NEUROTUBULE ASSEMBLY AT SUBSTOICHIOMETRIC NUCLEOTIDE LEVELS USING A GTP REGENERATING SYSTEM*

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Summary

Tubulin derived from cold depolymerized bovine microtubules has been gel filtered to obtain a tubulin preparation with only 3% of the tubulin dimers containing exchangeable [^{3}H]-guanine nucleotide. In the presence of acetyl-P and bacterial acetate kinase, this preparation polymerizes to form microtubules which are morphologically indistinguishable from microtubules formed in the presence of excess GTP. The extent of microtubule formation at substoichiometric nucleotide levels using the GTP regenerating system exceeds the extent of assembly obtained with excess GTP. It is concluded that the exchangeable guanine nucleotide site can be virtually unoccupied in intact neurotubules and this finding indicates that GDP can "catalyze" tubule assembly in the presence of a GTP regenerating system.

Tubulin, the principal protein component of neurotubules, binds two molecules of guanine nucleotide per 110,000 molecular weight αβ dimer (1,2). One molecule is noncovalently fixed on the protein in a nonexchangeable fashion, and the other rapidly exchanges with the medium; these sites are commonly designated as the N-site and E-site, respectively. There is a growing body of evidence suggesting that certain phosphotransferase reactions which are associated with microtubules are important in their assembly (3,4). The formation of tubules in the presence of GTP is attended by hydrolysis of nucleotide at the N- and E-sites (3). Yet it is also clear that E-site binding of GTP analogs such as 5′-guanylylmethylene diphosphonate (6) or Cr(III)GTP (7), leads to polymerization without hydrolysis. Nonetheless, the detailed role of the nucleotide interactions at

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these tubulin binding sites is not clear, and we have initiated studies to define the minimum requirements for tubule assembly. Along this line, we have found that one can advantageously employ an acetate kinase GTP-regenerating system to demonstrate tubule assembly with the E-site virtually unoccupied by nucleotide.

METHODS

Beef brain tubulin was purified as described elsewhere (8,9) but the polymerization buffer contained 100 mM potassium glutamate, 20 mM MES, 1 mM EGTA, 1 mM $MgCl_2$, 0.2 mM GTP, 10 mM acetyl-P, and E. coli acetate kinase (0.15 units/ml of polymerization medium). Immediately prior to use, tubulin was further purified by a polymerization/depolymerization cycle, and the polymerization buffer contained [^{3}H]-GTP (2.24×10^6 cpm/ml). This was followed by gel filtration to remove E-site guanine nucleotide. The amount of bound guanine nucleotide in the tubulin sample was estimated by liquid scintillation counting.

Polymerizations were conducted at 37°C and absorbancy changes at 350 nm were monitored in a Cary 118C recording spectrophotometer (0-0.2 absorbance scale). The justification for using turbidity to follow polymerization has been presented (2).

Electron micrographs of the tubules fixed on carbon coated grids and negatively stained were prepared as described elsewhere (7). Protein concentration was estimated by the Lowry method (10) using bovine serum albumin standards.

RESULTS

Jacobs and Caplow (4) recently reported that porcine tubulin will assemble to form microtubules if ATP and low levels of GDP (0.1 μM) are present in the assembly medium. At 37° with the bovine brain tubulin, we have consistently found that ATP will support the polymerization without added GDP. When tritiated GTP was included in the final polymerization/depolymerization cycle prior to our assembly experiments, we found that 2-3% of the E-sites contained

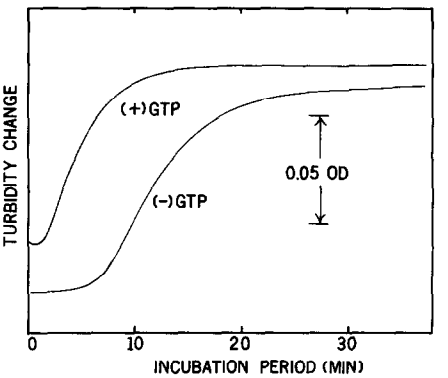


Fig.1 : Extent of polymerization with time as measured by increase in optical density at 350 nm.
Upper curve: Tubulin was 1.02 mg/ml, acetate kinase 0.25 unit, acetyl phosphate 2 mM, GTP 1 mM, free Mg^{2+} 1 mM.
Lower curve: Same conditions except no added GTP.

bound guanine nucleotide after gel filtration. It was of interest to determine whether ATP supported polymerization by binding to tubulin or as a result of GDP phosphorylation. Jacobs and Caplow (4) favor the latter for the porcine system but their evidence was indirect and inconclusive. To answer this question, we decided to utilize a GTP-regenerating system which did not require the presence of any other nucleotides. From earlier work (13), we noted that the E. coli acetate kinase can utilize GDP or ADP as phosphoryl acceptors from acetyl-P. Since the formation of GTP is thermodynamically quite favorable, it was clear that rephosphorylation of GDP by this system could readily provide an answer about tubule assembly at substoichiometric guanine nucleotide levels. As shown in Fig.1, bovine neurotubular protein will reassemble over a 15-20 min period to give neurotubules. This occurs to roughly the same final extent whether or not GTP is added to the medium (compare the amplitudes of curves A and B). Furthermore, qualitatively similar results are observed with a pyruvate kinase regenerating system. GDP is a poorer substrate than ADP with rabbit muscle pyruvate kinase; so the acetate kinase system is considerably more useful to us. In any case, polymerization occurs without any added nucleotide, and this finding suggests that intact neurotubules can form with the E-site lacking an equivalent level of nucleotide.

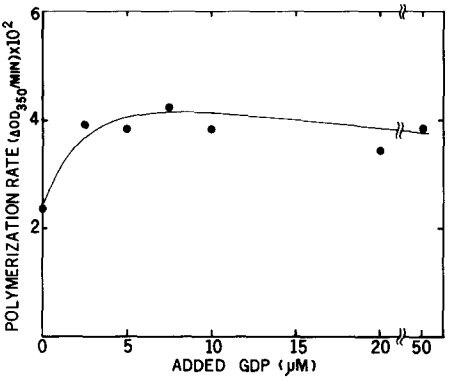


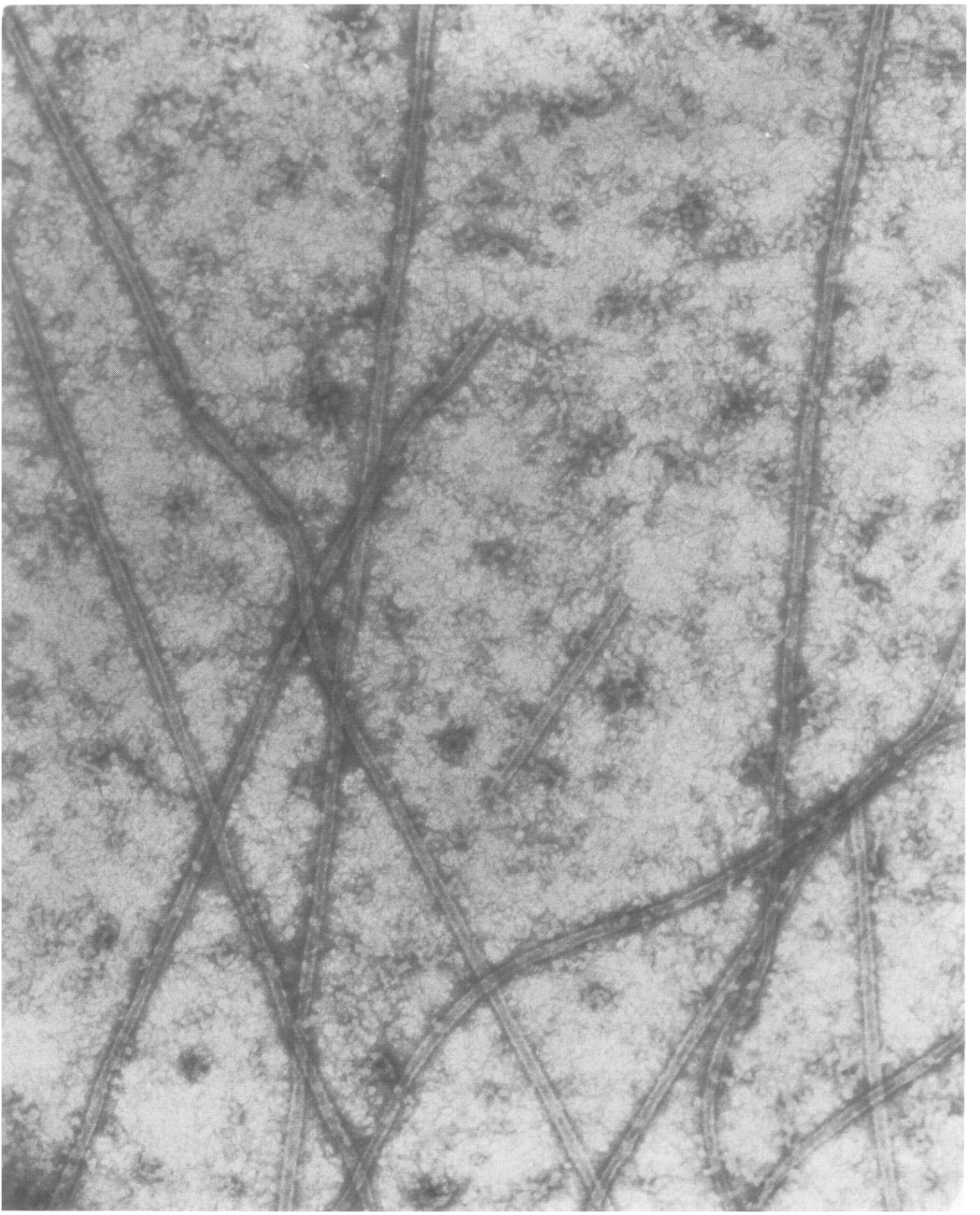
Fig.2 : Effect of added GDP on acetate kinase-acetyl phosphate stimulated polymerization. Acetate kinase was 0.25 unit, acetyl phosphate 2 mM. Tubulin was 1.10 mg/ml (10 μM). Temperature was 37°. Rates corrected for loss of polymerization competency in tubulin stock solution.

Figure 2 shows that added GDP can stimulate the rate of acetate kinase induced tubule assembly. The amount of stimulation is only about two-fold. Since the tubulin levels were approximately 10 μM, the amount of endogenous E-site guanine nucleotide corresponds to 0.2-0.3 μM. This is roughly equivalent to the value of the dissociation constant (0.15 μM) for the tubulin·GDP complex (4), and the partial stimulation by GDP accords with these data.

Finally we have obtained electron micrographs (Fig.3) of tubules assembled at substoichiometric nucleotide levels using the GTP-regenerating system. These are to be compared with those formed in GTP-supported assembly. It is noteworthy that these neurotubules have no unusual morphological features. The fields shown do not reflect the length and abundance of tubules on the EM grids. Cursory inspection of the sample grids suggests that the regenerating system is in fact very effective in the assembly process.

DISCUSSION

Taken together, these data provide strong evidence against the requirement of nucleotide binding at the E-site in reassembled neurotubules. Two possible mechanisms by which the small levels of guanine nucleotide permits assembly are



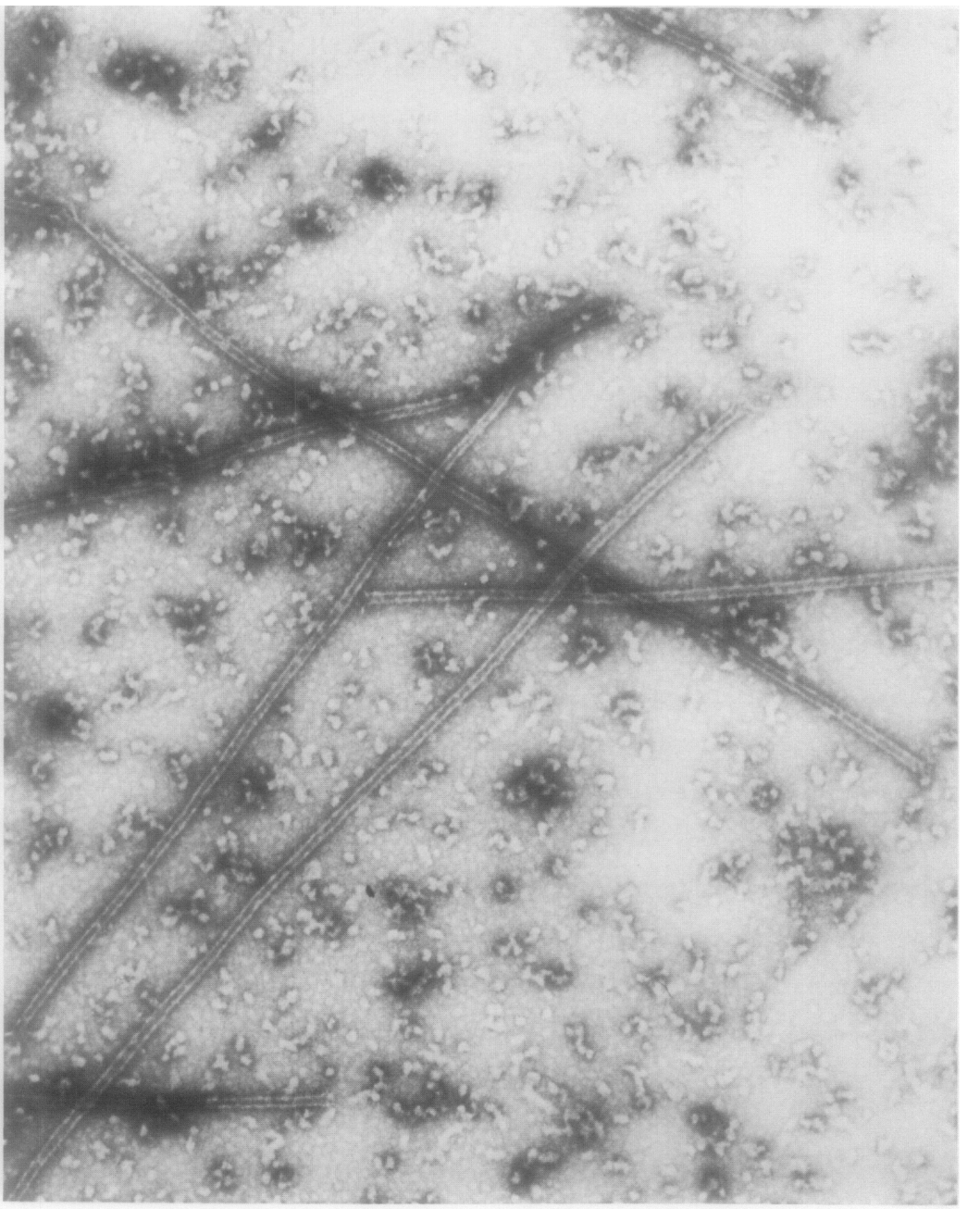
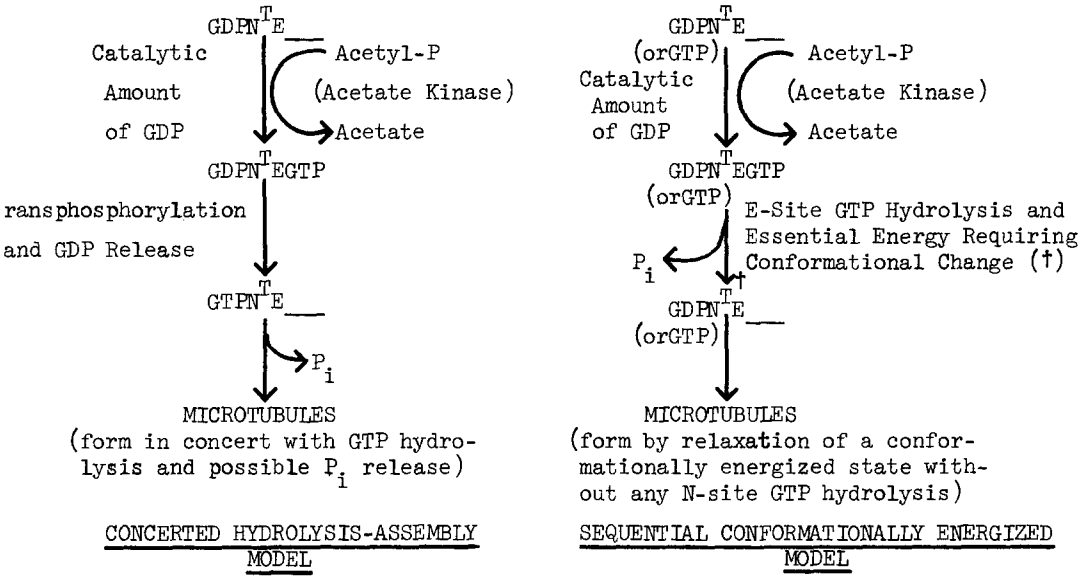


Fig.3 : Electron micrographs, magnification 100,000X.
Left: Tubulin was 0.87 mg/ml, GTP was 1 mM.
Right: Tubulin was 0.87 mg/ml, acetate kinase 0.50 unit, acetyl phosphate 2 mM. Polymerization at 37°.



SCHEME I

summarized in the following scheme. The first of the two most plausible models involves N-site hydrolysis of GTP occurring in concert with tubulin insertion into microtubules. The second model only requires a conformational change at the E-site to give a new energized form of tubulin (T*) which drives the polymerization process to form microtubules. In this case, N-site hydrolysis is not requisite to assembly. Work is in progress to take advantage of this new finding on the microtubule assembly requirements to distinguish between the two mechanisms.

Acknowledgement

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