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Assembly of Chick Brain Tubulin onto Flagellar Microtubules from *Chlamydomonas* and Sea Urchin Sperm

(*in vitro* assembly/flagella/electron microscopy)

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ABSTRACT **Flagellar microtubules from *Chlamydomonas* and sea urchin sperm were used as *in vitro* assembly sites for chick brain tubulin. Brain microtubules assembled onto the A-tubules, central tubules, and, to a limited extent, onto the B-tubules of flagellar axonemes. Assembly occurred onto the distal ends of the axonemes at low tubulin concentrations and onto distal and proximal ends at high tubulin concentrations; however, the rate of assembly onto the distal end was always greater. The rate of neurotubule assembly onto axonemes was shown to be dependent upon tubulin concentration and a forward rate constant for assembly was determined.**

It has been possible to study a variety of aspects of microtubule protein synthesis and assembly *in vivo* by the use of systems of flagellar regeneration in flagellated protozoans (1, 2). Among these studies were those which demonstrated by use of light and electron microscopic autoradiography that the flagellum, and specifically the flagellar microtubules, assembled by the addition of subunits to their distal ends during flagellar elongation (tip growth) (1–3). Additional studies on the mechanisms controlling the directionality, rate, and initiation of flagellar microtubule assembly would be facilitated if means were available to assemble flagellar microtubules *in vitro*. As yet, this has not been possible. However, it has been possible to assemble brain microtubules (neurotubules) *in vitro* (4) and, because of the chemical similarity of tubulin isolated from a variety of organisms (5), studies were initiated on the assembly of brain tubulin subunits onto flagellar microtubules (axonemes) from *Chlamydomonas* and sea urchin sperm.

MATERIALS AND METHODS

Axoneme Isolation. Chlamydomonas reinhardi (strain 21 gr) was grown to a cell density of 1×10^6 cells per ml in Medium I of Sager and Granick (6) at 25°. Growth was with continuous aeration on a cycle of 13 hr of light and 11 hr of dark. The cells were harvested by centrifugation and washed once in 10 mM Tris·HCl buffer, pH 7.8, at 25°. All subsequent steps were carried out at 25°. The cells were then resuspended in either 0.5% Triton X-100 or 0.5% Nonidet P-40 (Shell), 1 mM ATP, 2 mM $MgSO_4$, 1 mM EDTA, 10 mM Tris, pH 7.8, or in 0.04% Nonidet P-40, 5 mM $MgSO_4$, 0.35 mM dithiothreitol, 0.5 mM EDTA, and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), pH 7.5. Treatment with either solution caused simultaneous flagellar detachment and demembranation. Five milliliters of the suspension of cells and axonemes were underlayed with 3 ml of 10% sucrose in 10 mM Tris, pH 7.8, and centrifuged on an IEC model HN centrifuge at 350 × *g* for 3 min, sedimenting the cells and leaving the axonemes above the sucrose. The layer above the sucrose was drawn off and centrifuged at 35,000 × *g* for 20 min to sediment the axonemes, which were resuspended in PM [1 mM GTP, 2 mM ethyleneglycol-bis-(β-aminoethylether)-*N*-*N'*-tetraacetic acid (EGTA), 1 mM $MgSO_4$, 100 mM piperazine-*N*-*N'*-bis(2-ethanesulfonic acid) (Pipes), pH 6.9] (4, 7) at 4°.

Spermatozoa from the sea urchin *Arbacia punctulata* were washed in calcium-free sea water and suspended in a solution containing 5 mM Tris, 0.5% Nonidet P-40, 2 mM $MgSO_4$, 1 mM ATP, 1 mM EDTA, pH 7.6. The flagella were detached and demembranated by this solution and the axonemes were then isolated by differential centrifugation (8) and suspended in PM.

Microtubule Protein Isolation. Crude (S-1) and partially purified (S-2) preparations of microtubule protein were obtained from brain homogenates of 1- to 3-day-old chicks by *in vitro* assembly as previously described (28). Quantitative sodium dodecyl sulfate/polyacrylamide gel electrophoresis (9–11) showed that S-1 contained about 25% tubulin while S-2 contained about 50–60% tubulin.

Assembly of Microtubules Onto Axonemes. Aliquots of axonemes from either *Chlamydomonas* or sea urchin spermatozoa were added to various concentrations of either crude (S-1) or purified (S-2) tubulin preparations. Incubation was carried out at 37° when S-1 was used as a source of tubulin subunits, and at 30° when S-2 was used. Samples taken at 2, 5, and 10 min during incubation were negatively stained directly or were fixed prior to negative staining.

Negative staining was carried out with either 1% uranyl acetate or neutralized 4% phosphotungstic acid in 0.4% sucrose. Fixation in 2% glutaraldehyde, 50 mM Pipes, pH 6.9, was employed to prevent breakage of the neurotubules which had assembled onto the axonemes and to permit phosphotungstic-acid-negative staining of the normally phosphotungstic-acid-labile neurotubules.

Negatively stained microtubules were observed with a Philips 201 electron microscope and lengths of neurotubules assembled onto the axonemes were measured from tracings made from enlarged negatives.

RESULTS

Identification of axonemal microtubules

Flagellar axonemes are composed of nine outer doublet microtubules surrounding a central pair of single microtubules. In negatively stained preparations of intact *Chlamydomonas*

Abbreviation: Pipes, piperazine-*N*-*N'*-bis(2-ethanesulfonic acid).

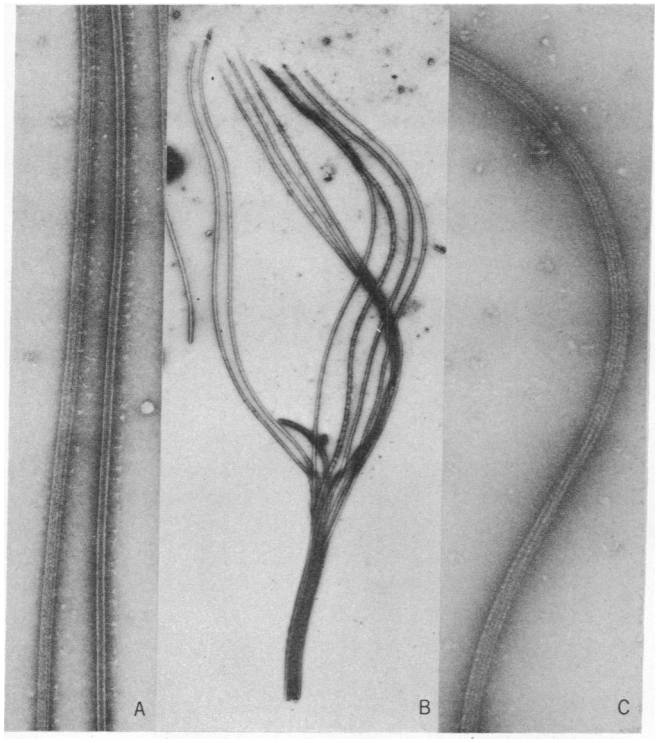

FIG. 1. Electron micrographs of flagellar microtubules from *Chlamydomonas* negatively stained with uranyl acetate: (A) outer doublet microtubules showing radial links on the A-tubule; (B) whole axoneme with frayed distal end and squared-off proximal end; (C) central pair microtubules with striated "sheath".

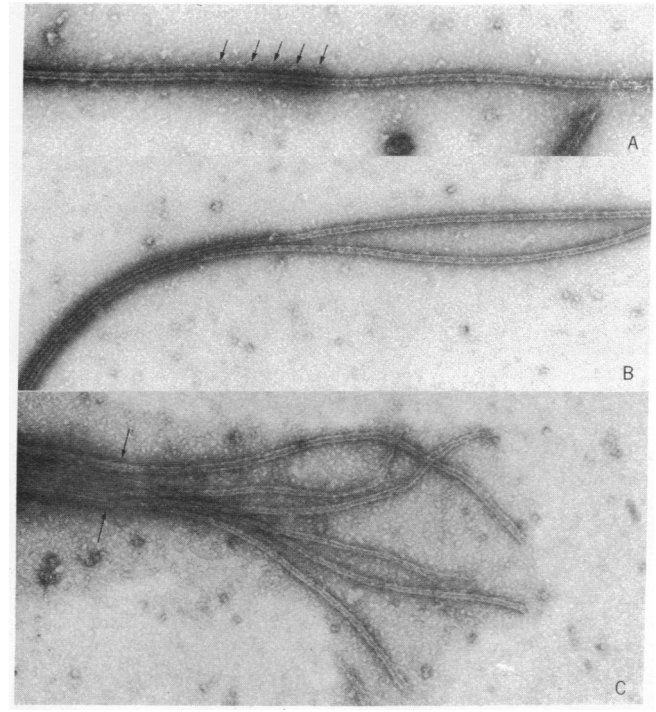

FIG. 2. Junctions of neurotubules with *Chlamydomonas* flagellar microtubules: (A) A-tubule of the outer doublet continuous with a lighter-staining neurotubule; the arrows indicate radial links on the A-tubule; (B) lighter-staining neurotubules assembled onto striated central pair microtubules; (C) neurotubules assembled onto the proximal end of a flagellar axoneme (arrows). Uranyl acetate negative stain.

axonemes, the outer doublet and central microtubules appeared splayed out only at the distal end of the axonemes (Fig. 1B) while at the proximal end the tubules remained evenly bundled together at the point of detachment from the basal body microtubules (12). The outer doublets could be differentiated from the central tubules by their double nature and by the presence of radial links attached to the A-tubules (Fig. 1A) (13). The central pair microtubules could be distinguished by small projections which gave them a striated appearance (Fig. 1C) (14).

Neurotubule assembly onto flagellar axonemes

When flagellar axonemes were incubated with chick brain tubulin, neurotubules assembled onto the axonemes. The neurotubules could be distinguished from the flagellar microtubules because the neurotubules dissociated upon treatment with cold or 5 mM $CaCl_2$ (Table 2) (4), and because they lacked the accessory structures characteristic of flagellar microtubules (radial links and striations; Fig. 1A and C). Moreover, flagellar tubules were always more densely stained then neurotubules (Fig. 2A and B; Fig. 3).

Neurotubule assembly occurred primarily onto the distal ends of *Chlamydomonas* axonemes when they were incubated with low concentrations of chick brain tubulin (S-1 or S-2) (Fig. 4A; Table 1). At higher concentrations, assembly occurred onto both proximal and distal ends of the axonemes, with distal assembly occurring at a greater rate (Fig. 4B; Table 1). Neurotubule assembly onto axonemes could be more clearly observed when preparations of purified tubulin (S-2) were used as sources of subunits rather than S-1, in which the high concentration of non-tubulin proteins interfered with negative staining.

Similar results were obtained when pieces of sea urchin axonemes were incubated with S-2 tubulin subunits. Assembly occurred primarily onto the distal ends of the axonemes at low tubulin concentrations and onto both ends if the subunit concentration was increased (Table 2).

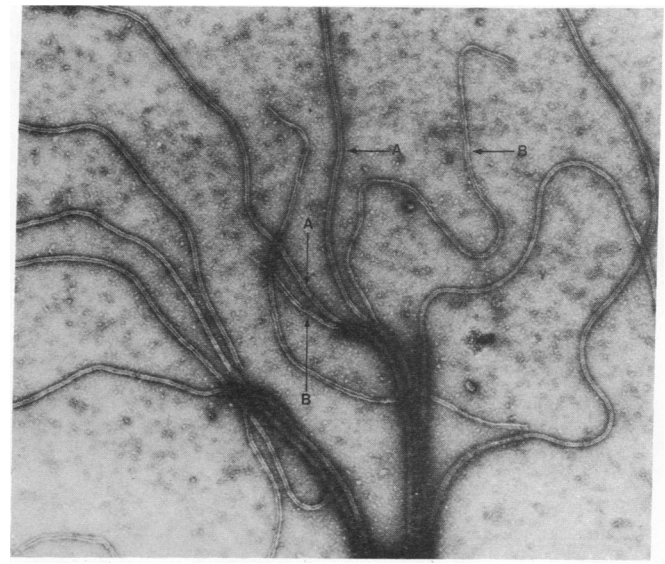

FIG. 3. Neurotubules (arrows) assembled onto the A- and B-tubules of flagellar outer doublets.

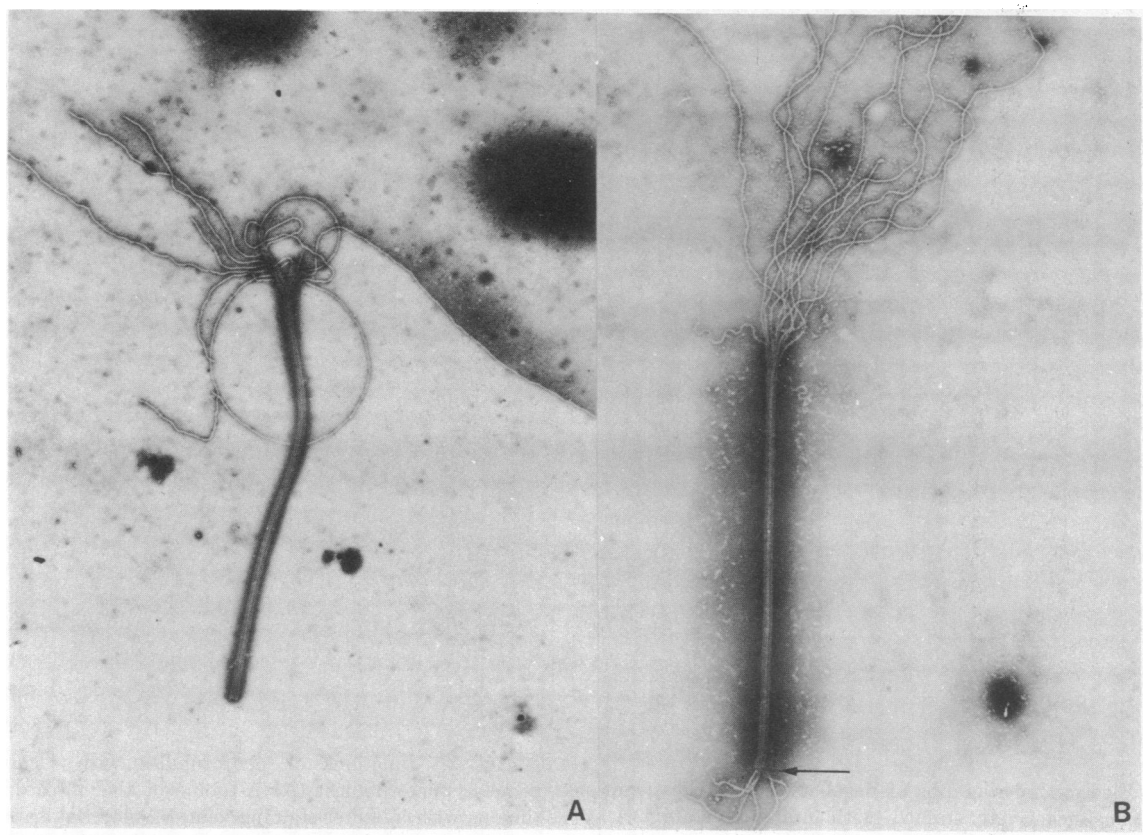

FIG. 4. *Chlamydomonas* axonemes incubated with: (A) low concentration of S-1 tubulin, showing distal assembly only; (B) high concentration of S-1 tubulin, showing bi-directional assembly with the distal end favored (see Table 1). Arrow indicates proximal end assembly.

Neurotubule assembly could be detected on the A- and central tubules of the axonemes (Fig. 2A and B). Neurotubules also assembled onto the B-tubules of the outer doublets, but these tubules had a smaller diameter than those assembled onto the A- and central tubules and were best preserved in glutaraldehyde-fixed preparations (Fig. 3).

Rates of neurotubule assembly onto sea urchin axonemes

To measure the rates of assembly of neurotubules onto both distal and proximal ends of sea urchin axonemes, constant amounts of axonemes were incubated with different concentrations of S-2 tubulin. At a tubulin concentration of 5 mg/ml, the neurotubules assembled onto the distal ends at a rate five times greater than onto the proximal ends; at 2.5 mg/ml, the rate of assembly onto the distal ends was eight times greater than onto the proximal ends (Fig. 5). The rate of neurotubule assembly onto the distal ends of axonemes was then investigated in greater detail. At each of four tubulin concentrations, the rate of distal assembly was constant (Fig. 6) and, because the rates were a linear function of tubulin concentration, the assembly of neurotubules onto the distal ends of axonemes could be expressed by the following relationship:

$$[M_p] + [S] \underset{k_1}{\overset{k_2}{\rightleftharpoons}} [M_pS] \qquad [1]$$

where $[M_p]$ is the concentration of distal ends of the individual flagellar microtubules; $[S]$ is the molar subunit concentration; k_1 is the reverse rate constant; and k_2 is the forward rate constant. It follows from this equation that the length change with time, dL/dt, is a direct function of the tubulin concentration, $[S]$, such that:

$$dL/dt = k_2[M_p][S]. \qquad [2]$$

Using this equation, the forward rate constant, k_2, could be calculated if it were assumed that: (*a*) The rate of depolymerization was very slow, such that reaction (1) proceeded almost entirely in the direction of polymer formation. (*b*) There are 13 protofilaments composing a microtubule (15) and, therefore, thirteen 110,000 dalton dimers per each 8 nm of microtubule [8 nm being the length of a single tubulin dimer (16)]. The

TABLE 1. *Directionality of assembly of brain tubulin onto Chlamydomonas flagellar axonemes*

Protein concentration (mg/ml)	Percent of axonemes with brain microtubules on distal end	Percent of axonemes with brain microtubules on proximal end
6.5	100	100
4.4	100	53
2.2	100	13
1.1	100	3

Chlamydomonas flagellar axonemes were incubated with crude brain tubulin (S-1, see *Methods*) for 5 min at 37°. At least 40 axonemes were scored at each protein concentration.

TABLE 2. *Directionality of assembly of brain tubulin onto axonemes from sea urchin sperm*

Concentration of brain tubulin (mg/ml)	Percent of axonemes with microtubules on distal end	Percent of axonemes with microtubules on proximal end
5	100	100
2.5	100	95
1.67	100	86
1.25	100	35
1	100	4
0.83	100	0
$CaCl_2$ added after assembly	0	0

Sea urchin sperm axonemes were incubated with brain tubulin (S-2, see *Methods*) for 5 min at 30°. At least 25 axonemes were scored at each tubulin concentration.

results in Table 3 show that the forward rate constant, k_2, is independent of tubulin concentration; therefore, one can take an average and calculate that the rate constant for distal neurotubule assembly onto axonemes is $1.53 \times 10^6\ M^{-1}\ sec^{-1}$.

DISCUSSION

The results in this report, showing that tubulin from chick brain assembles onto flagellar microtubules of the green alga *Chlamydomonas* and sea urchin sperm, demonstrate the evolutionary stability of tubulin subunit assembly sites. This has also been shown in recent studies in which brain tubulin was assembled onto the isolated, microtubule-depleted mitotic apparatuses of marine eggs (17, 18) and HeLa cells (19) as well as onto the isolated flagellar basal bodies of *Chlamydomonas* (20).

Neurotubule assembly onto flagellar microtubules was directional, proceeding principally from the distal end. At sufficiently high tubulin concentrations in both crude (S-1) and purified (S-2) preparations, neurotubules also assembled onto the proximal ends of axonemes, although this assembly proceeded at a much slower rate than distal assembly.

The distal directionality of neurotubule assembly onto axonemes *in vitro* supports the results obtained on the directionality of microtubule assembly during flagellar regeneration in *Ochromonas* and *Chlamydomonas* (1, 3). In these regeneration studies, it was shown by light and electron microscopic autoradiography that flagellar elongation occurred principally by distal (tip) assembly. In the present studies, there was also a small amount of proximal assembly of neurotubules onto axonemes at high tubulin concentrations; similar results were obtained when brain tubulin was assembled onto

TABLE 3. *Rate of assembly of brain tubulin (S-2) onto sea urchin flagellar axonemes*

Subunit concentration (mg of tubulin/ml)	Subunit concentration (μM)	Rate (μm/min)	k_2 ($\times 10^{-6}\ M^{-1}\ sec^{-1}$)
2.5	22.7	1.29	1.53
1.67	15.2	1.02	1.81
1.25	11.36	0.58	1.38
0.83	7.55	0.39	1.40

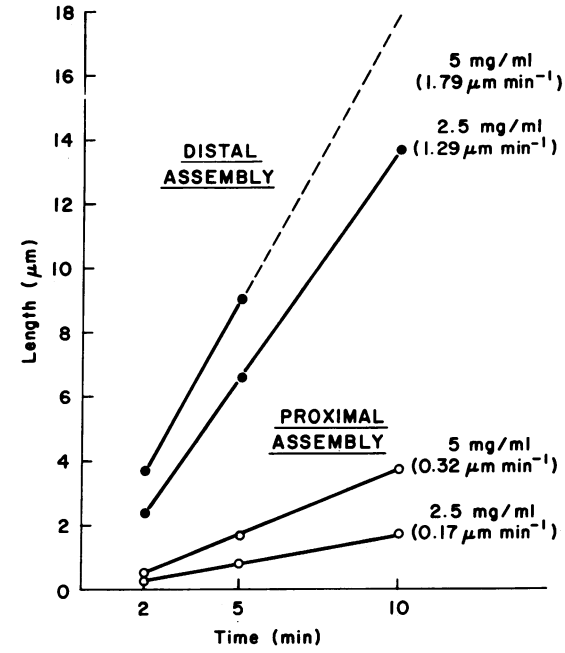

FIG. 5. Rates of assembly of neurotubules onto distal (●) and proximal (○) ends of sea urchin flagellar axonemes. The broken line represents an extrapolation, since the neurotubules at this time and concentration were too long to measure accurately.

isolated *Chlamydomonas* basal bodies (20). The significance of this *in vitro* proximal assembly in regard to the formation of flagellar microtubules *in vivo* is not known.

Neurotubule assembly occurred onto the axonemal A- and central microtubules, which contain 13 protofilaments, as well as onto the B-tubules of the outer doublets, which contain only 10 or 11 protofilaments (15) and are in the form of a C rather than a complete tubule. The neurotubules that assembled onto the B-tubules had a smaller diameter than those that assembled onto the A- or central tubules, suggesting that they had fewer than 13 protofilaments. It will be inter-

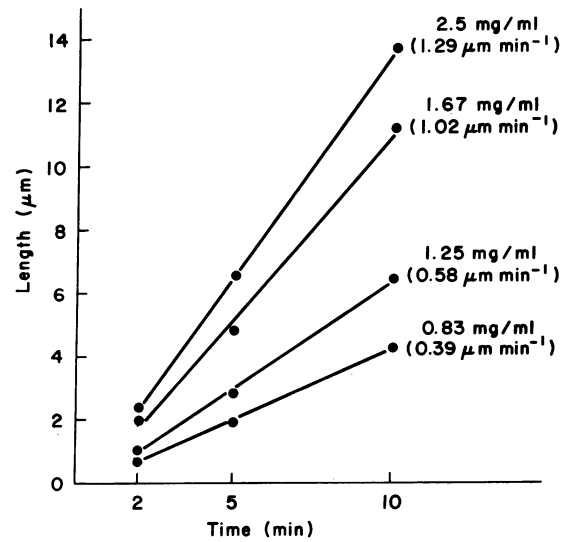

FIG. 6. Rates of neurotubule assembly onto distal ends of sea urchin axonemes at four tubulin concentrations.

esting to determine how many protofilaments B-tubule-associated neurotubules actually contain, since most current models of microtubule structure are based on an odd number of protofilaments (15, 21).

In no case did the neurotubules that assembled onto the A- and B-tubules of the outer doublets (Fig. 3) form intact doublet microtubules, i.e., fused large and small diameter neurotubules sharing a common wall of three protofilaments (22). This suggests that at least one class of assembly sites present on the flagellar tubulins does not exist on the tubulins from chick brain. These may be the sites on the outside of the A-tubule protofilaments that are responsible for the lateral attachment of the B-tubule protofilaments to the A-tubule.

Although chick brain tubulin can assemble onto flagellar microtubules, there are some obvious differences in the microtubules from the two sources. The most prominent of these is the stability of flagellar tubules to a variety of treatments (cold, calcium, and pressure) (23) in comparison to neurotubules, which are labile under the same conditions. Also, different types of accessory structures are attached to the flagellar tubules and neurotubules. The flagellar tubules have arms and links (13, 14) while the neurotubules have instead, a fine, filamentous coating both *in situ* (24–27) and *in vitro* (28, 29). It will be interesting to determine if these accessory structures are interchangeable between flagellar tubules and neurotubules.

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