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# Comparison of the Microtubule Proteins of Neuroblastoma Cells, Brain, and *Chlamydomonas* Flagella

**(tubulin/flagellar outer doublets/electron microscopy/acrylamide gel electrophoresis)**

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**ABSTRACT** **Intact A microtubules isolated from outer doublet microtubules of *Chlamydomonas* flagella contain two separable proteins (tubulins) that differ in molecular weight and in amino-acid composition. The microtubule protein isolated from brain or neuroblastoma cells also has two electrophoretically distinct tubulins. Although the two tubulins of brain and neuroblastoma cells are electrophoretically similar to each other, only one of these tubulins migrates with the flagellar tubulins. This is the first evidence that (*a*) isolated, morphologically intact, single microtubules from flagella contain at least two different tubulins, and (*b*) at least one of these tubulins differs from tubulins that are isolated from other sources.**

Recent work has stressed both the ultrastructural and biochemical similarity of microtubules found in such diverse sources as the mitotic apparatus, flagella and cilia, or brain (1–3). However, microtubules from these different sources show different degrees of stability to physical and chemical treatment. For example, most cytoplasmic microtubules dissociate rapidly after exposure to cold, pressure, or colchicine (4–7); in contrast, ciliary and flagellar microtubules are stable to these same treatments (6, 8–10). This report suggests that some of these variations in lability of microtubules may be accounted for by differences in microtubule protein (tubulin) composition. We have found that (*a*) single microtubules (A tubules), isolated from the outer doublet microtubules of flagella, contain at least two electrophoretically-separable tubulins¶ that have different molecular weights and amino-acid compositions; (*b*) purified microtubule protein isolated from brain or neuroblastoma cells also contains two tubulins; (*c*) the two tubulins of brain and neuroblastoma cells have similar electrophoretic mobilities, but only one of the two tubulins derived from neuroblastoma cells comigrates with a flagellar tubulin. Therefore, these results demonstrate the presence of three tubulins: one shared by neural microtubules and flagella, one unique to flagella, and one unique to neural tubules.

## MATERIALS AND METHODS

### Cell culture

*Neuroblastoma.* A murine neuroblastoma tumor line (C1300) was adapted to *in vitro* culture in 1967‡. A cloned line of this tumor (Neuro 2a) has been maintained in suspension or monolayer culture in Dulbecco-Vogt modification of Eagle's medium containing 10% newborn-calf serum (11).

*Chlamydomonas.* Wild-type *Chlamydomonas* (21 gr) was grown synchronously in Medium I of Sager and Granick (12) in aerated 5-liter diphtheria toxin bottles.

### Microtubule protein

*Neuroblastoma.* Microtubule protein was isolated from neuroblastoma cells by the vinblastine precipitation method described by Olmsted *et al.* (11, 13), or by the following batch DEAE-cellulose procedure. Neuroblastoma cells were homogenized in PK buffer [0.01 M potassium phosphate buffer (pH 6.8)–0.1 M KCl]. The homogenate was centrifuged at 105,000 × *g*. Two volumes of the supernatant were added to one volume of packed DE-52 cellulose equilibrated in PK buffer. Fractions were obtained by batchwise elution of the DEAE with 0.1, 0.3, 0.5, 0.7, and 1.0 M KCl in PK buffer. The 0.5 M fraction was used as a partially purified source of microtubule protein.

Radioactive microtubule [$^{14}$C]protein was obtained by feeding monolayered cultures with 1 $\mu$Ci/ml of [$^{14}$C]amino-acid mixture (New England Nuclear), 5 and 3 days before harvesting the cells and isolating the microtubule protein.

*Chlamydomonas flagella.* 12 liters of *Chlamydomonas* were harvested and the flagella were detached and purified§ (14). The flagellar membranes were removed and the 9 + 2 microtubular axonemes were fractionated to isolate the outer doublet microtubules and the single A tubules of the outer doublets§¶ (14). Radioactive microtubule [$^{3}$H]protein was isolated from flagella by deflagellation of cultures of *Chlamydomonas* (9) followed by complete regeneration in 100 $\mu$Ci/ml of [$^{3}$H]acetate. The radioactive flagella were then detached, isolated, and fractionated. By use of this procedure, flagellar tubulins of high specific activity were prepared.

*Brain.* Microtubule protein from porcine brain was purified by the method of Weisenberg *et al.* (15) and was the gift of Dr. M. Shelanski.

### Electrophoresis

*Urea gels.* Microtubule protein from flagella, brain, or neuroblastoma cells was reduced and alkylated by standard

Abbreviations: SDS, sodium dodecyl sulfate; PK buffer, 0.01 M potassium phosphate buffer (PH 6.8)–0.1 M KCl.

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‡ Klebe, R., and F. Ruddle, *J. Cell Biol.*, **43**, 69a (1969).

§ Witman, G. B., K. Carlson, J. Berliner, and J. Rosenbaum; submitted to *J. Cell Biol.*

¶ Witman, G. B., *J. Cell Biol.*, *47*, 229a (1970).

procedures (16) in the presence of 8 M urea. Electrophoresis was performed on 7.5% acrylamide gels containing 8 M urea [6 × 70 mm, 2.5 mA/gel (pH 8.3), at room temperature]. The gels were stained with Fast Green (17).

*SDS–urea gels.* The discontinuous gels ystem described above was used with the inclusion of 0.1% sodium dodecyl sulfate (SDS) in all gel solutions and buffers. Protein samples were reduced and alkylated as before, and SDS was added to 0.1%. SDS–urea gels were stained with Coomassie Blue.

**Densitometry and counting of gels**

Gels were scanned with a Joyce-Loebl microdensitometer (17) or a Gilford spectrophotometer with a gel-transport system

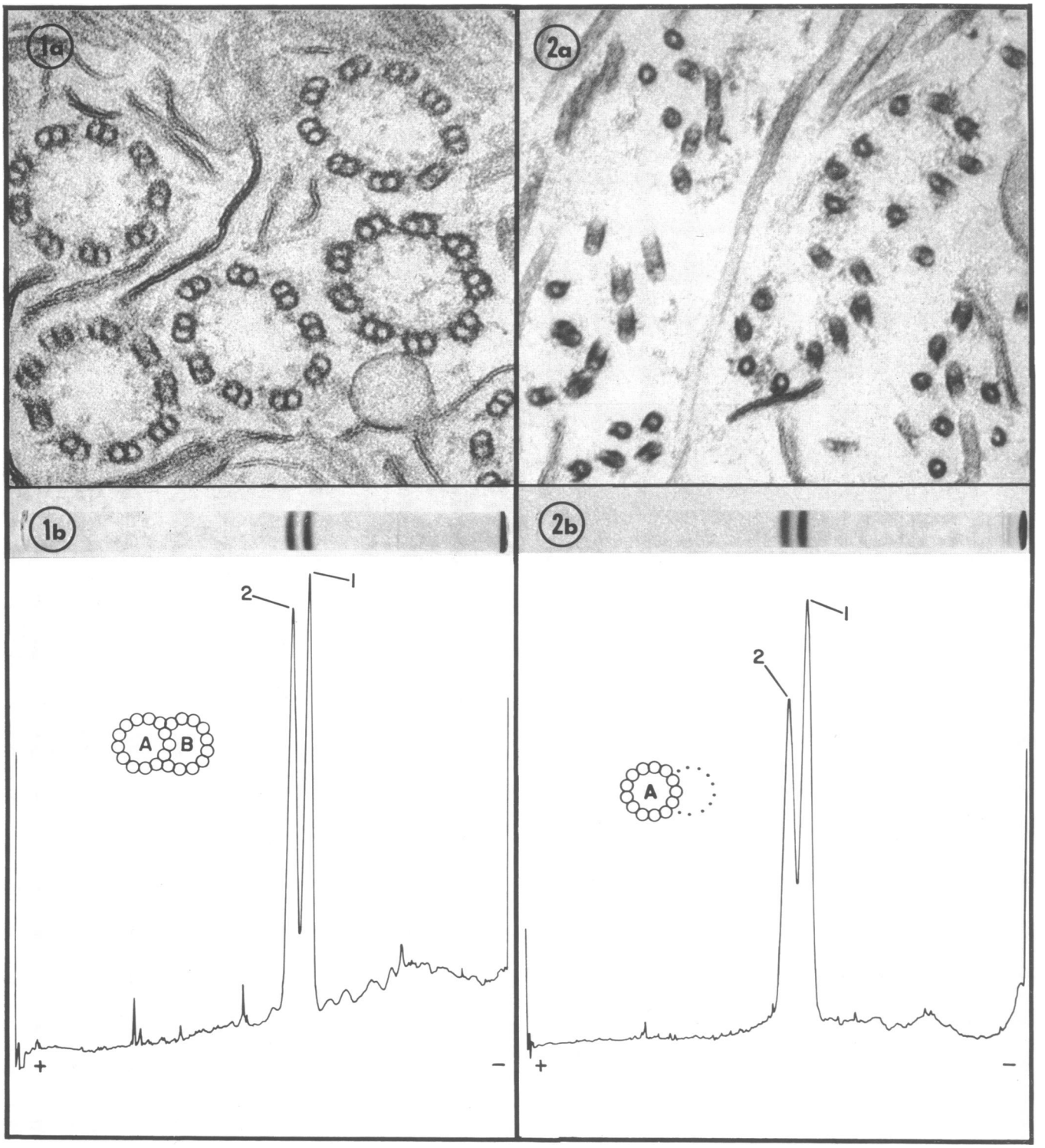


FIG. 1. Isolated intact outer doublets of *Chlamydomonas* flagella. (*a*) Electron micrograph, ×155,000. (*b*) Photograph and densitometric tracing of outer doublets on urea–acrylamide gel.

FIG. 2. Isolated intact *A*-tubules of *Chlamydomonas* outer doublets. (*a*) Electron micrograph, ×155,000. (*b*) Photograph and densitometric tracing of A-tubules on urea–acrylamide gel.

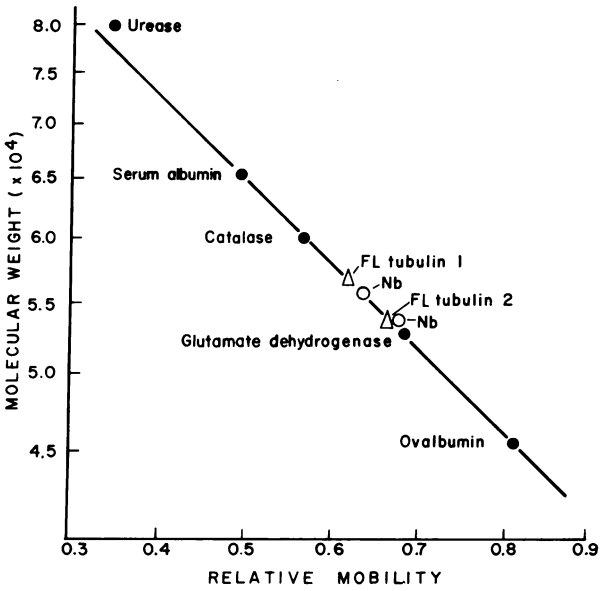


FIG. 3. Molecular weight determinations on SDS–urea acrylamide gels. Tubulin 1 and 2 isolated from *Chlamydomonas* outer doublets (*FL*); Nb is the tubulin isolated from neuroblastoma cells.

After densitometry, gels were sliced into 1-mm discs, solubilized with Nuclear Chicago Solubilizer (NCS) (18), and counted in Liquifluor (Nuclear-Chicago, Inc.).

### Separation of tubulins 1 and 2 of flagella

Urea-solubilized flagellar tubulins 1 and 2 were separated by cutting the appropriate bands out of urea-containing acrylamide gels, after locating the proteins with a fluorescent dye (19). More efficient separation of the tubulins could be obtained on DEAE cellulose–urea columns‖.

### Electron microscopy

Flagellar fractions were sedimented at 105,000 × *g* and the pellets were fixed *in situ* for 1 hr with 2.5% glutaraldehyde [in 5 mM phosphate buffer (pH 7.0), at 0°C], then with 1% $OsO_4$ in the same buffer. The intact pellets were flat-embedded in epon§ and the thin sections were examined in a Philips 200 electron microscope.

## RESULTS

The presence of two microtubule proteins in the outer doublets of cilia was first observed by Renaud *et al.* (16). Later work on flagella by Stephens (20) and by Jacobs and McVittie (21) suggested that one of the microtubule proteins ("A" tubulin) composed the *A* tubule of the outer doublets, while the other ("B" tubulin) made up the *B* tubule. Recent findings in our laboratory have confirmed that preparations of intact outer doublets (Fig. 1*a*) migrate as two distinct proteins on urea acrylamide gels (Fig. 1*b*). However, different results on the distribution of these tubulins within the outer doublets have been found with the use of selective solubilization procedures in conjunction with quantitative ultrastructural analysis and quantitative gel electrophoresis§¶ (14). These combined methods have clearly shown that *the two tubulins that can be separated by electrophoresis are found in both the A and B tubules.*¶ For example, pure preparations of intact *A* tubules (Fig. 2*a*) that were obtained by fractionation of the outer doublet microtubules can be seen to contain two tubulins when analyzed on acrylamide gels (Fig. 2*b*). Similarly, electrophoretic analysis of the microtubule protein of the *B* tubule, which is solubilized during the preparation of intact *A* tubules, also indicates that the two tubulins are present§ (14). Because of these findings, reference to these proteins as tubulin "A" and "B" according to the earlier terminology of Stephens (20), and Jacobs and McVittie (21) would be confusing. Therefore, we propose calling these proteins tubulins 1 and 2.

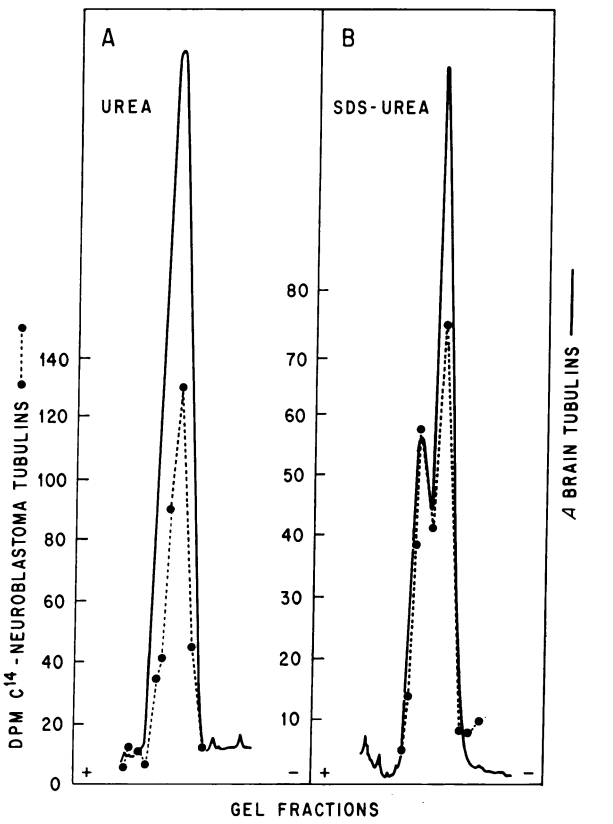


FIG. 4. The electrophoresis of brain and neuroblastoma microtubule protein on (*A*) urea and (*B*) SDS–urea acrylamide gels. Brain tubulin absorbance (——); $^{14}C$-labeled neuroblastoma tubulin (●—●). Radioactivity plotted as dpm/1-mm gel slice.

Tubulins 1 and 2 from intact *A* tubules or from outer doublets have also been separated on DEAE cellulose–urea columns. Analysis of the separated tubulins has shown that they have different amino acid compositions§. Molecular weight determinations by the method of Hedrick and Smith (22), on the SDS gels (23), or on SDS–urea gels (Fig. 3) indicate that the tubulins also have different molecular weights (about 56,000 for tubulin 1, and 53,000 for tubulin 2).

In contrast to flagellar outer doublets, which separate as two tubulins on urea gels, brain and neuroblastoma microtubule protein both migrate as a single band. However, the neutrally-derived proteins resolve into two bands that migrate closely when run on SDS–urea gels. Electrophoresis of unlabeled brain microtubule protein with $^{14}C$-labeled neuroblastoma microtubule protein shows that these tubulins have identical mobilities both urea (Fig. 4*a*) and SDS–urea gels (Fig. 4*b*).

‖ Carlson, K., and A. Telser, unpublished results.

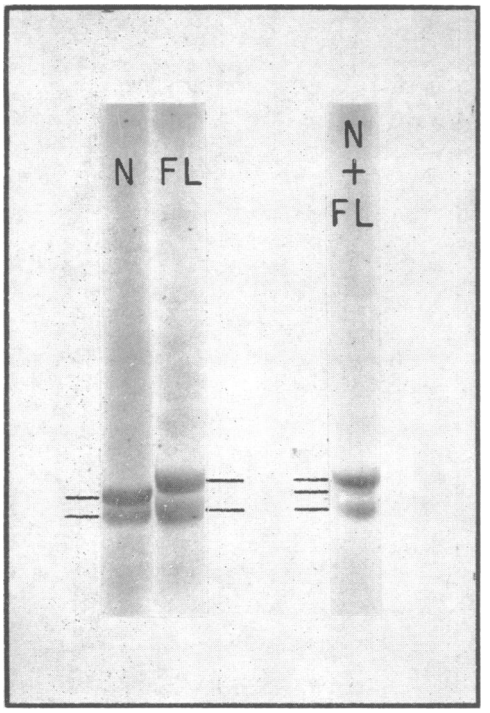


FIG. 5. Comparison of the migration of *Chlamydomonas* outer doublet and neuroblastoma microtubule proteins on SDS–urea acrylamide gels. *FL*—*Chlamydomonas* outer doublet tubulins 1 and 2; *N*—neuroblastoma tubulins; *N + FL*—coelectrophoresis of neuroblastoma tubulins with *Chlamydomonas* tubulins 1 and 2. Lines indicate migration distance of outer doublet and/or neuroblastoma tubulins.

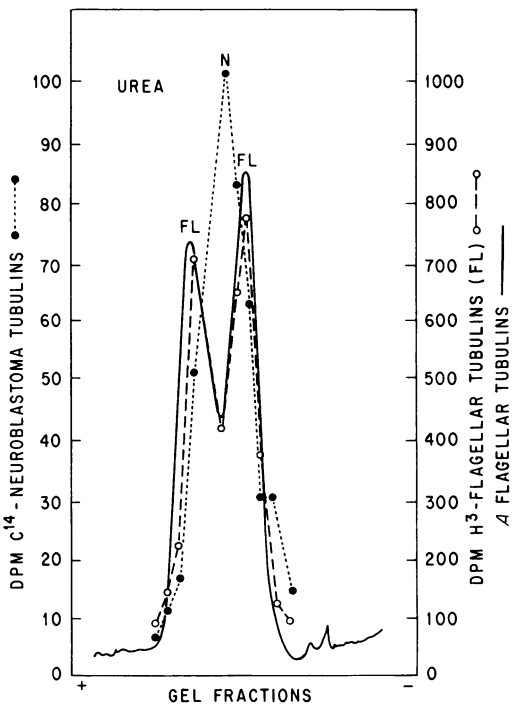


FIG. 6. Electrophoresis of *Chlamydomonas* outer doublet and neuroblastoma microtubule proteins on urea–acrylamide gels. *Chlamydomonas* tubulins: absorbance (——), dpm $^{3}H$ (O---O). Neuroblastoma tubulins: dpm $^{14}C$ (●---●). Radioactivity plotted as dpm/1-mm gel slice.

Although both the neural and flagellar microtubules have two tubulins, it was evident that the tubulins from flagella were more-widely separated than the tubulins from neuroblastoma cells or brain when these proteins were analyzed by electrophoresis under identical conditions (Fig. 5). In order to show this difference clearly, and to determine if either of the neural and flagellar tubulins comigrated, electrophoresis experiments were conducted with $^{14}C$-labeled neuroblastoma microtubule protein and $^{3}H$-labeled flagellar protein on urea and SDS–urea gels.

When very small, nonstaining quantities of $^{14}C$-labeled neuroblastoma tubulin were coelectrophoresed on urea gels with $^{3}H$-labeled flagellar tubulins, the pattern shown in Fig. 6 resulted. Neuroblastoma microtubule protein moves as one band on these urea gels, and the $^{14}C$ radioactivity clearly migrates *in between the $^{3}H$-labeled tubulins 1 and 2 of flagella***.

When $^{14}C$-labeled neuroblastoma and $^{3}H$-labeled flagellar tubulins were analyzed by electrophoresis on SDS–urea gels and stained with Coomassie blue, three tubulins were resolved. They consisted of the distinct tubulins 1 and 2 of flagella and a third tubulin that was resolved as a shoulder of the slower-moving tubulin 1 of the flagella. The radioactivity patterns of these gels confirm that there are three separate tubulins: one $^{3}H$-labeled flagellar tubulin, one $^{14}C$-labeled neuroblastoma tubulin, and one peak that contains both $^{14}C$-labeled tubulin and $^{3}H$-labeled flagellar tubulin 2 (Fig. 7). Comparison of the electrophoretic pattern of tubulins of flagella and neuroblastoma cells (or brain) therefore demonstrates that microtubule proteins from each source have one tubulin in common and one that is unique.

** It was originally reported that neuroblastoma microtubule protein comigrated with tubulin 2 on urea–acrylamide gels (see ref. 14). The corrected result reported is due to a refinement of the urea–acrylamide gel procedure.

## DISCUSSION

The localization of two tubulins in both the A and B tubules of flagellar outer doublets and in microtubule protein isolated from brain or neuroblastoma cells (also, mitotic apparatus, see ref. 24) raises the question of how tubulins are distributed within individual microtubules. Bryan and Wilson (25) have found that the two tubulins occur in equal amounts in microtubule protein purified from embryonic brain, and they have therefore suggested that these two tubulins may comprise the protein dimer of the microtubule. However, these results cannot exclude the possibility that individual brain microtubules might be made up of only *one* of the tubulins. We have therefore studied the problem of tubulin distribution within the microtubules of intact flagellar outer doublets. By use of sequential solubilization techniques, quantitative analysis of electron micrographs, and acrylamide gels, we have found that both tubulins 1 and 2 occur in almost equal amount in the A and B tubules§. These results would appear to support the suggestion of Bryan and Wilson that the two tubulins comprise the protein dimer of the microtubule. However, other findings indicate that *the three protofilaments of the A tubule wall that are shared with the B tubule (partition) contain only one tubulin*§¶ (14). Therefore, in at least the partition

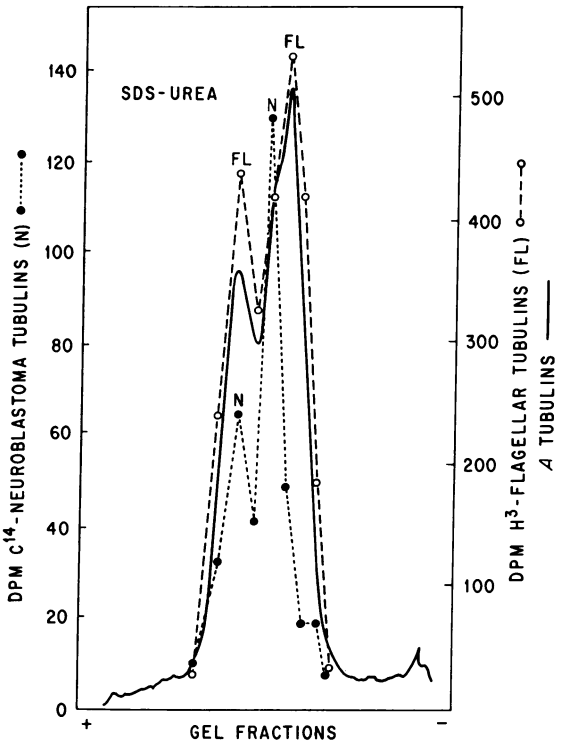


FIG. 7. Electrophoresis of outer doublet tubulins 1 and 2 and neuroblastoma tubulins on SDS-urea acrylamide gels. Tubulin absorbance (——); dpm $^3H$ outer doublet tubulins (O—O); dpm $^{14}C$ neuroblastoma tubulins (●—●). Radioactivity expressed as dpm/1-mm gel slice.

region of the *A* tubule, it does not appear that both tubulins could compose the protein dimer of the microtubule. However, since the flagellar outer doublets have a unique geometry and contain several specific attachment sites for accessory structures, the distribution of tubulins in the outer doublets may be different from that found in cytoplasmic microtubules.

The results presented in this paper indicate that the microtubule protein of both flagella and neural tissue consists of two tubulins. By electrophoretic criteria, one of these two tubulins is common to both microtubules sources and the other is unique. We suggest that the different stability properties of the two types of microtubules that were investigated may be partially accounted for by this diversity in tubulin composition.

## NOTE ADDED IN PROOF

Microtubule proteins from the outer doublets of *Chlamydomonas* flagella have recently been analyzed by isoelectric focusing on urea–acrylamide gels. This method demonstrates that the outer doublets contain at least five different proteins. The three "partition.' protofilaments are composed solely of one of these proteins (Whitman, G. B., K. Carlson, J. L. Rosenbaum, in preparation).

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