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Membrane-bound Tubulin in Brain and Thyroid Tissue

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Brain and thyroid tissue contain membrane-bound colchicine-binding activity that is not due to contamination by loosely bound cytoplasmic tubulin. This activity can be solubilized to the extent of 80 to 90% by treatment with 0.2% Nonidet P-40 with retention of colchicine binding. Extracts so obtained contain a prominent protein band in disc gel electrophoresis that co-migrates with tubulin.

Membranes, and the solubilized protein therefrom, exhibit ligand binding properties like tubulin; for colchicine the K_A is $\sim 1 \times 10^6$ M^{-1} in brain and $\sim 0.6 \times 10^6$ M^{-1} in thyroid; for vinblastine the K_A is $\sim 8 \times 10^6$ M^{-1} for both tissues; and for podophyllotoxin the K_i is $\sim 2 \times 10^{-6}$ M for both tissues. Displacement by analogues of colchicine is of the same order as for soluble tubulin.

Although membrane-bound colchicine-binding activity shows greater thermal stability and a higher optimum binding temperature (54° *versus* 37°) than soluble tubulin, this appears to be the result of the membrane environment since the solubilized binding activity behaves like the soluble tubulin.

Antibody against soluble brain tubulin reacts with membranes and solubilized colchicine-binding activity from both brain and thyroid gland.

We conclude that brain and thyroid membrane preparations contain firmly bound tubulin or a very similar protein.

In recent years many studies have implicated the participation of cytoplasmic microtubules in secretory phenomena (see Ref. 1 for review). The great bulk of this work has relied on the inhibitory effect on secretion of colchicine or vinblastine. These compounds disaggregate microtubules, bind to tubulin, the protomer of the microtubule, and interfere with *in vitro* polymerization of tubulin (2–5). They also cause readily visible structural changes in various cells. While colchicine can be a highly specific agent when used at low concentrations (1), both the requirement for low concentrations and the need to prove such specificity have frequently been disregarded and the interpretation of such studies may thus be open to doubt. Even when these precautions have been taken into account, it has been difficult to propose satisfactory models for the precise contribution and microtubule may make to the exo- or endocytosis of "packaged" material.

In the thyroid gland low concentrations of colchicine block thyroglobulin endocytosis in an apparently specific manner (1). However, microtubules do not extend to the apical plasma membrane where the initial steps of this process occur (6, 7). It has thus been difficult to explain satisfactorily the effect of colchicine on these early steps of secretion through the simple participation of cytoplasmic microtubules.

Although soluble tubulin constitutes the bulk of the colchicine-binding activity of many tissues (8, 9), it has been shown that colchicine binding may also be associated with particulate cell fractions from several tissues (10–13). It has not been unequivocally established whether this binding activity is intrinsic to these particles or appears there during the course of preparation. We have, therefore, examined the possibility that colchicine may exert its effect on secretion by interacting with a membrane component rather than the cytoplasmic microtubule. To this end we have compared colchicine binding protein in the plasma membranes of rat brain and beef thyroid with their cytoplasmic (microtubular) counterparts. The results suggest that a protein very similar to cytoplasmic tubulin is bound firmly to such membranes.

EXPERIMENTAL PROCEDURES

Methods

Preparation of Membranes—Both rat brain (Sprague-Dawley females) and beef thyroid membranes were isolated by a modification of the procedure of Wolff and Jones (14). In this method the tissue was briefly homogenized in a Polytron device and subsequently by eight strokes with a loose fitting Dounce homogenizer. The homogenate was centrifuged for 10 min at 200 × *g* to remove debris, and the resulting supernatant solution was centrifuged at 37,000 × *g* for 10 min. The resulting pellet was resuspended and washed twice with the homogenizing media (containing 0.25 M sucrose, 1 mM dithiothreitol, and 3 mM Tris-HCl buffer, pH 7.4) and centrifuged at 37,000 × *g* for 10 min. The upper, nearly white, portion of the pellet so obtained was removed without disturbing the lower pellet and resuspended in the same media and layered on a discontinuous sucrose gradient with layers of 30, 40, and 45% sucrose. Centrifugation was carried out with the SW 27 rotor at 27,000 rpm for 180 min or the SW 41 rotor at 41,000 rpm for 120 min. Bands at the interfaces were withdrawn from the top by syringe, diluted with water, and centrifuged for 15 min at 37,000 × *g*. Each pellet was divided into two portions: one was resuspended in the

homogenizing media for the adenylate cyclase assay; the other was dispersed in 0.25 M sucrose, 10 mM magnesium chloride, 10 mM phosphate buffer (pH 6.5), and 0.1 mM GTP for the colchicine binding assay.

In the early stage of our investigation we followed the particle fractionation procedure to Gray and Whittaker (15) with slight modification. The modification involved the mild (3 s) sonication of the crude mitochondrial fraction and three final washings with 0.32 M sucrose to ensure complete removal of trapped soluble colchicine-binding protein before putting the sample on the gradient. This procedure did not give a clear-cut separation of colchine-binding activity, possibly because sonication of the crude fraction disrupted the synaptosomes, yielding synaptosome fragments and ghosts as well as membranes (10). Therefore, most of our experiments were carried out with the membranes prepared by the first method.

Assay of Colchicine-binding Activity—Bound [^{3}H]colchicine in both soluble and particulate fractions was determined by using the DEAE-impregnated filter paper technique of Weisenberg *et al.* (16) modified by Williams and Wolff (17). For membrane fractions this DEAE-filter paper method was verified by an alternate, discontinuous gradient method of Moore and Wolff (18). The membrane suspension also was sedimented into a pellet with a microfuge running for 5 min. The supernatant solution was aspirated from the top and the pellet was counted. All three procedures gave identical results, indicating that little free colchicine was trapped on the filter papers.

Disc Gel Electrophoresis—The sodium dodecyl sulfate polyacrylamide gels were run as described by Weber and Osborn (19) with phosphate buffer (pH 7.1). The gel contained 5% acrylamide and 0.2% bisacrylamide. In all gels, polymerization was initiated by the addition of *N,N,N',N'*-tetramethylethylenediamine and ammonium persulfate. The samples were run at a current of 4 mA/tube at 23° until the tracking dye reached the end of the gel. The gels were stained with 0.02% Coomassie brilliant blue in 50% methanol and 9% acetic acid and destained in 5% methanol and 7.5% acetic acid.

Protein was determined by the method of Lowry (20) using bovine serum albumin as standard. [^{14}C]Leucine-labeled tubulin was prepared from the soluble fraction of rat brain by the method of Eipper (21). The specific activity was 18,000 cpm/mg of protein.

Radioimmunoassay—Two rabbits were injected at multiple sites with 0.5 mg of purified rat brain tubulin (mixed with 4 ml of complete Freund's adjuvant) weekly for 2 weeks, and then at 2-week intervals for 3 months. The serum was stored at −20°. The titers were assayed by radioimmunoassay with ^{125}I-tubulin as described below.

Iodination of tubulin was carried out according to a modification of the method of Hunter and Greenwood (22) using one-tenth the recommended amount of chloramine-T and sodium metabisulfite. The labeled ^{125}I-tubulin was separated from free ^{125}I on a Sephadex G-75 column.

The assay mixture contained: (*a*) 0.1 ml of diluted antiserum (in 0.1% albumin and 0.2 M phosphate buffer pH 7.0); (*b*) 10 μl of ^{125}I-tubulin diluted in albumin buffer in order to give 5000 cpm; and (*c*) 10 μl of cold antigen in the same buffer. Volumes were made up to 0.5 ml by adding the same albumin buffer. Samples were incubated for 1 hour at 37° and 12 hours at 4°. Then 0.1 ml of normal rabbit serum (diluted 1:100) was added to each tube, followed by 0.2 ml of undiluted sheep anti-rabbit γ-globulin antiserum and the incubation was continued for 12 hours at 4°. The tubes were centrifuged for 15 min at 4000 × *g* to separate free and antibody-bound ^{125}I-tubulin. The supernatant fluid was then aspirated and the pellet was counted. Controls used in these experiments contained nonimmune serum in place of anti-tubulin serum.

Adenylate Cyclase—Adenylate cyclase was assayed by the method of Salomon *et al.* (23) using incubation conditions described earlier (24).

Materials

Preparation of Tritiated Vinblastine—Radioactive vinblastine was prepared by an exchange method using [^{3}H]trifluoroacetic acid in a variation of the method of Owellen and Doring (25). Five milligrams of vinblastine sulfate were dissolved in 0.2 ml of trifluoroacetic acid. To this were added 25 mg of 5% rhodium on Al_2O_3 as catalyst and 10 Ci of tritiated water. After 2 days at room temperature labile tritium was removed *in vacuo* with 0.2 ml of methanol and 2.0 ml of ethanol after filtration from the catalyst. The compound was taken to dryness from methanol twice more. The yield of crude tritiated products was 108 mCi. Of this approximately 35 to 40% behaved chromatographically as vinblastine. The crude product was repeatedly purified by chromatography on silica plates (Q1-Quantum Industries) in chloroform/methanol (9/1). The final product had a radiochemical purity of 93 to 95%. Specific activity was not determined but was calculated to be >19 mCi/μmol, *i.e.* sufficiently high not to contribute significant amounts of carrier in the present experiments.

GTP (Grade IIS) was obtained from Sigma. Tritiated colchicine (ring C, *methoxy*-^{3}H), a product of New England Nuclear Corp., had a specific activity of 2.5 Ci/mmol. Brij 35 SP was purchased from Atlas Chemical Industries and Nonidet P-40 was obtained from BDH, England. All other detergents were obtained from commercial sources. Vinblastine sulfate was a gift from Eli Lilly and Co. The colchicine analogues used were gifts of Dr. Colin Chignell. Podophyllotoxin was the gift of Dr. W. J. Gensler of Boston University.

RESULTS

Membrane Preparation and Distribution of Colchicine-binding Activity—In rat brain homogenates prepared by the method of Gray and Whittaker (15), 50 ± 5% of the colchicine-binding activity was found in the soluble fraction, while the crude nuclear and mitochondrial fractions accounted for 15% and 35 ± 5%, respectively. When the crude nuclear fraction was further purified in 2.4 M sucrose containing 1 M $MgCl_2$ at 50,000 × *g* for 1 hour, there was almost no activity in pure nuclei and more than 90% of the activity was recovered in the soluble phase. This suggested that essentially all the binding activity of the crude nuclear fraction was due to contamination by soluble or weakly bound colchicine-binding protein (Table I). On the other hand, the bulk of the activity in mitochondrial suspensions disrupted by mild sonication and repeated washing was still bound to particles (Table I).

Further purification of this crude mitochondrial fraction (15) did not give a clear-cut enrichment of the colchicine binding in one particular fraction. While the brief sonication may have removed trapped colchicine-binding activity, it made the composition of the synaptosome fraction less specific. Membranes were, therefore, purified by a different method (14) based on enrichment of the F^--activated adenylate cyclase in the case of brain, and thyrotropin-stimulated adenylate cyclase in the case of thyroid. It is clear from Fig. 1 that the fraction recovered at the 40% sucrose interface showed the highest

TABLE I

Fractionation of colchicine-binding activity in brain and thyroid

Subcellular fractionation was carried out by a slight modification of the procedure of Gray and Whittaker (15). The crude mitochondrial fraction was briefly sonicated and washed three times with 0.32 M sucrose to ensure removal of trapped soluble protein.

Tissue fraction	Colchicine-binding activity	
	Brain	Thyroid
	% of total homogenate	
Nuclei (M_1)		
Crude	15	10
Purified	1	0
Washings from crude nuclei	14	10
Mitochondria (M_2)		
Crude	30	18
"Myelin"	8	4
"Synaptosomes"[a]	15	9
Mitochondria	7	5
Soluble cytoplasmic	50–55	70–75

[a] This fraction would also contain all those membranes produced by sonication.

specific activities for colchicine binding. In thyroid membranes this coincided with the highest specific activity for adenylate cyclase, whereas in brain the enzyme activities were equal at the 40% and 45% sucrose interfaces. The fraction at the 40% sucrose interface was, therefore, used in subsequent studies.

In comparison to brain, thyroid is a poor source for colchicine-binding protein (26). Nevertheless, the amounts present readily permitted an estimate of about 18 to 20% of the total colchicine-binding activity in this tissue as particle-bound (Table I). The distribution of colchicine-binding activity in the subcellular fractions was roughly the same as in brain by both methods of fractionation (Table I). Enrichment for colchicine-binding activity in the same fractions as adenylate cyclase suggested that much of this binding protein was in the plasma membranes of thyroid and brain.

Is this colchicine-binding protein of membrane fractions due to tubulin tightly linked to the membrane or to nonspecific adsorption of soluble tubulin during homogenization? When [^{14}C]leucine-labeled tubulin (prepared from rat brain) (21) was added before homogenizing the brain, more than 97% of the radioactivity appeared in the crude soluble fraction and less than 0.1% of the radioactivity was associated with any of the fractions after separation by discontinuous sucrose gradient centrifugation (Table II). Similar data were obtained using the method of Gray and Whittaker (15); no significant amount of label was associated with the purified particulate fractions (data not shown). It seems unlikely, therefore, that the colchicine-binding activity of membrane fractions was due to the contamination by a soluble tubulin or an artifact of separation.

Next we attempted to determine whether the colchicine-binding activity of particulate fractions was due to tubulin present in membranes or to the presence of other colchicine-binding proteins. Solubilization of colchicine-binding activity was attempted by use of detergents. At the same time, we tested how much each detergent affected colchicine binding to soluble brain tubulin (Table III). Although sodium dodecyl sulfate and sodium deoxycholate solubilized large portions of the membrane protein, these detergents abolished all binding activity even at 0.05%. It was therefore necessary to find a detergent that did not interfere with colchicine binding to soluble tubulin but was still able to solubilize such activity from the membranes. Among the detergents tested, the extent of over-all solubilization of membrane protein decreased in the order Triton X-100 (0.1%) > Lubrol PX (0.1%) > Tween 20 (0.1%) > Brij 35 (0.1%) > Tween 40 (0.1%) > Nonidet P-40 (0.1%). This was the reverse order of the recovery of colchicine-binding activity in the solubilized fraction (Table III). The soluble fraction prepared with Nonidet P-40 was highest in relative colchicine-binding activity. The proteins solubilized from rat brain membranes by sodium dodecyl sulfate and by Nonidet P-40 are shown in Fig. 2. It is clear that many fewer bands appear in the Nonidet P-40 preparation but also that one of the two major bands migrates with the same mobility as purified soluble rat brain tubulin. Similar results were obtained with detergent extracts from beef thyroid membranes (data not shown).

We therefore chose Nonidet P-40 as the most useful detergent for the present purposes. Fig. 3 shows the effect of the concentration of Nonidet P-40 on the extent of extraction of the active colchicine-binding protein from both brain and thyroid membranes. Since the presence of 0.2% Nonidet P-40 during the long incubation at 37° decreased the extent of colchicine binding by the solubilized protein, we precipitated the solubilized protein with 55% $(NH_4)_2SO_4$ after extraction with Nonidet P-40. No differences were noted in the extent of solubilization of binding activity from brain and thyroid.

Affinity for Colchicine—Analysis of tritiated colchicine binding to membrane-bound colchicine-binding protein, solubilized binding protein, and soluble tubulin was performed by Lineweaver-Burk plots (Fig. 4). The binding constants for membrane-bound and solubilized colchicine-binding protein from brain (1.0×10^6, 0.7×10^6 M^{-1}) are very similar to that obtained for soluble brain tubulin (1.4×10^6 M^{-1}) (8, 29, 30); for thyroid the constant was 0.6×10^6 M^{-1} for both membrane and solubilized colchicine-binding activity compared with

TABLE II

Distribution of added [^{14}C]tubulin in different subcellular fractions of rat brain

[^{14}C]Tubulin, 18,000 cpm, was added during the homogenization of brain. Fractionation was carried out according to Wolff and Jones (14).

Tissue fraction	^{14}C
	cpm
Homogenate	17,540
Soluble supernatant	17,100
30%	15.4
40%	3.6
45%	<1.0
Pellet	<1.0

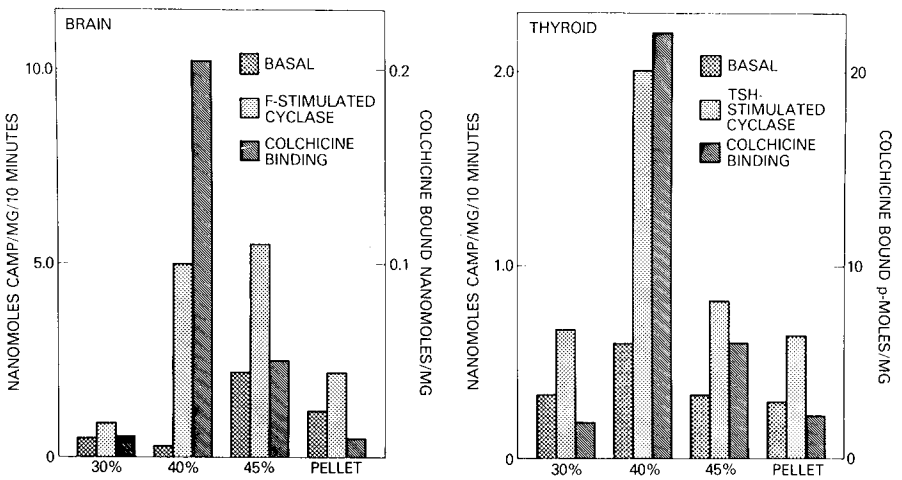

FIG. 1. Distribution of adenylate cyclase and colchicine-binding activities in different sucrose gradient fractions. Fluoride (10 mM) was used as stimulant for brain, and bovine thyrotropin (*TSH*) (200 milliunits/ml) for thyroid adenylate cyclase. For the colchicine-binding assay, the membrane protein concentrations were 1.3 mg/ml for brain and 1.7 mg/ml for thyroid tissue. Samples were incubated at 37° for 1.5 hours with 1.0×10^{-5} M [^{3}H]colchicine and binding activity was measured according to Williams and Wolff (17).

TABLE III

Solubilization of membrane-bound colchicine-binding protein with various detergents

Detergents	Per cent of total protein solubilized		Per cent of colchicine-binding activity solubilized		Per cent inhibition of colchicine-binding activity in tubulin	
	Brain	Thyroid	Brain	Thyroid	Brain	Thyroid
Sodium dodecyl sulfate, 0.05%	93.0	53.2	0	0	100	100
Sodium deoxycholate, 0.05%	64.6	38.4	0	0	100	100
Triton X-100, 0.1%	40.4	23.1	7–10	8	75	72
Lubrol PX, 0.1%	34.8	23.0	9	10	60	64
Tween 20, 0.1%	24.9	21.4	10	11	53	60
Brij 35, 0.1%	21.6	19.4	14	11.5	48	52
Tween 40, 0.1%	18.0	19.0	20	13	41	50
Nonidet P-40, 0.2%	16.4	18.5	80–90	85	0	0

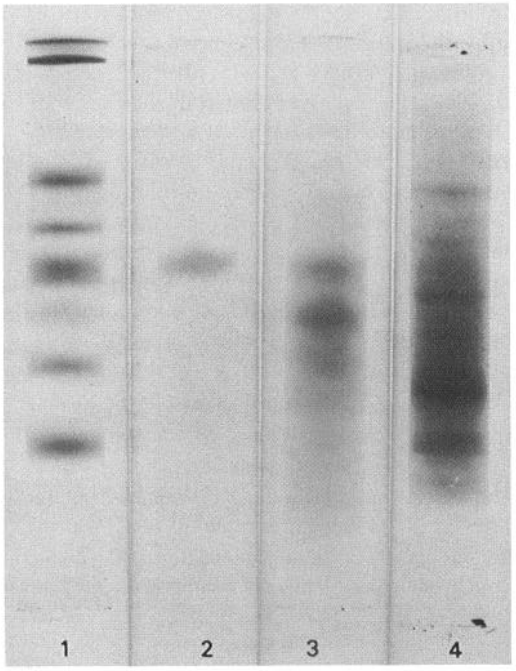

FIG. 2. Sodium dodecyl sulfate acrylamide disc gel electrophoresis. The gels, pictured from *left* to *right*, are: *1*, protein standards, from *top* to *bottom*, 19 S and 12 S thyroglobulin, phosphorylase *b*, transferrin, glutamic dehydrogenase, aldolase, carbonic anhydrase, and myoglobin; *2*, purified rat brain tubulin; *3*, Nonidet P-40-treated rat brain membrane fraction; and *4*, sodium dodecyl sulfate-treated rat brain membrane fraction.

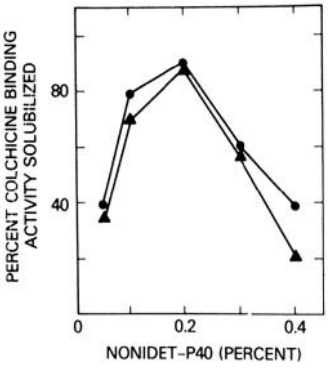

FIG. 3. Release of colchicine-binding protein from rat brain (●——●) and beef thyroid membranes (▲——▲) with Nonidet P-40. The rat brain membrane (1.5 mg/ml) and beef thyroid membrane (1.8 mg/ml) were suspended in various concentrations of Nonidet P-40 at 4° for 30 min and then centrifuged at 37,000 × *g* for 20 min at 4°. Solubilized colchicine-binding protein of the supernatant solution was precipitated with 60% ammonium sulfate for 30 min at 4°, and centrifuged for 20 min at 37,000 × *g*. The pellet was resuspended in 0.25 M sucrose/10 mM sodium phosphate (pH 7.0)/10 mM $MgCl_2$/0.1 mM GTP buffer, and the colchicine-binding activity was measured as described.

0.5×10^6 M^{-1} for soluble thyroid tubulin (17, 26).

We were also able to compare the colchicine-binding activity of both membrane-bound and solubilized colchicine-binding protein with that of soluble tubulin using the various analogues of colchicine. Analogues like colcemid and colchiceine reduced binding of radioactive colchicine by membrane-bound and solubilized protein by 55 and 20%, respectively, when present in 10-fold excess. These displacements are comparable to those observed with soluble tubulin (1, 27). On the other hand, a 10-fold excess of colchicoside and isocholchicine did not prevent labeled colchicine from binding to soluble tubulin, membrane-bound, or solubilized colchicine-binding protein of both brain and thyroid preparations. This is in agreement with the known effects of these analogues[1] (see Ref. 1).

Another similarity between the soluble tubulin and the colchicine-binding protein present in the membrane is their interaction with podophyllotoxin, a known competitive inhibitor for colchicine binding (28, 29). Podophyllotoxin inhibited colchicine binding to membrane proteins and its solubilized form with K_i values (Fig. 5) similar to those obtained for soluble tubulin. In brain these constants were 3.5, 3.8, and 2.0 $\times 10^{-6}$ M respectively, for membrane, solubilized, and soluble colchicine-binding activity. For thyroid tissue K_i values were 1.8, 2.5, and 1.8 $\times 10^{-6}$ M, respectively, for membrane, solubilized, and soluble colchicine-binding activity.

Vinblastine Binding—Another alkaloid, vinblastine, binds

[1] Another method of assessing binding of colchicine to tubulin is by fluorescence. Although colchicine does not fluoresce significantly in nonpolar solvents, fluorescence of colchicine is promoted upon binding to soluble tubulin (29). We were therefore surprised that no fluorescence could be elicited when colchicine was bound to membranes. Three possible reasons for this difference are: (*a*) association with the membrane puts constraints on tubulin that permits binding but not the promotion of fluorescence. This seems unlikely since affinity constants for colchicine in membranes of both thyroid and brain are the same as for their soluble counterparts. However, we cannot rule this out until the reason for the promotion of fluorescence is better understood. (*b*) The protein in the membrane is different. (*c*) Some membrane component other than the bound tubulin quenches the fluorescence. This appears, at present, the most likely explanation since fluorescence of colchicine in the presence of soluble tubulin is abolished by addition of membranes (unpublished results).

tubulin, but at a site different from the colchicine-binding site (28, 30). Table IV shows the distribution of vinblastine-binding activity in different membrane fractions for both rat brain and beef thyroid tissue. Like the colchicine-binding activity, vinblastine-binding activity is highest in the membrane fraction at the 40% sucrose interface. But unlike the colchicine-binding activity, there is also very high vinblastine-binding activity in the fraction at the 45% sucrose interface. In the thyroid, the pellet also contained a substantial amount of binding activity. It seems probable from these results, as well as the fact that vinblastine is a cation at neutral pH, that vinblastine binding is less specific than colchicine binding. Nevertheless, it appeared to be useful to compare binding constants for vinblastine in membrane, solublized, and soluble protein preparations from brain and thyroid. The association constants for tritiated vinblastine to membranes, solubilized protein, and soluble tubulin were determined by Lineweaver Burk plots as shown in Fig. 6. The association constants obtained were $\sim 8 \times 10^6$ M^{-1} for all preparations and are very similar to that obtained by Owellen *et al.* for pig brain tubulin (30).

Stability—A characteristic of the colchicine-binding site of the tubulin is its lability at 37° (28). In the presence of vinblastine and sucrose, this labile colchicine-binding site can be protected (28, 31). Fig. 7 shows the decay characteristics of colchicine binding at 37°. In contrast to soluble tubulin (*Curve 4*), membrane-bound colchicine-binding protein from both brain and thyroid was relatively stable at 37° (*Curve 3*). Decay of colchicine-binding capacity also was diminished by vinblastine and sucrose (*Curves 2* and *1*). When the colchicine-binding protein was solubilized from the membrane, it decayed at the same rate as did soluble tubulin (*Curve 5*). Thus, the environment of the membrane may play the same role that sucrose and vinblastine do for protecting the binding site against decay at 37°. This increased stability also indicates that there is fairly tight binding between membrane and colchicine-binding protein rather than simple physical trapping. In contrast, tubulin entrapped in chick brain synaptic vesicles exhibits normal decay rates for colchicine-binding activity (32).

Temperature Optimum for Colchicine Binding—Colchicine-binding activity in tubulin from brain or thyroid tissue is strongly influenced by temperature. There is no binding at 0° (17, 28, 29); binding is maximal at 37° and equilibration is slow. Above 37°, binding falls off sharply. In contrast to soluble tubulin, membrane-bound tubulin from both brain and thyroid shows an optimum temperature for colchicine binding at 54° for both brain and thyroid membranes (Fig. 8). When membrane-bound protein was solubilized, it showed the same

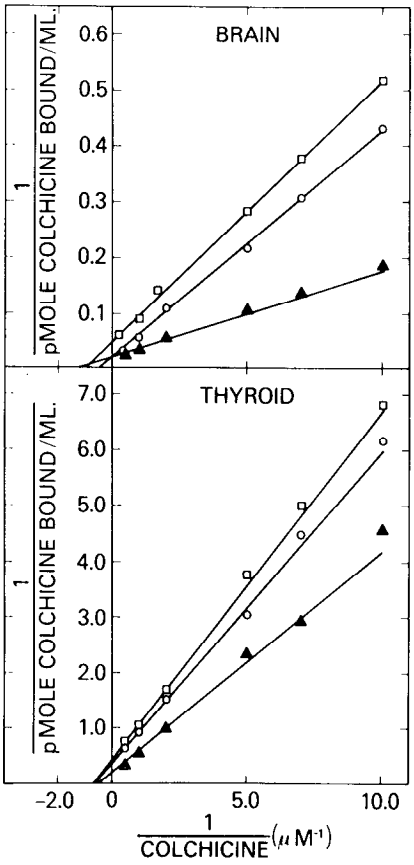

FIG. 4. Lineweaver-Burk plots of the binding of [^{3}H]colchicine to tubulin. For brain the protein concentrations were 200 μg, 1.05 mg, and 220 μg/ml for soluble (▲——▲), membrane-bound (□——□), and solubilized (○——○) colchicine-binding protein, respectively. For thyroid these values were 240 μg, 1.31 mg, and 248 μg/ml for soluble, membrane-bound, and solubilized protein, respectively. Samples were incubated at 37° for 1.5 hours.

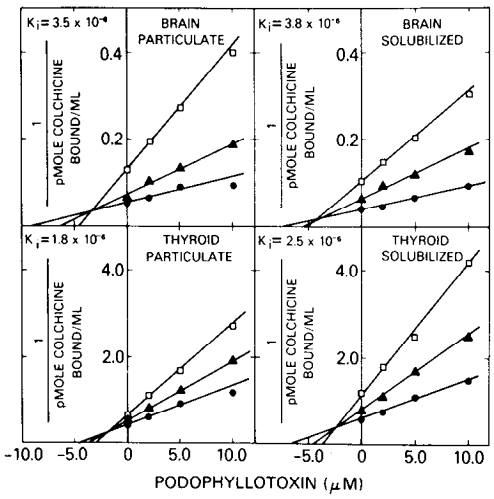

FIG. 5. Modified Dixon plot demonstrating the effect of podophyllotoxin on the colchicine-binding reaction. The concentration of podophyllotoxin present is plotted against the reciprocal of bound colchicine at several concentrations of colchicine: □——□, 1.0 μM; ▲——▲, 2.2 μM; ●——●, 3.5 μM. The protein concentrations were 1.1 mg and 242 μg/ml for particulate and solubilized protein in the case of brain and 1.31 mg and 252 μg/ml for thyroid. All samples were in 0.25 M sucrose/10 mM sodium phosphate (pH 7.0)/10 mM $MgCl_2$/0.1 mM GTP buffer, incubated at 37° for 1.5 hours.

TABLE IV

Distribution of vinblastine-binding activity in different tissue fractions

Aliquots of each tissue fraction for both brain and thyroid were incubated with 1×10^{-5} M [^{3}H]vinblastine for 1 hour at 37° and the bound vinblastine was determined by filter disc method as used in case of colchicine-binding (17). Protein concentrations are 500 μg/ml in all fractions for brain and 250 μg/ml for each fraction in thyroid.

Tissue fraction at interface	Specific activity	
	Brain	Thyroid
	pmol/mg	
Sucrose		
30%	2.2	5.4
40%	17.4	26.1
45%	13.8	22.1
Pellet	2.0	8.3

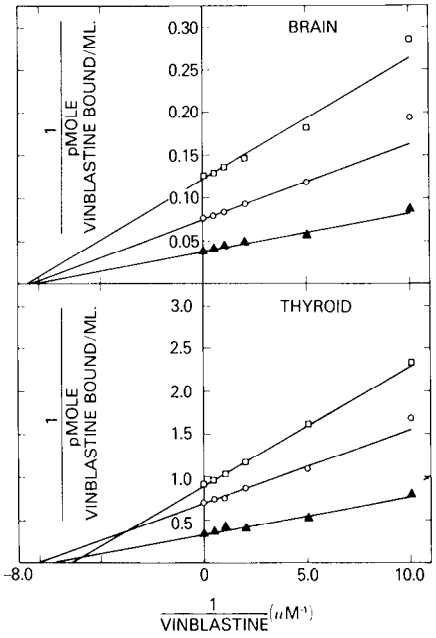

FIG. 6. Lineweaver-Burk plots of the binding of [^{3}H]vinblastine to tubulin. In brain the protein concentrations were 200 μg, 1.05 mg, and 220 μg/ml for soluble (▲——▲), membrane-bound (□——□), and solubilized (○——○) tubulin. For thyroid these values were 210, 200, and 248 μg/ml in case of soluble, membrane-bound, and solubilized tubulin. Samples were incubated at 37° for 1 hour and bound vinblastine was determined by the same filter disc method as used in the case of colchicine binding (17).

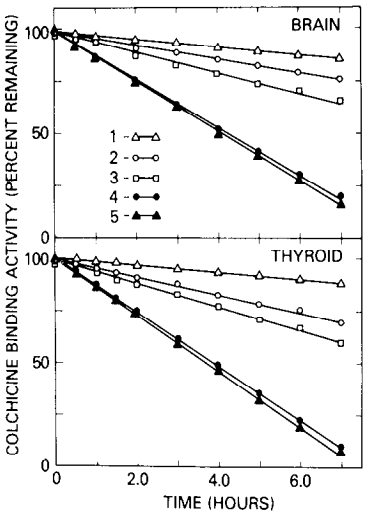

FIG. 7. Loss of [^{3}H]colchicine binding of soluble tubulin, membrane-bound, and solubilized colchicine-binding protein from rat brain and beef thyroid membranes as a function of incubation time at 37° and conditions of incubation. Conditions for both brain and thyroid are: *Curve 1* (△——△), soluble tubulin in 10 mM sodium phosphate (pH 7.0)/10 mM $MgCl_2$/0.1 mM GTP solution (PMG buffer) containing 1 M sucrose; *Curve 2* (○——○), soluble tubulin in PMG buffer containing 1×10^{-4} M vinblastine; *Curve 3* (□——□), membrane-bound colchicine-binding protein in PMG buffer; *Curve 4* (●——●), soluble tubulin in PMG buffer; and *Curve 5* (▲——▲), solubilized colchicine-binding protein in PMG buffer. Protein concentrations are: 240 μg/ml for *Curves 1, 2,* and *4* in the case of brain and 262 μg/ml in thyroid; *Curve 3,* 1.1 mg/ml for brain and 1.25 mg/ml for thyroid; *Curve 5,* 205 μg/ml for brain and 282 μg/ml for thyroid. Samples were preincubated at 37° and the colchicine-binding activity was measured for 1.5 hour at 37° at the end of the preincubation period.

temperature optimum for colchicine binding (37°) as does soluble tubulin. Fig. 8 also shows that the temperature optimum for soluble tubulin changes to higher temperatures in the presence of stabilizing agents such as sucrose and vinblastine. This close parallelism between the sucrose and vinblastine effects on the one hand, and membrane binding on the other, with respect to the temperature optimum for colchicine binding, suggests that the colchicine-binding site is similarly protected in all cases.

Tubulin Antibodies—An additional method for comparing soluble and membrane-bound tubulin was provided by use of antibodies directed against soluble rat brain tubulin. Although antiserum titers were relatively low, they permitted identification of cross reactivity of membrane-bound and solubilized material with soluble brain tubulin.

To determine the optimal dilution of antiserum for the radioimmunoassay, the ^{125}I-tubulin bound to antibody at various dilutions of antiserum was measured (Fig. 9). A dilution of 1/100 precipitated about 50 to 60% of the labeled tubulin. Only 8% of the label was precipitated in this double antibody technique when nonimmune serum was used, and 5% when buffer alone was used. Using this dilution of antiserum, various amounts of unlabeled tubulin were added to the assay mixture, and free and bound radioactivity were determined. Unlabeled tubulin competed with the iodinated tubulin and prevented the latter from binding to antibody (Fig. 10). Membrane-bound colchicine-binding protein and the solubilized protein from brain and thyroid membranes displaced label in the same fashion as did the soluble tubulin. It is interesting that anti-brain tubulin antibody binds to membrane-bound colchicine-binding protein from both brain and thyroid, showing that there is little species or tissue specificity, in agreement with earlier studies (33, 34).

DISCUSSION

In the present study we report the presence of substantial amounts of particulate colchicine-binding activity in brain and thyroid tissue. Brain contains about twice the proportion of the total activity in the particulate form as thyroid. These findings on brain are in general agreement with earlier reports (10, 12, 28, 35, 36). The subcellular distribution of colchicine-binding activity has revealed the highest binding to be associated with the synaptosome fraction, which, in our procedure, would also contain membranes derived by sonication (10, 12, 37). An additional site for colchicine binding has been found in the nuclear membrane of liver cells and *Lilium* meiocytes (13, 38, 39). However, in contrast to soluble tubulin, the liver membrane binds lumicolchicine as well as colchicine. It seems probable that such binding is nonspecific since lumicolchicine binding in brain was not displaced by colchicine or podophyllotoxin and was not temperature-dependent (32). In the present study, direct isolation of membranes has shown that enrichment for adenylate cyclase is accompanied by enrichment in colchicine-binding activity. This suggests that the plasma membrane is a major contributor to particulate colchicine binding (Table I, Fig. 1).

Characterization of the membrane-bound colchicine-binding activity can be accomplished by detailed kinetic and analogue studies of colchicine binding, by various forms of chemical characterization of protein extracted from membranes, or by immunological techniques. To our knowledge, the present report is the first where all of the approaches have been combined, thus leading to considerably greater confidence in the identification of the membrane-bound colchicine-binding activity.

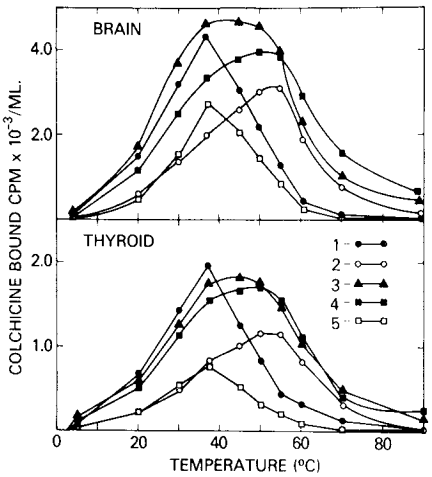

FIG. 8. Determination of the optimum temperature for colchicine binding in different incubation conditions. Incubation conditions for both thyroid and brain are: *Curve 1* (●——●), soluble tubulin in 10 mM sodium phosphate (pH 7.0)/10 mM $MgCl_2$/0.1 mM GTP solution (PMG buffer); *Curve 2* (○——○), membrane-bound colchicine-binding protein in PMG buffer; *Curve 3* (▲——▲), soluble tubulin in PMG buffer containing 1×10^{-4} M vinblastine; *Curve 4* (■——■), soluble tubulin in PMG buffer containing 1 M sucrose; and *Curve 5* (□——□), solubilized colchicine-binding protein in PMG buffer. Protein concentrations are: *Curve 1*, 280 μg/ml; *Curve 2*, 700 μg/ml; *Curve 3*, 180 μg/ml; *Curve 4*, 253 μg/ml; and *Curve 5*, 193 μg/ml for brain tissue. For thyroid these values are: *Curve 1*, 1.4 mg/ml; *Curve 2*, 2.8 mg/ml; *Curve 3*, 1.20 mg/ml; *Curve 4*, 1.20 mg/ml; and *Curve 5*, 480 μg/ml. Samples were incubated at the designated temperatures for 1.5 hours.

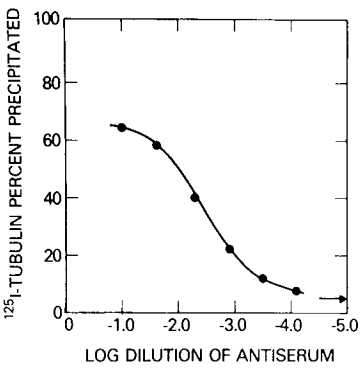

FIG. 9. Standard curve for the antigen-antibody reaction between ^{125}I-tubulin and antitubulin antiserum. The per cent of ^{125}I-tubulin bound to antibody is plotted against the log of the dilution of antiserum in the assay mixture. ^{125}I-tubulin (approximately 5000 cpm in 10 μl) was incubated with 0.1 ml of several dilutions of antiserum. After 1 hour at 37° and 12 hours at 4°, 0.1 ml of a 1:100 dilution of normal rabbit serum was added, followed immediately by 0.2 ml of undiluted sheep anti-rabbit γ-globulin. After 12 hours further incubation at 4°, free and antibody-bound ^{125}I-tubulin were separated by centrifugation and counted.

Affinity constants for the binding of colchicine, vinblastine, or podophyllotoxin (as K_i) to membranes have not, heretofore, been measured. As shown in Figs. 4 to 6, the affinity was the same for soluble tubulin, purified membranes, and binding protein solublized from such membranes. This was true for both rat brain and beef thyroid tissue. Analogues of colchicine acted equally on colchicine-binding protein from all three sources. Thus colcemid and colchiceine showed activity against colchicine binding by membranes, whereas colchicoside and isocolchicine were inactive, as is the case with soluble tubulin (27, 29). For both brain and thyroid tissue, podophyllotoxin yielded K_i values that did not vary with the source of the

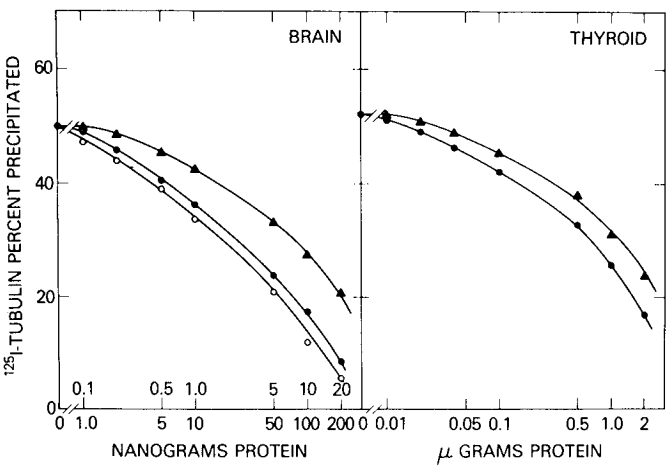

FIG. 10. Displacement of ^{125}I-tubulin from antibody by unlabeled colchicine-binding protein. The radioimmunoassay was performed using an antiserum dilution of 1:100. A 10-μl aliquot of soluble tubulin (○——○), membrane-bound (▲——▲), and solubilized (●——●) colchicine-binding protein was added to the reaction mixture as in Fig. 9. Note that for soluble brain tubulin only, a different range of concentration is shown above the *abscissa*.

binding activity. Bamburg *et al.* (32) also have shown that podophyllotoxin inhibits binding of colchicine by chick brain particulate fractions. Finally, while vinblastine appears to be a less specific agent for tubulin than colchicine (40), it is nevertheless a useful adjunct in the identification of tubulin-like proteins. Affinity for vinblastine was the same for soluble, membrane, and solubilized protein. On the basis of binding criteria, therefore, the colchicine-binding activity of brain or thyroid membranes, and the solubilized protein derived from these membranes, resemble soluble tubulin in every respect so far examined.

The chemical characterization of membrane colchicine-binding activity has frequently required extraction procedures that destroyed binding activity. Thus Feit *et al.* (37) showed that membranes contained a protein of the same electrophoretic mobility (in polyacrylamide gels) as soluble tubulin. This protein also shows considerable similarity in peptide maps, although not all peptides of pure tubulin were demonstrated in membrane-derived material (41). Following the procedure of Hotta and Shepard (39) we were able to treat brain and thyroid membranes with detergents that extracted only a few proteins. Among these was nearly all of the colchicine-binding activity. One of the major protein bands of this extract showed a molecular weight of 55,000 in disc gel electrophoresis, *i.e.* like the subunit molecular weight of soluble tubulin from these tissues (Fig. 2). While it is not yet possible to show coincidence of this protein band with the colchicine-binding activity of the extracts, the appearance of both of these properties of tubulin in the membrane extract supports the suggestion that the binding activity of the membranes is, in fact, tubulin.

Additional evidence for the tubulin-like nature of the membrane-bound colchicine-binding activity was provided by immunologic means. It had been shown previously that brain particulates showed the best complement fixation reaction with antibodies against soluble tubulin (42). As shown in the present study (Figs. 9 and 10), both brain and thyroid membranes, and the solubilized colchicine-binding activity, precipitated with anti-brain tubulin in a double antibody technique. This again suggests the considerable similarity

between soluble and membrane colchicine-binding activity.

Certain apparent differences have been observed between the behavior of membrane and soluble tubulin that merit attention. Thus membrane tubulin is far more stable than soluble tubulin and exhibits a colchicine-binding optimum near 54°. That this probably results from the bound state and the resultant change in the environment of the protein is demonstrated by two observations. (*a*) When tubulin is extracted from either brain or thyroid membranes its stability characteristics revert to that of soluble tubulin. (*b*) If soluble tubulin is kept in 1.0 M sucrose or vinblastine, its stability is very near that of membrane tubulin. We believe, on the basis of this evidence, that the stability difference is not an intrinsic property of the protein in the membrane but rather of its environment. The change in thermal stability of membrane tubulin also argues against simple entrapment of cytoplasmic tubulin inside membrane vesicles. Therefore, on the basis of binding, chemical, and immunological properties, we conclude that the colchicine-binding activity of membranes is very similar to, if not identical with, soluble tublulin.

An important consequence of the membrane locus for tubulin is that while colchicine effects on various cellular processes, such as secretion (1), may be specific for tubulin, they are not necessarily specific for microtubules. Since such secretory processes as endo- and exocytosis intimately involve membranes, the interpretation of colchicine effects as operating through the participation of microtubules cannot be made unambiguously, even when the structure of microtubules is seen to be altered. Similar considerations apply to the effects of colchicine on the mobility of membrane surface constituents (43, 44) and they may be pertinent to the effects of this drug on transport processes (45). By what means colchicine binding to membrane tubulin may alter the function of such membranes remains to be determined.

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