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Polycation-induced assembly of purified tubulin

(microtubules/microtubule-associated proteins/high-molecular-weight proteins/tau factor)

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ABSTRACT **Several different polycations have been found that can substitute for the microtubule-associated proteins, or tau factor, in facilitating assembly of tubulin that has been purified by ion exchange chromatography. In low concentrations of the polycation diethylaminoethyl-dextran, 7 mg of tubulin is pelleted per 1 mg of polycation added. Under conditions favorable to microtubule assembly the entire pellet is seen by electron microscopy to consist of "double wall microtubules," which are essentially identical to normal microtubules in subunit structure and arrangement. When assembly is inhibited approximately the same amount of tubulin is pelleted, but it is in the form of clusters of curved sheets or filaments apparently related to tubulin rings. When conditions are changed to favor assembly, the tubulin within these clusters appears to reassemble to form the double wall microtubules.**

When microtubules are purified by assembly and centrifugation they carry with them a number of microtubule-associated proteins (MAP). These amount to between 5 and 20% of the total protein and appear as a set of high-molecular-weight bands on sodium dodecyl sulfate gels (1). In the initial work on microtubule assembly it was tempting to treat these MAP as contaminants, which somehow attached to assembled microtubules and were thereby carried into the pellet. One could then assume that assembly involved only the self-assembly of tubulin subunits by a process of helical polymerization.

Murphy and Borisy (2, 3) found that the MAP could be separated from the tubulin on an anion exchange column, and demonstrated that microtubules could be assembled from the purified tubulin only if the MAP were added back. Weingarten *et al.* (4) confirmed these results for tubulin purified on a cation exchange column. Bryan *et al.* (5) demonstrated that assembly could be inhibited by addition of RNA, which they suggested was binding and removing a polycationic protein that was required for assembly.

Weingarten *et al.* (4) suggested that there was a protein factor, which they called "tau," among the MAP, which was required for microtubule assembly. The idea of a specific protein that could play a role in regulating assembly was attractive, since reversible assembly and disassembly of microtubules is an important part of many cellular processes. Attractive as the idea is, however, little evidence has been obtained that there is such a specific factor among the MAP. On the contrary, the assembly-promoting activity seems to be a property of several different fractions among the MAP, as separated on Sepharose columns (unpublished data from our laboratory). Furthermore, this activity is retained after heating the MAP to 100° for several minutes (4, 5) and even after moderately extensive degradation of the protein by proteolysis (no bands remaining on sodium dodecyl sulfate gels) (6). Finally, Lee and Timasheff (7) have recently demonstrated that microtubules can be assembled from highly purified tubulin subunits in certain ionic conditions. These observations argue against the hypothesis that a specific factor among the MAP is an essential component of the assembly reaction.

Tubulin behaves as a strongly anionic protein, and the MAP as weaker cationic proteins, on ion exchange columns. Thus tubulin binds tightly to the anion exchanger DEAE- (diethylaminoethyl) cellulose (3), and does not bind to the cation exchanger phosphocellulose at an ionic strength as low as 0.025 M (4). The MAP, although they appear on sodium dodecyl sulfate gels to be a heterogeneous group of from two to 15 proteins, are all removed by binding to the phosphocellulose. One common feature of these proteins, therefore, must be an arrangement of positively charged groups that allows them to bind to the negatively charged phosphocellulose. Since the surface of microtubules presumably carries the negative charge of the tubulin subunits, the MAP could bind to the microtubules by the same nonspecific electrostatic forces. During assembly and centrifugation, the microtubules could thus be acting as a cation exchanger in effecting the batch purification of the MAP.

Since the majority of these proteins might be unrelated to microtubule function, the possibility was considered that their role in assembly might also involve a nonspecific electrostatic interaction. We found that histone and RNase A, both basic proteins, and the polycation DEAE-dextran could, in fact, substitute for the MAP in facilitating assembly of microtubules from purified tubulin (8, 9). In this paper we describe more quantitative measurements of assembly in DEAE-dextran, which was effective at the lowest concentration of any of the factors tested.

MATERIALS AND METHODS

The standard assembly buffer used for preparation of tubulin and for assembly experiments was 50 mM Mes [2-(*N*-morpholino)ethanesulfonic acid], 1 mM EGTA [ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetra acetic acid], 0.5 mM $MgSO_4$, and 0.5 mM GTP, adjusted to pH 6.5 at room temperature with NaOH. Microtubules were prepared from pig brain homogenate as described by Shelanski *et al.* (10), except that the 50 mM Mes buffer used in our experiments was half the concentration used in that work. In most preparations, ATP was substituted for GTP in the initial assembly from the crude supernatant. The tubulin obtained after one cycle of assembly and centrifugation was stored in glycerol for up to 3 weeks (10), and was either reassembled and pelleted, or simply dialyzed against buffer, before ion exchange chromatography.

Purification of the tubulin and MAP on phosphocellulose was essentially as described by Weingarten *et al.* (4). The 5 ml bed volume was equilibrated with 25 mM Mes, 0.25 mM $MgSO_4$, 0.5 mM EGTA, at 4°. Samples containing 5–10 mg of protein in 2–3 ml of 25 mM Mes buffer were loaded on the column and eluted at a rate of 2–5 ml/hr. Fractions containing the tubulin were pooled and centrifuged at 100,000 × *g* for 30 min at 4°.

Abbreviations: MAP, microtubule-associated proteins; DEAE, diethylaminoethyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid.

Mes and GTP were then added to the concentration in the standard assembly buffer.

For quantitative assay of microtubule assembly, samples were incubated at 35° for 30 min and centrifuged at 50,000 × *g* for 30 min at 30°–35° and the amount of protein in the pellet and supernatant was determined (11). Some pellets were prepared for electron microscopy by fixing in 2½% (wt/vol) glutaraldehyde in Mes buffer for 2 hr at room temperature. The pellets were post-fixed in osmium tetroxide, dehydrated, and embedded in Epon. Thin sections were stained with 7% uranyl acetate followed by Sato's lead strain.

DEAE-dextran was obtained from Sigma Chemical Co. It was stated to be 2,000,000 average molecular weight, but we found that the fraction active in promoting assembly eluted from a Sepharose 6B column as a 100,000 molecular weight fraction (partition coefficient $K_{av} = 0.5$) containing about half of the total dextran. A chloride analysis (12) indicated one basic group per three glucose units. Phosphocellulose (fibrous powder, P11) was obtained from Whatman Products.

RESULTS

Induction of tubulin assembly by DEAE-dextran

We found that tubulin purified by phosphocellulose chromatography produced no microtubules or related structures at the highest tubulin concentrations obtained, about 3 mg/ml, as was previously reported by Weingarten *et al.* (4). When low concentrations of DEAE-dextran (0.003–0.03 mg/ml) were added to the purified tubulin (0.3–1 mg/ml), structures which we called "double wall microtubules" (9) were formed. These consist of an apparently normal microtubule (the inner wall) surrounded by a sheet of protofilaments, essentially identical to a microtubule wall (13). In this outer wall the protofilaments are inclined at a 38° angle to the microtubule axis, and thus form a spiral wrapping. Fig. 1a and b shows images in which the outer wall is incomplete and the structure is revealed directly. We have confirmed from optical diffraction patterns that the intact double wall microtubules (Fig. 1c) have the same structure.

Jacobs *et al.* (14) have used the term "duplex microtubule" to describe a similar structure formed after addition of polycations to assembled microtubules. In their structure the protofilaments in the outer wall appear to form a very shallow helix, essentially equivalent to the coiled protofilaments or rings we have described previously (15). This is significantly different from the structures we have obtained, and we will continue to use the term "double wall microtubules" for our structures, in which we have determined that both walls are composed of subunits on the same two-dimensional lattice as the normal microtubule wall.

The extent of assembly in different concentrations of tubulin and DEAE-dextran was determined by centrifuging the assembled double wall microtubules. Fig. 2 shows that the pelleting of tubulin was maximal at a certain optimal concentration of DEAE-dextran, and that more of the tubulin remained in solution as the DEAE-dextran concentration was increased or decreased. Negatively stained specimens, prepared after assembly in low concentrations of DEAE-dextran (the left part of the curve in Fig. 2), showed a homogeneous spread of double wall microtubules in the electron microscope. At a DEAE-dextran concentration of approximately 0.1 mg/ml, at which 80% of the tubulin was pelleted, the micrographs showed large dense clumps of material, but few regular structures. At still higher concentrations of DEAE-dextran (the right-hand end of the curve in Fig. 2), a mixture of rings, coils, and spiraling sheets of protofilaments was found, which we describe elsewhere (9).

The number of double wall microtubules seen in the negatively stained specimens corresponded approximately to the amount of tubulin pelleted, but it was important to determine more convincingly how much of the pelleted tubulin was in the form of these microtubules. For this we fixed, embedded, and sectioned pellets obtained after assembly or precipitation under various conditions. The most important observation was that, after assembly in a low concentration of DEAE-dextran, the pellet consisted entirely of double wall microtubules. The image shown in Fig. 3 is typical of the entire thickness of the pellet. Such pellets were obtained whenever the DEAE-dextran concentration was less than that required to pellet 75% of the tubulin, and conditions were favorable to microtubule assembly. At 0.1 mg/ml of DEAE-dextran (maximum pelleting, Fig. 2), the pellets consisted primarily of large dense aggregates, and at higher concentrations structures were seen which probably corresponded to the large tubes and decorated microtubules seen previously (9) in negatively stained specimens.

Since assembly in the presence of low concentrations of DEAE-dextran produced only double wall microtubules, we explored this region of the curve in Fig. 2 in more detail. The results presented in Fig. 4 show that under these conditions the amount of tubulin pelleted was directly proportional to the amount of DEAE-dextran added until about 75–85% of the tubulin was pelleted. From the slope of the line we see that 7 mg of tubulin was pelleted per 1 mg of added DEAE-dextran. This value was obtained in a number of different experiments, using different tubulin preparations, and thus does not seem to be affected by such uncontrolled variables as trace contamination with MAP.

The aggregation of tubulin by DEAE-dextran when microtubule assembly is inhibited

The curve in Fig. 2 resembles that for the aggregation of mixtures of gelatin and gum arabic (16), or the precipitation of serum albumin by DEAE-dextran (12), and this suggested that the pelleting of tubulin might not require the assembly of microtubules. We therefore investigated the pelleting of tubulin under four conditions which are known to block microtubule assembly: (*a*) the reaction mixture was kept at 4°; (*b*) 5 mM calcium was added; (*c*) 0.1 mM colchicine was added; and (*d*) GTP was removed by dialyzing the tubulin for 2 hr. Electron microscopy showed that no microtubules were formed under any of these conditions. The precipitation of tubulin, however, as indicated by the turbidity of the solution and by determination of the amount of protein pelleted, was similar to that obtained under conditions favorable to assembly. For conditions *c* and *d*, the pelleting was determined for a range of concentrations of DEAE-dextran, and essentially the same curve as in Fig. 2 was obtained.

The tubulin aggregates that were induced by DEAE-dextran in conditions unfavorable to assembly were not, however, amorphous precipitates, but were seen to have a characteristic substructure in negatively stained specimens. The grids showed large aggregates, 1–10 μm in size, which consisted of tangled clusters of curved filaments or sheets 40–45 nm outside diameter, similar to tubulin rings. In the first few minutes of assembly, negatively stained specimens showed double wall microtubules growing from these clusters, but the structural relationships and pathway of assembly are not clear.

We conclude from this that addition of a small amount of DEAE-dextran to a solution of purified tubulin causes the tubulin to aggregate into dense clusters, within which the tubulin

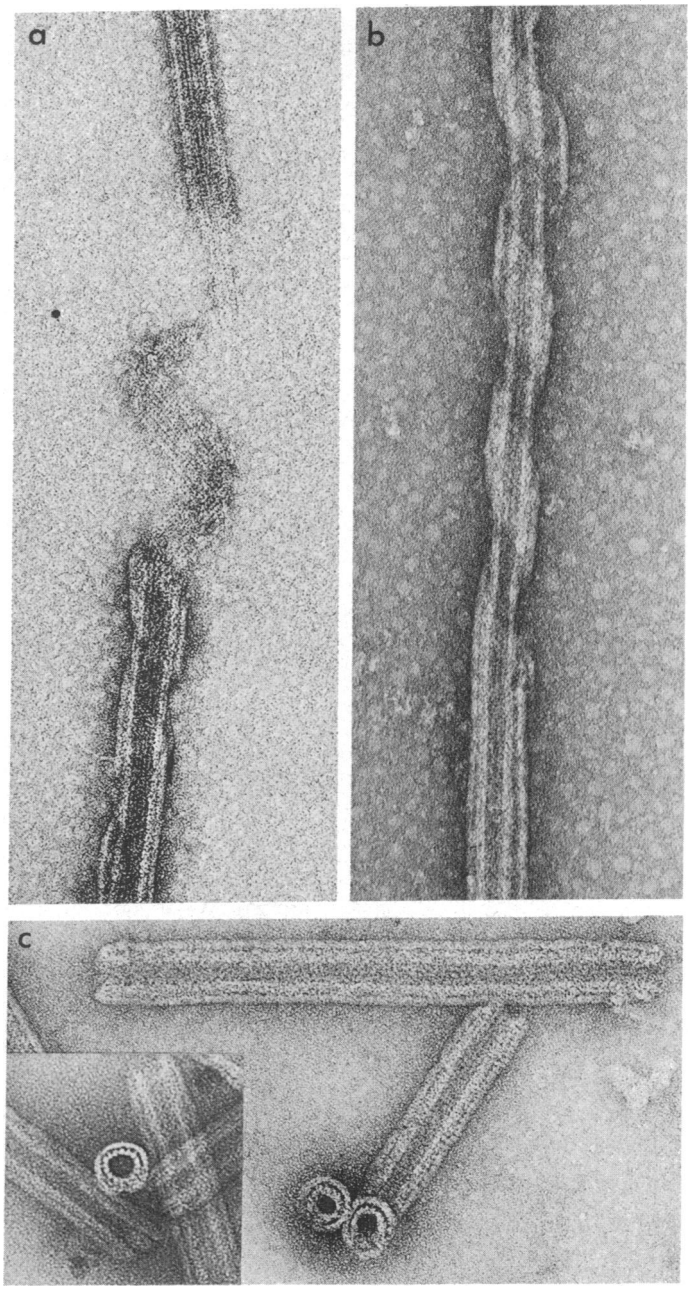

FIG. 1. Negatively stained double wall microtubules. Structures assembled in RNase A (a and b) frequently had an incomplete outer wall, which revealed the structure of the inner microtubules and the spiral sheet of protofilaments. The intact double wall microtubules in (c), assembled in DEAE-dextran, occasionally stuck to the carbon film and broke off, giving cross-sectional views of the small pieces. The outside diameter measured from these cross sections was 45.5 nm. The inner microtubule in these specimens appears to have 16 protofilaments, and is correspondingly larger in diameter than the normal microtubule with 13 protofilaments. Optical diffraction of the longitudinal views confirmed that the outer wall was a sheet of protofilaments with the same lattice as the microtubule wall. ×150,000.

is assembled into curved structures apparently is related to that of tubulin rings. When solution conditions are changed to favor assembly, the tubulin within these aggregates is reassembled into double wall microtubules.

DISCUSSION

Most previous studies of microtubule assembly have used tubulin preparations in which the MAP were playing a crucial role. Some or all of the characteristics that have been determined for the assembly reaction, such as ionic strength and pH dependence, the effect of tubulin concentration (including the observation of a critical concentration), and the effects of temperature and pressure, may reflect primarily the interaction of tubulin and the MAP, rather than the self-association of tubulin subunits. Consequently, we believe that these determinations, and measurements of kinetic and thermodynamic parameters, will not be meaningful until the polycations involved in the reaction are characterized and their role understood.

The main conclusion that we want to draw from the structural analysis is that the double wall microtubules, which appear abnormal in terms of gross morphology, are composed of tubulin subunits arranged on the same lattice as the normal microtubule wall. Thus, at the level of subunit structure and in

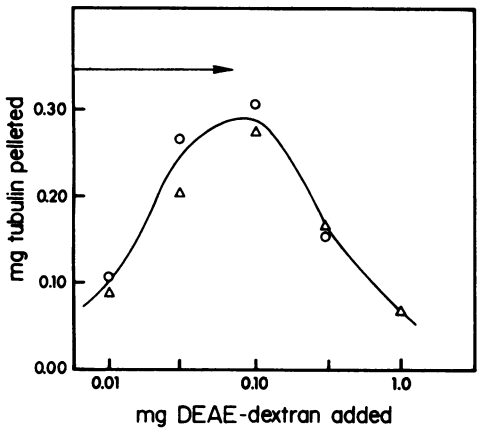

FIG. 2. Pelleting of tubulin after assembly in the presence of different concentrations of DEAE-dextran. Each sample contained 0.34 mg/ml of tubulin (arrow) in the standard assembly buffer. The amount of tubulin pelleted per 1 ml sample was determined by Lowry assays on the pellets (△) and supernatants (○).

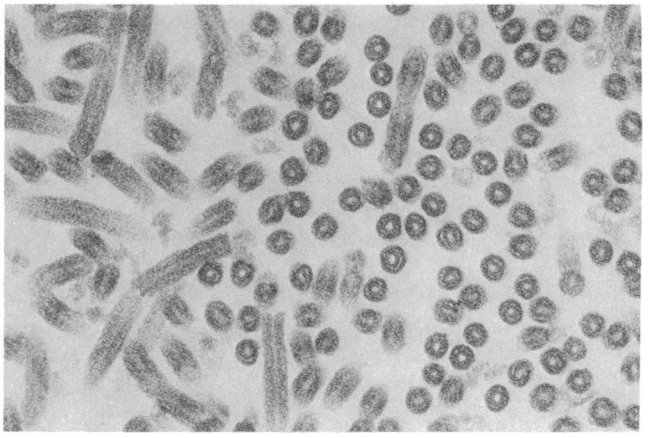

FIG. 3. A pellet of double wall microtubules was fixed with glutaraldehyde, embedded, and sectioned, after assembly of 0.55 mg/ml of tubulin in 0.03 mg/ml of DEAE-dextran. The entire pellet consisted of double wall microtubules, 46 nm outside diameter, as seen in this representative image. ×71,000.

particular of the contacts or bonds between subunits, the double wall microtubules involve only a small distortion from the normal microtubule structure. We believe, therefore, that the reaction we have investigated is essentially that of normal microtubule assembly. In support of this we would like to recall that assembly induced by RNase was mostly of normal microtubules (9). In addition we have recently prepared DEAE-dextran with a lower density of charged groups (12), and in preliminary experiments this material also induced assembly of normal microtubules.

The 46 nm outside diameter of the double wall microtubule is approximately the same as that of the tubulin rings and coils that have been observed in a variety of conditions (1, 15, 17, 18). These rings consist of a protofilament uniformly curved to form a circle or shallow helix (15, 17) and it appears that this curvature is retained in the protofilaments in the outer wall (the helix is much steeper, however, in this structure). We would like to note that, although the cross-sectional images of the double wall microtubules (Fig. 1c) bear a superficial resemblance to the double rings that have been described in specimens of tubulin plus MAP (17) or magnesium (18), we believe they are quite different structures. In the tubulin rings, including the ones that appear doubled, the protofilament lies approximately parallel to the grid surface (15), while in the cross-sectional images protofilaments are perpendicular to the grid in the inner wall, and obliquely spiral in the outer wall.

Lee and Timasheff (7) have recently demonstrated that assembly of microtubules may be obtained from tubulin purified by the ammonium sulfate, DEAE-Sephadex method (19). Since this purification scheme involves ion exchange chromatography this tubulin should be free of polycations. We would suggest that this is, in fact, the reason why tubulin purified by this method will not assemble in the buffer conditions normally used for assembly experiments with brain homogenates. The buffer in which Lee and Timasheff succeeded in obtaining assembly was quite different from that used in our work. The concentration of monovalent ions was lower (10 mM phosphate versus 50 mM Mes in our system), 3.4 M glycerol was necessary for optimal assembly, and the magnesium concentration that produced optimal assembly was much higher (16 mM versus 0.5 mM in our system). We have repeated their experiment using tubulin purified by phosphocellulose chromatography and have confirmed that both tubulin rings and microtubules are formed from our purified tubulin in their buffer conditions. We believe that the tubulin we have purified by cycles of assembly followed by phosphocellulose chromatography is equivalent in purity and activity to that obtained by the ammonium sulfate, DEAE-Sephadex procedure.

We would like to suggest two speculative hypotheses that might explain the facilitation of assembly. The first is based on the concept of complex coacervation of polyelectrolytes (16). The primary effect of the polycation appears to be to induce an aggregation of tubulin, which precedes microtubule assembly and can occur under conditions where assembly is inhibited. We imagine that the tubulin subunits within these aggregates are crosslinked by electrostatic attachments to the polycation molecules. If the packing is close but still loose and flexible, the tubulin subunits could still interact with each other as in solution, but the effective concentration of the tubulin within the aggregates would be greatly increased over the av-

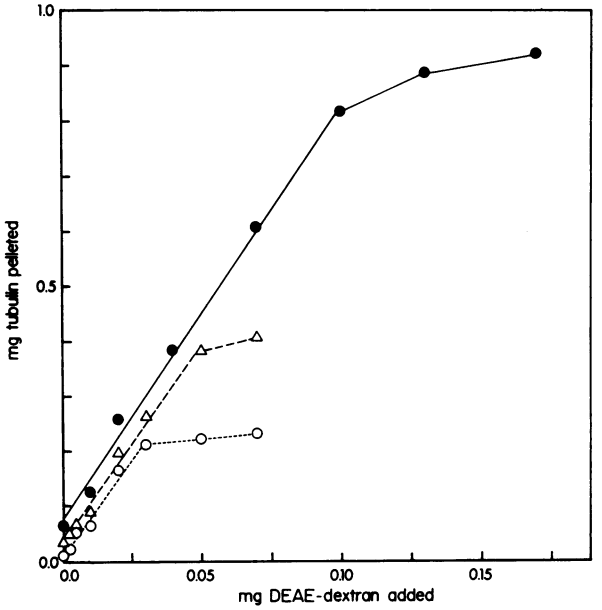

FIG. 4. Assembly of double wall microtubules determined by assay of protein pelleted. Three different tubulin concentrations were investigated: 1.14 mg (●), 0.485 mg (△), and 0.263 mg (○) per 1 ml sample.

erage value for the solution. We will assume further that the critical concentration for assembly of tubulin is on the order of 10–50 mg/ml, values which obtain in cell cytoplasm but which are an order of magnitude higher than those that have been explored in assembly experiments *in vitro*. We can then postulate that the concentration of tubulin is too low to support assembly in the *in vitro* system, but within the aggregates the effective concentration is increased to above the critical concentration.

This hypothesis might also apply to assembly of tubulin in magnesium and in MAP. We note that the magnesium concentration that was effective in promoting assembly in the phosphate buffer (7) is very close to that which causes tubulin to precipitate (20). Some degree of tubulin aggregation in the presence of MAP is indicated by the significant amount of tubulin that is always pelleted after centrifugation at 4°. Thus, the possibility that an aggregation or precipitation of tubulin may facilitate assembly by increasing the effective concentration should be considered in all of these assembly experiments.

As an alternate hypothesis we would like to consider that the primary phenomenon involved in these reactions may be the formation of tubulin rings, and that facilitation of assembly results because these rings are especially active in microtubule assembly. It is known that MAP induce the formation of tubulin rings, and our own work (15) and similar experiments of Kirschner *et al.* (17) have suggested a pathway in which rings may be an intermediate in microtubule assembly. In the system of Lee and Timasheff (7) microtubule assembly in high magnesium is accompanied by formation of rings, and under these buffer conditions rings are in fact formed before assembly is initiated by raising the temperature to 37° (ref. 18, and our own observations). In our experiments with DEAE-dextran, curved sheets or filaments, apparently related to the ring structure, were formed prior to assembly, i.e., under conditions where assembly was inhibited. The observation that rings or related structures are formed prior to or accompanying assembly in each of these cases is suggestive that they might have a role in assembly, and is consistent with the hypothesis that polycations or magnesium facilitate assembly by inducing ring formation. We should caution, however, that it has not been established for any case that rings are an obligate intermediate, and although there is evidence that rings are incorporated into growing microtubules (15, 17) the interpretation is ambiguous.

The observation that the extent of assembly may be limited by the concentration of DEAE-dextran, and a similar finding with MAP (6), suggests that the polycation is incorporated into the microtubule structure in a precise stoichiometric relation to the tubulin. Another mechanism may be suggested, however, for the apparent stoichiometry. We have already postulated that the polycations bind to the surface of the microtubules by nonspecific electrostatic interactions, and it is reasonable to suppose that this binding would be stronger than that to unassembled tubulin subunits. Thus, as microtubules assembled they would remove the polycations from the solution, and the reaction would cease when the number of microtubules assembled was sufficient to bind all of the polycations. In this interpretation the stoichiometry is really a measure of the amount of polycation that will bind to the surface of the microtubules.

We feel that it is premature to speculate on the role of these phenomena in the regulation of microtubule assembly *in vivo* until the mechanism is better understood and the MAP are characterized. The observation that facilitation of assembly *in vitro* can be obtained with a variety of nonspecific polycations leads us to question the simplest hypothesis that there is a factor among the MAP specifically involved in regulating microtubule assembly. Our results suggest that if the MAP are involved in assembly they may be acting through electrostatic interactions as we have discussed here for the *in vitro* system, and this is at least a possible mechanism for regulation *in vivo*.

Finally, we might speculate that similar phenomena could be important in other systems of protein interaction and assembly. In particular we note that actin is similar to tubulin in being an acidic protein, and that it assembles into fibers that should carry a negative charge on their surface. It has recently been found that high-molecular-weight proteins attach to actin after polymerization in cell homogenates, and appear to play a role in binding actin filaments into larger fibers and cytoplasmic gels (21–24). A cell membrane fraction, presumably because it contains such a protein, has been observed to facilitate actin assembly *in vitro* (25). These proteins may be related to the MAP and the interactions may be similar to those discussed in this report.

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