reassembly was most evident in the earliest rewarming stages. After 2 min at 25°C, intact fixed cells appeared somewhat less wrinkled than at 0°C, usually with a smooth outline at one end of the ellipse (Fig. 5 a). In phase contrast, many of the cytoskeletons were observed to have extremely thin fibers emanating from the centriolar vicinity, appearing to end 3.5–8 μm from the centrioles (Fig. 5 b and c). This gave the cytoskeletons a pointed appearance, with the centrioles at the "pole". It should be noted that phase contrast at such early time points revealed only MT bundles of sufficient thickness, leaving individual MTs invisible and exaggerating the degree of organization actually present. In thin-sectioned material, MTs were limited almost entirely to the vicinity of the centrioles (Fig. 5 d and e). In one instance MTs extended approximately half-way along the length of the cytoskeleton, but they were never present at the end of the cytoskeleton opposite the centrioles. Cytoskeleton outlines, as defined by the SAC, were similar to those seen in the 0°C samples (Fig. 5 d).

After 5 min of rewarming, intact cells were usually smoother at one end than at the other (Fig. 6 a). In phase contrast, thin MBs could now be found that extended from the centriolar point around the opposite end of the cytoskeleton. These MBs were more readily observed by viewing through an air bubble to enhance contrast (Fig. 6 b). Thin sections revealed the presence of MTs in all regions of many cytoskeletons, focusing on the centrioles in greater numbers and with longer profiles than
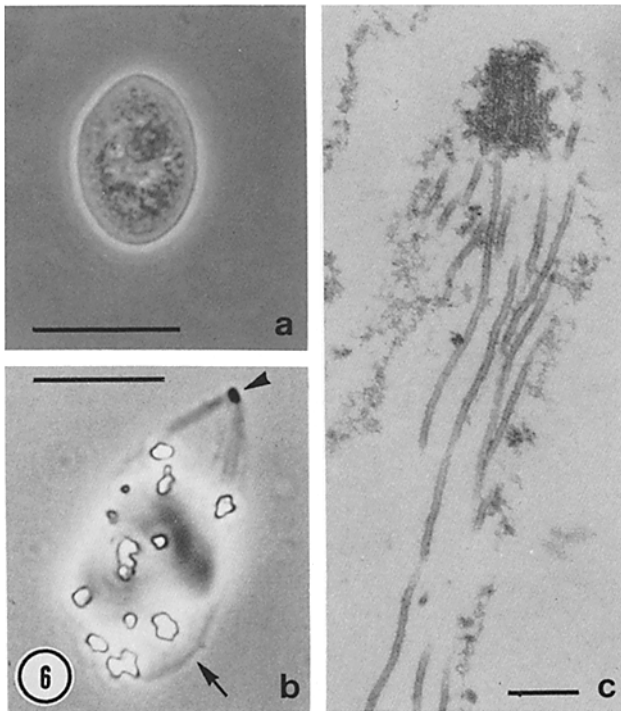
FIGURE 7   N. ponderosa. MB reassembly in cells rewarmed 15 min. at 25°C. (a) The intact fixed cells have smooth outlines, and many are somewhat pointed at one end of the ellipse (arrow). (b) Thin tangential section of a right-angle centriole pair in association with the MB (part of another cytoskeleton nearby). (c) In longitudinal thin section, long parallel MTs comprise the MB. (d) The MB at this time point consists of loosely packed MTs located in SAC protrusions. Few, if any, cross-bridges are visible. (a) Phase contrast: bar, 10 μm; × 960. (b–d) TEM: bar, 0.2 μm; × 46,400.

FIGURE 6   N. ponderosa. MB reassembly in cells rewarmed 5 min at 25°C. (a) Intact fixed cell with fairly smooth flattened, elliptical contour. (b) Cytoskeleton viewed through an air bubble in phase contrast to permit better visualization of the fibrous, pointed MB and apical centrioles (arrowhead). The MB is continuous distal to the centrioles (arrow). (c) Thin section showing centriole associated with relatively long MT profiles. (a and b) Phase contrast: bar, 20 μm; × 960, and bar, 10 μm; × 1,700, respectively. (c) TEM: bar, 0.2 μm; × 45,700.
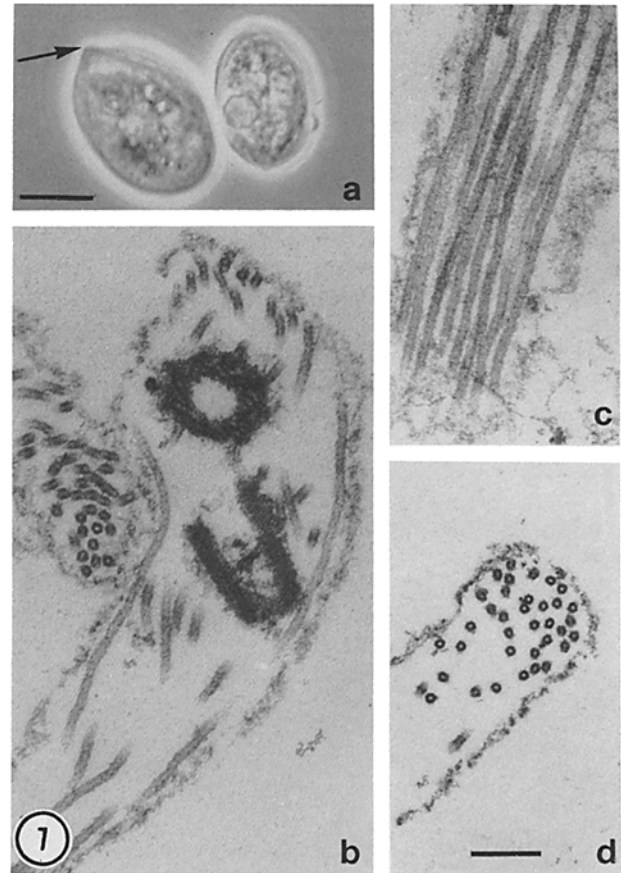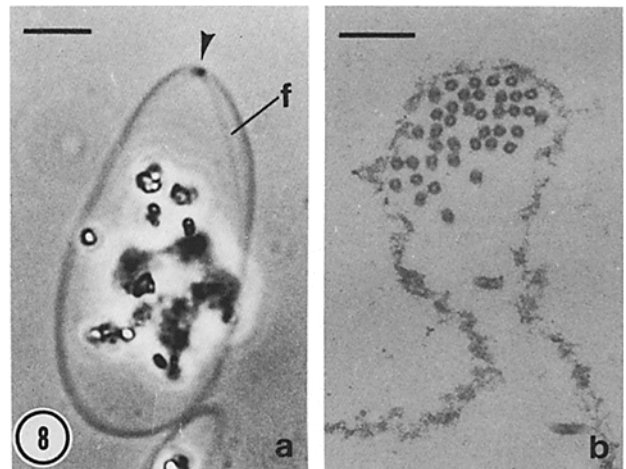
FIGURE 8   N. ponderosa. MB reassembly in cells rewarmed 120 min at 25°C. (a) Cytoskeleton in phase contrast. The MBs are usually elliptical, with centrioles (arrowhead) lcoated at or near one end of the ellipse. A thin fiber (f) emanates from the vicinity of the centrioles, joining the MB some distance away. (b) Cross-section of MB in a SAC protrusion. The MTs are more closely packed than at earlier stages, and some cross-bridges are evident (arrow). (a) bar, 5 μm; × 1,700. (b) TEM: bar, 0.2 μm; × 49,400.
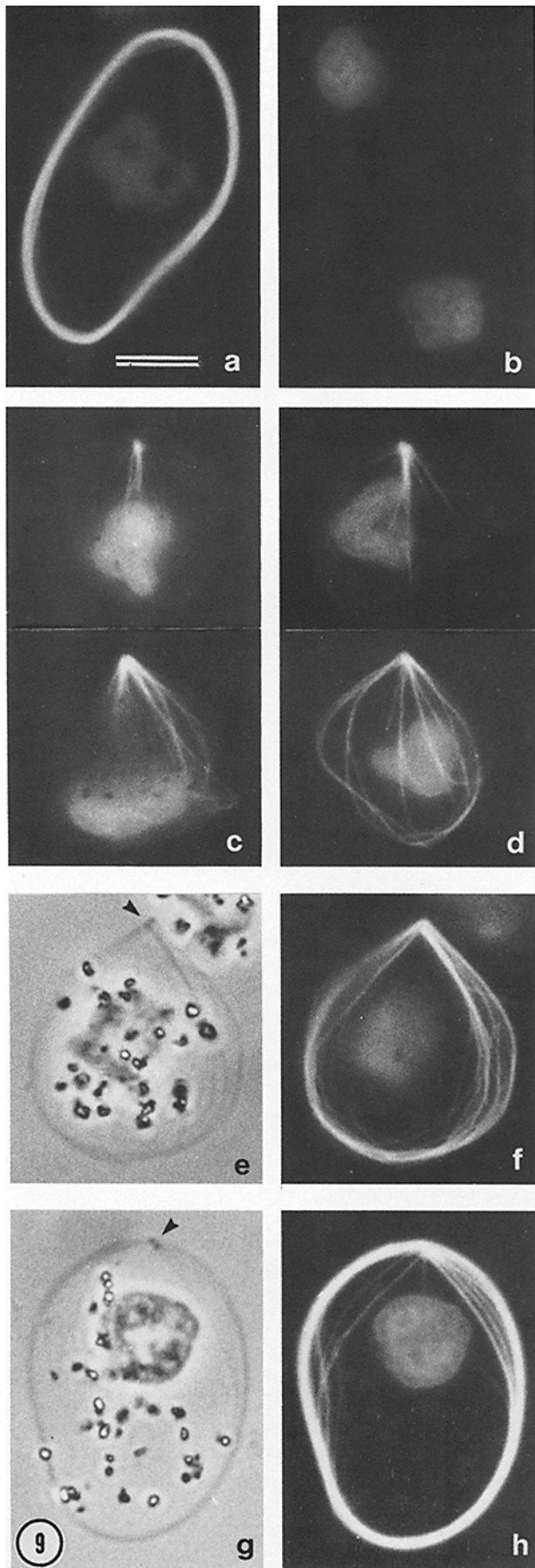
in the 2 min samples (Fig. 6 c). Nascent continuous MBs were present but not yet organized into well-defined bundles.

Cells rewarmed for 15 min and fixed intact had smooth edges and occasionally a small point at one end of the ellipse (Fig. 7 a). Under phase contrast, cytoskeletons had continuous MBs that were often somewhat pointed. Thin sections confirmed the reassembly of discrete MBs and their association with centrioles (Fig. 7 b–d). As counted in cross section, MBs contained fewer MTs than noncooled controls, ranging from 33 to 38. In addition, there appeared to be fewer connections between adjacent MTs and between peripheral MTs and the SAC as compared with controls (Fig. 7 d vs. Fig. 2).

N. ponderosa erythrocytes rewarmed at 25°C for 120 min appeared to have normal shape and elliptical MBs with associated centrioles. In some of these, fibers could be seen extending from the centrioles out to the MB (Fig. 8 a). In thin section, discrete MB cross-sections were located in SAC protrusions similar to those of controls (Fig. 8 b vs. Fig. 2a), and bridges were present between some of the MTs (Fig. 8 b). Centrioles were closely associated with MT bundles, and the number of MTs per MB ranged from 31 to 41, still considerably fewer than in noncooled controls.

MB Reassembly: Antitubulin Immunofluorescence Observations

Indirect immunofluorescence with monoclonal antitubulin was employed to verify the reassembly sequence suggested by the above observations. After incubation at 0°C for 4 h, >99% of cells counted contained no microtubules (Noetia; Fig. 9, a vs. b). Cells were rewarmed at 18°C and studied in time-course samples. Reassembly was not tightly synchronized, so that various stages were present in a given sample. At the earliest time points (2–10 min), all of the cytoskeletons in which reassembly had begun contained microtubules focused on the centrioles, producing a pointed or polelike appearance (Fig. 9 c and d). The number of such cytoskeletons reached a maximum at 5–10 min, after which time recognizable MBs began to appear (Fig. 9 e, f and g, h), many of which were still pointed (as in Fig. 9 e, f). Centriole presence at the point of microtubule "focus" was verified in each case by phase-contrast microscopy (as in Fig. 9 e and g).

Effect of Colchicine on MB Reassembly

After 1 h of rewarming at ambient temperature in the presence or absence of 0.1 mM colchicine, very thin MBs had formed in both control and experimental erythrocytes (N.

FIGURE 9   N. ponderosa. MB disassembly and reassembly as visualized by antitubulin immunofluorescence of cytoskeletons. (a) Noncooled control; faint fluorescence of nucleus is a background effect due to the secondary antibody. (b) Disappearance of MB and all MTs after incubation of cells for 4 h at 0°C; only nuclei are visible. (c and d) Cytoskeletons after rewarming of cells at 18° for 5 and 10 min, respectively. Early stages of MB reassembly, with MTs emanating from centriolar "points." Tight synchrony is not observed, so that the extent of assembly varies within a given time point. In the lower example in (d) there appears to be an extremely thin MB on the verge of continuity. (e and f) Phase contrast/fluorescence photomicrograph pair, 20 min rewarming. The MB is continuous at end of cell distal to centrioles, but it is still pointed. The centrioles lie at the point (e, arrowhead), with part of another cytoskeleton nearby. (g and h) Phase/fluorescence pair, 45 min rewarming. As observed by immunofluorescence (h), the MB is no longer pointed; it approximates the control (a) in appearance except that MTs still radiate from the centrioles (g, arrowhead) to join MB some distance away. bar, 5 μm; × 2,500.

ponderosa). However, MBs of all of the colchicine-treated cells had a point at one end, with the centrioles located at the tip. Longer rewarming periods results in progressively thicker elliptical MBs in the control samples, while those reassembled in the presence of colchicine remained flimsy and pointed (Fig. 10).

MB Reassembly: Whole Mounts

The temperature-induced reassembly sequence was also examined by transmission electron microscopy of uranyl acetate-stained cytoskeleton whole mounts. Observations on erythrocyte cytoskeletons of *A. ovalis* showed that MB disassembly/reassembly could also be induced in this species by temperature cycling. Whole cytoskeletons of noncooled controls exhibited an electron-dense MB, densely stained centrioles, and a collapsed SAC in addition to the nucleus and some other organelles. After incubation of cells at 0°C for 4 h, the MBs and MTs had disappeared from most of the cells, but the SAC remained. As counted on grids and verified by immunofluorescence, ~75% of the cytoskeletons in this species now contained no MTs while 25% contained one or a few MTs near the periphery, some of which traversed most of the cell circumference. These were perhaps cold-stable MB remnants. The centrioles were not readily identifiable in any of the cytoskeleton whole mounts from 0°C-incubated cells.

After 2 min rewarming at 25°C, ~50% of the *A. ovalis* cytoskeletons contained MTs. When visible, the centriole pairs were situated at a "pole" of MT convergence similar to that observed by phase contrast and immunofluorescence in Noetia (Fig. 11 *a*). In some cases MTs clearly originated at the longitudinal surface of the centriolar cylinder, as opposed to its ends (Fig. 11 *b*). After 5 min of rewarming, nearly all cytoskeletons had MTs associated with a centriolar "pole" (Fig. 11 *c* and *d*). Both centrioles were active in the sense that MTs were independently associated with them, and microtubules from the two centrioles were observed to join in common bundles (Fig. 11 *d*). Thin but complete MBs appeared after about 20 min, and a few hours of rewarming produced well-developed elliptical MBs, though thinner than normal (Fig. 11 *e*). It was evident that the centrioles were still intimately involved in formation of these MBs, as numerous MTs emanated from them and joined the primary MB bundle at distant points. Careful inspection of cytoskeleton whole mounts at all time points revealed instances in which MTs originating at each of the centrioles went off in the same direction, joining into a common bundle (as in Fig. 11 *d*) or joining the MB some distance away. In some of the latter cases there was a suggestion of preferential MT direction (as in Fig. 11 *e*).
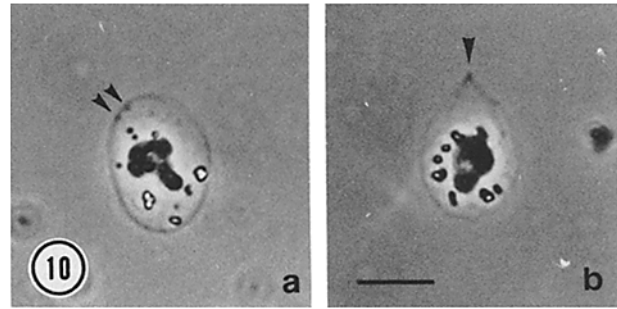

FIGURE 10 *N. ponderosa*. Effect of 0.1 mM colchicine on MB reassembly. Erythrocytes were cooled for 2 h at 0°C at which time colchicine was added to the experimental samples. After 2 or more h rewarming at ~20°C, controls (no colchicine) had normal elliptical MBs, whereas experimentals contained pointed MBs with the centrioles located at the points. (*a* and *b*) Control and experimental cytoskeletons, respectively, after 2.5 h rewarming. Arrowheads denote location of centrioles. Phase contrast: Bar, 10 μm; X 1,000.

DISCUSSION

### Centriole Function in MB Reassembly

The observations strongly support the proposal that the MB-associated centrioles of Arcidae erythrocytes function as MT-organizing centers for MB formation. That centrioles are involved in nucleation of MT assembly is strikingly evident at the earliest stages of reassembly. Furthermore, they are intimately associated with the nascent MBs at all times. Both centrioles of the pair are probably active, since MTs emanate from each of them throughout reassembly. The serial sections indicate that MTs are in contact with amorphous material on the exterior surface of the centrioles, and not continuous with the centriolar triplets. This is further supported by the observation that at least some centrioles lie at right angles to the MB, with no evidence of MTs oriented so as to meet their ends (as in Fig. 3). In addition, some views of whole mounts show MTs arising from the sides rather than the ends of the centriolar cylinder (Fig. 11 *b*), or orthogonally to one end. Nucleation of MTs for MB construction in this cell type is thus presumed to be a function of pericentriolar material, as in various other MT systems (10, 22, 35), with the centrioles serving to organize and/or anchor this material. As the evidence for involvement of pericentriolar material is circumstantial however, with data on in vitro function not yet available, we prefer not to apply the general term "centrosome" to the blood clam MB-organizing center at this time.

If the centrioles and associated material are responsible for MT initiation, what mechanisms are involved in establishing and maintaining the MT bundle? Although direct analysis of

FIGURE 11   *A. ovalis*, cytoskeleton whole mounts. MB reassembly at 25°C after disassembly at 0°C for 4 h (*a*) 2 min rewarming. A few MTs project from the centriole pair (arrowheads), but do not extend all the way around the cytoskeleton periphery. (*b*) Higher magnification view of the centrioles in (*a*). A microtubule projects from centriole no. 1 at right angles to the centriolar cylinder. (*c*) 5 min rewarming. Microtubules focus at the centrioles (arrowheads), forming a "point." (*d*) The centriolar region of (*c*) at higher magnification. Microtubules apparently arising from each of the centrioles (arrows) fuse into a single fiber (*fi*). (*e*) 4 h rewarming. A thin continuous MB is present. Fortuitous displacement of the centrioles from the MB in this cytoskeleton discloses individual fibers (MT bundles) emanating from each centriole and going off in the same direction to join the MB at distant points. (*f*) Centrioles of (*e*) at higher magnification, showing MTs arising from their surfaces (arrows). The centrioles appear to be joined by a discrete structure, possibly a MT. (*a, c,* and *e*) TEM: bars, 1 μm; X 12,400, 14,000, and 5,200, respectively. (*b, d,* and *f*) Bar, 0.2 μm; X 90,000.
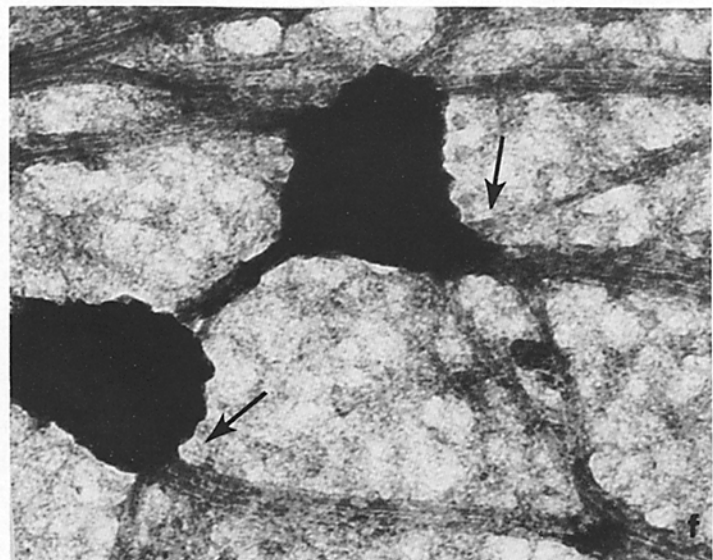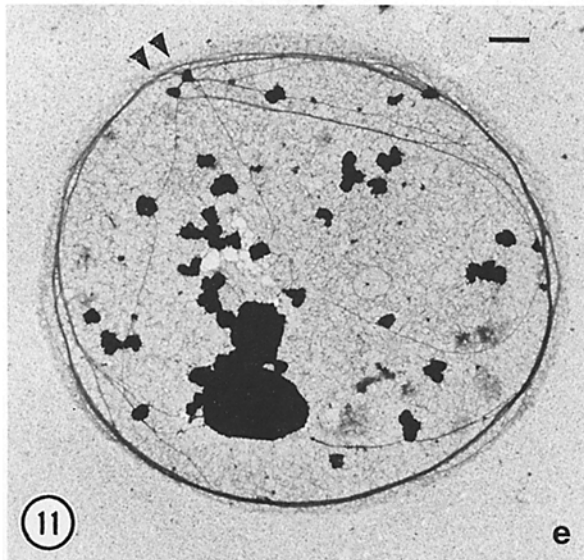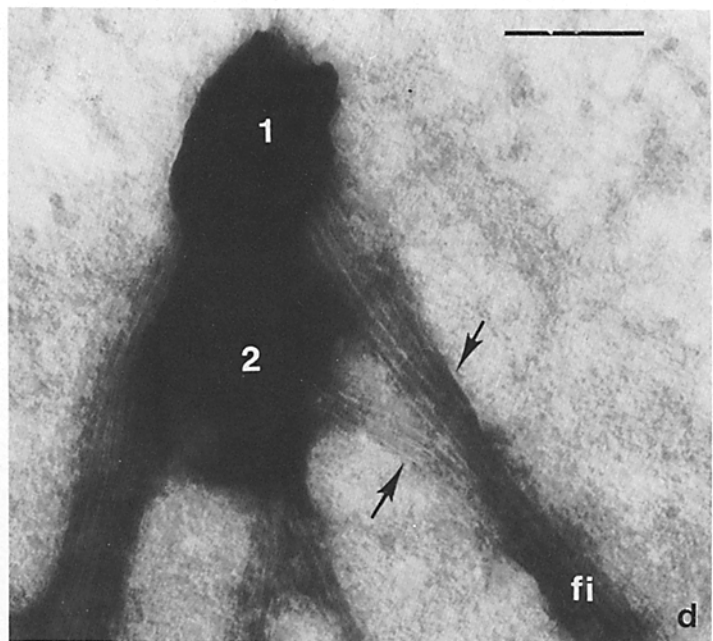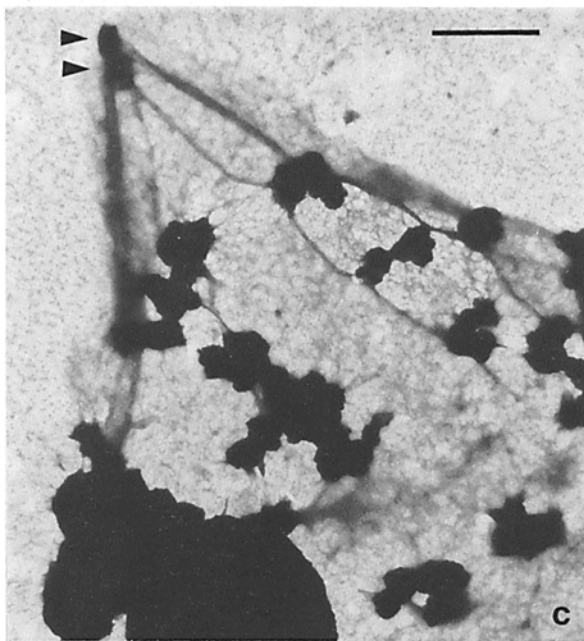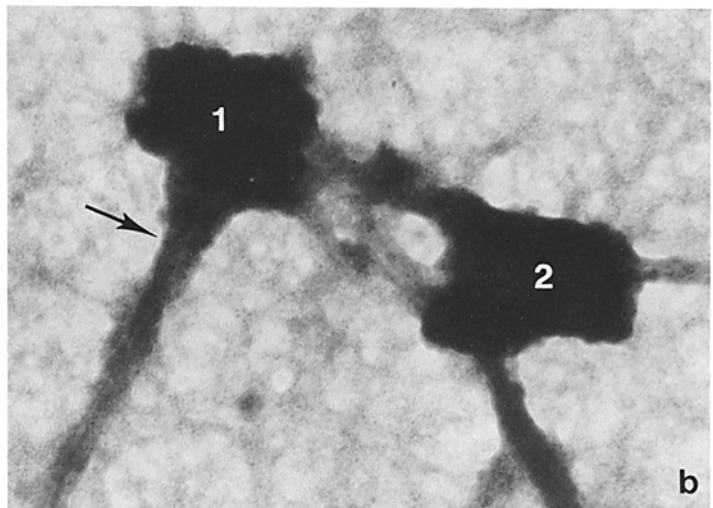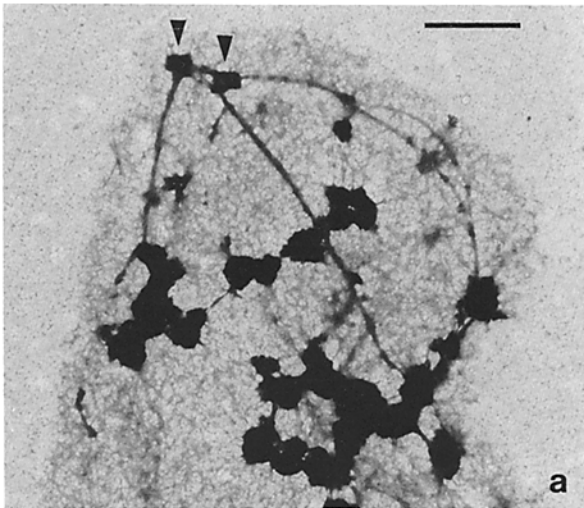
this problem is lacking, a few observations may be relevant. The MBs of noncooled control cells contain relatively closely spaced MTs, many of which are cross-bridged to neighbors (Fig. 2), whereas the forming MBs contain fewer and more widely separated MTs that are not cross-bridged (Fig. 7 *d*). This suggests that MT initiation and growth occurs in the absence of extensive inter-MT bridging, and that progressive cross-bridging of MTs occurs later at points of close encounter.

Might centrioles and pericentriolar material act as organizing centers for MB formation in blood cells of other species? The data are sparse at present. Centrioles are associated with the MBs of sea cucumber erythrocytes (18), but their function has apparently not yet been studied. Based upon preliminary observations, centrioles also appear to be associated with the MBs of at least some dogfish erythrocytes (29). A pair of centrioles is present in many (possible all) of the erythrocytes of the amphibians *Rana catesbiana* (bullfrog) and *Xenopus laevis* (African clawed toad); however, they are located adjacent to the nucleus in these species (16, on poster; 19). For a true assessment of centriole function during MB assembly in vertebrate erythrocytes, examination of erythropoietic tissues may be required. Many centrioles are also present in megakaryocytes, apparently the product of numerous mitoses without cytokinesis (6). However their number (perhaps 64, maximum, for typical 16–32n megakaryocytes) is far below the several thousand platelets produced by a single megakaryocyte (31, 37), and they are rarely observed in circulating platelets (8, 40). Behnke (5) found electron-dense material, but not centrioles, associated with re-forming platelet MBs. Thus, if centrioles have any role in platelet MB biogenesis, it is probably quite an indirect one involving pericentriolar material.

MB Formation in the Arcidae

A relatively simple model for MB formation in Arcidae erythrocytes is presented in Fig. 12. MT assembly initiates at the centriole pair and MTs grow in two directions as defined by the right-angle centriolar relationship ("initiation stage"; Fig. 12 *a*). Tubulin addition is assumed to occur distal to the organizing center, in accordance with the analysis of other MT systems (9, 15, 26). Although the simplest model would be one in which MTs from each centriole grew only in one direction, observations on whole mounts (for example, Fig. 11 *c–e*) suggest that the centrioles can contribute jointly to microtubule bundles, as illustrated. The growing MTs eventually cross in opposite directions distal to the centrioles ("pointed stage"; Fig. 12 *b*), and continue to grow circumferentially, passing peripherally to the centrioles when they reach their region of origin ("elliptical stage"; Fig. 12 *c*). The centrioles thus retain a location internal to the forming MB, as observed in the whole mounts. As the MTs continue to elongate, they eventually cross-bridge to pre-existing ones to form and thicken the MB. In its simplest form, this model predicts that the Arcidae MBs will contain approximately equal numbers of MTs of opposite polarity. However, whole-mount views suggest that there may be a preferential growth direction in at least some cells (for example, Fig. 11 *e*). In addition, some bullfrog MBs appear to contain MTs of predominantly one polarity while others contain both polarities (16), and the platelet MB may consist of a single coiled MT (8, 28) for which uniform polarity would be predicted. This aspect of the model (Fig. 12) thus awaits experimental examination.

The results obtained with colchicine tend to support the sequence illustrated, with MB reassembly in treated cells ar-
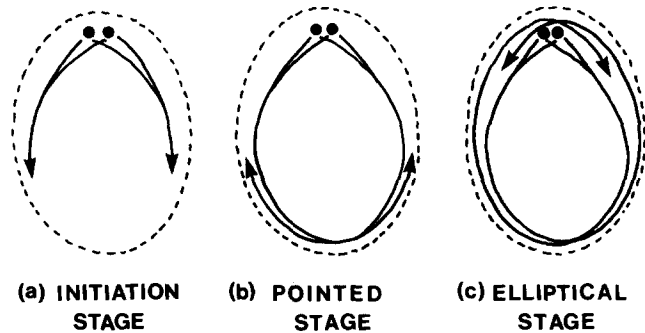

(a) INITIATION STAGE   **(b) POINTED STAGE**   **(c) ELLIPTICAL STAGE**

FIGURE 12   Proposed stages of MB reassembly in Arcidae erythrocytes. (*a*) "Initiation stage." MTs emanate from the centriolar region, with fibers growing in divergent directions toward the cell surface. Observations on whole mounts suggest that MTs nucleated by each of the centrioles can join to form the fibers, as illustrated. (*b*) "Pointed stage." MTs of opposite polarities meet and pass one another at the end of the cell opposite the centrioles. Cytoskeletons prepared at this stage have a pointed appearance in the centriolar region. (*c*) "Elliptical stage." MTs continue to grow around the cell periphery past their place of origin. MT passage external to, or behind the centrioles closes the ellipse, and continued elongation of MTs gradually thickens the MB. Note that the figure is simplified for diagrammatic purposes; the arrows indicate general growth direction only, not specific numbers of MTs.

rested at the "pointed stage" (Fig. 12). Under the conditions of the experiment, the intracellular colchicine concentration was apparently too low to block assembly completely, but sufficient to prevent completion of the MB.

While the model represents our hypothesis concerning stages of MB reassembly, the mechanisms remain unexplained. Why do the MTs grow with curvature, rather than simply producing straight bundles protruding from the cell? Why does the MB reassemble in a plane, rather than a more three-dimensional array such as an aster? We can only speculate that interactions between MTs and between MTs and the SAC somehow induce their curvature, and that the MB plane is (or was originally) defined by the centriole pair. Since the cells retained a flattened elliptical shape throughout MB disassembly/reassembly, the MB must re-form in its original plane. Otherwise a variety of cell shapes would be generated as it grew at various angles to the original plane of flattening. This may mean that there is some kind of track along the inner surface of the SAC which guides MB reassembly in the mature cell. Structural evidence of individual MT tracks that could function in this manner has recently been presented for chicken erythrocytes (23). Alternatively, the centrioles may assume a prefixed position as they determine the plane of MB formation, or possibly long, growing MTs automatically "find" and accumulate in the narrow edge of an already flattened cell for mechanical reasons.

MB Formation and Cellular Morphogenesis

As noted above, the MBs of the Arcidae erythrocytes do not seem to be required for maintenance of cell flatness and ellipticity under the conditions employed (0°C, nonflow). Similar observations have been made for MBs of nonmammalian vertebrate erythrocytes (reference 7, chicken; reference 13, dogfish). It appears possible, therefore, that cell "maturation" entails modification of the SAC material such that it no longer requires the MB for shape maintenance under the experimental conditions. This could also account for the fact that, as MBs

passed through the "pointed stage" during cell rewarming, there was little effect on cell shape other than the slight pointedness observed in some cells (Fig. 8). If the SAC were more elastic or fluid in immature cells, then it might be deformed and shaped by the growing MTs within.

We visualize the sequence of events during erythropoiesis as follows: the shape of the SAC and the cell coincide (13), with the cell initially being roughly spherical (2) or perhaps amorphous. Cells would begin to flatten at the "initiation stage" as MTs made contact with the SAC and applied pressure against it. Further growth of MTs to the "pointed stage" could elongate the SAC along the axis from the centrioles to the opposite side of the cell. Concurrently the SAC would constrict in the same plane in a direction perpendicular to that axis, resulting in a flattened teardrop-shaped cell. Subsequently, by passing peripherally to the points at which the centrioles are located, the MTs could close the ellipse and attenuate cell pointedness. The naturally occurring pointed cells and MBs of these species, particularly evident in the "*Anadara spp.*" (Fig. 2), are interpreted according to the Model (Fig. 12) as variants in which the MB fails to progress beyond the "pointed stage".

Fundamental mechanisms in cellular morphogenesis and the biogenesis of MT systems remain to be elucidated. The MB-centriole system described here is a relatively simple one which should be of further value in studying spatial control of MT arrays. Since the cell type involved is a differentiated hemoglobin-containing erythrocyte, the centrioles and associated material are unlikely to have further multiple functions such as participation in mitosis or ciliogenesis. The system should be amenable to analysis by a variety of techniques beyond those employed in the present work.

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