ISOLATION AND REACTIVATION OF THE AXOSTYLE

Evidence for a Dynein-like ATPase in the Axostyle

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ABSTRACT

The contractile axostyle is a ribbon-shaped organelle present in certain species of flagellates found in the hindgut of wood eating insects. This organelle propagates an undulatory wave whose motion, like flagella and cilia, is related to microtubules. Unlike the axoneme of cilia and flagella, however, the axostyle is composed of singlet microtubules linked together in parallel rows. Axostyles were isolated from *Cryptocercus* gut protozoa with Triton X-100. Normal motility of the isolated axostyle could be restored with adenosine triphosphate (ATP); the specific conditions necessary for this reactivation were essentially identical with those reported for the reactivation of isolated flagella or whole sperm. ATPase activity of the isolated axostyle was comparable to the values reported for ciliary or flagellar axonemes. The axostyle was reasonably specific for ATP. Most of the proteins of the isolated axostyle comigrated with proteins of the ciliary axoneme on sodium dodecyl sulfate (SDS) polyacrylamide gels (i.e. equivalent molecular weights). These included the following: the higher molecular weight component of dynein, tubulin, linkage protein (nexin), and various secondary proteins. Evidence for dynein in the axostyle is presented and a model proposed to explain how repeated propagated waves can be generated.

INTRODUCTION

Although many instances have now been described relating microtubules to motility, there is remarkably little known about the mechanics of this motility or, for that matter, what proteins besides tubulin are involved in this motion. The best understood example of microtubule related motility is, of course, that of cilia and flagella. The ATPase (dynein) involved has been isolated and characterized and the motion has been analyzed through reactivation studies of the isolated axoneme. Yet even for cilia and flagella there are many unexplained facets about the mechanism of motility: why, for example, an isolated axoneme does not go into rigor upon the addition of ATP but instead propagates a wave; how the dynein arms might induce the sliding of doublets past each other; and whether or not conformational changes might occur as well as sliding.

While the mechanism of motion of the ciliary and flagellar axoneme is poorly understood, there are many other motile systems also involving microtubules of which virtually nothing is known about the mechanism of motion. Excluding instances involving changes in cell shape, examples include the following: chromosomal movement during mitosis or meiosis, pigment migration in melanophores of teleosts (Bikle et al., 1966), undulatory motion of certain insect and flatworm sperm which do not contain or use flagella (Robison, 1972; Silveira and Porter, 1964), rapid contraction of the cell body of certain ciliates (Huang, 1970), cytokinesis in *Chlamydomonas* (Johnson and Porter, 1968), cytoplasmic streaming such as occurs in the *Suctoria* (Rudzinska, 1965), and motion of certain organelles present in protozoa such as the cytopharyngeal basket of *Nassula* or *Phascolo-*

don (Tucker, 1968, 1972) or the contractile axostyle of flagellates living in the hindgut of wood eating roaches or termites (Grassé, 1956; Grimstone and Cleveland, 1965). Although the microtubules in all of the above systems appear as singlets, not as doublets as in cilia and flagella, the motion generated in many of the systems is similar to the undulatory motion characteristic of cilia and flagella. Furthermore, the tubules such as those in the undulatory axostyles are not arranged radially, as in flagella, but rather in linear rows.

Obviously one would like to know if there are biochemical similarities between the above systems and the 9 + 2 axoneme. More specifically, does dynein, or an ATPase comparable to that present in cilia and flagella, exist in any of the above systems? Furthermore, in order to approach the mechanism of motion we would like to be able to reactivate one of the above systems in vitro as has been done for the axoneme. Hopefully such a comparison would add to our present understanding of microtubule-related motility. We chose as our experimental material the contractile axostyle because, unlike many of the other systems mentioned above, workable amounts of material could be isolated. We have found that the axostyle is strikingly similar in its behavior and biochemistry to the axoneme. This suggests that the motion in these two organelles, and perhaps in many of the above systems as well, has a similar molecular basis.

MATERIALS AND METHODS

Collection and Maintenance of Cryptocercus

Cryptocercus punctulatus was collected from the Mountain Lake Biological Station, Mountain Lake, Va., most frequently from partially rotted hemlock and chestnut logs, although small numbers were present in oak as well. In the laboratory they were housed in plastic boxes to which partially rotten wood had been added. Moisture was kept high by covering the rotten wood with moist filter paper which had to be replenished as it is eaten by *Cryptocercus*.

Isolation of Protozoa from the Hindgut

Several days before sacrifice, *Cryptocercus* were kept in finger bowls containing moist filter paper as a food source. The hindguts of two to four roaches were removed and the protozoa freed by agitation of the gut in 15 ml of isolation media. The medium consisted of 23 mM Na_2HPO_4, 15 mM NaH_2PO_4, 10 mM KCl, and 1 mM $(NH_4)_2SO_4$, to which 11 ml of 1% $CaCl_2$ and 1.2 ml of 0.1% $MgSO_4$ were added dropwise to each liter of the above. The suspension of protozoa was spun at low speed to remove gut fragments, large particulate matter, and the larger species of flagellates which do not contain axostyles, e.g. *Barbulanympha*. The remainder was then pelleted from the supernatant in an International clinical centrifuge (1500 rpm for 2 min) (International Equipment Company, Needham Heights, Mass.). The pellet was washed and repelleted as above. This washing procedure was repeated several times and was critical in keeping bacterial contamination to a minimum.

Isolation of the Axostyles

(All operations at 4°C.) The pellet of gut protozoa was suspended in 15 ml of *Tx* solution: 1.0% Triton X-100, 3 mM $MgCl_2$, 30 mM Tris-HCl, pH 7.5 (Stephens and Linck, 1969). This procedure disrupts the cells, leaving a suspension of cellulose fibers, cell debris, and axostyles free of their limiting membranes. The axostyles were isolated from this suspension using a simple centrifugation procedure. The bulk of the particulate matter was removed from the suspension by a low speed centrifugation in the clinical centrifuge (900 rpm for 2 min). Most of the axostyles remained in the supernatant. The pellet, however, also contained some axostyles; these were recovered by resuspending the pellet in *Tx* solution and repeating the above centrifuge procedure until most of the axostyles remained in supernatant fractions. These supernatants were then combined and the axostyles collected by centrifugation in the clinical centrifuge (3200 rpm for 10 min). The resulting pellet consisted of large numbers of axostyles; the only contaminant was cellulose or wood fibers, depending on the roaches' diet.

SDS Polyacrylamide Gel Electrophoresis

The isolated axostyle preparation, isolated scallop ciliary axonemes (prepared after Stephens and Linck, 1969), axonemes from sea urchin sperm, and proteins of known molecular weights as standards, were heated for 2 min at 100°C in 1.0% SDS, 1.5% mercaptoethanol, 1.0 mM phenyl methyl sulfonyl chloride, and 10 mM phosphate buffer at pH 7.0 (Shapiro et al., 1967; Weber and Osborn, 1969) and run on 5% polyacrylamide gels containing 0.1% SDS. The gels were calibrated with bovine serum albumin (mol wt 68,000 and 136,000), chick actin (46,000), aldolase (40,000), and ciliary or flagellar axonemes (tubulin, 55,000, and dynein, 460,000 and 500,000 [Linck, 1971]). Bromphenol blue was used as a tracking dye. Gels were stained with Coomassie blue (Weber and Osborn, 1969). The following preparations were run as controls: (*a*) whole organisms from the gut which include

organisms with axostyles and those without, (*b*) *Barbulanympha*, a large flagellate containing numerous flagella but no contractile axostyle.

Axostyles Individually Isolated and Run on SDS Gel Electrophoresis in Capillary Tubes

Before we arrived at the isolation procedures described above we were concerned about contamination from the flagella of organisms such as *Barbulanympha*. To eliminate this problem axostyles were individually collected with a micropipette under the high power of the dissecting microscope. This was carried out in a 2°C cold room. Isolated axostyles (800–1000 axostyles per 0.01 ml solution) were run on 4% SDS polyacrylamide gels cast in 1-mm diameter tubing. Gel preparations of the axostyles and standards were carried out essentially as above. Approximately 3–5 λ of solution was layered on each gel. Protein standards were used in concentrations of less than 0.01 mg/ml. Electrophoresis was conducted at a running voltage of 1.25 v/tube. Gels were stained as above in Coomassie blue for 30 min.

Reactivation of the Axostyle

Axostyles were isolated as above and stored on ice as a suspension in 0.2 mM ethylenediaminetetraacetate (EDTA), 10 mM Tris-HCl (Schwarz/Mann, Orangeburg, N. Y.), pH 7.5. A drop of this suspension was placed on a slide at 22°C and the following reactivating solution, developed by Gibbons et al. (1970) and Gibbons and Gibbons (1972), was perfused through the slide mount: 2.0 mM nucleotide (adenosine triphosphate [ATP], guanosine triphosphate [GTP], inosine triphosphate [ITP], or adenosine diphosphate [ADP]), 4.0 mM divalent cation ($MgCl_2$, $MnCl_2$, or $CaCl_2$), 0.15 M KCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, and 10 mM Tris-HCl, pH 7.5. Sodium pyrophosphate was substituted for nucleotide in one experiment. The pH range of reactivation was determined by varying the Tris-HCl buffers from pH 6.5 to pH 8.5.

A second method was also used to reactivate axostyles, primarily for the study of axostylar motility. This method consisted of mixing drops of protozoan suspension and *Tx* solution on a perfusion slide mount. When the axostyles on the slide appeared free of limiting membranes and all motion had ceased, a drop of the above reactivating solution was added to the slide mount. This method differed from the first in that *cell lysis material was present*

ATPase and GTPase Activity of the Isolated Axostyle

Samples of the axostyle preparation (0.2 ml) were pipetted into 5 ml of assay mixture consisting of 0.9 mM ATP or GTP, 1.2 mM $MgSO_4$, 0.15 mM EDTA, 27 mM Tris-HCl, pH 7.8 at 22°C (Gibbons, 1966). Samples (2.0 ml) were taken at 0 and 20 min and enzymatic activity stopped by addition of 0.2 ml of 50% trichloracetic acid. Inorganic phosphate was determined by the method of Taussky and Schorr (1953). The protein concentration of the axostyle preparations was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Both the axostyle preparations and standards were made in 1.0% SDS and heated for 2 min at 100°C. The axostyle preparation was then centrifuged to remove insoluble materials (e.g. cellulose) which could interfere with the accuracy of the assay.

Light Microscopy

Living specimens and the isolated axostyle were studied with polarization, phase contrast and Nomarski interference microscopy using Zeiss optics. Movies of beating axostyles both in living organisms and during reactivation were taken with a 16 mm Bolex camera (Paillard Bolex, Sainte-Croix, Switzerland) attached to the Zeiss microscope. With a mercury arc lamp sufficient illumination was present to use either phase contrast or Nomarski interference at camera speeds of 18–64 frames per second.

Electron Microscopy

The isolated axostyles were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.6 for 30 min. They were postfixed in 1% OsO_4 in 0.1 M cacodylate at pH 7.5 for 45 min, dehydrated rapidly in acetone, and embedded in Araldite. Thin sections were cut on a Servall Porter-Blum ultramicrotome II, stained with uranyl acetate and lead citrate, and viewed with a Philips EM 200 electron microscope.

RESULTS

Purity of the Isolated Axostyle Preparations

Preparations were assessed for purity with light (Fig. 1) and electron microscopy (Fig. 2). The major contaminant was either wood chips or cellulose depending on the diet of the roaches. Bacteria sometimes contaminated the preparations. Thorough washing of the gut protozoa before treatment with *Tx* greatly reduced this contamination. Electron microscopy of the isolated axostyle pellet revealed that most of the axostyles (about 80%) are free from their limiting membranes. The four flagella which are attached to the axostyle at the anterior end of the organism often remain attached

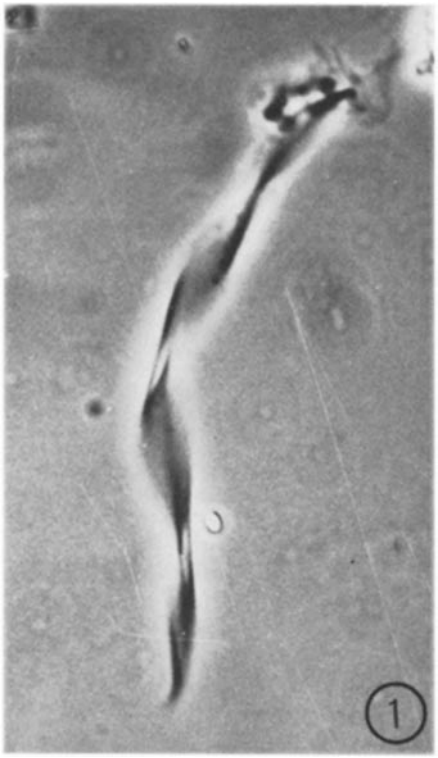

FIGURE 1 Light micrograph of an isolated axostyle taken with phase contrast microscopy. Flagella project from the anterior end of the axostyle (upper right). × 1000.

to the axostyle and comprise the major flagellar contaminant of the preparations. The amount of this contamination relative to the size of the axostyle is less than 1%. Fragments of *Barbulanympha*, which would increase this contamination, rarely occurred in our preparations. Even so, we had an internal check for contamination: on SDS gels of ciliary or flagellar axonemes there are two dynein bands, but the isolated axostyle has only one. This will be discussed in more detail below

SDS Gel Electrophoresis of the Isolated Axostyle

It is obvious from Fig. 3 that 5% SDS gels of isolated axostyles (on the left) and ciliary axonemes (on the right) are quite similar in that most axostylar proteins comigrate with proteins on the axonemal gel. The approximate molecular weights of the principal axostylar proteins (i.e. prominent bands on the gel) are as follows: 500,000 (same molecular weight as the higher molecular weight subunit of dynein; see Linck, 1971), 120,000, 80,000, 55,000, 30,000–35,000, and 20,000–25,000. There are also very faint bands corresponding to molecular weights of about 250,000, 125,000, and 100,000. The very intense band on the axostyle gel (and the cilia gel) is tubulin. This tubulin band often separates into two distinct bands of 55,000 and 52,000 when phenyl methyl sulfonyl chloride (a proteolysis inhibitor) is omitted in preparing the protein samples. The 120,000 band is probably a dimer of tubulin because this band increases greatly in intensity when the protein sample is not reduced before electrophoresis. Gels of *Barbulanympha* reveal two prominent bands, 500,000 and 460,000; these bands have been attributed to dynein on gels of ciliary or flagellar axonemes (Linck, 1971).

Small Gel Electrophoresis in Capillary Tubes

In order to insure that the above results were not simply artifacts of contamination, individually isolated axostyles were run on small SDS polyacrylamide gels. Two bands were observed: one corresponding to tubulin, the other to the upper band of dynein of cilia or flagella. These gels were similar to standard size gels of axostylar preparations of low protein concentration.

Reactivation of the Axostyle

Nearly 100% reactivation of isolated axostyles could be achieved using a reactivating solution containing ATP and Mg^{++} (Gibbons and Gibbons, 1972). Some characteristics of this reactivation, including pH range, nucleotide and cation requirements, are as follows.

PH RANGE: Reactivation could be achieved over a wide pH range, pH 6.5–pH 8.2, with an optimum range of pH 7.5–pH 8.0. At pH's above or below the effective range the axostyles began to fall apart.

DIVALENT CATION REQUIREMENTS: No motion could be generated in the absence of divalent cations. Mg^{++} (in the presence of ATP) gave the best reactivation both in quality and quantity of axostyles reactivated. Ca^{++} was generally ineffective and inhibited Mg^{++} activated reactivation. Mn^{++} gave results intermediate between those of Ca^{++} and Mg^{++}.

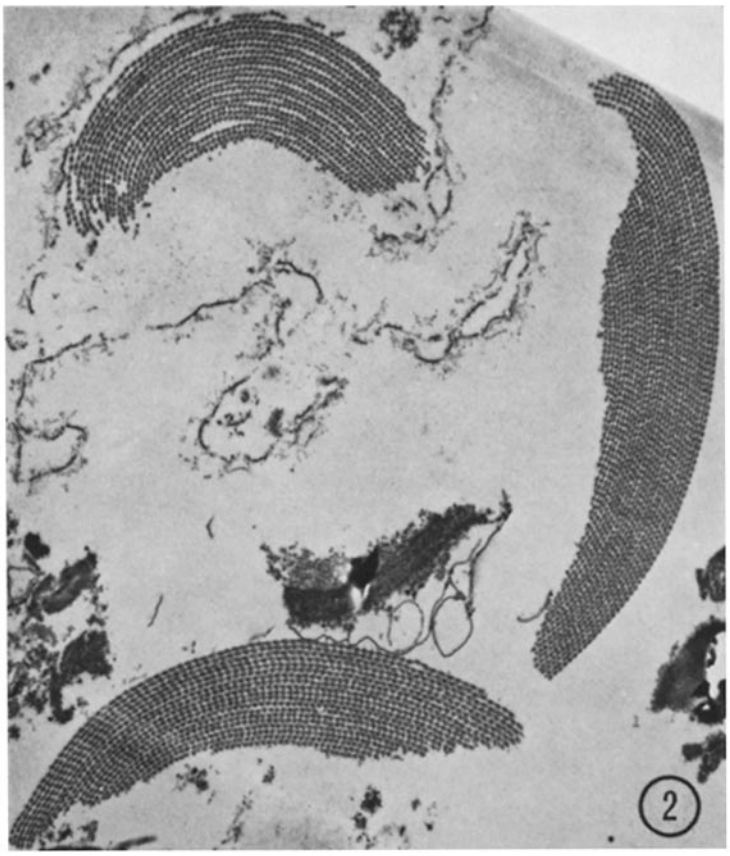

FIGURE 2 Low magnification electron micrograph of transverse sections cut through three isolated axostyles. Notice that the axostyles are cupped-shaped. The debris near the center of the field is a wood chip. × 12,000.

NUCLEOTIDE REQUIREMENT: ATP was most effective in both types of reactivation, i e. reactivation in the presence of cell lysis products and reactivation of isolated axostyles. Good reactivation could also be achieved with ITP or GTP provided these nucleotides were added to axostyles *in the presence of lysis material* However, neither of these nucleotides was effective in generating motion in isolated and purified axostyles that had been stored for some time (10 min to 2 hr) in 0.2 mM EDTA, 10 mM Tris-HCl, pH 7 5 ITP and GTP had a very weak effect immediately after isolation (local twitching of some axostyles in a preparation); this effect ceased within 10 min after suspension of the axostyles in the above Tris-HCl solution. ATP, on the other hand, effectively reactivated axostyles up to 2 hr after their isolation ADP, ADP plus GTP, and pyrophosphate were all ineffective reactivators. ADP inhibited ATP reactivation.

CHARACTERISTICS OF MOTION: The motion of reactivated axostyles mimics that of axostyles in live organisms (Fig 4) There are several species of flagellates in the hindgut which contain contractile axostyles, and the axostyles in these various organisms all have distinctive patterns of beat. This is also true of isolated axostyles which are reactivated with ATP and Mg^{++}. These various beat patterns can be grouped into two classes of motion The first class is characteristic of most of the axostyles in a preparation. it consists of an undulatory wave which is propagated periodically (several beats per second) from the anterior end of the axostyle, although it can be initiated from the posterior end as well This wave is propagated in three dimensions, and the

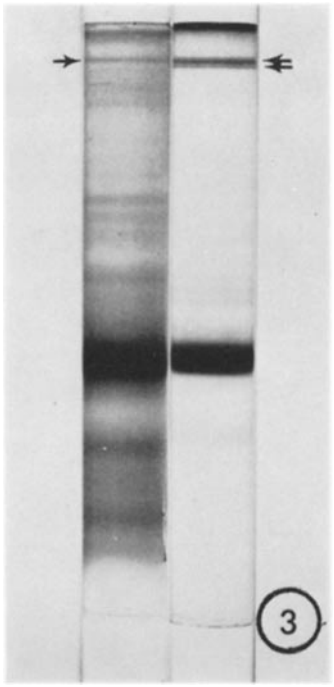

FIGURE 3 5% SDS polyacrylamide gel electrophoresis of the isolated axostyle (left gel) and of axonemes from scallop cilia (right gel). The arrows indicate dynein.

axostyle rotates as the wave passes down its length (see Fig. 4). The second type of motion is found only in the largest axostyles which probably come from a single species, *Saccinobaculus doroaxostylus*. This motion is more complex than the former and much more violent. It consists of a periodic supercoiling, then relaxation of the axostyle This motion is often coupled with a propagated wave which causes the axostyle to rotate

Axostyles reactivated in the presence of cell lysis products continued to beat normally for considerable periods of time (up to 30 min) without adding additional ATP to the slide mount Preparations of axostyles isolated from cell lysis material reactivated well initially, but normal beat patterns ceased after a much shorter period of time Normal motion could be restored, however, by perfusing additional reactivating solution through the slide mount

Isolated axostyles which had been stored for over 2 hr on ice no longer were capable of normal beat patterns The motion observed was in the form of localized contractions or twitching This type of motion was also indicative of older preparations of axostyles which had been beating for some time Like the normal beat patterns previously described, this localized twitching was periodic and was usually initiated in the anterior region of the axostyle. However, many instances were seen of local contractions initiated at other positions along the axostyle as well In one movie sequence of such a local contraction, the anterior end of the axostyle first straightened, then *elongated*, and finally relaxed to its initial position. This movement was much like the "head tossing" behavior seen in mating gulls and ducks where the head straightens, is thrust forward, and finally returns to its initial position (see Tinbergen, 1960)

Nucleotidase Activity of the Isolated Axostyle Preparation

ATPASE ACTIVITY: The average ATPase activity measured in seven tests was 0.19 μmoles of phosphate liberated per minute per milligram of protein (Table I) The values had a range of 0 13–0.29 μmoles of phosphate split per minute per milligram of protein Both Ca^{++} and Mg^{++} activated the ATPase activity equally well It was also ob-

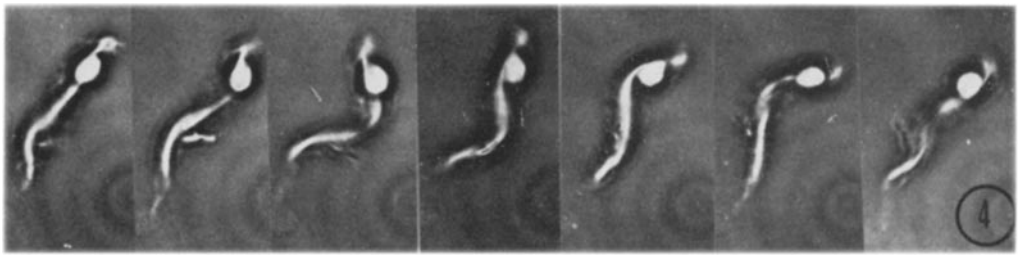

FIGURE 4 Consecutive frames taken from a movie sequence of an axostyle which had been reactivated with ATP. Phase contrast microscopy. The movies were taken at 24 frames/sec.

TABLE I

Values for Mg^{++} Activated Nucleotidase Activity of the Isolated Axostyles

ATPase	GTPase
0.16	0 07
0 14	0 03
0 13	0.07
0.20	0.07
0.29	0.11
0.18	0 09
0.20	0.08
0 19 (average rate)	0 07 (average rate)

Units are in μmoles of phosphate liberated per mg min at 22°C.

served that the enzymatic activity decreased as a function of the age of the preparations used.

GTPASE ACTIVITY: The average GTPase activity measured in seven tests was 0.07 μmoles of phosphate liberated per minute per milligram of protein or about 40% of the ATPase activity (Table I).

Morphology of the Isolated Axostyle

The isolated axostyle maintained the ribbon-like form consistently seen in living organisms which are momentarily inactive (see Fig. 352 of Cleveland et al, 1934). The axostyle is twisted or spiralled so that some portions of it are viewed from the side while others are viewed from the edge. Since protozoa containing axostyles appear in a continuous spectrum of sizes, isolated axostyles of varying lengths and diameters are routinely found Even though some may be two and a half to three times longer than others, the number of spiral turns per axostyle appears constant, each ribbon forming one and a half to two complete spiral revolutions (Fig. 1). The anterior end of the axostyle can be identified in most cases by the flagella which remain attached. The posterior end is quite sharp and here the ribbon appears rounded If organisms are viewed during treatment with Tx, the frequency of beat of the axostyle increases rapidly as the cell lyses. As the membrane dissolves the axostyle often continues to beat rapidly, although the mode of beat is erratic and less periodic than in the untreated organism. Within about 30 sec the beating gradually ceases and with time most of its associated cytoplasm and its nucleus disappear. The morphology of the isolated axostyle closely resembles the description of Grimstone and Cleveland (1965). The description presented below emphasizes certain features of the morphology which were not described before or which are necessary for subsequent discussion.

The isolated axostyle is crescent- or cupped-shaped in transverse section (Figs. 2 and 5 a). It is composed of microtubules arranged in rows running across the width of the axostyle. The number of rows and the number of tubules per row is not constant The convex surface, which corresponds to the outer surface in untreated organisms (the nucleus being attached to the concave side), generally contains a complete row of microtubules; the concave side on the other hand, frequently consists of a short row of tubules which do not extend the width of the axostyle (Fig. 5 a) The spacing of the tubules within each row is remarkably uniform having a center-to-center spacing of 405 A (Fig 5 b) Adjacent tubules in each row appear to be connected by bridges each measuring about 160 A in length and 30 A in width. Rather than lying on a line running between the centers of adjacent tubules in a row these bridges are tilted so that they make a 10–15° angle with this line The centers of adjacent rows are separated by approximately 320 A. The tubules in one row do not occupy a fixed position with respect to those in the next so that if one follows two adjacent rows the tubules exist both in hexagonal packing and rectilinear packing as one scans across the width of the axostyle. This change in packing appears to be a product of the cupping of the axostyle (see arrows in Fig 5 b). Sometimes several rows seem to behave as a unit forming what looks like a crystal dislocation with respect to adjacent rows Bridges are also present connecting tubules in adjacent rows, but these are far less regular than those within a row. The angle they make with the axis of the row seems variable. Bridges can also be seen extending outward from the peripheral rows of tubules on the concave and convex surfaces of the axostyle

In longitudinal section the bridges are easily seen as periodic structures whose period measures about 140–150 A (Fig 6) Since the axostyle is ribbon-like, we can tell whether longitudinal sections are cut parallel or perpendicular to its width If the sections are cut parallel to its width or parallel to a row, the periodic bridges show up clearly On the other hand, in longitudinal sections cut through the narrow axis of the axostyle, the peri-

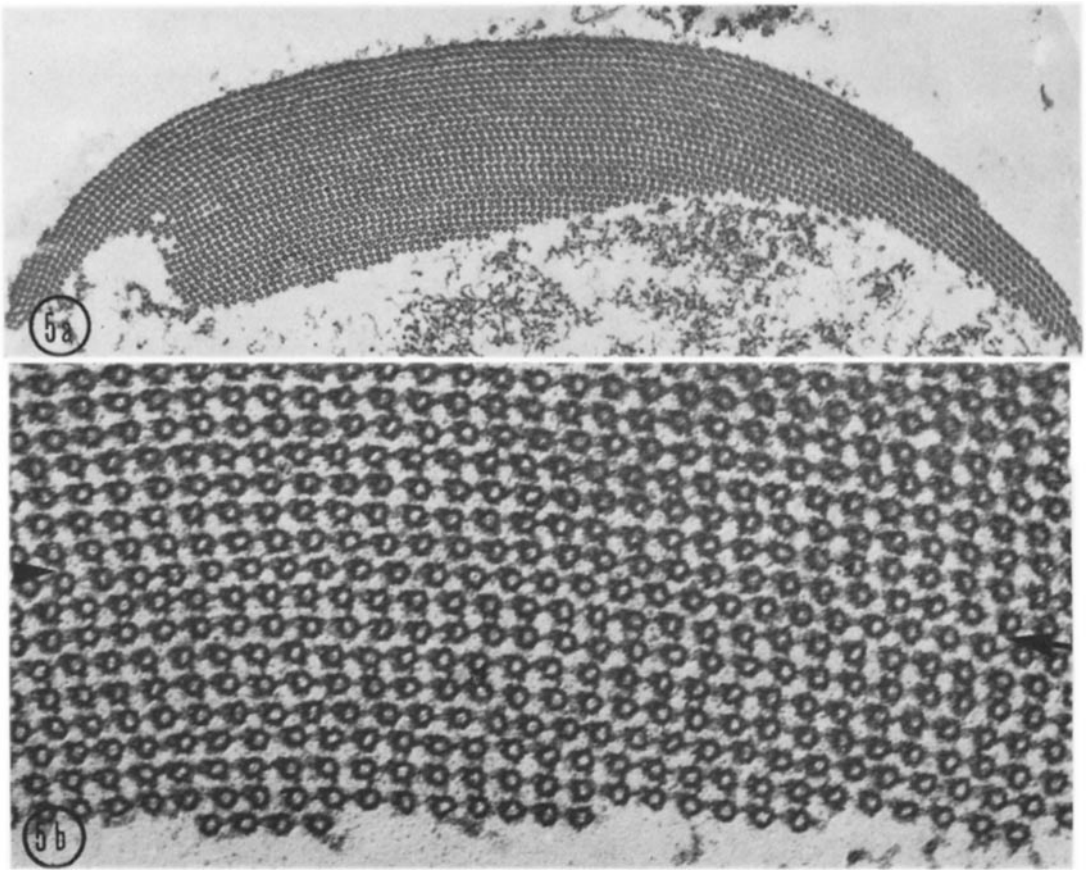

FIGURE 5 a Low magnification electron micrograph of an isolated axostyle Note that the tubules are in rows. × 30,000.

FIGURE 5 b Higher magnification of a portion of the axostyle seen in Fig. 5 a Note that the tubules are connected by bridges, and that the packing of adjacent microtubules in an individual row changes from rectilinear to hexagonal packing as one follows a row from left to right. (See arrows). × 120,000.

odic nature of the bridges is not obvious Most frequently, of course, longitudinal sections are cut at some angle with respect to its width In these cases the 150 A period can be seen as a portion of the surface of a tubule or as short processes extending from the tubules.

DISCUSSION

The Homology between the Axostyle and the 9 + 2 Axoneme

A comparison of the axostyle with the 9 + 2 axoneme demonstrates the following similarities. (*a*) Both are autonomous in the sense that the machinery necessary for generating motion is present in the isolated organelle and not dependent upon the rest of the cell. (*b*) Like the axoneme, the axostyle contains an ATPase whose properties are similar to dynein in both its specificity for ATP and its pH and cation requirements. (*c*) The proteins of the axostyle are homologous to those of the axoneme in terms of molecular weight; most importantly, this includes a protein with the same molecular weight as the enzymatic subunit of dynein. (*d*) The spacing between adjacent rows of microtubules, speculated to be the site of axostylar ATP, corresponds well with what is known about the dimensions

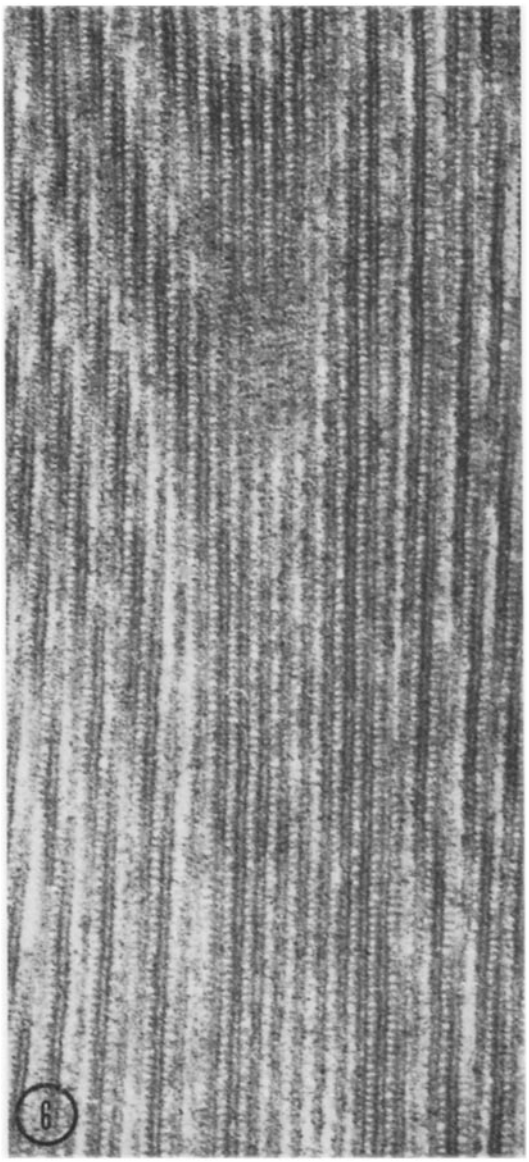

FIGURE 6 Longitudinal section cut through an isolated axostyle. The section is cut across the width of the axostyle. Since the axostyle is cupped, the section cuts through one row, then another. Of particular interest is the periodic nature of the bridges. × 85,000.

of dynein subunits. All of these points will be discussed in detail below.

COMPOSITION OF THE AXOSTYLE. SDS gel electrophoresis studies indicate that the ciliary or flagellar axoneme is composed of numerous proteins with a wide range of molecular weights A number of these have been identified with corresponding structural components of the axoneme These include the microtubule protein(s), tubulin (mol wt 55,000), the linkage protein, nexin (165,000, [Stephens, 1970] or 80,000 [Stephens, 1971]) and dynein (which consists of two molecular weight components, 500,000 and 460,000 [Linck, 1971]) SDS gels of isolated axostyles reveal that among the principal proteins are those with the same molecular weight as tubulin, nexin (80,000), and the enzymatic component of dynein (There is recent evidence discussed in a later section which indicates that only the higher molecular weight (500,000) subunit is enzymatic; the other is assumed to be structural) In addition, most of the other axostylar proteins also comigrate with proteins of the ciliary axoneme. These include a protein which comigrates with a 32,000 mol wt protein in cilia or flagella which has been tentatively identified as an adenylate kinase (Stephens, 1971) This striking correlation of molecular weights suggests that the axostylar proteins are homologous to ciliary proteins by other criteria. This is particularly true of the axostylar protein with the same molecular weight as the enzymatic subunit of dynein It should be pointed out that dynein is a unique molecule in terms of its size; virtually no other protein has a subunit size this large (myosin, for example, is 220,000). Therefore, the unusual size of the dynein subunits can be considered almost diagnostic for this protein

REACTIVATION OF THE AXOSTYLE: Reactivation of the axostyle demonstrates that (*a*) the axostyle behaves as an autonomous unit that is not dependent on the rest of the cell for its motion, (*b*) the motion of the axostyle is completely dependent on the presence of nucleotide; (*c*) the characteristics of axostylar reactivation (cation cofactors, pH range, and nucleotide specificity) are similar to those found for reactivation of flagella or whole sperm (Gibbons et al , 1970, Gibbons and Gibbons, 1972) Some of the characteristics of flagellar or whole sperm reactivation are as follows Divalent cations must be present: Mg^{++} provides the best results, while Ca^{++} is ineffective and inhibits Mg^{++} activated reactivation, Mn^{++} does reactivate, but less effectively than Mg^{++} Flagella or whole sperm can be reactivated over a wide pH range (7.5–9 6) with an optimum around

pH 8.3 ATP is the most effective nucleotide. Others such as ITP or GTP have only a slight effect, if any.

The cation requirements for axostylar reactivation are essentially identical to those found by Gibbons for reactivation of flagella or whole sperm. The axostyle can also be reactivated over a wide pH range, but its optimum range (7.5–8 0) is less basic than that reported for flagella or whole sperm However, this seems to be a function of the organelle's instability at higher pH's rather than a failure of the ATPase to generate motion As long as the structure of the axostyle remains intact, it can usually be reactivated to some degree at pH's from 6.5 to 8.3 Thus, the ATPase involved in generating motion is similar to dynein in its ability to act over a broad pH range.

Like flagellar reactivation the axostyle can best be reactivated with ATP. Axostyles which are first isolated from cell lysis material are also quite specific for this nucleotide ITP and GTP yield only very poor reactivation (localized contractions) and this effect can be generated only immediately after axostylar isolation. The fact that these two nucleotides do yield good reactivation in the presence of cell lysis material suggests that there may be auxiliary enzymes present generating ATP from them, e.g. nucleotide diphosphate (NDP) kinase, rather than the direct utilization of these nucleotides by the axostylar ATPase. This is supported by the fact that reactivation in the presence of cell lysis material continues much longer than in the isolated axostyle system, to which additional ATP must be added periodically to maintain normal beat patterns. This suggests that there may be some recycling of nucleotide into ATP in the former reactivation system The fact that there is an initial positive response to ITP or GTP in the reactivation of isolated axostyles allows for the possibility that auxiliary enzymes exist in the axostyle as well as extraneously in the cell lysis material; i e. there may be residual ADP bound to the axostyle immediately after isolation which is gradually released with washing. This would allow the generation of small amounts of ATP by NDP kinase from ITP or GTP, thus explaining the initial positive response of the axostyle to these nucleotides. This explanation seems more reasonable than assuming that the axostylar ATPase can use other nucleotides in addition to ATP, because ATP reactivates isolated axostyles long after others become ineffective, indicating that the ATPase is still quite healthy The possibility of auxiliary enzymes in the axostyle is a very significant one because the specificity of dynein for ATP is a unique characteristic of this enzyme, and any claim regarding the involvement of dynein in motility must include a consideration of nucleotide specificity.

These reactivation studies clearly demonstrate that the motility of the axostyle is similar in nature to that of the axoneme and thus point to the involvement of an ATPase similar to dynein This conclusion is supported by the fact that the motile properties of the axoneme are directly related to the ATPase properties of dynein (Gibbons et al , 1970; Gibbons and Gibbons, 1972; Gibbons, 1966; Summers and Gibbons, 1971).

NUCLEOTIDASE ACTIVITY OF THE ISOLATED AXOSTYLE: No attempt was made to fully define the parameters of axostylar nucleotidase activity because of the difficulty involved in obtaining workable quantities of axostyles. Despite their limited scope, these studies on the ATPase and GTPase activity of the isolated axostyle are of value in the implication of dynein as the axostylar ATPase. The results demonstrate the following. (*a*) because the axostyle is capable of splitting ATP, it must contain an ATPase, (*b*) the axostyle splits ATP at a rate comparable to that reported for cilia or flagella which is 0 11–0 15 μmoles phosphate split per milligram of protein per minute for cilia and flagella (Stephens and Levine, 1970; Gibbons et al , 1970) and 0.19 μmoles for the isolated axostyle; (*c*) the axostyle splits ATP more effectively than GTP, although with less specificity for ATP than reported for cilia or flagella (40% vs 10%).

The first point is clearly the most significant By proving the existence of an ATPase in the axostyle, these studies confirm the presumed involvement of an ATPase in the reactivation of the axostyle

The second point may or may not be significant. ATPase activity is measured in terms of total axonemal or axostylar protein It is, therefore, important to know the amount of ATPase protein relative to the total protein in the axoneme or axostyle before a meaningful comparison of ATPase rates can be made. Since the axostylar ATPase has not yet been isolated this ratio is not available However, it is interesting to note that the relative amount of dynein (upper band) to tubulin, in terms of band intensity on SDS gels, of ciliary axonemes is roughly the same as the "dynein" to "tubulin" ratio on axostyle gels

The final point can be interpreted in two ways Although the figures indicate that the axostyle prefers ATP but is less specific for ATP than either cilia or flagella, the reactivation studies point to the possible involvement of auxiliary enzymes which could mask specificity for ATP

FINE STRUCTURAL ANALYSIS A POSSIBLE LOCATION FOR DYNEIN IN THE AXOSTYLE: A glance at the fine structure of the axostyle indicates that it consists predominantly of microtubules, the bridges that link the microtubules together in rows, and the less regular bridges between rows In order to generate motion in the axostyle there must be interactions between tubules of adjacent rows. Thus, the ATPase, in order to effect motion, must interact with the tubules in adjacent rows. From our calculations, the average separation of tubules in adjacent rows is approximately 80 A (320 A–240 A) This value, 80 A, must be considered a minimum estimate since this would be the length of dynein if the tubules of adjacent rows were rectilinearly packed If they were hexagonally packed, this distance could be maximally 160 A, assuming of course, that the dynein does not reach down the longitudinal axis of the axostyle as well as across. Gibbons (1965) has demonstrated that dynein from *Tetrahymena* cilia exists as 14 S and 30 S molecules. The 30 S species, when observed after shadow casting, consists of rods 70–90 A in width and 400–5000 A in length (Gibbons and Rowe, 1965). These were interpreted as polymers of the 14 S dynein which by negative staining measures 85–90 A in width and 140 A in length: therefore, the 30 S polymer is composed of globular units which repeat every 140 A Single particles 70 A in diameter and 100 A in length were observed next to isolated and negatively stained flagellar axonemes by Grimstone and Klug (1966). These were interpreted as the 14 S dynein of Gibbons and Rowe (1965) Therefore, the distance between adjacent rows of microtubules in the axostyle (80–160 A) corresponds well with what is known about the linear dimensions of 14 S dynein (100–140 A).

Allen (1968), using an image reinforcement technique on transverse sections of cilia, has demonstrated that the outer dynein arms are really composed of two units. a linear portion and a hooked portion. It seems reasonable to expect that these two portions correspond to the two bands seen on SDS gels of axonemes, which have been identified as dynein If this were true, then one would expect to see only a single dynein band on gels of axostyles because the bridges seen between adjacent rows of microtubules are single lobed and span *half* the distance (about 100 A) that is required of the dynein arms in the axoneme to make contact between adjacent doublets (250 A) This interpretation could be correct because there is, as predicted, only a single "dynein" band on axostyle gels If this 500,000 mol wt band does correspond to a dynein-like ATPase in the axostyle, then it points out the fact that the lower molecular weight band (460,000) seen on axonemal gels may not be needed for the enzymatic activity of dynein because the axostyle functions without it This implies, of course, that only one of the lobes in the dynein "arm" in the axoneme is enzymatic, the other, presumably structural

There is also considerable evidence from work on both cilia and flagella that supports the claim that only the higher molecular weight component of dynein is enzymatic (*a*) Almost all the dynein in flagellar axonemes can be extracted with high salt (0 6 M KCl) (Gibbons and Gibbons, 1972) When dynein from flagella of *Strongylocentrotus* sperm is extracted in this way, it runs as a *single* band (500,000) on SDS gels (Stephens, personal communication). (*b*) When isolated ciliary axonemes are dialyzed in the presence of low salt and EDTA, 50% of the ATPase activity is extracted from the axonemes and goes into solution; the other 50% remains in the axonemal pellet after dialysis. When this pellet is run on gels, only a single band at 500,000 is present; the supernatant shows both bands in a ratio of 1:1 rather than 2:1 (500,000 and 460,000) as is the case in unfractionated axonemes Thus, there is an equivalent amount of higher molecular weight component in both fractions, and both have the same ATPase activity Therefore, the 460,000 mol wt component is not needed for ATPase activity (Linck, 1971) (*c*) Stephens (1972) has shown that the upper dynein band is present in gels of unfertilized sea urchin eggs Presumably this is the ATPase present in these eggs described by Weisenberg and Taylor (1968). The lower band is synthesized at a uniform rate after fertilization.

The above discussion points out the following: (*a*) The axostyle contains bridges in the right position to generate motion by causing interactions between adjacent rows of microtubules. (*b*) These bridges (and the distance they span) correspond well with what is known about the size of a single dynein subunit. (*c*) The axostyle contains a pro-

tein with the same molecular weight as the enzymatic subunit of dynein in cilia or flagella

A Model for Axostylar Motility

One of the most intriguing features of the isolated axostyle and the isolated axoneme of cilia or flagella is that these organelles contain all the information necessary to undergo repeated movements essentially identical to those in vivo The isolated myofibril, on the other hand, is not autonomous but can undergo only a single contraction in the presence of ATP and divalent cations. Tetanus occurs, relaxation being impossible in the absence of their sarcoplasmic reticulum. Thus, the isolated microtubule systems appear to contain within them a mechanism which can regenerate motion The following model gives a partial explanation of how repeated propagated waves could be generated by the axostyle and the axoneme.

We have already pointed out that the tubules in adjacent rows of the nonactive axostyle are not homogeneously packed. Instead, if we compare the packing of microtubules in two adjacent rows, we find some hexagonally packed, others rectilinearly packed. This appears to be due to the cupping of the axostyle which in turn is probably a product of the relationship of the bridges to adjacent tubules It was noticed first by Grimstone and Cleveland (1965), and obvious in our micrographs as well, that the bridges between microtubules within a row are tilted The explanation for this tilting has not been suggested but perhaps it is related to the tubule substructure, i e to an odd number of subunits At any rate, due to the cupping of the nonactive axostyle the spacings between the tubules of one row and those in an adjacent row vary and thus cannot allow maximum bonding Maximum bonding between tubules in adjacent rows can only occur when the axostyle is uncupped, i e when the ribbon is flattened across its width Based on thin sections cut, presumably, through the active region of the axostyle, McIntosh et al. (1970) have stated that in this region the axostyle is flattened; elsewhere it is cupped Thus, when an active wave passes down the axostyle the axostyle appears to become flattened in the active region which, in turn, would allow for maximum interaction between the microtubules of adjacent rows There is evidence which demonstrates that, if part of the axostyle is flattened in vivo, active movement is propagated from this point usually in both directions, suggesting that one of the key controls for active propagation is the initiation of maximum bonding between the microtubules in adjacent rows The evidence is as follows. If an organism is severely flattened by a cover slip, that part of the axostyle in contact with and directly flattened by the cover slip induces an active wave at the point of contact (George Langford, personal communication). Inoué (personal communication) has also described an instance in which an organism containing an axostyle was rammed by a second organism, thus flattening the axostyle locally. This outrage induced the axostyle in the first organism to propagate an active wave in both directions from the point of contact. In both the above examples, therefore, what presumably happens is a flattening of the axostyle which, in turn, allows for the maximum association of adjacent rows of tubules and thus beating is initiated. We presume that once the wave begins to progress from the point of initiation the elastic recall of the axostyle to the cupped position takes over so that behind the wave the axostyle relaxes. Again we presume the cupped position is related to the prominent bridges connecting adjacent tubules within a row. Thus, during relaxation adjacent tubules between rows become both hexagonally and rectilinearly packed

The propagation of repeated waves down the axoneme of cilia and flagella can be explained by a similar mechanism. Gibbons and Grimstone (1960) and Gibbons (1961) were the first to demonstrate that the outer doublets on which dynein is known to be attached (Gibbons, 1965) are twisted inwards 5–10°. This fact has been used repeatedly to identify the A tubule of the doublet This twisting is presumably caused by a bridge (nexin) which connects the inner surfaces of adjacent A tubules (Stephens, 1971) If, as already described, the length of dynein is fixed, proper association of the dynein arms with the nearest B tubule could only occur if the outer doublets were untwisted and thus lay exactly on the circumference of a circle This untwisting, we presume, would proceed in a stepwise fashion, i.e , from 5 to 6, then from 6 to 7, then from 7 to 8, etc. For example, if doublet 5 untwists so that it can make contact with 6, the dynein of 5 can now connect to the B tubule of 6. We presume that part of the active function of the dynein of 5 would be to untwist doublet 6, the dynein of 6 now in a position to bind to the B tubule of 7 and so forth. Thus a wave would propagate in a helical way, assuming

the motion to travel down the axoneme as well as around As in the axostyle, once the wave had passed from doublet 5 to 7 or 8, then the elastic recall of the nexin bridges would retwist doublet 5 so that relaxation would occur.

This model for the ciliary and flagellar axoneme could account for repeated helical waves which could pass down the axoneme Of course, some flagella and cilia elicit nearly planar waves. Such a variation from the true helical wave might be accounted for by the relative rigidity of the central pair and/or associated structures

In this model, for both the axostyle and the axoneme of cilia and flagella, repeated propagated waves could be generated; relaxation being the result of the elasticity of certain bridges which connect adjacent tubules. It is of further interest, as stated before, that the molecular weight of ciliary nexin is similar to the molecular weight of a major protein of the axostyle which we believe to be the bridge We also should mention that the lengths of the bridges in these two systems are multiples of each other, e g. 150 A for bridges of the axostyle and 300 A for nexin of cilia. Both, of course, function by being connected to adjacent microtubules asymmetrically.

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