20. T. Ohsawa, J. R. Knox, S. Khalifa, J. E. Casida, *J. Agric. Food Chem.* **23**, 98 (1975); G. Zweig, E. L. Pye, R. Sitlani, S. A. Peoples, *ibid.* **11**, 70 (1963); P. B. Bush, J. T. Kiker, R. K. Page, N. H. Booth, O. J. Fletcher, *ibid.* **25**, 928 (1977).
21. M. A. Saleh and J. E. Casida, *ibid.*, in press.
22. B. N. Ames, *Science* **204**, 587 (1979).
23. D. Kennedy *et al.*, Eds., *Pest Control: An Assessment of Present and Alternative Technologies* (National Academy of Sciences, Washington, D.C., 1975).
24. A major cotton pest, the tobacco budworm (*Heliothis virescens*), has developed resistance to toxaphene in areas of prolonged and extensive use [*Environment* **19**, 23 (1977)].
25. D. Brusick (Litton Bionetics) has independently found that toxaphene is mutagenic without requiring metabolic activation on *Salmonella* strains TA98 and TA100 (unpublished data).
26. We thank D. Maron and J. A. Katzenellenbogen for their considerable help on the project. Supported by Department of Energy contract EY-76-S-03-0034-PA156 to B.N.A. and NIH grant PO1 ES00049 and EPA grant R-803913 to J.E.C.

9 March 1979; revised 30 March 1979

Endogenous Inhibitor of Colchicine-Tubulin Binding in Rat Brain

Abstract. *A competitive inhibitor of colchicine binding to tubulin has been found in rat brain. Most of the inhibitor is associated with microsomes but some inhibitor, with an apparent molecular weight of approximately 250,000, is found in the cytosol. Both the microsomal and cytosol inhibitors are heat- and trypsin-sensitive, indicating that a protein moiety is required for activity. The microsomes bind tubulin directly; the microsomal and cytosol fractions both inhibit microtubule assembly in vitro. The inhibitor may function in the living cell to bind and sequester nonpolymerized tubulin. Regulation of tubulin attachment to microsomes could then control the concentration of cytosolic tubulin available for microtubule assembly.*

It is generally believed that microtubules in living cells are in a dynamic equilibrium with their subunit proteins. The number and distribution of assembled microtubules can change rapidly during the cell cycle or in response to external stimuli. Perhaps the most dramatic shift occurs when cells enter mitosis, at which time cytoplasmic microtubules disassemble and their protein constituents reassemble into microtubules of the mitotic spindle. Since Weisenberg's discovery in 1972 (*1*) of conditions for assembly of microtubules from brain supernatants, much has been learned about the factors that participate in or affect polymerization in vitro. Microtubule-associated proteins, Mg^{2+}, and guanosine 5′-triphosphate (GTP) stimulate and calcium inhibits tubulin assembly in vitro (*1*-*7*), but it is not known whether these or other factors regulate microtubule formation and dissolution in cells.

Colchicine, an alkaloid derived from *Colchicum autumnale*, inhibits microtubule assembly both in vitro and in vivo by binding with high affinity to tubulin (*8*-*10*), the major protein constituent of microtubules. Because colchicine is not found in the animal kingdom or in most plants, so far as is known, it has not been considered a candidate for regulation of microtubule assembly. However, it seemed possible that the colchicine-binding site on tubulin might have evolved as an attachment site for an endogenous material, which would be a natural regulator of tubulin assembly in living cells. Previous studies of the colchicine-tubulin binding reaction prompted the suggestion that a factor in brain cytosol lowered the affinity of tubulin for colchicine (*10*). In this report, evidence is presented for the existence of a trypsin-sensitive competitive inhibitor of colchicine binding in the microsomes and cytosol of rat brain.

Microtubule protein was prepared from weanling rat brains by two cycles of assembly-disassembly, using the method of Shelanski *et al.* (*11*). In electrophoresis on sodium dodecyl sulfate polyacrylamide gels (*12*), more than 90 percent of the protein was present in the tubulin band and the remaining 5 to 10 percent was found in the high-molecular-weight region. At saturation, a typical preparation of purified microtubule protein bound 4 pmole of colchicine per microgram. To prepare brain cytosol, rat brains were homogenized at 4°C in 10 m*M* Na_2HPO_4, *p*H 7.0, or 50 m*M* 4-morpholineethanesulfonate (MES), 1 m*M* [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid (EGTA), and 1 m*M* $MgCl_2$, *p*H 6.6 (1 ml/g), using a Tekmar tissue disruptor (model SDT), and centrifuged for 90 minutes at 100,000*g*. Unlabeled colchicine was obtained from Sigma and [^{3}H]colchicine from Amersham.

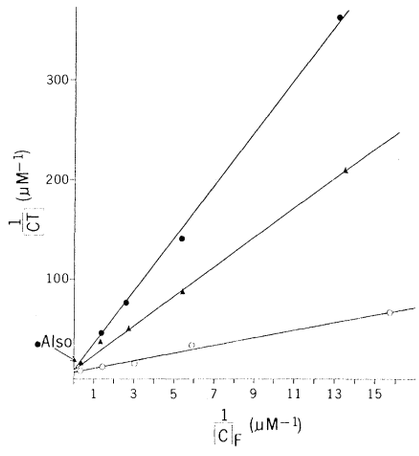

Fig. 1. Effect of brain cytosol inhibitor, partially purified by $(NH_4)_2SO_4$ fractionation (●), and of resuspended microsomal pellet inhibitor (▲) on colchicine-binding affinity of purified tubulin. Open circles represent colchicine binding by tubulin in the absence of inhibitor; $[C]_F$ represents concentration of free colchicine and $[CT]$ the concentration of bound colchicine at the end of the incubation.

Colchicine binding was determined by the charcoal separation method previously described (*13*). In a typical assay, unlabeled colchicine or a putative inhibitor sample was preincubated with tubulin (6 μg per 95 μl) for 2 hours at 37°C before adding 20 μl of labeled colchicine (final concentration, 0.48 μ*M*), followed by incubation for an additional 2 hours. In the kinetic study (Fig. 1), various concentrations of labeled colchicine were incubated with tubulin at 37°C for 4 hours before charcoal separation.

Native tubulin in brain cytosol has a lower affinity for colchicine than does purified tubulin. Addition of purified tubulin to brain cytosol results in a homogeneous class of low-affinity colchicine-binding sites (*10*). These data suggested that the lower colchicine-binding affinity of tubulin in brain cytosol was due to an endogenous factor which inhibits colchicine-tubulin binding. However, in fresh brain cytosol, endogenous tubulin has substantial colchicine-binding activity, which makes it difficult to measure precisely the inhibitory activity of the preparation. We were able to eliminate this problem because the colchicine-binding activity of tubulin is labile, whereas inhibitory activity is stable. Thus, after dialysis of brain cytosol for 48 hours at 4°C (500 volumes of 10 m*M* Na_2HPO_4, *p*H 7.0, three changes) all colchicine-binding activity was lost and inhibitory activity persisted. In multiple experiments, the inhibitor in the dialyzed brain extract decreased the apparent affinity of tubulin for colchicine, but had no effect on the maximum binding, and thus behaved as a competitive inhibitor. Inhibitory activity was precipitated from undialyzed brain extracts between 30 and 60 percent $(NH_4)_2SO_4$. The 60 percent pellet was resuspended in one-fifth the volume of the original supernatant and dialyzed for 48 hours at 4°C. This preparation also behaved as a competitive inhibitor of colchicine binding by tubulin (Fig. 1).

The inhibitor is inactivated by heating

0036-8075/79/0810-0593$00.50/0

at 60°C for 15 minutes (Table 1). No activity is detected in the presence of 1*M* NaCl, but it is completely restored after removal of the NaCl by dialysis. The inhibitory activity was abolished by incubation with trypsin (Table 1), indicating that a protein moiety is required for inhibition of colchicine binding to tubulin. The inhibitor present in the dialyzed brain cytosol or $(NH_4)_2SO_4$ fractions was completely retained after fivefold concentration across an Amicon XM 100 membrane (nominal molecular weight cutoff, 100,000). No inhibitory activity was detected in the filtrate and all activity was recovered after dilution of the fivefold concentrated retentate back to its original volume. A sample of a redissolved $(NH_4)_2SO_4$ fraction from cytosol was applied to a Sepharose 4B column and inhibitory activity in the fractions was measured. The inhibitor eluted with an apparent molecular weight of 250,000 (Fig. 2).

To determine the subcellular distribution of inhibitory activity, we prepared a postmitochondrial supernatant from rat brain by centrifuging a homogenate at 8000*g* for 60 minutes at 4°C. Cytosol and microsomal fractions were derived from the postmitochondrial supernatant by centrifugation at 100,000*g* for 60 minutes at 4°C. After resuspension of the microsomes and precipitation of the cytosol inhibitor by 60 percent $(NH_4)_2SO_4$, the three fractions were dialyzed for 48 hours at 4°C. The inhibitory activity of the fractions was then determined. If 1 unit of inhibitor is defined as the amount necessary to decrease binding of [^{3}H]-colchicine in the preincubation assay by 50 percent, then the postmitochondrial supernatant derived from 1 g of brain contained 187 units. The cytosol and microsomal fractions derived from the postmitochondrial supernatant contained 35 and 177 units per gram, respectively, confirming that most of the activity is particulate. The resuspended microsomal pellet competitively inhibits colchicine binding in a manner indistinguishable from that of the cytosol inhibitor (Fig. 1). In addition, the microsomal inhibitor is heat- and trypsin-sensitive (Table 1). These observations support the hypothesis that the cytosolic and microsomal inhibitors are similar or identical. Perhaps all of the inhibitory protein is associated in situ with subcellular organelles and a small fraction is solubilized during homogenization. Both the microsomal and cytosol fractions inhibit microtubule assembly in vitro at concentrations comparable to those used to inhibit colchicine binding in the preincubation assay (Fig. 3). Heat or trypsin treatment destroys the assembly-inhibiting activity of these fractions (data not shown). However, until each molecule has been purified, it is not possible to know whether the molecule which inhibits tubulin assembly is the same as that which inhibits colchicine binding.

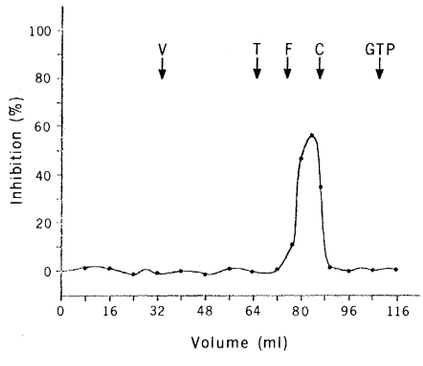

Fig. 2. Gel filtration chromatography of cytosol inhibitor. A 3-ml portion of a 60 percent $(NH_4)_2SO_4$ precipitate dissolved in 50 m*M* MES, 1 m*M* EGTA, 1 m*M* $MgCl_2$, *p*H 6.6, was applied to a Sepharose 4B column (1.5 by 67 cm) equilibrated in the same buffer at 4°C. Eluted fractions were assayed for inhibition of colchicine binding and the values plotted on the ordinate as the percentage of decrease from binding in the presence of buffer alone. The void volume is indicated by the arrow marked *V*; the elution volume of GTP indicates the salt volume. Arrows marked *T*, *F*, and *C* indicate the elution volumes of thyroglobulin (molecular weight, 669,000), ferritin (molecular weight, 440,000), and catalase (molecular weight, 232,000), respectively.

Table 1. Effect of heat and trypsin treatment on inhibitor. In experiment 1 the cytosol or microsomal fraction was heated for 15 minutes at 60°C. Controls were incubated at room temperature (25°C). In experiment 2 the cytosol or microsomal fraction was incubated with trypsin (100 μg/ml) for 3 hours at 37°C, after which lima bean trypsin inhibitor (400 μg/ml) was added to stop digestion. Controls were incubated with inactivated trypsin (trypsin plus trypsin inhibitor) for 3 hours at 37°C. Inhibitory activity was measured by the preincubation assay.

Condition	Colchicine bound (pmole)
Experiment 1	
No inhibitor	5.82
Cytosol	0.32
Cytosol*	4.86
Microsomes	0.53
Microsomes*	6.46
Experiment 2	
No inhibitor	6.14
Cytosol treated with inactivated trypsin	1.57
Cytosol treated with trypsin	5.42
Microsomes treated with inactivated trypsin	1.38
Microsomes treated with trypsin	6.82

*After 15 minutes at 60°C.

The molar concentration of inhibitor sites is approximately equal to the molar concentration of total tubulin in brain. In the preincubation assay, approximately 60 pmole of unlabeled colchicine are required to inhibit binding of labeled colchicine by 50 percent. Thus 1 unit of inhibitor is equivalent to 60 pmole of colchicine. Since we measure approximately 200 units of inhibitor per gram of brain, there are at least 12,000 pmole of inhibitor sites per gram of brain. From previous colchicine-binding measurements (*10*, *13*), there are about 25,000 pmole of tubulin per gram of rat brain. Thus there is approximately 0.5 mole of inhibitory sites per mole of tubulin. Allowing for loss of inhibitor activity during preparation, the number of inhibitor sites appears to be sufficient to bind virtually all of the tubulin molecules present in brain.

The calculation of the number of inhibitor sites on microsomes (which, as noted, comprises approximately 85 percent of the total number of sites) by inhibition of labeled colchicine binding is substantiated by measurement of the depletion of tubulin from supernatant after incubation and centrifugation in the presence of microsomes. In these experi-

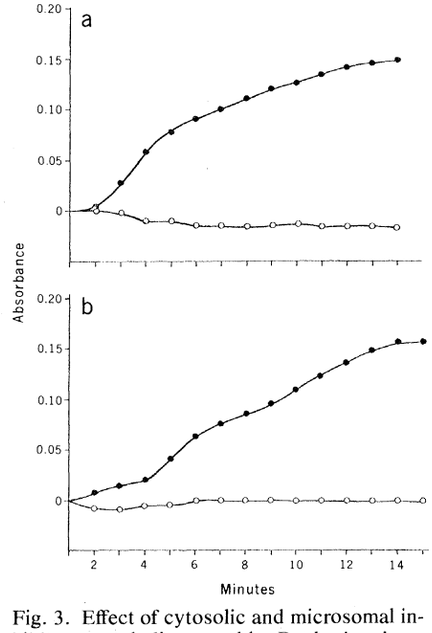

Fig. 3. Effect of cytosolic and microsomal inhibitors on tubulin assembly. Rat brain microtubule protein (3.5 mg/ml) was incubated for 30 minutes at 22°C in reassembly buffer alone (●); in reassembly buffer (a) containing 10.5 units of cytosolic inhibitor (○); or (b) 31 units of microsomal inhibitor (○). Assembly was initiated by the addition of GTP to a final concentration of 1 m*M* and was monitored by the change in absorbance at 350 nm.

ments microsomes were incubated with tubulin for 120 minutes at 37°C, centrifuged at 100,000*g* for 60 minutes at 25°C, and the supernatant assayed for tubulin content by colchicine binding. Control tubes contained tubulin but no microsomes. The amount of tubulin bound to the microsomes was calculated as the difference between the tubulin remaining in the supernatant in control tubes and that in tubes incubated with microsomes. The supernatant from microsomes incubated and centrifuged in buffer alone had no effect on the colchicine binding by tubulin. Microsomes derived from 1 g of brain bind approximately 12,000 pmole of tubulin. The value predicted on the basis of inhibition of colchicine binding is 10,600 pmole/g. Inactivation of microsomal colchicine-binding inhibition by heat or trypsin results in a concomitant loss of ability to bind tubulin. The close agreement between the number of inhibitory sites calculated by the two methods supports the notion that the inhibition of colchicine binding to tubulin by brain proteins reflects a specific ligand-receptor type interaction.

It is curious that a highly specific, high-affinity colchicine-binding site on tubulin should have evolved. The existence in animal tissue of a protein that competitively inhibits colchicine binding to tubulin may provide an answer to this puzzle. Because most if not all of the inhibitor is particulate, it could function to bind tubulin and maintain it in an insoluble form, preventing polymerization. Regulation of tubulin binding to the inhibitor might then control the local concentration of cytosolic tubulin available for assembly into microtubules (*14*).

PETER SHERLINE
KAREN SCHIAVONE
SUSAN BROCATO

Division of Endocrinology and Metabolism, Department of Medicine, University of Connecticut Health Center, Farmington 06032

References and Notes

1. R. C. Weisenberg, *Science* **177**, 1104 (1972).
2. J. B. Olmsted and G. G. Borisy, *Biochemistry* **12**, 4282 (1972).
3. ———, *ibid.* **14**, 2996 (1975).
4. D. B. Murphy and G. G. Borisy, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2696 (1975).
5. M. D. Weingarten, A. A. Lockwood, S. Hwo, M. W. Kirschner, *ibid.*, p. 1858.
6. J. B. Olmsted, in *Cell Motility*, R. Goldman, T. Pollard, J. Rosenbaum, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1976), pp. 1081-1092.
7. R. C. Weisenberg, W. J. Deery, G. Dickinson, *ibid.*, pp. 1123-1131.
8. R. C. Weisenberg, G. G. Borisy, E. W. Taylor, *Biochemistry* **7**, 4466 (1968).
9. L. Wilson, J. R. Bamburg, S. B. Mizel, L. M. Grisham, K. M. Creswell, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 158 (1974).
10. P. Sherline, J. T. Leung, D. M. Kipnis, *J. Biol. Chem.* **14**, 5481 (1975).
11. M. L. Shelanski, F. Gaskin, C. Cantor, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 765 (1973).
12. D. M. Neville, Jr., *J. Biol. Chem.* **246**, 6328 (1971).
13. P. Sherline, C. K. Bodwin, D. M. Kipnis, *Anal. Biochem.* **62**, 400 (1975).
14. While this manuscript was in review, a paper appeared that describes the presence of three peptide inhibitors of colchicine binding in rat brain supernatants [A. Lockwood, in *Cell Reproduction*, E. R. Dirksen, D. M. Prescott, C. F. Fox, Eds. (Academic Press, New York, 1978)]. The relationship of these peptides (all of which have molecular weights less than 40,000) to the inhibitor described in the present work should be clarified by further research.
15. We thank B. Kream for her careful reading of the manuscript. This work was supported by NIH grant GM 22497 and by NIH research career development award AM00456 to P.S.

8 December 1978; revised 9 April 1979

Reptiles and Mammals Use Similar Sensory Organizations in the Midbrain

Abstract. *Striking similarities were observed between the overlapping visual and tactile maps of the mammalian superior colliculus and of its homolog in reptiles, the optic tectum. This topographic pattern probably represents a plan of sensory representation that existed in ancient reptiles and that was retained during the evolution to mammalian forms more than 180 million years ago.*

The ability to focus attention on a stimulus and to orient toward and follow that stimulus is critical for the survival of many species. Refinements of this ability are evident in the stalking and attack behavior of hunting mammals. Although the specific neural mechanisms that underlie attentive and orienting behavior are not fully understood, cells of the superior colliculus must be involved (*1*).

Natural sensory stimuli excite superior colliculus neurons that, in turn, activate brainstem motor centers (*2*) to produce orientation of the eyes, ears, head, and limbs. Because natural stimuli affecting various sensory modalities may produce similar orientation changes via the circuitry of the superior colliculus, the manner in which these sensory representations are organized in the colliculus has been of considerable interest.

Stein *et al.* (*3*) have shown that the visuotopic organization of the cat superior colliculus is in register with deep-layer topographic somatic (somatotopic) representation. Although some differences in laminar distribution exist, parafoveal visual receptive fields are found in the same areas of the superior colliculus as tactile receptive fields on the face. Cells with either superior, inferior, or temporal visual receptive fields are found near somatic cells with receptive fields on the superior, inferior, or caudal regions of the body, respectively. Such topographic register between modalities [a similar one has been described in rodents (*4*)] seems to represent a general mammalian

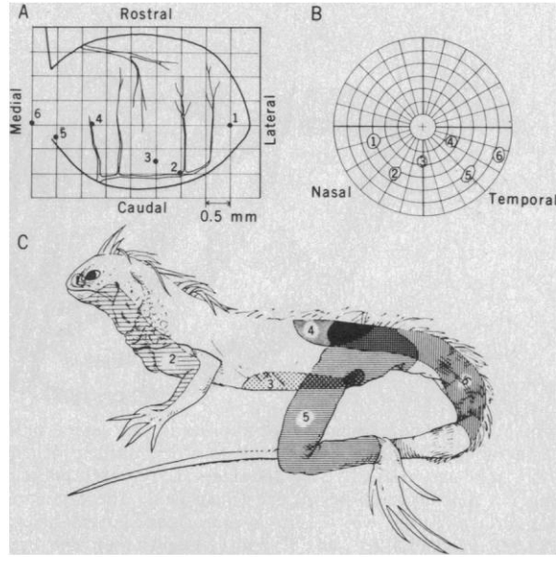

Fig. 1. Visual and somatic receptive fields. (A) Six electrode penetrations from a series extending across the lateral-medial axis of the right tectum. The surface vasculature and the grid system are also shown. Penetrations were made perpendicular to the tectal surface at separations of 0.5 and 0.25 mm along grid lines. (B) Visual receptive fields mapped in each of these penetrations in the central 70° of the left visual field. The field is divided by 10° concentric circles. (C) Somatic receptive fields recorded in these penetrations. Cells with nasal visual receptive fields were found at or just superficial in the tectum to cells with somatic receptive fields on the face (lateral tectum), whereas cells with temporal visual receptive fields were located near somatic cells with receptive fields on the tail (medial tectum). Similarly, inferior visual receptive fields corresponded to ventral somatic receptive fields (caudal tectum) and superior visual receptive fields to dorsal somatic receptive fields (middle-rostral tectum).

0036-8075/79/0810-0595$00.50/0