The distribution of tubulin in soluble, reversibly stabilized (assembled) and insoluble forms has been determined in neuroblastoma cells undergoing microtubule-dependent neurite elongation. Procedures were developed to obtain reproducible tubulin fractions and to assay total tubulin. Radioimmunoassays showed that both differentiated and nondifferentiated cells contained ~4 pg of tubulin per cell, of which 3–10% was in an insoluble, particulate form. The amount of tubulin assembled in differentiated cells was four to five times greater than in nondifferentiated cells, constituting 48–63% and 11–16% of the total tubulin pool in the respective cell types. Calculation of the concentration of soluble tubulin in differentiated cells (~0.8 mg/ml) and nondifferentiated cells (~1.6 mg/ml) indicates that a critical concentration of subunits probably does not limit the induction of microtubule formation during neurite elongation.

The cellular distribution of tubulin and the mechanisms by which this protein participates in a variety of cellular functions have been under investigation for a number of years. For mitosis, it was originally proposed that spindle function might be mediated by the localized assembly and disassembly of microtubules (9). A shift in the equilibrium between soluble and assembled tubulin was also postulated to be involved in the modulation of events such as secretion (4, 8) and phagocytosis (1); this hypothesis was largely derived from studies that demonstrated that the antimitotic drug, colchicine, interfered with these processes. The earliest morphological studies also made clear that the assembly and orientation of microtubules was a factor in determining cellular shape and that tubulin had a major cytoskeletal function.

In the last several years, procedures have been developed which made possible the examination of the equilibrium for tubulin assembly in vitro (10) and also the analysis of tubulin distribution in vivo. Several groups (20, 22, 27) devised methods for assessing the organization of tubulin in soluble and stabilized forms and have used colchicine binding for quantitation. Pipeleers et al. (22) developed a detailed procedure and assayed the distribution of tubulin in a variety of tissues (23). In their study, the fraction of assembled tubulin was found to vary from 30 to 90%, the highest being in human platelets. Studies on the partitioning of tubulin during lymphocyte activation by phytohemagglutinin (PHA) demonstrated that the percentage of formed microtubules paralleled the increase in total tubulin pool upon activation; the maximum fraction assembled was 30–40% of the total tubulin measured (31). Varying reports have appeared on changes in tubulin distribution occurring during virus-induced transformation of cultured cells, with some studies suggesting an increase in pelletable tubulin upon transformation (26) and others detecting no change (6). More recently, Ostlund and co-workers (21) examined the distribution of microtubules in cultured cells as a function of plating time and found that the percentage of tubulin assembled increased with time of plating from 5–58% of the total assayed.

We have been concerned with the regulation of tubulin distribution and assembly in cultured neuroblastoma cells. These cells undergo microtubule-dependent neurite extension upon plating on a suitable substrate, and our previous studies demonstrated that postmitochondrial supernates from differentiated cells contained amounts of tubulin equivalent to those from nondifferentiated cells (33). The present report describes the quantitation of tubulin in soluble, reversibly stabilized (assembled) and insoluble forms and outlines how the total distribution of tubulin in these fractions varies as a function of cellular differentiation.

MATERIALS AND METHODS

Cell Culture and Fractionation

Mouse neuroblastoma cells (NB2a) (12) were subcloned in our laboratory (AB-1) and this clone was maintained in F12 medium (Grand Island Biological Co., Grand Island, New York) with 10% fetal calf or calf serum. Cells were grown either in monolayer cultures (to densities of 1 × 10⁶ cell/ml) or in suspension cultures (to 2 × 10⁶ cells/ml) in a humidified 5% CO₂ atmosphere at 37°C.

Cell fractionation was carried out using modifications of the method originally described by Pipeleers et al. (22) in which buffers either stabilized (MTS) or depolymerized (TS) microtubules. The efficacy of the buffers in stabilizing microtubules and not inducing polymerization under the modified conditions was tested using purified brain microtubule protein in quantitative sedimentation assays (10). Cells grown in monolayer for 5–7 d were harvested at room
temperature by gently rinsing three times with phosphate-buffered saline (PBS) (6 mM Na-KPO, buffer, pH 7.2, 171 mM NaCl, 3 mM KCl), and once with either TS buffer (10 mM PIPES, pH 6.9, 0.5 mM MgCl, 0.5 mM GTP, 1.0 mM EGTA) or MTS buffer (10 mM PIPES, pH 6.9, 0.5 mM MgCl, 0.5 mM GTP, 1.0 mM EGTA, 50% (vol/vol) glycerol, 5% (vol/vol) dimethyl sulfoxide. Cells were then suspended in the fixatives in TS or MTS by gentle agitation. Suspension cultures were harvested by centrifugation for 4,500 g-min, washed once with PBS, and then washed two additional times with MTS or TS. Because the viscosity of MTS was ~7.2 times that of TS at 18°C, cells from either fixatives or suspension cultures were pelleted for 4,500 g-min in TS, or 3,150 g-min in MTS. In a typical experiment, cells were resuspended to a final concentration of 4 x 10^6 cells/ml and homogenized with a glass-Teflon homogenizer or sonicated with a microprobe until 2-90% of the cells were lysed, but the nuclei remained intact. Further fractions were obtained by centrifuging MTS-WC at 18°C for 6.3 x 10^4 g-min, and TS-WC lysates for 10^4 g-min. Resulting pellets (MTS-P, or TS-P) were resuspended in TS and left for 1 h at 0°C. Further fractions (S1, P1) were obtained by centrifugation for 10^5 g-min at 0°C; repetition of pellet resuspension in TS and centrifugation yielded S2 and P2 fractions. In all cases, pellets were resuspended to the original volume of the centrifuged fraction. Data presented in Results have been corrected to account for samples removed for analyses.

**Gel Electrophoresis**

One-dimensional gel electrophoresis on SDS-containing polyacrylamide gels was carried out according to the method of Laemmli (13) and two-dimensional gel electrophoresis was performed according to the method of Waring et al. (35). Gels were stained with Coomassie Blue (7). For RIAs and parallel protein determinations, samples were boiled in SDS buffer without mercaptoethanol, and the precipitation procedure was modified to give a more concentrated sample. The boiled samples (20 µl) were mixed with either 50 µl of 1.0 M or 100 µl of 500 mM KPO4 buffer, pH 7.2, and (b) 0.1 ml of the supernate resulting from the phosphate precipitation step was mixed with 1.0 ml of commercially prepared Coomassie Blue-G reagent (BioRad protein reagent, 1:5 dilution; Bio-Rad Laboratories, Richmond, Calif.) and absorbances were read at 595 nm. The standard was bovine serum albumin.

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**Sample Preparation and Protein Determinations**

Samples were treated in a similar fashion to obtain material for one-dimensional gels, protein determinations, and radioimmunooassay (RIA). Samples were initially mixed with an equal volume of 2x SDS sample buffer (13) which lacked mercaptoethanol and sucrose (final concentrations in sample: 0.08 M Tris, pH 6.8, 2% SDS). For gel analysis and routine protein assay, aliquots of the SDS-treated samples were made to 5% with mercaptoethanol before boiling for 3-5 min. Typically, protein assays on these SDS-treated samples were carried out as described by Zaman and Verwilghen (37) with the following modifications: (a) a final volume of 20 µl of SDS-treated sample was mixed directly with 0.5 ml of 90 mM KPO4, buffer, pH 7.2, and (b) 0.1 ml of the supernate resulting from the phosphate precipitation step was mixed with 1.0 ml of commercially prepared Coomassie Blue-G reagent (BioRad protein reagent, 1:5 dilution; Bio-Rad Laboratories, Richmond, Calif.) and absorbances were read at 595 nm. The standard was bovine serum albumin.

**Immunological Procedures**

The tubulin antisera used for all procedures was raised in rabbits against electrophoretically purified tubulin from Tetrahymena and was characterized as described previously (13, 34). RIA were carried out as published (34). Standard curves were obtained by treating purified hog brain tubulin or neuroblastoma tubulin suspended in MTS or TS with SDS as described. Identical curves were obtained for MTS and TS samples, and for both neuroblastoma and hog tubulin. For RIAs, parallel protein determinations, and radioimmunooassay (RIA), samples were boiled in SDS buffer without mercaptoethanol, and the precipitation procedure was modified to give a more concentrated sample. The boiled samples (20 µl) were mixed with either 50 µl of 1.0 M or 100 µl of 500 mM KPO4 buffer, pH 7.2, such that the ratio of SDS to potassium phosphate remained the same as for the original protein assay (37). After standing for 15 min at room temperature, samples were centrifuged for 30,000 g-min and the supernates removed. Protein assays of the supernates were carried out using a microassay procedure (typically 1-10 µl of supernate, 0.8 ml of water, 0.2 ml of concentrated BioRad protein reagent) with appropriate blanks and standards. After correction for dilution, these assays gave protein concentrations identical to those of the more diluted samples. In addition, gel electrophoresis of these samples after dialysis and retreatment with SDS showed profiles identical to those of the original gel samples, indicating that no preferential loss of protein occurred using this modified procedure.

**Distribution of Cellular Proteins in MTS and TS Solutions**

To analyze the distribution of total tubulin in various cellular fractions by RIA, it was necessary to have highly reproducible methods for obtaining fractions and for determining total protein concentrations. To obtain consistent fractions from various experiments, cell lysates were made with 4 x 10^7 cells/ml of lysing buffer. A summary of the distribution of total tubulin antigens was determined and the results were compared to the original lysates.

**RESULTS**

**Morphological Characteristics of Neuroblastoma Cells**

The subclone of neuroblastoma cells used in this study undergoes microtubule-dependent neurite outgrowth in the presence of a suitable substrate and without changing medium conditions or inhibiting cell division (33). As shown from previous electron microscope observations (18, 25) and also in Fig. 1, differentiated cells possessed elongate neurites that contained numerous microtubules. In contrast, cells grown in suspension culture were rounded, contained no neurites, and showed only diffuse fluorescence when stained with antitubulin antibody (not shown). Differentiated cells lost the microtubule pattern when treated with 10^-5 M colchicine for 1 h at 37°C, or for 2 h at 4°C; retraction of neurites under these conditions occurred more slowly. Neurite formation was blocked completely in the presence of colchicine or at 4°C and microtubule networks did not form. Thus, as previously inferred from other studies on neuroblastoma cells and neurons (5, 28, 29), these immunofluorescence observations demonstrated that the elaboration of neurites and the formation of microtubules appeared to be parallel events.

A qualitative evaluation of the effect of TS and MTS solutions on the morphology and microtubule networks of the neuroblastoma cells was made. Differentiated cells were exposed to either TS or MTS for various lengths of time, examined by phase microscopy, and then fixed for immunofluorescence. As shown in Fig. 1 c, within 5 min, cells in TS had become swollen, and a majority had started to round. Inclusion of isotonic sucrose in the TS solution prevented the swelling but did not alter the change in morphology. In contrast, cells treated with MTS maintained their elongate appearance (Fig. 1 c) but became less phase dense. This morphology was maintained even after the cells were released from the substrate. Immunofluorescence demonstrated that few formed microtubules remained after 2 min of treatment with TS (Fig. 1 f), whereas microtubules in MTS-treated cells were maintained for up to 3 h, even at 4°C (Fig. 1 d, inset). Although observation of MTS-treated cells showed a transient shrinkage during the initial 30 s of treatment, no change in the distribution of microtubules in the MTS-treated cells as compared to controls could be ascertained by immunofluorescence. These data suggest that there was no qualitative induction or depolymerization of microtubules upon treatment with MTS.
protein from a number of experiments is shown in Table I. The error for replicate samples for a given determination was <5%, and the standard deviation shown in the table represents variation in protein concentrations from experiment to experiment. These data demonstrate that the MTS solution caused the release of some protein which was less soluble in TS solution. However, cells from suspension and differentiated cultures showed no consistent differences in protein distribution when either MTS or TS fractions were compared. Lysing the cells at more dilute concentrations (0.5, 1.0, or 2.0 × 10^7 cells/ml) resulted in distributions of total protein essentially identical to those shown in Table I.

Qualitative studies on the distribution of tubulin in the various fractions were carried out using one-dimensional gel analyses. Fig. 2 shows results from a typical experiment on differentiated cells. The bulk of material migrating with tubulin appears in the S fraction of the TS material, but in the S2 fraction of the MTS preparation. This is consistent with the solubilization of microtubules in TS, and the stabilization of microtubules in NITS lysates, which are then depolymerized upon shift to 0°C and resuspension in TS.

Quantitative Analyses of Tubulin Distribution

A quantitative analysis of tubulin distribution in various fractions was undertaken using RIA. In particular, accurate determination of the fraction of tubulin present in soluble and cold-reversible form would give an indication of the equilibrium distribution of tubulin in the two cell types. In addition, by carrying out analyses on all fractions, it could be determined whether a substantial fraction of tubulin existed in an insoluble form. Conventional colchicine-binding assays have been problematical because of the lability of the binding activity, and it has also not been feasible to measure insoluble material reliably by this method. One-dimensional gel analyses also are not adequate for precise estimates of tubulin in heterogeneous
FIGURE 2. SDS PAGE of MTS and TS fractions from differentiated cells. Cells were grown in monolayer culture for 5 d and prepared according to the fractionation scheme outlined in Materials and Methods. 7.5% acrylamide gel, stained with Coomassie Blue. Slots from left to right: Partially purified neuroblastoma tubulin; WC; S₁; P₁; S₂; P₂; S₃; P₃. (A) MTS treatment; (B) TS treatment.

samples. As described in Materials and Methods, a modified version of the sample preparation procedure used for protein assays on SDS-containing samples (37) was employed to prepare samples for RIA. This allowed both the direct calculation of protein concentrations on all samples being assayed and the determination of tubulin concentrations by RIA in fractions which were normally partially (WC) or totally (P₃) insoluble in aqueous buffers.

The results from typical experiments quantitated by RIA are shown in Tables II and III. In differentiated cells (Table II), 89% of the total tubulin in the cell lysates was present in the soluble supernate (TS-S₁) under depolymerizing conditions. Rewashing the pellet fraction resulted in the release of amounts of tubulin which, if present, were below the limit of detection of the assay (0.2 μg/ml). However, assay of the P₂ and P₃ fractions indicated that 6–8% of the total tubulin was present in an insoluble form. If purified labeled tubulin was added before cell lysis, <0.2% of the total counts were recovered in P₃ fractions, indicating that the presence of tubulin in this pellet fraction was not caused by trapping during fractionation. As shown in Table III, similar results were found for nondifferentiated cells treated with TS.

In contrast to the results with TS buffer, cells lysed in MTS buffer showed markedly different distributions of tubulin, depending on the state of differentiation. In suspension cultures (Table III), soluble tubulin (MTS-S₁) represented 75% of the total measured, and cold-reversible tubulin constituted 15%. An insoluble fraction, that was not reduced by repeated washing, represented <7% of the total (P₃). In contrast, differentiated cells (Table II) had a substantial amount of tubulin that fractionated as polymerized microtubules. Tubulin that was initially soluble (MTS-S₁) represented 43% of the total, and an additional 54% of the total was released when pelleted material (MTS-P₁) was placed under depolymerizing conditions. Due to the release of tubulin from the pelleted material, 54% of the total tubulin was released when pelleted material (MTS-P₁) was placed under depolymerizing conditions. For seven experiments on differentiated cells, the amount of cold-reversible tubulin ranged from 48 to 63% of the total tubulin. Electron microscope examination of these fractions showed that tubules were present in lysates and P₁, resuspended in MTS, but lacking from S₂ fractions. In both differentiated and nondifferentiated cells, <5% of the tubulin was insoluble; this percentage was consistently less than that for the equivalent fraction derived from cells fractionated with TS buffer. Two-dimensional gel analyses of the particulate fractions demonstrated that faint spots comigrating with purified neuroblastoma tubulin were present.

The redistribution of tubulin after treatment of differentiated cells with colchicine is also shown in Table II. No change was seen in fractions from cells fractionated with TS. However, the amount of tubulin assayed in the S₁ fraction from MTS-treated cells increased to 90% of the total tubulin. This is consistent with the depolymerization of microtubules by colchicine in situ. Similar data were obtained using cold treatment to depolymerize the tubules. A fraction of the total tubulin (5.3%) was still recovered as insoluble material.

DISCUSSION

Neuroblastoma cells have served as a useful model system for studying the events that accompany microtubule-dependent shape changes. In examining the distribution of tubulin in neuroblastoma cells as a function of differentiation, we thought it necessary to devise methods that would enable quantitation of tubulin in all cellular fractions, particularly those that had not readily been assayed by colchicine binding. A modified sample preparation procedure and RIA were therefore used to quantitate fractions that were insoluble and to obtain an estimate of the total tubulin content and distribution in these two cell types.

Both differentiated and nondifferentiated cells were found to contain ~4 pg of total tubulin per cell. Of this total, ~85–90% of the tubulin was recovered in the soluble fraction when either cell type was lysed in TS buffer. These results are consistent with previous reports that indicated that >90% of the total tubulin was solubilized in aqueous buffers at 0°C (14, 18, 33). All these data also demonstrate that the total pool of tubulin does not change during differentiation.

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Differentiated cells isolated in MTS had a soluble pool that ranged from 35 to 50% of the total tubulin; except for ~5%, all of the remaining tubulin (48–63%) was solubilized under de-
These data can be compared to the estimate made from electron micrographs that indicated that up to 70% of the total tubulin was polymerized in neuroblastoma cells which differentiated upon serum withdrawal (14). In contrast, only 12-15% of the tubulin pool was in stabilized form in nondifferentiated cells. Because the amount of tubulin/cell was the same for either cell type, these data indicated that approximately four to five times more tubulin was polymerized in differentiated cells compared to rounded cells. Although stabilized tubulin might exist in nonmicrotubule form, treatment of differentiated cells with cold or colchicine before fractionation suggested that the majority of tubulin measured as stabilized was in a form that was sensitive to these microtubule-stabilizing agents.

In both cell types, a small fraction of total tubulin appeared to be insoluble. This represented 6-10% of the total in TS samples, and 3-5% in the MTS samples. Repeated washing of these pellets, and experiments in which exogenous labeled tubulin was added before cell lysis, indicated that this did not consist of trapped tubulin. However, MTS-treated samples consistently contained less tubulin in this fraction. It is postulated that this difference might result from the partial solubilization of membranes by glycerol in the MTS buffer, and/or its release from undefined compartments that might not be soluble in more aqueous solutions. It is currently unknown whether this fraction represents a unique type of tubulin. However, there have been other reports of particulate forms of tubulin (36) and also data on the existence of membrane-associated or bound tubulin (2, 11, 38). Nath and Flavin (17) recently reported a particulate form of tubulin in neuroblastoma-glioma hybrid cells. They estimated that this constituted 10% of the tubulin in this cell type and demonstrated that tyrosylation of this fraction could occur in vivo.

Although it is clear that neurite formation results in a change in the total distribution of tubulin in neuroblastoma cells, the factors that induce this assembly remain unknown. In the clone used for these studies, it has been demonstrated that this change occurs without alteration of the cell cycle (33) and with no net change in the total tubulin pool (these data). Therefore, other factors that might regulate polymerization have to be considered. One possibility is that the total concentration of subunits changes such that a critical concentration for assembly is achieved. From in vitro studies, it has been calculated that a critical concentration of 0.2 mg/ml is required for brain microtubule formation (10). Based on an estimate of 2.0 mg/ml for the concentration of tubulin in neuroblastoma cells (33), our data indicate that the concentration of free subunits would be ~0.8 mg/ml in differentiated cells and 1.6 mg/ml in suspension cells. Assuming that all the tubulin in that is measured as soluble in MTS-treated cells is competent to assemble, it is apparent that factors other than the total concentration of subunits must determine the extent of polymerization in the two cell types.

In a number of in vitro systems, tubulin polymerization is augmented by specific proteins, termed microtubule-associated proteins (3, 15, 16, 32). Seeds and Maccioni (30) originally reported a fraction from serum-induced differentiated neuroblastoma cells which contained activity that promoted the polymerization of brain tubulin. An equivalent fraction from

| TABLE II | Distribution of Tubulin in Differentiated Cells Determined by RIA |
|----------|---------------------------------------------------------------|
| Treatment | Fraction | Tubulin (µg/ml) | % Total protein | Tubulin distribution % | Tubulin distribution colchicine* |
|----------|----------|-----------------|-----------------|------------------------|---------------------------------|
| MTS      | WC       | 192.0 ± 16.9    | 1.8             | 153                    | 100.0                           |
|          | S1       | 92.9 ± 7.2      | 1.6             | 66                     | 43.1                            |
|          | P1       | 108.0 ± 6.2     | 2.0             | 87                     | 55.9                            |
|          | S2       | 119.7 ± 8.2     | 6.3             | 82                     | 53.6                            |
|          | P2       | 7.7 ± 2.3       | 0.2             | 6                      | 3.9                             |
|          | P3       | 8.4 ± 0.6       | 0.3             | 7                      | 4.3                             |
| TS       | WC       | 143.3 ± 11.9    | 1.7             | 139                    | 100.0                           |
|          | S1       | 125.4 ± 5.4     | 3.8             | 124                    | 89.2                            |
|          | P1       | 103.3 ± 0.2     | 0.2             | 10                     | 6.9                             |
|          | S2       | None detected   | —               | —                      | —                               |
|          | P2       | 9.4 ± 2.1       | 0.3             | 11                     | 7.8                             |
|          | P3       | 8.1 ± 1.1       | 0.2             | 9                      | 6.2                             |

* Cells were treated with 10-8 M colchicine for 2 h at 37°C before being fractionated.

| TABLE III | Distribution of Tubulin in Nondifferentiated Cells Determined by Radioimmunoassay |
|-----------|-----------------------------------------------|
| Treatment | Fraction | Tubulin (µg/ml) | % Total protein | Tubulin distribution % |
|-----------|----------|-----------------|-----------------|------------------------|
| MTS       | WC       | 93.4 ± 6.2      | 1.5             | 131                    |
|           | S1       | 96.6 ± 10.1     | 2.5             | 103                    |
|           | P1       | 23.9 ± 0.7      | 0.6             | 29                     |
|           | S2       | 16.1 ± 0.5      | 2.3             | 18                     |
|           | P2       | 6.8 ± 1.4       | 0.2             | 8                      |
|           | P3       | 10.0 ± 1.6      | 0.2             | 7                      |
| TS        | WC       | 149.2 ± 5.9     | 1.8             | 155                    |
|           | S1       | 139.5 ± 7.6     | 3.9             | 136                    |
|           | P1       | 14.3 ± 0.8      | 0.3             | 16                     |
|           | S2       | None detected   | —               | —                      |
|           | P2       | 11.5 ± 1.2      | 0.3             | 14                     |
|           | P3       | 13.2 ± 0.9      | 0.4             | 15                     |

Experimental fractions for each treatment were obtained from a total of 4 x 10^7 cells. Tubulin concentrations (µg/ml) are presented as the mean ± SD for values determined from the appropriate standard curve, and are corrected for dilution during sample preparation.

Experimental fractions for each treatment were obtained from a total of 4 x 10^7 cells. Tubulin concentrations (µg/ml) are presented as the mean ± SD for values determined from the appropriate standard curve, and are corrected for dilution during sample preparation.
undifferentiated cells caused no polymerization. Using the clone of neuroblastoma cells described in this report, we have recently defined conditions for the self-assembly of tubulin (19). In this system, in vitro polymerization proceeds readily in extracts from differentiated cells but is poor in extracts from nondifferentiated cells. In differentiated cells, we have found a high molecular weight protein (215,000) which copurifies with tubulin by assembly-disassembly; this protein is much reduced, and perhaps absent, in nondifferentiated cells (19). Because the distribution of tubulin in neuroblastoma cells can be reproducibly quantitated, we can now determine whether the equilibrium is directly affected by specific microtubule-associated proteins that appear during neurite elongation.

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