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# The Cytoskeletal System of Nucleated Erythrocytes. I. Composition and Function of Major Elements

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**ABSTRACT** We have studied the dogfish erythrocyte cytoskeletal system, which consists of a marginal band of microtubules (MB) and trans-marginal band material (TBM). The TBM appeared in whole mounts as a rough irregular network and in thin sections as a surface-delimiting layer completely enclosing nucleus and MB. In cells incubated at 0°C for 30 min or more, the MB disappeared but the TBM remained. MB reassembly occurred with rewarming, and was inhibited by colchicine. Flattened elliptical erythrocyte morphology was retained even when MBs were absent. Total solubilization of MB and TBM at low pH, or dissolution of whole anucleate cytoskeletons, yielded components comigrating with actin, spectrin, and tubulin standards during gel electrophoresis. Mass-isolated MBs, exhibiting ribbonlike construction apparently maintained by cross-bridges, contained four polypeptides in the tubulin region of the gel. Only these four bands were noticeably increased in the soluble phase obtained from cells with 0°C-disassembled MBs. The best isolated MB preparations contained tubulin but no components comigrating with high molecular weight microtubule-associated proteins, spectrin, or actin. Actin and spectrin therefore appear to be major TBM constituents, with tubulin localized in the MB. The results are interpreted in terms of an actin- and spectrin-containing subsurface cytoskeletal layer (TBM), related to that of mammalian erythrocytes, which maintains cell shape in the absence of MBs. Observations on abnormal pointed erythrocytes containing similarly pointed MBs indicate further that the MB can deform the TBM from within so as to alter cell shape. MBs may function in this manner during normal cellular morphogenesis and during blood flow in vivo.

Marginal bands of microtubules (MBs) are a major structural feature of all nonmammalian vertebrate erythrocytes and various other blood cell types (3, 15, 16, 22, 23, 31). Although MBs are probably simpler than many other microtubule systems, fundamental questions concerning them remain unanswered. Little is known of their formation during morphogenesis (2, 12). The number, length, and polarity of MB microtubules, their molecular composition, and the ultrastructural and molecular components responsible for their bundling have not yet been described. Moreover, while previous data for erythrocytes support a role in genesis but not maintenance of cell shape (2, 5, 13), MB function is not well understood. It is now clear that MBs should be considered in relation to the cytoskeletal system as a whole, which includes trans-MB material of unknown

composition [TBM; (11)]. However, the interaction between MB and TBM, and the possible assignment of shape maintenance function to the latter, have not been explored.

Ready experimental access to such problems requires a cell type that is (a) relatively large, with the MB easily visible in phase contrast, (b) physiologically accustomed to laboratory temperatures, for convenient handling, (c) available in quantity for cytoskeletal fractionation and biochemistry, and (d) amenable to MB disassembly and reassembly in vivo. In this paper we show that these criteria are met by erythrocytes of the smooth dogfish, *Mustelus canis*, and we report studies on the structure, molecular composition, and function of MB and TBM in these cells.

## MATERIALS AND METHODS

### Living Material

Blood was freshly drawn for each experiment and either heparinized (20–50 U/ml) or immediately diluted at least 1:10 into Elasmobranch Ringer's solution (9). Cells were washed by four or more centrifugation/resuspension cycles in Ringer's, carefully removing the leukocyte (upper) layer each time, after which there was <1% leukocyte contamination. Alternatively, essentially complete leukocyte removal was achieved by layering heparinized blood (1:1 in Ringer's) onto 25% Ringer's containing 0.5 M sucrose. Erythrocytes were sedimented and washed in Ringer's. Dogfish of moderate size (~0.9 m) readily survived withdrawal of 40 ml of blood, containing ~10 ml of packed erythrocytes.

### Nucleated Cytoskeletons

Erythrocyte pellets or suspensions were diluted at least 1:10 with lytic medium (LyM) consisting of 100 mM PIPES, 1 mM MgCl<sub>2</sub>, 5 mM EGTA, 10 mM *p*-tosyl arginine methyl ester (TAME), and 0.4% Triton X-100, pH 6.8 with KOH (25, 32). As in other species, LyM immediately cleared the cells of hemoglobin, revealing MB, nucleus, and TBM (11).

### Anucleate Ghosts and Cytoskeletons

Erythrocytes were lysed osmotically in 50% LyM (without Triton) containing 0.05 mM diisopropyl fluorophosphate (DFP). The preparation was drawn three times through a 22-gauge syringe needle, producing a suspension of anucleate ghosts and free nuclei, plus some nucleated ghosts and unlysed cells. After centrifugation for 3 min at ~750 g (top speed, International HN, International Equipment Corp., Needham Heights, MA), the supernate contained only anucleate ghosts. These were sedimented in 10 min at ~11,000 g (Sorvall RC-2B, SS-34 rotor; DuPont Co., Wilmington, DE) at 15°–20°C. The upper part of the pellet was resuspended in the same medium, and the lower part, containing both anucleate ghosts and contaminating particles, was discarded. Anucleate cytoskeletons were obtained by washing anucleate ghosts in LyM (without Triton) containing 0.1 mM DFP, extracting in the same medium containing 0.4% or 0.5% Triton X-100, and washing twice in medium without Triton.

### Mass Isolation of MBs

All steps were at 20°–22°C unless otherwise noted. In method I, erythrocytes from 1 vol of blood were suspended in 10 vol of 100 mM PIPES, 1 mM MgCl<sub>2</sub>, 5 mM EGTA, and 2% Triton X-100, pH 6.8 (isolation medium), and the nucleated cytoskeletons sedimented in 1 min at ~750 g. After resuspension in the same medium, the preparation was gently poured between beakers, and the resulting release of both MBs and nuclei was monitored in phase contrast. Additional fluxing or vortex mixing was used if necessary. The suspension was centrifuged for 5 min as before to sediment free nuclei and any intact cytoskeletons. The supernate, containing free MBs, was slowly removed to a fresh tube via a 1-mm wide Pasteur pipette (to minimize MB breakage), and centrifuged as before to remove additional nuclei. The supernate was transferred to 50-ml polycarbonate tubes and centrifuged for 10 min at ~11,000 g. Pellets consisted largely of intact and broken MBs, plus some contamination (see Fig. 4). MB sedimentation and subsequent washes in isolation medium lacking Triton were tested both at 0°–4°C and at 15°–20°C; no major MB dissolution occurred in either case.

This procedure (method I) appeared to be facilitated by increased leukocyte contamination, presumably a source of protease activity. Initial removal of essentially all leukocytes prevented MB release, yielding nucleated cytoskeletons which were now stable. In an alternative procedure (method II) these stable cytoskeletons were incubated in isolation medium containing 0.5 U/ml elastase (Sigma Chemical Co., St. Louis, MO) which released MBs and nuclei in ~10 min at room temperature. The nuclei were removed and the MBs sedimented and washed as in method I. The MBs in these preparations were remarkably intact and free of contamination (see Fig. 11), and remained intact in the presence of elastase for at least 1 h.

### pH 3 Extracts

Erythrocyte pellets (1 vol) were suspended in ~10 volumes of the same medium used for MB isolation and centrifuged for 2 min at room temperature (International HN, 3/4 speed). The nucleated cytoskeleton pellets were suspended uniformly in 10 volumes of McIlvaine 0.08 M citrate–0.04 M phosphate buffer at pH 3.0 (9), after which only intact nuclei were present (see Fig. 8). The nuclei were removed (10 min, ~750 g) and the supernate was adjusted to pH 7.3 with NaOH. Ice-cold TCA was added to 5% final concentration, and the preparation

stored on ice for 1 h. The precipitate was collected (10 min, ~750 × g), washed twice with ice-cold acetone, and air-dried before storage at –20°C.

### Cell Lysates before and after MB Disassembly

Control cells (room temperature) and cells incubated for 60 min at 0°C to disassemble MBs were lysed in LyM. As a further precaution against proteolysis, LyM contained 1 mM benzamide, 64 μM pepstatin, 100 μg/ml soybean trypsin inhibitor, and 25 KIU trasylol/ml. Equal numbers of cells (0.09-ml packed vol) were lysed in 0.45 ml LyM + inhibitors at 0°C. Preparations were centrifuged for 2 min at ~750 g, and the supernates were analyzed by SDS PAGE.

### Cell and Cytoskeletal Morphology

Cell morphology was assessed by phase-contrast examination of both living and fixed cells. For the latter, ~25 μl of cell suspension was diluted into 0.5 ml of fixation medium consisting of 1 vol of 10% glutaraldehyde, 9 vol of Ringer's, which faithfully preserved cell morphology. Fixed samples were a convenience for observation, and, more importantly, avoided "glass effects" which can grossly distort living erythrocyte shape. For standardized surveys of cytoskeletal structure, ~25 μl of cell suspension was diluted into 0.5 ml of LyM. A Zeiss phase-contrast microscope equipped for photomicrography was used throughout. For some critical phase-contrast observations, samples were viewed through small air bubbles intentionally produced under the cover slip, enhancing contrast considerably (see Fig. 11). Specimens under bubbles are effectively in miniature moist chambers, and do not dry out.

### Electron Microscopy

Isolated MBs were fixed in isolation medium containing 1% glutaraldehyde (18 h, room temperature), washed twice in isolation medium followed by 10 mM NaH<sub>2</sub>PO<sub>4</sub> at pH 6.0 (buffer), postfixed in cold buffer containing 0.5% OsO<sub>4</sub> (30 min, 3°C), washed twice in cold buffer, dehydrated in ethanol, and embedded in Epon. For whole mounts, isolated MBs on Formvar-coated grids were rinsed with isolation medium, fixed in the same medium containing 1% glutaraldehyde (5 min), rinsed with water, and stained with 2% aqueous uranyl acetate. Nucleated cytoskeletons on grids were rinsed twice with LyM (lacking Triton and TAME), fixed in the same medium containing 2.5% glutaraldehyde (15 min), rinsed as before and once with water, and stained with 2% uranyl acetate. For anucleate cytoskeletons, anucleate ghosts were extracted on grids with LyM (2 min) and processed as described above for nucleated cytoskeletons, except for fixation (5 min). To improve retention of material in some cases, Formvar-coated grids were treated for 5 min with 1% polylysine solution (Sigma Chemical Co.; >400,000 mol wt), washed, and dried before use.

Simultaneous lysis and fixation (12) was used to examine cytoskeletal structure in thin section. In temperature-cycling experiments, samples included controls at room temperature, cells incubated for 1.5 h at 0°C, and similarly cooled cells rewarmed for 1.5 h at room temperature. Cells were sedimented at their respective temperatures and the pellets resuspended in LyM containing 1% glutaraldehyde at room temperature (1:20). A loose hemoglobin gel formed in which the cytoskeletons were suspended, eliminating the need for centrifugation during subsequent steps. After 48 h at room temperature small pieces of the gel were washed twice in 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, pH 6.0, postfixed for 1 h in the same medium containing 1% OsO<sub>4</sub>, washed again in the 0.1 M buffer, and dehydrated and embedded as described above.

Thin sections were cut on a Sorvall MT-2 ultramicrotome with diamond knives, and stained with uranyl acetate and lead citrate. Specimens were examined in the Hitachi HS-8 transmission electron microscope operating at 50 kV or the JEOL 100B at 60 kV.

### Gel Electrophoresis

SDS PAGE was carried out on 20 × 30 cm slabs using a modified Laemmli system (19) in 5–12% or 5–15% gradient form, in which polypeptides of molecular weights from 12,000 to >300,000 were resolved. Gels were stained with Coomassie blue and destained by diffusion. Standards included: (a) crude dogfish brain tubulin [the C<sub>1</sub>S fraction, as described by Langford (20)] containing high molecular weight microtubule-associated proteins (MAPs) corresponding to MAPs 1 and 2 of mammalian brain tubulin; (b) bovine brain tubulin, twice cycled according to the method of Shelanski et al. (28), containing MAPs 1 and 2; (c) polypeptides from human erythrocyte ghosts prepared by hypotonic hemolysis in phosphate-buffered saline, including spectrin and erythrocyte actin; (d) rabbit muscle actin (Sigma Chemical Co.); and (e) 14,000–212,000 mol wt standards (Bio-Rad Laboratories, Richmond, CA).

## RESULTS

### *Living Dogfish Erythrocytes, and Nucleated Cytoskeletons*

The erythrocytes of *M. canis* are flattened, elliptical, and nucleated (Fig. 1a), with biconvexity due to the nuclear bulge, typifying erythrocyte morphology in all nonmammalian vertebrates (1). They are relatively large, with major and minor axes in the range of 17–22  $\mu\text{m}$  and 13–15  $\mu\text{m}$ , respectively. Cell morphology is stable during washes and several hours of

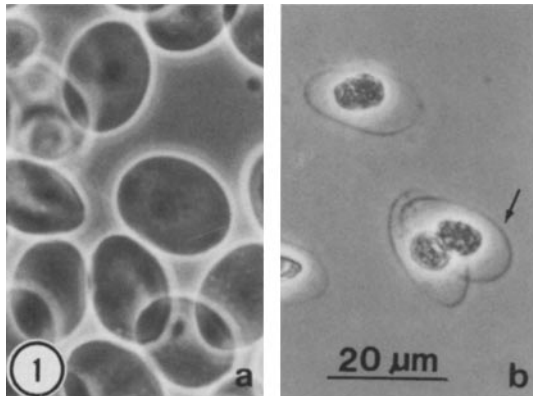


FIGURE 1 Dogfish erythrocytes and their cytoskeletons. (a) Living cells in fresh blood sample, with flattened elliptical morphology typical of all nonmammalian vertebrate erythrocytes. (b) Nucleated cytoskeletons prepared by Triton X-100 lysis under microtubule-stabilizing conditions (LyM; see Materials and Methods). Nuclei and MBs (arrow) become visible immediately as hemoglobin is lost. Phase contrast,  $\times 825$ .

storage in Elasmobranch Ringer's solution at room temperature (20°–22°C). When the cells are lysed in LyM, MB and nucleus immediately become visible in phase contrast (Fig. 1b). The material between MB and nucleus (TBM) is essentially phase-transparent but is readily visualized by electron microscopy of whole mounts and thin sections. In thin section (Fig. 2a), the TBM is seen as a surface-delimiting component that completely encloses nucleus and MB. In tangential section the TBM appeared to be a continuous layer. Whenever MB cross sections were found (as in Fig. 2a), they characteristically occupied tapered TBM extremities. In whole mounts (Fig. 2b) the TBM appeared as a rough irregular network completely spanning the space between MB and nucleus. The MB was electron dense, with individual microtubules sometimes visible.

### *MB Isolation and Structure*

The first mass MB isolation procedure (method I) was developed empirically based upon the spontaneous appearance of free MBs in some cytoskeleton preparations. The mechanism of MB release from cytoskeletons was investigated by testing variations on the successful procedure. Free MBs were not obtained when the isolation medium was used at 0°C (though MBs remained stable), or when it contained TAME in very high concentration (0.1 M). This suggested that proteolysis was involved, with contaminating leukocytes as a possible source of activity. When essentially all leukocytes were removed by sucrose density centrifugation before cell lysis, the erythrocyte cytoskeletons were highly stable and MBs could not be released even with excessive mechanical agitation. However, incubation with elastase produced intact free MBs in a few minutes at room temperature, and these MBs were stable for at least 1 h in the presence of elastase (method II).

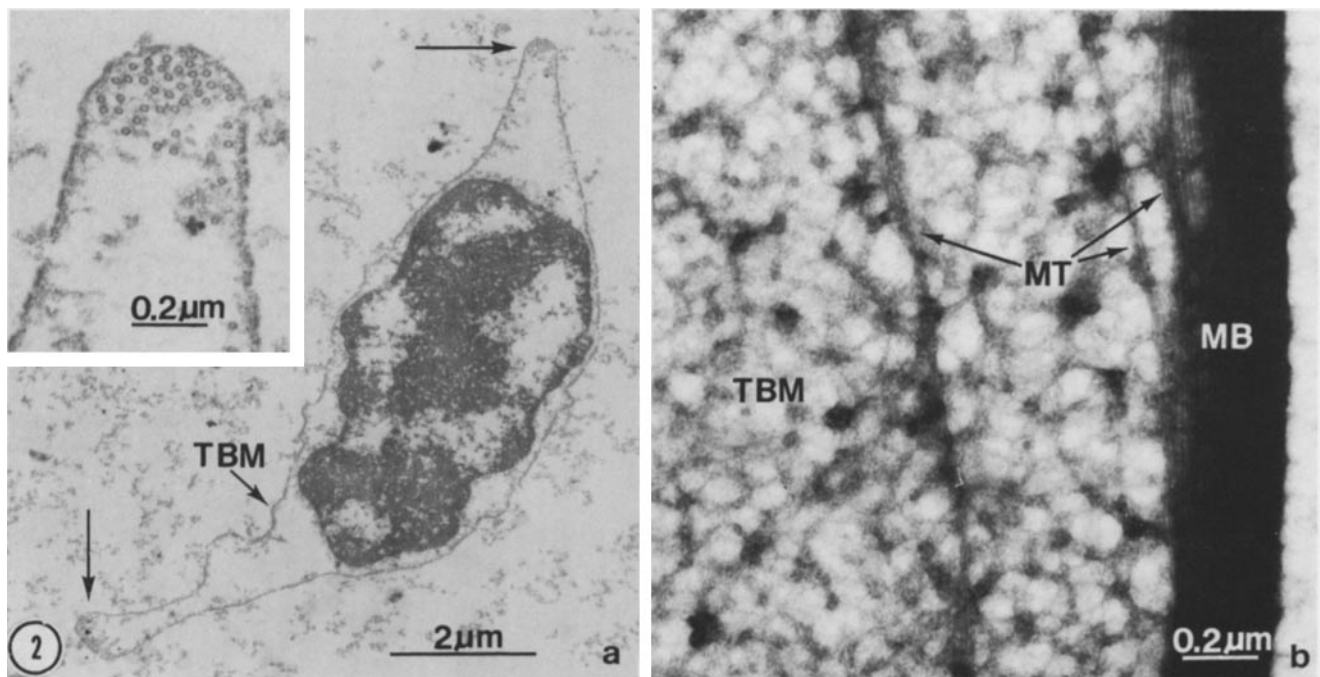


FIGURE 2 The cytoskeletal system of the dogfish erythrocyte in thin section and whole mount. (a) Thin section of simultaneously lysed and fixed erythrocyte. The trans-MB material (TBM) is seen as a surface-delimiting layer completely enclosing nucleus and MB, with the MB characteristically lysing in its tapered extremities (unlabeled arrows). Inset, higher-magnification view of upper MB cross section, showing the microtubule bundle in close proximity to the TBM. Fuzzy background material is dispersed hemoglobin. TEM,  $\times 9,500$ ; inset,  $\times 50,000$ . (b) Part of a nucleated cytoskeleton whole mount. The trans-MB material (TBM) appears as a rough, irregular network occupying all of the space between MB and nucleus. Individual microtubules (MT) are evident in some places within the densely stained MB, and are occasionally splayed from it. Uranyl acetate staining, TEM,  $\times 50,000$ .

Isolated MBs of this species are large enough to permit preselection of whole mounts on grids under phase contrast (Fig. 3 *a* and *b*). In TEM they appeared ribbonlike, with closely applied microtubules (Fig. 3 *c* and *d*). Thin sections of MB pellets (method I) contained microtubule bundles in all orientations, plus some contaminating fibrillar and granular ma-

terial (Fig. 4 *a*). Typically, 40–50 microtubules were counted in well-separated cross sections, although lower numbers were frequently encountered. At high magnifications, cross-bridges were evident between many of the microtubules (Fig. 4 *b*). No filaments of other kinds or internal “dots” (4) were observed in regular association with the microtubules.

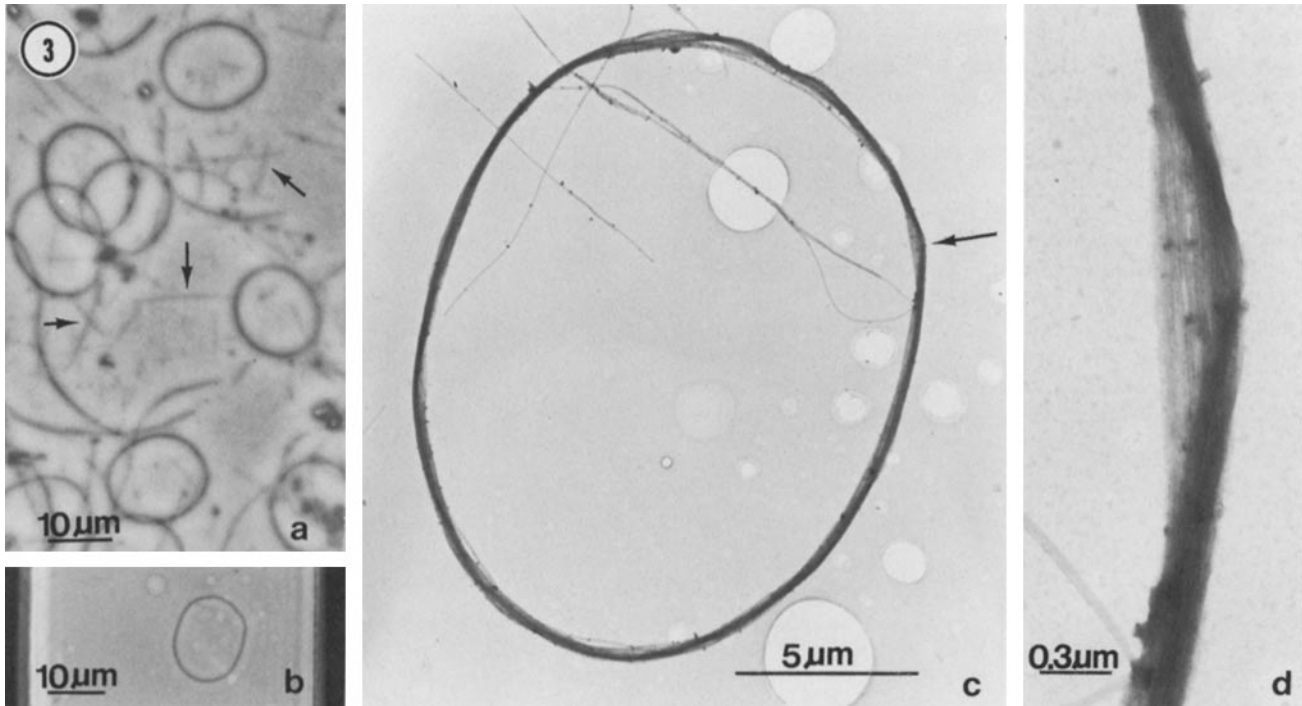


FIGURE 3 Isolated dogfish erythrocyte MBs (method I). (*a*) Sample from MB pellet, viewed through air bubble, showing whole MBs plus smaller and thinner MB segments (arrows); phase contrast,  $\times 880$ . (*b*) Whole mount of one isolated MB on Formvar-coated grid after uranyl acetate staining; phase contrast,  $\times 760$ . (*c* and *d*) Same MB as shown in *b*, viewed in the electron microscope. The MB is a continuous bundle of microtubules which appears ribbonlike in construction. A “fold” or buckled point (*c*, arrow) is shown at higher magnification in *d*. TEM,  $\times 4,800$  and  $\times 32,000$ , respectively.

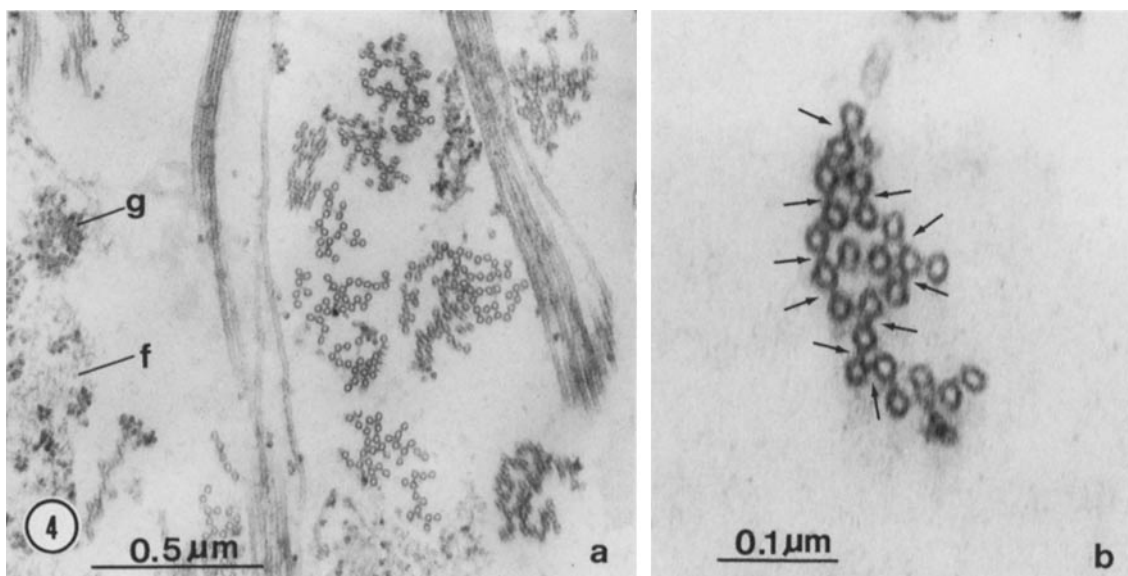


FIGURE 4 Isolated MB pellet (method I) in thin section. (*a*) Microtubule bundles are present in various orientation, plus some contaminating granular (*g*) and fibrillar (*f*) material. Separate cross sectioned bundles typically contain 40–50 microtubules, though lower numbers are encountered. Cross-bridges are frequently observed between adjacent microtubules of the isolated MBs (*b*, arrows). TEM,  $\times 44,500$  and  $\times 160,000$ , respectively.

## MB Disassembly and Reassembly in Living Cells

When cells were incubated in Ringer's at 0°C for 30 min or more, cell morphology remained normal but the MBs disappeared (Fig. 5*a* and *b* vs. *c* and *d*). Upon rewarming, MBs reassembled with much the same appearance as noncooled controls (Fig. 5*e* and *f* vs. *a* and *b*). The MB of this species is thus low-temperature labile *in vivo*. Multiple disassembly/reassembly cycles were also obtained, and even after five cycles many MBs were of normal appearance. Erythrocytes could be stored in Ringer's for 21 h at 0°C (longer periods not tested), with essentially normal MB re-formation occurring in most cells after rewarming.

Colchicine ( $10^{-3}$  M) and Colcemid ( $10^{-3}$  M,  $10^{-4}$  M) did not produce MB disassembly after 1–2 h at room temperature but inhibited MB reassembly after 0°C-induced disassembly, as illustrated for colchicine in Fig. 5*g–l*. After 1 h at room temperature, the appearance of both the cell and the cytoskeleton was normal (Fig. 5*g* and *h*), and after 1 h at 0°C the MBs disappeared as in controls (*i* and *j* vs. *c* and *d*). However, in the presence of colchicine (*k* and *l*), MBs failed to reappear after rewarming, in contrast to controls (*e* and *f*). Inhibitor-treated cells retained flattened elliptical morphology throughout, though wrinkling of the cell surface was sometimes observed.

Cytoskeletal structure during temperature cycling was further examined by electron microscopy (Fig. 6). In whole mounts, typical structure was evident in noncooled controls (Figs. 6*a* and *b*), with the TBM most apparent where it overlapped itself due to MB twisting. After incubation of cells at 0°C, the MB was gone, leaving the TBM collapsed inward into a denser mass (Fig. 6*c* and *d*). Upon rewarming (Fig. 6*f* and *g*), cytoskeletal structure again resembled that of controls. Thin sections of simultaneously lysed and fixed material confirmed the presence of the TBM after incubation of living cells at 0°C (Fig. 6*e*). Neither MBs nor the TBM tapering typical of controls (Fig. 2*a*) were found in any sections of cooled cells, and no microtubules were observed anywhere in these lysed cells examined at higher magnification. Rewarmed cells regained essentially normal cytoskeletal structure (Fig. 6*h*; compare with Fig. 2*a*).

## "Spindle Cell" Formation

When dogfish erythrocytes were stored in Ringer's at room temperature for long periods (4 h or more) with or without prior temperature cycling, increasing numbers of single- and double-pointed cells appeared with time. A few single-pointed cells can usually be found in fresh erythrocyte suspensions, but

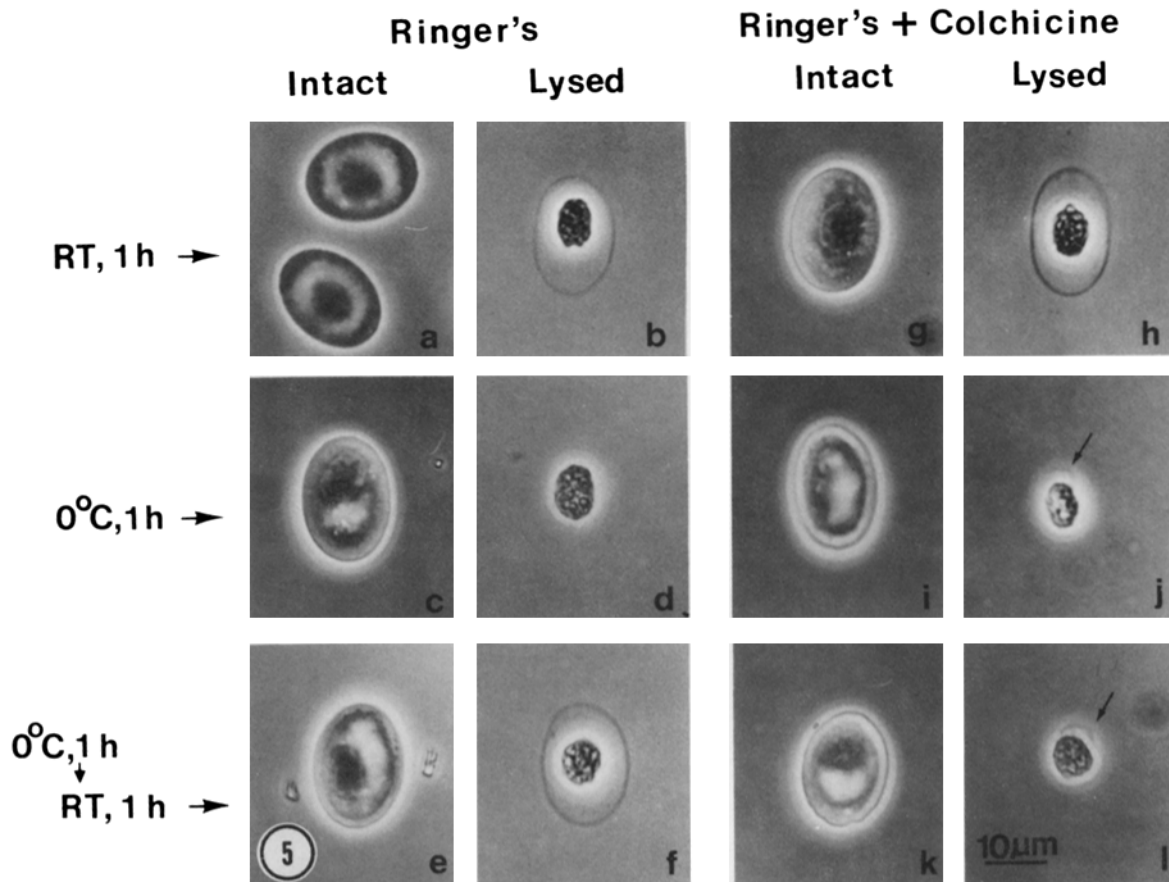


FIGURE 5 Effect of temperature cycling (0°C/room temperature) and colchicine (1 mM) on cell morphology and the cytoskeleton. Erythrocytes were incubated in Ringer's (*a–f*) or in Ringer's containing colchicine (*g–l*). Paired photographs show representative intact cells and cells lysed in LyM. *a–b* and *g–h*, incubation at room temperature, 1 h; MBs are not disassembled by colchicine. (*c–d* and *i–j*) 0°C, 1 h; MBs disassemble with or without colchicine. Faint cytoskeletal remnants are sometimes detectable near nuclei (*j*, arrow; see also Fig. 6*c*). (*e–f* and *k–l*) 0°C, 1 h, transferred to room temperature, 1 h. MBs reassemble in the absence (*f*) but not presence (*l*) of colchicine. Note cytoskeletal remnant adjacent to nucleus (*l*, arrow). Flattened, elliptical cell morphology is retained in all cases. Except for *a*, which shows living cells, intact cells were fixed in Ringer's containing 1% glutaraldehyde. Phase contrast,  $\times 800$ .

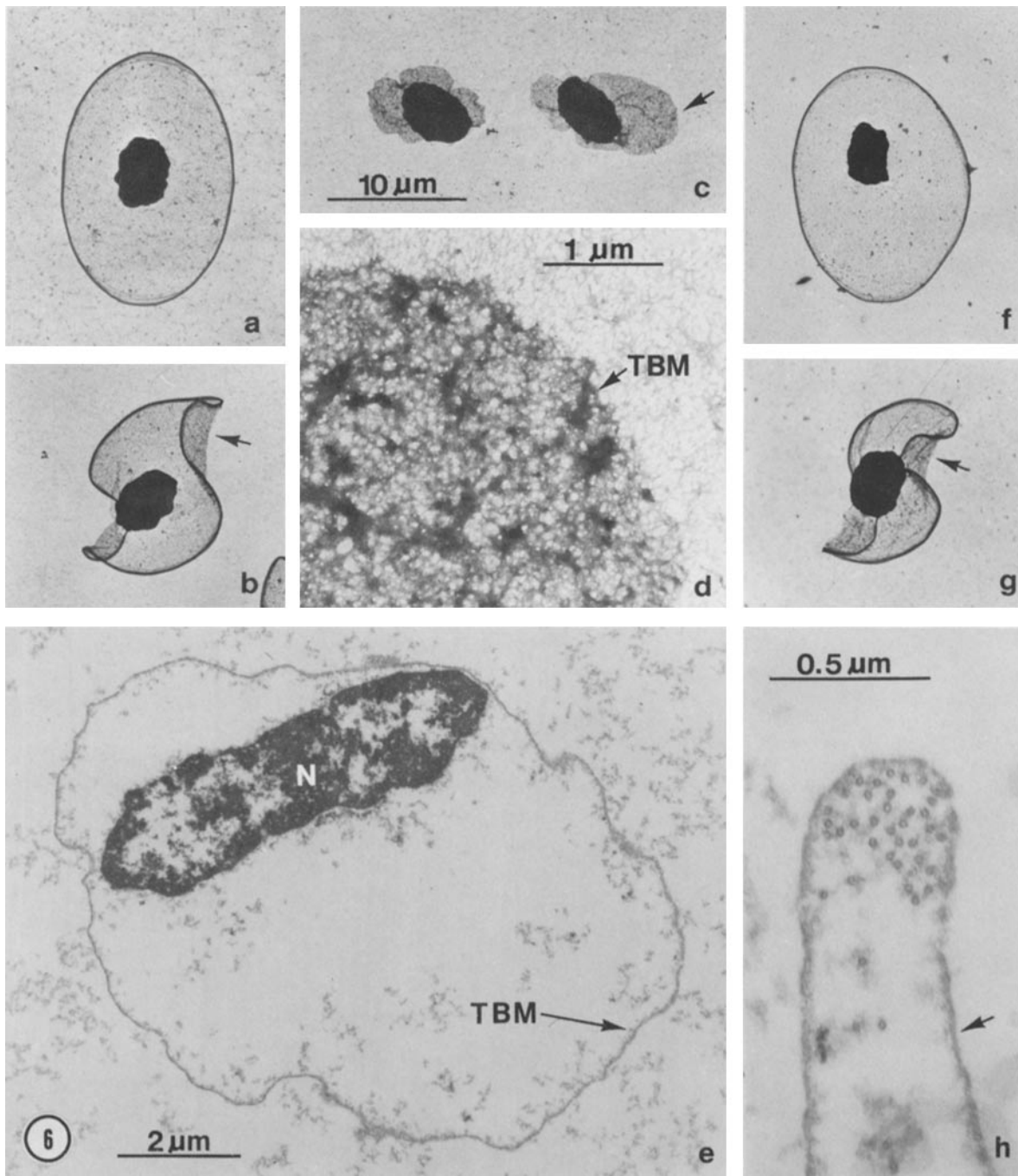


FIGURE 6 Effect of temperature cycling as observed in whole mounts and thin sections, TEM. Whole-mount controls: (a) cells lysed at onset of experiment, or (b) after incubation for 5 h at room temperature. When MBs are twisted, TBM visibility is enhanced where it overlaps itself (b, arrow). Twisting is common in all whole-mount preparations and is not peculiar to long-term incubations. Comparable thin-sectioned material is shown in Fig. 2 a. Bars as in c;  $\times 2,100$ . (c) Incubation for 3 h at  $0^{\circ}\text{C}$ ; MBs have disappeared, but the TBM remains collapsed into a relatively dense mass around nuclei. Area near arrow is shown at higher magnification in d.  $\times 2,100$  and  $18,000$ , respectively. (e) Thin section of cell simultaneously lysed and fixed after incubation at  $0^{\circ}\text{C}$  for 1.5 h. The TBM is present encompassing the nucleus (N), but the MB is absent. Examination at higher magnification shows that no microtubules are present, and the TBM no longer displays tapered extremities in any view (compare with Fig. 2 a). Fuzzy background material is dispersed hemoglobin.  $\times 9,500$ . (f and g) Whole mounts of cells incubated for 3 h at  $0^{\circ}\text{C}$  followed by rewarming for 2 h at room temperature; cytoskeletons are comparable to controls (a and b), with the TBM again more apparent at overlap (g, arrow). Background contamination in these and other whole mounts (a–d) is due to polylysine coating on Formvar. Bars as in c;  $\times 2,100$ . (h) Part of thin section of cell incubated for 1.5 h at  $0^{\circ}\text{C}$  followed by rewarming for 1.5 h at  $20^{\circ}\text{C}$ ; the MB has reassembled, and tapered morphology of the TBM (arrow) is restored (compare with Fig. 2 a, inset).  $\times 50,000$ .

the double-pointed “spindle cells” are rare. In some stored preparations, however, spindle cells represented  $>20\%$  of the population (Fig. 7 a and b). Lysed samples contained a similar

percentage of anomalous double-pointed MBs (Fig. 7 c and d). These were not continuous but consisted of separated fibers (microtubule bundles) of varying thickness converging at cell



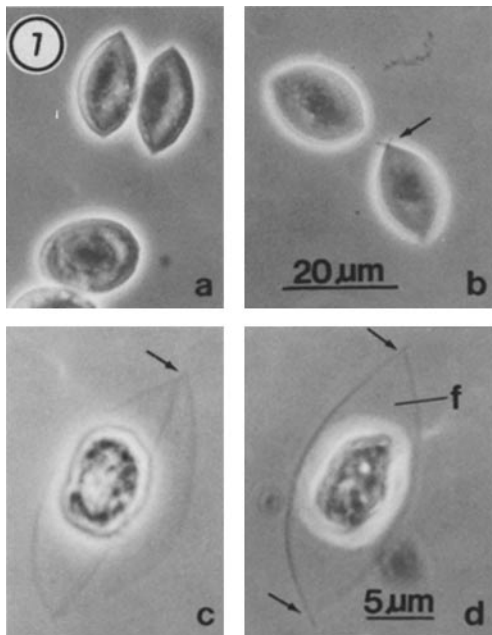


FIGURE 7 "Spindle cells" and their cytoskeletons. Double pointed erythrocytes are rare in fresh blood samples but appear in increasing numbers with long-term storage in Ringer's at room temperature (4 h or more). (a) Two spindle cells. Like normal erythrocytes (cell at lower left) the spindle cells are flattened. Bar as in b. (b) Two spindle cells, one with two antennalike projections at one end (arrow). Note that projection angles follow cell surface contours. (c) A spindle cell cytoskeleton with three major fibers (microtubule bundles) meeting at the "poles" and crossing at upper pole (arrow; crossed fibers very thin and faint). Bar as in d. (d) A spindle cell cytoskeleton with two major fibers crossing at "poles," and a third very thin fiber (f) visible. Phase contrast,  $\times 750$  (a and b) and  $\times 1,700$  (c and d).

"poles." These fibers were essentially planar, and, correspondingly, the pointed cells retained their flatness. Occasionally, the fibers could be traced slightly beyond the polar convergence point (Fig. 7c and d), presumably accounting for polar projections observed in some of the intact spindle cells (Fig. 7b). Single-pointed teardrop-shaped MBs were also found, corresponding to single-pointed cell morphology. Would normal cell shape be regained if these anomalous MBs were disassembled at  $0^{\circ}\text{C}$ ? Cell suspensions were incubated at room temperature until significant numbers of spindle cells were present, and samples were placed at  $0^{\circ}\text{C}$ . Ellipticity was restored as nearly all points disappeared within 1–2 h, while being retained in room temperature controls.

### Molecular Components of the Cytoskeletal System

Total cytoskeletal polypeptide composition could not be determined by simple dissolution of nucleated cytoskeletons in SDS sample solution because nuclei rapidly swelled and produced chromatin gels. A method was therefore sought for selectively solubilizing MB and TBM while keeping nuclei intact. Various salt solutions also produced chromatin gels, but very low pH proved effective. When we used LyM in which 0.1 M citrate was substituted for PIPES, MB and TBM were present at pH 6, pH 5, and pH 4. Increased MB twisting occurred with pH reduction, and at pH 4 the cytoskeleton appeared to be collapsed tightly around the refractile nuclei.

However, lysis at pH 3, or transfer of nucleated cytoskeletons from normal LyM (pH 6.8) to pH 3 medium, immediately dissolved MB and TBM, leaving morphologically intact nuclei (Fig. 8). The latter procedure eliminated hemoglobin and other soluble components in the initial lysate, simplifying the "pH 3 extract" before further processing as described in Materials and Methods.

SDS-PAGE analysis of pH 3 extracts (Fig. 9, lane 7) revealed polypeptides comigrating with human erythrocyte spectrin (two bands) and actin, and with three bands in the  $C_1S$  fraction containing dogfish brain tubulin (20) [bands a (59,500), b (57,000), and d (52,500)]. Another "tubulin region" band was present [c (54,000)] plus several other major components (unidentified) and hemoglobin (Fig. 9, lane 7 vs. lanes 6 and 8). High molecular weight bands corresponding to MAPs 1 and 2 (lane 1) were not observed.

Although pH 3 extraction of nucleated cytoskeletons appeared to solubilize MB and TBM selectively, the nuclei could not be dismissed as a possible source of extract components. The method for mass preparation of anucleate cytoskeletons consisting only of MB and TBM (Fig. 10a–c; see Materials and Methods) was therefore developed as an independent approach to cytoskeletal composition. SDS PAGE of anucleate cytoskeletons dissolved directly in SDS sample solution confirmed the presence of polypeptides comigrating with tubulin, spectrin, and actin in the MB and TBM (Fig. 10d).

### Major Molecular Components of Isolated MBs and MBs Disassembled *In Vivo*

Isolated MBs (method I) exhibited the same four tubulin region bands found in the pH 3 extract (Fig. 9b, lane 4; bands a–d), three of which comigrated with major bands in the  $C_1S$  fraction containing dogfish brain tubulin (lane 6). Isolated MB pellets (method I) did not contain spectrin, but various other bands were present. Most of these, including some actin, were judged to be contaminants because they were also present in a pellet obtained by further centrifugation of the supernate after MBs had been sedimented (Fig. 9a, lane 4 vs. 5). High molecular weight MAPs were not observed in these isolated MB preparations, even on overloaded gels.

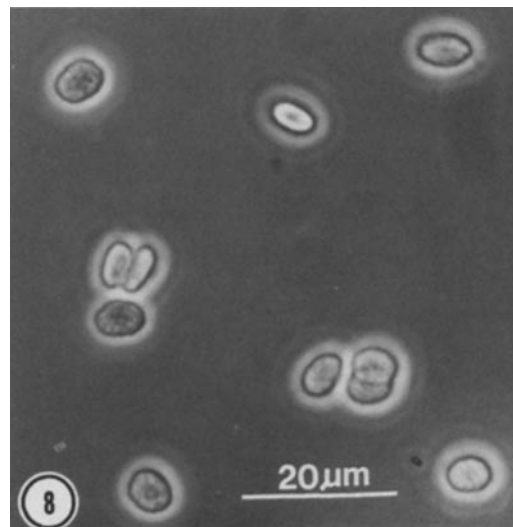


FIGURE 8 Effect of pH 3 media on nucleated cytoskeletons; MB and TBM are solubilized, leaving free nuclei. Phase contrast,  $\times 1,000$ .



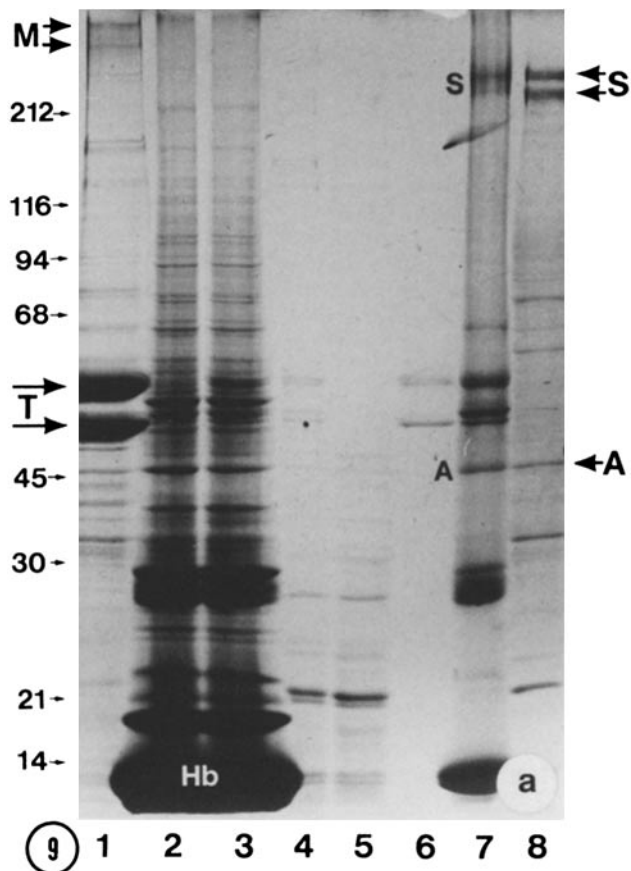
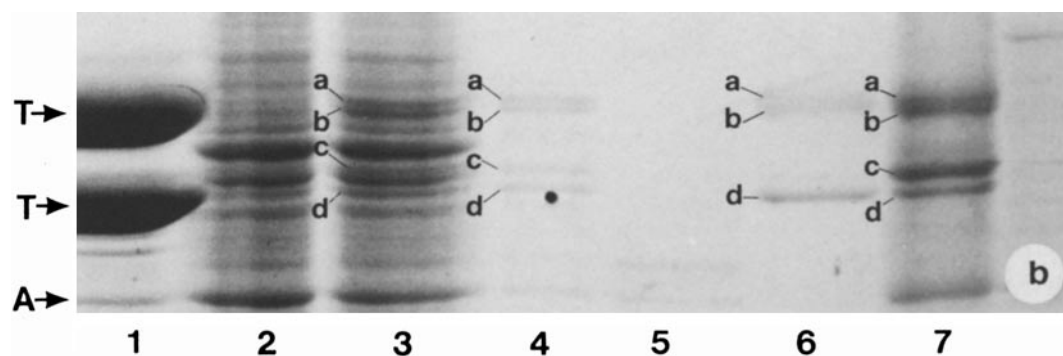


FIGURE 9 SDS-PAGE separation of dogfish erythrocyte cytoskeletal polypeptides. (a) 5–12% gradient gel. Lanes: (1) C,S (crude) fraction of dogfish brain tubulin (*T*; 20) overloaded to show high molecular weight MAPs (*M*), (2) control cell (room temperature) lysate supernate, (3) lysate supernate from cells incubated for 2 h at 0°C to disassemble MBs, (4) isolated MB pellet (method I) containing four “tubulin region” (*T*) bands, (5) pellet obtained by further centrifugation (~11,000 g, 60 min) after MB removal (11,000 g, 10 min). This is the probable contaminant fraction for lane 4. (6) C,S fraction of dogfish brain tubulin, underload, (7) pH 3 extract containing solubilized components of nucleated cytoskeletons (*S*, dogfish erythrocyte spectrin; *A*, erythrocyte actin), (8) human erythrocyte ghost standards (*S*, spectrin; *A*, erythrocyte actin). Molecular weight standards (mol wt  $\times 10^{-3}$ ) are given at left. (b) Higher magnification of the “tubulin region” (*T*) of lanes 1–7. Four bands (*a*–*d*) were identified by enhancement in the lysate supernates from 0°C-incubated cells (lane 3) vs. controls (lane 2). Enhancement of bands *a* and *b* is most evident here, with *c* and *d* more apparent in other experiments. The four bands identified by *in vivo* MB dissolution (lane 3) comigrate with the four tubulin region bands of isolated MBs (lane 4), and are not present in the isolated MB contaminant pellet (lane 5). Bands *a*, *b*, and *d* are also evident in the C,S fraction of dogfish brain tubulin, in the pH 3 extract (lane 7) containing both MB and TBM with a trace of band *c* (lane 6). All four bands (*a*–*d*) are prominent components.



MBs isolated by elastase treatment of nucleated cytoskeletons (method II; Fig. 11) were nearly all intact, and the preparations were relatively free of visible contaminants. Their SDS-PAGE pattern was correspondingly simple, even on overloaded gels (Fig. 12, lane 3), consisting almost entirely of protein in the tubulin region. There were no polypeptides comigrating with high molecular weight MAPs (*M*, lane 1), spectrin (*S*, lane 2), or actin (*A*, lane 2).

An independent *in vivo* approach to identification of major MB components was also utilized. Control cells at room temperature were lysed in LyM and the solubilized proteins were compared with those of an equal number of cells lysed after MB disappearance at 0°C (60 min). Of the numerous bands present, only four were enhanced in supernates from cells with 0°C-dissociated MBs (Fig. 9b, lane 3 vs. 2; bands *a*–*d*). These correlated closely with the four tubulin region bands of isolated MBs (lane 4). Enhancement of bands *a* and *b* is apparent in the gel shown (Fig. 9b, lane 3 vs. 2), while bands *c* and *d* were more evident in other experiments. The actin band was not

noticeably affected, and spectrin did not appear in either supernate (lanes 2 and 3).

## DISCUSSION

### *The Dogfish Erythrocyte Cytoskeletal System*

The major components of the dogfish erythrocyte cytoskeletal system are the MB and TBM. The latter was initially described in Triton-lysed amphibian and goldfish erythrocytes. It is nearly transparent in phase contrast, but in edge view appears to correspond to the cell surface (11). In thin sections of whole cells the TBM is invisible due to the electron density of hemoglobin, but in lysed cells it is seen as a thin surface-delimiting layer which is external to the MB and nucleus. MB cross sections are always found in tapered regions of the TBM, giving the impression that MB pressure against the TBM flattens the cytoskeleton in the plane of the MB. In lysed cell whole mounts observed by electron microscopy, this surface layer collapses onto itself from opposite sides and appears as a

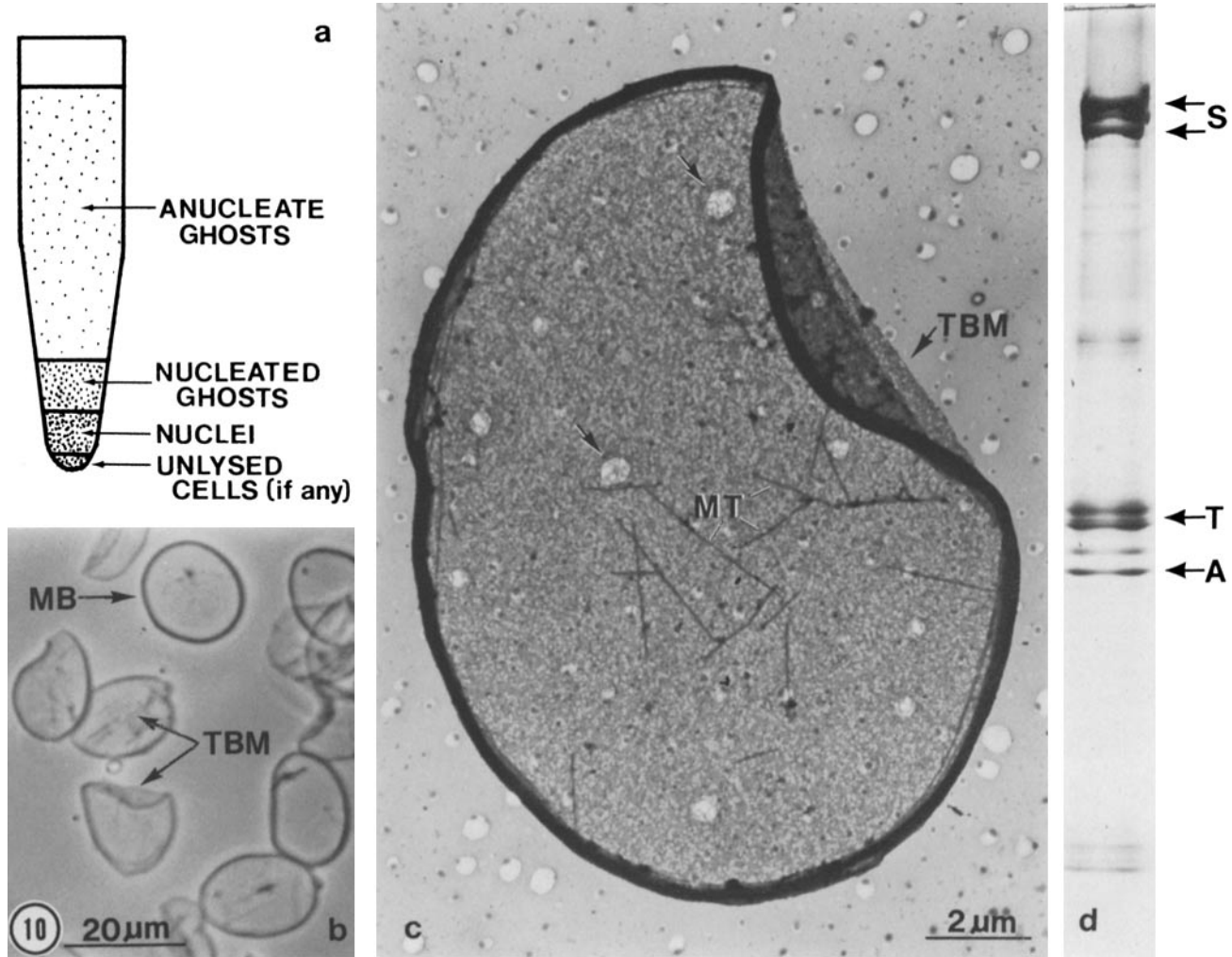


FIGURE 10 Structure and composition of anucleate cytoskeletons. (a) After shearing and centrifugation of osmotically lysed cells, suspension components sediment according to density. Anucleate ghosts are collected from the supernate and extracted in LyM, yielding anucleate cytoskeletons. (b) Anucleate cytoskeletons in phase contrast (viewed in air bubble), showing MB and TBM;  $\times 850$ . (c) Anucleate cytoskeleton whole mount, TEM. The anucleate cytoskeletons consist only of MB and TBM, with broken microtubule segments sometimes present (MT, verified at higher magnification). Note netlike appearance of TBM over holes in Formvar (unlabeled arrows). Uranyl acetate staining, TEM,  $\times 7,300$ . (d) SDS-PAGE pattern from whole anucleate cytoskeletons (5–15% gradient). Major proteins comigrated with the two bands of human erythrocyte spectrin (S; location of standards denoted by arrows), human erythrocyte actin (A), and bovine brain tubulin (T); several additional polypeptides are present.

rough planar network traversing the space between MB and nucleus. Therefore, in terms of cellular location, the TBM is quite likely the nonmammalian counterpart of the “membrane cytoskeleton” that underlies the mammalian erythrocyte membrane bilayer.

The data on molecular components of the dogfish erythrocyte cytoskeleton are clearly consistent with this morphological picture. Both the pH 3 extracts of nucleated cytoskeletons and the whole anucleate cytoskeletons contain polypeptides comigrating with human erythrocyte spectrin and actin, in addition to tubulin, whereas isolated MBs contain tubulin but not spectrin or actin. In addition, when the MB (but not the TBM) is disassembled at  $0^{\circ}\text{C}$  in living cells, only polypeptides in the tubulin region of gels are noticeably increased in cell lysates. Therefore the dogfish erythrocyte spectrin and actin appear to be in the TBM, with tubulin in the MB.

#### MB Isolation and Properties

The problem of MB isolation is defined by the morphology

of the cytoskeletal system. Since the MB is enclosed within the TBM, the latter must be removed selectively. As a first approach to mass isolation, TBM proteolysis has been satisfactory. Whole MBs are released, and many survive as such during centrifugation, with the microtubules presumably stabilized into ribbonlike arrays by the cross-bridges. Ribbonlike construction has also been observed in amphibian MBs (6, 11). Apparent cross-bridges have been noted previously in platelet and amoebocyte MBs (4, 23), but nothing is known of their three-dimensional distribution or composition. Tubulin preparations from whole mammalian platelets contain several high molecular weight proteins as minor components (8), but it is not known whether these are actually present in platelet MBs. A “MAP-2” has been reported in amphibian erythrocyte MBs on the basis of antibody binding (29), but polypeptides migrating with or near MAP-1 and MAP-2 standards were not observed in the present work, even on gels overloaded with samples from apparently intact isolated MBs. The problem of potentially protease-sensitive minor components of isolated

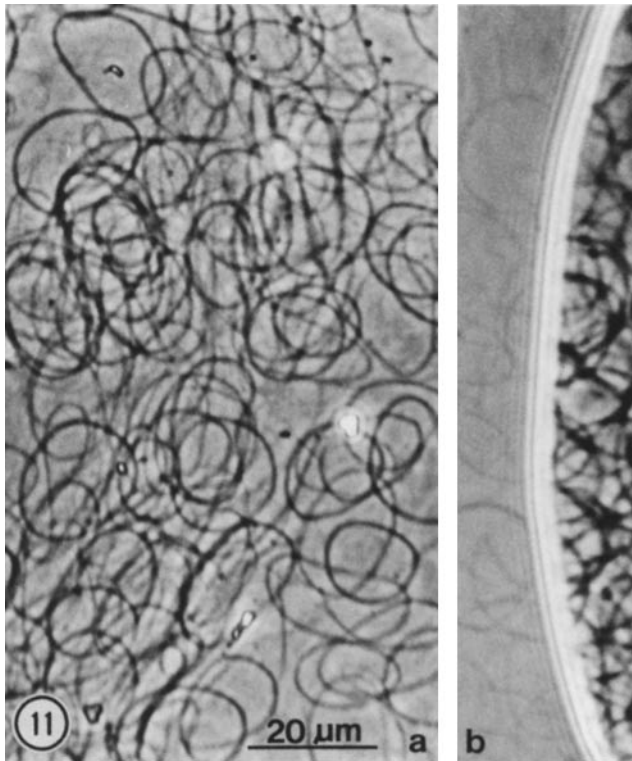


FIGURE 11 MBs mass-isolated by elastase treatment of nucleated cytoskeletons (method II). (a) Sample from MB pellet, viewed in air bubble in phase contrast. Most MBs are intact, with no apparent contamination by TBM or nuclei. (b) Illustration of contrast enhancement in bubble: liquid medium at left, air at right. Phase contrast,  $\times 850$ .

MBs, such as MAPs and bridges, should yield as other conditions are discovered for selective TBM dissolution.

The four tubulin-region proteins found in isolated MBs and other cytoskeletal preparations were detectable with underloading of 5–12% gradient gels (Fig. 9). Although our identification of all four as tubulins must be regarded as tentative, their number is not surprising, as multiple  $\alpha$ - and  $\beta$ -tubulins have been found in other systems.

In living cells, the dogfish erythrocyte MB undergoes temperature-induced disassembly/reassembly, with colchicine inhibiting reassembly after cooling but not inducing disassembly before cooling. The former property is similar to that of mitotic spindles (18), while the latter resembles that of flagella (2, 26). Low temperature presumably alters the state of tubulin molecules throughout MB microtubules, whereas colchicine-induced disassembly would be a function of the number of microtubule free ends, of which there may be few in the MB.

MBs of avian erythrocytes and mammalian platelets have been reported previously to be cold-labile (2, 4, 5), while those of amphibian erythrocytes are cold-stable (5). The low-temperature lability of the dogfish erythrocyte MB refutes a possible correlation of MB lability with homeothermy and stability with poikilothermy. Though cold-labile in vivo, the dogfish erythrocyte MBs were relatively stable to washes and storage at low temperature after isolation, raising a problem for further investigation.

### Function of Erythrocyte Cytoskeletal Components

Dogfish erythrocytes retain approximately normal morphol-

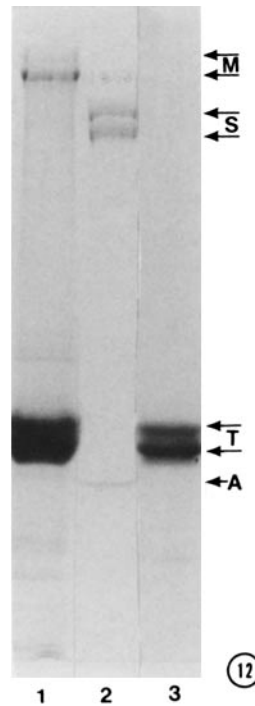


FIGURE 12 SDS-PAGE pattern from elastase-isolated MBs (see Fig. 11). Lanes: (1) twice-cycled bovine brain tubulin (T) prepared by the method of Shelanski et al. (28), overloaded to show high molecular weight MAPs (M). (2) Human erythrocyte spectrin (S) and actin (A). (3) Elastase-isolated MBs, overloaded. The gel is dominated by tubulin (T); proteins comigrating with high molecular weight MAPs (M), spectrin (S), and actin (A) are not present. 5–15% gradient gel.

ogy at low temperature in the absence of MBs. This is also the case for chicken erythrocytes, on which basis Behnke (5) proposed that something other than the MB was responsible for cell shape maintenance in at least some species. Our observations raise the possibility that shape maintenance at low temperature is a function of the TBM, which remains present throughout temperature cycling. We suggest that nucleated erythrocyte shape corresponds to that of the TBM at all times, with the MB acting as a frame upon which TBM shape is generated during cellular morphogenesis. If this subsurface material “set” to an equilibrium shape as the erythrocyte matured, normal morphology would be maintained in living cells when MBs were experimentally disassembled. The loss of normal tapered TBM shape in cells lysed after MB disassembly at  $0^{\circ}\text{C}$  (Fig. 6) is admittedly a discrepancy in this scheme, one which is set aside for the present. Extraction of a frozen lipid bilayer contributing to shape maintenance would not account for it, since shape is also maintained in living cells that lack MBs after rewarming in colchicine.

“Spindle cells” similar to those observed here also occur naturally in avian blood (21), and may be the erythrocyte equivalent of spindle-shaped platelets produced by cooling and rewarming (4, 33). They demonstrate the close correlation between cell and MB morphology. Our interpretation is that the TBM is subject to elastic deformation by the MB from within;  $0^{\circ}\text{C}$  disassembly of the anomalous pointed MBs removes the deforming forces, permitting TBM reversion to normal elliptical morphology.

Spectrin and actin are the major components of the sub-bilayer cytoskeleton or “Triton shells” of mammalian erythrocytes (14, 27, 30). The flattened elliptical shape of camel erythrocyte ghosts, unique among mammals, is lost only upon complete spectrin extraction (24). Spectrin or spectrinlike polypeptides have also been reported for chicken erythrocytes (7, 10), where they could account for cell shape retention at low temperature (2, 5) as postulated here for dogfish erythrocytes. Taken together, the observations support a general role for spectrin in erythrocyte shape maintenance. The mammalian

erythrocyte is perceived as a gross modification of the non-mammalian one, in which MB and nucleus are eliminated but a spectrin-actin subsurface layer is retained.

Nearly all studies of erythrocyte MBs support a morphogenetic role and deny a shape maintenance role (2, 5, 13, 17), yet MBs are present in most (possibly all) of the circulating erythrocytes of nonmammalian vertebrates. Is their retention simply economical? A broader view shows the morphogenesis vs. maintenance problem to be illusory: erythrocytes are designed for conditions of blood flow, with appropriately adapted mechanical and rheological properties. When MBs are experimentally disassembled in dogfish or chicken erythrocytes (2, 5), retention of normal cell morphology is meaningful only for the static conditions of microscopic examination. It says nothing about morphological or mechanical properties during flow or osmotic stress *in vivo*. Thus, caution must be exercised so that the data are not overextended. In the normal erythrocyte environment, MBs may well have an important continuing influence on cellular mechanics and shape maintenance.

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