ALTERATIONS IN NUMBER OF PROTOFILAMENTS IN MICROTUBULES ASSEMBLED IN VITRO

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ABSTRACT

Tubulin from bovine brain was polymerized in vitro using a variety of assembly conditions. Many of the formed microtubules were shown to contain 14 wall protofilaments. The number of microtubules containing 14 protofilaments increased with consecutive repetitions of cold-dissociation followed by reassembly in vitro.

KEY WORDS protofilaments · protofilament number · protofilament alterations · microtubules · microtubule assembly · microtubules in vitro

Microtubules have been routinely reassembled from tubulin in vitro since Weisenberg (16) and Shelanski et al. (14) described conditions necessary for polymerization. Many characteristics of in vitro microtubule assembly have been well-defined in numerous studies (e.g., 4, 7, 11), mostly involving use of negative-staining methods to visually assay for the presence of microtubules or their precursors. Several workers have used glutaraldehyde-fixed, thin-sectioned preparations to examine microtubules formed in vitro, but their examinations were limited in ultrastructural detail since individual protofilaments are not clearly visualized by this method (3, 9). Few studies have involved examination of microtubules formed in vitro using tannic acid staining (8), which is the only method available providing accurate definition of protofilament number in thin-sectioned material (2, 5, 12, 15). Tilney et al. (15) showed that microtubules reassembled in vitro contained 13 wall protofilaments, as did microtubules from a variety of cell types. Also, tubulin from *Drosophila melanogaster* polymerized in vitro forms microtubules having an "average" of 13 protofilaments (5). Recently, we reported that microtubules assembled in vitro from tubulin isolated from crayfish nerve cord contain different numbers of protofilaments, depending upon incubation temperature (12); this is the only report suggesting that certain microtubules formed in vitro might contain other than the expected 13 protofilaments. To date, the assumption has been made that all microtubules reassembled in vitro retain the same structural fidelity, as assayed by protofilament number, that they possess in the intact tissue. We now report that large proportions of in vitro reassembled microtubules contain 14 protofilaments rather than the expected 13, depending on solution conditions and the number of purification cycles.

MATERIALS AND METHODS

Microtubules were isolated from freshly slaughtered bovine brains by the method of Shelanski et al. (14). Five different isolation and assembly buffers were used: 0.1 M 2-[*N*-morpholino]ethane sulfonic acid (MES), pH 6.5 (4); 0.1 M MES, pH 6.9; 0.1 M piperazine-*N*,*N*′-bis[2-ethane sulfonic acid](PIPES), pH 6.5; 0.1 M PIPES, pH 6.9 (11); and 20 mM MES at pH 6.5 supplemented with 70 mM NaCl (7). All buffers contained 0.5 mM $MgCl_2$, 1.0 mM ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.0 mM guanosine 5′-triphosphate (GTP), and 4 M glycerol. Microtubules were isolated through three cycles of disassembly-reassembly, each cycle consisting of cold homogenization of the intact tissue or sedimented micro-

tubules from the previous cycle, followed by centrifugation at 0°C for 1 h at 100,000 *g*; the resultant supernant fluid containing the soluble tubulin fraction was incubated at 37°C in the presence of 1.0 mM GTP and 4 M glycerol for 30 min, then centrifuged at room temperature for 1 h at 100,000 *g*, yielding a sediment containing the reassembled microtubules. Portions of the sedimented material were taken for electron microscopy; the remainder of the microtubule sediment was carried through a total of three cycles.

Samples taken for electron microscopy were fixed in a solution of 3% glutaraldehyde and 8% tannic acid in 0.05 M sodium phosphate, which was adjusted to a final pH value of 6.8 with saturated NaOH as described by Burton et al. (2). After fixation for 1 h, samples were washed briefly with three changes of 0.05 M sodium phosphate at pH 6.8, then postfixed with 1% OsO_4 in 0.05 M sodium phosphate for 1 h at room temperature. Samples were dehydrated through an acetone series and embedded in Araldite. Silver-to-gray sections were obtained with a diamond knife and collected on uncoated 400-mesh copper grids, and then stained with methanolic uranyl acetate for 3 min followed by lead citrate staining for 10 min (13). Material was examined with a Philips 300 electron microscope.

Only microtubules which clearly displayed all their protofilaments were included in this survey, and micrographs were obtained of microtubules which presented a distinct profile when viewed in cross section. Protofilament number was determined by examination of photographic enlargements.

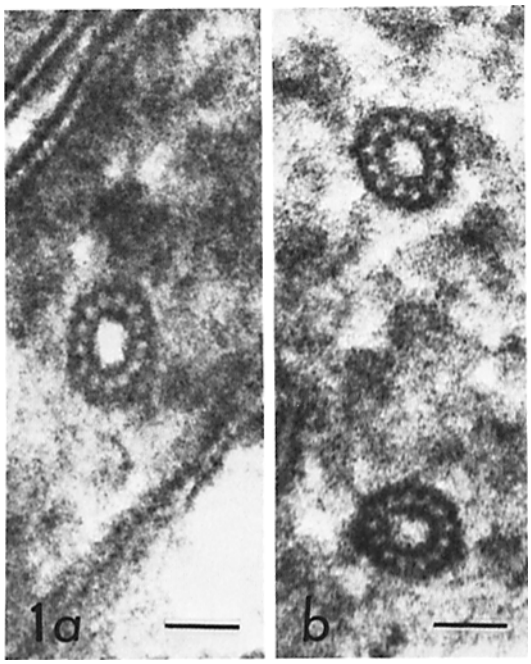


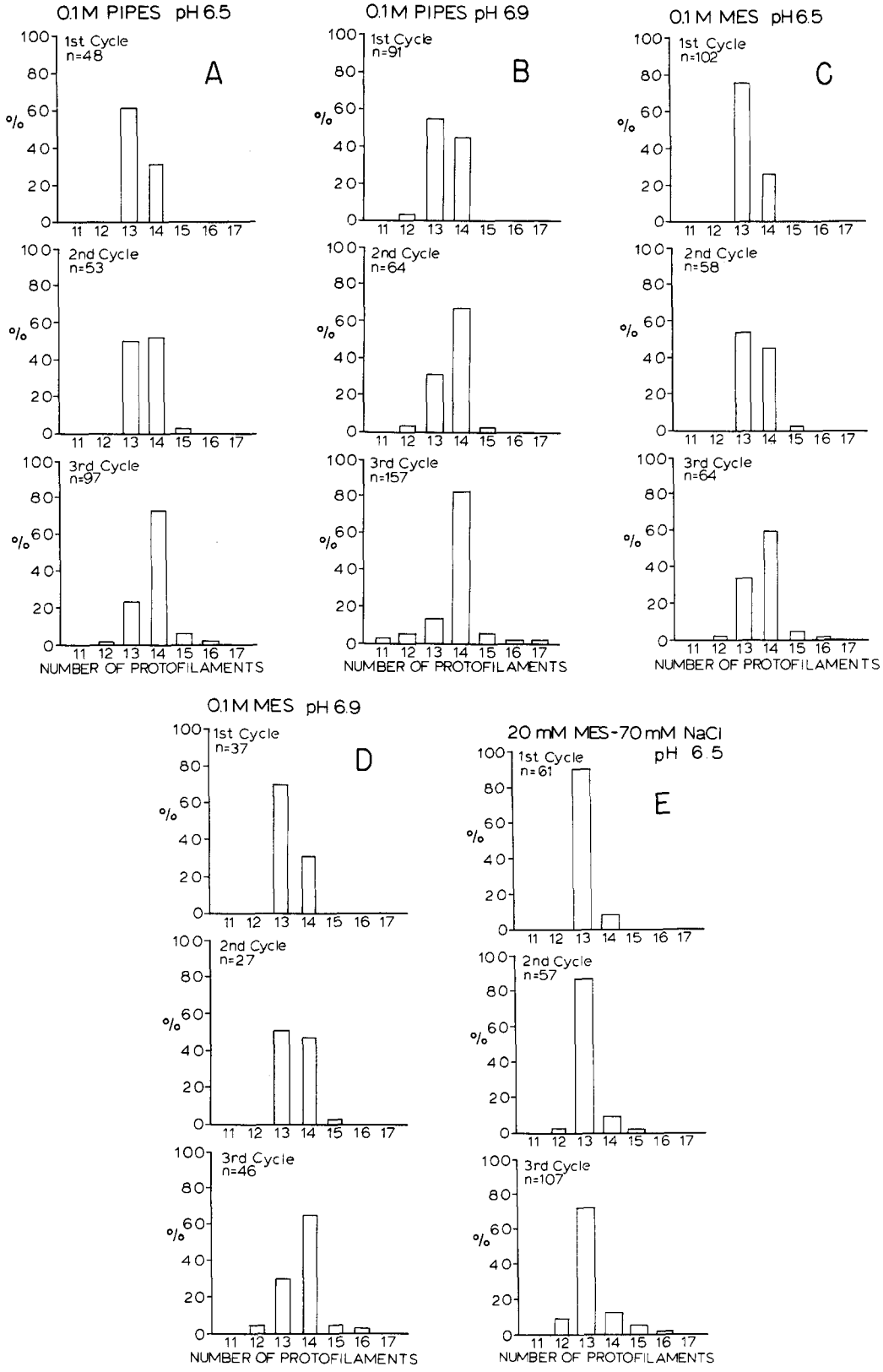
FIGURE 1 *a* and *b* Transverse sections through glutaraldehyde- and tannic acid-fixed microtubules in axons of bovine cerebrum. Note that 13 wall protofilaments are present in all cases. Bar, 0.02 μm. × 448,798.

RESULTS

To our knowledge, the number of protofilaments in microtubules of bovine brain cells has not been published. Since this information is germane to our results, pieces of fresh bovine brain (cerebrum) were fixed in glutaraldehyde-tannic acid; only microtubules with 13 wall protofilaments were seen in sections of the several samples examined. Most of the microtubules in which distinct wall subunits were seen appeared to be of axonal origin (Fig. 1).

After observing a large number of microtubules with 14 protofilaments in some of our preparations obtained by polymerizing bovine brain tubulin in vitro, we made a systematic investigation of the effect of purification through three cycles of disassembly-assembly using five different buffers. The results are presented in Fig. 2. In all five buffer systems the percentage of 13-protofilament microtubules decreases with each successive cycle of purification. The decrease in number of microtubules with 13 protofilaments occurs to a greater extent in 0.1 M MES or PIPES buffer (Fig. 2 A–D) than in buffer containing 20 mM MES-NaCl (Fig. 2 E). In 0.1 M PIPES at pH 6.9

FIGURE 2 Microtubules were isolated through several cycles of disassembly-assembly. After assembly, microtubules were sedimented by centrifugation. Samples were removed from sedimented microtubules after 1, 2, and 3 cycles of disassembly-assembly, and fixed with glutaraldehyde-tannic acid for ultrastructural examination. Transverse sections of microtubules were examined and protofilament number was determined. Buffer systems used included: (A) 0.1 M PIPES, pH 6.5; (B) 0.1 PIPES, pH 6.9; (C) 0.1 M MES, pH 6.5; (D) 0.1 M MES, pH 6.9; and (E) 20 mM MES-70 mM NaCl; pH 6.5. Except in 20 mM MES-70 mM NaCl (pH 6.5), many microtubules show both 13 and 14 wall protofilaments after each cycle of polymerization, although after the third cycle of polymerization greater variation in protofilament number is seen. Also, greater numbers of microtubules with 14 wall protofilaments appear with each successive cycle of polymerization.



(Fig. 2 B), for example, 45% of the microtubules contained 14 protofilaments after the first cycle of purification, and 80% of the microtubules showed 14 subunits after the third cycle. In the MES-NaCl buffer (Fig. 2 E), 70% of the microtubules contained 13 protofilaments after the third cycle of purification. By the end of the third cycle with all five buffer systems, small numbers of microtubules with 11, 12, 15, 16, and 17 wall protofilaments were observed with variable frequency. The difference between pH 6.5 and 6.9 and the presence or absence of glycerol (data not shown) had no apparent effect on the results

The effect of the kind of buffer and subsequent cycles of purification on protofilament number is illustrated in another way in Fig. 3. Changes in the mean number of wall protofilaments obviously trend upward with subsequent purification cycles, even in 20 mM MES-NaCl, the buffer system which provided for the most microtubules with the "normal" number of wall protofilaments.

Figs. 4–11 show representative cross sections through microtubules obtained by polymerizing bovine brain tubulin as described. After three cycles of purification, microtubules with variable numbers of wall protofilaments may be seen alongside one another in the same sample, indicating that microtubules did not stratify on the basis of protofilament number during centrifugation. On the contrary, microtubules with different protofilament numbers were found throughout the same sample, suggesting that they were heterogeneous with regard to the different types of microtubules.

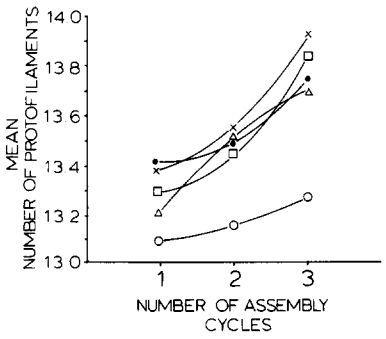


FIGURE 3 This graph shows the mean number of protofilaments in microtubules from each assembly cycle for the five different buffer conditions. The curves represent: (○) 20 mM MES-70 mM NaCl, pH 6.5; (△) 0.1 M MES, pH 6.5; (●) 0.1 M MES, pH 6.9; (□) 0.1 M PIPES, pH 6.5; and (×) 0.1 M PIPES, pH 6.9. The data plotted are from information provided in Fig. 2 A–E, and the graph shows that after successive cycles of disassembly-assembly, only with 20 mM MES-NaCl does the number of wall protofilaments remain relatively constant.

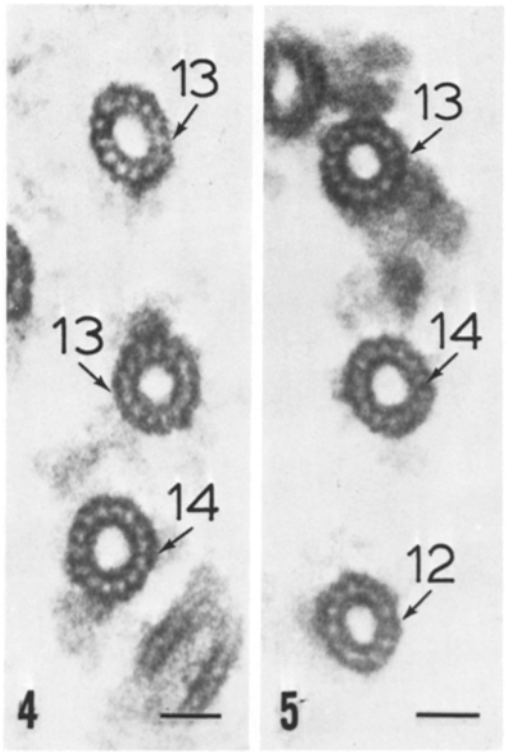


FIGURE 4 Glutaraldehyde-tannic acid-fixed microtubules with 13 and 14 protofilaments formed in 0.1 M PIPES buffer at pH 6.9 after three cycles of assembly-disassembly. Bar, 0.02 μm. × 384,684.

FIGURE 5 Microtubules with 12, 13, and 14 protofilaments formed in 0.1 M MES buffer at pH 6.5 after three cycles of disassembly-assembly. Bar, 0.02 μm. × 384,684.

DISCUSSION

The results reported here show that large proportions of in vitro reassembled microtubules contain 14 protofilaments rather than 13 as found in fresh brain tissue. Thus, the fidelity of protofilament number found in microtubules existing in the intact tissue appears to be lost during reassembly in vitro. The samples examined in these experiments contained not only a large number of microtubules with 14 protofilaments but also a number of microtubules with 11, 12, 15, 16, and even 17 protofilaments. Although the frequencies of these microtubules in the sample were relatively low, there was an increase in their number with

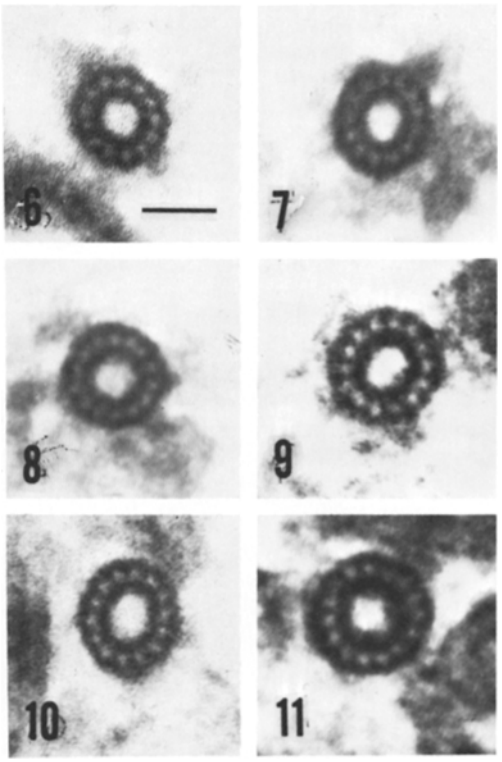


FIGURE 6 Microtubule with 13 protofilaments collected after the first disassembly-assembly cycle in 0.1 M MES buffer at pH 6.5. × 448,798. Bar, 0.02 μm in all cases.

FIGURE 7 Microtubule with 13 protofilaments formed in 0.1 M PIPES buffer at pH 6.5 and obtained after the first assembly-disassembly cycle. × 448,798.

FIGURES 8 and 9 Microtubules with 14 wall protofilaments formed in 0.1 M PIPES buffer at pH 6.9; these microtubules were collected after three cycles of disassembly-assembly. × 448,798.

FIGURE 10 Microtubule with 14 wall protofilaments obtained after two cycles of disassembly-assembly using 0.1 M PIPES at pH 6.5. × 448,798.

FIGURE 11 Microtubule with 15 protofilaments obtained after three cycles of disassembly-assembly in 0.1 M MES at pH 6.5. × 448,798.

each cycle of polymerization (Fig. 2). The controls responsible for protofilament number and conformational fidelity existing in vivo are apparently lost during in vitro assembly. There are several possible explanations as to how these effects are achieved. Two are presented below.

Microtubule-associated proteins (MAPs) which copurify with tubulin are capable of stimulating the self-assembly of tubulin (3, 9). Although the precise role of such MAPs remains unclear, the alterations observed in protofilament numbers of in vitro assembled microtubules might be the result of a decrease in the amount of these proteins. Possibly the MAPs confer conformational fidelity upon microtubules observed in the intact tissues. Another possibility is that the tubulin dimer may undergo a conformational change during repeated cycles of disassembly and assembly, and such an alteration in protein structure could be caused by the incubation conditions. It is noteworthy that the decrease in 13-protofilament microtubules was much less when a low concentration of MES was used (Fig. 2). We have found that the ethane sulfonate buffers have unusual effects on the assembly reaction (6). At high concentrations (0.4 M and above), tubulin assembles into complex branched ribbons rather than microtubules, indicating a change in binding domains. Perhaps at lower concentrations less drastic changes in structure occur, leading to microtubules with more than the expected number of protofilaments.

Our results may have relevance to the control of the number of protofilaments in microtubules in vivo. Although the 13-protofilament arrangement is by far the most common, microtubules with 11 (12), 12 (1, 2), and 15 (2, 10) protofilaments have been observed. Perhaps cytoplasmic environmental conditions control the number of protofilaments in a microtubule. Our work should be a warning to others in this field that in most cases microtubules assembled from purified tubulin in vitro do not have precisely the same ultrastructure as those found in vivo.

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