TUBULIN CONSTANCY DURING MORPHOLOGICAL DIFFERENTIATION OF MOUSE NEUROBLASTOMA CELLS

JANET L. MORGAN and NICHOLAS W. SEEDS

From the Department of Biophysics and Genetics and the Department of Psychiatry, University of Colorado Medical Center, Denver, Colorado 80220

ABSTRACT

Clonal cell lines N18 and N103 of the mouse neuroblastoma C1300 possess an undifferentiated neuroblast morphology under optimal growth conditions; however, when deprived of serum, N18 can be induced to extend long neurites. Although initial neurite outgrowth is rapid, very long fibers are found only after several days. Both initial outgrowths and established neurites contain microtubules; however, the number and density of these polymerized tubules increase markedly during this time. Optimum conditions have been established for assessing the colchicine-binding activity of neuroblastoma sonicates. A time-decay colchicine-binding assay was used to make a comparative study of the tubulin content of both undifferentiated and differentiated N18 as well as the nondifferentiating N103 and the rat glioma C6. Both morphologies of clone N18 possessed similar concentrations of tubulin (130–140 pmol/10^6 cells). Although cells of clone N103 contain 20% less tubulin than N18 cells, this is considerably more tubulin than is present in the glioma C6 (30 pmol/10^6 cells) which has a similar generation time. Quantitative densitometry of neuroblastoma extracts electrophoresed on SDS-polyacrylamide gels confirmed the constancy of tubulin. Radiolabeled proteins from neuroblastoma cells subjected to both growth conditions show that neurite outgrowth does not create a disproportionate demand for tubulin synthesis. Thus, the morphological differentiation of neuroblastoma cells probably reflects the regulation of tubulin storage and microtubule polymerization.

The extension of axons is a characteristic feature of nerve cell differentiation. Axonal outgrowth is accompanied by the elaboration of many long, well organized microtubules oriented parallel to the axonal axis. These microtubules are thought to function as a cytoskeleton (12, 14) since dissolution of microtubules by drugs such as colchicine and vinblastine prevents axon outgrowth and promotes axon retraction (6, 22). However, the precise role that these structures and their protein subunit, tubulin, play in nerve cell differentiation is not clear.

Most studies of the role of microtubules in axon outgrowth have utilized ganglia preparations (7, 18) containing mixed cell types. Several years ago we described a homogeneous cell system of clonal cell lines from the mouse neuroblastoma C1300, with which entire populations could be rapidly induced to extend axonlike outgrowths (16). These outgrowths, which for the lack of functional correlates have been called neurites, contain many microtubules (13, 15). Neurite formation occurred within minutes and was cold sensitive, and inhibited by low concentrations of colchicine and vinblastine (16). Furthermore, initial neurite outgrowth (0–6 h) was independent of de novo protein

synthesis, suggesting that a pool of preformed tubulin subunits was available (16).

After several days these neuroblastoma cells possessed very long neurites often exceeding 1 mm in length; however, it was not known whether the formation of these very long neurites required a significant increase in the tubulin pools. Therefore, a comparison of tubulin levels in these two distinct morphologies of neuroblastoma was initiated, and the results of this study are presented here. A brief communication of these results has appeared (10).

MATERIALS AND METHODS

Clonal cell lines of mouse neuroblastoma, N18 and N103, have been described by Seeds et al. (16). The rat glioma clonal cell line, C6, was obtained from the American Type Culture Collection (Rockville, Md.) and was described by Benda et al. (3). Cells were grown in plastic tissue culture dishes (Falcon Plastics, Division of Bioquest, Oxnard, Calif.) containing Dulbecco's modification of Eagle's medium (DMEM) (Grand Island Biological Co., Grand Island, N.Y.) and fetal calf serum (Reheis Co., Inc., Berkley Heights, N.J.) in an incubator at 37°C with an atmosphere of 10% CO_2 and 90% air.

For determination of the amount of DNA and protein/cell, neuroblastoma clones N103 and N18 without neurites and glioma C6 were grown in DMEM plus 10% fetal calf serum while N18 with neurites were cultured 4 days in DMEM with 0.2% fetal calf serum. Differentiated and undifferentiated N18, N103, and C6 were grown on 60-mm intergrid plastic tissue culture dishes. Cells were washed twice with saline I (0.138 M NaCl, 5.4 mM KCl, 1.1 mM Na_2HPO_4, 1.1 mM KH_2PO_4) plus 0.001 M $CaCl_2$. Cell counts (>1,000/plate) were performed by microscope observation of quadruplicate plates for each cell type, and the number of cells per plate was determined from the number of cells per unit area. Then 1 ml of 0.01 M NaOH was added to each plate to dissolve the cells. The amount of protein per plate was determined by the method of Lowry et al. (9). The amount of DNA per plate was determined by the method of Burton (5). The amounts of protein and DNA per cell were calculated to be 470 pg (±10%) and 41 (±12%), respectively, for N18 cells with neurites, 480 pg (±9%) and 65 pg (±18%) for N18 without neurites, 480 pg (±5%) and 57 pg (±8%) for N103, and 180 pg (±5%) and 19 pg (±9%) per C6 cell.

The colchicine-binding assay for microtubule protein is a variation of that described by Weisenberg et al. (20). Unless indicated otherwise, samples of 50 μl were incubated with 2.4×10^{-5} M [^{3}H]colchicine (New England Nuclear, Boston, Mass.) at 37°C for 2 h. The reaction was terminated with 1 ml of ice-cold IM buffer (0.01 M imidazole chloride, pH = 6.8, 0.005 M $MgCl_2$) containing 2×10^{-5} M colchicine (Sigma Chemical Co., St. Louis, Mo.), filtered by gravity through two no. DE81 Whatman filter disks (2.5 cm) (W and R Balston, Ltd., England), and washed under gentle suction with 15 ml of ice-cold IM buffer. The filters were homogenized in 3 ml of H_2O; 10 ml of Triton X-100 (Packard Instrument Co., Inc., Downers Grove, Ill.): toluene: Permafluor (Packard Instrument Inc. Co.) (1:2:0.135) was added, and the sample was counted in a liquid scintillation counter.

To determine the amount of microtubule protein per cell, clonal cell lines, N18, N103, and C6, were grown in 150-mm plastic tissue culture dishes (Falcon Plastics). Cells from clone N103 and clone N18 without neurites were removed from the tissue culture dishes with Ca^{++}-free saline I, washed twice in saline I to remove serum proteins, and then suspended in a small volume of 0.15 M NaCl, 0.01 M imidazole chloride, pH = 6.8, 0.004 M $MgCl_2$, 0.001 M GTP, 10% glycerol (vol/vol). Plates of cells from clone N18, which had been induced to extend neurites with low serum, and cells from clone C6 were washed twice with saline I containing 0.001 M $CaCl_2$ and once with 0.15 M NaCl, 0.01 M imidazole chloride, pH = 6.8, 0.005 M $MgCl_2$, 0.001 M GTP, 10% glycerol (vol/vol) (IMGG buffer). The cells were removed from plates by scraping and placed in a small volume of IMGG buffer. All cells were broken at the same time in a Raytheon sonic oscillator (Raytheon Co., Waltham, Mass.) at a setting of 1 A for 4 min at 4°C and then placed in an ice bath.

A time-decay colchicine-binding assay very similar to that of Wilson (21) and Bamburg et al. (1) was used to determine the initial amount of colchicine-binding activity present in sonicates of these cells. Within 15 min of sonication, 0.05-ml or 0.1-ml samples were placed in a water bath at 37°C, and [^{3}H]colchicine (1 μCi/1.2 mmol), final concentration of 2.4×10^{-5} M, was added to some samples immediately and to others after 2, 4, or 6 h of incubation at 37°C. The sonicates were incubated with colchicine for 2 h at 37°C, and the reaction was terminated with 1 ml of ice-cold IM buffer. The microtubule protein-colchicine complexes were collected on two no. DE81 Whatman filter disks and washed with about 15 ml of IM buffer. The filters were homogenized and counted as described above. From the four time points, 0, 2, 4, and 6 h of preincubation at 37°C before the addition of colchicine, the decay rate of the colchicine-binding ability of the solution was obtained, and its initial colchicine-binding activity (pmol of colchicine bound/10^6 cells) was determined by extrapolating the line back 2 h.

The amount of microtubule protein was also quantitated from densitometric tracings of 7.5% polyacrylamide 0.1% sodium dodecyl sulfate (SDS) gels. Sonicates of "differentiated" and "undifferentiated" N18 and N103 were centrifuged at 100,000 *g* for 1 h at 4°C. The supernates were brought to a final concentration of 0.02 M sodium phosphate, pH = 7.2, 2% SDS, 10% glycerol, 0.7 M β-mercaptoethanol, and 0.005% bromphenol blue, placed in boiling water for 1 min, and electrophoresed on SDS-polyacrylamide gels (17). The gels were stained overnight in 1% amido blueblack (Sigma Chemical Co.), 7.5% acetic acid, and 40% ethanol. The gels were then

destained by diffusion in 7.5% acetic acid and 5% ethanol, and scanned with a Joyce, Loebl (Joyce, Loebl and Co., Gateshead-on-Tyne, England) or Beckman model 25 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). This stain was found to be linear with protein from 2 μg to 120 μg protein per band.

The proteins of clone N18 with and without neurites were labeled with radioactive proline. Cells from clone N18 were cultured in 150-mm plastic tissue culture dishes with DMEM containing 10 μCi [^{3}H]proline (33.8 Ci/mmol, New England Nuclear) to label the proteins of N18 with neurites. The proteins of N18 without neurites were labeled by growing the cells in DMEM plus 10% fetal calf serum containing 3 μCi [^{14}C]proline (232 mCi/mmol, New England Nuclear). After 2 days, the cells were washed in saline I, removed from the dishes, and frozen at −70°C. The cells were thawed and homogenized. Samples from both homogenates containing approximately the same number of counts per minute were mixed together and sonicated in a Raytheon sonic oscillator. The sonicate was then centrifuged at 100,000 *g* for 1 h at 4°C. The 100,000-*g* supernate was dialyzed and lyophilized, resuspended in 0.02 M sodium phosphate pH = 7.2, 2% SDS, 10% glycerol, 0.7 M β-mercaptoethanol, and 0.005% bromphenol blue, placed in boiling water for 2 min, and electrophoresed on 5% polyacrylamide-SDS gels (17). The gels were processed into 1-mm slices on a Gilson Aliquogel fractionator (Gilson Medical Electronics, Inc., Middleton, Wisc.) and counted in Triton X-100: toluene:Permafluor (2:1:0.135).

RESULTS

The clonal cell line N18 of the mouse neuroblastoma can be induced to undergo morphological changes similar to those of normal neuron differentiation. When maintained in logarithmic growth in culture medium containing 10% fetal calf serum, clone N18 appears "undifferentiated", possessing a spherical morphology characteristic of a neuroblast (Fig. 1 *a*). Environmental modifications, such as a reduction in the concentration of fetal calf

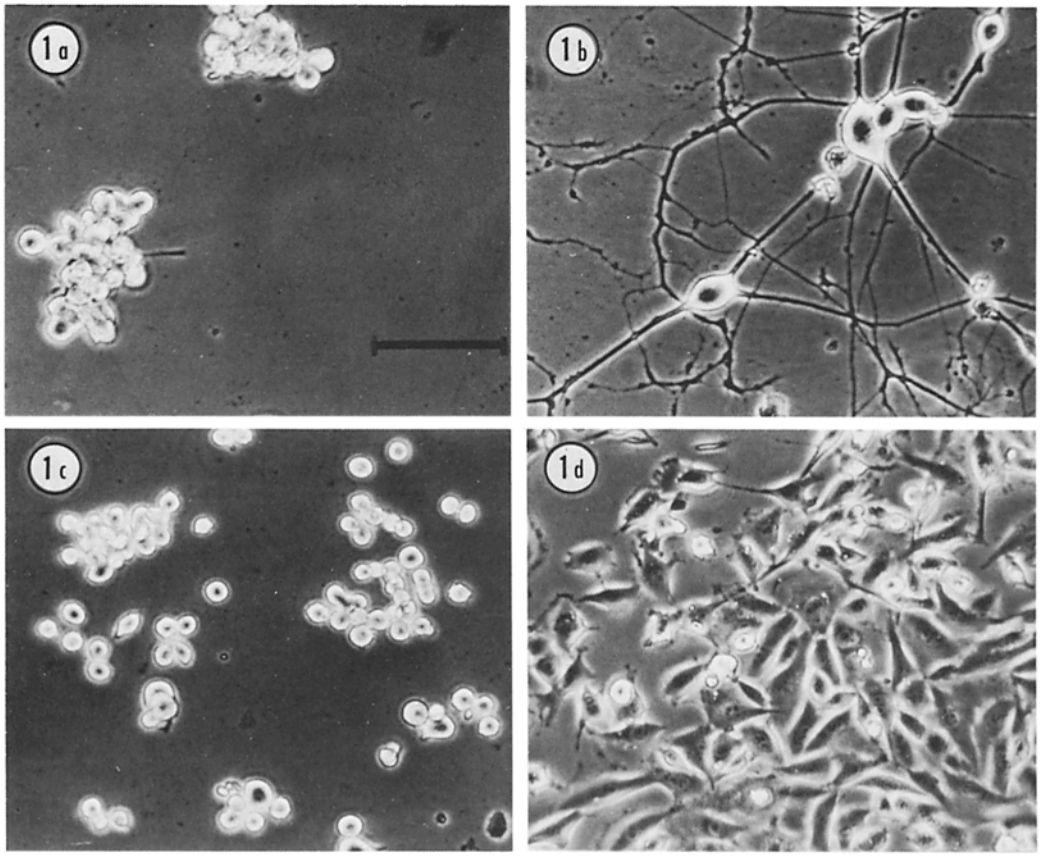

FIGURE 1 Phase contrast photomicrograph of neuroblastoma and glioma. (*a*) Neuroblastoma clone N18 without neurities; (*b*) neuroblastoma clone N18 with neurites, cultured in medium containing 0.2% fetal calf serum for 4 days (most cells are bipolar with neurites about 150 μm long); (*c*) neuroblastoma clone N103, and (*d*) glioma C6. The bar represents 100 μm. Enlargement is × 170.

serum in the medium, that enhance attachment of the cells to the culture dish and retard cell division, allow these N18 cells to extend very long neurites and assume a "differentiated" morphology similar to that of a mature neuron (Fig. 1 *b*). Not all clones of the mouse neuroblastoma show this morphological transition. One such clone, N103 (Fig. 1 *c*), attaches to the culture dish but fails to extend neurites under a variety of conditions that often influence changes in cellular morphology: serum withdrawal, dibutyryl cyclic 3',5'-AMP, bromodeoxyuridine or cytosine arabinoside. In contrast, the glial cell line, C6, is well attached to the culture dish during logarithmic growth, and the cells have an elongated shape (Fig. 1 *d*).

The extension of neurites by the neuroblastoma cell is blocked by colchicine and vinblastine (16), thus implicating microtubules in the formation and maintenance of these structures. Electron microscope observations show that there are relatively few microtubules in the cytoplasm of the undifferentiated N18 cells (Fig. 2 *a*). These tubules are short and randomly arranged, often occurring adjacent to the plasma membrane. Removal of the culture medium and its replacement with serum-free medium brings about the rapid extension of neurites that are 25–100 μm in length by 30–60 min. A longitudinal section through a typical N18 cell 2 h after induction shows that these initial cellular extensions contain numerous microtubules generally aligned parallel to the long axis of the neurite (Fig. 2 *b*). Neurite outgrowth continues for several days with neurites up to 1 mm in length and extensive networks being formed by 4 days in reduced serum medium (Fig. 1 *b*). These established neurites contain long, well-organized microtubules and neurofilaments (Fig. 2 *c*). In cross section, tubules and filaments as well as cross bridges between microtubules are apparent (Fig. 2 *d*). The most notable feature of these micrographs is the apparent large increase in the number of microtubules that accompany morphological differentiation of the mouse neuroblastoma.

Initial neurite outgrowth occurs in the virtual absence of protein synthesis and suggests that these early microtubules arise from a preformed pool of tubulin subunits (16). However, the use of cycloheximide to block protein synthesis in the neuroblast cell is only a valid tool for 6–8 h, after which the cells begin to detach and die, presumably due to the lack of short-lived proteins necessary for life support. Thus, a direct measure of tubulin protein is necessary for the highly differentiated cells (Fig. 1 *b*) present after several days of culture.

The specific binding of 1 mol of colchicine to 1 mol of tubulin dimer (110,000 daltons) can be used as an index of tubulin concentration in these cells. The [^{3}H]colchicine-binding assay originally described by Weisenberg et al. (20) was made optimal for neuroblastoma sonicates as shown in Fig. 3. A concentration of [^{3}H]colchicine that would saturate all the available colchicine-binding sites was determined (Fig. 3 *a*). With a saturating concentration of 2.4×10^{-5} M [^{3}H]colchicine, maximal binding was reached by 60 min of incubation at 37°C (Fig. 3 *b*). Under these conditions, the colchicine-binding activity increases linearly with protein up to 1.1 mg of neuroblastoma sonicate per 50-μl sample (Fig. 3 *c*).

The colchicine-binding activity of tubulin in solution is very labile, and decay rates vary from one extract to another (2). Although the presence of 1 mM GTP and 10% glycerol in the sonicates reduces the decay rate by 75%, we found it is necessary to use a time-decay colchicine-binding assay which defines the initial colchicine-binding capacity of the sonicate. The initial colchicine-binding activity is not affected by factors that influence the decay rate of the tubulin and therefore is an accurate measure of the amount of tubulin present in an extract (1, 2, 21). The initial colchicine-binding capacity of undifferentiated N18, differentiated N18, N103, and glioma C6 sonicates is shown in Fig. 4. The glial cell C6 was chosen as a representative of rapidly dividing, non-neuronal cells with a generation time of 16–20 h. This generation time is close to that of neuroblastoma N18 and N103 and should require a similar tubulin pool for mitotic spindle formation. Cells from clone N18 with and without neurites (±n) contain nearly the same amount of colchicine-binding protein (Fig. 4) which represents approximately 5% of the total protein or 12% of the soluble protein in these cells. Cells from clone N103 that are incapable of neurite formation contain about 80% of the colchicine-binding protein found in the larger N18 cells. Yet, N103 cells still have four times more colchicine-binding protein than other rapidly dividing cells, as represented by the glioma C6. Thus, the elaboration of extensive neurite networks by clonal neuroblastoma cells, and the assembly of large numbers of microtubules do not necessitate an increased concentration of colchicine-binding protein, since all neuroblastoma cells normally possess high levels of this protein.

A second approach was undertaken to confirm the results obtained from the initial colchicine-

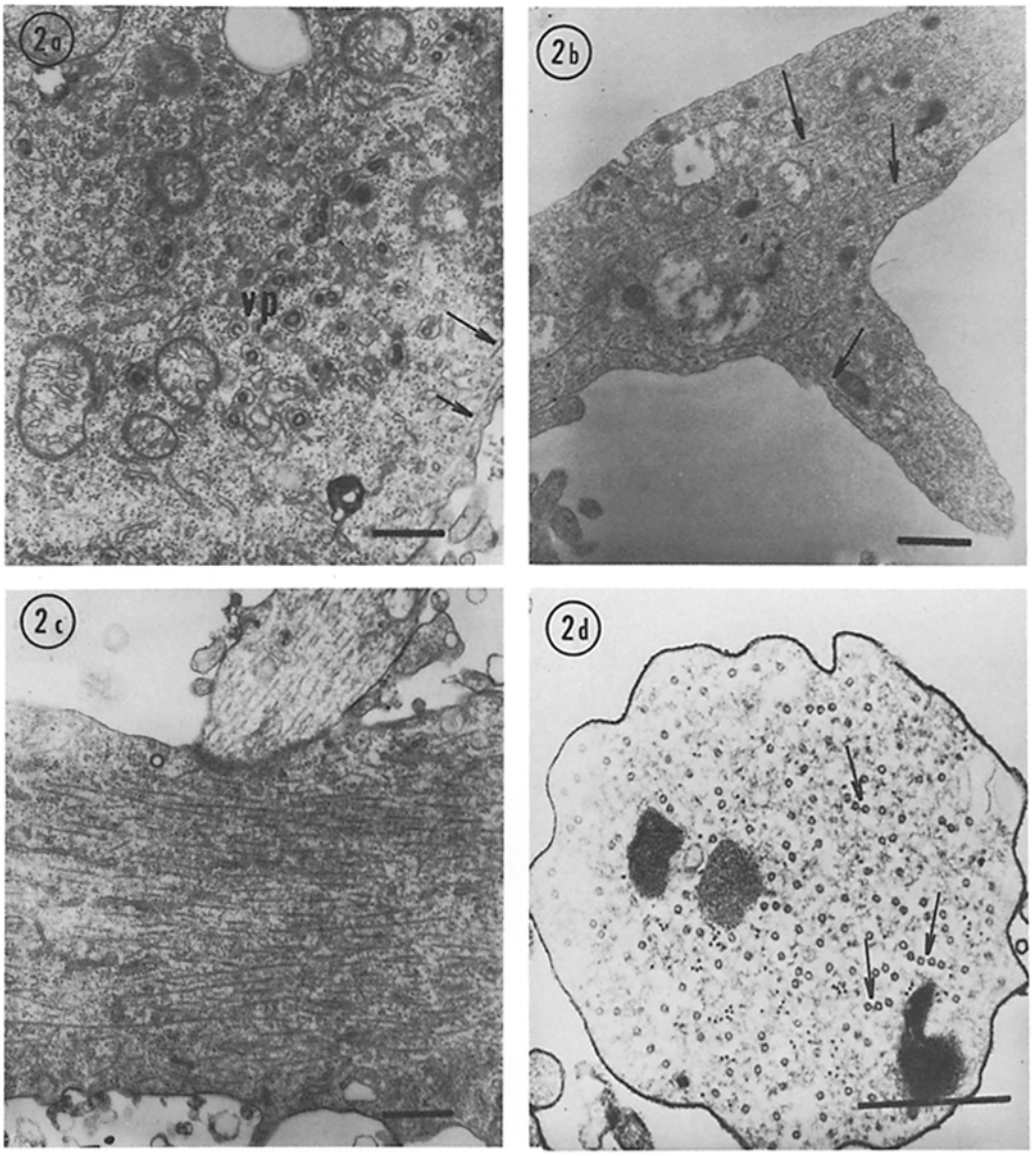

FIGURE 2 Electron micrographs of neuroblastoma N18 microtubules. (*a*) Neuroblastoma clone N18 (without neurites) possessing a few short microtubules (*arrows*) and virus particles (*vp*) in the perikaryon; (*b*) newly formed neurite 2 h after induction by serum withdrawal possessing microtubules (*arrows*) in both branches; (*c*) neurite of differentiated cell 4 days in low serum medium; and (*d*) cross section of a neurite, similar to Fig. 1 *c*, where neurofilaments, microtubules, and cross bridges (*arrows*) between tubules are apparent. The bar on each figure represents 0.5 μm, and enlargement is × 18,600 for Fig. 2 *a–c* and × 40,000 for Fig. 2 *d*.

binding activity (Fig. 4). Since approximately 87% (±9%) of the colchicine-binding activity in all sonicated neuroblastoma cells is recovered in the 100,000-*g* supernate, densitometric studies after electrophoresis of the respective 100,000-*g* supernates on SDS-polyacrylamide gels reflect the amount of tubulin present in the cells. Several concentrations of protein were electrophoresed for each supernate to insure linearity of the tubulin peaks with increasing supernate. Densitometric

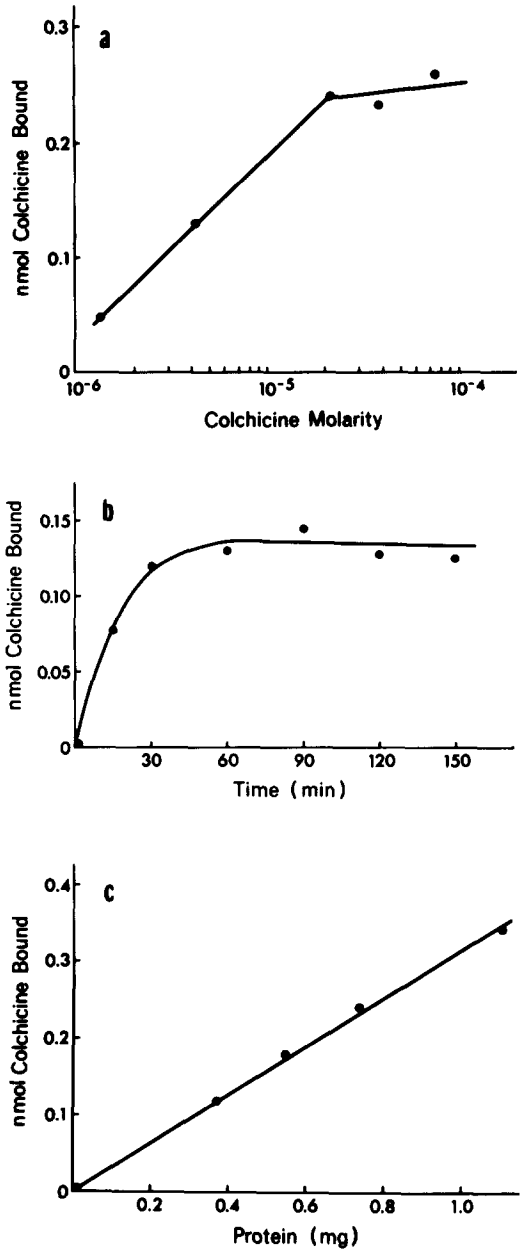

FIGURE 3 Optimum conditions for colchicine binding to neuroblastoma microtubule protein. (*a*) Optimum colchicine concentration for maximum binding. Identical samples of sonicated neuroblastoma cells (0.74 mg protein/samples) were incubated for 2 h at 37°C with increasing concentrations of [^{3}H]colchicine from 1.2×10^{-6} M to 7.5×10^{-5} M [^{3}H]colchicine. (*b*) Optimum incubation time for colchicine binding. Identical samples of sonicated neuroblastoma cells (0.63 mg protein/sample) were incubated with 2.4×10^{-5} M [^{3}H]colchicine at 37°C for varying lengths of time. (*c*) Linearity of colchicine binding with increasing protein concentration. Portions of sonicated neuroblastoma cells containing the indicated amounts of protein were incubated with 2.4×10^{-5} M [^{3}H]colchicine at 37°C for 2 h. All samples were filtered and counted as described in Materials and Methods.

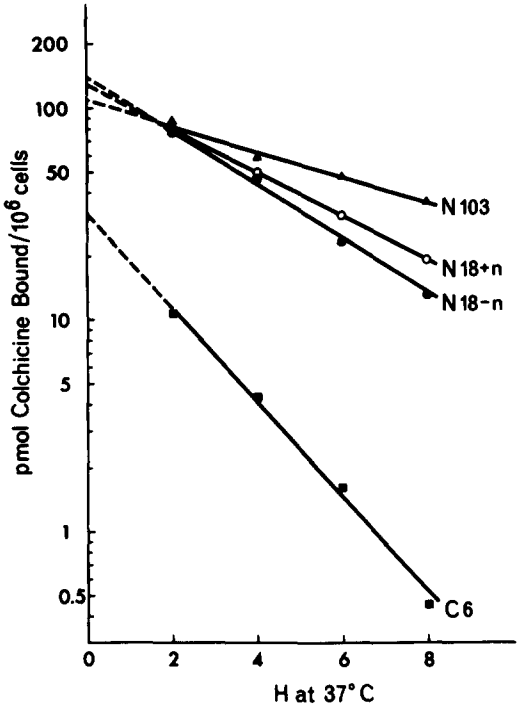

FIGURE 4 Time-decay colchicine-binding assay for neuroblastoma and glioma cells. Sonicates of N18 cells with neurites (+n) (cultured 4 days in low serum), N18 cells without neurites (−n) (cultured 4 days in high serum), N103 cells, and C6 cells were prepared as described in Materials and Methods. Portions of the sonicates were placed at 37°C, and 2.4×10^{-6} M [^{3}H]colchicine was added to some samples immediately and to others after 2, 4, or 6 h. Samples were incubated with the [^{3}H]colchicine for 2 h, and processed as described in Materials and Methods.

tracings of these polyacrylamide gels representing N18 ± n and N103 are shown in Fig. 5. Tubulin is found in the peak corresponding to a mol wt of 55,000 daltons. Integration of these gel profiles indicates that the 55,000-dalton peak represents 19–21% of the total protein in the 100,000-*g* supernate of all three neuroblastoma cell types, regardless of morphology. However, only part of this 55,000-dalton peak probably represents tubulin or colchicine-binding protein. When all the colchicine-binding activity has been removed from

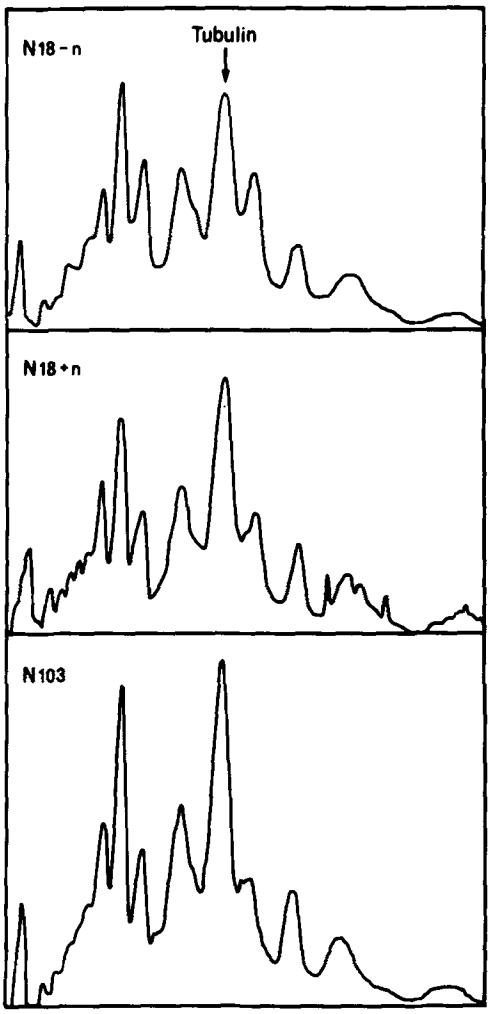

FIGURE 5 Densitometric tracings of SDS-polyacrylamide gels of 100,000-*g* neuroblastoma supernates. The 100,000-*g* supernate from sonicates of N103 and N18 cells with and without neurites (±n) was electrophoresed on SDS-7.5% polyacrylamide gels at neutral pH. Each gel, containing about 75 μg of protein, was stained with amido blueblack as described in Materials and Methods, and the gel was scanned with a recording spectrophotometer. The amount of tubulin, relative to the other proteins present, was determined by measuring the area of the tubulin band and comparing this to the total area of all bands on the gel. Protein migration was from left to right.

the neuroblastoma supernates by affinity chromatography (8, 11), the 55,000-dalton band still contains approximately 42% of the original amount, suggesting that only 58% of the total protein in the 55,000-dalton band corresponds to colchicine-binding protein. With this 0.58 factor, the densitometry studies indicate that 11% of the soluble protein can bind colchicine in N18 (+n), 12% in N18 (−n) and N103, thus giving values similar to those found with the initial colchicine-binding assay.

The possibility that neurite outgrowth placed an increased demand on the synthesis of tubulin relative to other soluble neuroblastoma proteins was also investigated. Rapid neurite formation and outgrowth was initiated by placing the N18 cells in culture medium containing no fetal calf serum. Long neurites were common after 1 day, and by 2 days these cells resembled those cultured for 4 days in medium containing 0.2% fetal calf sera (Fig. 1 *b*). The proteins of cells grown without serum were radiolabeled with [^{3}H]proline for the entire 2-day period. The undifferentiated control cultures contained [^{14}C]proline during the same period. The incorporation of radioactive amino acid into proteins is naturally greater in the logarithmic undifferentiated cultures, where the number of cells has increased two- to fourfold during the labeling period. However, similar amounts of radioactivity from both cell types were sonicated, centrifuged, and the soluble proteins were coelectrophoresed on 5% polyacrylamide-SDS gels. The radioactive profile of such a gel is shown in Fig. 6 where gel slice 43 corresponds to the position of tubulin in this gel system. There is neither an appreciable increase in the amount of tubulin synthesized relative to other soluble proteins in differentiated cells, nor a substantial difference in the relative synthesis of tubulin compared to the undifferentiated neuroblastoma cells. The ratio of ^{14}C to ^{3}H radioactivity in gel slices is fairly constant throughout the gel, except in the region of proteins with mol wt greater than 150,000 daltons (fractions 1–14).

DISCUSSION

Three different methods have been used to directly determine the total amount of tubulin in morphologically differentiated and undifferentiated neuroblastoma cells. Although there is an apparently large increase in the number of microtubules in these cells (Fig. 2) as a result of this morphological transition, all three methods indicate that the tubulin concentration in neuroblastoma N18 cells is relatively constant during this transition (Figs. 4, 5, and 6). These studies lend support to the observations reported by several other laboratories that neurite outgrowth induced by nerve growth factor (NGF) in chick dorsal root ganglia cultures

(23) or superior cervical ganglia of neonatal rats (18) does not require an increase in the tubulin concentration. However, studies by Hier et al. (7) indicate that NGF produces a twofold increase in the amount of tubulin per ganglia even when neurite outgrowth is blocked by vincristine, although no increase was observed when neurites were induced by dibutyryl cyclic 3',5'-AMP. These differences may reflect environmental influences on the growth or death of several cell types in the ganglia. Furthermore, all three studies (7, 18, 23) determined tubulin levels in their respective systems with a single time-point colchicine-binding assay.

Since microtubule protein is a labile protein and expresses considerable variation in the decay rate of its colchicine-binding activity, attempts to measure tubulin with a single [^{3}H]colchicine incubation can be misleading (2, 21). As shown in Fig. 4, the decay rates of the colchicine-binding activity of N103, N18, and C6 are quite different. The half-lives of active tubulin in sonicates of N103, N18 (−n), N18 (+n), and C6 cells are 4.3, 2.2, 3.0, and 1.5 h, respectively. A single time-point binding assay would suggest that N103 cells have slightly more tubulin than N18 cells and eight to nine times more tubulin than C6 cells; furthermore, later time-points would greatly magnify these differences. In fact, N103 cells have about 15–20% less tubulin than N18 cells and only four times more tubulin than C6 cells. Thus, extracts with a slow decay rate could appear to have artificially more tubulin than solutions with a rapid decay rate when single incubations with [^{3}H]colchicine are used. Even greater discrepancies have been found with single incubations of chick brain extracts (2).

Since the colchicine-binding activity was carried out for 2 h, the decay curve was extrapolated back 2 h to determine the initial colchicine-binding capacity (Fig. 4) as described previously (21). It may only be valid to extrapolate the line back 1 h since the colchicine concentration was made optimal and the binding reached completion in 60 min (Fig. 3 *b*). However, this change would not significantly alter the results as presented in Fig. 4. One major factor that influences decay rates is the concentration of active tubulin (2); however, C6 sonicates still show a very rapid decay even after the decay curves are normalized by tubulin concentration. This difference may reflect a functional and structural difference in tubulin from neurons and glia.

Densitometric studies of 100,000-*g* supernates from neuroblastoma cell sonicates showed that there was no increase in the relative amount of protein that migrated in the 55,000-dalton band on SDS-polyacrylamide gel electrophoresis after the morphological differentiation. Furthermore, affinity chromatography of these supernates on agarose columns containing deacetyl- and isodeacetylcolchicine removed about 58% of the protein from the 55,000-dalton band of each gel, thus agreeing with the initial colchicine-binding activity data indicating that tubulin represents 11–12% of the soluble protein in neuroblastoma cells. However, this calculation assumes that all tubulin can bind colchicine or colchicine derivatives (11), which may not necessarily be true. In this regard, a comparison of peptide maps for the residual 42% of 55,000-dalton proteins and tubulin could prove interesting.

Both the densitometric and initial cholchicine-binding studies have shown that morphologically undifferentiated N18 and N103 cells contain higher concentrations of tubulin than most non-neuronal cells, yet neither cell type has a well organized network of long microtubules. Furthermore, there is no significant increase in the amount of microtubule protein when N18 cells differentiate and become bipolar cells with neurites about 150 μm. An approximate calculation of the amount of tubulin in the microtubules of neuroblastoma neurites can be made from the microscope studies (Figs. 1 and 2). Cross-sectional electron micrographs show about 130 microtubules per average neurite. Assuming that each average microtubule extends the entire length of the neurite (150 μm), each cell has 10^8 molecules of tubulin in neurites. From the initial colchicine-binding activity (Fig. 4), the same cell has a total of 1.44×10^8 molecules of tubulin, i.e. about 70% of the cell's tubulin is in its neurites. However, the localization of this large tubulin pool in the undifferentiated neuroblastoma cell is intriguing and may be elucidated by fluorescent antibody studies (19).

Although neurite extension does not require an increased tubulin concentration within the cell, it may require increased synthesis that is coupled with an increased turnover under the environmental conditions used to induce neurite outgrowth. Such turnover studies are complicated by several factors including the death of some cells after several days in the low serum medium; however, our preliminary studies indicated that in both cell

morphologies the tubulins have similar half-lives. In low serum medium, tubulin shows a half-life of about 4.2 days; in high serum medium, tubulin shows a half-life of 3.2 days while the total proteins turn over with a slightly longer half-life of 4.4 days. Neurite outgrowth does not require a disproportionate synthesis of tubulin for its initial phases (unpublished observation) or the entire transition (Fig. 6). This result, in conjunction with the densitometric studies and initial colchicine-binding data, suggests that, while it is probably necessary to have large tubulin pools to support neurite outgrowth during neuronal differentiation, the regulation of microtubule formation is not solely controlled by the size of the tubulin pool. Regulation may occur at several levels, including microtubule assembly. Several high mol. wt proteins ($> 3 \times 10^5$ daltons) have recently been implicated in microtubule assembly in vitro (4). In this regard, Fig. 6 is especially interesting since the only major difference between the proteins synthesized in the two morphologies of neuroblastoma occurs in the high mol wt region, where there is a relatively greater synthesis of protein in the differentiated cells. Although the radioactivity is low (30–120 cpm) and there may be several reasons for this difference, it certainly warrants further experimentation. In addition, we hope that some neuroblastoma clones that fail to form neurites, such as N103 and subclones of mutagenized N18, may serve as models for studying the regulation of microtubule formation both in cell culture and, hopefully, in cell extracts.

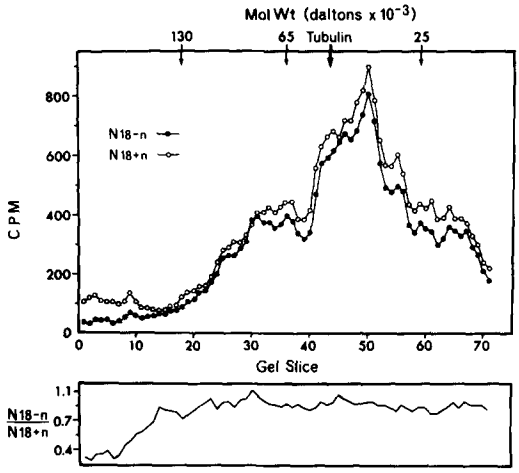

FIGURE 6 SDS-polyacrylamide gel profile of radioactive-soluble proteins from differentiated and undifferentiated neuroblastoma cells. Proteins of neuroblastoma cells in logarithmic growth (N18 −n) and those in serum-free medium (N18 +n) were labeled with [^{14}C]-proline and [^{3}H]proline, respectively, for the 2 days required for extensive neurite formation in the latter culture. The 100,000-*g* supernates from a sonicate of mixed cells were electrophoresed on SDS-5% polyacrylamide gels at neutral pH. The gel was sectioned at 1-mm intervals, sliced, and counted as described in Materials and Methods. Proteins of known mol wts served as reference standards. The ratio of radioactivity (^{14}C counts per minute/^{3}H counts per minute) for each gel slice is shown in the lower portion of the figure. Protein migration was from left to right.

We would like to thank Susan Haffke and Barbara Fonda for their expert technical assistance during these studies and Drs. R. Lasher and J. Brown for the use of their Joyce-Loebl spectrophotometer and Gilson Aliquogel fractionator, respectively. The electron micrographs were done in collaboration with Drs. Alfred Gilman, Marshall Nirenberg, and William Hall at the National Institutes of Health.

Dr. Morgan was a trainee of the United States Public Health Service grant 5 T01-GM00781, and Dr. Seeds is Career Development Awardee of the United States Public Health Service grant K04-GM40170. This work was supported in part by United States Public Health Service research grant R01-NS10709.

Received for publication 11 April 1975, and in revised form 27 May 1975.

REFERENCES

1. BAMBURG, J. R., E. M. SHOOTER, and L. WILSON. 1973. Developmental changes in microtubule protein of chick brain. *Biochemistry*. **12**:1476–1482.
2. BAMBURG, J. R., E. M. SHOOTER, and L. WILSON. 1973. Assay of microtubule protein in embryonic chick dorsal root ganglia. *Neurobiology (Copenh.)*. **3**:162–175.
3. BENDA, P., J. LIGHTBODY, G. SATO, L. LEVINE, and W. SWEET. 1968. Differentiated rat glial cell strain in tissue culture. *Science (Wash. D. C.)*. **161**:170–171.
4. BORISY, G. G., J. B. OLMSTED, J. M. MARCUM, and C. ALLEN. 1974. Microtubule assembly *in vitro*. *Fed. Proc.* **33**:167–174.
5. BURTON, K. 1956. A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**:315–323.
6. DANIELS, M. P. 1972. Colchicine inhibition of nerve fiber formation in vitro. *J. Cell Biol.* **53**:164–176.
7. HIER, D. B., B. G. W. ARNASON, and M. YOUNG. 1972. Studies on the mechanism of action of nerve growth factor. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 2268–2272.
8. HINMAN, N. D., J. L. MORGAN, N. W. SEEDS, and J.

R. CANN. 1973. Isolation of brain tubulin by affinity chromatography. *Biochem. Biophys. Res. Commun.* **52**:752–758.

9. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275.
10. MORGAN, J. L., and N. W. SEEDS. 1973. Properties of tubulin isolated by affinity chromatography. *J. Cell Biol.* **59**(2, Pt. 2):233*a*. (Abstr.).
11. MORGAN, J. L., and N. W. SEEDS. 1975. Properties of tubulin prepared by affinity chromatography. *Ann. N. Y. Acad. Sci.* **253**:260–271.
12. OLMSTED, J. B., and G. G. BORISY. 1973. Microtubules. *Annu. Rev. Biochem.* **42**:507–540.
13. OLMSTED, J. B., K. CARLSON, R. KLEBE, F. RUDDLE, and J. ROSENBAUM. 1970. Isolation of microtubule protein from cultured mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U. S. A.* **65**:129–136.
14. PORTER, K. R. 1966. Cytoplasmic microtubules and their function. *In* Principles of Biomolecular Organization. G. E. W. Wolstenholme and M. O'Connor, editors. Little, Brown, & Co. Inc., Boston, Mass. 308–345.
15. SCHUBERT, D., S. HUMPHREYS, C. BARONI, and M. COHN. 1969. In vitro differentiation of a mouse neuroblastoma. *Proc. Natl. Acad. Sci. U.S.A.* **64**:316–323.
16. SEEDS, N. W., A. G. GILMAN, T. AMANO, and M. W. NIRENBERG. 1970. Regulation of axon formation by clonal lines of a neural tumor. *Proc. Natl. Acad. Sci. U. S. A.* **66**:160–167.
17. SHAPIRO, A., E. VINUELA, and J. MAIZEL. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **28**:815–820.
18. STÖCKEL, K., F. SOLOMON, U. PARAVICINI, and H. THOENEN. 1974. Dissociation between effects of nerve growth factor on tyrosine hydroxylase and tubulin synthesis in sympathetic ganglia. *Nature (Lond.).* **250**:150–151.
19. WEBER, K., R. POLLACK, and T. BIBRING. 1975. Antibody against tubulin: the specific visualization of cytoplasmic microtubules in tissue culture cells. *Proc. Natl. Acad. Sci. U. S. A.* **72**:459–463.
20. WEISENBERG, R. C., G. G. BORISY, and E. W. TAYLOR. 1968. The colchicine binding protein of mammalian brain and its relationship to microtubules. *Biochemistry.* **7**:4466–4479.
21. WILSON, L. 1970. Properties of colchicine binding protein from chick embryo brain. Interactions with vinca alkaloids and podophyllotoxin. *Biochemistry.* **9**:4999–5007.
22. YAMADA, K. M., B. S. SPOONER, and N. W. WESSELLS. 1970. Axon growth: roles of microfilaments and microtubules. *Proc. Natl. Acad. Sci. U. S. A.* **66**:1206–1212.
23. YAMADA, K. M., and N. W. WESSELLS. 1971. Axon elongation (effect of nerve growth factor on microtubule protein). *Exp. Cell Res.* **66**:346–352.