

THE DYNEIN ELECTROPHORETIC BANDS IN AXONEMES NATURALLY LACKING THE INNER OR THE OUTER ARM

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ABSTRACT

Two unconventional sperm models (all motile) have been studied. The first one has only the outer arm on the doublets (the gall midge, *Diplolaboncus*); the second one, has only a well-developed inner arm (the eel, *Anguilla*). Both are devoid of central tubules and radial spokes. The gall midge sperm yields a single electrophoretic band migrating similarly to the sea urchin dynein band A; a major high-molecular-weight band is obtained from eel sperm which co-migrates with the sea urchin dynein band B. The present picture is consistent with the localization of dynein in the axoneme—namely, of an A-like band in the outer arm, and of the B band in the inner arm. Moreover, the D band is present only in the eel, where γ -links are present. ATPase activity was localized histochemically and found to be associated with both inner and outer arms, as well as with the γ -links.

KEY WORDS dynein bands localization · axoneme · spermatozoa · ATPase · motility

Recent work on the dynein protein complex in axonemes has been aimed at the topographic localization of the different isozymes and polypeptide components in the various axoneme compartments. Dynein was described in sea urchin sperm as an adenosine triphosphatase (ATPase) enzyme of 600,000 dalton mol wt (9). This protein was extracted from and reconstituted to flagellar axonemes, reappearing as the arms of the doublet microtubules in various flagella (9, 20). Subsequently, Linck (13) demonstrated in scallop cilia and flagella that this protein is made up of two components, each characterized by different electrophoretic mobilities in sodium dodecyl sulfate-(SDS)-polyacrylamide; they were indicated as A and B (with 500,000 and 400,000 mol wt, respectively). Burns and Pollard (5) named the two components in clam flagella α - and β -dyneins.

In sea urchin, Gibbons et al. (8) were able to distinguish five electrophoretic bands, indicated in order of decreasing mol wt (from 600,000 to 300,000) as C, A₁, A₂, D, and B. Quite recently, Warner et al. (21), in a different mollusc and in a protozoan, have distinguished six bands (from 360,000 to 300,000) named 1, 2, 3, 4, 5, and 6. Numbers 3 and 4 are predominant and correspond to the A and B of the previous authors.

From the beginning of the studies on dynein, it has been evident that this protein is an ATPase enzyme. After its resolution into several polypeptide bands, the problem arose as to which of them possessed the ATPase activity. Most of the activity was demonstrated to be present in the salt-extracted component A₁ of band A (11), while the situation with the B band was not clear. In fact, while Burns and Pollard (5), Mabuchi and Shimizu (14), and Mabuchi et al. (15) described ATPase activity in the B-band of mollusc and echinoderm sperm, and protozoan flagella, Gibbons et al. (8), and Ogawa and Gibbons (18) did

not confirm this observation. These authors have indeed attributed ATPase activity to the D polypeptide and have distinguished two dynein isozymes: 1 (band A), and 2 (band D).

The problem of the localization of the different polypeptide chains has been investigated by selective extraction procedures. Kincaid et al. (11) first demonstrated in sea urchin that 65% of the ATPase and one-half of the protein present in band A (A_1 component) is located on the outer arm of the doublets; this has been repeatedly confirmed by the reactivity of whole or partially extracted flagella against an anti-dynein 1 serum (7, 17, 19). Nothing is known of the localization of the other polypeptide bands or of their ATPase activities.

We have approached the problem by investigating naturally occurring motile axonemes lacking radial spokes, spoke heads, and central tubules (structures displaying ATPase activity, references 8, 11), and also lacking either the inner or the outer arm. We used the cecidomyid fly, *Diplolaboncus tumorificus*, which has only outer arms (3), and the eel *Anguilla anguilla*, having only inner arms.

MATERIALS AND METHODS

Material

Pure spermatozoa from the gall midge, *Diplolaboncus tumorificus*, were obtained by dissecting the deferens of adult males in Hoyle's medium (0.375 g KCl, 3.750 g NaCl, 0.110 g CaCl_2 , 0.205 g MgCl_2 , 0.170 g NaHCO_3 , 0.415 g NaH_2PO_4 , 500 cm^3 H_2O). Whole cells were used. Eel spermatozoa were obtained by squeezing adult males. The spermatozoa were suspended in 0.9% NaCl in 0.01 M Tris(tris[hydroxymethyl]aminomethane)-HCl pH 7.5 and decapitated with a loosely fitting pestle in a Dounce (Kontes Co., Vineland, N. J.) homogenizer. Flagella were purified by centrifugation at 26,000 rpm for 3 h through a 25–62% sucrose gradient prepared in Beckman (Beckman Instruments, Inc., Fullerton, Calif.) SW 27.1 rotor tubes.

Dynein Extraction

Whole *Diplolaboncus* spermatozoa were demembrated by treatment for 15 min at 2°C with 1% Triton (Rohm and Haas Co., Philadelphia, Pa.) in Hoyle medium. Axonemes were recovered by centrifugation at 30,000 g for 10 min at 2°C and dynein was solubilized by dialysis for 48 h at 2°C against 2 mM Tris-HCl, pH 8 with 0.5 mM EDTA. Extraction with NaCl was attempted with 0.5 and 1 M solutions, in 0.01 M Tris-HCl, pH 7.5, from 15 min to 48 h. The sediment and supernatant fractions were obtained by centrifugation at 30,000 g for 10 min at 2°C.

SDS-Polyacrylamide Gel Electrophoresis

Whole and extracted *Diplolaboncus* spermatozoa and purified eel flagella were dissolved by boiling for 2 min in 0.062 M Tris-HCl, pH 6.8, containing 2% SDS, 5% mercaptoethanol, 0.005% bromphenol blue, and 0.03 mg/ml phenylmethylsulphonyl fluoride (PMSF). Before electrophoresis, samples were diluted with glycerol to 10% original concentration. Electrophoresis was performed on 4% acrylamide gels (0.5 × 10 cm, or 0.5 × 12 cm) by the discontinuous pH method described by Laemmli (12). Staining and destaining were performed with Coomassie blue in methanol, as described by Meizel (16).

Electron Microscopy

Gall midge spermatozoa, either native, or after dialysis or NaCl treatment (see above), were fixed in Karnovsky's fixative (10) at pH 7.2 in cacodylate buffer to which 2% tannic acid was sometimes added.

Eel spermatozoa were fixed in 2.5% glutaraldehyde in 0.05 M cacodylate buffer containing 2% tannic acid. In some cases the material was pretreated with 1% Triton X-100 in cacodylate buffer for 15 min. The material was rinsed in buffer and post-fixed in 1% OsO_4 for 1 h, dehydrated and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate.

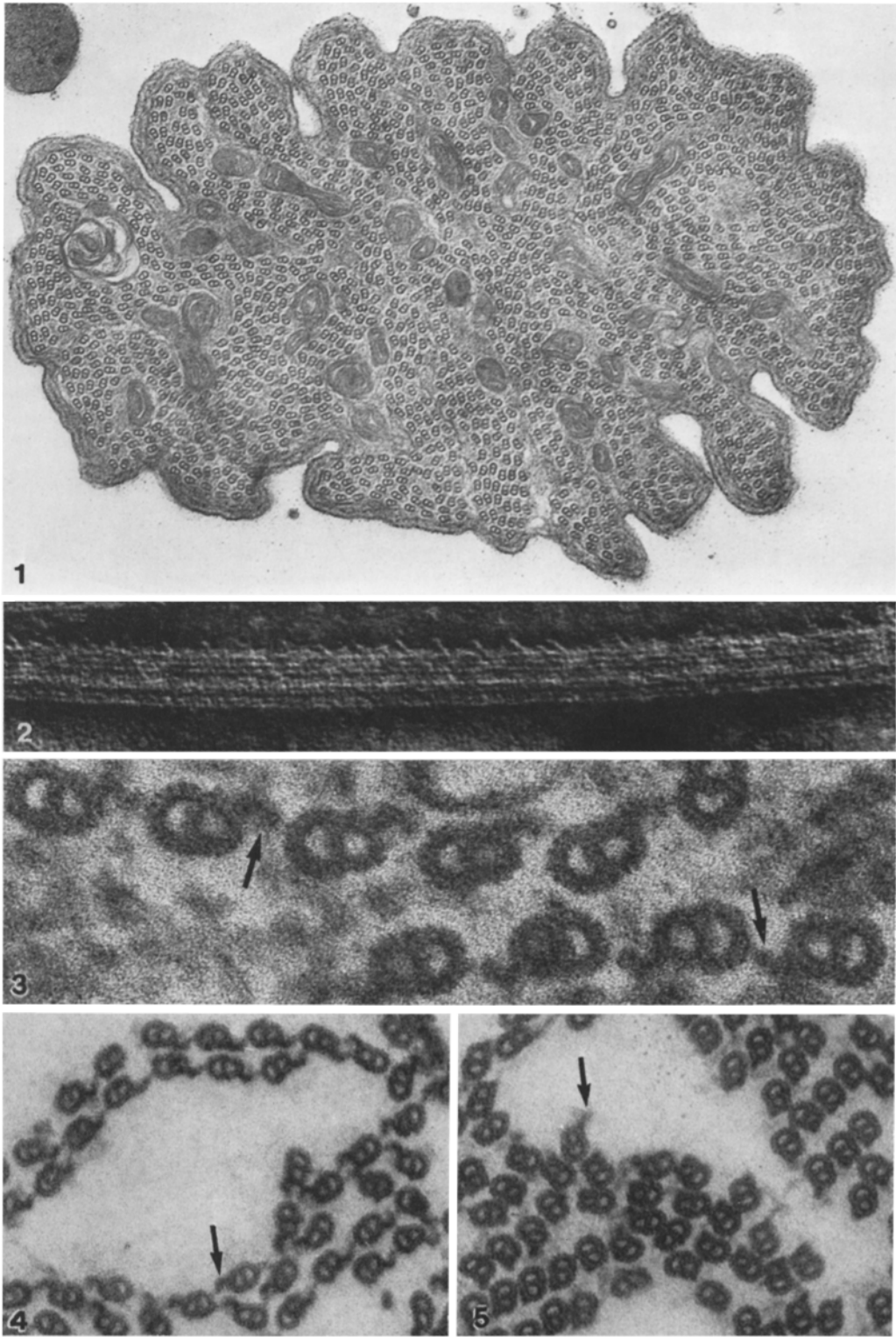
For negative stain preparations a drop of sperm suspension in distilled water was placed on a coated grid. A drop of 5% uranyl acetate pH 4.5 was added, the excess fluid removed after 10 min, and the preparation allowed to air dry.

For ATPase activity the unfixed or briefly fixed (1.5% glutaraldehyde for 10 min) spermatozoa were incubated in a modified Wachstein and Meizel medium for 30 min. Controls were carried out either by omitting ATP or by adding 0.33 M NaF to the medium.

RESULTS

Diplolaboncus sperm has up 2,000 doublet microtubules (Fig. 1) arranged in bundles. No other kinds of tubules are present. Each doublet possesses only one row of arms—the outer one—as shown by their position in the bundles. Yet this one-armed spermatozoon is motile. In physiological fluids, the spermatozoa recovered from mated females beat actively with waves propagating along their whole length (Fig. 7). In longitudinal view after negative staining, the arms appear obliquely oriented and spaced with a repeat of 22 nm (Fig. 2). In cross sections after tannic acid impregnation, they show an angular profile made up of two segments 10 nm long. No radial spokes are present (Fig. 3).

After Triton treatment the outer membranes and mitochondria are removed from the sperm,



and only the bundles of doublet microtubules, with well preserved arm structures, are present. The subsequent treatment with 0.5 M NaCl, even for a prolonged time, leaves the arms completely unchanged (Fig. 4). Dialysis at low ionic strength affects all the arms and results in the removal of a major distal portion while an extremely short basal segment remains attached to the doublet (Fig. 5).

Electrophoretic analysis (Fig. 6) shows that in *Diplolaboncus* sperm only a single band with migration is similar to that of sea urchin A band is detected. Comigration experiments (Fig. 6) show that this polypeptide is slightly lower in mol wt than the sea urchin A polypeptide. This band is also present in the axonemes after treatment with Triton and with 0.5–1.0 M NaCl. Dialysis-extracted axonemes do not show any band in the dynein region; on the other hand after dialysis the supernate contains the A-like band.

The eel spermatozoon has a "9 + 0" axoneme (Fig. 8) made up only of doublet tubules which lacks central tubules as well as any system of radial connections (Figs. 8). These spermatozoa move quite actively and their tail beats with large frequent waves propagating backwards, similar to marine spermatozoa (Fig. 12). All doublet tubules possess only the row of inner straight arms which are about 20 nm long (Fig. 10). A very short segment of outer arm is present in the proximal segment of the axoneme (Fig. 10). Short γ -links bind doublets to the membrane (Fig. 10). The reaction of Wachstein and Meisel for ATPase demonstrated a large precipitate both on the inner arms and the γ -links (Fig. 11).

Electrophoretic analysis (Fig. 6) demonstrates

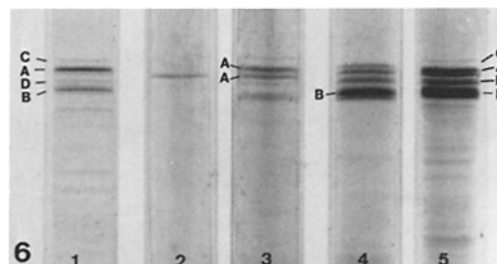


FIGURE 6 The dynein bands after polyacrylamide gel electrophoresis in several sperm models. 1: sea urchin in which the classical bands can be seen; 2: *Diplolaboncus*, showing only a band in the region of the A sea urchin band; 3: coelectrophoresis of *Diplolaboncus* and sea urchin: the *Diplolaboncus* upper band migrates closely to the sea urchin A band, (the upper A); 4: *Anguilla* showing a prominent B band; 5: comigration of *Anguilla* and sea urchin showing a perfect correspondence between the two B bands.

in *Anguilla* axonemes a group of bands in the gel region where sea urchin dynein bands migrate. The major eel band has the same mobility as the sea urchin B band. Minor bands are also present; two of them clearly correspond to the sea urchin A and D bands.

DISCUSSION

From the results presented in this paper, four main conclusions may be drawn, each concerning one of the most important polypeptide chains attributed to dynein.

(a) In an axoneme carrying only the outer arm, such as that of *Diplolaboncus*, only the A-like electrophoretic band is detected. On the other hand, the same band is very faint in an axoneme

FIGURE 1 Cross section of the sperm tail in *Diplolaboncus tumorificus*. Up to one thousand doublet microtubules can be observed. Each doublet has only one arm which is known to be the outer one by the position in the peripheral doublets. $\times 45,000$.

FIGURE 2 Longitudinal view of a doublet microtubule in *Diplolaboncus*, negatively stained with 5% uranyl acetate. The outer arms can be seen with a regular, repeating pattern spaced every 22 nm along the upper edge, tilted obliquely. $\times 200,000$.

FIGURE 3 Cross section of *Diplolaboncus* doublets after Karnovsky 2% tannic acid fixation. The arm (arrow) present on each doublet has the classical angulate profile. $\times 510,000$.

FIGURE 4 Cross section of *Diplolaboncus* doublets after Triton-NaCl treatment and Karnovsky 2% tannic acid fixation. The arms (arrows) are still evident. $\times 170,000$.

FIGURE 5 Cross section of *Diplolaboncus* doublets after dialysis at low ionic strength and Karnovsky 2% tannic acid fixation. A short basal segment (arrow) of the outer arm remains attached to the doublet, but the main segment of the arm has been removed. $\times 150,000$.

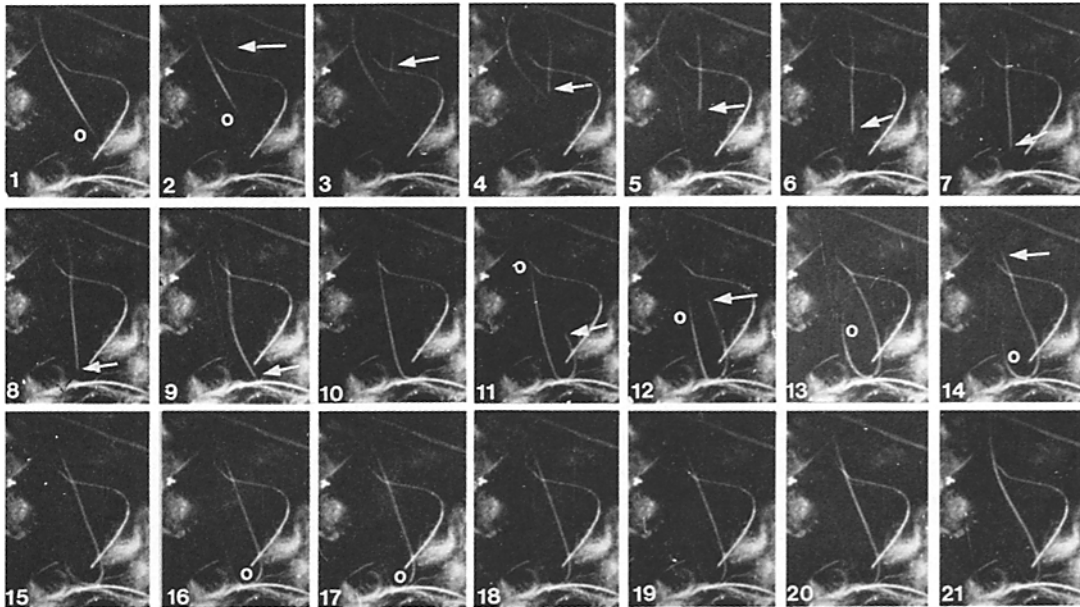


FIGURE 7 The movement of *Diplolaboncus* sperm. The central spermatozoon is moving by consecutive waves. The base of the flagellum is marked with a circle, the tip with an arrow. Images were taken every $\frac{1}{12}$ s. $\times 200$.

having only an extremely reduced system of outer arms, as in *Anguilla*. We conclude that this band is localized on the outer arm. Because *Diplolaboncus* sperm are actively motile, the presence of the outer arms, and therefore of the A dynein band only (probably Dynein 1 of Gibbons et al., [8]), could be sufficient for motility.

This conclusion agrees with the view of Kincaid et al. (11). The *Diplolaboncus* outer arm is, however, different from the corresponding structure of sea urchin, *Tetrahymena*, or *Unio*, because of its resistance to NaCl extraction. Furthermore, only a portion of *Diplolaboncus* outer arm which is constituted by the A-like polypeptide is removed by dialysis at low ionic strength. The nature of the dialysis-resistant segment is at present obscure. In this connection it may be recalled that Warner et al. (21) and Burns (4) have questioned the suggestion that the outer arm is made up of a single polypeptide chain, and also Ogawa and Gibbons (18) suggest a heterodimeric structure both in dynein 1 and 2.

Even if the A-like band from *Diplolaboncus* does not have all the classical characteristics of the Dynein A (it has a lower mol wt and cannot be extracted with NaCl), we attribute it to the Dynein A category of isozymes because it is located in the

outer arm and it is clearly distinct from the D band. For these reasons, Dynein 1 seems to vary in its characteristics between different phyla, and even between species, as pointed out by Mabuchi et al. (15) in their comparison of sea urchins and starfishes, and by Mabuchi and Shimizu (14), who have examined *Tetrahymena* cilia.

(b) The B-like electrophoretic band is predominant among the other dynein bands in an axoneme having only a well developed inner arm (*Anguilla*). We conclude that the predominant band derives from the predominant structure. On the other hand, because the same band is absent when the inner arm is missing (*Diplolaboncus*), we conclude that the B band is localized on the inner arm. Because *Anguilla* sperm are actively motile, the presence of the inner arm, and therefore of the predominant B band, is sufficient to support motility. In fact the very reduced eel outer arms, present only in an extremely short basal segment of the axoneme which bends along its whole length. Furthermore, ATPase activity is histochemically detectable on the eel inner arm.

This conclusion is particularly relevant to the discussion of dynein, since works performed on

conventional axoneme patterns have not yet clearly established the localization of the B band and its involvement in dynein ATPase activity. It

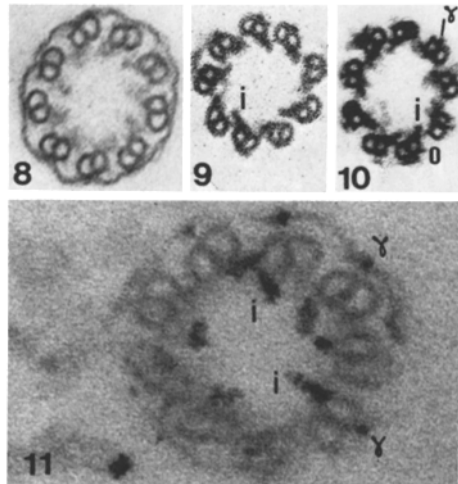


FIGURE 8 Cross section of the sperm tail in *Anguilla anguilla*. Only nine doublets, each one with only the inner arm, are evident. $\times 120,000$.

FIGURE 9 Cross section of the *Anguilla* axoneme after Karnovsky 2% tannic acid fixation. The inner arm (*i*) is evident. $\times 100,000$.

FIGURE 10 The basal segment of the *Anguilla* axoneme, in cross section. The doublets bear not only the inner arm (*i*) but also a short segment of outer arm (*o*) and the γ -link (γ). Radial connections are absent. $\times 100,000$.

FIGURE 11 Cross section of the *Anguilla* sperm tail, showing ATPase activity (lead precipitates) both on the inner arms (*i*) and on the γ -links (γ). $\times 200,000$.

is unlikely that the predominant band of *Anguilla* sperm corresponds to sea urchin bands A and D, which have been demonstrated to be carriers of ATPase activity, rather than to sea urchin B bands; in fact, the electrophoretic pattern of *Anguilla* sperm shows, in addition to the predominant B-like band, minor bands which migrate with or close to sea urchin A and D bands. Therefore, on the basis of our studies on the eel sperm axoneme the B band can be included in the dynein group, as it is endowed with ATPase activity and is involved in axoneme motility.

(c) Our results indirectly touch on the localization and function of the D band. This band is missing in *Diplolaboncus*, whose axoneme contains only doublet tubules and outer arms; it is present in the eel, where radial spokes and all the central structures are also missing, but γ -links are present. In this region ATPase is histochemically detectable. The D band is the dynein 2 of Ogawa and Gibbons (18), for which these authors suggested a localization on the radial spokes. We suggest here that the γ links are the source of the D band. These structures, so called by Baccetti and Afzelius (2), may be responsible for the diffuse ATPase activity detected by Dentler (6) in protozoan flagella.

(d) The C band is constantly lacking in the axoneme models we have studied. The only characteristic common to both is the lack of the central system of singlets, projections, radial spokes, and spoke heads. These structures could be the source of C dynein band in conventional (sea urchin) axonemes.

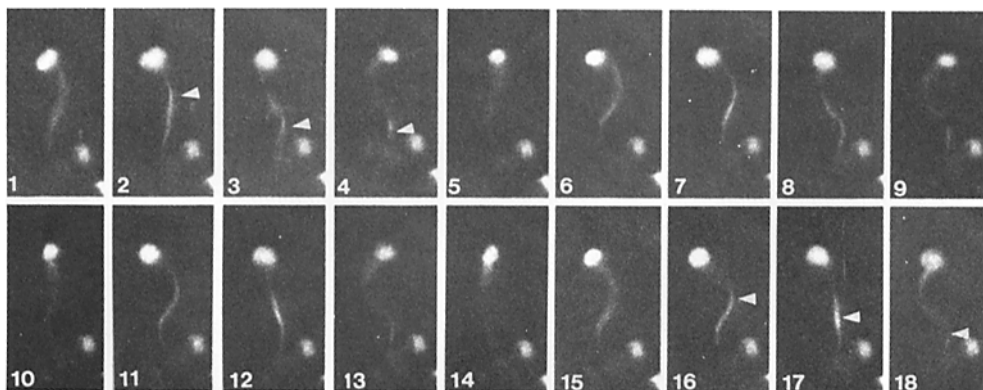


FIGURE 12 The movement of *Anguilla* sperm. The images must be observed from the left to the right. The spermatozoon is moving by consecutive waves (arrows). The time interval is $1/38$ s. $\times 1,000$.

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