# PURIFICATION OF TUBULIN FROM NEUROBLASTOMA CELLS: ABSENCE OF COVALENTLY BOUND PHOSPHATE IN TUBULIN FROM NORMAL AND MORPHOLOGICALLY DIFFERENTIATED CELLS

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## 1. Introduction

The state of assembly of cytoplasmic microtubules is believed to be an important factor in the regulation of a number of cell functions [1]. The microtubules are composed primarily of tubulin, a 120 000 molecular weight heterodimeric protein. Morphological differentiation of neuroblastoma cells [2] or Chinese hamster ovary cells [3], and the accompanying appearance of microtubules, does not require protein synthesis. The assembly of microtubules from a pool of preformed tubulin subunits could be controlled by a covalent modification of the tubulin to a form with a different ability to assemble. Since phosphate has been found associated with tubulin [4,5] and since appropriate phosphokinase activities have been found in brain extracts [6], phosphorylation has been considered to be a possible control mechanism. Alternatively, assembly could be controlled by a non-covalent interaction of the subunits with some other cellular component [7,8].

To attempt to distinguish between these possibilities, we have prepared tubulin from neuroblastoma treated with a glial cell conditioned medium. This medium induces a high per centage of the cells to extend processes, but unlike other techniques does not affect the growth rate of the cells [9], making possible study of the effects of differentiation alone. We describe here a procedure for purification of neuroblastoma tubulin to approximately 85% homogeneity, with essentially quantitative recovery, under conditions where the colchicine binding activity of the tubulin is stabilized [10,11]. Covalently-bound phosphate is not detected in tubulin from either normal or differentiated cells, suggesting that phosphorylation is not essential for the control of microtubule assembly.

## 2. Materials and methods

Mouse neuroblastoma cells (NB-2a) and rat glioma cells (C-6) were grown as previously described [9]. For these experiments, $3 \times 10^5$ neuroblastoma cells were grown on 10 cm Falcon tissue culture dishes in normal medium for 16 h. The medium then was removed and either fresh medium or medium removed from 72 h cultures of C-6 cells was added. After 48 h under these conditions, about 5.0% of the cells in normal medium develop processes, while more than 75% of the cells in glia conditioned medium develop processes [9].

To prepare tubulin, the medium, was removed and the plates washed twice with 5 ml of 0.32 M sucrose, $10^{-3}$ M Tris-HCl, $5 \times 10^{-3}$ M $MgCl_2$, pH 7.0 (0°C). The cells were suspended with a rubber policeman in $5 \times 10^{-2}$ M pyrophosphoric acid (Fluka Chemicals), $1.9 \times 10^{-1}$ M NaCl, 1.0 M sucrose, adjusted to pH 7.0 with NaOH (PPSS buffer). After Dounce homo-

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genization and low speed centrifugation, a 100 000 *g* supernatant containing all the colchicine binding activity was prepared [10]. In PPSS buffer, the colchicine binding activity is stabilized and decays with a half-time of 220 h at 4°C [10,11]. Protein was measured by the method of Lowry [12]. QAE-Sephadex A-25 was prepared by suspending 0.25 g of the powdered gel in 10 ml PPSS and heating in a boiling water bath for 2 h. The suspension was cooled to room temperature under vacuum and centrifuged for 1 min at 2000 *g*. The pellet was resuspended in fresh cold PPSS and packed in a column of 0.9 cm diameter, and washed at 4°C with degassed PPSS for 1 h at 30 ml/h.

All the following operations were also performed at 4°C. A 3.0 ml aliquot of the supernatant was desalted on a Sephadex G-25 column (0.5 × 25 cm), equilibrated with degassed PPSS buffer at a flow rate of 20 ml/h and then loaded on the QAE-Sephadex column. The column was washed with 1.5 ml of PPSS buffer, and a linear gradient of total volume 15.0 ml, between PPSS buffer and the same buffer adjusted to pH 5.0, was applied, followed by 10.5 ml of the final buffer. Fractions of 1.5 ml were collected, and adjusted to $5.0 \times 10^{-3}$ M $MgCl_2$, $1.0 \times 10^{-4}$ M GTP, then neutralized to pH 6.95 ± 0.05 with 2 M Tris-HCl, pH 8.0. A 100 microliter aliquot of each fraction was assayed for colchicine binding activity [13].

To measure phosphate incorporation, the normal or glia conditioned medium added after 16 h incubation contained, in two different trials, 6.05 or 10.8 Ci $^{32}PO_4^{2-}$ (Eidg. Institut fur Reaktorforschung, Wurenligen, Switzerland) per mole of phosphate. Purification of both samples was completed within 8 h of harvesting cells. The fractions containing tubulin were dialyzed against 6 changes of 1000 vol of water at 4°C in 24 h, then lyophilized.

The mol. wt. of the tubulin was determined on SDS-polyacrylamide gels [14]. The phosphate incorporation was determined after electrophoresis on SDS-urea polyacrylamide gels [15] which separate the two polypeptide chains of the heterodimer [5]. The gels were stained in Coomassie Blue and scanned at 610 nm. Radioactive gels were cut into 1.1 mm slices and counted in 10 ml toluene-based scintillation fluid containing 3.5% Protosol (New England Nuclear), 4.3 g/liter PPO and 0.48 g/liter POPOP. The efficiency of this counting system was 78% as determined by polymerizing $^{32}P$-labelled protein (a gift from Dr E. D. Wachsmuth) into a gel.

## 3. Results and discussion

High speed supernatants prepared as described above from normal and morphologically differentiated cells have identical specific activities of colchicine binding, about $6 \times 10^5$ cpm/mg protein [10]. The half-lives and molecular weights of the binding activities in the two extracts are identical, (data not shown).

Desalting the supernatants does not affect the specific activities, but it is necessary for obtaining reproducible results on the ion exchange chromatography. Fig.1a shows the measured pH values of the eluting buffer as it enters the QAE column, and of the recovered fractions. The pH values shown are reproducible within 0.05 pH units. Fig.1b shows the elution of colchicine binding activity from the column. Most of the protein does not bind to the column. Recoveries of colchicine binding activity from the column vary between 75% to 100% without affecting the elution pattern.

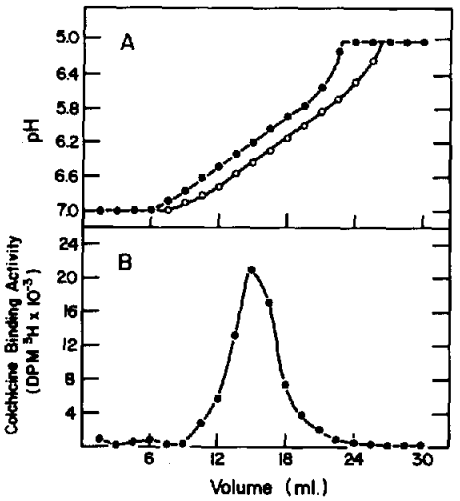


Fig.1. Elution of colchicine binding activity from QAE-Sephadex. An aliquot of high speed supernatant from morphologically differentiated cells was desalted as described, and then placed on the column. (A) The pH of the eluting buffer as it enters the column (closed circles) and of the fractions (open circles). (B) The colchicine binding activity in each fraction, from an aliquot assayed after neutralization.

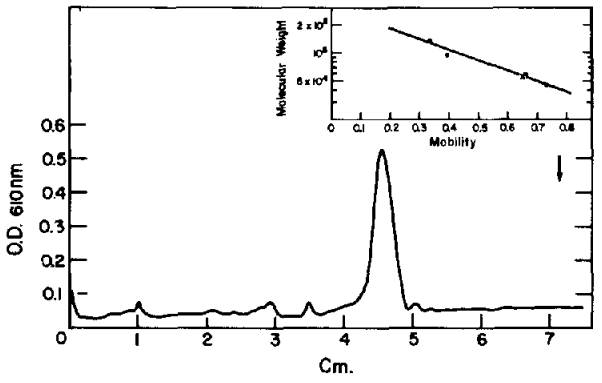


Fig.2. SDS-gel electrophoresis of QAE-Sephadex purified tubulin from differentiated cells. Peak fractions from the ion exchange elution in fig.1 were dialyzed, lyophilized, and then denatured in SDS. The gel was stained in Coomassie Blue, and cleared in 7.5% acetic acid, 10% methanol. (Inset) Mol. wt. determination of the major band eluted from QAE-Sephadex. The mobility of the major peak in fig.2 (×) was compared with the mobilities of standard proteins run simultaneously on a separate gel (○): cytochrome *c* (mol. wt. 11 300) ovalbumin (43 500), phosphorylase A (94 000) and β-galactosidase (136 000).

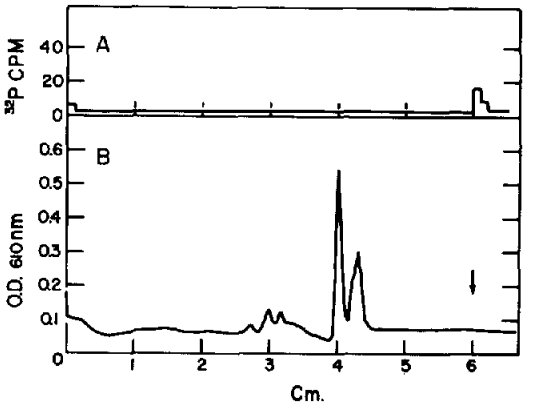


Fig.3. Urea-SDS polyacrylamide gels of tubulin from $^{32}$P-labelled differentiated neuroblastoma cells. Tubulin from the laterunning part of the peak (2.6 μg) was prepared for electrophoresis, and the gels treated as in Materials and methods. (A) Counts of $^{32}$P in 1.1 mm slices of the same gel. (B) Optical density scan, showing resolution of the two tubulin polypeptide chains.

SDS-gel electrophoresis of the fractions containing tubulin (fig.2) gives one major peak with a mobility corresponding to a mol. wt. of 55 000, which agrees well with the value reported elsewhere for the subunit chains [15]. Fractions taken from the peak in fig.1 and from the trailing edge of this peak have generally fewer contaminants than fractions from the leading edge.

Fig.3 shows the scan of a urea-SDS gel and the corresponding pattern of radioactive phosphate eluted from gel slices. This gel is of material eluted later from the ion-exchange column (fig.1, 15–18 ml) prepared from morphologically differentiated cells. Using the colchicine binding activity of the material as a measure of the amount of tubulin applied, and the radioactivity incorporated into the acid-precipitable nucleic acid as a measure of the specific activity of the phosphate, mole/mole incorporation of phosphate into tubulin would have given 210 cpm associated with the tubulin bands. In four separate experiments, and testing both early and late fractions from the ion exchange column, neither normal nor morphologically differentiated cells showed any phosphate associated with tubulin. We estimate, within the limits of the experiment, that there is less than 0.1 mol of phosphate per mole of tubulin from either cell type. This result suggests that phosphorylation of tubulin is not essential to microtubule assembly.

The difference between our result and the findings of others that tubulin is phosphorylated [4,5] can be explained by several possibilities. First, tubulin extracted from brain cells which are no longer proliferative may be phosphorylated for reasons not connected with their state of assembly. Second, the phosphate found associated with the tubulin may in fact be associated with a contaminant [16]. Third, there are reports of an in vitro phosphokinase activity which can utilize tubulin as an acceptor [6,17,18], and this activity may account for the phosphorylation found. Alternatively, loss of phosphate during purification cannot be ruled out. A phosphatase which can hydrolyze in vitro phosphorylated tubulin has been discovered [19], although the amount of that activity has been questioned [20]. The presence of such an enzyme in neuroblastoma at higher levels compared to brain and active under our experimental conditions is unlikely.

This method of purification with quantitative recovery of the colchicine binding activity represents a tool for further biochemical studies of free and polymerized tubulin from neuroblastoma cells.

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