Microtubule Formation in vitro in Solutions Containing Low Calcium Concentrations

Abstract. *Isolated rat brain tubulin can be repolymerized in vitro in solutions containing adenosine triphosphate or guanosine triphosphate, magnesium ions, and a good calcium chelator. The extreme sensitivity of tubulin to calcium ions explains the failure of previous efforts to obtain polymerization and suggests a possible mechanism for regulation of microtubule polymerization in vivo.*

Although microtubules are present continuously in many cell types, they also may be formed or broken down as needed by the cell. The most obvious example of such a cyclic formation and breakdown of microtubules occurs during mitosis, but it may also occur during developmental processes involving changes in cell shape. It is important to determine the mechanism of microtubule polymerization and how it might be controlled by the living cell, but it is this aspect of microtubule chemistry that has proved most difficult to solve. Although purified microtubule subunit protein has been available for several years, there have been no reports of its successful repolymerization under physiological conditions. I now report the successful repolymerization of isolated rat brain tubulin. The formation of microtubules is strongly inhibited by calcium ions, and calcium appears to be a logical candidate as a regulator of microtubule polymerization in vivo.

Young Sprague-Dawley rats, weighing about 200 g, were decapitated and the whole brains were quickly removed. The brains were weighed and 1.5 ml of homogenizing medium was added per gram of tissue. Homogenization was performed in a glass homogenizer with a Teflon pestle operated at about 100 rev/min, at least ten passes of the pestle being used. Purification of tubulin was performed essentially by the batch DEAE (diethylaminoethyl) method of Weisenberg and Timasheff (*1*), except that the initial ammonium sulfate fractionation and the final magnesium precipitation were eliminated. This produces a preparation which is about 90 percent pure tubulin. Because of the instability of tubulin (*2*), a number of experiments were performed with a simple one-step ammonium sulfate precipitation. For this procedure the brain was homogenized in 0.1*M* MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (*3*) and 1 m*M* guanosine triphosphate (GTP) at *p*H 6.5 and was centrifuged at 20,000 rev/min for 20 minutes at 2°C. The supernatant was removed and an equal volume of a saturated solution of ammonium sulfate was added. The precipitate was recovered by centrifugation at 10,000 rev/min for 10 minutes and resuspended in 1 ml of MES buffer. Excess ammonium sulfate was removed by passing the protein through a column of Sephadex G-25 equilibrated with MES buffer.

To prepare samples for electron microscopy, it was necessary to dilute the samples in a microtubule stabilizing medium, such as a Kane's spindle isolation solution (*4*). In general, two drops of the tubulin sample were added to 0.5 ml of a solution containing 1*M* hexylene glycol and 0.01*M* sodium phosphate at *p*H 6.2. A drop of this mixture was then placed on a grid coated with 0.3 percent Formvar; after 1 minute the drop was rinsed off with hexylene glycol solution and the grid was negatively stained with 0.5 percent uranyl acetate.

In an early series of experiments the ability of microtubules to form in a crude, unfractionated homogenate was investigated. Microtubules could be observed after homogenization in 0.1*M* sodium phosphate at *p*H 6.5 and incubation for an hour at 35°C. Microtubules were essentially absent after incubation at 0°C, but reappeared when the temperature was raised to 35°C, which indicates that microtubule polymerization was occurring. Sodium phosphate worked as well as potassium phosphate, but no tubules were observed when sodium maleate was used as the buffer. In order to determine whether there was a specific requirement for phosphate, a number of the hydrogen ion buffers developed by Good *et al.* (*3*) were investigated for their ability to allow polymerization. Of the four buffers that Good developed which I studied, MES, ADA [*N*-(2-acetamido)-iminodiacetic acid], ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid], and BES [*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid], only one, ADA, was observed to allow polymerization, and in terms of the number and length of tubules observed ADA was clearly superior to phosphate.

Since the only obvious characteristic

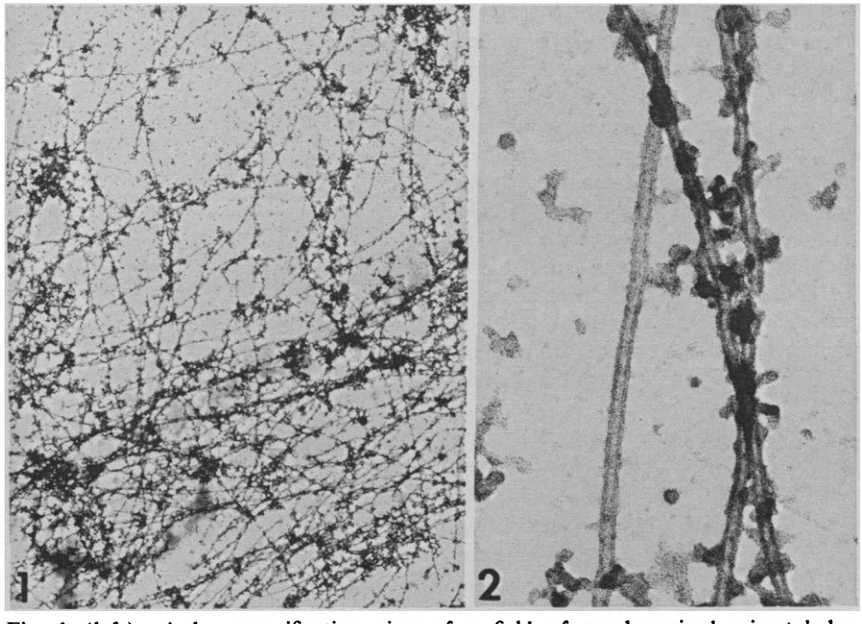

Fig. 1 (left). A low-magnification view of a field of repolymerized microtubules. Tubulin was purified by the batch DEAE method (*1*) and contained 1 m*M* GTP, 1 m*M* EGTA, and 0.5 m*M* $MgSO_4$ in 0.1*M* MES at *p*H 6.5. The sample was incubated for 1 hour at 35°C (× 2,900). Fig. 2 (right). A high-magnification view of repolymerized microtubules from the same grid as used for Fig. 1. A smooth-walled microtubule and a pair of highly decorated microtubules are present in the same field (× 103,000).

Table 1. Effects of GTP, Mg, and EGTA on microtubule polymerization. Tubulin was partially purified by ammonium sulfate precipitation. All samples were in 0.1*M* MES buffer at *p*H 6.5. The final concentrations were: GTP, 1 m*M*; EGTA, 1 m*M*; $MgSO_4$, 0.5 m*M*; and $CaCl_2$, 1.5 m*M*. The protein concentration was about 2.5 mg/ml.

Additions	Polymerization
GTP	No
$MgSO_4$	No
EGTA	No
GTP + $MgSO_4$	Slight
GTP + EGTA	No
GTP + $MgSO_4$ + EGTA	Yes
ATP + $MgSO_4$ + EGTA	Yes
GTP + $MgSO_4$ + EGTA + $CaCl_2$	No

of ADA which was different from the other buffers tested was its ability to bind calcium and magnesium ions, an investigation was made of the effects of these ions on polymerization. The addition of 0.01*M* $MgCl_2$ to 0.1*M* ADA appeared to give slightly better polymerization, while the addition of 0.01*M* $CaCl_2$ nearly eliminated polymerization. The addition of Mg and Ca together also prevented polymerization. Since the estimated concentration of free Ca produced by 0.01*M* Ca in 0.1*M* ADA was only about 6×10^{-6} (based upon a stability constant of 10^4), a very low concentration of Ca was sufficient to block polymerization.

To investigate the role of Ca and Mg further the effects of the chelators ethylenediaminetetraacetate (EDTA) and ethylenebis(oxyethylenenitrilo) tetraacetate (EGTA) on microtubule polymerization were also determined. The EDTA alone, at 0.05*M*, did not allow polymerization to occur, but polymerization was observed if 0.01*M* $MgCl_2$ was added to the EDTA solution. Also EGTA alone, at 0.05*M*, allowed polymerization to occur to roughly the same extent as did ADA alone, or EDTA plus Mg.

On the basis of the results obtained with the crude, unfractionated homogenate, the ability of partially purified tubulin to polymerize in various mediums was determined. As shown in Table 1, microtubule formation could be obtained in a medium containing a nucleotide triphosphate [GTP and adenosine triphosphate (ATP) were equally effective], $MgSO_4$, and EGTA. None of these agents were effective alone, nor were any pair of them (the exception was GTP with $MgSO_4$ in which a few relatively short microtubules were observed). Polymerization could also be obtained with a more highly purified tubulin (Fig. 1), and again both GTP and ATP were effective in promoting polymerization.

Polymerization proceeds rapidly, appearing to be nearly complete within about 30 minutes at 35°C. Incubation at 0°C, after preincubation at 35°C for an hour, greatly decreases the amount of polymer, as does incubation in the presence of 0.1 m*M* colchicine. The ultrastructure of the repolymerized tubules appears normal, with the stain either penetrating the core or staining only the surface (Fig. 2). A noteworthy feature is the frequent presence of "decorated" tubules with large numbers of heterogeneous projections along the length of the tubule. The presence of these projections appears to depend upon the way the microtubule is stained and is probably a staining artifact, but could also represent a true property of the microtubule. It is also important that microtubule formation is spontaneous, and requires the addition of no nucleating centers. The possibility that some tubules survive the process of purification cannot be absolutely ruled out, but has to be considered extremely unlikely. No tubules are seen prior to the induction of polymerization.

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RNA Metabolism in Tracheal Epithelium: Alteration in Hamsters Deficient in Vitamin A

Abstract. *The electrophoretic pattern of RNA molecules that are synthesized in vitro in tracheal epithelium from hamsters deficient in vitamin A differs from that of RNA synthesized in normal, pair-fed control hamsters. There is less RNA of low electrophoretic mobility in the epithelial cells deficient in vitamin A. This alteration is reversed after the deficient animals have been treated with vitamin A.*

The state of differentiation of the tracheal epithelium is determined by vitamin A (*1*, *2*). In vitamin A deficiency, the normal columnar tracheal epithelium, composed of ciliated or mucus-producing cells, which act as a barrier to infectious and other noxious agents (see cover), is replaced focally by squamous cells, which are neither ciliated nor mucus-producing. The extent of replacement by squamous cells (squamous metaplasia) generally parallels the severity and duration of vitamin A deficiency. After feeding vitamin A to previously deficient animals, new ciliated and mucus-producing cells repopulate the epithelium. We report here studies on the effect of vitamin A deficiency on RNA metabolism in this tissue. These studies show that the electrophoretic pattern of RNA molecules in tracheal epithelium from vitamin A–deficient hamsters differs from the pattern in normal, pair-fed controls. The percentage of newly synthesized, high-molecular-weight RNA species with low electrophoretic mobility is reduced in tracheal epithelium deficient in vitamin A.

The effect of vitamin A on RNA metabolism in the liver and intestine of the rat has been studied. Decreased isotopic labeling of RNA in the intestine of vitamin A–deficient rats has been shown to result from decreased synthesis, rather than from differences of entry of radioactively labeled uridine into intracellular uridine pools (*3*); a decrease in total cellular transfer RNA was also observed. Administration of vitamin A stimulated incorporation of [^{3}H]uridine (*4*) or [^{3}H]orotic acid (*5*) into RNA in the intestine of vitamin A–deficient rats. Studies of the incorporation of [^{3}H]uridine into nuclear and cytoplasmic fractions of the livers of vitamin A–deficient rats demonstrated that incorporation of [^{3}H]uridine into nuclear RNA was reduced by approximately 50 percent when compared to pair-fed controls (*6*). However, in a number of the above-mentioned experiments (*4–6*), the effects of vitamin A on the incorporation of uridine or