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Isolation of Microtubule Protein from Cultured Mouse Neuroblastoma Cells

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Abstract. The addition of vinblastine to high-speed supernatants derived from homogenates of cultured mouse neuroblastoma cells results in the formation of a precipitate which has been characterized as microtubule protein by the following criteria: colchicine-binding activity, molecular weight, amino acid composition, and electrophoretic mobility. The method therefore permits the rapid isolation of microtubule protein from crude supernatants of neuroblastoma cells.

Recent work has shown that the application of vinblastine or vincristine to various cell types results in the breakdown of microtubules into filaments and the formation of large cytoplasmic crystals which appear to be composed of microtubule protein and bound vinblastine.[1–7] Since vinblastine caused this crystallization of microtubule protein *in vivo*, it seemed reasonable to determine if this drug would also cause the aggregation of microtubule protein *in vitro*.[7, 8] In this report, we describe studies in which the treatment of high-speed supernatants of microtubule-rich neuroblastoma cells with vinblastine causes the precipitation of colchicine-binding protein. In addition to a high specific activity of colchicine binding, the precipitate has been characterized as microtubule protein by its molecular weight, amino acid composition, and electrophoretic mobility similar to that of microtubule protein isolated from *Chlamydomonas* flagella.

Materials and Methods. Cell culture: A murine neuroblastoma tumor line was adapted to *in vitro* culture in November 1967, and was maintained in either suspension or monolayer culture.[9]

Electron microscopy: Electron microscopy of the cultured cells was carried out using the fixation, flat-embedding, and staining procedures described by Brinkley *et al.*[10]

Cell fractionation: Cells from suspension cultures were harvested by low-speed centrifugation at room temperature and were washed once with ST (0.24 *M* sucrose, 0.01 *M* Tris, pH 7.0); all succeeding operations were performed at 4°C. Cells, 3×10^8, were homogenized in 3.0 ml ST or ST with 20 mM $MgCl_2$ (SMT), centrifuged at $2500 \times g$ for 10 min, the pellet discarded, and the supernatant centrifuged at $35,000 \times g$ for 30 min. The resulting supernatant was centrifuged at $150,000 \times g$ for 90 min, and the $150,000 \times g$ supernatant used for the isolation of microtubule protein.

Precipitation of microtubule protein with vinblastine: Vinblastine[11] was added to the $150,000 \times g$ supernatant to a final concentration of 2×10^{-3} *M*. If cell homogenization was carried out in ST, $MgCl_2$ was added to a final concentration of 2.5 mM following the addition of a vinblastine. The solution became turbid immediately but

was allowed to incubate at 4°C for at least 30 min; the precipitate was then sedimented at 35,000 × *g* for 30 min. The supernatant was removed and the pellet resuspended in 1.0 ml of SMT or SGT (ST with 0.1 mM GTP) to a final protein concentration of 1.5 to 2.0 mg/ml. Reprecipitation for further purification of the protein could be carried out by repeating the procedure described above.

Assay for microtubule protein: The colchicine-binding assay for microtubule protein was carried out according to the method of Borisy and Taylor.[12] The ^{3}H-colchicine had a specific activity of 2.54 Ci/mM (New England Nuclear). The assay was optimized for ^{3}H-colchicine concentration (5×10^{-6} *M*), protein concentration (about 1.5 mg/ml), and incubation time (1.5 hr at 37°C). Protein-bound ^{3}H-colchicine was separated from free ^{3}H-colchicine either on Sephadex G-50 columns or DEAE Whatman DE 81 filter disks.[13] The elution buffer used for both procedures was 0.01 *M* Na phosphate buffer, pH 7.0. Radioactivity in the column fractions or on disks was determined by liquid scintillation counting in Triton X-100 fluor[14] and protein was assayed by the Lowry method.[15]

Acrylamide gel electrophoresis: Protein samples were reduced and alkylated by standard procedures.[16] Electrophoresis was carried out on 7.5% acrylamide gels containing 8.0 *M* urea (6 × 70 mm, 3 ma/gel, pH 8.3 at room temperature). Gels were stained with fast green for quantitative determination of protein.[17] Counts on the gels were analyzed following the procedures of Gray and Steffensen.[18] Molecular weight determinations on the microtubule protein were carried out using sodium dodecyl sulfate (SDS) acrylamide gel electrophoresis as described by Shapiro *et al.*[19]

Amino acid analysis: Twice-precipitated microtubule protein was dialyzed against water overnight and lyophilized. The protein was dissolved in a minimum volume of 0.02 *N* NaOH, made to 6 *N* HCl with constant boiling HCl, and hydrolyzed at 110°C for 24, 48, 72, and 96 hr in sealed, evacuated tubes. Amino acid analyses were performed according to the procedures of Spackman *et al.*[20]

G-200 Sephadex columns: Cells were labeled for at least 20 hr in culture medium containing 0.15 μc/ml of ^{14}C-amino acid mixture. The ^{14}C-labeled microtubule protein was isolated by vinblastine precipitation in the absence of $MgCl_2$ and was chromatographed with appropriate molecular weight markers on 80 × 1.5 cm G-200 Sephadex columns equilibrated with 0.01 *M* Na phosphate buffer containing 0.1 mM GTP (pH 7.0).

Flagellar microtubule protein: *Chlamydomonas* flagellar outer fiber protein was used as a reference microtubule protein for acrylamide gel electrophoresis. Methods of isolating and fractionating flagellar axonemes from *Chlamydomonas* will be described elsewhere.[21]

Results. Cell culture: Neuroblastoma cells grown in suspension cultures are rounded and fine structural analysis indicates that they contain few microtubules and filaments. Cells transferred to monolayer cultures will rapidly adhere to the substrate and extend long, branching processes (neurites). These extensions are birefringent (Fig. 1*A*) and electron micrographs show them to contain numerous microtubules (Fig. 1*B*). When monolayer cultures are incubated for two hours in the presence of 10^{-6} *M* vinblastine, large bundles of aligned 50–60 Å filaments are formed in the cell cytoplasm and in the neurites (Fig. 1*C*); these are similar to filaments described by others in vinblastine-treated cells.[1–3] Treatment of the monolayered neuroblastoma cells with 10^{-5} *M* vinblastine results in the appearance within 30 minutes of crystals which are birefringent by polarization microscopy and which have a lattice spacing of 280 to 300 Å (Fig. 1*D*). These crystals have been previously observed and are thought to be composed of microtubule protein on the basis of ultrastructural analysis.[3, 4]

Isolation and characterization of microtubule protein: It has been shown that

^{3}H-colchicine binding can be used as an assay for microtubule protein.[12] In neuroblastoma cell homogenates, the majority of colchicine-binding protein is found in the 150,000 × *g* supernatant and the specific activity of colchicine binding in this supernatant is 35 times that of the total homogenate. When vinblastine is added to the 150,000 × *g* supernatant of cells homogenized in ST, the

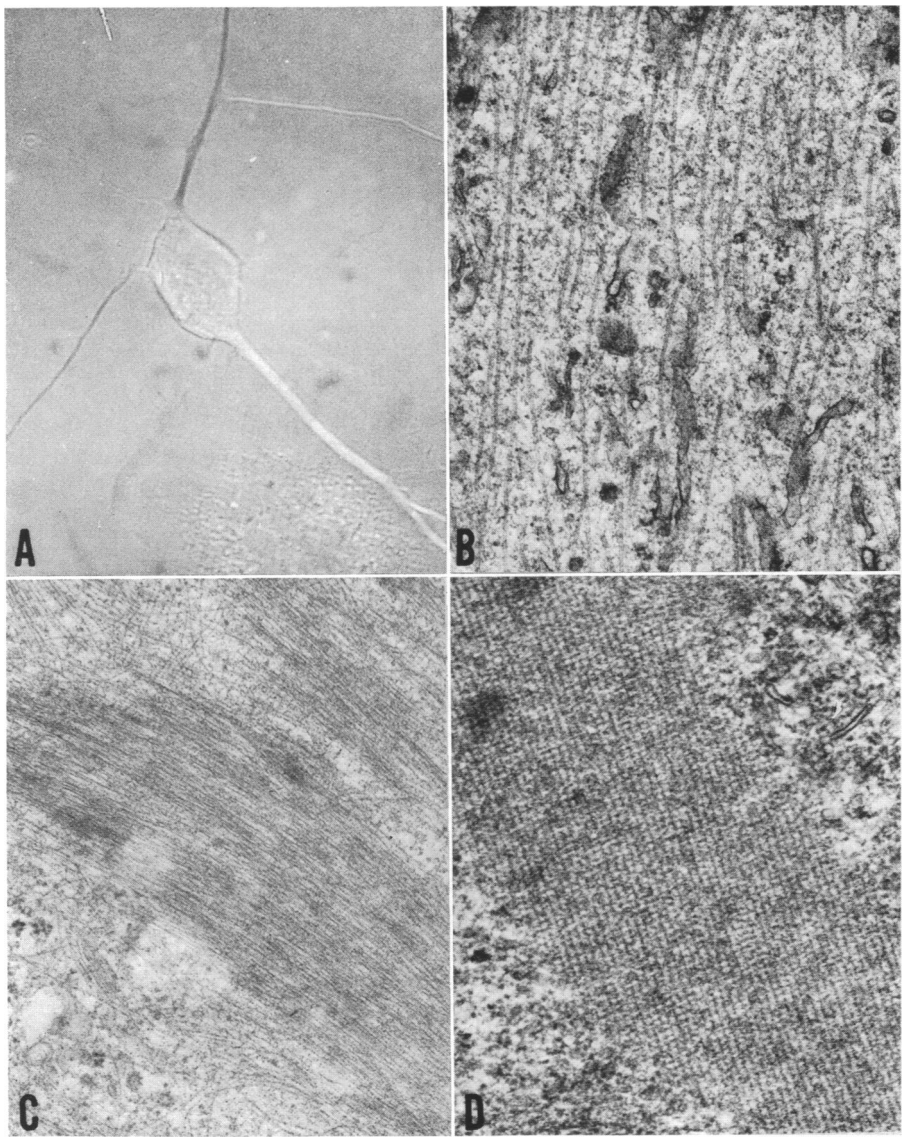

FIG. 1.—Micrographs of control and vinblastine-treated neuroblastoma cells. (*A*) polarization micrograph showing birefringence in neurites of control cell (×570); (*B*) neurite of control cell with large number of microtubules (×29,300); (*C*) bundles of 50 to 60 Å filaments in cytoplasm after treatment with 10^{-6} *M* vinblastine for 2 hr (×31,200); and (*D*) crystals formed after treatment with 10^{-5} *M* vinblastine for 2 hr (×38,500).

precipitate which forms represents 10–12 per cent of the total 150,000 × *g* supernatant protein. Regardless of whether or not $MgCl_2$ is included in the homogenization medium, about 65 per cent of the total colchicine-binding activity of the 150,000 × *g* supernatant is sedimented at 35,000 × *g* after vinblastine precipitation (see *Discussion*); the supernatant remaining after the removal of the precipitated protein contains very low levels of binding (Fig. 2). Using ^3H-

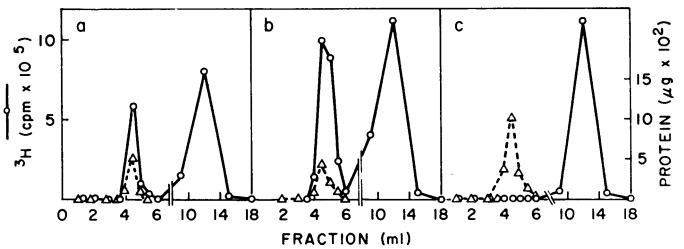

FIG. 2.—Sephadex G-50 chromatography showing ^3H-colchicine binding to neuroblastoma 150,000 × *g* supernatant and vinblastine precipitate. (*a*) 150,000 × *g* supernatant; (*b*) vinblastine-precipitated protein; and (*c*) supernatant remaining after removal of vinblastine-precipitated protein.

colchicine at the same specific activity (2.5 Ci/mM) as was used by Weisenberg *et al.*,[13] we obtain a specific activity of colchicine binding for the resuspended vinblastine-precipitated protein of 2.0 to 3.0 × 10^6 cpm/mg (5.0–7.5 × 10^6dpm/mg) as compared to 1.2 × 10^6 cpm/mg for their extensively purified microtubule protein from brain (Table 1).[13] Calculated from the number of counts bound, our specific activity represents 0.1–0.2 moles colchicine bound per 110,000 *g* of the vinblastine-precipitated protein.

TABLE 1. *Specific activity of colchicine binding of neuroblastoma fractions.*

Fraction	Specific activity (cpm × 10^5/mg protein)
150,000 × *g* supernatant	8.25
Vinblastine-precipitated protein	28.40
Supernatant after removal of vinblastine-precipitated protein	0.074
Purified brain microtubule protein[13]	12.6

Neuroblastoma protein fractions obtained from cells homogenized in ST.

Polyacrylamide gel electrophoresis of the 150,000 × *g* supernatant shows that many proteins are present of which one is prominent. After treatment with vinblastine, this prominent band is found entirely in the vinblastine-precipitated fraction and is missing from the vinblastine-treated supernatant (Fig. 3). In addition, the isolated protein has an electrophoretic mobility similar to that of the faster moving of the two proteins characteristic of *Chlamydomonas* flagellar outer fiber microtubule protein (Fig. 4). When electrophoresis of ^{14}C-labeled vinblastine-precipitated protein is carried out with unlabeled flagellar outer fiber protein, the counts are also found to migrate with the faster moving band. Molecular weight determinations on SDS acrylamide gels show that both vinblastine-

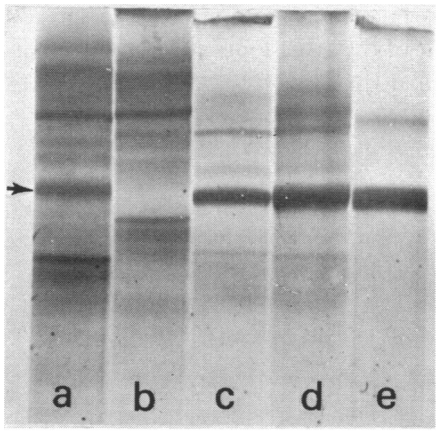

FIG. 3.

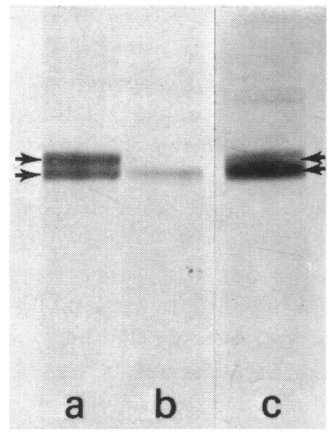

FIG. 4.

FIG. 3.—Urea-acrylamide gels of neuroblastoma proteins and *Chlamydomonas* flagellar outer fiber microtubule protein; fast green stain. *Arrow*: microtubule protein band. (*a*) 150,000 × *g* supernatant before vinblastine treatment; (*b*) supernatant after removal of vinblastine-precipitated protein; (*c*) 1X-vinblastine-precipitated microtubule protein; (*d*) flagellar outer fibers + 1X vinblastine-precipitated protein; and (*e*) flagellar outer fibers.

FIG. 4.—Urea-acrylamide gels of vinblastine-precipitated protein and *Chlamydomonas* flagellar outer-fiber microtubule protein. Double arrows indicate double band characteristic of flagellar outer fibers. (*a*) *Chlamydomonas* flagellar outer fibers; (*b*) 2X-vinblastine-precipitated microtubule protein; (*c*) flagellar outer fiber protein + 2X-vinblastine-precipitated protein showing migration of vinblastine-precipitated protein with the faster moving band. NOTE: When ^{14}C-labeled vinblastine-precipitated protein is run with unlabeled outer fiber microtubule protein, the counts also migrate with the faster moving of the two bands. The double band has not been seen in vinblastine-precipitated microtubule protein of neuroblastoma cells, although it has been reported in some polyacrylamide gels of purified microtubule protein from brain.[13]

precipitated protein from neuroblastoma cells and *Chlamydomonas* outer fiber microtubule protein have electrophoretic migrations corresponding to a subunit molecular weight of about 55,000 (Fig. 5).

Vinblastine-precipitated, ^{14}C-labeled microtubule protein chromatographed on G-200 Sephadex appears in the void volume of the column and is probably an aggregate with a molecular weight higher than 250,000. This aggregate is still present even after the vinblastine-precipitated protein suspended in SGT is dialyzed for 30 hours against SGT.

The amino acid composition of the vinblastine-precipitated protein can be seen to be very similar to the analyses of microtubule protein from several

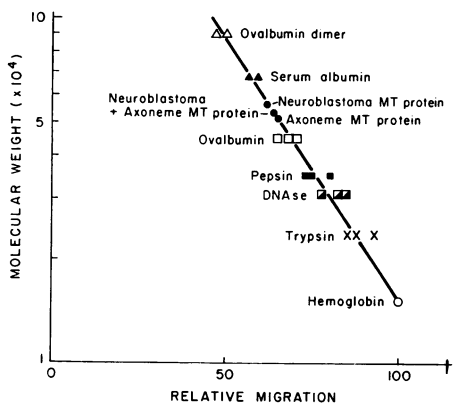

FIG. 5.—SDS gel electrophoresis determination of monomer molecular weight. Microtubule (MT) protein isolated from *Chlamydomonas* flagellar outer fibers; neuroblastoma microtubule (MT) protein isolated by vinblastine precipitation.

sources (Table 2, Fig. 6).[13, 16, 22, 23] Leucine is the only amino acid which differs by more than 10 per cent from at least one of the other four microtubule proteins.

Discussion. Recent reports have shown that the addition of vinca alkaloids to various cells causes the formation of crystals which appear to be composed of microtubule protein.[3–6] Our work on the ultrastructure of vinblastine-treated neuroblastoma cells supports these observations and shows that vinblastine causes the breakdown of microtubules into 50–60 Å filaments, the massing of filaments into bundles, and the formation of crystals.[7] A similar process of crystallization may be occurring *in vitro* since the addition of vinblastine to high-speed supernatants from rabbit brain has been shown to cause the formation of lattice structures resembling those found in cells treated with vinblastine.[8] Our work and that of Marantz *et al.*[8] indicates that the protein which is precipitated by vinblastine treatment has colchicine-binding activity. In addition, we have shown in this report that the precipitate is microtubule protein on the basis of having the same electrophoretic mobility as flagellar outer fiber protein, a subunit molecular weight of around 55,000, and an amino acid composition similar to microtubule protein isolated from several sources.

A preliminary report by Weisenberg and Timasheff[24] has shown that Mg^{+2} is significant in the vinblastine precipitation of purified microtubule protein from brain and that Mg^{+2} concentration influences the size of the aggregates formed. We have found that precipitation of microtubule protein from 150,000 × *g* supernatants of neuroblastoma cells can occur without added $MgCl_2$. The vinblastine-precipitated microtubule protein resuspended in SGT is excluded from G-200 Sephadex even after extensive dialysis in the absence of Mg^{+2}; this indicates that the colchicine is binding to a high molecular weight aggregate. Although this aggregate is calculated to bind up to 0.1 mole of colchicine per 110,000 gm protein, it is not known what influence the presence of bound vinblastine might have on its colchicine binding activity. For example, it has been

TABLE 2. *Amino acid composition.*

Amino acid	Mole Percentage: Vinblastine-precipitated protein	Mole Percentage: Sea urchin sperm outer fibers[23]
Lysine	6.45	5.70
Histidine	2.38	2.53
Arginine	5.57	5.58
Aspartic acid	10.10	10.57
Threonine*	6.35	6.21
Serine*	5.45	5.54
Glutamic acid	13.37	13.38
Proline	5.17	5.03
Glycine	8.17	7.36
Alanine	8.07	7.54
Half Cysteine	1.78	1.66
Valine	6.64	6.53
Methionine	2.33	3.12
Isoleucine	5.02	4.81
Leucine	8.70	7.97
Tyrosine	3.01	3.38
Phenylalanine	4.04	4.27

Mole percentage for vinblastine-precipitated protein represents averages of duplicate sets of 24-, 48-, and 72-hour hydrolysates.

* Serine and threonine were determined by extrapolation to zero hydrolysis time.

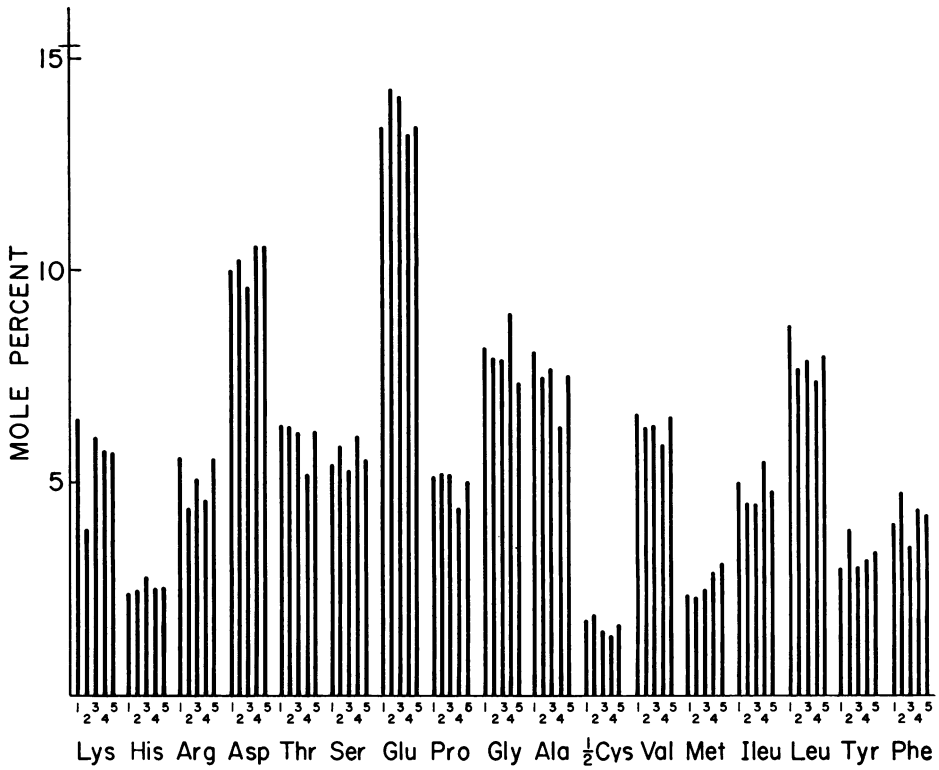

FIG. 6.—Amino acid composition of microtubule protein from several sources. (*1*) Vinblastine-precipitated protein from neuroblastoma cells; (*2*) purified brain microtubule protein;[13] (*3*) central pair microtubule protein from sea urchin sperm;[22] (*4*) outer fiber protein from *Tetrahymena* cilia;[16] and (*5*) outer fiber microtubule protein from sea urchin sperm.[23] Mole percentages for 4 and 5 were calculated from the number of residues reported in the respective papers.

found that vinblastine may stabilize[25] or enhance[26] the binding of colchicine to high-speed supernatants. Our work shows that increasing concentrations of vinblastine do not inhibit colchicine binding to 150,000 × *g* supernatants.[7] These results suggest that vinblastine and colchicine are not binding at the same site.

When colchicine binding is carried out on the microtubule protein after precipitation with vinblastine and sedimentation at 35,000 × *g*, only 65–70 per cent of the total binding activity of the 150,000 × *g* supernatant is detected in the precipitate. However, only a small percentage (5–10%) of the remaining total binding activity can be found in the supernatant from which the precipitate is taken. On the other hand, if colchicine is bound to the 150,000 × *g* supernatant *prior* to vinblastine precipitation of the protein (in the presence or absence of 2.5 mM $MgCl_2$), and the protein is then sedimented by centrifugation, 90–95 per cent of the binding activity of the original supernatant is removed (*cf.* Marantz *et al.*[8] for similar results). These results suggest that some colchicine-binding sites may become masked during vinblastine precipitation.

We have found that the presence of GTP and/or $MgCl_2$ stabilizes colchicine binding to vinblastine-precipitated protein.[7] GTP stabilization has previously

been reported for microtubule protein isolated from brain.[13] When vinblastine-precipitated protein is resuspended in ST, bound to ^{3}H-colchicine, and placed in the cold for 24 hours, 85 per cent of the original binding activity is retained in the presence of GTP and/or Mg, whereas only 50 per cent of the activity remains in the absence of either or both of these factors. Although we have been unable to detect the presence of bound guanine nucleotides on vinblastine-precipitated protein, the protein will bind some ^{3}H-GTP *in vitro*.[7]

The results presented in this paper indicate that neuroblastoma cells are exceedingly rich in microtubule protein, and that vinblastine can be used to isolate this protein from high-speed supernatants of these cells. The ease and rapidity of this method should facilitate further studies on the mechanism of action of vinblastine, the kinetics of GTP binding to isolated protein, the turnover of microtubules *in vivo*, and the synthesis and assembly of microtubule protein in both *in vivo* and *in vitro* systems.

Note: It has been found that some other proteins can be quantitatively precipitated with vinblastine,[25] although the results in this report show that vinblastine causes an almost exclusive precipitation of the microtubule protein of neuroblastoma cells.

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