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Polarity of some motility-related microtubules

(cilia/flagella/axopodium/melanophore)

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ABSTRACT **We have investigated the structural polarity of microtubules from several systems in which these fibers are thought to contribute to cell motility. By using a method for displaying microtubule polarity in the electron microscope, we find that both the A and B subfibers of *Tetrahymena* ciliary outer doublets and the inner pair of single microtubules are all oriented with their plus ends (i.e., their fast-growing ends) distal to the basal body. All of the microtubules in the axopodia of the heliozoan *Actinosphaerium* and all of the microtubules in the processes of melanophores from the angelfish *Pterophyllum* are likewise oriented with their plus ends distal to the cell centers. These results suggest that cellular systems for motility, and even those capable of bidirectional motility, can be constructed from microtubules of a single polarity.**

Numerous machines for cellular and subcellular motility include microtubules as a major fibrous component (reviewed in refs. 1–4). Cilia and flagella are the best-studied examples. In these organelles microtubules constitute a framework upon which a variety of enzymes and crosslinks are assembled to build a machine that transduces the chemical energy of ATP into the mechanical work necessary to slide the microtubules (5). The sliding movement is in turn converted to a bending movement by the radial crosslinks (for a review, see ref. 6). The mitotic spindle and many structures associated with intracellular granule motions are also composed in part of microtubules, but in these cases the mechanisms of force generation are far from clear (reviewed in refs. 4, 7, and 8). The paradigm for many cases of cellular motility has grown from studies on vertebrate striated muscle in which one type of fiber includes an ATPase (myosin) that will interact cyclically with a protein component of an adjacent fiber (actin) to release chemical energy and generate a force for fiber sliding. In this system, the orientation of the structural macromolecules is critical for their interaction and capacity to do work. Huxley (9) was able to determine the relative orientations of actin and myosin within the sarcomere and, thereby, to define constraints on the permissible ways to think about the mechanochemistry of muscle. An analogous approach has been adopted by students of cell motility, who have used Huxley's discovery that proteolytic fragments of myosin will bind to actin-containing fibers and reveal their polarity (10). The resulting determinations of microfilament polarity have been of considerable importance in defining the machinery built from muscle-like proteins in nonmuscle cells (11–13).

Microtubules are constructed by a head-to-tail polymerization of asymmetric subunits in a fashion reminiscent of actin, so they too possess an intrinsic structural polarity (14–18). Just as the study of actin fiber polarity has provided information about mechanism in muscle-related biological machines, it is plausible that the study of microtubule polarity could help to elucidate microtubule-related motility. Unfortunately, microtubule polarity is not visible directly by electron microscopy, and some additional information has been necessary to determine which way a microtubule points. Many microtubules are initiated by organizing centers visible by electron microscopy, such as mitotic centers, and orientation relative to specific structures has been used as one indicator of microtubule polarity (for example, see ref. 19). Microtubules grow faster at one end than at the other (20, 21), and this property has been used as an experimental probe for microtubule polarity (17, 18, 22). Microtubules of cilia and flagella sometimes bind the ATPase dynein in an asymmetric orientation which imparts a visible polarity to the aggregate (21, 23). This property has been used by Haimo *et al.* (24) to initiate the use of flagellar dynein as a probe for the polarity of cytoplasmic microtubules. None of these methods has reached the levels of convenience and dependability offered by the use of myosin fragments to decorate actin filaments.

More recently, a simple method for revealing microtubule polarity has been described in which neurotubulin is used to elongate and decorate cytoplasmic microtubules under unusual polymerization conditions (25). These conditions promote the formation of an abnormal junction between microtubule protofilaments (26) whereby cytoplasmic microtubules bind C-shaped fragments of neurotubule wall (27). In cross section, the decorated cytoplasmic microtubules appear as circles with one or more hooks protruding from their surfaces and curving either clockwise or counterclockwise. We have found that when a microtubule is in transverse view looking towards its minus (slowly growing) end (15, 18), about 90% of all hooks seen curve clockwise. The method has been applied successfully to basal bodies and the mitotic aster (25), the midbody of animal cells, and the phragmoplast of plant cells (27) to determine the polarities of their component microtubules. This paper describes the results of using the method to look at three subcellular structures in which microtubules are associated with motility: cilia, heliozoan axopodia, and melanophore cell processes. We have chosen these well-studied systems because we hope that a knowledge of their microtubule polarity, combined with other data already available from them, may help to elucidate the contribution that microtubules make to cellular motility.

MATERIALS AND METHODS

Tubulin Preparation. Microtubule protein was prepared from bovine brain by a modification of the method of Shelanski *et al.* (28). A high-speed supernate of microtubule protein depolymerized in 0.5 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.9/1 mM EDTA/1 mM $MgCl_2$/1 mM GTP (buffer A) (27) was used in all hook-growing experiments. We will call this preparation "tubulin."

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Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate.

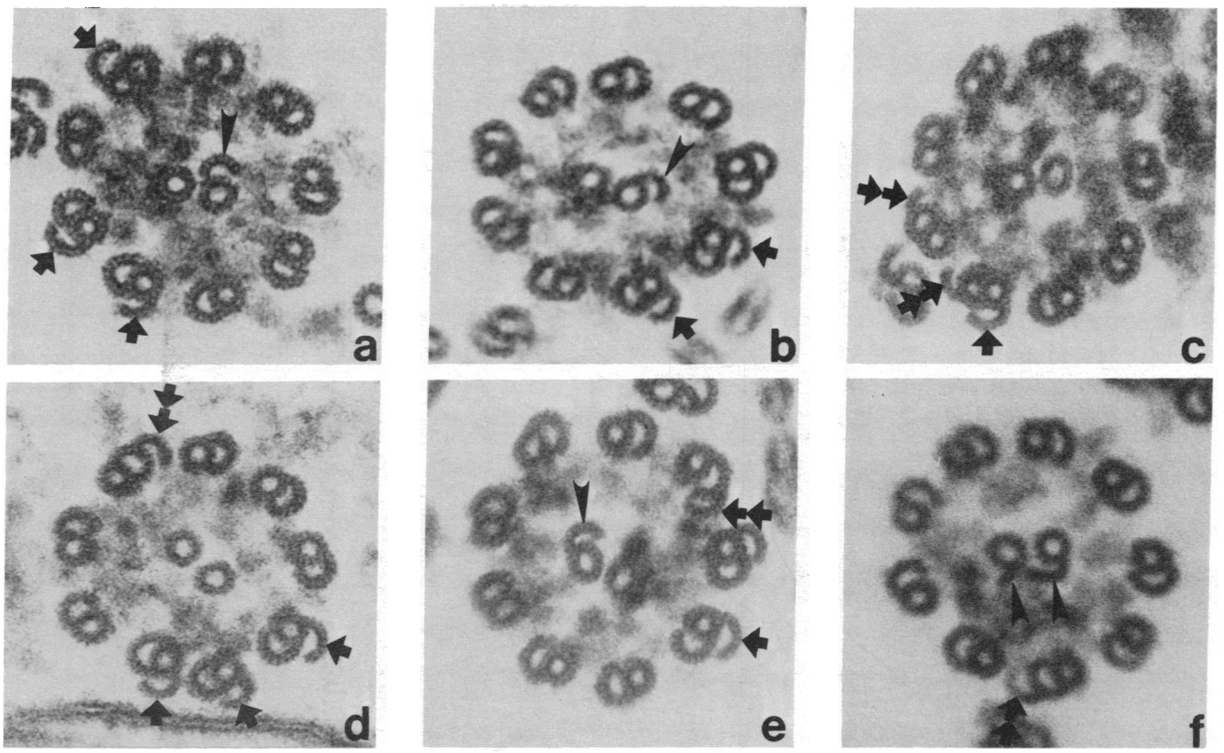

FIG. 1. Transverse sections of six cilia, each viewed looking toward the basal body. Hooks on A subfibers (arrows; *a–e*); hooks on B subfibers (double arrows; *c–f*); hooks on central-pair microtubules (arrowheads; *a, b, e,* and *f*). All hooks curve clockwise. (×190,000.)

Ciliary Microtubules. *Tetrahymena pyriformis* was grown to midlogarithmic phase by standard methods (27) and deciliated by treatment with 4 mM dibucaine in fresh medium (29). Isolated cilia were washed in 0.1 M Pipes/1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate (EGTA)/1 mM $MgCl_2$ (buffer B) either with or without Triton X-100. For growth of hooks, the cilia were incubated in buffer A, 2.5% (vol/vol) dimethyl sulfoxide, and 2.0 mg of tubulin per ml for 20 min at 37°C.

Axopodial Microtubules. *Actinosphaerium nucleofilum* was obtained from Carolina Biological Supply (Burlington, NC). Individual animals were selected under a stereomicroscope and transferred by pipette to a hook-forming medium at 25°C for about 20 min. The final composition of this buffer was 0.5 M Pipes/1 mM $MgCl_2$/40 mM EGTA/1 mM GTP/1.5% Triton X-100/2.5% dimethyl sulfoxide/1.5 mg of tubulin per ml. The high EGTA concentration prevented rapid disruption of the axopodia by the lysis conditions.

Melanophore Microtubules. Melanophores were isolated from scales of the angelfish *Pterophyllum scalare* by published procedures (30). Cells attached to coverslips were rinsed in buffer B and then incubated in buffer A containing 0.5 mg of tubulin per ml, 2.5% dimethyl sulfoxide, 1% Triton X-165, 0.1% deoxycholate, and 0.2% sodium dodecyl sulfate (25) at 37°C for 20 min.

Electron Microscopy. After their incubation in hook-forming buffers, cilia, heliozoa, and melanophores were fixed and embedded as described (27). All specimens were sectioned on a Sorvall ultramicrotome and observed in a JEOL 100C electron microscope. From sectioning to printing, we were careful to invert the specimen an even number of times to preserve the correct handedness of the structures. The direction of hook curvature was scored on prints with a final magnification of ×30,000–60,000.

RESULTS

Cilia. Any transverse view of a cilium can be identified unambiguously as a view either toward or away from the basal body on the basis of its fine structure: looking toward the basal body all the B subfibers lie on the clockwise-facing surface of their respective A subfibers (31). In the same view, according to the results of Heidemann and McIntosh (25), hooks should curve

Table 1. Number of hooks on ciliary microtubules

Cilia	A subfiber	B subfiber	Central-pair
a	2	1	
b	1	2	
c	3	1	
d	2	1	
e	4	2	2
f	3	1	1
g	3		1
h	4		1
i			1
j	3		1
k	2	1	
l	1		1
m	2	2	
n	2		1
o	2	1	1
p	2		1
q	3		1
r	1		1
s		1	1
Total	40	13	14

Data from 19 different cilia. Only cilia with hooks on either B subfiber or central-pair microtubules were taken into account. All cilia were viewed looking toward the basal body; all hooks were curved clockwise.

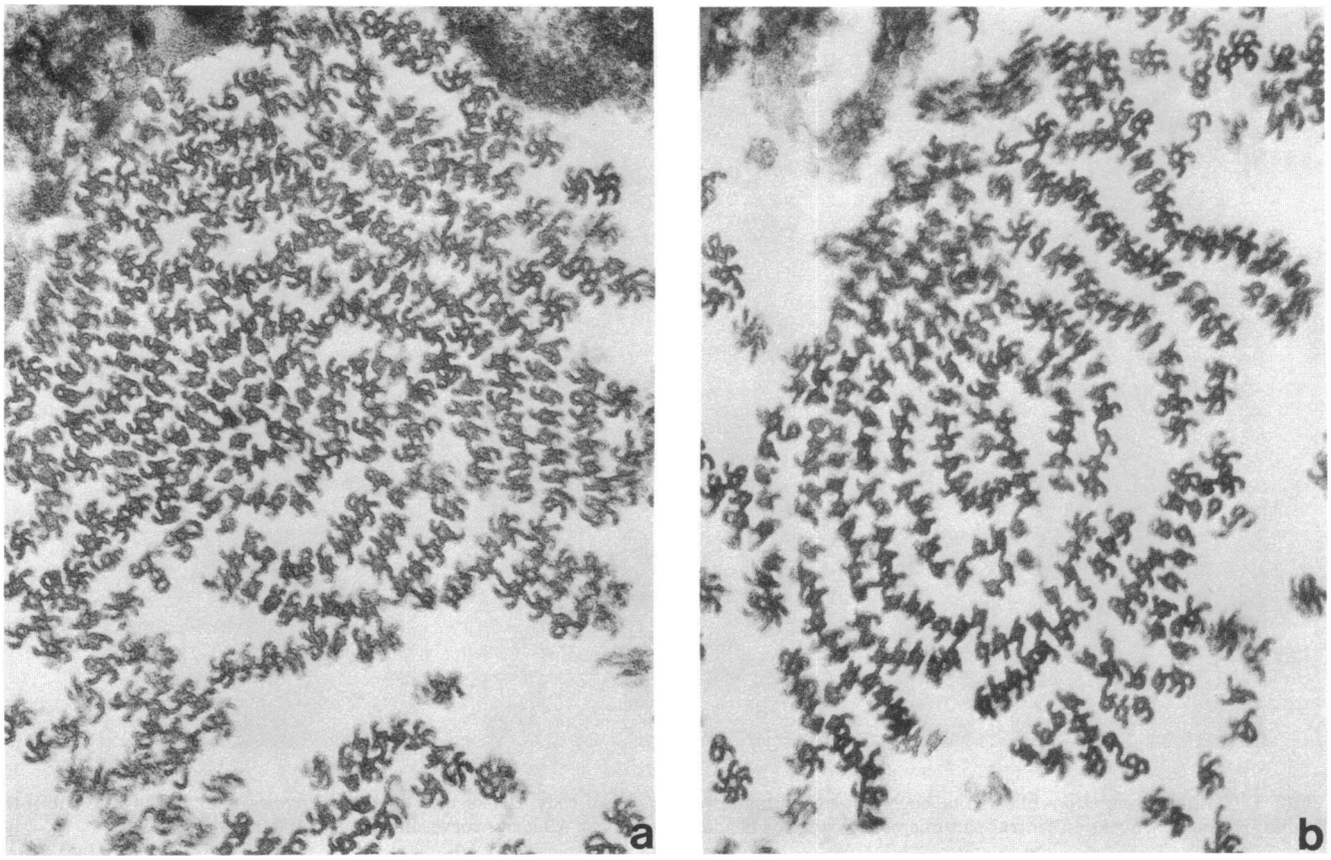

FIG. 2. Transverse sections from two axopodia. Both are cut on the way toward the center of the cell. All hooks curve clockwise. (×68,000.)

clockwise about the A subfiber. Fig. 1 is a set of ciliary cross sections. All images are presented in such a way that one is looking toward the basal body. One or more of the microtubules in each cilium are decorated by a hook-shaped fragment of neurotubule wall. Most of the hooks are found attached to A subfibers (data not shown). Hooks on central-pair microtubules and B subfibers are less frequent, but all hooks that we have found on any ciliary microtubule curve clockwise when the cilium is viewed looking toward its basal body (Table 1). We conclude that all the ciliary microtubules (A subfiber, B subfiber, and central-pair microtubules) have the same polarity: the plus (or fast-growing) ends are distal.

Axopodia. Fig. 2 shows two cross sections of lysed axopodia of *Actinosphaerium*. The lysis buffer preserves much of the native axoneme geometry, although in the seconds before death, many cells withdraw their axopodia at least part way—a characteristic shock response in this organism. The view shown in Fig. 2 is that seen looking along the axis of the axopodium toward the cell center. Most hooks curve clockwise, suggesting that the plus ends of the microtubules in the axopodium are distal (Table 2). For unknown reasons, we have found a high frequency of decoration and a very consistent hook curvature in all the axopodia.

Table 2. Number of hooks on axopodian microtubules

	Counter-clockwise hooks		Clockwise hooks		
Cell	No.	%	No.	%	Total
a_i	0	0	122	100.0	122
a_o	684	98.3	12	1.7	696
b_i	7	0.3	2696	99.7	2703
c_i	4	0.7	608	99.3	612

Data from three cells: a, b, and c. The subscripts i and o indicate the region of the cell body under examination: i implies sections taken on the way in toward the cell center, o implies sections cut on the way out.

Melanophores. Fig. 3 shows transverse sections through the process of melanophores in which the granules are dispersed. The view is outward from the cell center, and the prevalent direction of hook curvature is counterclockwise. Table 3 presents our observations on the frequencies of hook curvature from several cells. We have examined both the microtubules that lie immediately beneath the plasma membrane and those that are nearer the axis of the cell process. There is no distinction between these microtubules on the basis of their relative frequency of bearing clockwise or counterclockwise hooks. We conclude that by our assay, almost all of the microtubules in the process of the *Pterophyllum* melanophore are oriented with their plus ends distal to the cell center.

DISCUSSION

Our results indicate that in the three systems studied, all of the microtubules are oriented with the same end distal to the center of the cell. In cilia, it has been determined that the distal end is the "plus end" [i.e., the fast-growing end (20, 21)], so we infer that all of the microtubules studied here are oriented with their plus ends distal to the cell center.

The A and B subfibers of cilia are transiently bridged by the ATPase dynein to generate a sliding force. These parallel tubules must possess the right geometry to allow the specific molecular interactions that link dynein to tubulin in a force-producing manner (5). At first sight, the active development of force between parallel microtubules might seem to be a violation of the symmetry considerations from which the potential

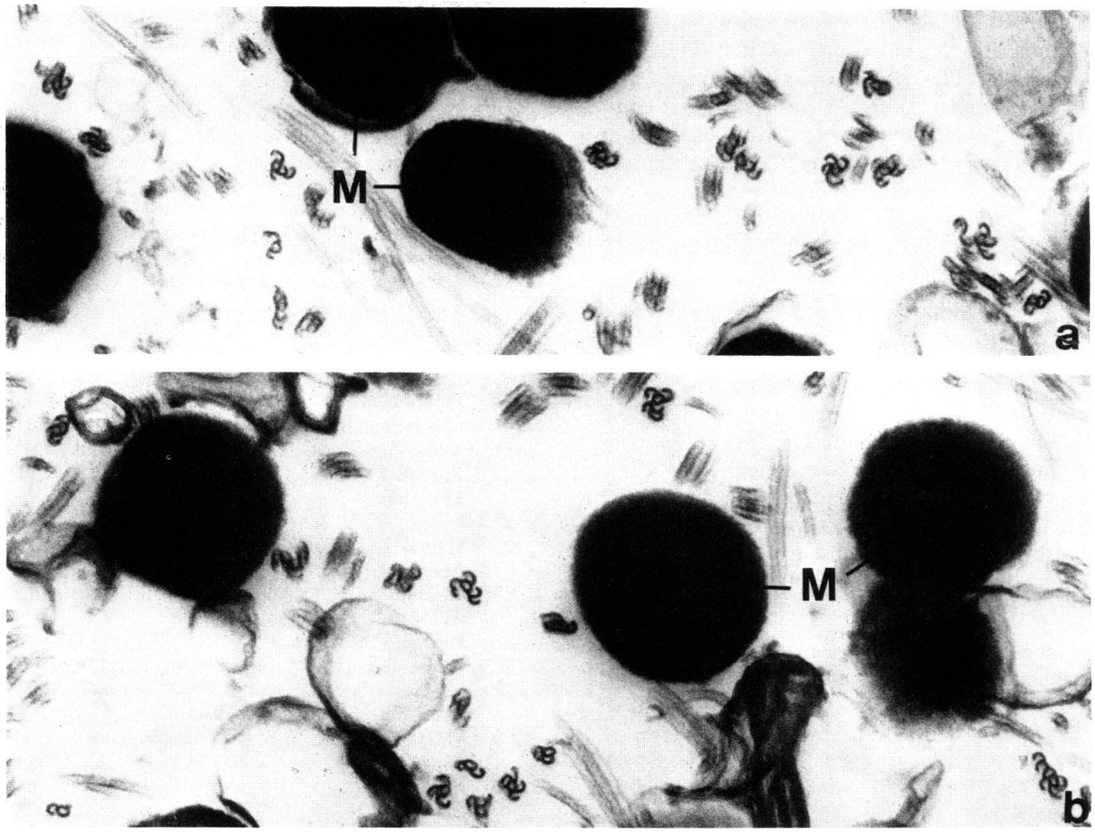

FIG. 3. Transverse sections through two melanophore cell processes, cut on the way out from the cell center toward the periphery. All hooks curve counterclockwise. M, melanosomes. (×50,000.)

importance of antiparallel microtubules for mitosis has been inferred (14). By these arguments, it is impossible for two identical, parallel microtubules interconnected by mechanochemical links to generate a net force on one another; it would be like two standing people of identical strength pushing down on each other's shoulders to try to lift themselves up into the air. Cilia probably overcome this constraint by the dissimilarity of the proteins that make up their microtubules. Stephens (32) has shown a difference in the amino acid compositions of the polypeptides comprising the A subfibers, B subfibers, and central-pair microtubules of sea urchin flagella. We infer that these proteins are different in such a way that their binding sites for the two ends of the relevant crosslinking structures are distinct. The fact that dynein exists as an arm protruding from the A subfiber in a relaxed cilium confirms the dissimilarity of functionally significant dynein-bonding domains on the tubulins of the A and B subfibers.

Table 3. Number of hooks on melanophore microtubules

Cell	Counter-clockwise hooks		Clockwise hooks		Total
	No.	%	No.	%	
a_i	17	7.3	216	92.7	233
a_o	301	94.7	17	5.3	318
b_i	16	7.9	187	92.1	203
b_o	169	90.4	18	9.6	187
c_i	14	8.0	161	92.0	175
c_o	314	96.0	13	4.0	327

Data from three cells: a, b, and c. The subscripts i and o indicate the cell region under examination: i implies sections taken on the way in toward the cell center, o implies sections cut on the way out.

Kirschner (33) has proposed that all cellular microtubules which comprise significant cytoplasmic structures are initiated by microtubule organizing centers and that the microtubules will be found to be oriented with their plus ends distal to the organizing centers. This proposal is derived from his realization that the "treadmill" behavior of microtubules *in vitro* (34) should render free microtubules unstable in comparison to microtubules with their minus ends blocked by association with a microtubule organizing center. Our results on the polarities of microtubules appear to agree with Kirschner's prediction. The central pair of microtubules from cilia may, however, be an exception. The distal ends of these microtubules are often embedded in a membrane-associated bit of amorphous, darkly staining stuff resembling the pericentriolar material of the cell center (35). This fact, combined with the persistent observation that during regrowth of flagella from isotopically labeled *Chlamydomonas* a small amount of label is incorporated at the proximal end of the flagellum (36), has led to the suggestion that the central pair of ciliary microtubules might grow by addition of tubulin at the proximal end (37). Our polarity studies identify this as the slowly growing end of these microtubules. If subunits really add at the minus end, the central-pair microtubules of cilia would differ from Kirschner's prediction.

The action of dynein in flagella and the obvious mechanical analogy between microtubules with bound ATPase and the myosin-containing thick filament of muscle have led to the proposal that granule motion in association with microtubules might be accomplished by a cyclic binding and release of an appropriate receptor on the granule membrane by a dynein-like molecule associated with cytoplasmic microtubules (14). A theme in such a model is that microtubule polarity would define the direction in which the granule is pushed. In both the heliozoan and the melanophore, granules move in two directions

along bundles of microtubules that we can now identify as possessing a single polarity. We must conclude that the action of a single set of flagellum-like dynein molecules cannot account for granule motility. Indeed, Edds (38) has shown that granule motion in heliozoa does not require a microtubule-built axopodium at all. Experiments with melanophores, on the other hand, implicate microtubules as one piece of a multicomponent motility system. Colchicine applied to these cells in culture removes their capacity for organized granule movement (39). Upon stimulation of granule migration in the presence of this drug, there is an increase in the local granule activity, but the absence of microtubules seems to disrupt the system to the point that there is little net movement. This evidence, combined with our observations on microtubule polarity, suggests that the microtubules define a framework upon which the granule-moving system can be organized. The microtubules may quite literally play a "cytoskeletal" role, constituting a set of vectorial bones, whereas other cell components such as actin microfilaments, microtubule-associated proteins, or microtrabeculae (40) constitute the cellular muscle (41, 42). Elucidation of the interactions between all these components promises to be a challenging but rewarding task.

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