Direct incorporation of microtubule oligomers at high GTP concentrations

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Chick brain microtubule protein consists primarily of a mixture of MAP2:tubulin oligomers and dimeric tubulin. The assembly of this protein is described by a single pseudofirst-order reaction at 20 μM GTP, but by the summation of two pseudofirst-order reactions at 1 mM GTP. The protein contains two GTP-binding species, corresponding to the tubulin dimers and the oligomers, and conditions which alter the dimer:oligomer equilibrium, affect the kinetics of microtubule assembly. The results indicate that the oligomers are only direct assembly intermediates at high GTP concentrations.

Microtubule assay *GTP binding* *Tubulin oligomer*

1. INTRODUCTION

GTP is required, under most conditions, for the assembly of brain microtubule protein in vitro. The GTP binds to an exchangeable site on the tubulin dimer [1,2] and is hydrolysed to GDP during assembly [3]. The binding constant of GTP to the tubulin dimer has been determined to be in the range $0.1-125 \times 10^{-6}$ M [2–5]. However, although complete microtubule assembly is observed on addition of a stoichiometric amount of GTP, the maximal rate of assembly only occurs at 1 mM GTP [6]. This suggests that the promotion of microtubule assembly by GTP may involve a mechanism in addition to the binding to the tubulin dimer.

Microtubule polymerisation consists of two phases: the initial formation of a nucleation seed, and a second elongation phase. The linear condensation model has been shown to describe the elongation kinetics in that the rate of elongation depends upon the concentration of polymer ends, equal to double the number of nucleation events, and upon the concentration of 'subunit protein' [7].

Cold dissociated microtubule protein consists of a mixture of tubulin dimers and monomers, the microtubule associated proteins (usually MAP2 and tau), and tubulin-MAP oligomers. A wide variety of oligomeric forms have been described, but they usually consist of either single or double gyre rings sedimenting at 36 S and 30 S, respectively [8,9], and they can form at least 50% of the total protein. Kinetic studies have shown that the number of such rings decreases rapidly during the early stages of microtubule polymerisation [10], indicating that they may be involved in nucleation.

The complex composition of the cold dissociated protein has meant that the identity of the effective 'subunit' has remained elusive. Indeed, in the definitive study of the kinetics of microtubule assembly [7], the cold dissociated protein was pretreated by extensive high-speed centrifugation to remove oligomeric material, in order to prevent self-nucleation, and the data was calculated in terms of the addition of tubulin dimers.

The identity of the effective subunit has, however, been recently examined using time-resolved synchrotron X-ray diffraction [11]. This method indicated that the tubulin:MAP2 rings

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dissociate on warming into an oligomeric intermediate which is then incorporated into the elongating microtubule; i.e., both the tubulin dimer and an oligomeric complex smaller than the rings can participate directly in microtubule assembly. In fact, an earlier temperature-jump ultracentrifugation study had suggested that 36 S rings break down to a 9 S component prior to incorporation into the elongating microtubule [12].

We show here that the GTP concentration has little effect upon the equilibrium level of polymerisation but that it significantly affects the kinetics of assembly, and we conclude that a tubulin oligomer is only utilised as an assembly intermediate at high GTP concentrations.

2. METHODS

Microtubule protein was purified from the brains of one-day-old chicks as in [13]. The protein was further purified by assembly for a third cycle at 37°C, pelleting through a cushion of 30% sucrose (120000 × *g*, 30 min, 30°C), and cold dissociation and clarification (65000 × *g*, 30 min, 4°C). This additional step was necessary to remove unbound nucleotides: the resulting cold dissociated protein contained 1 mol GTP and 1 mol GDP per mol dimeric tubulin. It is assumed that the GTP is bound to the non-exchangeable site and the GDP to the exchangeable site.

2.1. *Microtubule assembly*

Assembly was assayed by measuring the change in absorption at 350 nm using a Beckman DU-8 spectrophotometer fitted with a temperature-controlled cuvette holder. The assay mixture (500 μl), containing 1 mg·ml^{-1}, 3 times recycled microtubule protein in reassembly buffer, was degassed, transferred to the cuvette and incubated at 37°C for 5 min, at which time polymerisation was initiated by the addition of 10 μl of the appropriate concentration of GTP. When appropriate, seeds were prepared as described and added after the pre-incubation [14].

2.2. *Oligomer–dimer separation*

In order to determine the fraction of oligomeric protein, microtubule protein was incubated at 37°C for 30 min, in the absence of GTP, at which time the protein was fixed by the addition of glutaraldehyde to a final concentration of 0.1%. The fixed protein was separated on a Sephacryl S-300 column (10 × 0.9 cm) equilibrated with assembly buffer, and the volume and protein concentration of each fraction were measured. The fixation by glutaraldehyde blocked the dissociation of the oligomers to dimers which is normally observed, and so permitted the fraction of protein existing in the oligomeric form at 37°C to be accurately determined. The use of Sephacryl S-300 permitted the complete separation of the oligomers and the dimers from the free glutaraldehyde, which otherwise interfered with the determination of the protein contents.

2.3. *[^{14}C]GTP binding*

Microtubule protein (100 μl, 1 mg·ml^{-1}) was added to [^{14}C]GTP and immediately (within 10 s) layered over a 1-ml Sephadex G-50 column equilibrated with assembly buffer, and centrifuged at 2500 × *g* for 2 min. The elutant was assayed for [^{14}C]GTP binding and protein content.

2.4. *Protein estimation*

Protein concentrations were determined as in [15]. The method was scaled down to a final volume of 505 μl for determinations of the oligomer:dimer equilibrium, thereby permitting the sensitivity to be increased to 20 μg·ml^{-1} protein. Bovine serum albumin was used as the standard.

2.5. *Other methods*

Proteins were fractionated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and scanned as in [16]. Aliquots of the cold dissociated microtubule protein were examined by electron microscopy after negatively staining with 1% uranyl acetate, using Formvar/carbon-coated grids, and were viewed with a Philips 300 electron microscope. The nucleotide content of the microtubule protein was determined by precipitating the protein with 5% perchloric acid, and fractionating the neutralised supernatant on a PEI-cellulose column (6 × 0.9 cm) with a 40 ml , 0–4 M LiCl gradient prepared in 20 mM KH_2PO_4.

2.6. *Materials*

Biochemicals were purchased from Sigma London Chemical, [^{14}C]GTP (47 mCi·mmol^{-1}) from

Amersham PLC, and Sephacryl S-300 from Pharmacia Fine Chemicals. All other reagents were of Analar grade.

The assembly buffer consisted of 0.1 M 4-morpholineethanesulfonic acid, 2.5 mM EGTA, 0.5 mM $MgSO_4$, 0.1 mM EDTA, 1 mM dithiothreitol (pH 6.4) with KOH.

3. RESULTS

The microtubule protein used in these studies consists of approx. 18% MAP2 and 80% tubulin, as in [16,17] and comprises an equilibrium mixture of MAP2:tubulin oligomers, tubulin monomers and dimers, and free MAP2. Electron microscopy shows that the principle oligomeric species of cold dissociated microtubule protein is a double gyre ring similar to those in [9].

The oligomer:dimer distribution of tubulin at 37°C has been determined as a function of the concentration of microtubule protein, by fixing with glutaraldehyde and fractionating on Sephacryl S-300. The proportion of the total tubulin eluting as an oligomer is almost independent of the protein concentration (fig.1). The amount of oligomeric tubulin has been calculated from the protein content of the void and correcting for the total MAP2 content, since all the MAP2, whether free or associated with the oligomer, coelutes in the void.

However, the tubulin dimer is also in equilibrium with the monomers (K_d = 0.8 × 10^{-6} M at 4.6°C, pH 6.9 [18]). Correcting for the dimer dissociation reveals that the fraction (54%)

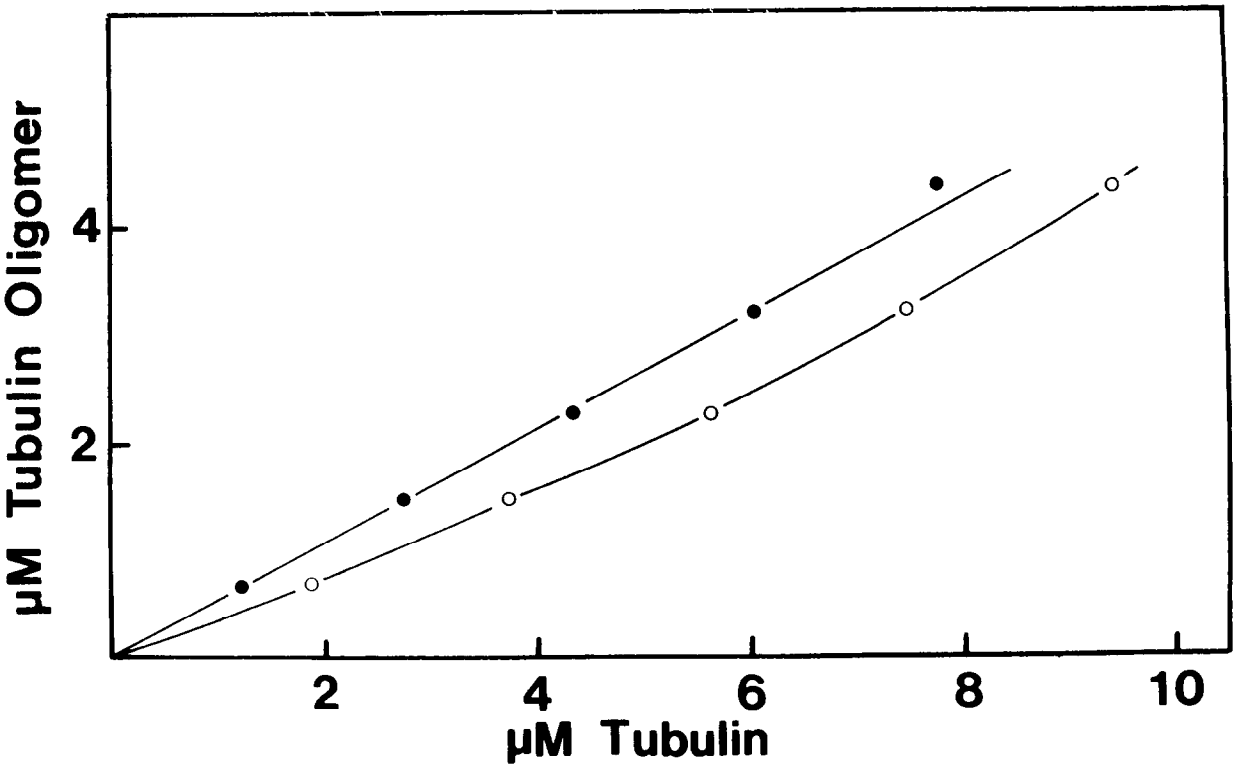

Fig.1. The amount of oligomeric tubulin eluting in the void fraction from a Sephacryl S-300 column as a function of the total tubulin concentration (○) and the concentration of tubulin oligomer + dimer (●). The oligomeric tubulin content has been calculated by subtracting the total MAP2 content from the protein content of the void fraction, and correcting for the M_r of tubulin (100000). The concentration of tubulin oligomers + dimers has been calculated by subtracting from the total tubulin content the calculated concentration of tubulin monomers, determined from the total amount of co-eluting tubulin dimers and monomers and the dimer dissociation constant (0.8 × 10^{-6} M [18]).

of tubulin eluting as an oligomer is directly proportional to the total dimer concentration (fig.1).

Microtubule assembly at 20 μM and 1 mM GTP shows the characteristic nucleation lag phase followed by elongation to a final equilibrium plateau value (fig.2). Although the plateau level is similar for both GTP concentrations, the maximum rate of assembly is, as in [6], substantially faster with 1 mM GTP. The kinetics at 20 μM GTP are described by a single pseudofirst-order reaction, while those at 1 mM GTP are more complex (fig.3a). The approach to equilibrium at 1 mM GTP is described by the summation of two reactions: in the early stages of polymerisation the apparent rate constant (8.75×10^{-3} s^{-1}) is considerably greater than that at 20 μM GTP (2.8×10^{-3} s^{-1}), while as assembly approaches equilibrium, the rate at 1 mM GTP is identical to that at 20 μM GTP. Consequently, the kinetics at 20 μM GTP are described by the linear condensation model of microtubule assembly [7], but those at 1 mM GTP are not consistent with the assembly of a single component.

As the rate of elongation is proportional to the concentration of nucleation sites as well as the subunit concentration [7], the faster initial rate at 1 mM GTP might be due to a higher seed concentration. However, this could not account for the biphasic kinetics. Indeed, the extent of self-nucleation at both 20 μM and 1 mM GTP was approx. 0.7 nM (at 1 $mg \cdot ml^{-1}$), determined by adding increasing concentrations of pre-assembled seeds and extrapolating the calculated pseudofirst-order rate constant to zero (not shown).

The difference in the kinetics is therefore not due to an effect on nucleation, but instead in-

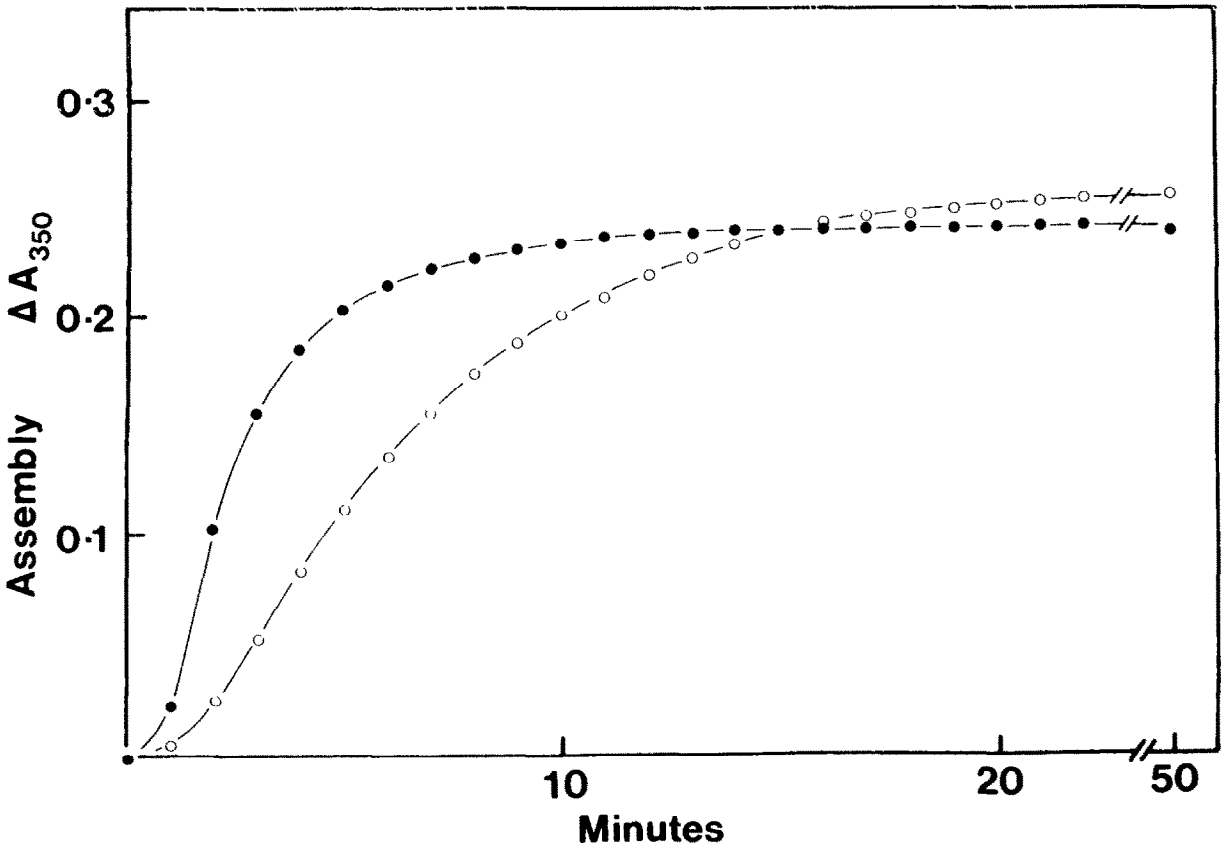

Fig.2. Kinetics of microtubule assembly at 37°C induced by (○) 20 μM GTP and (●) 1 mM GTP. Microtubule protein (1 $mg \cdot ml^{-1}$) was pre-incubated at 37°C for 5 min, at which time GTP was added to 20 μM or 1 mM GTP. The assembly was monitored at $A_{350\,nm}$.

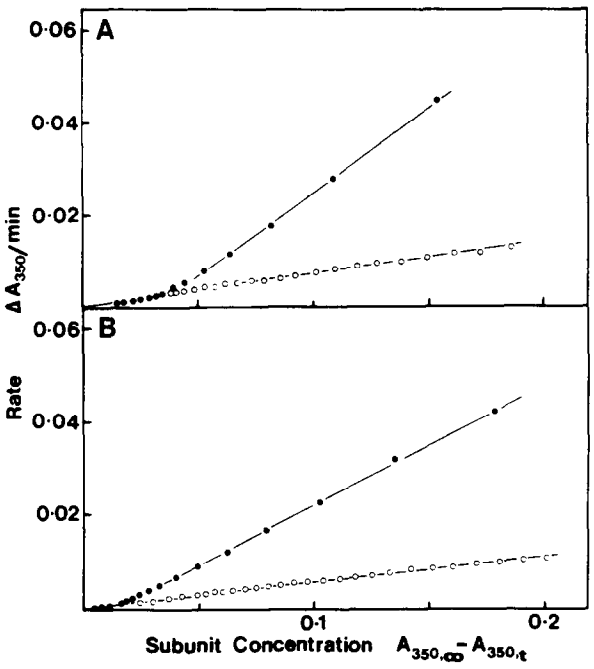

Fig.3. Calculation of the apparent first-order rate constant for assembly at (○) 20 μM and (●) 1 mM GTP. The rate of assembly ($A_{350\,nm} \cdot min^{-1}$) is plotted against the instantanous free subunit concentration ($A_{350\,nm,\infty} - A_{350\,nm,t}$). Data collected during the nucleation phase has been omitted, and for clarity only representative points are presented as the assembly approaches equilibrium. (A) Kinetics of assembly at 20 μM (○) and 1 mM (●) GTP for unfractionated microtubule protein. The increase in absorption as a function of time is shown in fig.2. (B) Kinetics of assembly at 20 μM (○) and 1 mM (●) GTP for protein which had been enriched for MAP2 by collecting the void fraction of microtubule protein maintained at 37°C and eluted through a Sephacryl S-300 column. Note that the free subunit concentration for the inflexion in the rate at 1 mM GTP is significantly lower than for the unfractionated protein (fig.3A).

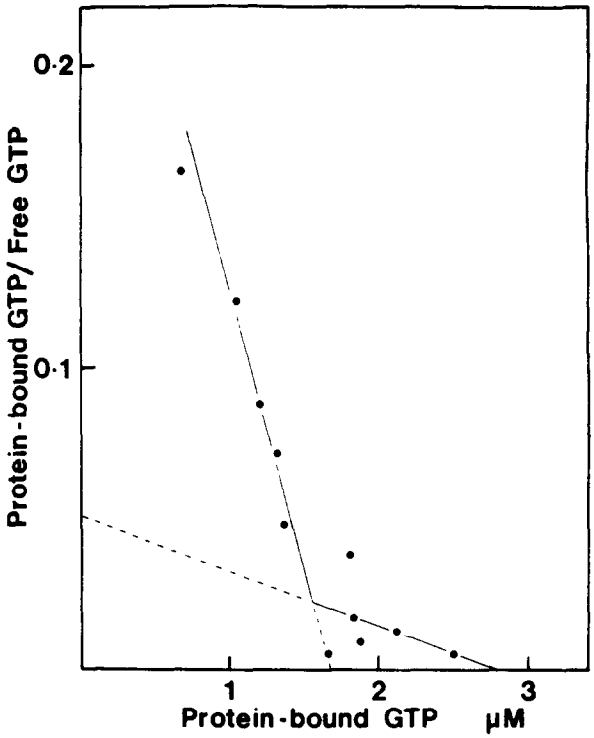

Fig.4. Eadie–Scatchard plot of [^{14}C]GTP binding at 37°C to unfractionated microtubule protein. Microtubule protein (1 mg·ml^{-1}) was incubated (10 s) with increasing concentrations of [^{14}C]GTP and then separated from the free GTP by centrifugal filtration through Sephadex G-50. The high affinity component has an apparent dissociation constant of 5.4×10^{-6} M. The apparent dissociation constant of the low affinity site cannot be determined (see text for details).

dicates that a low affinity GTP-binding component is present. The possible identity of this additional component is indicated by the observation in [19], from a Hummel–Dryer experiment, that the tubulin-MAP2 oligomer binds significantly less GTP (at 25–50 μM GTP) than the tubulin dimer.

The binding of [^{14}C]GTP has been examined as a function of the GTP concentration to unfractionated microtubule protein (fig.4). There are two distinct GTP-binding species: a high affinity component (apparent $K_d = 5.4 \times 10^{-6}$ M) which is assumed to be the tubulin dimer, and a second low affinity component. The observed scatter prevents any calculation of the apparent binding constant of this second component. This scatter is due in part to the need to fractionate the protein away from the free GTP before any significant dimer : oligomer exchange occurs. Ligand binding is normally examined under equilibrium conditions, but clearly in these experiments it was necessary to distinguish between the direct binding of GTP to the oligomers and the oligomer-labelling resulting from the exchange of labelled dimers.

There is an additional low affinity GTP-binding component, and the results in [19] indicate that this additional component is oligomeric.

This, together with the effect of the GTP concentration on the rate of assembly (figs 2,3a), strongly suggests that the oligomeric species is incapable of participating directly in microtubule assembly at low GTP concentrations. This has been tested by: (i) increasing the stability of the oligomers by raising the MAP2 concentration; (ii) changing the ionic strength of the assembly buffer such that the oligomers are fully dissociated but microtubule assembly is only partially inhibited.

Microtubule protein was fractionated, without fixation, on a Sephacryl S-300 column equilibrated with assembly buffer. As the void fraction contains both the MAP2:tubulin oligomer and free MAP2, the stoichiometry of MAP2:tubulin is raised above the value of 12:1 found in reassembled microtubules [17]. The MAP2 concentration was increased by a factor of 1.4, consistent with the elution of about 40% of the total protein as the tubulin dimer (fig.2).

The assembly of this MAP2-enriched protein behaved as a single reaction with pseudofirst-order kinetics at 20 μM GTP, although the rate was significantly (27%) slower than that of the unfractionated protein (fig.3). Similarly, the assembly at 1 mM GTP behaved as the summation of two pseudofirst-order reactions, each rate being inhibited by 33%. However, the intercept, or critical concentration, for the faster of these two reactions was 2.7-times lower than for the unfractionated protein. As the MAP2:tubulin oligomer is in equilibrium with its subunits, the presence of additional MAP2 favours oligomer formation. An increased oligomer content should, if they participate directly in microtubule assembly, reduce the apparent critical concentration. This is observed for 1 mM GTP.

In the second test, microtubule protein was preincubated in assembly buffer containing 75 mM NaCl at 37°C for 30 min. Fixation and separation on a Sephacryl S-300 column showed the absence of oligomers from the void fraction, and this was confirmed by chromatography of unfixed protein by SDS–PAGE: MAP2 but no tubulin was detected in the void fraction.

Unfractionated microtubule protein in the assembly–75 mM NaCl buffer is capable of polymerisation, and the rate of assembly is directly proportional to the free subunit concentration at both 20 μM and 1 mM GTP (fig.5). Dissociation of the oligomers abolishes the second, faster, phase observed in the normal assembly buffer at 1 mM GTP (fig.3).

4. DISCUSSION

The results show: (i) that microtubule protein at 37°C in the absence of GTP consists of an oligomer:dimer:monomer equilibrium (fig.1); (ii) that this mixture contains a high affinity GTP-binding component, presumed to be the tubulin dimer, and a low affinity component (fig.4); (iii) that the assembly kinetics at 20 μM GTP are described by the linear condensation model of polymerisation; but that (iv) the assembly at 1 mM GTP conforms to the summation of two pseudofirst-order reactions but only when the protein contains a mixture of tubulin oligomers and dimers (figs 3,5).

As authors in [19] have shown that the tubulin oligomer fails to bind significant amounts of GTP at 25–50 μM GTP, these results indicate that the biphasic kinetics at high GTP concentrations are due to the binding of the nucleotide to a low affinity site on the tubulin oligomers.

However, complete assembly is observed at both 20 μM and 1 mM GTP (fig.2). The assembly at low GTP concentrations involves the dissociation of the oligomers to tubulin dimers, the subsequent binding of GTP, followed by their incorporation into the elongating microtubule. By contrast, the assembly at high GTP concentrations appears to entail the direct incorporation of both the tubulin dimer and an oligomeric species. The geometry of the microtubule precludes this oligomer being the double gyre ring observed in cold dissociated protein, but both X-ray diffraction [11] and temperature-jump ultracentrifugation [12] studies have indicated that the rings dissociate into an oligomeric assembly intermediate. The results indicate that such oligomeric species are only direct assembly intermediates at high GTP concentrations.

The GTP:ATP ratio of C6 glial cells has been shown to be about 1:4–7 [20,21], suggesting that the total GTP concentration is probably 250–500 μM if the total ATP concentration is about 1–2 mM. The free GTP concentration will be somewhat lower, as the total concentration will include, for example, GTP bound to the non-

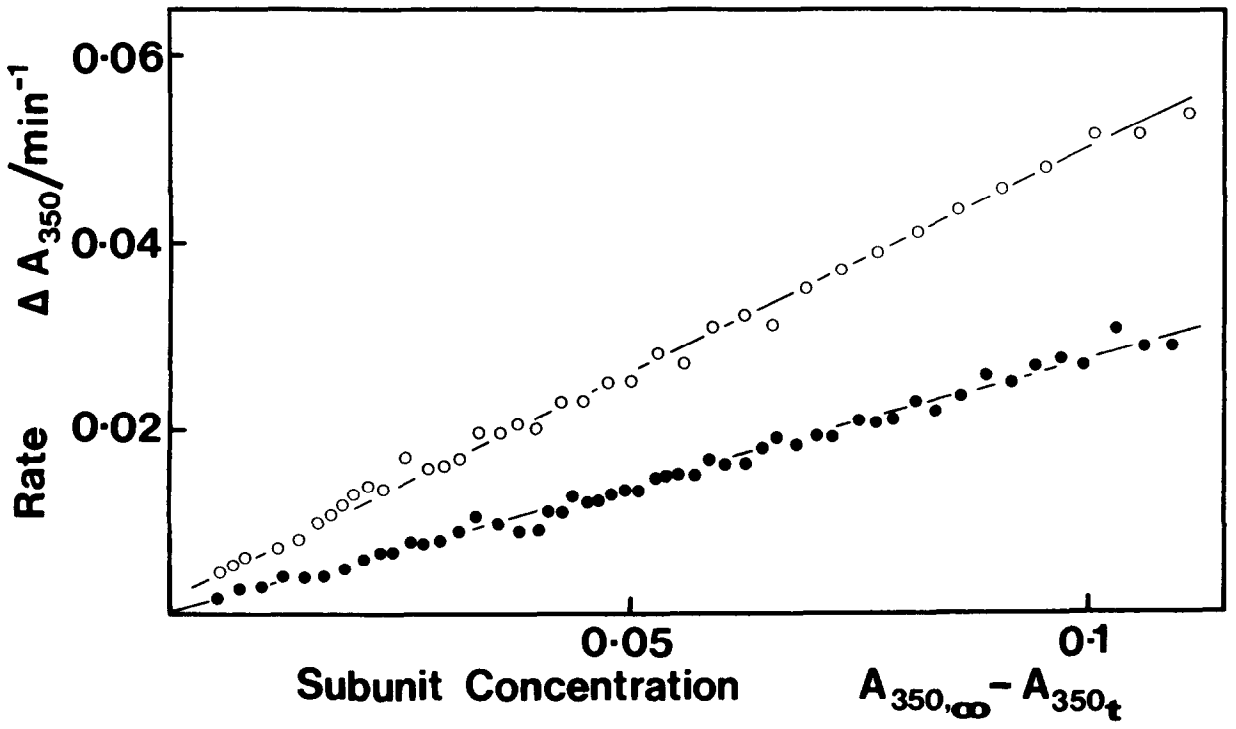

Fig.5. Calculation of the apparent first-order rate constant for the assembly of unfractionated microtubule protein in assembly buffer containing 75 mM NaCl at 20 μM (○) and 1 mM (●) GTP.

exchangeable site on tubulin. While the minimum GTP concentration required for the direct in vitro incorporation of tubulin oligomers is not yet known, the Eadie-Scatchard plot of GTP binding (fig.4) indicates that significant incorporation would not be expected below approx. 50–100 μM GTP. Regulation of the in vivo GTP concentration may therefore represent an important cellular control on the rate, but not the extent of microtubule assembly.

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REFERENCES

[1] Jacobs, M., Smith, M. and Taylor, E.W. (1974) J. Mol. Biol. 89, 455–468.
[2] Arai, T., Ihara, Y., Arai, K. and Kaziro (1975) J. Biochem. (Tokyo) 77, 647–658.
[3] David-Pfeuty, T., Erickson, H.P. and Pantaloni, D. (1977) Proc. Natl. Acad. Sci. USA 74, 5372–5376.
[4] Jacobs, M. and Caplow, M. (1976) Biochem. Biophys. Res. Commun. 68, 127–135.
[5] Maccione, R. and Seeds, N.W. (1977) Proc. Natl. Acad. Sci. USA 74, 462–466.
[6] Olmsted, J.B. and Borisy, G.G. (1975) Biochemistry 14, 2996–3005.
[7] Johnson, K.A. and Borisy, G.G. (1977) J. Mol. Biol. 117, 1–31.
[8] Vallee, R.B. and Borisy, G.G. (1978) J. Biol. Chem. 253, 2834–2845.
[9] Kirschner, M.W., Williams, R.C., Weingarten, M.D. and Gerhart, J.C. (1974) Proc. Natl. Acad. Sci. USA 71, 1159–1163.
[10] Olmsted, J.B., Marcum, J.M., Johnson, K.A., Allen, C. and Borisy, G.G. (1974) J. Supramol. Struct. 2, 429–450.
[11] Bordas, J., Mandelkow, E.-V. and Mandelkow, E. (1983) J. Mol. Biol. 164, 89–135.
[12] Weisenberg, R.C. (1974) J. Supramol. Struct. 2, 451–465.
[13] Burns, R.G. and Islam, K. (1981) Eur. J. Biochem. 117, 515–519.

[14] Burns, R.G., Islam, K. and Chapman, R. (1984) Eur. J. Biochem., in press.
[15] Hartree, E.F. (1972) Anal. Biochem. 48, 422–426.
[16] Islam, K. and Burns, R.G. (1981) FEBS Lett. 123, 181–185.
[17] Burns, R.G. and Islam, K. (1984) Eur. J. Biochem., in press.
[18] Dietrich, H.W. and Williams, R.C. (1978) Biochemistry 17, 3900–3907.
[19] Caplow, M. and Zeeberg, B. (1980) Arch. Biochem. Biophys. 203, 404–411.
[20] Franklin, T.J. and Twose, P.A. (1977) Eur. J. Biochem. 77, 113–117.
[21] Brostrom, C.O., Bocckino, S.B. and Brostrom, M.A. (1983) J. Biol. Chem. 258, 14390–14399.