Comparison Of The Effects Of Different Adenosine And Guanosine Nucleotides On The Assembly Of Bovine Neurotubules

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Summary: Crude brain tubulin was purified in the presence of two non-hydrolyzable guanosine triphosphate analogues. While crude tubulin extracted in 5-guanylylimidodisphosphate was capable of assembly without added nucleotide, that extracted in β,γ-methyleneguanosine 5'-triphosphate required additional nucleotide to promote assembly. Adenosine diphosphate promoted the assembly of the latter tubulin; however, the same diphosphate slightly inhibited the assembly of tubulin which had been extracted in guanosine triphosphate and which was otherwise capable of assembly without added nucleotide.

Introduction: Although it has been reported that 1 mole of GTP is hydrolyzed/mole of 6S tubulin polymerized in vitro (1,2) with a heat of hydrolysis on the order of $\sim$5-7 kcal/mole of GTP (3), the ΔH of the chain propagating step in the polymerization of bovine tubulin was recently found to be 0 ± 1 kcal/mole of 6S tubulin (4). There appear to be three possible explanations for these findings: (1) exact cancellation of the exothermic heat of hydrolysis by an endothermic reaction(s) of equal magnitude; (2) nucleation rather than chain propagation linked GTP hydrolysis; and (3) microtubule assembly proceeding independently of GTP hydrolysis. It was found that the ΔH of chain lengthening was unaffected by temperature (4), so the first possibility would have required the additional equality of the heat capacity changes of the separate exo and endothermic reactions. As this is unlikely, experiments relating to the two latter possibilities were undertaken using non-hydrolyzable GTP analogues. Similar studies were previously done by Olmsted and Borisy (5) and by Gaskin, Cantor and Shelanski (6) who reported that assembly does not occur if

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β,γ-methyleneguanosine 5'-triphosphate (GMPPCP) is substituted for GTP, a result which has been disputed by Lockwood, Penningroth and Kirschner (7). These latter workers (7) and Arai et al (8) found that assembly is possible in the presence of a different GTP analogue, 5-guanylylimidodisphosphate (GMPPNP). Moreover, Shelanski Gaskin and Cantor (9) polymerized tubulin in 4M glycerine in the absence of exogenous nucleotide. Kobayashi (10) found the ratio of tubulin bound GTP/GDP to be unaffected by polymerization.

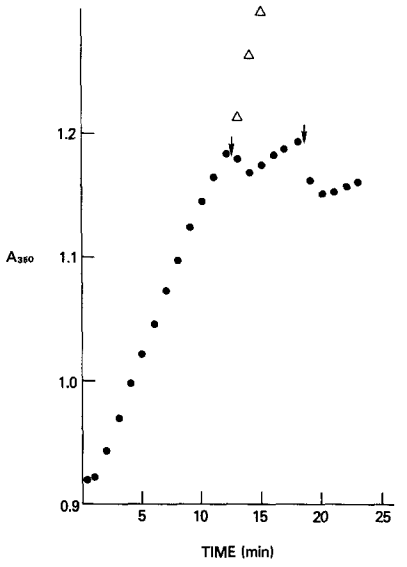
In view of the apparent discrepancy between the results of Ref. (1,2) on the one hand, and Ref. (4) on the other, it was felt to be desirable to ensure that tubulin extracted by the purification procedure used in the calorimetric experiments was capable of assembly in the absence of hydrolyzable nucleotide. Accordingly, two types of experiments were done: (I) the experiment of Ref. (9) was repeated in which tubulin assembly was attempted in 4M glycerine in the absence of exogenous nucleotide, and (II) tubulin purified in non-hydrolyzable nucleotide was tested for polymerizability.

Materials and Methods: All experiments were done using calf brain tubulin except those performed with GMPPNP where rat brain was used. It is considered unlikely that the use of brain from two different species would have qualitatively affected the results. Tubulin was extracted by the procedure of Shelanski et al (9) with minor modifications. The buffer was 0.1M Pipes (pH 6.9), 1mM $MgCl_2$, 2mM EGTA and 1mM nucleotide except as noted otherwise. Nucleotides were from Sigma Chemical Company except for GMPPCP (P-L Biochemicals) and GMPPNP (Boehringer-Mannheim).* Assembly was verified by electron microscopy. The assembly of tubulin in 4M glycerin in the absence of exogenous nucleotide was performed as in Ref. (9), while experiments with GTP analogues were performed with material extracted from brain in a buffer containing 1mM analogue but no GTP. Tubulin pellets so obtained were depolymerized at 0°C and clarified by centrifugation. Protein concentrations were measured by a modified Lowry procedure (11).

Results: (I) It was found by turbidity and verified by electron microscopy that tubulin prepared in the absence of exogenous nucleotide assembled over a period of hours in 4M glycerine, as stated in Ref. (9).

(II) It was found [Fig. 1] that 1mM GMPPNP was capable of promoting the assembly of tubulin at a concentration of 4mg./ml. without additional nucleotide. The

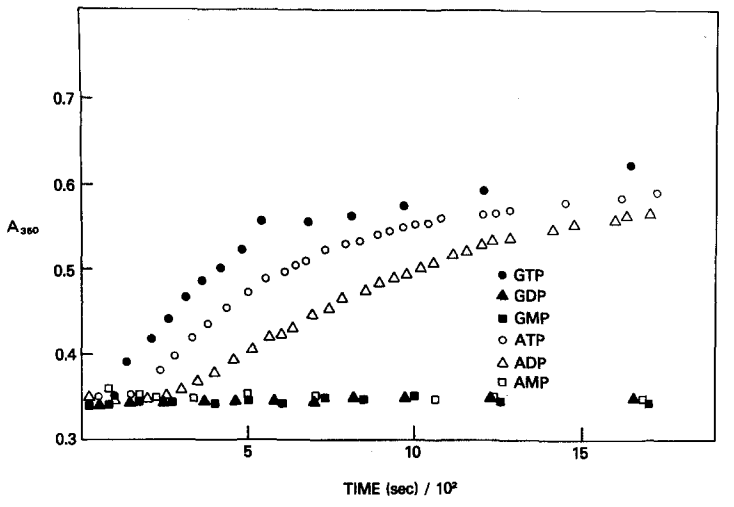
*We thank the referee for noting that commercially available ADP may contain approximately 5% ATP.



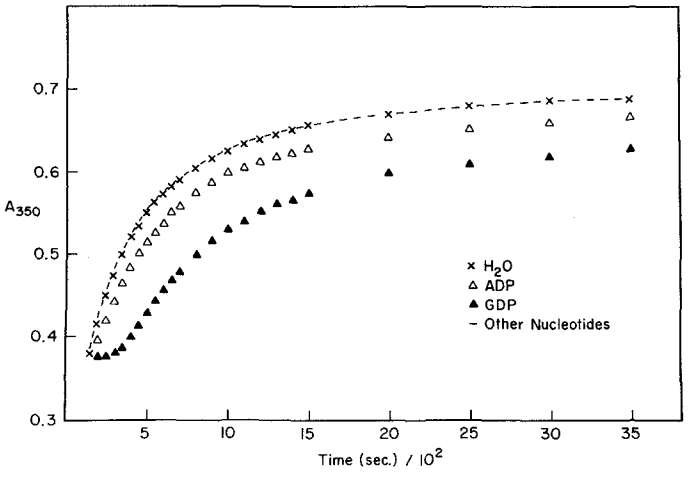
1. Effect of Ca^{+2} and GTP on tubulin prepared in 1mM GMPPNP. Two aliquots of a solution containing such tubulin (4 mg./ml.) were incubated at 23°C without additional nucleotide and monitored turbidimetrically. At points marked by arrow, one aliquot (Δ) was made 1mM in GTP; the other (•) was made first 3mM, and then 6mM in $CaCl_2$.

temperature induced turbidity increase in this preparation was not nullified by millimolar $CaCl_2$ as was that in a control prepared from the same tissue in GTP rather than GMPPNP. On the other hand, the assembly of tubulin prepared in GMPPCP required additional nucleotide before assembly occurred. The nucleotides GTP, ADP and ATP promoted assembly of this material, whereas GDP and the nucleotide monophosphates did not [Fig. 2]. These microtubules were depolymerized by 5mM $CaCl_2$.

To test whether nucleotide hydrolysis might be necessary only for nucleation, a pellet of microtubules extracted from brain in a buffer containing GMPPCP was suspended at 35°C in buffer containing GMPPCP. As noted previously, this material could not have contained sufficient nucleotide to support assembly. These microtubules were sheared by passage through a hypodermic needle. Microliter aliquots of these fragments failed to stimulate the assembly at 25°C of tubulin prepared and depolymerized in GMPPCP,although



2. Effect of 1mM nucleotides on tubulin prepared in 1mM GMPPCP. Solutions containing protein (3.5 mg./ml.) and glycerine (2M) were incubated at 25°C with nucleotides (1mM).



3. Competition effects between GTP (1mM) and other nucleotides (3mM). In contrast with tubulin of Figs. (1) and (2), tubulin was extracted from brain in presence of 1mM GTP rather than GTP analogue. Solutions containing protein (4.2 mg./ml.) and glycerine (2.7M) were incubated at 25°C with additional nucleotide. Of the nucleotides screened (GMPPCP, GDP, GMP, ATP, ADP and AMP), only GDP and ADP show any deviation from a blank (dashed line) in which only water was added.

equal aliquots of fragments did initiate immediate assembly in a tubulin control prepared in GTP without the usual lag time.

A competition experiment performed with tubulin extracted from brain in 1mM GTP but no GTP analogue revealed that millimolar ADP and GDP slightly inhibited assembly [Fig. 3]. Further evidence of nucleotide diphosphate inhibition was obtained when microtubules prepared in 1mM GTP were dialyzed at 23°C versus buffers containing GTP, GDP, ADP and GMPPCP. After 24 hours, tubules dialyzed against GDP and ADP had disappeared whereas some remained following dialysis against GTP and GMPPCP according to electron microscopy.

Discussion: (I) These experiments confirm the ability of tubulin purified by the procedure used in the calorimetric experiments (4) to assemble in the absence of added hydrolyzable nucleotide. While these observations do not prove that hydrolysis cannot be linked to polymerization, they are consistent with the absence of any calorimetrically detectable polymerization linked GTP hydrolysis (4). It is not clear to what extent, if any, contamination of tubulin by exothermic materials from the crude brain homogenates (4) might have complicated the interpretation of the results of Refs. (1) and (2). The differences in polymerizability of tubulin prepared in the different GTP analogues is evidently due to weak binding of GMPPCP to tubulin as compared with GMPPNP (7,8).

(II) The apparent competitive inhibition by ADP of tubulin prepared in GTP [Fig. 3] is interesting since adenosine nucleotides are thought not to bind to tubulin (10,12). This raises the question as to the location of the GTP promotional site if it is not on the 6S dimer. Since at 25°C, GTP activates 36S rings which are known to contain high molecular weight proteins (13) and tau factor (14), these accessory proteins must be considered possible sites. The usual explanation for ADP promoted assembly involves enzymatic conversion of GDP to GTP (6):

$$2\ \mathrm{ADP} \xrightleftharpoons{\text{adenyl kinase}} \mathrm{ATP} + \mathrm{AMP}$$

$$\mathrm{ATP} + \mathrm{GDP} \xrightleftharpoons{\text{phosphoryl transferase}} \mathrm{ADP} + \mathrm{GTP}$$

Insofar as calorimetry failed to detect polymerization linked GTP hydrolysis (4)

and since microtubule assembly can be induced in the absence of hydrolyzable nucleotides, an alternative explanation for ADP enhanced assembly is simply an allosteric effect resulting from direct binding of ADP to the GTP promotional site. Clearly, however, much more detailed knowledge of the binding of nucleotides as well as of the ADP inhibition will be required to verify these hypotheses.

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