INCOMPLETE MICROTUBULES OBSERVED IN MAMMALIAN BLOOD PLATELETS DURING MICROTUBULE POLYMERIZATION

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Cytoplasmic microtubules are labile structures sensitive to environmental conditions, e.g. it has been shown that the microtubules in the axopods of *Actinosphaerium* (1) and in developing sperm tails of a crane fly (*Nephrotoma suturalis* Loew) (2) disappear when the cells are exposed to 4° and 0°C. In both cases the process was reversible (1),[1] the microtubules reappearing when the cold treatment was discontinued.

This work deals with the cytoplasmic microtubules in mammalian blood platelets. Unlike the microtubules of the axial filament complex of cilia and flagella, these microtubules are properly preserved only by glutaraldehyde fixation. In platelets the microtubules are arranged in a marginal bundle (3–5) which is thought to be responsible, at least in part, for the maintenance of the lenticular shape of the circulating blood platelet. The report presents some observations on cytoplasmic microtubules of the marginal bundle of mammalian blood platelets subjected to changes in environmental temperature, and shows that, like cytoplasmic microtubules in other cells, platelet microtubules disappear when platelets are placed in the cold, and that they reappear when the platelets are warmed again. Observations are presented which give indications of how microtubules are formed.

Changes in the shape of platelets concomitant with changes in the marginal bundle of microtubules will be dealt with in detail in a later report.

MATERIAL AND METHODS

Siliconized glassware was used in all experiments. Human and rat venous blood was anticoagulated with sodium citrate (2 ml of 2% sodium citrate at pH 6 and 37°C, added to 8 ml of blood), and platelet-rich plasma (PRP) was obtained by centrifugation at 150 *g* for 15 min while the temperature was maintained at 37°C. The pH of the PRP was 7.2–7.3.

1 ml samples of PRP were treated in the following ways: (*a*) maintenance at 0°C for 5, 10, 15, and 30 min; (*b*) maintenance at 0°C for 45 min and then warming to 37°C and maintainance at this temperature for 2, 4, 6, 8, 10, 12, 14, and 60 min; (*c*) maintenance at 37°C for 2 hr.

After treatment the platelets were fixed for electron microscopy by adding 4 ml of a 4% solution of glutaraldehyde (in 0.1 M cacodylate buffer at pH 6) to the 1 ml samples of PRP. The fixative was at room temperature. Platelets were isolated immediately after adding the fixative by centrifugation at 2000 *g* for 15 min. The resulting pellets were kept in glutaraldehyde for an additional 15 min and then post-fixed in OsO_4 and embedded in Epon. Sections were stained with uranyl acetate and lead citrate.

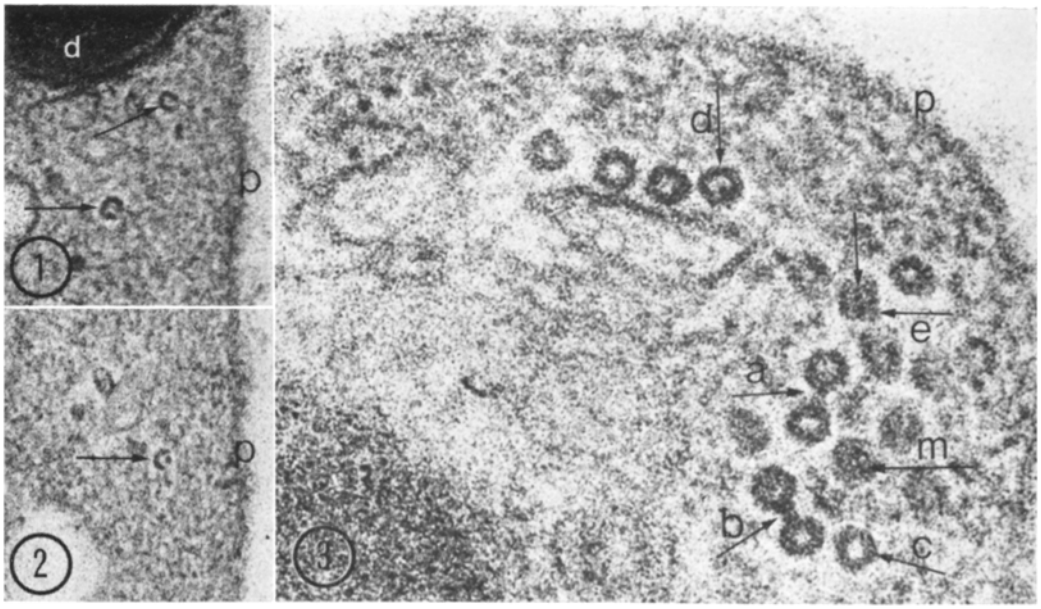
OBSERVATIONS

After exposure to 0°C for 5 min the majority of platelets contained no microtubules, although platelets with intact marginal bundles were occasionally seen. After exposure for 30 min microtubules were never identified. Platelets without marginal bundles generally assumed spherical or highly irregular shapes with numerous pseudopods.

Upon return to 37°C microtubules began to show up in the platelets after 2 min, the number of identifiable profiles increasing with time. The microtubules were in the initial stages of reformation oriented at random in the cytoplasm, and the platelets were spherical or irregular. After 60 min at 37°C the microtubules had become arranged again as a marginal bundle in most platelets, and these platelets had resumed the normal lenticular shape. Platelets maintained for 2 hr at 37°C had intact marginal bundles of microtubules and were lentiform; only a few platelets had formed pseudopods.

Generally the reformed microtubules were morphologically indistinguishable from microtubules in untreated platelets. In platelets fixed at all periods of microtubule reformation, however, a number of observations were made of figures which were identified as incomplete microtubules. It is thought that these figures represent cross-sections of microtubules fixed at various stages of the reassembly process. No microtubules could be identified as incomplete in longitudinal sections. Most of the incomplete microtubules seen were shaped like the letter C (Figs. 1, 2, 6, 7, 9–13),

[1] Behnke, O., and A. Forer. Data in preparation.



All illustrations shown are from blood platelets fixed during the stages of microtubule reformation, after the platelets had been exposed to cold. Figs. 1–3 and 8–13 are from human platelets; Figs. 5–7 and 14 are from rat platelets. In all figures, *p* indicates the plasma membrane.

FIGURE 1 Platelet reheated for 10 min. Two incomplete microtubules are seen (arrows). *d*, dense granule. × 95,000.

FIGURE 2 Platelet reheated for 8 min. An incomplete microtubule associated with a "dot" is seen (arrow). × 95,000.

The microtubules shown in Figs. 3–7 are from platelets reheated for 1 hr.

FIGURE 3 Arrows marked *a* and *b* indicate microtubules connected in pairs by a dense line passing from one microtubule to the other. *c*, complete microtubule; *d*, microtubule associated with dot placed excentrically; *e*, obliquely sectioned microtubule with a dense line in the center (unmarked arrow). This line, which appears to be made up of globules, probably corresponds to the dot seen in some microtubules in transverse section. *m*, mass of unidentified dense material. × 224,000.

but some were hook-shaped (Figs. 5 and 8) or appeared as fragments of microtubule wall material arranged in a circle. In a few platelets S-shaped figures were seen (Figs. 4 and 5), and sometimes two microtubules appeared to be connected by a dense line of the same dimension and density as the microtubule walls (Fig. 3). Round, dense masses of some unidentified material were occasionally observed near newly formed microtubules (Figs. 3, 9, and 11).

In normal platelets fixed instantaneously upon withdrawal from a vein a small "dot" is occasionally seen in cross-sections of some of the microtubules. Microtubules with dots were more frequent in the reformation period (Figs. 3 and 14). In obliquely sectioned microtubules the dot appeared to be a central filament made up of globular subunits (Fig. 3). Incomplete microtubules were often associated with dots (Fig. 2).

DISCUSSION

Naturally it might be argued that newly formed microtubules are very sensitive to the fixative, and that the incomplete microtubules are the result of improper fixation. After 2–4 min of rewarming, however, one finds complete and incomplete microtubules in the same platelet; this indicates that microtubules maximally 2 min "old" are fixed adequately with the technique used.

It thus appears that exposure of intact mammalian platelets to 0°C causes the microtubules to depolymerize or, at any rate, to vanish. It is at present unknown whether this is due to an effect of cold directly on the microtubules or whether it is a cellular response to cold, that is, a depression of enzymes, the function of which is necessary for the maintenance of microtubular integrity.

If it is assumed that microtubules depolymerize into subunits, what, then, are these subunits? In their first report on microtubules in plants Ledbetter and Porter suggested the presence of a

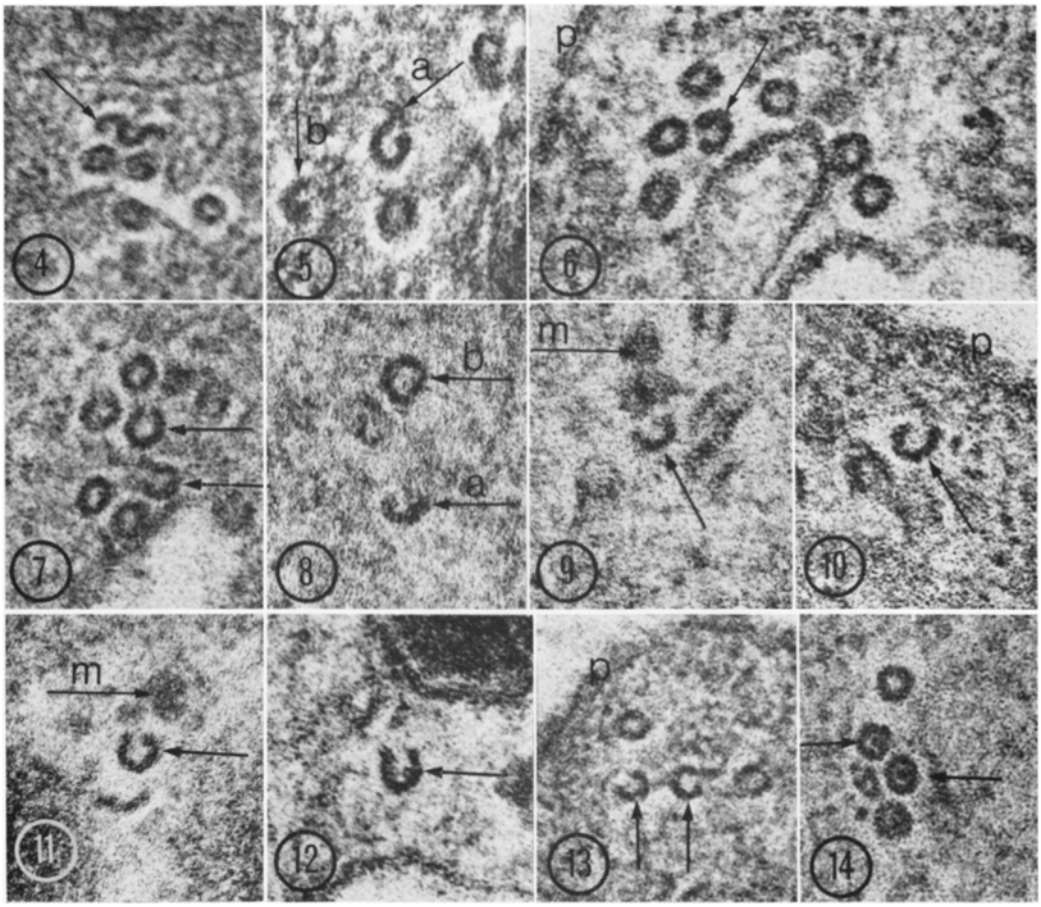


FIGURE 4 S-shaped piece of microtubule wall (arrow). × 148,000.

FIGURE 5 S-shaped piece of microtubule wall (arrow *a*) and hook-shaped, incomplete microtubule (arrow *b*). × 224,000.

FIGURES 6 and 7 C-shaped, incomplete microtubules are marked with arrows. Fig. 6, × 224,000; Fig. 7, × 189,000.

FIGURE 8 Platelet reheated for 8 min. A hook-shaped (*a*) and a complete microtubule (*b*) are seen. × 213,000.

FIGURES 9–13 Platelets reheated for 8, 6, 8, 2, and 12 min, respectively. Arrows indicate incomplete, C-shaped microtubules. The arrows marked *m* in Figs. 9 and 11 indicate masses of unidentified dense material. Figs. 9–12, × 213,000; Fig. 13, × 150,000.

FIGURE 14 Platelet reheated for 1 hr. Two microtubules with dots are indicated with arrows. × 189,000.

substructure (6), and, based on Markham rotation tests, they concluded that the walls of microtubules were constructed of 13 substructures spaced evenly around the circumference (7). Negative stain experiments on spindle microtubules (8, 9) have shown that spindle microtubules are constructed of filaments which in turn appear to be linear arrays of globular particles, approximately 35 A in diameter. Negative stain experiments on platelet microtubules (10) yielded similar results. In platelets it was sometimes observed that when some microtubules had adhered to the grid the constituent filaments broke into globules, about 35 A in diameter (11). Similar observations were made by Kiefer et al. on spindle microtubules (9). These observations agree with those of Tilney and Porter (12) who found in *Actinosphaerium* "a finely divided amorphous mass of material" when the microtubules of the axonemes had depolymerized completely in the cold. It is evident that, if microtubules in intact platelets break into 35-A particles, these would not be identified as such in sectioned material.

Chemical investigations on the proteins of isolated mitotic apparatuses by various workers have shown many differing components (see, for example, Kane (13) and Stephens (14) for recent reviews). It is at present not clear, however, how these different components relate to the information on microtubular substructure obtained with the negative stain procedure.

Platelet microtubules which had disappeard at 0°C reappear after transfer to 37°C. The reformation of microtubules must take place by assembly of subunits already present in the cytoplasm, for synthesis of new protein is unlikely: platelets are anuclear and virtually no DNA is present (see reference 15 cited in reference 16), ribosomes are an inconstant feature in platelet morphology, and only traces of RNA are present (see reference 15 cited in reference 16).

When microtubules reappear in the cytoplasm of platelets, they do so at random. This indicates that the subunits, into which the microtubules are proposed to break, are distributed more or less evenly in the cytoplasm, and that reassembly of subunits into microtubules is initiated all over the cytoplasm. Whether the dense masses (Figs. 3, 9, and 11) sometimes observed in the vicinity of reformed microtubules are collections of subunits or perhaps represent an initial stage in microtubule reformation is not known. The profiles of incomplete microtubules observed in the reformation stages seem to indicate that at least some of the microtubules reassemble by the formation of curved "sheets" which finally form the microtubule wall. The observations suggest that subunits align laterally into filaments which then align into sheets. Some support for this idea may be gained from a very recent report by Stephens (14) on purified mitotic apparatus protein with a sedimentation rate of 22–24S. Stephens showed that, under proper experimental conditions, the 22S protein formed filamentous aggregates, the individual filaments ranging from 40 to 60 A in diameter. The dense line sometimes observed between two microtubules might originate from a sheet which has grown too big (Figs. 4 and 5, the S-shaped sheets) so that two microtubules might form from the sheet, since they are interconnected by a piece of wall material. The possibility also exists that the incomplete microtubules are aberrant forms reflecting defects or errors in the reassembly mechanism.

Whether the microtubules that reappear after cold treatment are constructed exclusively of the material from the depolymerized microtubules or whether a pool of "microtubule protein" exists in platelets is unknown. If the latter alternative is true, then one must anticipate, in the normal circulating platelet, an equilibrium between monomeric microtubule protein of low molecular weight and the macromolecular structure, the microtubule, and that this equilibrium is influenced by temperature. Polymerization would be favored by higher temperatures and depolymerization by lower temperatures. This conception of the dynamics of marginal bundle microtubules would thus be in line with current views on the dynamic structure of the mitotic spindle (17).

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