($\sim$100 s^{-1}) in the reverse direction (data not shown). There is also scope for studying the properties and structure of the reverse reaction ternary complex of CAT, CoA, and 3-acetylchloramphenicol (Figure 1). Such information and the determination of all of the microscopic rate constants by stopped-flow fluorometry should allow a more comprehensive view of the conclusions arising from the present study.

ACKNOWLEDGMENTS

We thank the Parke-Davis Division of Warner-Lambert for the *p*-cyano-CM analogue, J. W. Williams and P. M. Cullis for the other mentioned chloramphenicol analogues, P. J. Day for the ethyl analogue of CoA, and G. C. K. Roberts for helpful suggestions with the manuscript. W.V.S. gratefully acknowledges the award of a research leave fellowship from the Wellcome Trust. The automatic titrator was constructed using funds awarded to C.R.B. and W.V.S. by the Molecular Recognition Initiative of the Science and Engineering Research Council.

Registry No. CAT, 9040-07-7; acetyl-CoA, 72-89-9; *p*-cyano-CM, 23885-61-2; ethyl-CoA, 70019-68-0; 3-deoxy-CM, 21735-83-1; 3-acetyl-CM, 10318-16-8; 3-iodo-CM, 133191-52-3; CM, 56-75-7.

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Identification of an Inhibitor of Microtubule Assembly Present in Juvenile Brain Which Displays a Novel Mechanism of Action Involving Suppression of Self-Nucleation†

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Received April 19, 1991; Revised Manuscript Received August 9, 1991

ABSTRACT: An inhibitor of microtubule assembly has been identified and partially purified from microtubule-depleted brain extracts from day-old chicks and 4-month-old calf. This inhibitor suppresses the self-nucleation of microtubules in vitro with minimal effect upon the final extent of assembly. It may have a developmental role in vivo as it is not detected in adult brain from either cattle or rabbit.

Cytoplasmic microtubules can be highly dynamic, with half-times of approximately 10 min (Schulze & Kirschner, 1987), and are not randomly distributed within the cell. They are generally nucleated by and radiate from specialized structures known as microtubule organizing centers (MTOCs; Brinkley et al., 1981; Karsenti et al., 1984). Assembly kinetics predict a free steady-state concentration of unpolymerized tubulin, yet various tissues contain a substantial concentration of free tubulin (Pipeleers et al., 1977) which exceeds the values observed in vitro.

Microtubule assembly may be regulated at a number of different levels. Endogenous inhibitors have been reported

† This work was supported by the Science and Engineering Research Council.

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which bind directly to tubulin, such as the 33 000-Da microtubule-assembly inhibiting factor (MIP) found predominantly in kidney (Kotani et al., 1988). Other potential mechanisms involve the microtubule-associated proteins (MAPs) and include the binding of phosphatidylinositol or estramustine phosphate to the tubulin binding domain of MAP2 or τ (Yamauchi & Purich, 1987; Burns, 1990) and various kinases modulating the phosphorylation status of the MAPs. Microtubule assembly is also inhibited by the various classes of antimitotic drugs [for review see Dustin (1984)], and brain extracts contain factors which inhibit both the binding of colchicine to tubulin and the assembly of microtubules (Sherline et al., 1979; Lockwood, 1979).

We have detected a component in young brain which inhibits microtubule assembly by the novel mechanism of the suppression of nucleation.

Materials and Methods

Materials. All reagents were obtained from Sigma Chemical Co., with the exception of glass fiber filters GF/C (2.1 cm) and phosphocellulose (Whatman), scintillation fluid (Beckman Ready Protein$^+$), [^{3}H]GTP (10.1 Ci/mmol; NEN Dupont), and Affi-Gel 15 and Bio-Gel P-100 (Bio-Rad Laboratories).

Preparation of Microtubule Protein. Microtubule protein was purified from day-old chick, adult rabbit, and 4-month-old calf brain by two cycles of assembly and disassembly (Burns & Islam, 1981). The protein ($\simeq$15 mg·mL^{-1}) was stored under liquid nitrogen in MES/NaCl buffer (100 mM MES, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 1.0 mM DTT, and 67 mM NaCl; pH 6.4 with KOH); the MES buffer has the same composition except that the NaCl was omitted. Bovine protein was purified similarly except that the assembly buffer for the second cycle of assembly consisted of 100 mM Pipes, 2.5 mM EGTA, 0.5 mM $MgCl_2$, 0.1 mM EDTA, and 1.0 mM DTT (pH 6.8 with KOH).

Preparation of Crude Inhibitor. The microtubule-depleted warm supernatant from the first cycle of assembly of the microtubule protein purification was adjusted to 30% ammonium sulfate. The precipitate was discarded and the supernatant adjusted to 60% ammonium sulfate. The resulting precipitate was dissolved in MES buffer to a protein concentration of $\simeq$5 mg·mL^{-1}, extensively dialyzed against MES buffer, and clarified (180000*g*, 120 min).

Assays of Inhibition of Microtuble Assembly. The kinetics of microtubule assembly were calculated from the increased turbidity at 350 nm, applying a scattering factor of 44 μM tubulin/absorbency unit (Burns, 1991b). The pseudo-first-order rate constant (k_{+1}^{GTP}[M], where k_{+1}^{GTP} is the association rate constant during elongation and [M] is the number concentration of assembled microtubules) and final extent of assembly were calculated from the instantaneous rate of subunit addition and the concentration of the assembled tubulin, thereby correcting for the critical concentration and assembly competency of the protein. This approach uses the kinetics of elongation at high free tubulin concentrations to calculate the final extent of assembly. This calculated extent of assembly is not achieved in practice due to the onset of dynamic instability at low free tubulin concentrations.

Microtubule assembly was also assayed by a rapid filtration assay (Wilson et al., 1982). Assays (150 μL) in the MES/NaCl buffer containing 100 μM [^{3}H]GTP (40.4 μCi·μmol^{-1}), 1 mM phosphoenolpyruvate (PEP), 60 units·mL^{-1} pyruvate kinase (PK), and approximately 10 μM tubulin were assembled at 37 °C for 10 min. Aliquots (50 μL) were removed, quenched with 1 mL of stabilizing buffer (100 mM MES, 1.0 mM EGTA, 1.0 mM $MgCl_2$, 5.0 mM ATP, 50% glycerol, and 20% DMSO) and carefully applied to a glass fiber filter, and the filter was washed with 15 mL of washing buffer (100 mM MES, 1.0 mM EGTA, 1.0 mM $MgCl_2$, 5 mM ATP, 25% glycerol, and 10% DMSO). The filters were air-dried and extracted with NaOH (0.1 M, 1 mL, 45 min), and the extract was counted (6 mL Beckman Ready Protein$^+$). Background levels were obtained by omitting the temperature jump.

Preparation of Axonemes from Tetrahymena thermophila SB255. Axonemes were prepared from cultures of *Tetrahymena thermophila* (100–150 mL) grown at 37 °C in a medium containing 2% (w/v) bacto-peptone, 0.2% (w/v) glucose, 0.1% (w/v) yeast extract, and 1 mg·mL^{-1} ampicillin. *Tetrahymena* were harvested by centrifugation (3000*g*, 5 min, 20 °C, no brake), washed twice (100 mL of medium), resuspended in medium (10 mL), and deciliated with dibucaine (1.5 mM, 1 min). After dilution of the dibucaine (20 mL, fresh medium), the deciliated cells were pelleted (3000*g*, 10 min, 4 °C, no brake) and the cilia were collected (25000*g*, 15 min, 4 °C) and demembranated by suspension in 2 mL of PEM buffer (100 mM Pipes, 1 mM EGTA, and 0.1 mM $MgSO_4$; pH 6.9 with KOH) containing Triton X-100 (0.2% v/v). Demembranated axonemes were collected by centrifugation (37000*g*, 15 min, 4 °C) and washed twice with PEM.

Chromatography. Approximately 10 mL ($\simeq$3 mg·mL^{-1} protein) of inhibitor was applied to either a phosphocellulose or a DEAE-cellulose ion-exchange column (15 × 1 cm) preequilibrated with MES buffer. The columns were washed with MES buffer at 4 °C followed by a linear 0–0.5 M NaCl gradient in MES buffer. The void and salt fractions, following extensive dialysis against MES buffer (12 h), were assayed for inhibitory activity by either the turbidimetric or the rapid filter assay.

The inhibitor (10 mL) was also applied to an inert agarose gel (2 mL) prepared by blocking the active groups of Affi-Gel 15 with 0.1 M ethanolamine for 4 h, which had been preequilibrated with MES buffer at 4 °C. The gel was eluted with 0.5 M NaCl/MES (1 mL), and after further washing with NaCl/MES (3 × 30 mL), a detergent fraction was eluted with 1% Nonidet P-40/MES (1 mL).

Assay of Protein Concentration. Protein concentrations were determined colorimetrically (Hartree, 1972). Since the identity and therefore molecular weight of the inhibitor are unknown, the concentration of inhibitor has been expressed as concentration of protein derived from the crude inhibitor preparation.

Results

The postmicrotubule warm supernatant from chick brain inhibits microtubule assembly in a dose-dependent fashion and has been fractionated by ammonium sulfate precipitation. Addition of increasing amounts of the material precipitated by adjustment to 30% ammonium sulfate and extensively dialyzed, only slightly reduced the elongation kinetics, while the extensively dialyzed 30–60% ammonium sulfate cut markedly inhibited assembly (Table I).

Addition of increasing amounts of the 30–60% ammonium sulfate cut prior to the initiation of assembly increased the initial lag phase and markedly reduced the rate of assembly (Figure 1a). Plotting the instantaneous rate of assembly against extent of assembly (Figure 1b) showed that the inhibitor has a much greater effect on the pseudo-first-order rate constant (k_{+1}^{GTP}[M]) than on the final extent of assembly (Figure 2). This strongly suggests that the inhibitor preferentially inhibits nucleation since the predicted maximum extent of assembly is goverened by the concentration of as-

Table I: Effect of Increasing Concentrations of the Ammonium Sulfate Cuts from Microtubule Protein Depleted Brain Extracts on the Pseudo-First-Order Rate Constants

conditions	$K_{+1}^{GTP}[M] \times 10^{-3}$ s^{-1}
control	15 ± 2
0–30% cut	
0.3 mg mL^{-1}	12 ± 1
0.6 mg mL^{-1}	12 ± 1
1.2 mg mL^{-1}	8.6 ± .8
30–60% cut	
0.6 mg mL^{-1}	8.5 ± .7
1.2 mg mL^{-1}	2.2 ± .2

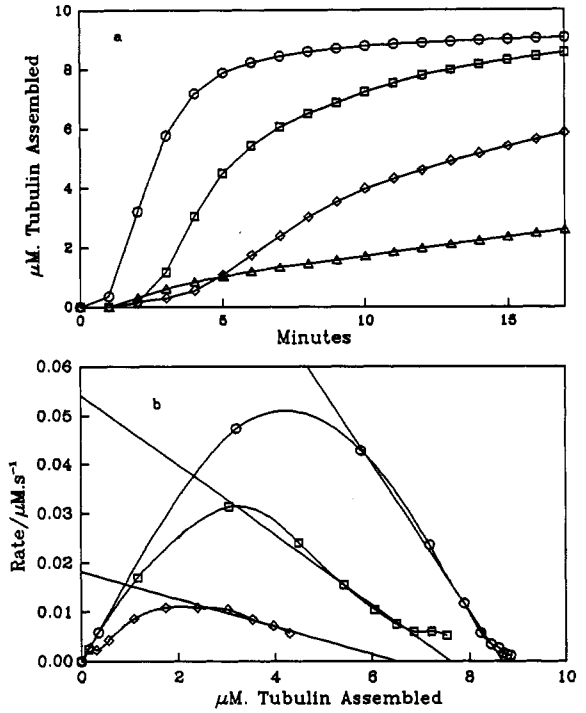

FIGURE 1: Assembly of microtubule protein in the presence of varying concentrations of crude inhibitor. Chick brain microtubule protein (equivalent to ≃12 μM tubulin) was assembled with 0 (○), 0.6 (□), 1.2 (◇), and 1.8 (△) mg·mL^{-1} inhibitor. (a) Extent of assembled tubulin with time and (b) instantaneous rate of assembly vs extent of assembled tubulin. The extent of assembly of microtubule protein is expressed as micromolar tubulin polymerized in this and all subsequent figures.

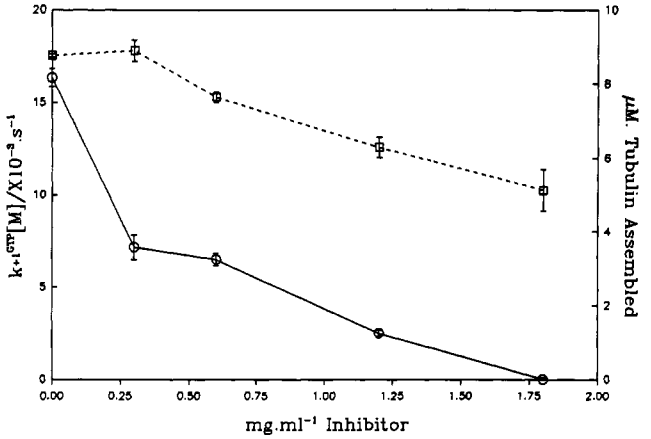

FIGURE 2: Effect of increasing concentrations of inhibitor from chick brain on the pseudo-first-order rate constant for microtubule elongation (○) and final extent of microtubule assembly (□). The values are derived from slopes and intercepts of the instantaneous rate of assembly against extent of assembly (e.g., Figure 1b). As the extrapolation of this plot is unreliable at low rates of assembly, the extent of assembly value at the highest inhibitor concentration may be inaccurate and its stated error underestimated.

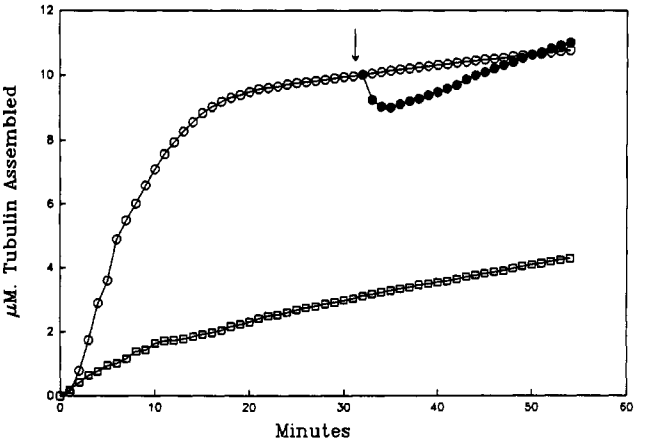

FIGURE 3: Effect of addition of crude inhibitor to steady-state microtubules. Chick brain microtubule protein (equivalent to ≃12 μM tubulin) was assembled in the presence (□) and absence (○) of 1.8 mg·mL^{-1} inhibitor. The inhibitor was also added to control microtubules assembled to steady state (↓) and their subsequent disassembly/assembly was monitored (●).

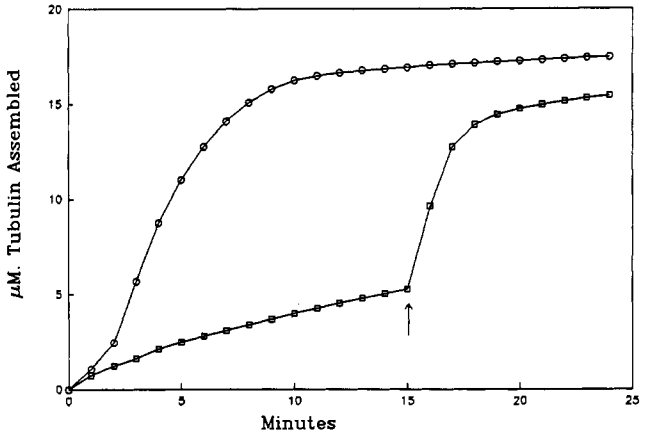

FIGURE 4: Effect of seeding on inhibited microtubule protein. Chick brain microtubule protein (equivalent to ≃18 μM tubulin) was assembled in the presence (□) or absence (○) of 1.8 mg·mL^{-1} inhibitor. A small volume (5 μL) of the microtubules assembled to steady state was removed, sheared, and added (↑) to the inhibited microtubules.

sembly competent tubulin and the critical concentration ($k_{-1}^{GTP}/k_{+1}^{GTP}$; Burns, 1991b) and is independent of the number concentration of microtubules. Indeed, the addition of 1.8 mg·mL^{-1} inhibitor to microtubules preassembled to steady state only caused a slight turbidity decrease compared with that when it was added prior to assembly (Figure 3). Similarly, there was rapid microtubule assembly when protein which had been inhibited by the addition of 1.8 mg·mL^{-1} of inhibitor was seeded by adding preformed microtubules (Figure 4).

Although the major effect of the inhibitor is on the nucleation of microtubules, there is also a noticeable reduction in the estimated final extent of assembly. This may be due to a real effect of the inhibitor either by altering the elongation rate constants or by binding to tubulin and thus reducing the concentration of assembly-competent free tubulin. The effect may also be artifactual since tubulin is a highly labile protein, with a consequential loss of assembly competency with increased time at 37 °C.

Seeding of microtubule assembly with *Tetrahymena* axonemes did not abolish the effect of the inhibitor. Sufficient axonemes were added to uninhibited microtubule protein to increase the pseudo-first-order rate constant from (4.5 ± 0.3) × 10^{-3} to (7.0 ± 0.2) × 10^{-3} s^{-1} (i.e., ≃ 80 pM axonemes).

Addition of the same concentration of axonemes to inhibited microtubule protein might be expected to increase the pseudo-first-order rate constant by a similar amount. However, there was no significant effect. The pseudo-first-order rate constant of assembly of microtubule protein in the presence of 1.8 mg·mL^{-1} inhibitor was (1.9 ± 0.3) × 10^{-3} s^{-1} without axonemes and (1.7 ± 0.5) × 10^{-3} s^{-1} with axonemes.

The observations that the activity of the inhibitor is abolished by seeding with fragments of preformed microtubules but not by axonemes might seem to be contradictory; however, there is evidence that these two methods of seeding are not identical. Sheared microtubules provide native growing ends for the further elongation of single microtubules, while axonemes are far more complex structures which can nucleate up to six microtubules in vitro (Walker et al., 1988). When microtubules are nucleated by axonemes in vitro, both the number of microtubules per axoneme (Burns, 1991a) and the percentage of axonemes bearing microtubules (Walker et al. 1988) show a sigmoidal relationship to tubulin concentration. Thus the growth of microtubules from axonemes requires some process prior to elongation, i.e., some form of nucleation, which is inhibited by this inhibitor.

Microtubule assembly is inhibited in a nonspecific fashion by assembly-incompetent tubulin and by polyanions such as nucleic acids (Bryan et al., 1975). As both may be present in microtubule-depleted brain extracts, the inhibitor was fractionated by ion-exchange chromatography. The void fractions from both the phosphocellulose and DEAE-cellulose columns (which would contain tubulin and polyanions, respectively) showed no inhibitory activity. However, the bound fractions from these columns (eluted with a 0–0.5 M NaCl gradient) also showed little inhibitory activity (results not shown). The inhibitory activity was not reconstituted by mixing void and bound fractions from the phosphocellulose column, suggesting that the inhibitor binds to the column by a nonionic interaction. This was further investigated by eluting the inhibitor through inert agarose beads. Microtubule assembly was completely inhibited by unfractionated inhibitor (1.6 mg·mL^{-1}). By contrast, the equivalent protein concentration following mixing with the agarose beads had no detectable effect on microtubule assembly. The inhibitor was not released by 0.5 M NaCl or detergent [1% (v/v) Nonidet P-40], assaying at equivalent concentrations to the unfractionated inhibitor.

Phospholipids such as phosphatidylinositol have been shown to inhibit microtubule assembly in vitro (Yamauchi & Purich, 1987) and to copurify with microtubules through cycles of assembly/disassembly (Hargreaves & McLean, 1988). These phospholipids do not inhibit microtubule assembly when solubilized by detergent (Surridge and Burns, unpublished results). The crude inhibitor, however, inhibits microtubule assembly in vitro in the presence of detergent (0.1% Nonidet P-40; results not shown).

Digestion of the inhibitor with either trypsin (14 μg·mL^{-1}, 37 °C, 15 min), chymotrypsin (15 μg·mL^{-1}, 37 °C, 15 min), DNase I (100 μg·mL^{-1}, 37 °C, 30 min), or RNase A (1 mg·mL^{-1}, 37 °C, 45 min) had no effect on its activity. While the activity of the inhibitor was not reduced by incubation at 37 °C for up to 45 min or storage at 4 °C, the activity was totally lost by storage either at −4 or −190 °C. The inhibitor did not affect the binding of colchicine to tubulin (data not shown).

The crude inhibitor from chick brain inhibited the assembly of microtubule protein from day-old chick, adult rabbit, and adult cattle. By contrast, no inhibitory activity could be detected in the adult rabbit or adult bovine brain postmicrotubule supernatants or the 30–60% ammonium sulfate cuts, when assayed with any of the sources of microtubule protein. Crude inhibitor could, however, be prepared from 4-month-old calf brain, which markedly inhibited microtubule assembly by reducing the pseudo-first-order rate constant without altering the final extent of assembly (Figure 5).

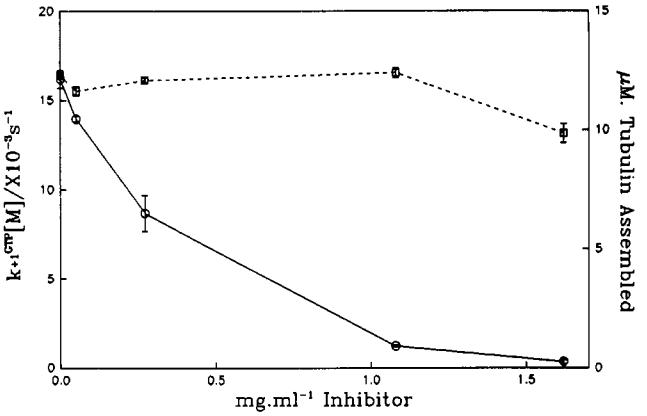

FIGURE 5: Effect of increasing concentrations of inhibitor from calf brain on the pseudo-first-order rate constant for microtubule elongation (○) and final extent of microtubule assembly (□); cf. Figure 2.

Discussion

The inhibitor reduces the pseudo-first-order rate constant of microtubule elongation while having little or no effect on the final extent of assembly (Figure 2). The inhibitor does not induce disassembly of preassembled microtubules (Figure 3) and therefore acts by reducing the effective number concentration of growing microtubule ends rather than the elongation kinetics of individual microtubules. The effective number of microtubule ends can be reduced either by the capping of growing microtubules or by the suppression of microtubule nucleation.

The effect of this inhibitor can be abolished by seeding with fragments of preformed microtubules (Figure 4), thus bypassing the process of self-nucleation by providing native microtubule ends for further elongation. The activity of the inhibitor was not abolished by seeding with *Tetrahymena* axonemes, which can be viewed as facilitating rather than bypassing nucleation (Walker et al., 1988; Burns, 1991a). As any capping factor would cap both self-nucleated and seeded microtubules, the inhibitor must suppress the processes of microtubule nucleation. Indeed, adding the chick brain inhibitor immediately after self-nucleation but before significant elongation had no effect upon the final steady-state extent of assembly (data not shown).

This is a novel mode of action. Previously identified inhibitors of microtubule assembly can be divided into two groups dependent on their mode of action. MIP (Kotani et al., 1988) and the antimitotic drugs such as colchicine and vinblastine inhibit microtubule assembly by binding directly to the tubulin dimer and altering the kinetics of elongation. Nonspecific polyanions, the polysaccharide derived from the cortices of unfertilized sea urchin eggs (Naruse & Sakai, 1981), phosphatidylinositol (Yamauchi & Purich, 1987) and estramustine phosphate (Burns, 1990) bind to MAPs and suppress the MAP-dependent polymerization. The inhibitor identified in chick brain appears to act directly on tubulin or with some microtubule-associated factor specifically involved with nucleation. Elucidation of the exact mechanism will require the purification of the inhibitor to homogeneity and the identification of the molecular structure.

The identity of this inhibitor is unclear. The enzyme digestion and ion-exchange chromatography show that it is neither nucleic acid nor assembly-incompetent tubulin. It differs from certain other inhibitors found in brain [e.g., Sherline et al. (1979) or Lockwood (1979)] by having no effect on colchicine binding and from MIP by having no depolymerizing effect on steady-state microtubules. Vesicular components, especially phospholipids, have been shown to both copurify with microtubules through cycles of assembly/disassembly (Hargreaves & McLean, 1988) and inhibit assembly (Reaven & Azhar, 1981; Yamauchi & Purich, 1987). The inhibitor is not a vesicular component since it is effective in the presence of detergent and since any vesicular components in the 30–60% ammonium sulfate cut would have been removed by the 180000g clarification. The retention of the inhibitory activity by inert chromatography media suggests that the inhibitor displays a potential for strong nonionic interactions.

The identification of the inhibitor in the brains of day-old chicks and 4-month old calf but not in adult brain from either rabbit or cattle is intriguing. There are considerable changes in the microtubule cytoskeleton associated with development, with significant differences in terms of the tubulin heterogeneity (Sullivan & Wilson, 1984), MAP1, and τ composition (Kumagai et al., 1985) of brain from embryonic and posthatching chicks. In addition, there are marked developmental changes in the MAP2 composition between embryonic and postnatal rat brain (Binder et al., 1984; Reiderer & Matus, 1985). We therefore suggest that this inhibitor of microtubule assembly may be concerned with the suppression of self-nucleation in developing brain.

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